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THE EFFECT OF MELATONIN ON HIPPO SIGNALLING PATHWAY IN DENTAL PULP STEM CELLS

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ABSTRACT

Baysal E., The Effect of Melatonin on Hippo Signaling Pathway in Dental Pulp Stem Cells, Hacettepe University Graduate School of Health Sciences Stem Cell Program Master Thesis, Ankara, 2020. Dental pulp stem cells (DPSCs) are heterogeneous multipotent adult stem cells with high capacity to differentiate to both neuronal and non-neuronal cell lineage. Hippo pathway regulates diverse cellular processes, including cell survival, proliferation, differentiation, and organ size. Yes-associated protein (YAP) is an important downstream effector of the Hippo signaling pathway involved in neuronal differentiation of neural progenitor cells. Melatonin has a regulatory role for the differentiation of neuronal lineage. Therefore, melatonin may have modulatory role in neurogenic differentiation by interacting with Hippo signaling pathway. In regard with this, DPSCs were incubated with growth and dopaminergic neuronal differentiation medium with or without melatonin (10 μM) for 21 days. The morphological changes were followed by phase contrast microscopy and differentiation of DPSCs was evaluated by immunofluorescence labelling with Neun, GFAP, TH and DMP1. Furthermore, we evaluated the presence of neural progenitor cells by nestin immunoreactivity. Hippo signaling pathway was investigated by evaluating the immunoreactivity of YAP and p-YAP^{Y357}. Our results were also supported by western-blot analysis and SOX2, PCNA and caspase-3 were also evaluated. The positive immunoreactivity for Neun, TH and negative immunoreactivity for GFAP showed the successful differentiation of DPSCs to neurons, not glial cells. Melatonin (10μM) addition to dopaminergic media induced TH and decreased significantly nestin expression. The expressions of PCNA and caspase-3 were also decreased significantly with melatonin addition into growth media. Melatonin treatment induced phosphorylation of YAP^{Y357} and reduced YAP expression. In conclusion, melatonin has potential to induce the neuronal differentiation and reduce proliferation of DPSC by increasing phosphorylation of YAP^{Y357} and eliminating the activity of YAP, which indicates the active state of Hippo signaling pathway.

Key Words: Dental pulp stem cells, Melatonin, Neurogenesis, Hippo signaling pathway, Phosphorylation of YAP

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ÖZET

Baysal E., Dental Pulpa Kök Hücrelerinde Melatoninin Hippo Sinyal Yolağı Üzerinde Etkisi, Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Kök Hücre Programı Yüksek Lisans Tezi, Ankara, 2020. Dental pulpa kök hücreleri (DPKH), nöronal ve nöronal olmayan hücre tiplerine dönüşebilen heterojen multipotent özellikte yetişkin kök hücrelerdir. Hippo sinyal yolağı, hücre canlılığı, çoğalması, farklılaşması ve organ büyüklüğü gibi çeşitli hücresel süreçlerin düzenlenmesinde görev almaktadır. Hippo sinyal yolunun önemli bir bileşeni olan Yes-ilişkili protein (YAP), nöral progenitör hücrelerin nöronal farklılaşmasında rol oynamaktadır. Melatonin hormonunun nöronal yönde farklılaşma üzerinde düzenleyici bir görevi bulunmaktadır. Bu nedenle, melatoninin Hippo sinyal yolu ile etkileşerek nörojenik farklılaşmada düzenleyici bir role sahip olabileceği düşünülmektedir. Bu bağlamda, DPKH'ler melatoninli (10 μM) ve melatoninsiz olmak üzere büyüme besiyeri, dopaminerjik nöronal farklılaşma besiyeri kullanılarak 21 gün boyunca kültüre edildi. Morfolojik değişikler faz-kontrast mikroskobu altında günlük olarak incelendi. DPKH'lerinin farklılaşması Neun, GFAP, tirozin hidroksilaz ve DMP1 immünoreaktiviteleri ile değerlendirildi. Nestin immünoreaktivitesi ile nöral progenitör hücrelerin varlığı, YAP ve p-YAP^{Y357} immünoreaktivitesi ile Hippo sinyal yolağı araştırıldı. SOX2, PCNA ve kaspaz-3 ekspresyonları ek olarak değerlendirilerek, sonuçlarımız western-blot analizleri ile desteklendi. DPKH'lerinin glial hücreler yerine nöronlara başarılı bir şekilde farklılaştığı Neun, TH pozitif immünoreaktivitesiyle ve GFAP negatif immünoreaktivitesiyle gösterildi. Dopaminerjik nöronal farklılaşma besiyeri içerisine melatonin (10 μM) eklenmesiyle, TH ekspresyonu önemli ölçüde artarken, nestin ekspresyonu istatiksel olarak anlamlı olarak azalmıştır. Büyüme besiyeri içerisine melatonin eklenmesiyle, PCNA ve kaspaz-3 ekpresyonları önemli ölçüde azalmıştır. Melatonin ile DPKH'lerin kültüre edilmesi sonrası, p-YAP^{Y357} fosforilasyonunu indüklenirken, YAP ekspresyonu azalmıştır. Sonuç olarak, melatonin Hippo sinyal yolağını aktive ederek, p-YAP^{Y357} fosforilasyonunun artmasını ve YAP aktivitesinin azalmasını sağlayarak DPKH'lerinin çoğalmasını azaltıp nöronal yönde farklılaşmayı indükleme potansiyeline sahiptir.

Anahtar Kelimeler: Dental pulpa kök hücreleri, Melatonin, Nörogenez, Hippo sinyal yolağı, YAP fosforilenmesi

Bu tez çalışması Hacettepe Bilimsel Araştırma Projeleri Koordinasyon Birimi tarafından desteklenmektedir. Proje no: TYL-2018-17575

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ABBREVIATION

µl	Mikrolitre
µM	Mikromolar
nM	Nanomolar
ALP	Alkaline phosphates
APS	Ammonium persulfate
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenic protein
BSA	Bovine Serum Albumin
cAMP	Cyclic adenosine monophosphate
DMP1	Dentin matrix protein
DNM	Dopaminergic neuronal differentiation medium
DFPCs	Dental follicle progenitor cells
DPBS	Dulbecco's phosphate-buffered saline
DPSCs	Dental pulp stem cells
DPSCGS	Dental Pulp Stem Cell Growth Supplement
DSPP	Dentin sialophosphoprotein
ERK	Extracellular signal-regulated kinase
ESCs	Embryonic stem cells
FBS	Fetal Bovine Serum
GA	Gentamicin/Amphotericin-B
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GM	Growth medium
GPRC	G-protein coupled receptors
ICM	Inner cell mass
LATS1/2	Large tumor suppressor 1/2
MAP2	Microtubule Associated Protein 2
MSC	Mesenchymal stem cells
MST1/2	Mammalian Ste20-like kinases 1/2
MT	Melatonin
MT1	MTNR1A

MT2	MTNR1B
Neun	The neuronal nuclear protein
NGF	Nerve growth factor
NSCs	Neural stem cells
OD	Optical Density
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PDLSCs	Periodontal ligament stem cells
PKA	Protein kinase A
PMSF	Phenylmethylsulfonyl fluoride
PPARγ	Proliferator-activated receptor gamma
pYAP^{Y357}	Phosphorylation of YAP on tyrosine residues at 357 (Y357)
RA	Retinoic acid
SCAP	Stem cells from the apical papilla
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHED	Stem cells derived from the dental pulp of human exfoliated deciduous teeth
SOX2	Sex-determining region Y-box 2
TEMED	Tetramethylethylendiamine
TH	Tyrosine hydroxylase
TE	Trophoectoderm
Ti	Titanium
YAP	Yes-associated protein

FIGURES

TABLES

1. INTRODUCTION

The stem cell research is one of the fascinating and promising field because of highly regenerative ability of the stem cells. They have potential to be used as a cure for the replacement of damaged cells in order to repair or regenerate organs (1). Recently, pre-clinical trials have been carried out for the treatment of neurological disease by using neural stem cells (NSCs) (2). However, NSCs are not applicable for cell-therapy in regenerative medicine because of limited proliferation rate, viability, and migration capacity. Also, ethical consideration restricts the application and isolation of NSCs. In this regard, alternative cell sources like dental pulp stem cell have been suggested as promising cell sources for the cure of neurodegenerative disease (3).

Dental pulp stem cells (DPSCs) are heterogeneous multipotent populations. They are isolated easily from permanent teeth with less ethical concern. They have the potential to differentiate neuronal and non-neuronal cell lineage such as osteogenic, chondrogenic, myogenic, and adipogenic lineage. Because of neuronal differentiation potential, ethical suitability, high proliferation rate, storage for a long time without losing functions, DPSCs have being explored as a seductive cell source for stem-cell treatment in neurodegenerative disease (3). DPSCs can mediate neuroregenaration in neurological disease by secreting growth and trophic factors, regulating angiogenesis and inhibiting apoptosis (4).

To use DPSCs more efficiently, there are many protocols for the differentiation of DPSCs to neurons (5, 6). Neurosphere formation and adherent monolayer cultures are used for differentiation of DPSCs to neuron (5, 7). After neuroectodermal differentiation, DPSCs show the features of neuronal cells in terms of morphology and spesific protein expression (8). They express the neuronal markers (The neuronal nuclear protein (Neun), Glial fibrillary acidic protein (GFAP), Tyrosine Hydroxylase (TH)) or neuroprogenitor marker (Nestin) after neurogenic induction (6, 9, 10). The expression of Sex-determining region Y-box (SOX)2 in DPSCs verifies the primitive or naïve nature of DPSCs (11) and maintains multipotency of the lineage committed progenitor or stem cells (12). Inactivation of SOX2 is required to complete neurogenesis of neuronal progenitor cells (13, 14). Moreover, SOX2 is completely

downregulated in MAP2 positive neurons which were differentiated from Ntera2/D1 neuron-like cells (15). In consequence, differentiation which is a switch from proliferation to specialization, involves the morphological and biochemical changes (16). Different molecules and signaling pathways can modulate the differentiation of DPSCs, but underlying mechanisms are not well known (17).

Evolutionarily conserved Hippo signaling pathway is one of these signalling pathways which has an important role in modulating the proliferation or differentiation of DPSCs (18, 19). Hippo signaling pathway interplays with several cascades such as Notch, Sonic Hedgehog, Wnt, and also regulates cell proliferation and neuronal differentiation. For instance, neuronal differentiation was inhibited in the chicken spinal cord by overexpression of Yes-associated protein (YAP) which is the final coactivator of the Hippo signaling pathway (20). YAP expression was detected in neural progenitor cells, but not in a mature neuron (21). Therefore, researchers proposed that YAP could undergo degradation during neuronal differentiation (21). Since the Hippo signaling pathway is regulated by several factors in different conditions, the role and modulatory mechanisms of the Hippo signaling pathway should be understood clearly in the neuronal system (22). Recent studies have reported that melatonin interplayed with the Hippo signaling pathway and activated several signaling pathways to regulate the physiological process in different cell lines (23).

Melatonin (MT) is a neurohormone that has a regulatory role in the cellular processes such as proliferation and differentiation (23). Melatonin increases the expression of neuronal markers by inducing differentiation of NSCs into neurons (24). Also, melatonin has a regulatory role in the differentiation of dental pulp stem cells. Melatonin induces differentiation of dental stem cells in different directions under various kinds of culture conditions (25-27).

Although the Hippo signaling pathway could regulate osteogenic/adipogenic differentiation of DPSCs (18, 19), there is not enough research on the interaction between neurogenic differentiation of DPSC and Hippo signaling pathway. Since melatonin induces the neuronal differentiation in NSCs, melatonin can modulate the neurogenic differentiation of DPSCs via the Hippo signaling pathway under proper conditions.

2. BACKGROUND

2.1. Stem Cells

Stem cell research has been a popular subject of study for decades because of its enormous potential in regenerative medicine and cell therapies (28). They are defined as the master cells of the body with the ability to multiply themselves and form different types of cell within the body such as a neuron, bone cell, hearth cell under proper conditions (29) (Figure 2.1.).

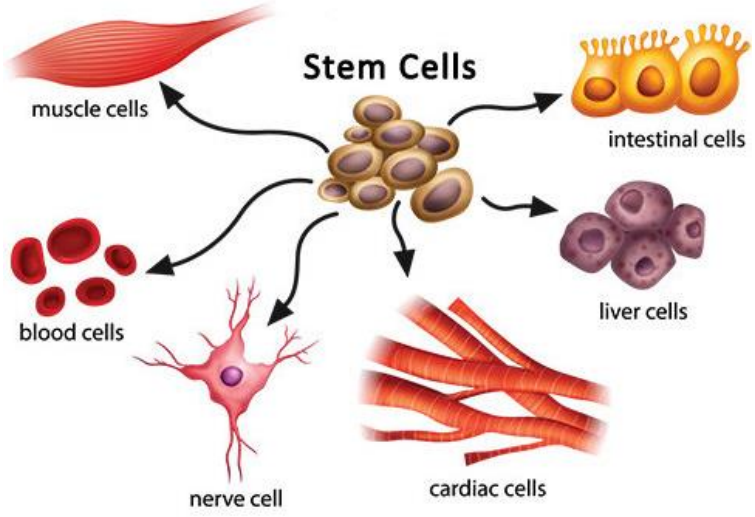

Figure 2.1. Specialized types of cells from stem cell differentiation (30).

2.1.2. Definition and Classification

In the early 1960s, the existence of the stem cell was proven by Ernest McCulloch and James Till (31). They reported the properties of stem cells that are still held today. Stem cell is defined as an unspecialized cell that has the self-renewable capacity and ability to differentiate into specialized cells (32).

Stem cells are categorized based on their origin and differentiation potential. Embryonic, fetal, and adult stem cells are the three main types of stem cells based on origin. According to their differentiation capacity, they are also classified as totipotent, pluripotent, multipotent, oligopotent or unipotent (32) (Figure 2.2.).

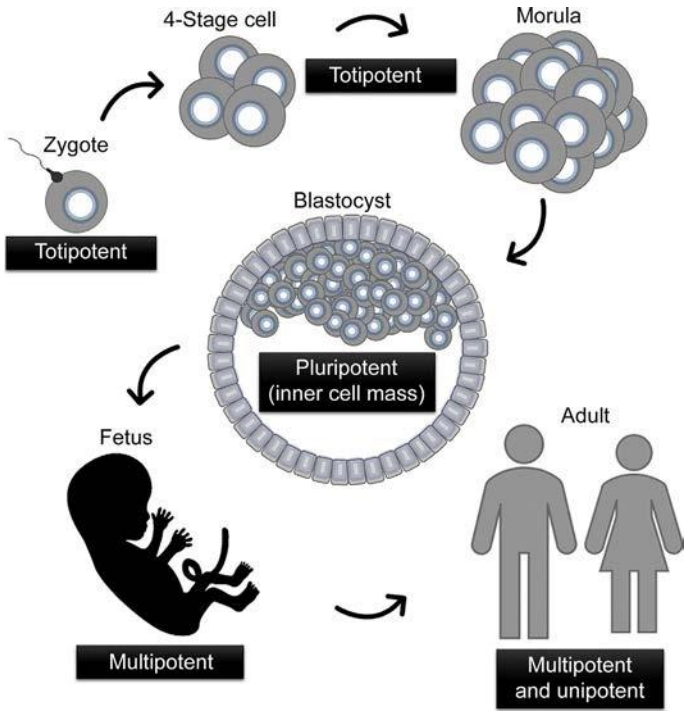

Figure 2.2. Classification of stem cells depends on origin and differentiation potential (33).

Embryonic stem cells (ESCs) can proliferate indefinitely under proper culture conditions. They have a remarkable capacitiy to differentiate to all type of cells and generate all three-germ layers (34). Even ESCs are a seductive source for multilineage differentiation capacity and regeneration of tissues, there are limitations to use of these cells in *in vitro* experiments as immunity resistance, rejection, the formation of tumors, and ethical issues (35). As a consequence of restrictions in using ESCs, scientists shift their focus from ESCs to adult stem cells.

Adult stem cells have multipotent, oligopotent or unipotent potential depending on their differentiation capacity. Multipotent stem cells have ability to generate cells, which are derived from the same germ layer (33). Although oligopotent adult stem cells can differentiate to two or more lineages within the specific tissues, the unipotent stem cell can differentiate only one specific cell type and form a single lineage. It is known that they can be isolated from organs and tissues belonging to all three germ layers, plus placenta including brain, teeth, hearth, liver, bone marrow, testis (36, 37).

Adult stem cells are present in dental tissues such as dental pulp stem cells (DPSCs), dental follicle progenitor cells (DFPCs) and the apical papilla stem cells

(SCAP) (35). DPSCs are gaining more importance in regenerative medicine, because they can be easily optained from teeth, have multi-lineage differentiation capacity and high proliferation potential (38).

2.2. Dental Pulp Stem Cell

Dental pulp tissue represents unique multipotent adult stem cells pool and highly proliferative cells (8). They were isolated by enzymatic digestion and characterized as DPSCs by Gronthos and his collaborators in 2000 (39). The localization of DPSCs in teeth is shown in Figure 2.3. These cells attach to solid surface and have fibroblast-like morphology like mesenchymal stem cells (MSC) (8, 40). DPSCs could be stored, passaged or cryopreserved for prolonged term without lacking their proliferation and differentiation potential (41).

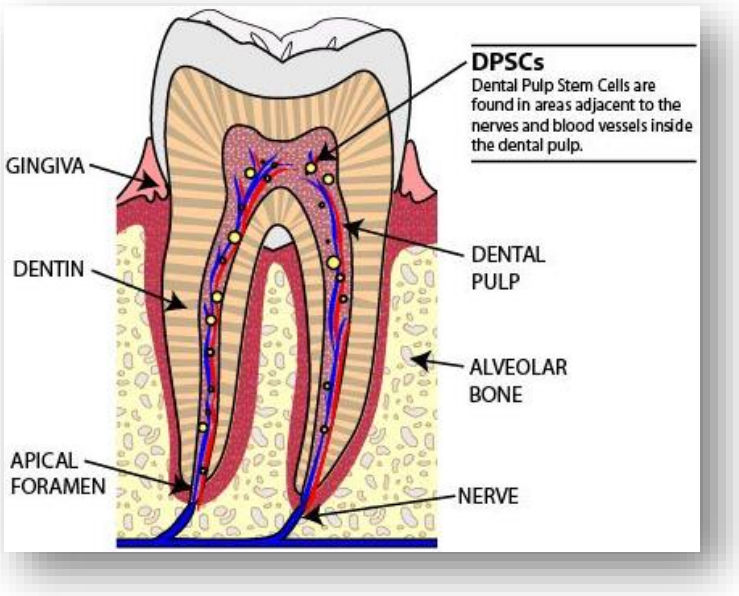

Figure 2.3. Diagram for localization of dental pulp stem cell in adult teeth (42).

2.2.1. Characterization and Differentiation Potential of DPSCs

Although the specific surface marker for DPSC is not yet defined, they express surface markers like MSCs such as Stro-1, CD271 CD166, CD146, CD105, CD90, CD73, CD44, and CD29. Also, they express hematopoietic (CD34 and CD117), stemness (OCT-3/4 and NANOG or SOX2) (41, 43) (Table 2.1.). They have potential to proliferate and differentiate to multiple lineages like osteoblast, chondroblasts, and cell types of adipose or neuronal tissue (8) (Figure 2.4.).

Table 2.1. Dental pulp stem cell properties (41).

	Location	**Surface Markers**		**Differentiation Potential**	
		Positive	**Negative**	**In Vitro**	**In Vivo**
DPSCs	The dental pulp of the permanent tooth	CD9 CD10 CD13 CD29 CD44 CD59 CD73 CD90 CD105 CD106 CD146 CD166 STRO-1 Nanog Oct-4 Sox-2	CD14 CD34 CD45 HLA-DR	Odontogenic Osteogenic Neurogenic Adipogenic Myogenic Chondrogenic	Dentin/pulp-like complex

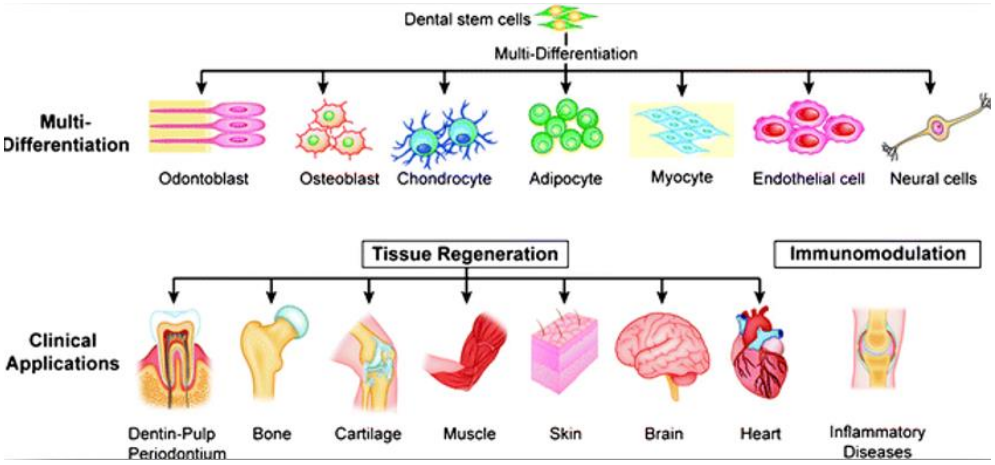

Figure 2.4. Differentiation potential of DPSCs and possible clinical applications (44).

Although DPSCs are important for forming dentin or bone-like complex, neuronal differentiation property also gives the unique therapeutic potential for the use

of these cells in nervous system injuries and neurodegenerative diseases in the future. There is much research about the differentiation of DPSCs to the neuronal side. The spontaneous expression of nestin was shown in undifferentiated DPSCs. They suggested that nestin expression could be related to the origin of DPSCs by the migration from the neural crest during development (45). Nestin is neural stem or progenitor marker and involves in axonal growth during neuronal differentiation. Its expression was downregulated during differentiation of central nervous system stem cells into neurons or glial cells (46, 47). The expression of nestin in undifferentiated DPSCs also supports the potential of neuronal differentiation of these cells. In another study, DPSCs were isolated from wisdom teeth and after culture under xeno-serum free conditions, neurospheres formation and differentiation to neurons were observed (48). Besides, DPSCs expressed neuronal markers of mature neurons and showed functional activity of voltage-gated sodium and potassium channels after neuronal differentiation induction (49). Recently, the therapeutic potential of secretome which is isolated from DPSCs have been reported for Alzheimer disease as it was highly enriched in neurotrophic factors, Aβ-degrading enzyme, and antiapoptotic factors (50).

Dental pulp stem cells have heterogeneous subpopulations, which express various surface markers for multilineage differentiation. As mechanisms for termination of differentiation are not known completely, more studies should be carried out based on a molecular basis.

2.2.2. Regulatory Role of Signaling Pathways on Differentiation of DPSCs

There are so many studies to support the interaction with signaling pathways for the determination fate of DPSCs. In 2017, researchers reported that activation of Wnt/Notch signaling is necessary for maintaining the expression of pluripotency markers such as OCT4a, SOX2, NANOG in DPSCs (51). Inhibition of these pathways led to a decrease in the stemness of DPSC. Also, the differentiation capacity of DPSCs to the adipogenic and osteoblastic side was enhanced by activation of these pathways (51).

Furthermore, osteogenic differentiation of DPSCs was modulated by sirtuin-1 treatment through Wnt/β-catenin signal. Deacetylated β-catenin by sirtuin-1

accumulated in the nucleus and promote the transcription of genes related to osteogenic features (52).

MAPK signaling pathway was activated by the upregulation of p38 MAPK and ERK1/2 with the treatment of epiregulin in DPSCs. Inhibition of epiregulin promoted the suppression of phosphorylation for these pathways and also the expression of genes such as DSSP, RUNX2 related to odontogenic differentiation (53).

Recently, researches have been carried out on the differentiation of DPSC to neurons via a signaling pathway. Feng et al. reported the enhacement of neuronal markers expressions (MAP2, TH, βIII-tubulin) by addition of human recombinant Wnt-1 to the medium. Therefore, they suggested that the Wnt/β-catenin signaling could modulate neuronal differentiation of DPSCs (54).

Besides the Wnt/β-catenin and MAPK signaling pathways, Hippo signaling pathway has an important role in the modulation of proliferation or differentiation of DPSCs (18, 19). As Hippo signalling pathway interacts with several signalling pathways, understanding the differentiation or stemness potential of DPSCs via Hippo signalling would open a new field for using these cells efficiently in stem-cell therapies or regenerative medicine.

2.3. Hippo Signaling Pathway

Evolutionarily conserved Hippo signaling pathway is a kinase cascade. It was firstly defined in *Drosophila melanogaster* in 1995 by genetic studies. Inactivation of genes related with Hippo signaling pathway in *Drosophila melanogaster* resulted in overgrowth of developing eyes and wings (55). Hippo signaling pathway has been shown as conserved within mammalians by using genetically modified mouse models (56).

The main function of this pathway is considered as restricting the tissue growth and modulating the proliferation, differentiation, and migration of cells. Yes-associated protein (YAP) is an important downstream effector in the Hippo signaling pathway involved in the cellular processes by activating the transcription of genes (57). Nuclear localization and binding of YAP to TEAD family transcription factors

promote cell differentiation, proliferation, or stem cell fate (58). For instance, YAP protein regulates the fate decision in the early development of mouse embryogenesis during the divison of blastocytes into the trophectoderm (TE) and the inner cell mass (ICM) as well as the differentiation of MSCs (59, 60).

Mammalian Ste20-like kinase 1/2 (MST1/2) is phosphorylated and activated Large tumor suppressor 1/2(LATS1/2) by phosphorylation after activation of Hippo signaling pathway. Activated LAT1/2 kinases suppress the activity of YAP by phosphorylation (61). Then, YAP undergoes cytoplasmic sequestration or degradation based on the phosphorylation site. The phosphorylation of YAP at S127 by LATS1/2 leads to cytoplasmic sequestration by creating binding sites for 14-3-3 protein. However, YAP phosphorylation at S381 induces the sequestration by ubiquitination (56) (Figure 2.5.).

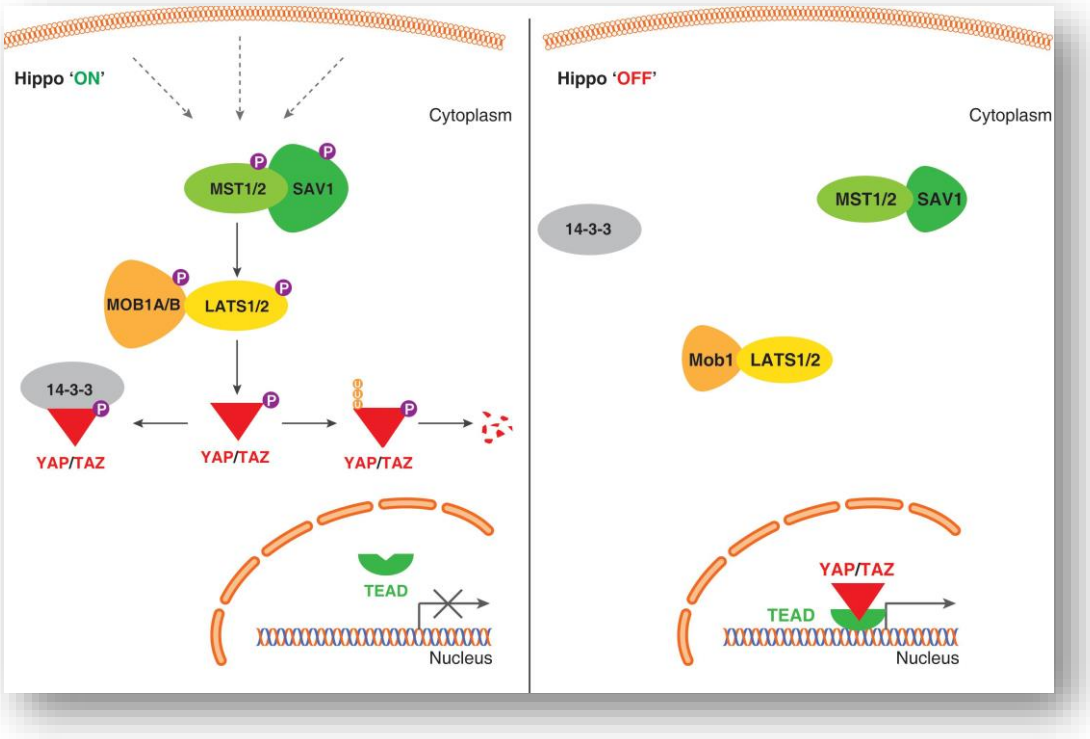

Figure 2.5. The schematic diagram for representing the Hippo Signaling Pathway (62).

Apart from the serine kinase phosphorylation, YAP can be phosphorylated on Tyr residues by Src family kinases. Phosphorylation on Tyr residues provides the regulation of YAP activity in the Hippo signaling- independent way (63). The role of

phosphorylation of YAP on tyrosine residues is not well known. Sugihara et al. reported that inhibitor of SRC family kinases leads to inhibition of tyrosine phosphorylation, redistribution the localization of YAP, and downregulate the expression of genes which are targeted by YAP (Figure2.6.) (64).

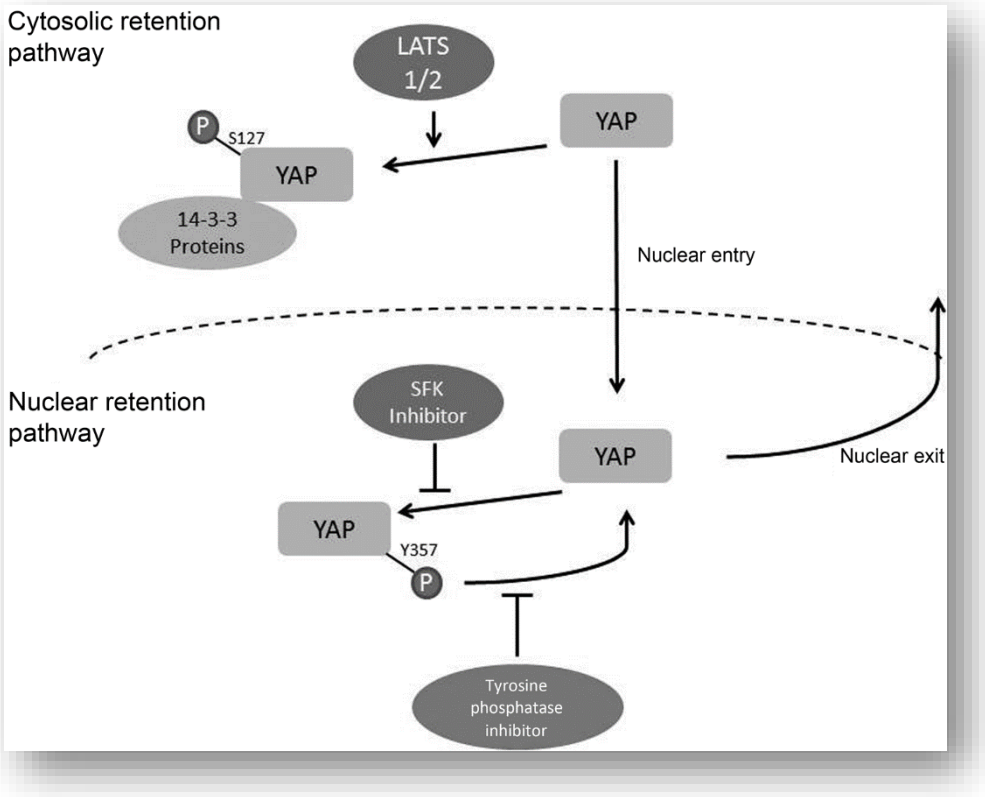

Figure 2.6. The model of nuclear and cytoplasmic retention of YAP (64).

2.3.1. The Role of the Hippo Signaling Pathway in Stem Cell

The regulatory role of the Hippo signaling pathway on stem cells has been recently shown in many articles by investigating YAP which is coactivator of the Hippo pathway (65).

Lian et al. showed the inactivation of YAP throughout the embryonic stem cell differentiation in 2010. OCT4, SOX2 (ESC pluripotent marker), and YAP protein expressions were examined under undifferentiated and differentiated conditions by western blot analysis (66). They found that the level of YAP expression and pluripotency marker were decreased after differentiation, and phosphorylation of YAP

on serine 127 was increased. In the same study, a critical role of YAP in maintaining stem cell pluripotency was reported (57).

YAP has a regulatory role not only in ESCs but also in progenitor cells. In submandibular glands ductal progenitors, ductal maturation was regulated by removing YAP from the nucleus by activation of LATS1/2 (67).

The regulatory effect of YAP in neuronal differentiation among stem cells has been recently investigated. Cao et al. demonstrated that overexpression of YAP with binding to TEAD caused expansion of neural progenitor cells and reduced neuronal differentiation in the chick neural tube. Also, *cyclin D1* expression was induced by suppressing MST1/2, LATS1/2, and activating YAP, and TEAD activity led to neural progenitor cell cycle progression (68). Since the expression of YAP was shown in NSC, not in mature neurons, Zaltsman et al. carried out a study about the function of YAP during neuronal differentiation. They observed that the total and nuclear YAP expression was decreased during neuronal differentiation. They proposed that YAP could be phosphorylated during neuronal differentiation. Then, the phosphorylated YAP can undergo degradation in the cytoplasm by ubiquitin mechanisms (21).

The function of YAP in tooth development has been investigated. A recent study showed that osteogenesis/odontogenesis, mineralization, proliferation, and migration of DPSCs were induced by the static magnetic field (18). They claimed that the expression of YAP was induced after applying the static magnetic field, but the phosphorylation of YAP was inhibited in DPSCs. Besides, the mineralization induced by a static magnetic field was decreased upon silencing YAP. In 2019, it was shown that F-actin activated the exclusion of YAP from the nucleus in DPSCs. YAP was predominantly expressed in the nucleus of DPSCs. By treatment with cytochalasin D which is an inhibitor of F-actin, YAP was excluded from the nucleus. Also, the osteo/odontogenic differentiation of YAP was examined by treatment with verteporfin which blocked the interaction between YAP and TEAD. Du *et al.* found that osteogenic differentiation was significantly decreased in DPSCs with verteporfin treatment (19). These findings support the interaction of YAP with TEAD and cytoskelatal filaments which resulted with osteogenic/ondotogenic differentiaition of DPSCs.

Various hormonal or mechanical stimulants modulate the cell fate decision of stem cells by interacting with Hippo signaling pathway. Recent in vitro studies reported the role of melatonin (MT) in differentiation and proliferation of embryonic and adult NSCs (69, 70) . Although, the role of Hippo signalling and MT on neuronal differentiation of neural progenitor cells is known, it has not been reported in DPSCs. Since DPSCs can express the neuronal lineage markers and differentiate to functionally active neurons, melatonin may have a regulatory role on Hippo signaling pathway during the induction or termination of the neuronal differentiation of DPSCs.

2.4. Melatonin

Melatonin (N-acetyl-5-methoxy tryptamine) (MT) was firstly isolated from the bovine pineal gland by Lerner and his colleges in 1958. It is an indolic neurohormone and not only released from the pineal gland, but also other peripheral organs such as gut, gonads, retina, ovarium, and salivary gland (23, 71). Production of MT is regulated by light/dark signals. While the amount of MT concentration is peaked during the night, the level declines relatively during the day. Also, the level of MT is higher in new-born and decreases by aging (23). The molecular structure of MT is shown in Figure 2.7.

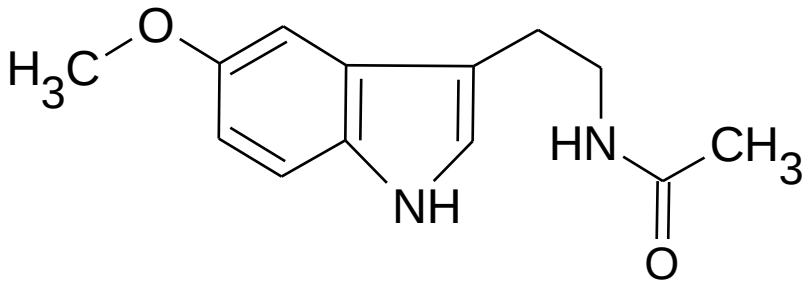

Figure 2.7. The molecular structure of melatonin.

Melatonin hormone plays several roles in the phycological process including regulation of blood pressure, oxidative stress, body temperature, circadian rhythm, memory formation, cell proliferation, and also differentiation. MTNR1A and MTNR1B (MT1, MT2 respectively), which belongs to G-protein coupled receptors (GPRCs) family are main two receptors for MT. They are expressed in the central nervous system, retina, immune cells, and arteries. When melatonin binds to its receptors, the signaling transduction pathways lead to different cellular responses or

potential crosstalk with other signaling pathways depending on cell or tissue type (23, 71).

2.4.1. The Role of Melatonin in Stem Cell

The function of MT on stem cell differentiation has been recently reported as inhibition of stemness-related genes expression and pathways (23).

The effect of MT on neuronal differentiation was proposed in 2009. Kong and colleagues isolated NSCs from embryonic day 14 rat pubs (70). They showed that the expression of TH was increased significantly and the level of GFAP was decreased after incubation with differentiation medium containing melatonin. TH is an essential enzyme for dopamine synthesis, so it is used as a marker of dopaminergic differentiation. Also, melatonin treatment resulted in increasing the expression of BDNF and GDNF with neurogenesis (70). Besides, in another study the neuronal fate of P19 cells which were pluripotent stem cells isolated from embryo-derived teratocarcinoma was regulated by MT treatment. They showed that nuclear localization of ERK 1/2 was induced and transcription of SOX2 was upregulated with melatonin treatment. Interestingly, the expression of mesodermal specific genes was suppressed (72).

Zhang et al. reported the role of MT in the differentiation of human MSC. They showed that the addition of MT to adipogenic/osteogenic medium inhibited adipogenic differentiation and induced significantly osteogenic differentiation by stimulating RUNX2 expression and suppressing proliferator-activated receptor gamma (PPARγ) (73). Also, the interaction between MT and Wnt- β catenin signaling pathway was examined in 2017 by Ping *et al.* Mice were exposed to low and high concentrations of MT after administration with titanium (Ti) which causes osteolytic disease. They found that while the osteolytic effect of Ti was decreased, the expression of alkaline phosphates (ALP), osteocalcin, and osteorix was increased. Furthermore, Dickkopf1 gene expression is a strong inhibitor of the Wnt- β catenin signaling pathway. After MT treatment, stronger β catenin staining and less Dickkopf1 gene expression were observed. These findings emphasize that MT has an osteogenic effect by activating Wnt- β catenin signaling pathway (74).

There are also studies that examine the differentiation potential of dental stem cells after melatonin tretament. Dental papilla cells were isolated from the first molar dental papilla of neonatal rats and incubated with osteogenic or basal media with the addition of increasing concentration of MT. After differentiation, they reported that dentin sialophosphoprotein (DSPP), ALP, DMP1 level were significantly peaked by 10 nM MT treatment. These findings proposed the effect of MT in dental papilla cells during odontogenic differentiation (25). Furthermore, the expression of osteogenic specific markers such as RUNX2, osteocalcin were increased by applying different osteogenic media supplemented with MT in DPSC (27). Also, DPSCs, which were isolated from premolar human pulp tissues were incubated in hepatogenic medium with or without MT for 21 days. They reported that MT induced hepatic differentiation by regulating the bone morphogenic protein (BMP), and extracellular signal-regulated kinase (ERK)(26).

Recently, researchers mentioned the possible interaction between the MT and Hippo signaling pathway in different cell lines (23).

2.4.2. The Interaction between Melatonin and Hippo Signaling Pathway

Hippo signaling pathway is regulated by several factors such as cell to cell contacts, the polarity of cells, actin-cytoskeleton, hormonal or mechanical signals by acting through GPRCs (23) . GPCRs signaling regulate activation or inhibition of YAP, based on which GPCRs or the subsequent Gα protein stimulated. For instance, LPA, S1P, and thrombin induced the Gαi, Gαq and Gα12/13 subunites and activated YAP by suppressing LATS1/2 and inducing dephosphorylation of YAP. However, glucagon, epinephrine, and dobutamine inhibit the activity of YAP by stimulating Gαs subunit. The activation of Gαs induce intracellular cAMP level and activate protein kinase A (PKA) and block RhoA/ROCK signaling. This causes the phosphorylation of LATS1/2 and YAP, and sequesteration of YAP in the cytoplasm (23).

MT activates Gαs by binding MT1 receptors in different cell lines and induces cAMP level with activation of PKA. Thus, MT may regulate YAP expression by inducing LATS1/2 with activation of Gαs and plays a role in the cellular processes (23) (Figure 2.8.).

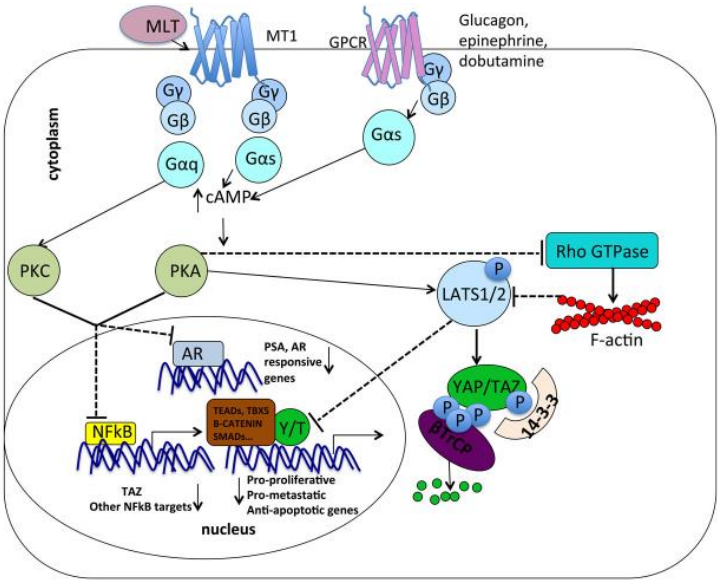

Figure 2.8. The model of MT and GPCR signaling on the regulation of YAP/TAZ (23).

2.5. Aim of the Thesis

DPSCs are remarkable cell sources in regenerative medicine due to their easily isolation protocol from teeth, less ethical consideration, highly proliferative and clonogenic ability and also multilineage differentiation capacity (3). Recently, DPSCs have been considered as alternative cell sources for neuronal repair and regeneration in neuronal disease due to neurogenic differentiation properties of these cells.

The role of Hippo signalling and MT on neuronal differentiation of neural progenitor cells is known, however it has not been reported in DPSCs and the mechanisms underlying the differentiation of DPSCs into neuronal lineage requires more clarifcations for using these cells efficiently in stem-cell therapies or regenerative medicine.

In regard with this, the aim of this study is to evaluate the effect of melatonin on Hippo signaling pathway through the expression of its downstream effector (YAP/ p-YAP^{Y357}) after the neuronal differentiation of DPSC with dopaminergic neuronal differentiation media. To determine the changes in the stemness, proliferation and apoptosis of DPSCs with neurogenic differentiation, we investigated the expression of SOX2, Proliferating cell nuclear antigen (PCNA) and caspase-3.

3. MATERIALS AND METHODS

3.1. Experimental Design

The effect of melatonin on Hippo signaling pathway in dopaminergic neuronal differentiation process of DPSCs were examined. Our research has ethical approval from the ethical committee of Hacettepe University Medical Faculty Non-interventional Clinical Investigations (2020/05/05-16969557-705). Firstly, the cell viability of DPSC with different concentrations of melatonin (1 nM-100 µM) was detected by WST-1 assay. Secondly DPSC were incubated with growth medium, and also dopaminergic neuronal differentiation medium to assess the neurogenic differentiation of DPSCs. Furthermore, DPSCs were incubated with 10µM melatonin containing growth medium, neuronal differentiation medium to evaluate the effect of melatonin. To demonstrate the differentiation of DPSC and alteration of the expression of YAP/pYAP, the immunocytochemistry and western blot analysis were carried out after 21 days incubation with/without melatonin containing media. Primary neuronal culture cells were also used to verify the YAP expression on mature neurons (P0). For the primary neuronal culture, procedure applied for the care and use of laboratory animals were based on the National Institutes of Health Guidelines in Norway (FOTS 20135149/20113133).

This thesis study was carried out within the scope of the Stem Cell Sciences Master Program, using the infrastructure, laboratory and technical equipment of Hacettepe University Faculty of Medicine Histology and Embryology Department, Medical Biochemistry Department and Bergen University Biomedicine Department. Work flow chart of this research is shown in Figure 3.1.

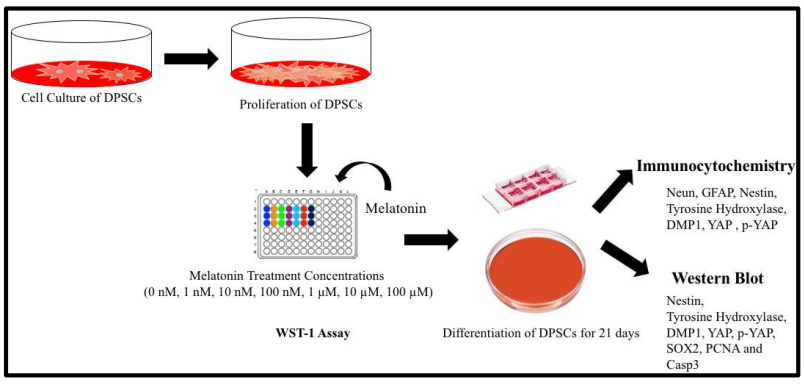

Figure 3.1. Work flow chart of the study.

3. 2. Cell Culture

DPSC and primary neuronal culture cells were used in this study. DPSC (Lonza, PT-5025, Basel, Switzerland) were obtained commercially before and primary neuronal culture cells (P0) were a gift from Dr Manja Schubert. DPSC were thawed, passaged and the cells belonging to passage 4 (when they reached adequate number) were used for experiments. Primary neuronal culture cells were used to verify the YAP expression on mature neurons.

3.2.1. Cell Culture for DPSCs

Thawing of DPSC

T-25 flask with DPSC growth medium (DPSC Bullet KitTM; Lonza, PT-3005) was cultured in a 37 °C, %5 CO2 incubator for 30 minutes. For thawing procedure, the cryovial containing the frozen dental pulp stem cells was taken from the liquid nitrogen tank and put into 37 °C water bath for 1-2 minute. Then cryovial was taken from water bath, outer surface was cleaned with 70% alcohol and taken in laminar cabin. The cell suspension was transferred into the tubes containing the pre-warmed growth medium (DPSC Bullet KitTM) and centrifuged at 1500 rpm (Eppendorf™ 5810R) for 5 min. Thereafter, the supernatant was discarded and pellet was resuspended with growth media. Cell suspension was seeded into T-25 flask containing pre-warmed growth medium. Then, DPSC were cultured at ambient conditions with %100 humidity, %5 CO2 at 37 °C overnight. The attachment of cells was controlled under phase contrast microscope and the medium was changed. For the following days, medium was changed every 2 days. When cell density reached %90 confluency, they were passaged and while the cells were passaged, some cells were frozen in freezing medium containing growth medium and 10% DMSO in the nitrogen tank (-196 °C) for later use, and the remaining cells were used for experiments.

Passaging of DPSC

Trypsin/EDTA (Sigma, T4049), Dulbecco's Phosphate-Buffered Saline (DPBS) (Sigma, D8537) and growth medium were taken from + 4°C refrigerator to water bath for reaching 37 °C . Then, they were cleaned with 70% alcohol and placed in the laminar cabin. The media in culture flasks were aspirated and cells were washed

with DPBS. After aspirating DPBS, 0.25% Trypsin/EDTA was added into flasks for detachment of the adherent cells from the surface. Flasks were incubated for 2-6 min in the incubator. Then, media containing FBS was added into flasks for neutralization the trypsin. After that, cell suspension was taken into tube and centrifuged at 800 rpm (Eppendorf™ 5810R), for 5 min. After centrifugation, the supernatant was removed, and pellet was mixed homogeneously in 1 ml of growth media. Then, number of DPSCs were determined by using trypan blue. According to result of the counting, 5,000-6,000 cells/ cm^2 were seeded into new flasks and kept in incubator for growing.

Cell Counting of DPSCs

To follow the optimization of protocols for cell culture and differentiation, cells were counted for each step. For counting the cells, 100 µl trypan blue solution (0.4%) and the same amount of the cell suspension were mixed by pipetting. Cell number was counted on the Neubauer cell count slide by adding 10 µl of cell suspension on both sides of the slide between the slide and the coverslip. Counts in each area were repeated with a new sample, so five separate counts were made totally. Then, the average number was calculated and the following formula is used for the number of cells in 1 ml (Figure 3.2).

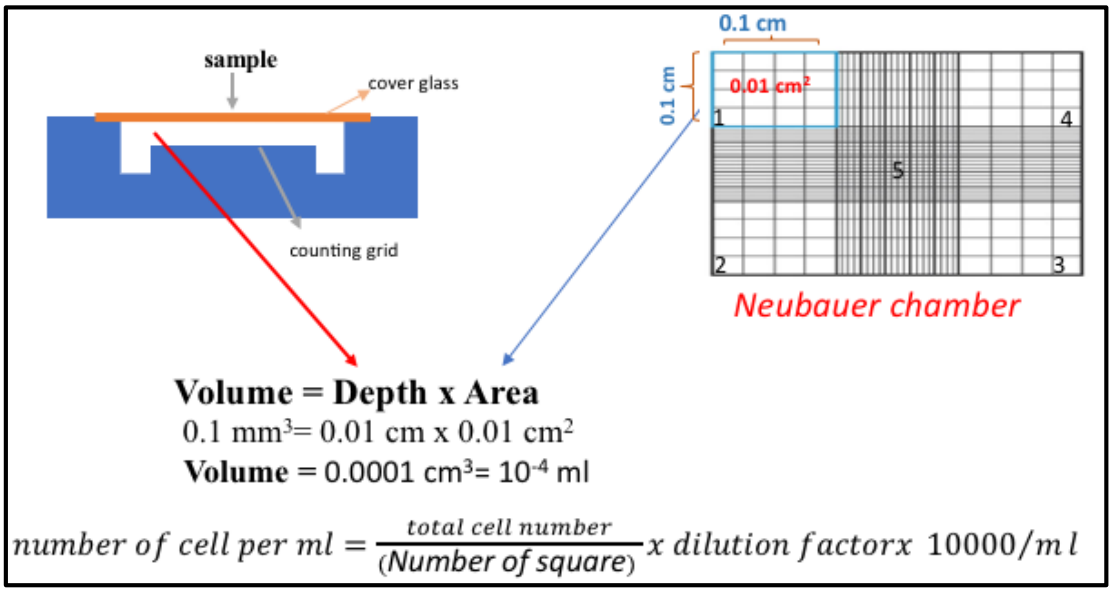

Figure 3.2. The formula used for the calculation of cell number per ml.

3.3. Assessment of Melatonin Concentration

3.3.1. Preparation of the Different Concentration Melatonin Solution

In this study, melatonin solution with different concentrations (1 nM-100 μM) was used in order to determine optimal dossage. Stock solution (0, 002 molar) was prepared by dissolving 25 mg melatonin (Sigma, 73-31-4) in 50 ml absolute ethanol. Intermediate stock solution (100 μM) was obtained by taking 500 μl from stock solution and adding into 9500 μl medium. Serial melatonin solutions were prepared by diluting intermediate stock solution with growth medium.

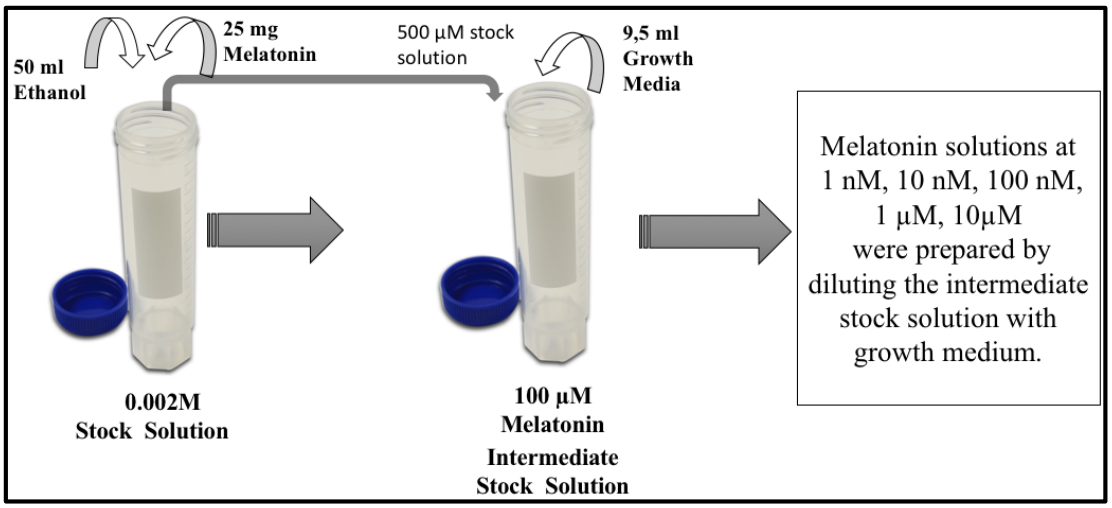

Figure 3.3. Preparation of stock solution and seires concentrations (1 nM-10 μM) of melatonin for cell cytotoxicity assay.

3.3.2. Cell Viability

Cell viability after melatonin administration was determined by WST-1 kit (Roche, Cat no 11 644 807 001). The principle of WST-1 assay is the conversion of tetrazolium salts into the colored dye by mitochondrial dehydrogenase enzymes. Basically, the assay measures the colored change which is related to the metabolic activity of cells. DPSCs were seeded into four 96-well plates at a density of 5 x 10^3 cells/well. After waiting 18 hours for the attachment of cells, growth medium was changed and melatonin with different concentrations (1 nM-100 μM) were applied (Figure 3.4). All plates were incubated overnight with growth medium containing melatonin. On the first day of melatonin treatment, 10μl of Wst-1 solution was added to plate one per well and incubated for 4 hours. After incubation, the optical density (OD) was measured at 450-600 nm by using micro plate reader (Versamax microplate

reader, Molecular Devices). OD was measured also for second plate on day 2, third plate on day 3 and fourth plate on day 4 by applying the same procedure as in plate one. Three replicates were performed in each test and each test was repeated three times. Reported values are the mean of three measurements. The experimental outline is shown in Figure 3.4. The cell viability was calculated by using the equation given in Figure 3.5.

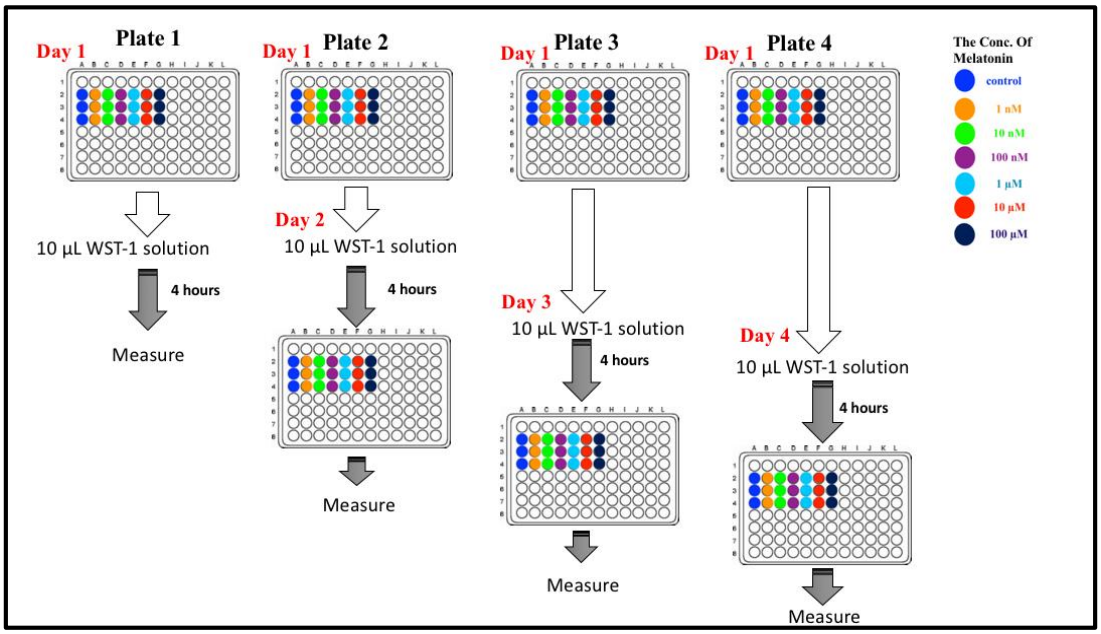

Figure 3.4. Experimental outline of WST-1 Assay. DPSC were seeded into four 96 well plates at a density of 5000 cells per well. After 18 hours, plates were exposed to growth medium with different concentration of melatonin (100 µM-1 nM) freshly added daily for 4 days. At day 1, 2, 3, and 4 in order to determine cell viability 10µl of Wst-1 solution was added to each well and after 4-hour incubation, OD was measured at 450-600 nm by using micro plate reader.

$$Cell\ \ Viability\ \ (\%) = (A_{experiment} - A_{blank}) / (A_{control} - A_{blank}) \times 100$$

A = absorbance

Figure 3.5. The formula used for the calculation of cell viability.

3.4. Procedure for DPSC Differentiation

For the neuronal differentiation of DPSCs coated plates are used (5, 75). According to the manufacture's instructions all the plates and slides used in the experiments were covered with cell matrix basement membrane gel (ATCC, ACS-3035).

3.4.1. Cell Matrix Basement Membrane Gel Coating

Before seeding the DPSCs onto chambers or dishes, Cell Matrix Gel Membrane that contains heparin sulfate, proteoglycan, laminin, entactin and collagen IV was used to promote attachment of cells. Briefly, the cell matrix basement membrane gel was thawed on ice in 4 °C refrigerator overnight. Then, it is divided into tubes in 250 µl on ice, so as not to be spoiled by repeated freezing thawing during experimental processes. The prepared tubes were quickly stored in -80 °C freezer for future usage. Before coating, 50 µl Cell Matrix gel was diluted with 4.950 µl cold DMEM/F12. Then, dishes and 8 well-chambered slides were coated with diluted gel (1000 µl, and 250 µl respectively). For the attachment of gel matrix to the surface, they were incubated in 37 °C incubator for 1 hour. After incubation, excess gel was aspirated and cells were seeded as 4000 cells/well, 80.000 on to the dish and incubated overnight for the attachment of cells.

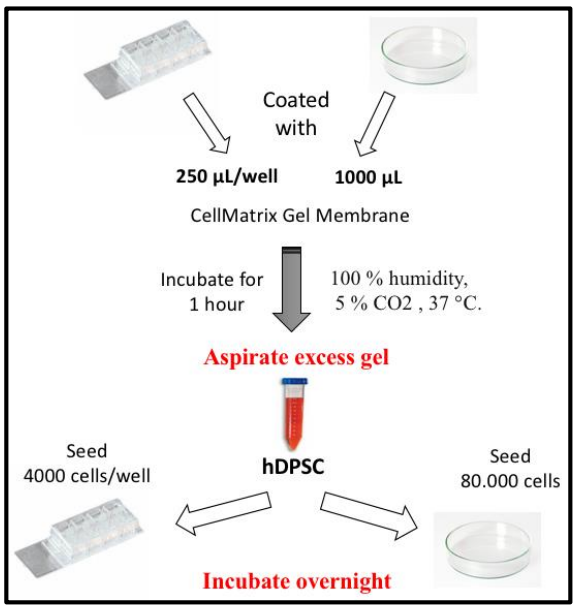

Figure 3.6. Preparation of wells and dishes for the attachment of DPSCs.

3.4.2. Growth and Differentiation of DPSC

DPSCs were incubated either in growth medium (DPSC Bullet Kit, Lonza, PT-3005) or dopaminergic neuronal differentiation medium (Neural progenitor cell Dopaminergic Differentiation Kit, ATCC, ACS-3004) with and without melatonin administration for 21 days to evaluate the effect of melatonin and YAP protein expression in differentiation (Table 3.1). Growth medium was used to maintain

undifferentiated state of DPSCs. Dopaminergic Neuronal Differentiation Kit, which was enriched with DMEM/F12 media and necessary supplements, was used in order to induce neuronal differentiation of DPSCs.

Culture Media used for Growth and Differentiation of DPSCs

The media were prepared based on the instructions of manufacturer. The components of culture media in the study were represented in Table 3.1.

Table 3.1. Culture media used for growth and differentiation of DPSCs.

Medium		**Cell Type**	**Company**	**Catalog.No.**	
DPSC Bullet KitTM **(Growth Medium)**	DPSC Basal Medium DPSC	DENTAL PULP STEM CELL (DPCS)	LONZA	PT-3005	PT-3927
	SingleQuotsTM Kit				PT-4516
Dopaminergic Neuron Differentiation Kit **(Neuronal Differentiation medium)**	L-Alanyl-L-Glutamine	DPSC (Differentiated group)	ATCC	ACS-3004	PCS-999-034
	Non-Essential Amino Acids Mix				PCS-999-006
	Ascorbic Acid				PCS-999-052
	Component A				PCS-999-006
	Component B				PCS-999-051
	Component C				PCS-999-057
	DMEM/F12				PCS-999-057
Melatonin		DPSC	Sigma		73-31-4

Growth Medium : Commercially DPSC Bullet Kit^{TM} (Lonza, PT-3005) was used as growth medium for DPSCs. SingleQuots™ Kit (Lonza, PT-4516) includes 50 ml Dental Pulp Stem Cell Growth Supplement (DPSCGS), 10 ml L-glutamine, 5 ml Ascorbic Acid, and 0.5 ml Gentamicin/Amphotericin-B (GA). This mixture was added to 500 ml DPSC basal medium (Lonza, PT-3927) and after mixing properly the growth medium was stored at 4 °C.

Dopaminergic Neuronal Differentiation Medium: Commercially purchased Dopaminergic Neuron Differentiation Kit was used as differentiation medium for DPSCs. During preparation of the differentiation medium, the kit taken from the freezer was kept at + 4 °C overnight. DMEM / F12 (237 ml) was taken in the laminar cabin, then 2.5 ml of L-Alanyl-L-Glutamine, 2.5 ml of Non-Essential amino acid, 0.5 ml of Ascorbic acid, 5 ml of Dopaminergic neuron differentiation kit component A, 2.5 ml Dopaminergic neuron differentiation kit component B and 0.5 ml Dopaminergic

neuron differentiation kit component C were added and mixed properly. It was wrapped in aluminum foil and stored at + 4°C.

DPSCs that were seeded over coated plates, were incubated with growth, neuronal differentiation medium with and without 10µM melatonin. The media of each group were changed and each group was photographed by a camera (Leica DFC7000 T) attached phase contrast microscope (Leica DMI6000B) every day for 21 days. At the end of the 21st day, immunofluorescence labelling was conducted on 8 chamber slides and samples in dishes were collected for western blot experiments to analyze the differentiation and related protein expressions of DPSCs. The outline of differentiation procedure was shown in Figure 3.7.

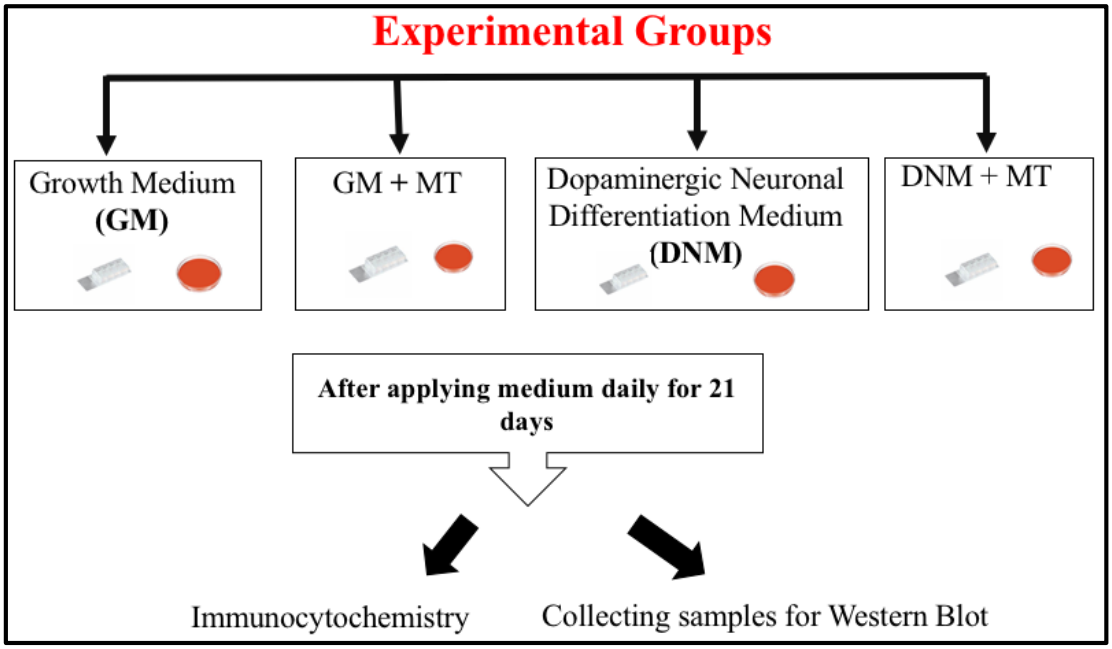

Figure 3.7. Outline of differentiation processes for experimental groups.

3.5. Immunocytochemistry (ICC)

3.5.1. Immunofluorescence Labeling of DPSC

For the immunofluorescence labeling of DPSCs, firstly, media in the wells were aspirated. After washing with DPBS, methanol (Sigma, STBH0775) was applied for 5 min to remove lipids from membrane and precipitate proteins. After fixation, cells were rinsed with 1x Phosphate Buffered Saline (PBS). The reagents used for preparing 1x PBS were given in Table 3.2. Next, 0,1 % Tween20 (Sigma, P1379) in

PBS was applied for 5 min to permeabilize the cells. After permeabilization, cells were blocked with 10 % serum +%1 Bovine Serum Albumin (BSA) (Sigma, A2153) in PBS for 1 hour. The species of serum depend on the host of the secondary antibody in order to avoid non-specific binding. After blocking, cells were incubated with primary antibodies (Table 3.3) in PBS containing 2.5% BSA at 4 °C, overnight. After washing wells 3 times with PBS, cells were incubated with secondary antibodies (Table 3.4) with 1:1000 dilution for 1 hour at room temperature. After incubating with secondary antibody, cells were washed with PBS three times for 5 minutes and mounted with fluorescence mounting medium containing DAPI (ThermoFisher, P36962). Images were taken with a camera (Leica DFC7000T) attached to a fluorescence microscope (Leica DM6B) using LASX program.

3.5.2. Immunofluorescence Labeling of Primary Neuronal Cells

For the immunofluorescence staining of primary neuronal culture cells, the cells were seeded on poly-L-lysine covered coverslips. For fixation, 4% paraformaldehyde (ThermoFisher, UK2883991) in PBS was applied for 20 min. Next, 0.1% Triton-X-100 in PBS (pH: 7.4) was used for 5 min to permeabilize the cells. After washing, 3 % BSA in PBS was applied for 1 hour to block non-specific binding. After incubation with blocking buffer and washing, YAP and Microtubule Associated Protein 2 (MAP2) antibodies in PBS containing 3 % BSA were applied overnight, at 4 °C for double staining to detect the YAP protein expression on mature neurons. After washing with PBS three times for 5 min, Prolong^{Tm} Diamond Antifade Mountant with DAPI was used for mounting. Images were taken with a fluorescence microscope (Zeiss AXIO Z1) using ZEN 2.3 program.

Table 3.2. Reagents used for preparing 10x PBS.

Reagent	Company	Amount to add (10x stock)	Final Concentration(10X)	Catalog no
NaCl	Sigma	80 g	1.37 M	7647-14-5
KCl	Sigma	2 g	27 mM	P-5405
Na_2HPO_4	Sigma	14.4 g	100 mM	30435
KH_2PO_4	Honeywell Fluka	2.4 g	18 mM	7778-77-0

Reagents, which were listed above were dissolved in 0.8 L of dH_20 in order to prepare 10X PBS solution. After mixing completely, 0.2 L dH_20 water was added to top up final solution to 1L. Then pH was adjusted to 7.00. In immunocytochemistry experiments 1X PBS is used as washing buffer. In order to make 1X PBS, 10X stock solution was diluted by adding 100 ml of the 10X solution into 900 ml H_20 and mixing properly.

Table 3.3. Primary antibodies used for immunofluorescence labelling.

Antibody name	Company	Cell Type	Catalog.No	Clonality	Clonal Number	Species	Dilution
Nestin	Abcam	DPCS	Ab18102	Monoclonal	2C1.3A11	Mouse	ICC 1:200
Anti-YAP	Abcam	DPCS	Ab52771	Monoclonal	EP1674Y	Rabbit	ICC 1:500.
	Cell Signalling	Primary Neuronal Cell	D8H1X	Monoclonal		Rabbit	ICC 1:100
Anti-YAP phospho	Abcam	DPCS	Ab62751	Polyclonal	Phospho Y357	Rabbit	ICC 1:1000
Anti-Neun	St Jones Laboratory	DPCS	STJ25311	Polyclonal		Rabbit	ICC 1:300.
Anti-GFAP	Abcam	DPCS	ab7260	Polyclonal		Rabbit	ICC 1:100
Anti-DMP1	Santa Cruz	DPCS	Sc-73633	Monoclonal		Mouse	ICC 1:100
Anti-Tyrosine Hyroxylase	Abcam	DPCS	Ab-6211	Polyclonal		Rabbit	ICC 1:1000
MAP2	EnCor Bio technology	Primer Neuronal Cell	CPCA-MAP2	Polyclonal		Chicken	ICC 1:3000

Table 3.4. Secondary antibodies used for immunofluorescence labelling.

Antibody Name	Company	Catalog.No	Clonality	Assay	Dilution
Anti-mouse IgG (whole molecule)-FITC antibody	Sigma	F9006	Polyclonal	ICC	1:1000
Anti-Rabbit IgG (whole molecule)–FITC antibody	Sigma	F6005	Polyclonal	ICC	1:1000
Donkey anti-Rabbit IgG (H+L) Secondary Antibody	Thermo Fisher	A10042	Polyclonal	ICC	1:1000
Donkey anti-Chicken IgG (H+L) CF488A Secondary Antibody	Biotum	20166	Polyclonal	ICC	1:500

3.6. Western Blot Analysis

SDS PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis *(SDS-PAGE)* is used to separate the proteins in terms of the molecular weight. Polyacrylamide gel is 3D matrix, which is formed by mixing acrylamine, bisacrylamide, dH2O, SDS, TEMED, APS and Tris-Cl. Acrylamide and bisacrylamide monomers can co-polymerase and provide matrix gel structure. The ratio of acrylamide and bisacrylamide lead to different pore size with different separating properties. TEMED acts as catalyst, increases the reaction rate by inducing free radicals of sulfate from ammonium persulfate (APS) and promote the polymerization of gel. Sodium dodecyl sulfate (SDS) is anionic detergent, which damage the tertiary structure of proteins and make them linear. Additionally, SDS covers up the protein charge by providing negative charge, so all proteins stay negative through the gel. All proteins migrate toward the positive side during the application of voltage and separated by molecular size, not on charge or shape.

Stacking gel (4%) and resolving gel (6 % for nestin, TH and 8% for YAP/pYAP, SOX2, PCNA, caspase3) were prepared with different concentration of

compounds and pH described in Table 3.5. The ratio of the acrylamide and bisacrylamide determines the pore size of the gel. Tris-Cl solution adjusted pH around 6.8 for stacking gel and 8,8 for separating gel. Stacking gel is used to stack mixture of proteins to seen as one band, so migration of proteins can be started at the same time in separating gel. After preparing resolving gel, approximately 3.4 ml gel was added to spacer and overlaid with water to remove the bubbles. Then, water is removed and stacking gel was added to top of well after polymerization of resolving gel for 30 min. Next, comb was placed in the gel and waited for 20 min to polymerization of stacking gel.

Table 3.5. Compunds used for the preparation of resolving and stacking gels.

Compounds	**Resolving gel (6 %)**	**Resolving gel (8 %)**	**Stacking gel (4 %)**
30% acrylamide/bis (BioRad)	3.0 ml	4.0 ml	1,3 ml
0.1M Tris-HCl pH 6.8	-	-	1.25 ml
1.5M Tris-HCl pH 8.8	3.8 ml	3,8 ml	-
10% SDS (Sigma)	150 µl	150 µl	100 µl
ddH_2O	7.9	6,9 ml	7.3 ml
10% Ammonium persulfate (APS, Sigma)	150 µl	150 µl	50 µl
Tetramethylethylendiamine (TEMED, Sigma)	12 µl	9 µl	10 µl

Western Blot

In order to collect the cells, DPSC were incubated with accutase for 5 min, then centrifuged at 500 g for 10 minutes to remove the accutase. Then, the supernatant was discarded and ice-cold lysis buffer (50 mM Tris, pH 6.8, 150 mM NaCl, 1 mM EDTA, 1% Triton x-100, 0.5% NP-40, 10% glycerol) containing protease inhibitor cocktail (Roche), phosphatase inhibitors (10 mM beta-glycerophospate, 10 mM NaF, 0.1 mM Na_3VO_4), and 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to the pellet of each sample. The samples were vortexed, and lysed on ice for 30 minutes. Cells which were lysed were centrifuged at 16,000xg for 10 minutes to discard cellular

debris. Supernatant was taken into tube and the protein assay kit (BioRad) was used to evaluate the protein concentration by using Bradford method (76) (Figure 3.8).

BL	BL	1	1
BL	BL	2	2
St1	St1	3	3
St2	St2	4	4
St3	St3		
St4	St4		
St5	St5		
St6	St6		

Figure 3.8. Determination of protein concentration. For the determination of protein concentration 96 well plate was loaded as shown in the template and after addition of the reactive (Bio-Rad Cat No: 5000 006), the absorbance of the wells were measured by a microplate reader (Molecular Devices Spectramax M2, USA) and the protein concentration of the samples were calculated according to the standards. BL: Blank, Standard 1 (St1)-Standard 6 (St6): 12,5-75 µg/ml BSA. Lysates of DPSC incubated for 21 days in **1;** Growth medium (**GM**), **2;** growth medium+Melatonin (10µM) (**GM+MT**), **3;** dopaminergic neuronal differentiation medium (**DNM**), **4;** dopaminergic neuronal differentiation medium+Melatonin (10µM) (**DNM +MT**).

After protein determination, Laemmli buffer was added to 30 µg protein-containing samples and they were loaded into each wells. Firstly, gel was run at 100V for 2 hour. PVDF membrane was washed with methanol for activation and rinsed with transfer buffer (Tris Glycine Solution). After running the proteins in the gels, proteins were transferred to PVDF membranes at 35 V overnight at 4°C. After transferring, the membranes were blocked with 5 % nonfat dry milk (for YAP (Abcam, Ab52771) , nestin (Ab18102), thyrosine hydroxylase (Ab-6211), SOX2(Abcam, ab184787) , PCNA (Invitrogen, 18-0110) and Caspase3(Cell Signalling, 4900s)) and with 5% BSA (for pYAP and SOX2) in TBS-T overnight at 4°C. Membranes were washed three times with TBS-T for 5 min. Next, membranes were incubated with primary antibodies (with a dilution of 1:5000 for YAP, 1:2000 for pYAP, 1:1000 for nestin, TH, SOX2, caspase-3 and for 1:100 for PCNA) overnight at 4 °C on shaker. The membranes were washed three times with TBS-T at room temperature and incubated with horseradish peroxidase conjugated secondary antibodies against mouse and rabbit (GE Healthcare NA934V and NA931V) at a concentrations of 1:10 000 (YAP, p-

YAP^{Y357}, nestin, TH) and 1:5000 (caspase-3, SOX2 and PCNA) for 1 hour at room temperature. After washing step, proteins were detected with western enhanced chemiluminescence (ECL) (Thermo scientific, 34095) and the images were taken by an imaging system (Bio-Rad, ChemiDoc Touch, USA) and analyzed by a software (Bio-RAD's ChemiDoc analysis software) to calculate the intensity of the bands. To evaluate multiple proteins, membranes were stripped and blotted again. β-actin (Sigma, A4700) was used as a loading control to normalize the levels of protein detected.

3.7. Statistical Analysis

Two-way ANOVA test was done to make comparations the viability of DPSCs with different melatonin concentrations. Two sample t-test were performed for comparations of Western Blot quantification between experimental groups. Data were given as Mean+SEM. The criterion for confidence interval for tests was 95 %. Statistically significant was considered as p value ≤ 0.05.

4. RESULTS

4.1. Cell Viability of DPSCs after Melatonin Treatment Was Assessed by Wst-1 Assay

To assess the non-toxic concentration of melatonin, we used a series of melatonin concentrations ranging from 1 nM to 100 μM on the DPSCs from day 1 to day 4. Melatonin with 1 to 10 nM concentrations did not show any difference in cell viability compared to control for all four days (Figure 4.1-A). We observed 19 % decrease in cell viability with 100 nM melatonin concentration on day 2, but 10 % increase on day 4 compared to control. Melatonin with 100 μM concentration was considered as non-tolerable to the DPSCs cells for long term incubation since there was a 27% decrease in the cell viability on day 4 (Table 1). At day 4, cells that were incubated with 100 nM and 10 μM melatonin showed similar increased cell viability compared to the rest (Figure 4.1).

The dose-dependent effect of melatonin on cell viability based on exposing days was analyzed with two-way ANOVA. According to two-way ANOVA analysis, there was no statistically significant effect of different melatonin concentrations on cell viability ($p \geq 0,05$). Even if there was no significant difference between groups, 10 μM concentration of melatonin was considered as an optimum tolerable dose and 10μM concentration of melatonin was chosen for further experiments.

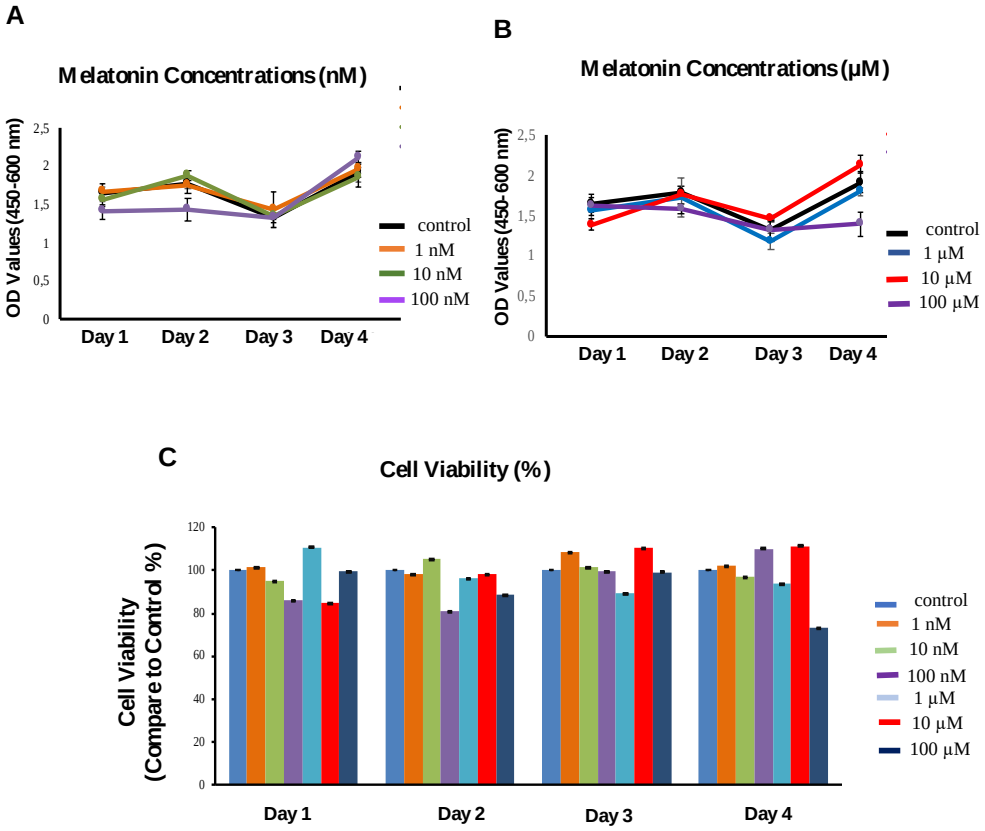

Figure 4.1. Cell viability of DPSCs treated with different concentrations of melatonin containing DPSC's growth medium at day1, 2, 3, and 4. **A, B-** Optical Density (OD) values with melatonin concentrations ranging from 1nM-100 nM, 1µM-100 µM respectively. **C**-The percentage of cell viability was normalized to control. OD of samples was given with ± standard error of the mean (SEM) at 450-600 nm.

Table 4.1. The percentage of the cell viability on DPSCs with serial concentrations of melatonin compared to control.

		Melatonin Concentrations					
		1 nM	10 nM	100 nM	1 µM	10 µM	100 µM
Cell Viability (%)	**1st Day**	102	95	86	111	85	100
	2nd Day	98	105	81	96	98	89
	3rd Day	108	102	100	89	110	99
	4th Day	102	97	110	94	112	73

4.2. Morphological Differentiation of DPSCs with Melatonin (10μM) Treatment during Differentiation Process

DPSCs were incubated with growth medium and dopaminergic neuronal differentiation medium containing with or without 10 μM melatonin for 21 days, and the media was changed on a daily basis. The cells were observed under a phase-contrast microscope and images were taken daily. The representative images of day 7, 14, and 21 were given in Figure 4.2 to show the morphological changes weekly. DPSC proliferated during 21-day culture and different morphological features of DPSC were detected due to addition of different media.

DPSCs that were treated with growth medium only, were broader and flattened with different size on day 7, and they became longer with spindle shape on day 21 (Figure 4.2-A). While DPSCs had fibroblastic morphology on day 7, they showed regular spindle shape on day 21 in growth medium containing melatonin (Figure 4.2-B).

Different size of spindle shaped cells was seen after treatment with dopaminergic neuronal differentiation medium at day 7 and they showed neuronal-like morphology with thin cytoplasmic extensions resembling neurite like features on day 21 (Figure 4.2-C). An addition of 10 μM melatonin to dopaminergic neuronal differentiation media gave rise to presence of cells with elongated morphology on days 7, 14, and 21(Figure 4.2-D).

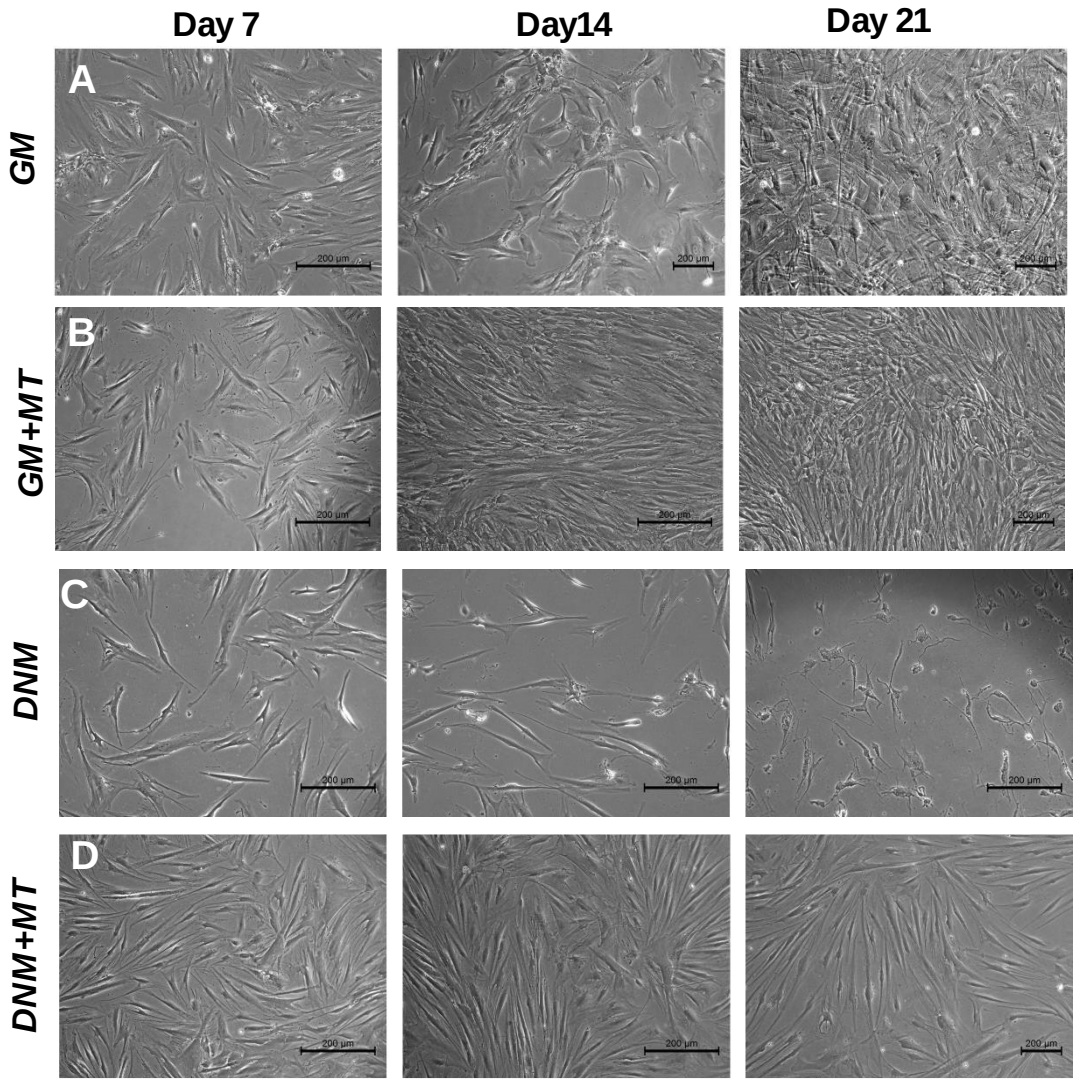

Figure 4.2. Morphological assessment of DPSCs with different culture media supplemented with 10µM melatonin for 21 days. The media were changed daily with fresh media, **A-** Growth medium (GM), **B-** GM+Melatonin (10µM), **C-** Dopaminergic neuronal differentiation medium (DNM), **D-** DNM+Melatonin (10µM), Images were taken by a phase-contrast microscope with 10X objective at 7th, 14th and 21st day to represent morphological changes weekly.

4.3. Differentiation of DPSCs into Neurons by Using Dopaminergic Neuronal Differentiation Medium

The differentiation of DPSCs towards neurogenic lineage was assessed by immunofluorescence labeling with Neun and GFAP. Positive immunostaining for Neun was observed after incubation with dopaminergic neuronal differentiation medium for 21 days (Figure 4.3-A). DPSCs that were treated with dopaminergic differentiation medium did not show positive staining for GFAP (Figure 4.3-B).

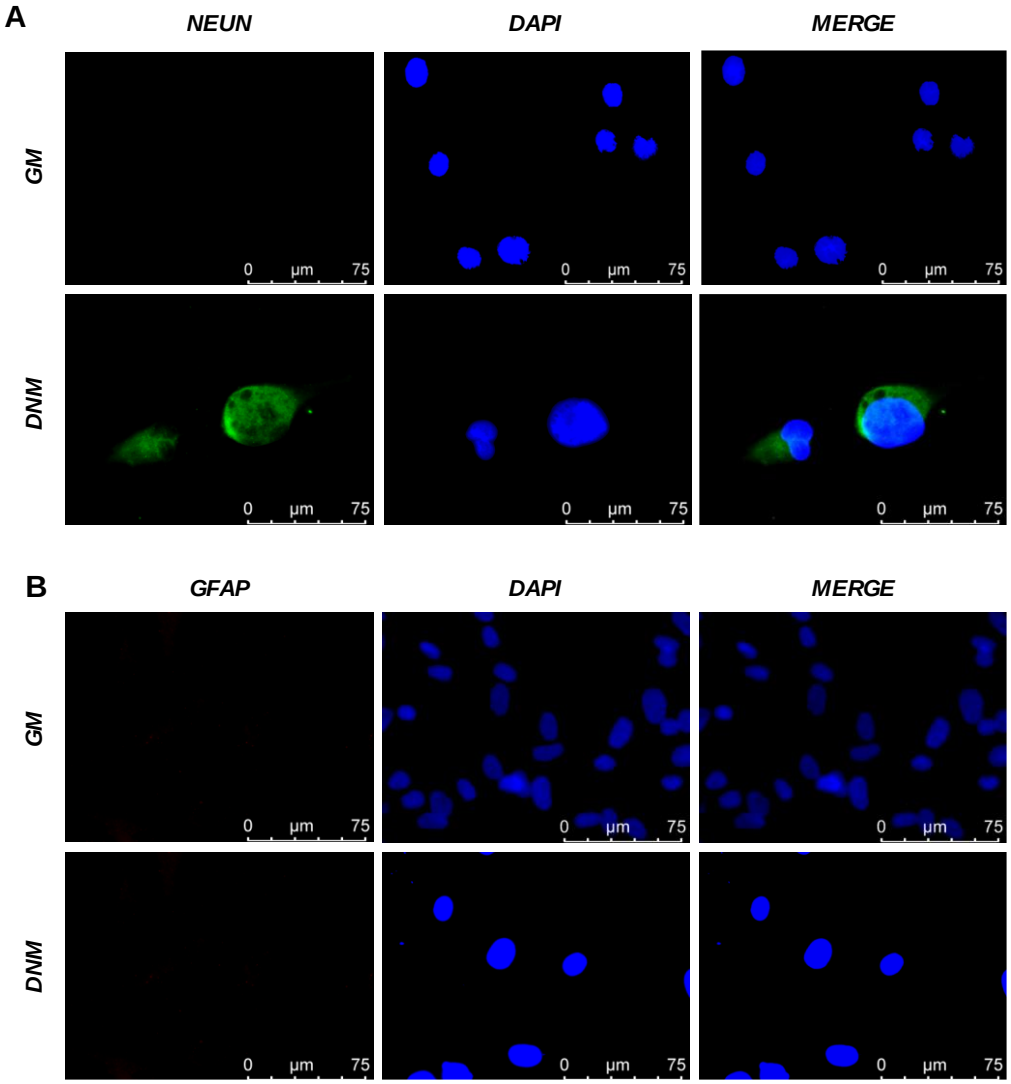

Figure 4.3. Assessment of neuronal differentiation through immunofluorescence labeling with Neun and GFAP. **A-**Immunofluorescence labeling with Neun (green) on DPSCs after incubation with growth medium (GM), dopaminergic neuronal differentiation medium (DNM) **B-** immunofluorescence labeling with GFAP (red) on DPSCs after incubation with GM, DNM. Images were taken by a 63X objective. Scale bar 75 µm.

4.4. The Effect of Melatonin (10μM) on Nestin Expression in DPSCs

Nestin expression was observed in groups, which were treated with growth medium and dopaminergic neuronal differentiation medium with or without melatonin on day 21. In growth media without melatonin, few cells were positive for nestin and the number of nestin positive cells was decreased by the addition of melatonin into the media. High number of nestin positive cells was observed in dopaminergic media and nestin positive cells were decreased with the addition of melatonin to dopaminergic media (Figure 4.4-A). Western blot analysis also supported the immunofluorescence results. The expression of nestin was high in dopaminergic media, and less in growth media without melatonin addition. The expression of nestin was decreased significantly by the addition of melatonin to growth media ($p<0.001$) and also to dopaminergic differentiation media ($p<0.05$). There was a 3,5-fold increase in the expression level of nestin in DPSCs which were treated with dopaminergic differentiation medium compared to growth medium only and this was statistically significant (Figure 4.4-B).

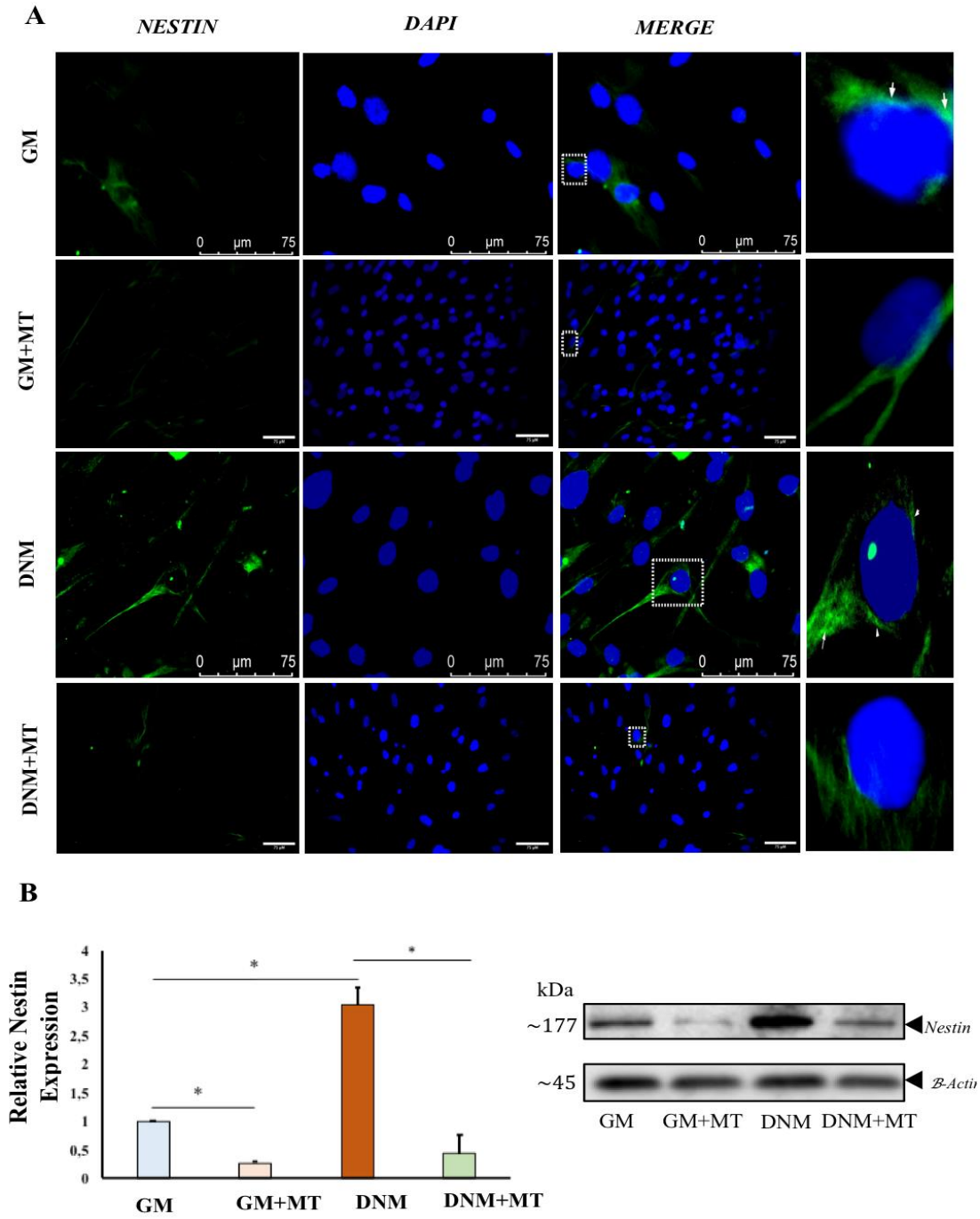

Figure 4.4. The effect of melatonin on the expression of Nestin in DPSCs by immunofluorescence and western blot analysis after incubation with GM, GM+MT, DNM, and DNM+MT for 21 days**. A–** Positive immunofluorescence labeling of nestin (green) on DPSCs (arrow). **B-** Nestin expression was assessed by Western blot analysis. β-Actin was used as the loading control. Bar graphs revealed the fold change of total Nestin quantification normalized to GM treated group. Data was given as mean+SEM (n=3). Scale bar 75 µm. Growth medium (**GM**), growth medium+Melatonin (10µM) (**GM+MT**), dopaminergic neuronal differentiation medium (**DNM**), dopaminergic neuronal differentiation medium+Melatonin (10µM) (**DNM +MT**).

4.5. Addition of Melatonin (10μM) to Dopaminergic Neuronal Differentiation Media Enhanced The Tyrosine Hydroxylase Expression in DPSCs

DPSCs that were incubated with growth media alone, showed a weak cytoplasmic immunoreactivity for TH, but an addition of 10 μM melatonin to growth media caused an increase in the expression of TH at day 21. DPSCs, which were incubated in dopaminergic differentiation media with or without melatonin, showed strong positive immunoreactivity for TH (Figure 4.5-A). Further analysis by western blot showed an increase (approximately 3,5-fold) in TH expression on cells treated with dopaminergic differentiation media supplemented with 10 μM melatonin compared to DPSCs incubated with growth media. The expression of TH on DPSCs was slightly increased by the addition of melatonin into the growth medium compared to growth medium only (Figure 4.5-B).

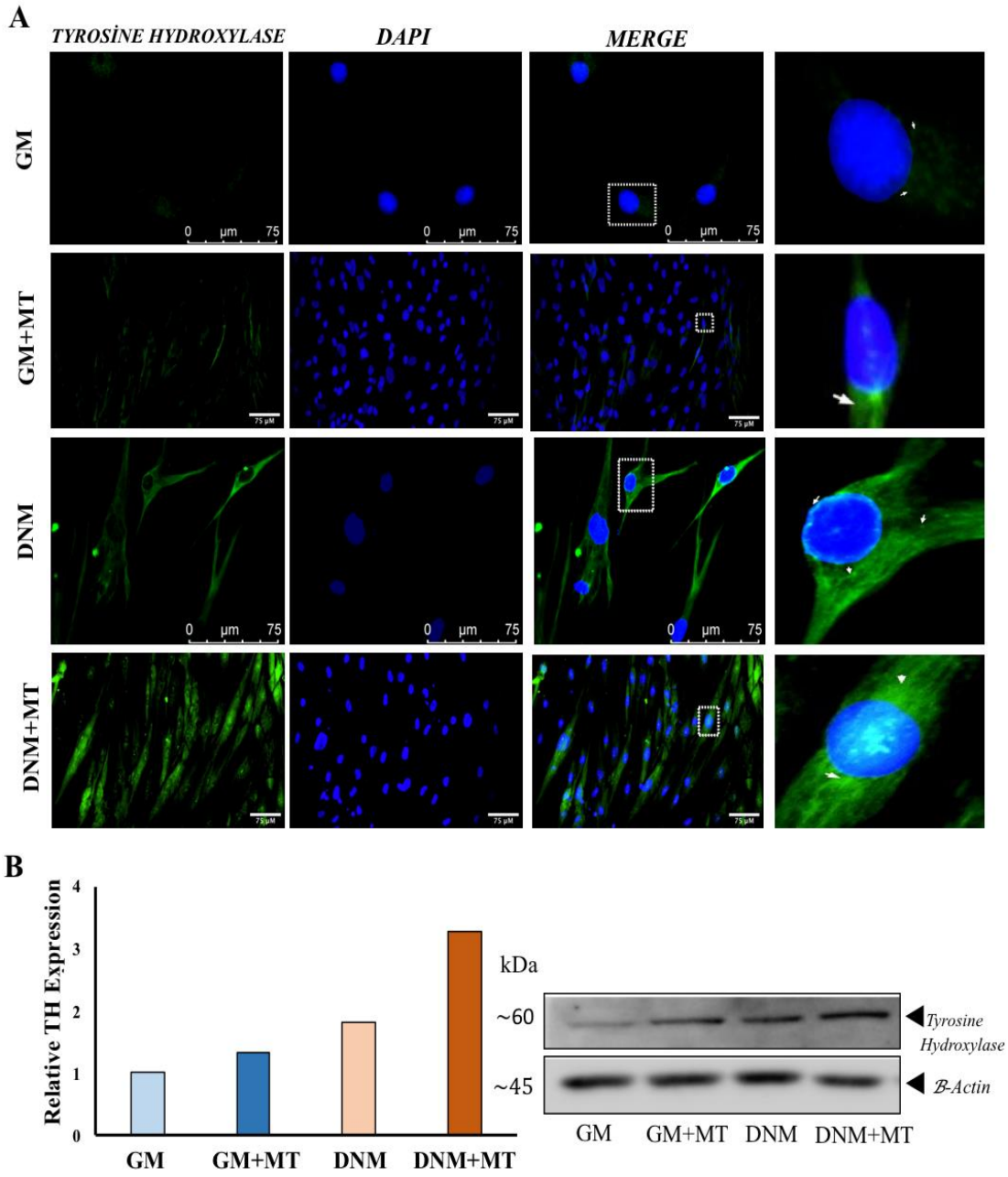

Figure 4.5. The effect of melatonin in the expression of TH on DPSCs by immunocytochemistry and western blot analysis after incubation with GM, GM+MT, DNM, and DNM+MT for 21 days. **A-** Positive immunoreactivity of TH is seen on DPSCs that were incubated with GM+MT, DNM, and DNM+MT for 21 days (arrow). **B-** TH expression was assessed by western blot analysis. β-Actin was used as the loading control. Bar graphs revealed the fold change of TH quantification normalized to GM treated group. n=2. Scale bar 75 µm. Growth medium **(GM)**, growth medium+Melatonin (10 µM) **(GM+MT)**, dopaminergic neuronal differentiation medium **(DNM)**, dopaminergic neuronal differentiation medium+Melatonin (10 µM) **(DNM +MT).**

4.6. Melatonin (10μM) Enhances The DMP1 Immunoreactivity in DPSCs Incubated with Growth Media

Positive immunoreactivity of DMP1 was observed on DPSCs incubated with growth media containing melatonin (10 μM) and this immunoreactivity was more intense compared to the immunoreactivity that is seen on DPSCs treated with growth media only. When DPSCs were incubated with dopaminergic neuronal differentiation media, there was almost no immunoreactivity of DMP1. However, positive DMP1 immune labeled cells were observed in dopaminergic differentiation media supplemented with melatonin (Figure 4.6-A). Further analysis with western blot showed a decrease in the expression of DMP1 in DPSCs incubated with dopaminergic media and melatonin addition to both media caused a slight increase in the expression of DMP1 compared to those incubated with growth media. No statistically significant difference was detected in the expression of DMP1 between groups (Figure 4.6-B).

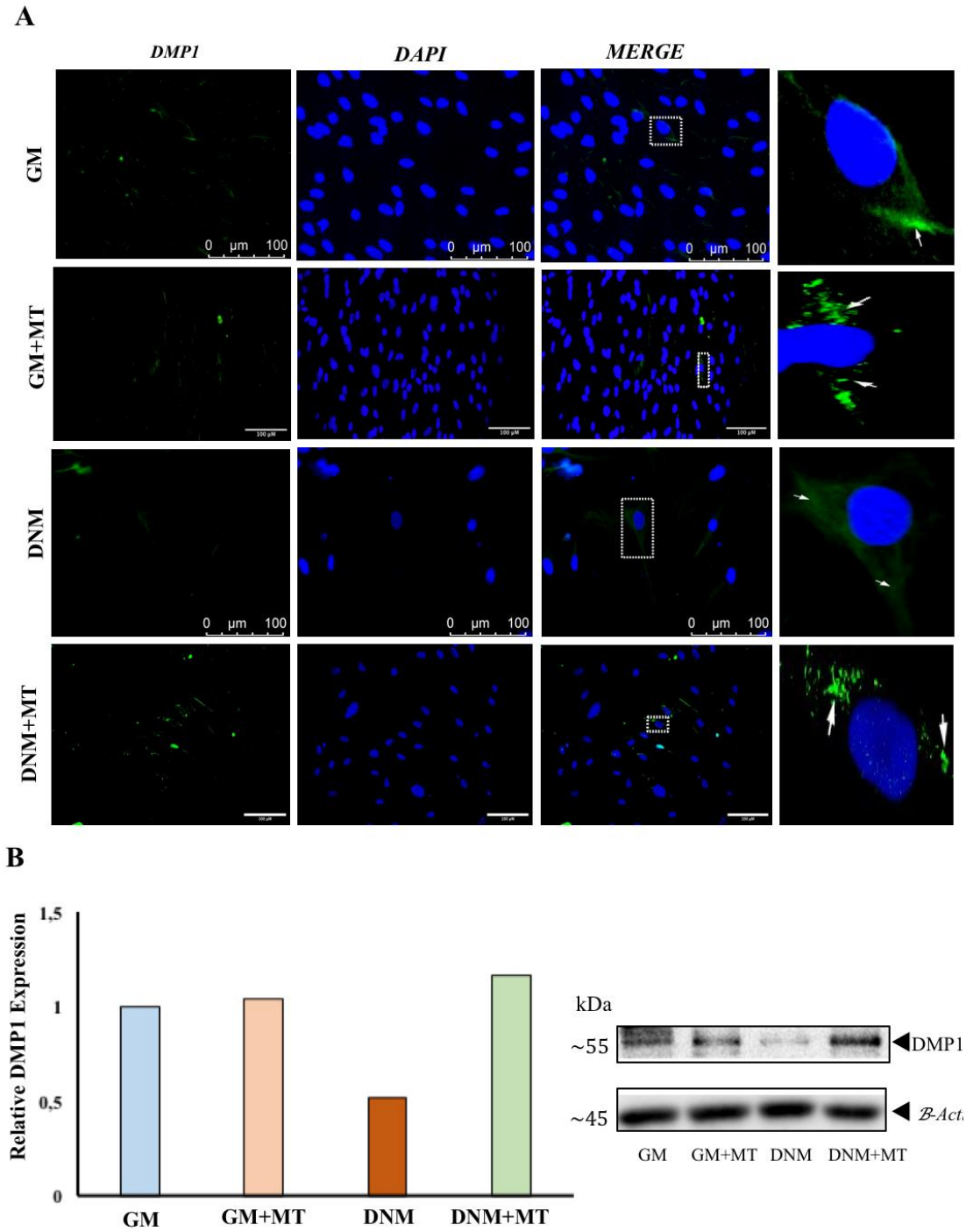

Figure 4.6. Assessment of the effect of melatonin in the expression of DMP1 on DPSCs by immunocytochemistry and western blot after incubation with GM, GM+MT, DNM, and DNM+MT for 21 days. **A** - Immunofluorescence labeling of DMP1 (green) on DPSCs (arrow). **B-** Western blot analysis of DMP1 expression. β-Actin was used as the loading control. Bar graphs revealed the fold change of total DMP1 quantification normalized to GM treated group (n=2). Scale bar 100 µm. Growth medium **(GM)**, growth medium+Melatonin (10µM) **(GM+MT)**, dopaminergic neuronal differentiation medium **(DNM)**, dopaminergic neuronal differentiation medium+Melatonin (10µM) **(DNM +MT).**

4.7. Expression of YAP and p-YAP^{Y357} on DPSCs and YAP Expression on Hippocampal Neurons (P0)

There was a prominent immunoreactivity for YAP in the nucleus, and a weak immunoreactivity in the cytoplasm of DPSCs, which were incubated with growth media only. By incubation with dopaminergic differentiation media, the immunoreactivity of YAP was detected only in the cytoplasm of DPSCs. An addition of melatonin to growth media reduced the YAP expression in the nucleus of DPSCs (Figure 4.7-A).

The positive immunostaining for p-YAP^{Y357} was detected in the cytoplasm of DPSCs. P-YAP^{Y357}expression was increased in DPSCs cultured with the dopaminergic neuronal differentiation media compared to those incubated with growth media. However, the immunoreactivity of p-YAP^{Y357} was less in DPSCs incubated with growth media and dopaminergic differentiation media compared to the melatonin added groups (Figure 4.7-C).

According to our western blot analysis, YAP expression was only detected in DPSCs incubated with growth media, but not in DPSCs that were incubated with dopaminergic differentiation media or melatonin added groups (Figure 4.7-B). The expression profile of p-YAP^{Y357} was opposite to the expression pattern of YAP. Low expression of p-YAP^{Y357} was detected in growth media, but it was increased with the addition of melatonin to growth media (Figure 4.7-C). Densitometric analysis of the bands showed similar p-YAP^{Y357} expression in growth media with melatonin and dopaminergic differentiation media with or without melatonin (Figure 4.7-D).

Additionally, hippocampal primary neuronal culture cells (P0) were used as a positive control to verify YAP activity in neurons. MAP2 was used as a neuronal marker. YAP expression was not detected in neurons, which were positive for MAP2. However, positive YAP immunoreactivity was detected in the cells, which were negative for MAP2 (Figure 4.7-E).

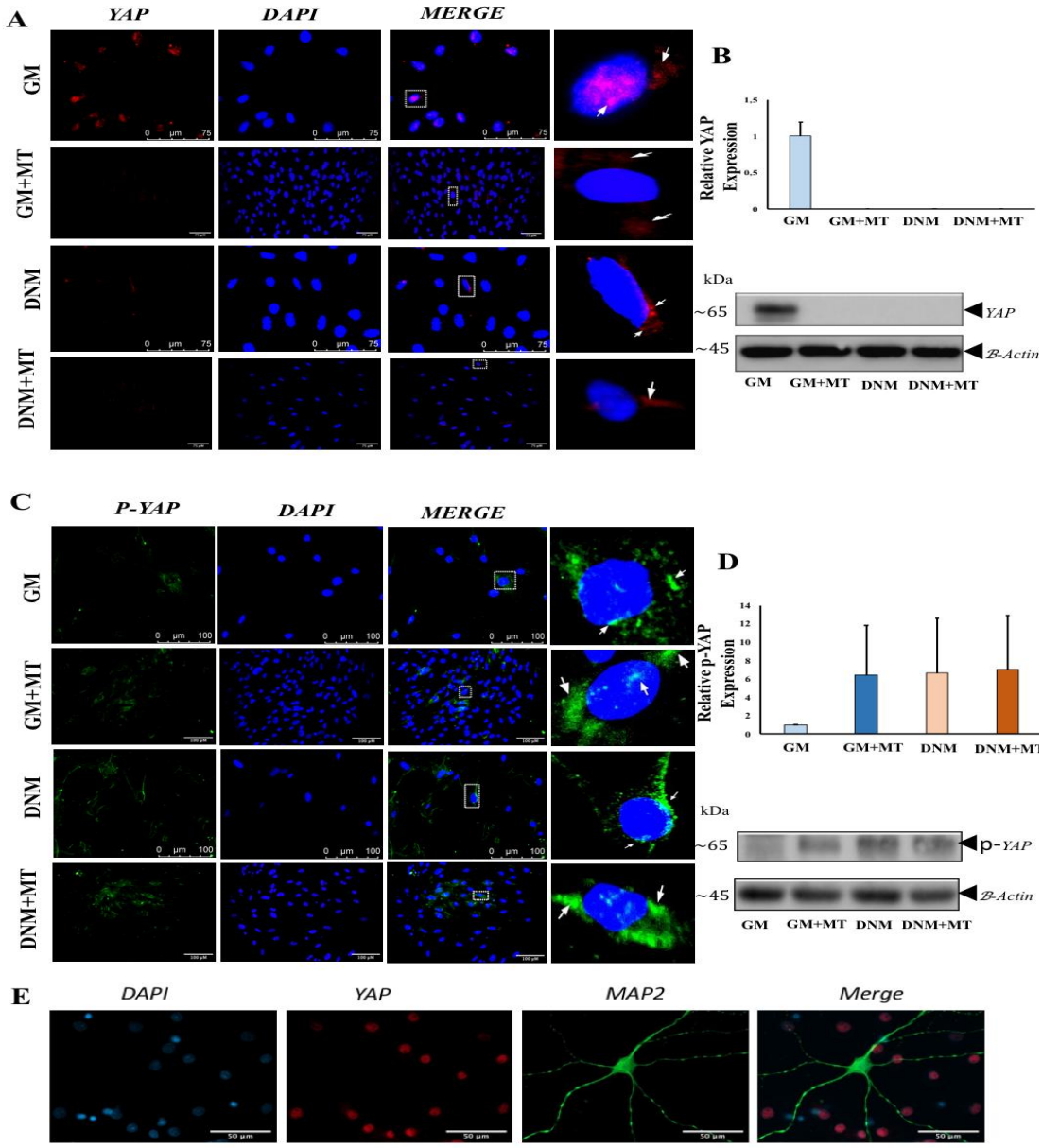

Figure 4.7. Assessment of the effect of melatonin in the expression of YAP and p-YAP^{Y357} on DPSCs by immunofluorescence and western blot analysis after incubation with GM, GM+MT, DNM, and DNM+MT for 21 days, YAP expression on hippocampal neurons (P0). **A–** Immunofluorescence labeling of YAP (red) on DPSCs (arrow). **B-** Western blot analysis of YAP expression. **C-** Immunofluorescence labeling of p-YAP^{Y357} (green) on DPSC (arrow). **D-** Western blot analysis of p-YAP^{Y357} expression. **E-** Double immunofluorescence labeling of YAP (red) and MAP2 (green) on mature neuron. β-Actin was used as the loading control. Bar graphs revealed the fold change of total interested protein quantification normalized to GM treated group. Data was given as mean+ SEM (n=3). Scale bar 100 µm (A-C), 50 µm (E). Growth medium **(GM)**, growth medium+Melatonin (10µM) **(GM+MT)**, dopaminergic neuronal differentiation medium **(DNM)**, dopaminergic neuronal differentiation medium+Melatonin (10µM) **(DNM +MT).**

4.8. The Co-localization of YAP with Nestin in DPSCs

Double immunofluorescence labeling for YAP and nestin was conducted to evaluate the expression of YAP in nestin positive cells. A strong immunoreactivity of YAP was observed in nestin positive DPSCs, which were incubated with growth medium, and also we observed colocalization of nestin and YAP. Although the highest nestin expression was observed in dopaminergic differentiation media, low expression of YAP as well as colocalization of them was observed. The addition of melatonin to growth media and dopaminergic media resulted with a decrease in the expression of YAP and nestin. (Figure 4.8.)

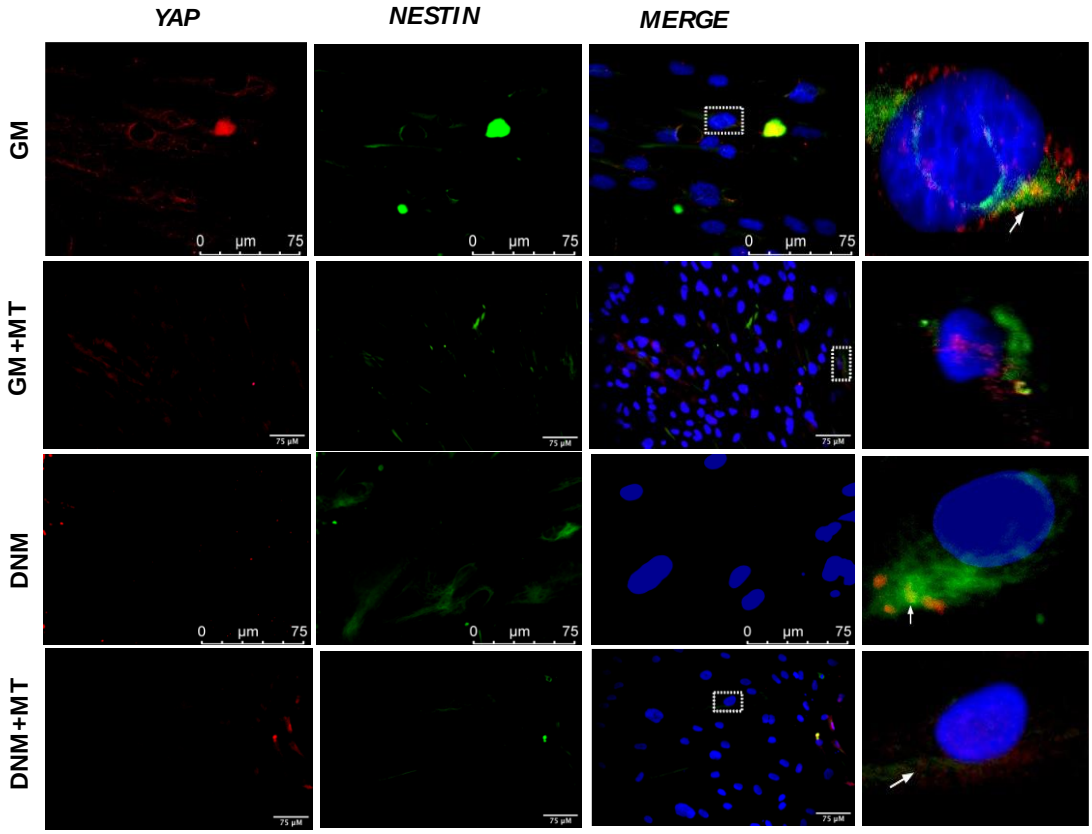

Figure 4.8. Assessment of the effect of melatonin on expression of YAP and Nestin in DPSCs by immunocytochemistry after incubation with GM, GM+MT, DNM, and DNM+MT for 21 days. **A –** DPSCs shows positive immunoreactivity for YAP (red) and Nestin (green) on DPSCs. Arrow shows the colocalization of YAP with nestin. Scale bar 75 µm. Growth medium **(GM)**, growth medium + 10 µM Melatonin **(GM+MT)**, dopaminergic neuronal differentiation medium **(DNM)**, dopaminergic neuronal differentiation medium + 10 µM Melatonin **(DNM +MT).**

4.9. The Effect of Melatonin (10 μM) on SOX2, PCNA, and Caspase-3 Expression

According to densitometric band analysis, the expression of SOX2 was decreased significantly in DPSCs which were incubated with dopaminergic differentiation medium with or without melatonin (10 μM) compared to those incubated with growth media ($p<0.05$). Approximately half -fold decrease was detected in the level of SOX2 in DPSCs cultured with dopaminergic neuronal differentiation medium compared to DPSCs incubated with growth medium. Furthermore, melatonin addition to both media caused a slight increase in the expression of SOX2 compared to cells incubated with growth media and dopaminergic media alone. This increase was not statistically significant (Figure 4.9-A).

The highest expression level of PCNA was detected in DPSCs incubated with growth medium. The level of PCNA was significantly decreased with dopaminergic differentiation medium and also melatonin addition to both media ($p<0.001$) (Figure 4.9-B).

Western blot analysis revealed that caspase-3 was highly expressed in cells treated with growth medium and slightly decreased by melatonin treatment ($p<0.05$). The level of caspase-3 was significantly decreased (more than a half fold) in cells treated with dopaminergic neuronal differentiation medium with and without melatonin (10 μM) compared to growth media ($p<0.001$). Melatonin addition to dopaminergic media caused a significant increase in the expression of caspase-3 compared to cells incubated with dopaminergic media alone. (Figure 4.9-C).

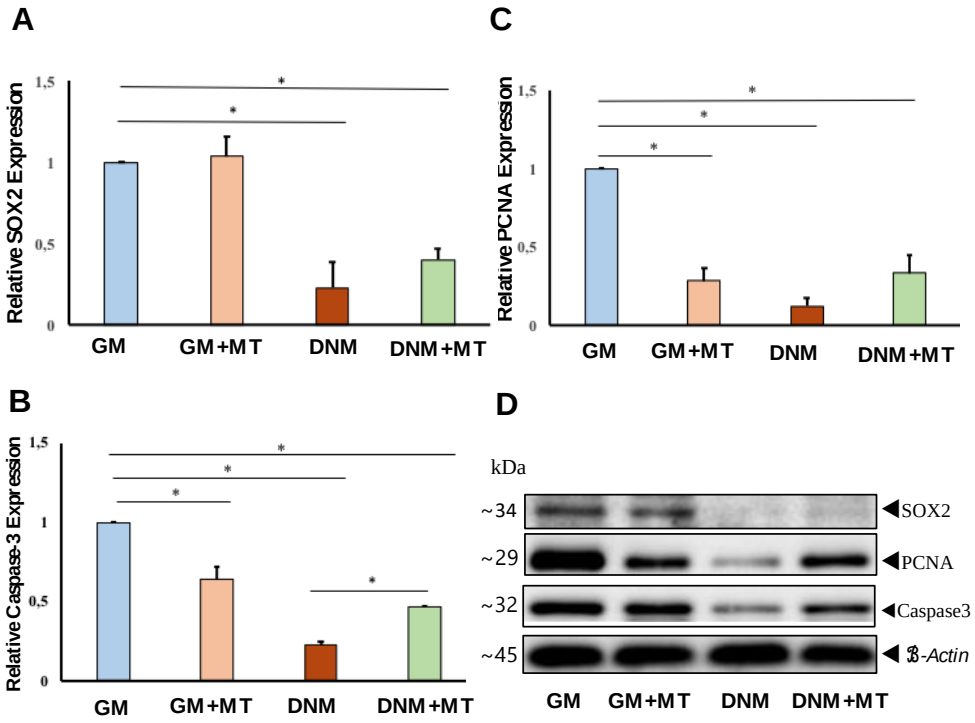

Figure 4.9. The effect of melatonin on expression of SOX2, Caspase-3 and PCNA in DPSCs by western blot after incubation with GM, GM+MT, DNM, and DNM+MT for 21 days. **A-B-C-** Bar graphs revealed the fold change of total quantification of SOX2, Caspase-3 and PCNA respectively normalized to GM treated group (n=3). β-Actin was used as the loading control. Growth medium **(GM)**, growth medium + 10 µM Melatonin **(GM+MT)**, dopaminergic neuronal differentiation medium **(DNM)**, dopaminergic neuronal differentiation medium + 10 µM Melatonin **(DNM +MT**).

5. DISCUSSION

In this thesis, the effect of melatonin on Hippo signaling pathway through the expression of its downstream effector (YAP/ p-YAP^{Y357}) in dopaminergic neuronal differentiation process of DPSCs was investigated. The positive effect of melatonin has been reported on neuronal differentiation of NSCs and odontogenic differentiation of human DPSCs (77, 78). Kong et al. showed that melatonin treatment (0.05, 0.1, 1 nM) for 3 days increased significantly the cell viability of NSCs (77). Further studies reported that 0.5, 1 and 5 μM melatonin treatment significantly increased the number of cells in iPSC-derived neuronal stem cells (79), and also melatonin treatment for 24 hours induced the cell viability in NSCs compared to control group (80). Fu *et al.* reported that the highest cell viability was observed in 100 nM melatonin treatment at 24 and 48 hours (80). In our study melatonin concentration of 100nM and 10 μM promoted the viability of DPSCs for 4 days, which is parallel to the findings of Fu *et al.* Although there was no significant difference in our results, with the highest cell viability, 10 μM melatonin concentration was used as a tolerable dose during differentiation of DPSCs.

DPSCs have capacity to differentiate into mature neurons, immature oligodendrocytes, Schwann cells or neuronal precursor cells (17). Several protocols have been reported for neuronal differentiation of DPSCs (5, 6). Neurosphere formation is one of the technique used for expanding NSCs and this technique is also used for differentiation of DPSCs to neuron (5). In a study DPSCs were incubated with neuroinductive medium for 7 days to form neurosphere structure and dissociated into single cells by pipetting. The single cells were seeded on collagen type IV covered dishes and incubated with neuroinductive medium including retinoic acid for 7 days. In another study, DPSCs were plated on non-adherent surface and incubated with NSC culture medium to induce neurosphere formation for 7-10 days. Then, NSC derived from DPSCs were collected and seeded onto poly-L-lysine surface and incubated with neurobasal media supplemented with N2 and B27 (75). Adherent monolayer method has been recently used and become more popular than neurosphere technique. It is claimed that heterogeneity of stem cell population and differentiation into glia cells are less in this method (7). In our study neurosphere formation and isolation of the

cells from the neurosphere was not carried out, only adherent monolayer technique was used. All the dishes or slides used in the experiments were coated with cell matrix basement membrane gel recommended for neural cell culture. DPSCs were seeded over the coated plates and incubated with dopaminergic differentiation media for 21 days. The possitive differentiaiton of DPSCs to neuron like cells was accomplished with this technique also.

Differentiation is a shift from proliferation to specialization, and involves the morphological and chemical alterations in the cells (16). During neurogenesis following neuronal commitment, alteration in cell morphology is seen. The rounded neuronal precursors switch the membrane sprouts to neurites. Then, extending neurites form neurite branches, leading to axon satellite or dendritic spindle (81). These alterations can be observed also in *in vitro* conditions. Atarri *et al.* reported that DPSCs looked like neuronal cells after neuroectodermal differentiation (8). Consistent with this, we have observed neuronal-like morphology in DPSCs with thin cytoplasmic extensions resembling neurite like features at day 21 under dopaminergic neuronal differentiation medium. Melatonin triggers the formation of neurite by regulating cytoskeletal arrangement and enhances the number of microfilaments by PKC and ROCK activation which results in enlargement of neurite. Melatonin induces the axonal formation by activating MT2 receptors or early neurite formation by activating MT1 in embryonic hippocampal neurons (82). Our results also demonstrated that DPSCs gained elongated morphology with axon-like feature and enlargement of the extensions was detected by melatonin administration into neuronal differentiation medium.

Morphologic features may give some clues about the commitment or differentiation of DPSCs on neurogenic side. However, morphological changes should be supported by the expression of specific proteins such as Neun, MAP2 or TH or negative expression of GFAP for neuronal differentiation.

Neun is one of the neuronal markers, which is localized in nucleus and perinuclear cytoplasm. It’s widely used in the immunocytochemical analysis to verify the neuronal differentiation (83). GFAP is also used as astrocyte specific marker and positive staining shows reactive astrogliosis (84). Luzuriaga *et al.* reported the positive

immunoreactivity for Neun and GFAP on DPSCs after incubation with Neurocult Differentiation Medium for 3 weeks (6). Kira´ly et al. showed expression of GFAP was increased in DPSCs with neuronal induction compared to non-differentiated cells. Moreover, they injected pre-differentiated DPSCs into the brain of 3-day old rats. After 4 weeks, they identified the localization of the most cells in the normal neuronal progenitor zones with the expression of Neun and GFAP (85). This finding indicates DPSCs can differentiate to neuronal like cells under appropriate circumstances. TH is widely used as dopaminergic neuronal marker because it is essential for synthesis of dopamine neurotransmitter (86). Incubation of DPSCs with human/mouse dopaminergic neuron differentiation kit or treatment with BDNF/NT3 in neuronal differentiation medium enhances the expression of TH significantly (6, 9) Consistently, positive immunoreactivity of Neun and TH on DPSCs that were incubated with dopaminergic media indicates the successful differentiation of DPSCs to neurons in our study. Moreover, negative staining of GFAP supports that DPSCs did not differentiated to glial cells with dopaminergic neuronal differentiation medium, which does not contain supplements to induce the gliogenesis.

Melatonin increases the expression of TH in NSCs and amniotic fluid MSCs (87). Several studies indicated the role of MAPK/ERK pathway in the regulation of TH expression and melatonin can induce the expression of TH by activating ERK1/2 in neuroblastoma cells (88). Although the relation between melatonin and TH in NSCs and MSCs have been reported, the effect of melatonin on neurogenic differentiation of DPSCs have not been shown in perivous studies. In our study, addition of melatonin into dopaminergic neuronal differentiation medium resulted with an increase in the expression of TH in DPSCs and supports the potential of melatonin to induce the dopaminergic neuronal differentiation.

Beside the neuronal markers, neuroprogenitor marker (Nestin) was also included to our study and the expression of Nestin was evaluated in DPSCs after neurogenic differentiation. Nestin is a cytoskeletal intermediate filament protein. It’s mostly expressed in NSCs and identified the neural crest derived cells (89). The expression of Nestin was reported in CD34+ DPSCs and considered as evidence for differentiation of DPSCs into neuronal lineage (36). Karamzadeh et al. showed the

expression of nestin in DPSCs by qPCR analysis (90). Particularly, our data are consistent with the findings reported by Li et al (10). They reported that weak expression of nestin in DPSCs was increased noticeably after neurogenic induction. In parallel with this study, we detected a significant increase in the expression of nestin in DPSCs after incubation with dopaminergic neuronal medium, which can be the evidence of expansion neural precursor cells that will differentiate into dopaminergic neurons.

Melatonin (0.1 μM) treatment for 14 days increased the expression of nestin in induced pluripotent stem cells (79). In contrast, incubation for 21 days with growth medium containing melatonin (10 μM) significantly decreased the nestin expression in our study. Consistently, Lee et al. showed that 1 mM melatonin treatment for 24 hours reduced the nestin protein in brain cancer stem cells (91). Furthermore, treatment with pharmacological concentration of melatonin (1-100 μM) reduced the neuronal differentiation of NSCs (92). These results indicated that the expression of nestin could be reduced by melatonin depending on concentration or exposure time.

SOX2 is one of the stemness markers and the existance of this molecule is seen in several embryonic or adult brain regions, and also in differentiated neurons (93). SOX2 as well as OCT4, NANOG which are transcription factors induce the self-renewal and pluripotency in pluripotent stem cells. The imbalance in these factors plays a role for the determination of cell fate. The upregulation of OCT4/NANOG induces the mesodermal differentiation, whereas the overexpression of SOX2 triggers the neuroectodermal differentiation and suppresses the mesodermal differentiation (94). However, the expression level of SOX2 is different in the undifferentiated state and during neurogenic differentiation or termination stage. Previous studies showed that inactivation of SOX2 were required to complete neurogenesis in neuronal progenitor cells (13, 14). Expression of SOX2 was completely downregulated in MAP2 positive neurons which were differentiated from Ntera2/D1 neuron-like cells (15). Besides the function of SOX2 on inducing and maintaining pluripotency in iPSCs and ESCs, and also fate decision in neuronal or epithelial stem/progenitor cells, it has a role in maintaining the multipotent lineage commitment in progenitor or stem cells (12). Yoon et al. proposed that knockdown of SOX2 expression led to reduce

significantly the multipotentiality and proliferation of MSCs (95). Recently, the effect of SOX2 in DPSCs has been investigated. The expression of SOX2 in DPSCs verified the primitive or naïve nature of DPSCs (11). Consistently, in our study the expression of SOX2 was higher in DPSCs which were incubated with growth media only, but it was significantly decreased in DPSCs, which were incubated with dopaminergic differentiation medium with and without melatonin (10 μM). These results showed that the stemness and multipotency of DPSCs was reduced by treatment with dopaminergic media plus melatonin which resulted in the neuronal differentiation. Furhermore, Liu et al. reported proliferation, adhesion and migration of DPSCs were enhanced with overexpression of SOX2 and decreased with SOX2-siRNA.(96). Decrease in the expression level of SOX2 in our study was also in line with the reduction in the proliferation of DPSCs besides losing multipotentiality of these cells. Decrease in the expression of PCNA in these groups confirmed this idea.

Our western blot data showing a slight increase in the expression of SOX2 in DPSCs that were incubated with growth media supplemented with melatonin (10μM) compared to growth media only. The same expression pattern was also shown in DPSCs by addition of melatoinin into dopaminergic differentiation media. Chen et al. showed that melatonin treatment resulted in enhancing the expression of SOX2 in P19 cells (72). In another study, the number of neural progenitor cells, which were labelled with SOX2 was increased in dentate gyrus by melatonin treatment (97). However, melatonin concentration used in our study did not cause a statistically significant increase in SOX2 expression.

DPSCs with heterogeneous subpopulations, express various surface markers for multilineage differentiation. DMP1 which is an extracellular matrix phosphoprotein and highly expressed in odontoblasts or osteoblasts was also evaluated in our study. DMP1 is present in the nucleus throughout the early differentiation of odontoblasts or osteoblasts and act as a transcription factor in preosteoblast for the induction of the osteoblast specific genes (98). In our study DMP1 expression in growth media supports the heterogenic population of DPSCs, which can differentiate to odontoblasts/osteoblasts. Decrease in the expression level of DMP1 supports neurogenic commitment instead of odonto/osteogenic differentiation under the

dopaminergic media. As reported in another study, the expression of DMP1 level was significantly increased in dental papilla cells with the addition of 10 μM melatonin into osteogenic or basal media. In parallel with previous studies, melatonin addition into growth media and dopaminergic media resulted in the increased expression of DMP1, but this was not statistically significant in our study.

Overall, our results demonstrated that we have successfully differentiated DPSCs into neurogenic side by using dopaminergic neuronal differentiation media and also with addition of 10 μM melatonin into this media. Next, we investigated the effect of melatonin on Hippo signaling pathway by evaluating YAP and p-YAP^{Y357} expression through neurogenic differentiation.

Regulatory role of the Hippo signaling pathway on stem cells has been shown in many articles by investigating YAP and p-YAP (65). The expressions of OCT4, SOX2 (ESC pluripotent marker), YAP protein decreases and phosphorylation of YAP on serine 127 increases after differentiation (66). It was shown that YAP expression was upregulated by inducing pluripotent markers in human fibroblast cells (66). In the chick neural tube, Cao and his team reported that overexpression of YAP with binding to TEAD led to an increase in the amount of neural progenitor cells and reduce neuronal differentiation (68). Zaltsman et al. reported that the total and nuclear YAP expression was decreased during neuronal differentiation (21). In our study decrease in the expression of YAP with neuronal differentiation media is consistent with the literature. Additionally, a prominent immunoreactivity of YAP in the nucleus and weak immunoreactivity in the cytoplasm of DPSCs that were treated with growth media only in immunofluorescence labeling supports the presence of YAP in the nucleus of naïve stem cells. In contrast, the cytoplasmic weak immunoreactivity of YAP by incubating DPSCs with dopaminergic media and also by the addition of melatonin to both media is consistent with the absence of YAP in the nucleus of cells after differentiation.

Melatonin activates Gαs by binding to MT1 receptors in different cell lines and induces intracellular cAMP level with activation of PKA. Activation of PKA results in phosphorylation of LATS1/2 which induces YAP phosphorylation and

sequestration in the cytoplasm (23). In our study decrease in YAP expression with addition of melatonin supports the counter relation between melatonin and YAP which shows that Hippo signalling pathway is active/on with melatonin administration.

As YAP shows the naïve state of stem cells and nestin is a NSCs marker and identifies the neural crest derived cells, we investigated YAP and nestin relation in DPSCs by double immunofluorescence labeling. YAP is considered as an essential protein for maintaining the stemness. In parallel with, knockdown of YAP was significantly decreased the SOX2 and OCT4 expression and brought losing pluripotency of embryonic stem cells (66). Due to the negative correlation between YAP expression and neuronal maturity, YAP is also considered as maintaining the neuronal stemness (99). There are few studies on relation between YAP and nestin; Huang et al. reported high level of YAP expression in nestin positive NSCs and they showed that this expression was necessary for astrocytic differentiation (100). In our study highest immunoreactivity for YAP and Nestin was detected in undifferentiated DPSCs that were incubated with growth medium and it was decreased after dopaminergic neruoanal differentiation. This finding can be explained as incubation of DPSCs with dopaminergic differentiation media decreases the stemmness features and induces the commitment of neurogenic differentiation, not astrocytic differentiation which resulted with a decline in YAP expression. The immunolabelling of neurnal markers (TH, Neun) and glial marker (GFAP) was also confirmed this finding. Decrease in YAP and nestin expression as well as colocalization with addition of melatonin supports the role of melatonin in neuronal differentiation by modulating YAP and Nestin interaction.

As reviewed previously, YAP expression was detected in nestin positive cells, but not in MAP2 positive neurons (100). Our result also verified that the YAP expression was not seen in mature neurons, which were labeled with MAP2. Overall, YAP expression was highest in the nucleus of DPSCs in undifferentiated state which indicates the role of YAP in stemmnes and also the expansion of DPSCs pool by binding transcription factors. However, phosphorylated YAP increases in the cytoplasm of DPSCs by incubation with dopaminergic neuronal differentiation media, as these cells lost their naïve state and committed to neuronal differentiation. Addition

of melatonin to both media reduces YAP expression in the nucleus of DPSCs and results in the degradation of YAP.

YAP activity is regulated by phosphorylation of LATS family kinases (63). Phosphorylation of YAP on S127 suppresses YAP activity by regulating cytoplasmic localization and degradation of YAP. Increased expression of p-YAP (S127) was observed in differentiated embryonic stem cells compared to undifferentiated cells. As expected, they detected few amounts of YAP in the nucleus, but cytoplasmic YAP was highly phosphorylated in differentiated cells (66). The function of serine phosphorylation on YAP activity is well known, however phosphorylation of YAP on tyrosine have not been shown in stem cells. Phosphorylation of YAP on tyrosine can modulate the localization or transcriptional activity of YAP in cancer cells. Recently, Sugihara et al. reported that inhibitor of SRC family kinases leads to inhibition of tyrosine phosphorylation of YAP and redistribution of YAP from nucleus to the cytoplasm, and downregulate the expression of genes targeted by YAP in cholangiocarcinoma cell line (64). Therefore, phosphorylation of YAP on Tyr residues also regulates YAP activity. In our study we investigated the expression of p-YAP which is phosphorylated on Tyr residues. The opposite expression profile of p-YAP^{Y357} was detected in DPSCs with differentiation compared to the expression profile of YAP. The low expression of p-YAP^{Y357} was detected in DPSCs incubated with growth media only, and using dopaminergic differentiation media as well as the addition of melatonin to growth media resulted with an increase in the expression of pYAP in DPSCs. In our study addition of melatonin into growth media and incubation with dopaminergic neuronal differentiation media induced the phosphorylation of YAP at Y357, while both of them caused a decrease in the expression of YAP in DPSCs. This supports the loss of stemness in DPSCs and induction the differentiation commitment with dopaminergic media and also with melatonin addition.

Proliferation and differentiation are inversely correlated processes in many cell types. Overexpression of YAP causes over proliferation, but reduces the differentiation in neural progenitor cells. Blocking of YAP and TEAD proteins also results in incomplete differentiation (68, 101). To verify the proliferation and apoptosis

in DPSCs during neurogenic differentiation, we investigated the expression of PCNA and caspase-3.

PCNA modulates replication of DNA and cell cycle (102). In our study, the high expression level of PCNA in DPSCs under growth medium demonstrates the high proliferation rate of undifferentiated DPSCs. Moreover, decrease in the expression of PCNA by incubating with neuronal differentiation medium as well as melatonin addition supports the decline in the proliferation rate of these cells with differentiation or melatonin addition.

Caspase-3 is a key factor in apoptosis (103). Apoptosis in stem cells is considered as a part of the mechanism to control the proliferation or self renewal of stem cells (104). High level of caspase-3 was shown in hematopoietic stem cells (HSCs) to regulate the cyclin dependent kinase activity and proliferation (105). Yan et al. showed active caspase-3 in highly proliferative regions of rat forebrain (106). In our study, high expression of caspase-3 along with PCNA in DPSCs which were incubated with growth media supports the role of caspase-3 in controlling the size of population. Significant decrease in the expression of caspase-3 on DPSCs by incubation with dopaminergic media suggests the downregulation of its expression with differentiation process. However, more studies are required to clarify these aspects.

Melatonin treatment decreases oxidative stress and apoptosis in the hippocampus of rats (107). In parallel with this, in our study, caspase-3 expression was decreased significantly by melatonin treatment compared to growth medium-treated group. Melatonin is a strong antioxidant and causes upregulation of antioxidant enzymes. Ataizi et al. showed that melatonin treatment significantly decreased caspase-3 activity and they reported that melatonin in hippocampus induced the anti-oxidant response, neutralization of reactive oxidant species and lipid peroxidation by providing enzymatic and non-enzymatic antioxidants and reducing apoptosis (108).

Since YAP is a main regulator of proliferation of cells and size of organ/tissue, there is a relation between YAP and caspase-3. A previous study demonstrated that inhibition of caspase-3 resulted with a decline in YAP activity and increase in p-YAP

(YAP S127) level in sebaceous gland cells (109). Consistently, we showed that YAP and caspase-3 expression was decreased after the neurogenic differentiation with and without melatonin treatment. Moreover, p-YAP^{Y357} was increased through this process. Decrease in the expression of YAP and caspase-3 after differentiation indicates the role of YAP/caspase-3 in the modulation of DPSCs proliferation or neuronal differentiation process.

Overall, we have differentiated DPSCs into neuron like cells and characterized the cells with the expression of neuronal markers such as Neun, TH and also by morphological features. We also showed that melatonin induced the expression of TH which indicates the potential effect of melatonin on neuronal differentiation of DPSCs. Then, we evaluated YAP and p-YAP^{Y357} expression after neurogenic differentiation. While YAP expression was decreased, p-YAP^{Y357} expression was increased after neurogenic differentiation. Melatonin treatment resulted with the same expression pattern of YAP/p-YAP^{Y357} in differentiation process. Therefore, we suggested that melatonin could trigger the neurogenic differentiation of DPSCs by reducing YAP expression, and inducing the phosphorylation of YAP on tyrosine residues. Moreover, diminished expression of YAP and SOX2 indicates the loss of stemness properties of DPSCs after neurogenic differentiation. Besides, decrease in the expression of PCNA and caspase-3 shows decline in the proliferation and apoptosis in DPSCs population with differentiation proceses.

Future Perspective

Nevertheless, this study has the preliminary results showing a relation between melatonin and YAP expression in the neurogenic differentiation of DPSCs. Further analysis with additon of melatonin receptor antagonist such as Luzindole or knockdown/overexpression of YAP is required to clarify the underlying mechanisms of melatonin in interaction with Hippo signaling pathway through neuronal differentiation of DPSCs.

It was reported that activation of Src kinases resulted in the phosphorylation of YAP on tyrosine residues at 357(110). Therefore, DPSCs which are incubated with melatonin and melatonin receptor antagonist in growth medium or dopaminergic

neuronal differentiation medium can be also treated with Src inhibitor. After evaluating the YAP and $pYAP^{357}$ expression, we can make more assumption about the relation between melatonin, Src kinases and phosphorylation of YAP after neurogenic differentiation.

Although we have successfully differentiated DPSCs into neurogenic side by using dopaminergic neuronal differentiation medium with addition of 10 μM melatonin, patch clamp analysis should be also included in our study to verify the activity of voltage gated channels, which indicates functionality of the differentiated neurons.

6. CONCLUSION

DPSCs are heterogeneous multipotent populations which have the potential to differentiate both neuronal and non-neuronal cell lineage. Because of neuronal differentiation potential, ethical suitability, high proliferation rate and long time storage without losing properties, DPSCs have being explored as a seductive cell source for stem-cell treatment in neurodegenerative disease (3). YAP is an important downstream effector of the Hippo signaling pathway and involves in neuronal differentiation of neural progenitor cells. Melatonin has a regulatory role for the neuronal differentiation of NSCs. In regard with this, the effect of melatonin on the downstream effector of Hippo signalling pathway (YAP/pYAP) after neurogenic differentiation of DPSCs was investigated in the scope of this master thesis.

The positive expression of neuronal markers (Neun, TH) and negative immunoreactivity for GFAP in DPSCs incubated with dopaminergic media was detected and this indicates the successful differentiation of DPSCs to neurons, not glial cells in our study. Presence of DMP1 on DPSCs incubated with growth media suggested the odontogenic differentiation potential of this pool. Decrease in the expression level of DMP1, along with the presence of spesific neuronal markers after incubation with dopaminergic media indicated the neurogenic commitment instead of odontogenic differentiation.

YAP expression in the nucleus of DPSCs incubated with growth media indicated the naive state of undifferentiated stem cells. Moreover, this finding shows that Hippo signalling pathway is not active (off) in undifferentiated DPSCs. However, weak positive immunoreactivity detected in the cytoplasm of DPSCs after incubation with dopaminergic neuronal differentiation media with/without melatonin suggests the exclusion of YAP from the nucleus and also activation of Hippo signalling pathway with differentiation process. YAP expression is also evaluated in primary neuronal culture in order to support our findings and YAP expression was not detected in mature neurons, which were positive for MAP2. Our findings showing increased p-YAP^{Y357} and decreased YAP expression in DPSCs indicates the induction of YAP phosphorylation on tyrosine residues and downregulation of YAP in DPSCs after incubation with dopaminergic neuronal differentiation media either containing

melatonin or not. Thus, our findings in DPSCs and mature neurons reveal that nuclear YAP expression is eliminated throughout dopaminergic neuronal differentiation.

Nestin is a neural precursor marker. High level of YAP expression in nestin positive NSCs considered as essential for astrocytic differentiation (100). In our study highest immunoreactivity for YAP and colocalization with nestin was detected in undifferentiated DPSCs that were incubated with growth medium and it was decreased after dopaminergic neuronal differentiation and also by melatonin addition. Addition of melatonin to dopaminergic media decreases the stemmness features and play a role in the commitment of neurogenic differentiation, not astrocytic differentiation. The potential effect of melatonin on neuronal differentiation of DPSCs was also supported with an increase in the expression of TH by the addition of melatonin into dopaminergic differentiation media.

Altough the expression of SOX2 was not significantly decreased after incubation with growth media containg melatonin, significant decrease in SOX2 expression was seen after incubation with neuronal differentiation media either containing melatonin or not compared to growth media alone. Decreased expression of SOX2 indicated the loss of stemness and multipotency of DPSCs after neurogenic differentiation.

Overall, we showed that melatonin treatment reduced the proliferation of DPSCs and induced the neurogenic differentiation. We suggest melatonin has potential to induce the neuronal differentiation and reduce proliferation of DPSCs by increasing phosphorylation of YAP at tyrosine residues and eliminating the activity of YAP, which indicates the active state of Hippo signaling pathway in this differentiation.

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