EVALUATION OF THE EFFECT OF CITRUS SINENSIS DERIVED EXOSOMES ON
NEUROGENIC DIFFERENTIATION OF HUMAN PERIODONTAL LIGAMENT
STEM CELLS

by
Oğuz Kaan Kırbaş

Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Master of Science in
Biotechnology

Yeditepe University
2019

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APPROVED BY:

Prof. Dr. Fikrettin Şahin
(Thesis Supervisor)
(Yeditepe University)

Assist. Prof. Dr. Bahar Soğutmaz Özdemir
(Yeditepe University)

Assist. Prof. Dr. Mehmet Ozansoy
(Medipol University)

DATE OF APPROVAL: ..../..../2019

ACKNOWLEDGEMENTS

First and foremost, I would like to sincerely thank my supervisor Professor Fikrettin Şahin for his encouragement and endless support and it has been a privilege to study under his supervision. I would like to thank my mentor Dr. P. Neslihan Taşlı for supporting me all the time and teaching everything that I know.

I would like to especially thank Assistant Professor Bahar Soğutmaz Özdemir for inspirational discussions mostly about studies and discovery of plant exosomes. I would like to thank literally all the people for coming into my life and in particular to members of the Molecular Diagnostics Laboratory.

Finally, I am honored to thank my lovely family, who is the most essential element that makes my life meaningful.

ABSTRACT

EVALUATION OF THE EFFECT OF CITRUS SINENSIS DERIVED EXOSOMES ON NEUROGENIC DIFFERENTIATION OF PERIODONTAL LIGAMENT STEM CELLS

The nervous system is often irreversibly damaged by both genetic and acquired diseases throughout life. Nowadays it is determined that in a vast majority of neurodegenerative diseases, completion of tissue regeneration are extremely challenging. As a consequence, not only the differentiation of neuronal stem cells in certain regions of the brain to mature neurons but also the differentiation of adult stem cells with multipotent characteristics to functional and healthy nerve cells at various developmental stages have a considerable potential to be used in future therapies. On the other hand, in the tissues that are involved in neurons, the vesicular structures, which are about 100 nanometers in size, undertake the role of a messenger in the provision of intercellular communication. Moreover, studies have shown that exosomes have a significant effect on cellular mechanisms. However, there are no studies on the effect of plant-derived exosomes on the differentiation of adult stem cells into mature neurons. As a result, the differentiation of periodontal ligament stem cells to mature neurons and an investigation of the possible effects of *Citrus sinensis* derived exosomes (CSDE) on the model are aimed. Accordingly, the cells were differentiated for 10 days by utilizing multi-stage inductive media. At the end of the differentiation protocol, differentiation success and efficiency were analyzed by various experiments in terms of both morphological and transcriptional-translational characteristics. As a result of the experiments, it was determined that the induction protocol used successfully differentiated the periodontal ligament stem cells into mature neurons and CSDE increased the cell viability during the differentiation process. Accordingly, a new induction protocol that can be used for the differentiation of stem cells into neurons has emerged and it is observed that plant-derived exosomes have a positive role in the differentiation process for the first time. In the future, it is extremely important to use the designed differentiation protocol in the modeling of neuronal diseases and to investigate the effects of plant-derived exosomes in these diseases.

ÖZET

CITRUS SINENSIS'DEN ELDE EDİLEN EKSOZOMLARIN PERİODONTAL LİGAMENT KÖK HÜCRELERİNİN NÖROJENİK FARKLILAŞMASI ÜZERİNE ETKİSİNİN ARAŞTIRILMASI

Sinir sistemi yaşam boyunca hem genetik hem de edinilmiş hastalıklardan geri dönüşümsüz olarak zarar görmektedir. Günümüzde nörodejeneratif hastalıkların çoğunda, doku rejenerasyonu sağlanmasının son derece zor olduğu belirlenmiştir. Sonuç olarak, beynin belirli bölgelerindeki nöronal kök hücrelerin sadece olgun nöronlara farklılaşması değil, aynı zamanda çeşitli gelişim safhalarındaki farklı potensilere sahip yetişkin kök hücrelerin fonksiyonel ve sağlıklı sinir hücrelerine farklılaştırılmasında kullanılması önemli bir potansiyele sahiptir. Öte yandan nöronlarında aralarında bulunduğu dokularda iletişim sağlanmasında haberci rolünü üstlenen yaklaşık 100 nanometre büyüklüğündeki eksozom adı verilen veziküler yapılar bulunmaktadır. Yapılan çalışmalarda eksozomların hücresel mekanizmalara önemli ölçüde etki ettikleri belirlenmiştir. Ancak bitkisel kaynaklı eksozomların, yetişkin kök hücrelerin olgun nöronlara farklılaşması üzerindeki etkisi ile ilgili hiçbir çalışma bulunmamaktadır. Bunun sonucunda, periodontal ligament kök hücrelerinin nöronlara farklılaştırılması ve *Citrus sinensis* kökenli eksozomların model üzerindeki olası etkilerinin araştırılması amaçlanmaktadır. Bu doğrultuda, kök hücreler, çok basamaklı indükleyici besiyerleri kullanılarak 10 gün süresince farklılaştırılmışlardır. Farklılaştırma protokolünün sonunda farklılaşma başarısı ve verimi, hem morfolojik ve hem de transkripsiyonel ve translasyonel karakteristik bakımından çeşitli deneyler ile analiz edilmiştir. Deneyler sonucunda, kullanılan indükleme protokolünün kök hücreleri başarıyla olgun nöronlara farklılaştırdığı ve eksozomların bu süreçte hücrelerin canlılığını arttırdıkları tespit edilmiştir. Elde edilen bulgulara göre, kök hücrelerin nöronlara farklılaşmasında kullanılabilecek yeni bir indükleme protokolü ortaya çıkmış ve ilk kez olarak bitkisel kökenli eksozomların süreçte pozitif rol oynadıkları belirlenmiştir. Gelecekte, geliştirilen farklılaşma protokolünün nöronal hastalıkların modellenmesinde kullanılması ve bitki kökenli eksozomların bu hastalıklardaki etkilerinin araştırılması oldukça önemlidir.

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LIST OF SYMBOLS/ABBREVIATIONS

°C	Degree centigrade
μg/cm^2	Microgram per centimeter square
μg/ml	Microgram per mililiter
ng/ml	Nanogram per mililiter
μM	Micromolar
nM	Nanomolar
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
ATRA	All-trans retinoic acid
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BMMSCs	Bone marrow mesenchymal stem cells
BMP-4	Bone morphogenetic protein 4
cAMP	Cyclic adenosine monophosphate
CDE	Caveolin dependent endocytosis
ChAT	Choline acetyltransferase
CME	Clathrin mediated endocytosis
CREB	cAMP response element binding protein
CREBBP	CREB binding protein
CSDE	*Citrus sinensis* derived exosomes
CTX	Cholera toxin
DAG	Diacylglycerol
db-cAMP	Dibutyryl cAMP
DFSCs	Dental follicle stem cells
DNA	Deoxyribonucleic acid
DPSCs	Dental pulp stem cells
DSCs	Dental stem cells
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinases

ESCRT	Endosomal sorting complex required for transport
EV	Extracellular vesicles
FBS	Fetal bovine serum
FGF8	Fibroblast growth factor
GalC	Galactocerebroside
GAP43	Growth-associated protein-43
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GMSCs	Gingival mesenchymal stem cells
HSP70	Heat shock proteins
HTERT	Human telomerase reverse transcriptase
IBMX	3-isobutyl-1-methylxanthine
IGF-I	Insulin-like growth factor
IP_3	Inositol 1, 4, 5-triphosphate
ISCT	International Society for Cellular Therapy
KCl	Potassium chloride
LIF	Leukemia inhibitory factor
MAP2	Microtubule-associated protein-2
MAPK	Mitogen-activated protein kinase
MBCD	Methyl-beta-cyclodextrin
MHC-1,2	Major histocompatibility complex
MSCs	Mesenchymal stem cells
MVB	Multivesicular bodies
NeuN	Neural nuclei antigen
NFH	Neurofilament heavy peptide chain
NGF	Nerve growth factor
NGFR	Nerve growth factor receptor p75
PDL	Poly-D-lysine
PDLSCs	Human periodontal ligament stem cells
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol (3, 4, 5)-triphosphate

PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PLL	Poly-L-lysine
PLO	Poly-L-ornithine
PS	Phosphatidylserine
RME	Raft mediated endocytosis
RNA	Ribonucleic acid
RRME	Receptor/raft-mediated endocytosis
Rtk	Receptor tyrosine kinase
SCAPs	Stem cells from the apical papilla
SHEDs	Stem cells from human exfoliated deciduous teeth
SHH	Sonic Hedgehog
TEM	Tetraspanin-enriched microdomains
TH	Tyrosine hydroxylase
TNF	Tumor Necrosis Factor
TSG101	Tumor susceptible gene 101
TUBB3	Tubulin beta 3 class III
VSP4B	Vacuolar protein sorting 4 homolog B

1. INTRODUCTION

1.1. CHARACTERISTICS AND NEUROGENESIS OF STEM CELLS

1.1.1. Classification of Stem Cells

In our body, one type of cells which are capable of self-renewal, colony formation, and differentiation into a wide range of different cells, i.e. neurogenesis, adipogenesis, odontogenesis, is defined as stem cells [1,2]. These cells, which has been active since the developmental part of human life, have two important characteristics. The first one of these is the functional ability of stem cells in order to renew themselves, which is sectionalized into the symmetric and asymmetric division in response to environmental stimulations [3–5]. The symmetrical division describes the equipotency of two new cells formed in mitosis. The emergent cells, therefore, may maintain their differentiation potential. Otherwise, these might have already differentiated [6].
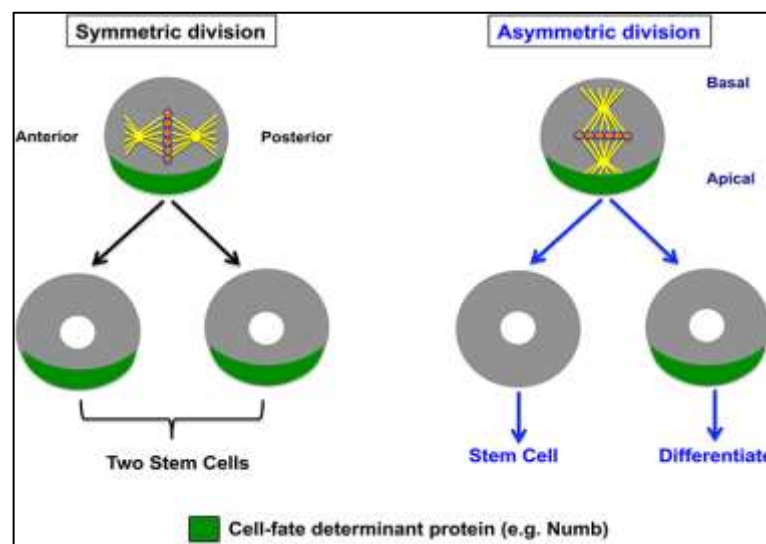
Figure 1.1. Symmetric and asymmetric division of stem cells and the influential role of centrosome positioning [7].

In asymmetric division, on the other hand, one of the formed cells shows identical to the parental cell, while the second one has differentiated. In terms of asymmetric division, the self-renewal of stem cells occurs dissimilar to the mitotic division of normal cells [8,9].

Thus, the stem cells don't lose their differentiation potential while generating new and differentiated cells. Furthermore, they have capable of maintenance of the balance between the differentiated cells and the stem cells through modulating the division of stem cells according to population status [10,11]. It is believed that the regulation of this may be controlled by the apical or basal orientation of the centrosome in dividing cells [12,13]. Depending on all these factors, there is an extremely important link between the division of stem cells and the preservation of differentiation potentials named potency. Potency, an important characteristic of the stem cells, determines the limit of the cell diversity in which cells can be transformed via differentiation [14,15].

Depending on the stage of development, the differentiation potentials of stem cells are categorized into several groups. Totipotent/omnipotent stem cells can form a complete living organism as a whole. After fertilization, the embryonic cells are totipotent in the first few divisions till the morula stage. After the morula stage, totipotent cells, which differentiate into specialized type cells, are transformed into the inner cell mass of blastocysts and into outer trophoblasts [16]. Thus, the cells named pluripotent stem cells which don't have the ability to transform into extraembryonic tissues and can only differentiate into embryonic tissues emerges. These cells have the ability to differentiate into all cells that can be originated from three germ layers [17–20]. Multipotent stem cells are progenitor cells that are found in developmentally close families and have the potential to differentiate into various cell types. These are abundant in tissues as cordon blood, adipose, bone marrow and dental [21,22]. Although there are limited differentiation potentials as progenitor cells, it has been proven in vitro that multipotent cells can transform into developmentally different tissues with recent studies [23–25]. Moreover, studies on the induction of pluripotency and the emergence of relevant molecular markers by epigenetically regulating multipotent stem cells have already involved in the literature [26]. It is postulated that unipotent or precursor cells, which differ from the normal cells in terms of self-renewal capability, can differentiate only in a single type of cell, i.e. skin cell progenitors [27]. In addition, in terms of neurogenic differentiation, dental multipotent stem cells in comparison with mesenchymal stem cells were found to be more successful owing to their expression of neuronal genes at basal levels [23,28]. It is determined that dental stem cells have a specific predisposition to the neurogenic differentiation owing to origination from neural crest structure [29].

1.1.2. Types of Dental Stem Cells and Their Sources

Dental stem cells (DSCs) are the important one of the multipotent stem cell groups and it is stated that they contain a great variety of subtypes of DSCs depends on source location and developmental stage of the tooth. These cells are stem cells from human exfoliated deciduous teeth (SHEDs), dental follicle stem cells (DFSCs), stem cell from apical papilla (SCAPs), gingival mesenchymal stem cells (GMSCs), dental pulp stem cell (DPSCs) and periodontal ligament stem cells (PDLSCs) [30–32].
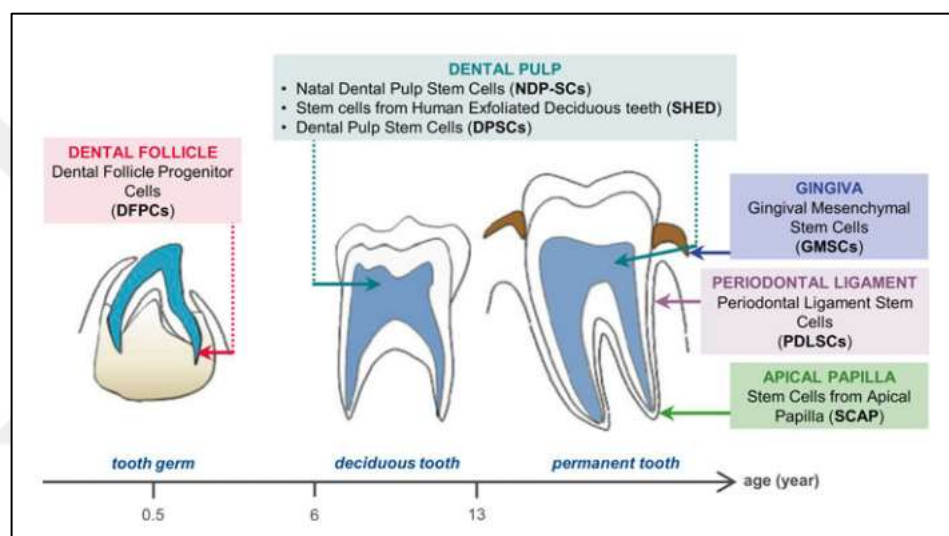
Figure 1.2. Localization of various DSCs during the tooth developmental phases [33].

Although all of these cells are generally close to mesenchymal stem cell characteristics, i.e. fibroblastic morphology and heterogeneity, there are significant differences in growth capacities, multilineage differentiation potentials, expressions of surface receptor and other marker genes such as pluripotent stem cell genes [34,35].

Despite these differences in DSCs, the characterization methods are based on three criteria that are determined by the Internal Society for Cellular Therapy (ISCT) regarding the identification of mesenchymal stem cells (MSCs) are extremely important [36]. These criteria are proposed for identifying MSCs:

  i.   Adherence to plastic in standard culture conditions
  ii. Genuine surface antigen expression (Positive for CD105, CD73, CD90 and Negative for CD45, CD34, CD14)

iii. Three-lineage differentiation potential (chondrogenesis, adipogenesis, osteogenesis demonstrated by staining in vitro cell culture)

Another important advantage of DSCs is that the tissue where the cells are located will be extracted from the body regardless of the purpose of the dental procedure. In addition, it is a substantial advantage that the extraction process is less invasive in dental tissue when the procedures regarding other tissues such as bone marrow are examined [37–39]. In contrast to the ethical problems of embryonic stem cells, which is a very contradictive subject related to stem cells studies, there are not any obstacles in terms of ethics in the studies of DSCs. Hence, it is thought to have a highly positive effect on studies [40,41].

It is envisaged that DSCs are not only incompatible with characteristics of MSC in some ways, but also have a superior potential to differentiate into the neuronal tissues owing to origination from neural crest in the early developmental stage [42]. Neural crest, a transient cells strips formed during embryogenesis, can be differentiated into a diverse range of lineages, including the dental mesenchyma, which contributes to the formation of the nervous system in the course of the developmental period [43,44]. Undoubtedly, DSCs are expected to have a more elevated expression of neural markers and neural differentiation potential than other MSCs in association with neural crest origin. Furthermore, it has been determined that DSCs are shown low-level expression without requiring any neuronal induction [45,46]. In the study performed by Martens et. al. (2013), it was investigated whether the hDPSCs without inducing via differentiation conditions expressed some of the neuronal markers. The expression levels of neuronal markers of these were evaluated through immunohistochemistry. In consequence of experiments, it was found that the cell population was uniformly positive immune-reactivity for tubulin beta 3 class III (TUBB3), S100 protein and synaptophysin, and the minor part of the population was shown the positive immune-reactivity for galactocerebroside (GalC), neurofilament heavy peptide chain (NFH) and nerve growth factor receptor p75 (NGFR) [47].

1.1.3. In vitro Neurogenic Differentiation of Periodontal Ligament Stem Cells

In terms of the neurogenic differentiation potential, the PDLSCs which have the heterogeneous population characteristics, have been proven several times that they have successfully differentiated into neurons through using a variety of protocols. Hence,

numerous studies regarding neuronal differentiation are found in the literature [48,49]. In these studies, the addition of growth factors, small molecules and other inducing cell culture supplements to the basal medium, coating the cell surfaces with special substrates, alterations of the cell number at the beginning of differentiation, the total differentiation time, single or multi-stage differentiation treatments and the induction of neurosphere formation at pre-inducing step are fundamental and influential factors caused the variations of differentiation protocols. However, it is observed in the literature that there are notable common points on the differentiation of PDLSCs. Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) were used as growth factors in most of the studies [50–52]. Although these factors aren't specifically associated with neuronal survivability and differentiation, it was observed that they were used more than neuron-specific growth factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). In differentiation protocols used in PDLSCs as cell source, induction of neurosphere formation in pre-differentiation stage or single/multi-stage differentiation protocols were used in similar ratios [48,49,53–59]. As the common extracellular matrix proteins are more frequently used in the coating of the cell culture surface, which is a remarkable factor in the differentiation studies, the more specific and infrequent proteins are also found in the literature [60,61]. In the differentiation protocols, Poly-L-Lysin (PLL), Poly-D-Lysin (PDL), Fibronectin, Laminin, Collagen derivatives, and various coating substrates were used. In literature, no studies have shown that the common or specific coating substrates have a direct effect on the neurogenic differentiation of PDLSCs. However, in studies on the differentiation of MSCs, the effect of surface coating substrates on neuronal fate was investigated. In the study performed by Qian (2004), PDL, PLL, collagen, laminin, fibronectin and matrigel substrates were evaluated in comparison with standard tissue grade polystyrene in terms of their effects on expansion and neuronal differentiation of MSCs [62]. As a result of experiments, it was observed that the use of Matrigel at a concentration of 5 $\mu g/cm^2$ did not induce MSCs expansion but increased the completion of neurogenic differentiation in terms of morphology. Furthermore, matrigel at a concentration of 50 $\mu g/cm^2$ induced successfully both expansion and neurogenic differentiation of MSCs. On the other hand, it was observed that the other substrates did not show a positive or negative effect on regarding criteria.

In the literature, almost all of the media composition which induced neurogenic differentiation did not contain fetal bovine serum (FBS) [63]. As a result, a significant part

of the studies was performed in serum-free medium and supplemental solutions like N2 supplement or B27 supplement were added to the medium to maintain the cell survivability [64,65]. Even more rarely, it was observed that sonic hedgehog (SHH) and fibroblast growth factor 8 (FGF8) were used in neurogenic differentiation of PDLSCs [58]. For example, in the study performed by Bahrami et al (2007), differentiation potency of PDLSCs and DFSCs used as a stem cell source which has high differentiation potential were compared for the induction and maintenance of neural regeneration [59]. In order to induce differentiation, single-step inducing medium composition containing dexamethasone, ascorbic acid and retinoic acid was used. In addition, any growth factor that was likely to be effective in the differentiation process wasn't included in the medium formulation. Analysis of differentiated neurons after medium administration for 14 days was mostly performed by examining the expression of Microtubule-associated protein-2 (MAP2) and TUBB3 genes. As a result, it was seen that MAP2 and TUBB3 expressions of the differentiated cells given neurogenic inducer medium were upregulated at both transcriptional and translational levels. However, as seen in the literature, PDLSCs were known to express some neural genes at specific levels and it was unlikely to determine that the cells differentiated effectively into neurons based solely on the expression of these two genes [45,66]. In addition, the cellular morphology presented in the study had uncertainty in terms of the success of differentiation. In the study of Trubiani et al (2016), a medium consisting of Neurobasal medium, B27 and bFGF was used to form neurospheres which are the important step for the neurogenic differentiation of PDLSCs [57]. After a period of 10 days, the neurospheres were trypsinized and seeded into collagen-coated plates according to the differentiation protocol. The neurogenic differentiation of the cells was examined in detail using western blot and high-end microscopy techniques. As a result, it was observed that nestin, growth-associated protein-43 (GAP43) and tyrosine hydroxylase (TH) expressions of PDLSCs were upregulated and also their localization, depended on differentiation, changed. In addition, spontaneous intracellular calcium oscillation of differentiated PDLSCs, one of the important evidence for neuronal activity, was demonstrated. In the experiment, while no action was observed in the non-calcium environment, calcium mobilization was significantly increased when potassium chloride (KCl) depolarization was performed. In another example, the study of Romeo et al (2018) was highly beneficial for investigating the effects of herbal extracts on neurogenic differentiation [48]. Any coating substrate was applied to the culture surface in the differentiation protocol and induction medium comprising of Neurobasal medium,

B27 and bFGF were given for 10 days. After the completion of the indicated treatment period, it was shown how the genes related to neurogenesis were changed and arranged by performing transcriptome analysis to investigate the possible effects of moringin on neurogenic differentiation. According to the experiment results, it was reported that there were alterations larger than 2 fold change in moringin at a concentration of 500 nM in 234 genes. In the study of Lee et al (2014), DPSCs, SCAPs and PDLSCs was used as a source of stem cells to investigate their differentiation potential for neural tissues [56]. It was observed that the DSCs had the potential as an alternative stem cell source in relation to the positive and promising experimental results. Laminin and poly-L-ornithine (PLO) coated cell culture plates were used for the neurogenic differentiation by commercial medium (promocell) as inducer. After a 24-hour incubation period, transcriptional and immunocytochemistry analysis were performed by harvesting and fixation, respectively, of differentiated cells. The morphology of the PDLSCs, therefore, were changed to emerge new neurite-like extensions. Although TUBB3 expression of PDLSCs significantly changed as a result of differentiation, no significant change in MAP2 expression was observed. Nevertheless, there was a significant upregulation in MAP2 expression in immunocytochemistry analysis. In the study performed by Malati et al (2018), it was determined that PDLSCs were used as neurogenic differentiation models to investigate the effects of platelet lysates [58]. They were cultured with basal growth medium containing human platelet lysates (PL) instead of FBS prior to induction of neurogenic differentiation. The PDLSCs grown on the PLL used as the coating substrate were differentiated on the two-step induction medium. In the first five days of the protocol, only bFGF was given and subsequently five days, bFGF, FGF8 and SHH containing maturation medium were given. In the study, the evaluation of differentiated cells was performed by analyzing only the alterations in gene expression. Although the transcription level of neural markers is an important factor in the success of the differentiation process, it will not be sufficient for the irrefutable characterization of differentiated cells. According to the results, there was a significant difference in the TUBB3, glutamic acid decarboxylase (GAD) and neuronal nuclei antigen (NeuN) genes of the cells differentiated with 5 percent PL. In the study of Tamaki et al (2012), several stem cells were isolated from the tooth and bone marrow [DPSCs, PDLSCs, DFSCs and bone marrow-derived stem cells (BMMSCs)] and their potential were examined for neurogenic differentiation during this analysis [53]. The differentiation protocol consisted of a single step medium which was applied for 7 days. All-

trans retinoic acid (ATRA), in addition to B27, ITS, bFGF and EGF, was used in the differentiation medium. As a result, immunocytochemistry was performed to analyze the morphology and translational expressions of the cells. Moreover, nestin, TUBB3 and NFH expressions were significantly upregulated. Owing to the neuronal differentiation protocol applied, the cells transformed into multipolar neuron structure. In addition to the protocol commonly used in PDLSCs studies, the effects of bone morphogenetic protein 4 (BMP4) and human telomerase reverse transcriptase (hTERT) gene expressions upregulated by plasmid transfection on neurogenic differentiation were also investigated. Single-step neuronal inducer medium containing bFGF, leukemia inhibitory factor (LIF) and forskolin were used for 72 hours to be used in the differentiation model. According to the experiment results, nestin and glial fibrillary acidic protein (GFAP) expressions, and the morphological changes occurring within a 24 hour period were clearly observed. With respect to the inducing protocol, it was found that these changes were observed due to the lack of any growth factors in the neuronal differentiation medium and the abrupt increment via small molecule inducers of cyclic adenosine monophosphate (cAMP). Consequently, it was investigated that the expression of GFAP, nestin and MAP2 proteins were upregulated significantly and relatedly, it was determined that neurogenic differentiation capacity of PDLSCs modulated with hTERT and BMP4 genes highly augmented.

1.1.4. Cellular Signalling in Neurogenic Differentiation

As in many of the metabolic and physiological events in the cells, the regulation of activation or deactivation of signaling mechanisms that regulate neurogenic differentiation of DSCs takes place under the control of various signaling pathways. A variety of important differentiation protocols allow the regulation of inducer either differentiation or maturation pathways by the use of small molecules [67,68]. While the DSCs differentiate into neuron-like cells, it takes part in the literature that it is possible to differentiate into the specific lineage with the necessary arrangements not only in the basic induction stage but also in the maturation stage [69]. Therefore, it is essential to investigate the properties of the known pathways and their effects on the neurogenic differentiation.

1.1.4.1. cAMP and PKA Signalling in Neurogenic Differentiation

The cAMP is one of the secondary messenger molecules involved in a great variety of vital and fundamental signal pathways in stem cells [70–72]. Secondary messengers are the common name given to intermediary molecules that have an important role in the perception of signals coming from extracellular milieu via receptors in cell membranes and converting them into intracellular signals. In the cAMP signaling mechanism, after an external stimulus or signal is detected by active g-coupled receptors, the membrane-bound adenyl cyclase enzyme is activated and begins to convert the ATP into cAMP. Thus, cAMP starting to increase its concentration in the cytoplasm catalyzes the phosphorylation of transcription factors such as cAMP responsive element binding protein (CREB) which is a regulatory step in signaling pathways by activating protein kinase A (PKA). After phosphorylation of CREB, it can be bound to CREB binding protein (CREBBP) [73,74].
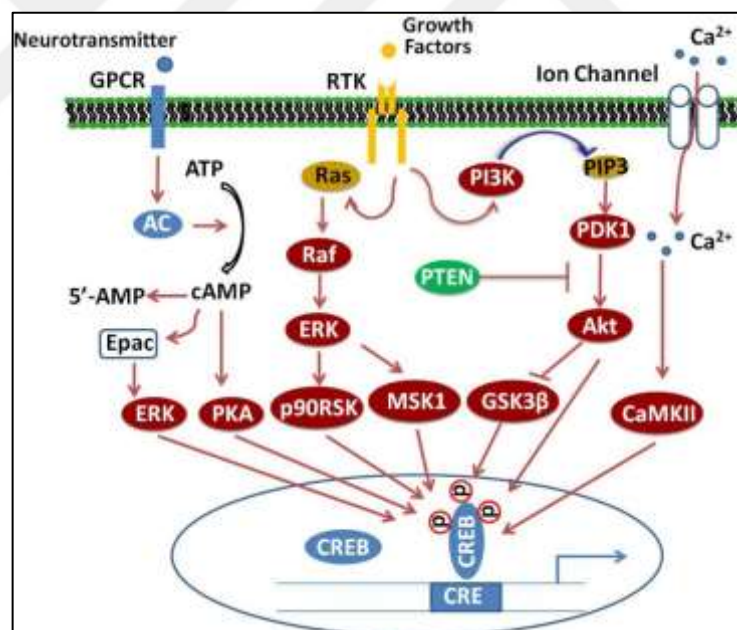
Figure 1.3. Cyclic-AMP dependent signaling in the neural survivability and differentiation processes [75].

Thus, the activated transcription factor regulates the expression of genes such as c-fos, BDNF or TH associated with induction of neurogenic differentiation or neuronal maturation. As a result, genes associated with neurogenic differentiation via cAMP are regulated by the signals from outside the cells [76–80]. However, maintaining the equilibrium of the concentration of cAMP in the cell has an important regulator in terms of differentiation

strategies. Adenyl cyclase inhibition or cAMP destruction are two key points involved in the regulation of the signaling pathway. Accordingly, there are differentiation protocols that are utilized in enhancing intracellular cAMP concentration by providing inhibition of cyclic nucleotide phosphodiesterases with small molecules used [81–83]. Considering all these, it has been determined that it is used greatly in the literature for utilizing in the neurogenic differentiation of stem cells. In DSCs, it has been found to be associated with the induction of neurogenic differentiation by increasing the amount of intracellular cAMP via the use of small molecules of activator or suppressor. For this purpose, the most widely used inductive medium approaches;

i. Increasing intracellular cAMP concentration using adenyl cyclase inducing molecules such as forskolin and cholera toxin (CTX) [67,84,85].

ii. Increasing the intracellular cAMP concentration inhibiting the phosphodiesterases via dibutyryl-cAMP (db-cAMP) and 3-isobutyl-1-methylxanthine (IBMX) [86–88].

Modulation of the cAMP and PKA signaling pathways is known to have an effective role in the neurogenic differentiation of DSCs. For instance, the study of Kiraly et al (2009) is quite important in terms of differentiation of DPSCs to mature and functional neurons via cAMP signalization [89]. In the study, the balanced and regular raise in the cAMP level of DPSCs was achieved by inducing with IBMX, forskolin and db-cAMP after epigenetic modulation. The resulting neurons were found to be functional in terms of both morphological and electrical membrane potential. In order to better understand the role of cAMP in the differentiation process, it was determined that the small molecule inducers were excluded from the medium in the maturation stage and so incomplete neuronal differentiation occurred due to decreased cAMP concentration. Therefore, the regulation of the cAMP concentration was found to be an important factor for neurogenic differentiation. In addition, the study performed by Jermavicule et al (2013) supported the previous one [90]. SHEDs, which were kept in serum-free and basic inducer medium, started to differentiate with the effect of db-cAMP and neural-related growth factors. When neurogenic differentiation was examined morphologically, it showed significant alterations with the application of db-cAMP, where the maturation stage started. According to the experimental result, both neurogenesis-related and cAMP-responsive genes were found to vary significantly between the control and sample groups. In the study of Lepski et al (2010), it was shown that MSCs could be

successfully differentiated by using BDNF and IBMX in conjunction with the cAMP pathway as a determining factor on neurogenic differentiation [91]. Afterward, it was shown that neurogenic differentiation would be adversely affected when various PKA inhibitors were used to show that actual changes occur by the effect of increased cAMP levels. In the patch clamp analysis, voltage values of Na^+, K^+ and Ca^{++} of successfully differentiated neuron-like cells were examined. Although a similar cellular yield was obtained with MSCs, differentiated neural stem cells were found to be phenotypically and functionally more stable in the maturation stage. The role of the cAMP pathway in neurogenic differentiation in undeniable, however it has been observed that some studies have been misleading though. In the study of Kadar et al (2009), three different induction protocols were used to differentiate the DPSCs. The first two of these protocols were associated with pseudo-neurogenesis in the literature and are characterized by an abrupt increase of cAMP or by the use of chemicals that damage the cell membrane and cytoskeleton [92]. As a result, the importance of the utilization of epigenetic regulation and neural-related growth factors for successful differentiation was further emphasized in this study. In the experiment, a rapid cAMP inducer medium was used to further elucidate the related matter. Although a visible morphological alteration was seen in the cells, it was observed that they began to turn back at the end of 24 hours and after the exclusion of small molecules, restored within 48 hours. In addition, in the study performed by Zhang et al (2011), forskolin and IBMX were used to differentiate BMMSCs in order to explain the capacity of cAMP to induce adult stem cells to mature and functional neurons [93]. They observed that the alterations in the neuron-like cell morphology occurred within hours that occurred just as seen in the previous article. These changes in morphology are thought to result from the damage that occurs in certain parts of the cytoplasm rather than a differentiation of neuronal pattern of stem cells at a level of transcriptional and translational expression. In addition, there was no change in neuronal morphology with the increase in the duration of differentiation medium treatment even if the fresh medium was replaced. Surprisingly, it was determined that the cAMP activators were increased the neuronal markers and calcium signalization of certain neuronal stimuli in the late stage of differentiation in contrast to alterations in the early stage induction. Furthermore, it was determined that the cAMP inducers can't achieve the differentiation to mature and functional neurons by itself because the responsive neuron-like cells aren't like primary neurons in any way [94].

1.1.4.2. Neurotrophin Factors Dependent Signalling in Neurogenic Differentiation

Neurotrophins are a family of proteins produced by various cells in the nervous system, which provide development, survivability, and regulation of multiple signalization pathways of neurons [95–99]. The major proteins in the family are NGF, BDNF and neurotrophin 3, 4/5, 6. The neurotrophin protein family activates to membrane receptors which belong to two different class as Trk family of tyrosine kinase receptors and NGFR family of tumor necrosis factor (TNF) [100]. Following trans-phosphorylation in the trk receptor, three important signaling pathways; mitogen-activated protein kinase (MAPK), phospholipase C (PLC) and phosphatidylinositol-3-kinase (PI3K), which control the neurogenic differentiation. Moreover, MAPK enzymes that are activated by various neurotrophins or neuronal stimulation are serine and threonine kinase that can translocate to the nucleus owing to their signal cascade [101–103]. After the activation of MAPK, which controls the translocation of the transcription factors to DNA during activation with a signal from extracellular milieu, the two protein kinases (Raf and Mek) mediates the extracellular-signal regulated kinase (ERK) activation. After the Raf is activated and phosphorylated Mek, ERK is activated by phosphorylation and phosphorylates the transcription factors necessary to regulate gene expression and so the extracellular signals are converted into the induction of gene expression. MAPK and ERK enzymes, which are highly expressed in the nervous system, are involved in several different processes including neuronal maturation, a continuation of vital activities and synaptic functions [104,105]. In addition to MAPKs, it is also important to induce the formation of phosphatidylinositol 4,5-bisphosphate (PIP_2), which is the phospholipid secondary messenger involved in various intracellular signaling pathways, by neurotrophins. The interaction of PIP_2 with PLC results in two different intermediate messenger molecule: Diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP_3). By increasing the intracellular concentration of these two messengers, they activate the pathways of protein kinase C (PKC) and calcium release and thus have a role in the neuronal functionality and survivability [106,107].
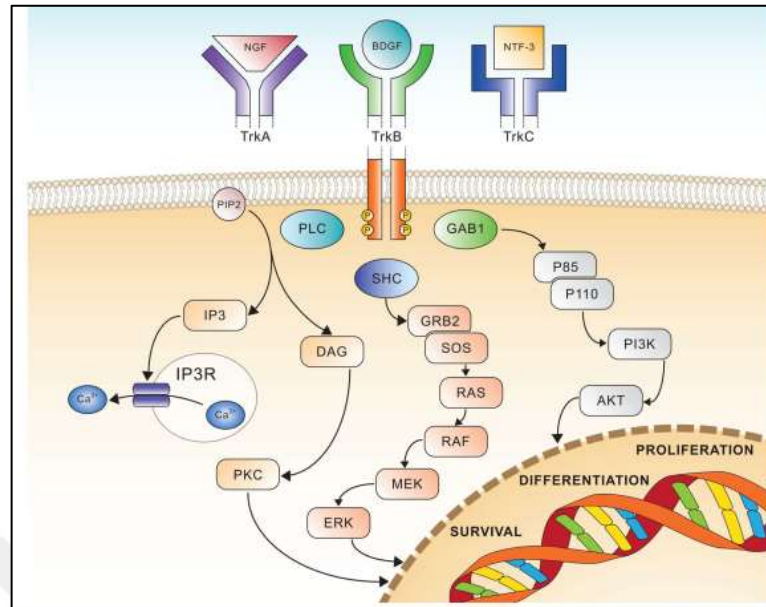
Figure 1.4. The neurotrophin dependent signaling with three core pathways implicated in neuronal differentiation and growth [108].

Finally, the PI3K/protein kinase B (Akt) pathway activated by neurotrophins has a place in the continuation of vital activities of neurons. The neurotrophin pathway can be activated by not only direct binding the PI3Ks to the neurotrophin/receptor tyrosine kinase (RTK) complex and also the membrane-bound PIP_2 transforming PI3K to phosphatidylinositol (3, 4, 5)-triphosphate (PIP_3) by phosphorylating and binding to Akt [109,110].

As a result of the importance of the pathway, neurotrophins have been found to be highly used in neurogenic differentiation of DSCs in the literature. Furthermore, they are also important in the lineage-specific neuronal approaches of differentiation protocols in addition to ensuring the continuity of functionalities and vital activities of neurons. For instance, the study performed by Sing et al (2017), the importance of neurotrophin treatment in the differentiation protocol was investigated by carrying out analysis on the neurogenic differentiation of DPSCs [23]. According to the induction protocol, the cells were treated with bFGF and BDNF for approximately 10 days. Neurogenic differentiation of DPSCs, which showed the highest neuronal differentiation potential among three cells lines used in the study, was evaluated by morphological, transcriptional and translational analysis. As a result, it was found that the DPSCs couldn't only be differentiated into functional and mature neurons but also gained fundamental dopaminergic features by utilizing BDNF in the inductive medium. The study of Chang et al (2014) has the importance in terms of

differentiation of DSCs into two different mature neurons types as motor and dopaminergic neurons by using neurotrophins [64]. In the induction protocol, BDNF, glial-derived neurotrophic factor (GDNF) and ascorbic acid were used to the genesis of dopaminergic neurons and insulin-like growth factor (IGF-1) and SHH was used for the motor neuron differentiation. As a result of the experiments, the modulations in the expression of neural marker genes draw the attention in addition to the noticeable morphological changes, which occur in consequence of neurogenic differentiation. TUBB3, nestin and GFAP expressions which are neural markers for differentiation were significantly upregulated in both groups. Moreover, the lineage-related functional factors such as TH and choline acetyltransferase (ChAT) were found to upregulate significantly in their own groups. Finally, NGF has been found to be associated with differentiation in cholinergic neurons.

1.2. THE BIOLOGY OF EXOSOME

1.2.1. Classification of Extracellular Vesicles

Intercellular communication and the regulation of the biochemical activity has a place in maintaining both the metabolic stability at the cellular level and physiological functions of the cell [111–113]. This information transfer system among the cells operates in general based on two essential processes. The first one is the passage of messenger biomolecules via physical contact of the membranes of adjacent cells [114–116]. Another is the transmission of soluble bioactive factors, such as hormones, through the circulatory system or tissue in order to communicate with the cells at a variable distance [117–120]. In the studies conducted in the last 20 years, it has been determined that the extracellular vesicles (EVs) have a crucial role in intercellular communication and even they are peerless for some biological systems. These unique vesicles, which can be effective by frequently paracrine mechanism, contain lipids, proteins, nucleic acids, receptor molecules and various effector molecules in their bilayer lipid membranes [121,122].
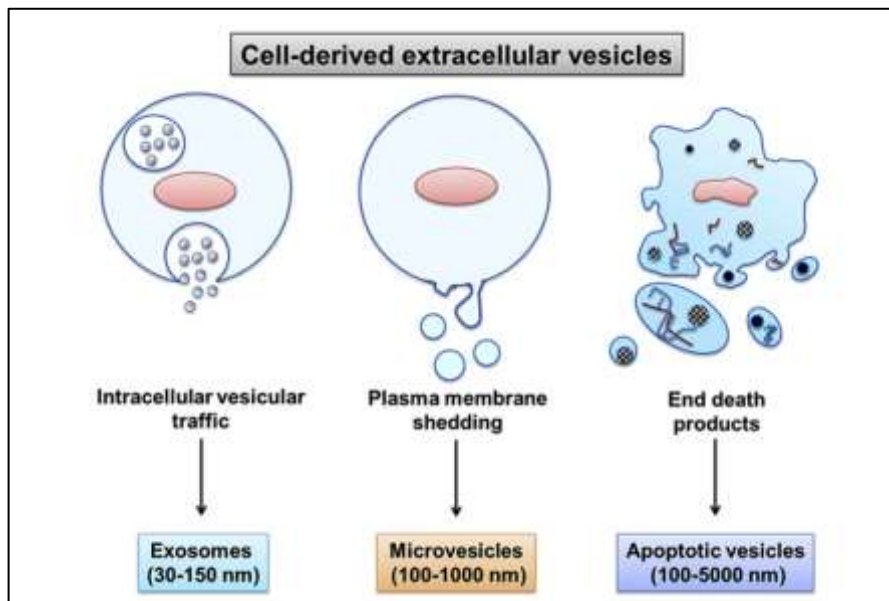
Figure 1.5. The categorization of extracellular vesicles as to the origin of biogenesis [123].

Moreover, it is ensured that the molecules are transferred among cells without losing their activity or disintegrating in the circulatory system due to the rigid lipid structure in comparison with the parental cell membrane [124,125]. In general terms, EVs are classified predominantly according to their origins in the biogenesis and dimensional features. Nowadays, EVs are divided into three different groups as microvesicles, exosomes, and apoptotic bodies [126]. First of all, ectosomes or microvesicles are emerged either by budding from the plasma membrane of the parental cells, or by regional fragments in the membrane. It is believed that EVs whose dimensions range from 100 nm to 1000 nm is responsible for the transition of specific lipid, nucleic acids, and proteins to recipient cells [127]. In fact, the exosomes, which comprise following the formation of microvesicles, are typically 40 nm to 100 nm and also provide the transmission of specific biomolecules among cells. Exosomes, which are secreted from only eukaryotic cells and present in almost every bodily fluid, are also biomarkers related to metabolic and pathophysiologic conditions thanks to the dynamic modulation of their original contents [128]. Finally, apoptotic bodies are the vesicles ranging in the size from 50 nm to 5000 nm, which are released from the cell in the process from that moment on the beginning of the dissemination of apoptosis signal. The apoptotic bodies, therefore, contain nucleic acids and histone proteins due to their known fragmentation process. Furthermore, it is determined that they activate the macrophages owing to these substances [129].

1.2.2. Exosome Biogenesis and Regulation

When the molecular mechanisms involved in the biogenesis of exosomes are examined, it has been determined that they have diversified properties in many ways by comparison with biogenesis pathways of microvesicles [130]. Typically, three different phases are observed in the cell during the biogenesis of exosomes [131]. In the biogenesis pathway, the emergence of intracellular vesicular structures which are formed by breaking away from the cell membrane, i.e. early endosomes, is observed [132]. Subsequently, early endosomes develop by the influence of ALIX, tetraspanins (CD9 and CD82), tumor susceptible gene 101 (TSG101) and vacuolar protein sorting 4 homolog B (VSP4B) proteins in order to generate late endosomes or multivesicular bodies (MVB) [133,134]. At this stage, there are three possible continuation pathways known today, and this point in the pathway is extremely important in order to elucidate the biogenesis mechanism of exosomes in detail. First one of them ends up by breaking up fully formed late endosomes combined with the lysosome in the cell and no exosomes are excreted to the extracellular milieu [135–137]. In the second pathway, according to recent studies, it has been determined that there was a connection between exosomal biogenesis and amphisome related autophagy. MVBs, a key structure in the biogenesis of exosomes, have been suggested to be disintegrated by transformation to amphisome and so autophagy process as well as by joining directly in the lysosome or secretion out of the cells [138–140]. In the study performed by Fader et al (2008) regarding this condition, it is found that the number of secreted exosomes was decreased sharply when autophagy was induced by starvation in the cells [141]. In addition, it is envisaged that the cells utilize the autophagy mechanism via MVBs as an alternative way of compensation due to a defect in lysosomes, the incurrence of damage or overloading of the lysosomal mechanism.
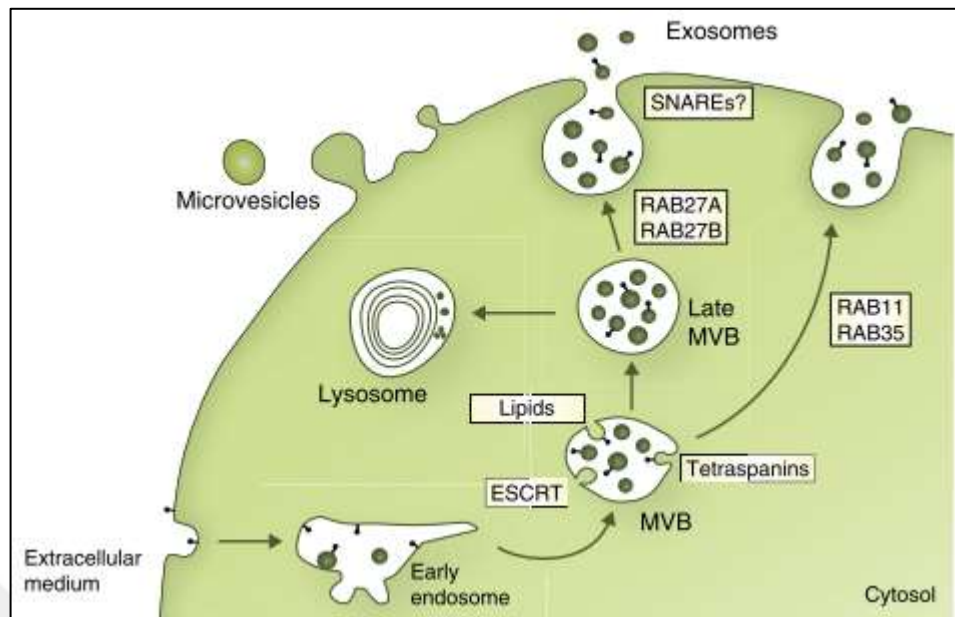
Figure 1.6. Intracellular pathways of exosomal secretion or degradation [142].

In the third pathway, the MVBs combine with the cell membrane to transfer the exosome vesicles out of the cell depending on the supervision of the Rab protein family, which contains Rab27 a/b and Rab 7, 11, 35 effectors, and SNARE proteins that administrate the secretion of intraluminal vesicles which are earlier form of exosomes found in the MVBs [143–146]. In addition, the endosomal sorting complex required for transport (ESCRT) family of proteins, which play a key role in exosome biogenesis, consists mainly of 4 soluble protein complexes. These protein complexes, which have different structures and functions in the biogenesis pathway, are called ESCRT-0, ESCRT-1, ESCRT-2, ESCRT-3. Studies on the regulation of vesicular secretion have shown a positive correlation between exosomal release and upregulation of ESCRT protein expression [147,148]. However, in the studies of reducing exosomal secretion by suppression of the ESCRT protein family, it is known that exosomes continue to secrete by a non-ESCRT-dependent mechanism, even if the expression of these proteins is suppressed [149]. Finally, in recent years, highly promising and informative studies have been made about the detailed mechanism of vesiculation and secretion from the cells [131]. However, it is essential to verify hypothetical information by conducting more detailed studies to elucidate this pathway due to the unspecified and hypothetical aspects of the biogenesis mechanism of exosomes.

1.2.3. Molecular Characteristic of Exosome

Exosomes which undertake the task of carrying information among the cells include the intermediate or end-product that are actively involved in the signaling pathways. Therefore,

they carry off the proteins, lipids, polysaccharides, nucleic acids that are highly specific to parental cells in vesicular structures in order to protect molecular stability and biological structures [150].

1.2.3.1. Protein Characteristic of Exosome

A great variety of exosomal proteins which has a vital role in the cellular signaling pathways have been found to be highly prevalent in accordance with biomolecular distribution in vesicle composition. According to the results of the different studies, it was observed that the protein composition was formed intensively by cytosolic proteins, heat shock proteins, cell skeleton related structural proteins and proteins working in the biogenesis of vesicles and receptor proteins on the cell membrane [151–153]. In addition, the proteins of the intracellular organelles were found to be much less than other types of proteins [154]. The studies on the functional and physiological properties of exosomes shown that each vesicle secreted from the parental cell contained a unique set of properties belonging to that cell. Moreover, it was determined that many factors such as the characteristics of the extracellular media, availability of the medium conditions, stimulation of cell death or proliferation created different protein compositions depending on the conditions, even if they were the same cell [155–157]. The specific surface receptors that are recognized by the immune system are particularly important in terms of the functional protein composition of exosomes [158,159]. Therefore, characterization of the exosomes is carried out using these high-level receptor proteins. The most common examples of marker proteins are tetraspanins (CD81, CD63, CD9), Major histocompatibility complex (MHC-1,2), specific stress proteins such as heat shock proteins (HSP-70), ALIX and annexin, flotillin and integrin proteins involved in cell membrane transition and adhesion process [160].

1.2.3.2. Lipid Characteristic of Exosome

The lipids found in the structures of exosomes have a role in the formation of the vesicles, regulation of the surface interactions with recipient cells and stiffness of the membrane structure by interacting with other molecules or by directly participating in the structure of vesicle membrane [161–163]. It has been determined that they have an important role in the

regulation of early endosome biogenesis when transferred from the extracellular milieu to the recipient cells [164]. In addition to these functions, the specific active lipids (prostaglandins, leukotrienes, fatty acids) in the cell have a direct or indirect effect on the activation of fundamental metabolic events due to their use in multiple signalization pathways. It was found that high levels of cholesterol, sphingomyelin, glycosphingolipids, phosphatidylserine (PS) were present in exosomes by lipidomic analysis [163,165]. According to the results of lipidomic analysis present in exosomes, important findings of the specificity of lipids and their similarities to the parental cell was obtained correlatively to the results of studies on exosomal protein composition [166]. In the recent studies, it was detected that the lipid species and quantities, which make up the composition in the exosome membrane, were not proportional to the abundance of lipid found in the membrane of the parental cells. In the study of Baig et al (2013), which is quite an informative one, the lipid types and quantities in the membrane structures of exosomes and parental cells obtained from placental tissues were compared [167]. As a result of the experiments, exosomes have been observed to be unusual in sphingomyelin and cholesterol in comparison with parental cells. Even so, in the biogenesis of exosomes, the membrane structures of vesicles are expected to show similar points with the lipid composition of cell membrane because typically the lipid-rich plasma membrane is provided by the parental cells. However, the membrane structures of exosomes were found to be rich in cholesterol and sphingomyelin compared to the phospholipid structure, which forms the majority of parental cell membranes [168]. As a result of these differences, the membrane structures of the exosomes were found to have a more resilient and rigid structure as opposed to the cell membrane structure as well as having lipid raft fragments in the lipid composition of the exosomal membrane.

1.2.3.3. *Polysaccharides Characteristic of Exosome*

Exosomal polysaccharides are typically incorporated in the structure of the cell membrane by interacting with other molecules, in particular, proteins, rather than in free form within the vesicles [169]. Unlike the lipids that lead to changes in the cell membrane structure, they function by complexing with proteins in order to emerge the glycoproteins and so highly specific epitopes on the exosomal surface [170,171]. The first studies on the presence of polysaccharide molecules in exosomes are about the examination of the lectin protein, which

has an important role in the realization of the protein glycosylation. It has been found that the exosomes have different glycosylation patterns than the motifs derived from the parent cell due to the binding of carbohydrates to lectin proteins that have high affinity to sugar molecules [172]. As a result of the analysis, exosomes are highly rich in epitopes that interact with high levels of mannose and in some fructose, including N-glycan and N-acetyllactosamine. Another characteristic feature of the polysaccharides found in the vesicular membrane is the interaction of the glycosylation motifs on the membrane surface with a number of specific ligands in order to the regulation of the uptake mechanism of exosomes [173,174]. As a result of inhibition of heparan sulfate proteoglycans through the addition of heparin to the medium, interruption of the exosomal uptake into the recipient cells is an important example of this case [175].

1.2.3.4. *Nucleic Acid Characteristic of Exosome*

Nucleic acids, one of the important biomolecules found in exosomes, are found in two different types, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) [176,177]. According to the results of the studies, we have more information on exosomal RNAs in terms of both structural information and functionalities than exosomal DNAs [178]. In recent studies about exosomal DNAs, it has been determined that apoptotic bodies contain high levels of oncogenic and mitochondrial DNA as well as single-stranded and double-stranded ones [179]. It has been suggested that exosomal DNA should be involved in a molecular application to detect the cancer disease in terms of diagnostics or progression after detecting that exosomal DNA from cancer tissue mostly carries and transmits cancer-related biomolecules such proteins and micro RNAs (miRNA) [180,181]. In addition, according to the current information, the suitability of exosomal DNA as a biomarker or the possible effects on the pathophysiological pathways is still not fully understood and agreed. Exosomal RNAs, of another type nucleic acid found in exosomes, exist in a great variety of RNA types which can be found in free form or in a protein complex within the vesicle [182]. There are various quantities and sizes of ribosomal RNA (rRNA), stable messenger RNA (mRNA), mRNA fragments, long non-coding RNA, miRNA, transfer RNA (tRNA) fragments and small RNA (sRNA). Exosomal RNAs play a role in the transmission of important properties of the vesicles in which they are secreted, to the recipient cell. In the studies, it has been observed that there are significant effects of miRNAs which are

transferred within the vesicle and they have a high capacity to affect gene expression [183–186]. Furthermore, the elimination of specific miRNAs transferred between cells and the transfer of miRNAs determined to be necessary by transferring them to the cells are the important examples about the regulation of exosomal RNAs.

1.2.4. Internalization Mechanisms of Exosome

It is known that exosomes secreted from parental cells have information about the cellular metabolism status through the biomolecules which are transferred among the cells and internalization of exosomes have a place in the signal transition. Therefore, it has been questioned whether exosomes were internalized by passive diffusion or by an uptake mechanism which required energy while taking the biological signals carried into the cell. In the study on this mechanism, a decline decrease was observed in the internalization of exosomes as a result of the decrease of 4°C in temperature [187,188]. In addition, the plasma membranes of the cells were fixed by 4 percent paraformaldehyde (PFA) and performed the exosomes treatment, there was a decline once again in the uptake of the vesicular structures [189,190]. According to the results of these studies, it was understood that internalization of the exosomes into the cell is not a passive process and requires significant amount of the energy. Therefore, it is known that there are a number of active internalization pathways that are important for the transition of the contents of the exosomes in the extracellular milieu to the cells [188,191–195]. As is known in the literature, exosomes can transfer the information to the cells either by taking into the cell as a vesicular form or by transferring over surface receptor as an extrinsic signal. Exosomes are extrinsically used to cleavage of the growth factors on the surface of the vesicle membrane by metalloproteases, namely soluble signaling, or direct binding of the membrane to the juxtacrine signaling pathways. In addition, membrane fusion, phagocytosis, macropinocytosis and receptor/raft-mediated endocytosis pathways are used to internalization of exosomes. Moreover, it was understood that the internalization pathways preferred by exosomes had an important place in the cellular responses of the transferred signals. Whereas it can transfer directly nucleic acid and protein molecules to the cell by fusion, it is known that it goes into a clearance in the internalization by phagocytosis. In contrast, in some cases, it was observed that the exosomes could not directly break down and emerge a cellular response after the uptake by macropinocytosis and endocytosis.
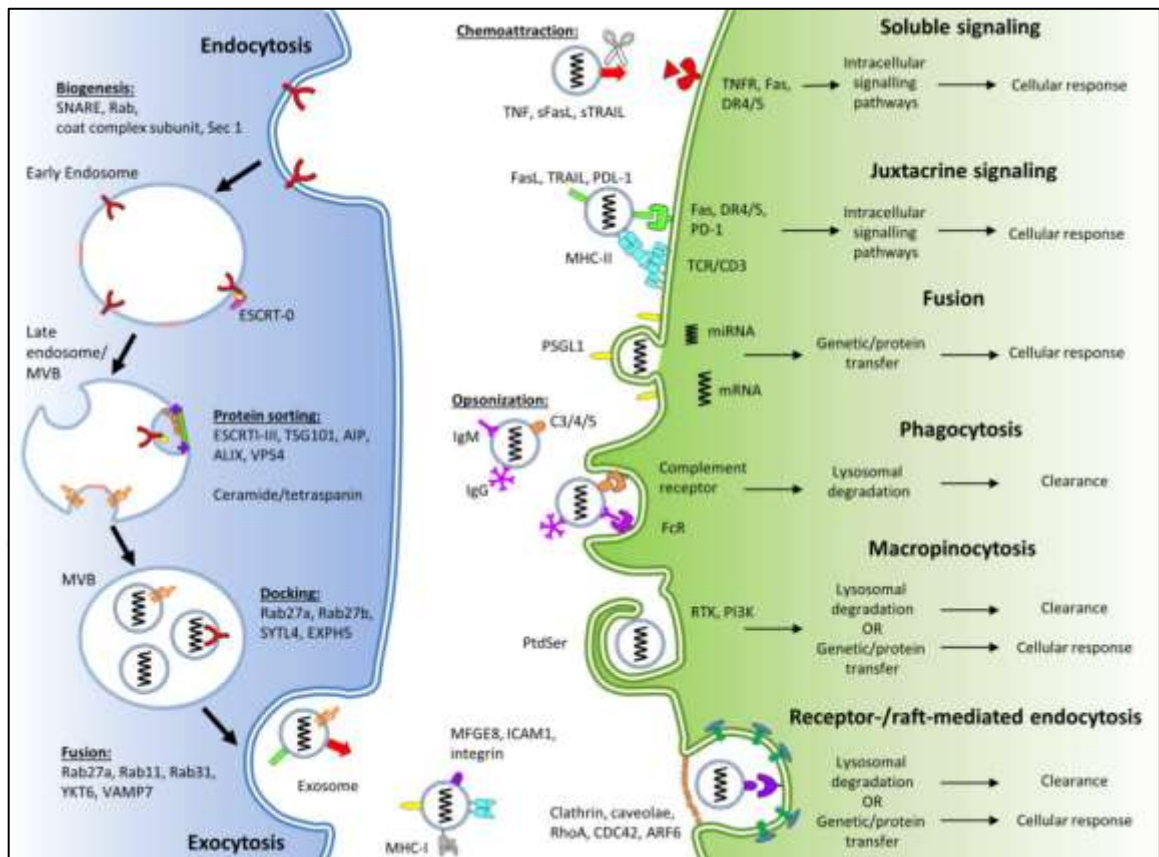
Figure 1.7. Biogenesis and internalization pathways of exosome [194].

1.2.4.1. Soluble Factor and Juxtacrine Mediated Internalization

In addition to the biomolecule-containing cargo carried by the exosomes during the biogenesis in the cell, it was discovered that a number of non-soluble membrane-bound proteins, especially growth factors on the vesicle membrane were present [196,197]. As a result, these membrane-bound growth factors are cleavaged from the membrane surface to the soluble form by the metalloprotease enzymes present in the extracellular milieu before being taken into the cell for information transfer between cells. As a result of the studies, membrane-bound TNF, TRAIL and FasL ligands were detected on the exosome surface [198–200]. An important example of the activity of these ligands was the investigation of the exosomes taken from the placenta and plasma of pregnant women [201,202]. In accordance with experimental results, it was found that the FasL and TRAIL proteins, which were present on the membrane or switched into a soluble form, induced the apoptosis of Jurkat T cells. Another example of the regulation of the immune system is the suppression of immune cells with similar membrane-bound factors found in exosomes originated from

cancer cells [203,204]. However, proteins bound to the membrane of exosomes are known to bind to each other by positioning with the receptors in the cell membrane without degradation [204,205]. According to the results of the studies, it was determined that the activities of apoptosis-inducing ligands such as FasL in the membrane-bound form were far higher than a soluble form of exosomal growth factors [206]. Therefore, the protection of membrane-bound proteins on the surface of exosomes during isolation and storage is extremely important in terms of the activity of receptor proteins.

1.2.4.2. Fusion Mediated Internalization

One of the important mechanisms used in the transfer of biomolecules carried by exosomes is the vesicular fusion and takes place with two functional modulations. In the first one, it was envisaged that membrane fragments from the exosomes were integrated into the membrane of the recipient cell and so they could transfer the characteristic fragments of the parental membrane [194,207,208]. In the second pathway, exosomes can transfer the carried biomolecules directly to the cytoplasm [209]. In the study of Zhang et al (2019), it was observed that fusion was included in the internalization pathways that multiple myeloma-derived exosomes used to get into the bone marrow cells [193]. Since the recognition and binding of the surface receptors are involved during the internalization of exosomes, it is contemplated that the choice of exosomal uptake by fusion can be determined and targeted by means of detection of fusion related receptor proteins. An important finding for the realization of this arrangement is the regulatory effect of ambient pH on the internalization pathway of the cell in the uptake of exosomes. In the study performed by Svensson et al (2009), it was observed that exosomes taken from metastatic melanoma preferred membrane fusion at low pH [192,195]. In addition, it has been found that caveolin-1 protein, which negatively affects endocytosis, is abundantly found on the surface of exosomes in an acidic environment. Although important information has been discovered about the main procurement mechanisms, the pathways for vesicle fusion is still not fully explained or understood. When the fusion pathway is activated in the cell, tetraspanin-enriched microdomains (TEM) have been found to be involved in multiple processes including cellular and vesicular fusion in the vesicle-cell interaction. Moreover, these protein complexes have increased the possibility of exosomal fusion due to processes involved in cell adhesion [210]. Tetraspanins, which are found in relatively high rate in comparison with

other protein ratios, are present in the structures of transmembrane receptors, adhesion molecules and tetraspanin clusters. They are located in raft-like complexes in the plasma membrane in order to control the fusion pathway.

1.2.4.3. Phagocytosis

Phagocytosis, which is a type of endocytosis that requires specific opsonization receptor and tool like receptor through actin proteins, is one of the vital functions of professional phagocytic cells, it can be performed by many common cell types though [211,212]. In the studies, it has shown that exosomes are internalized into the cell by phagocytosis. In the study of Feng (2010), K562 and MT4 derived exosomes were shown to be more internalized by phagocytic cells than non-phagocytic cells [213]. In addition, the exosome internalization due to actin skeleton and PI3K activation was determined to be inhibited by the suppression of dynamin 2. At the same time, it was stated that caveolae, macropinocytosis, and clathrin-coated vesicles related uptake pathways weren't linked with the internalization of exosomes. Although it is undeniable that phagocytosis is a very important pathway for exosome uptake, the experimental results have been found to be contradictory with studies in the literature [195,214–216]. In addition, it should be investigated whether exosomal uptake by phagocytosis is only a process related to clearance or it has a role in the communication among cells by means of vesicle transition.

1.2.4.4. Macropinocytosis

In addition to other exosomal uptake mechanisms, macropinocytosis, which is a type of endocytosis that non-specifically allows the introduction of exosomes, is another example of the actin-dependent uptake mechanism. Although internalization pathway hasn't been fully described like other ones in the literature, some findings have been obtained about macropinocytosis. In the study of Tian et al (2014), PC-12 derived exosomes were given directly to the bone marrow cells and uptake pathways were examined [215]. As a result of the experiments, it was determined that macropinocytosis and clathrin-mediated endocytosis were performed by analysis utilizing selective uptake inhibitors and special endocytosis markers. In addition, it was found that downregulated caveolin 1, flotillin 1, RhoA, Rac1

and PAK-I proteins brought to a standstill the internalization by macropinocytosis. Despite the elimination of exosomes internalized by phagocytosis, it was observed that exosomes taken by macropinocytosis didn't directly lead to clearance and eventuated as a cellular response. Accordingly, certain uptake mechanisms can be activated as desired, so that the internalization pathways of exosomes into the cell can be controlled and targeted. In a study performed by Nakase et al (2016), the membrane surface of exosomes was modulated in order to prefer the internalization of exosomes by macropinocytosis and increase the cellular response [214]. Therefore, it was determined that the exosomal uptake of the cells by macropinocytosis increased significantly after the modification of the membrane surface with stearylated octarginine.

1.2.4.5. *Receptor/Raft Mediated Endocytosis*

One of the most important and most studied mechanisms of exosomes internalization is the receptor/raft-mediated endocytosis (RRME). Obviously, there must be special ligands on the exosome surface that will interact with the receptors on the plasma membrane of the cell for the induction of RRME [173,188]. In addition, the presence of lipid raft structures formed by microdomains rich in cholesterol and sphingolipid in the vesicle membrane is quite important in terms of regulation of raft-mediated endocytosis (RME) [217]. According to studies, it was determined that the vesicles were taken into the cell within 15 minutes following the administration of exosomes via RRME, one of the most preferred pathways in the exosomal uptake of the cells [216]. Although several clathrin-independent endocytosis inducing pathways have been found in the RRME mechanism, it can be examined the pathways of general endocytosis in two ways. Two different mechanisms classified as clathrin-mediated endocytosis (CME) and caveolin-dependent endocytosis have been used to provide the internalization of exosomes to the cells [218,219]. The most important difference that distinguishes CME from others is that it requires clathrin complexes formed by scaffold proteins in the triskelion structure consisting of 3 heavy and light protein chain to the activation of internalization. Clathrin complexes in the CME mechanism polymerize around the cytoplasm of the endocytic sac that invaginates into the cells, allowing the vesicle to form without direct contact with the membrane [220]. In contrast, caveolin-dependent endocytosis (CDE), a clathrin-independent mechanism, is pit-like structures formed by invagination on the plasma membrane called caveolae similar to CME. The caveolae which

have a key role in the CDE mechanism are the sub-domains of glucolipid raft formed by cholesterol, sphingolipids and caveolins in the plasma membrane [221,222]. As a natural consequence of this, it was determined that endocytosis function was damaged when exposed to chemicals and environmental conditions like fumonisin B1, methyl-beta-cyclodextrin (MBCD) and simvastatin, which cause depletion and inhibition of occurrence of cholesterols or sphingolipids [223–225]. In addition to the important functions of lipid raft structure, it was determined that caveolin-1 proteins acted as key regulators in the emergence of caveolae formation. Moreover, it was determined that the accumulation of cholesterol levels increased and correlated with the complex formation of Caveolin 1 proteins which are activated functional status with the participation of dynamin 2 [195]. The effect of regulators proteins on exosomal uptake in each pathway can be investigated through the application of specific inhibitors in internalization phases.

1.2.5. Plant-derived Exosomes

Although the biogenesis and secretion pathway of exosomes in mammalian cells is not fully understood and explained, basically a few fundamental points are very important in the occurrence of vesicles. These are composed of two major points as the cells have a Golgi apparatus for exosomal secretion and the presence intracellularly of the functional multivesicular body [131,226,227]. As a natural consequence of these, it is expected inevitably that plants such as other eukaryotic cells can produce exosomes and secrete into the external environment. Accordingly, it is of utmost importance to show that the plant cells have the MVB in terms of verification of this important phenomenon [228]. In the past decade, it was observed that the studies on exosome-like structures originated from plants increased considerably. In the studies, it has been detected that the plant-derived exosomes have a number of similarities with mammalian vesicles and vice versa, significant differences in critical points. The noticeable common features of plant-derived exosomes are that they have similarities in both dimensional and vesicular morphology, double-layer lipid membrane transports, and similar biomolecule groups [229]. In addition, it is noteworthy that exosomal protein markers such as CD9, CD81, CD63 and membrane-bound HSP70 contained in the plasma membranes of vesicles are positive owing to their conserved protein sequences. Exosomes involved in mammalian cells responding to physiological events and intercellular communication also provide information about the metabolic status of parental

cells. It is shown that plant cell-derived exosomes have almost similar functions. In a study of Cai et al (2018), the exosomal secretion of *Arabidopsis thaliana* was investigated after fungal pathogen *Botrytis cinerea* infection [230]. As a result, it was determined that *Arabidopsis thaliana* transmitted the sRNA species which would silence pathogenic damaging genes against fungal pathogen via exosomes. Thus, it has been found that plant cells are actively responding to the stimulations arising extracellular milieu by exosomal structures and that exosomes can be used as a communication tool to cross-kingdom signaling. In addition, the functional effects of exosomes obtained from fruits were analyzed with the objective to investigate the therapeutic application of plant-derived exosomes. Accordingly, the exosomes were purified by differential ultracentrifugation and their functional characteristic was investigated on experimental animals. An extremely important phenomenon is that it is requisite to know that the purified final product won't consist solely of exosomes or extracellular vesicles due to the dispersion of intracellular structures during the purification of fruit derived exosomes. One of the important studies on the therapeutic effect of the plant-derived exosomes in the literature was aimed to investigate the activity of grape-derived exosomes on the DSS-induced colitis model [231]. As a result, it was reported that the generation of Lgr5$^+$ stem cells in the intestine was induced by grape-derived exosomes, thereby inducing a protective effect. In the study performed by Zhuang et al (2015), the effect of ginger-derived nanoparticles was investigated on the alcohol-induced liver damage in mice [232]. The aqueous extract from the ginger root was purified by exosomes by differential ultracentrifugation and sucrose gradient, and then applied to mice by gavage. As a result of the experiments, it was determined that ginger-derived nanoparticles, which seemed to inhibit alcohol-induced ROS, protected the cells from the liver damage caused by alcohol. Finally, in the study of Deng et al (2017), it was detected that the broccoli-derived nanoparticles ensured the homeostasis in the chronic inflammation related colitis model by modulating the immune cells of intestinal tract through oral administration [233]. Specifically, they induce the inhibition of dendritic cells by activation of AMP-activated protein kinase (AMPK) pathways. Thus inflammation related colitis damage was prevented. Therapeutic activity, as well as its use as a cargo carrier in the literature, are quite examples.

1.3. AIM OF THE STUDY

The purpose of the present study is to investigate whether *Citrus sinensis* derived exosomes (CSDE) has a functional effect on the in vitro neurogenic differentiation model of HPDLSCs. It is also within the scope of the aim to discover the potential effects of CSDE on which cellular signaling molecules affect the differentiation process in the cell.

In accordance with this purpose, the thesis consists of 3 main stages:

i. To characterize and evaluate the molecular features of exosomes isolated from fruit extract of Citrus sinensis.

ii. To establish of neurogenic differentiation model of HPDLSCs to functional and mature neurons using multiple chemically-defined inductive media.

iii. To explore whether CSDE has a significant effect on the neurogenic differentiation process.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Instruments

Table 2.1. Instruments utilized in all experiments.

	Brand	Item	Cat. No.
1	Esco	Cell Culture Hood	Class II BSC
2	Memmert	CO2 Incubator	INB-500
3	Sigma	Centrifuge	3-18 KS
4	Waring	Blender	8011 EB
5	Eppendorf	Vacuum Centrifuge	5301
6	Malvern	Nanosight	NS300
7	Zeiss	Scanning Electron Microscope	EVO LS 10
8	Zeiss	Inverted Microscope	Axio Vert.A1
9	Zeiss	Fluorescence Inverted Microscope	Axio Vert.A1
10	BioTek	Microplate reader	EL800
11	ThermoScientific	Varioskan LUX	VL0LA0D0
12	ThermoScientific	NanoDrop Spectrophotometers	2000
13	Bio-Rad	ChemiDoc	XRS+
14	BD Biosciences	Cell Analyzer	FACSCALIBUR

2.1.2. Chemicals

Table 2.2. Chemicals utilized for all experiments.

	Brand	Item	Cat. No.
1	Gibco	Dmem Low Glucose	11885084
2	Gibco	Fetal Bovine Serum	10500064
3	Gibco	PSA	15240062
4	Gibco	Trypsin-EDTA (0.25 percent)	25200056
5	Gibco	Dmem/F-12	10565018
6	Gibco	B27 supplement	17504044
7	StemCell	bFGF	78003.2

8	ThermoScientific	EGF	PHG0313
9	Sigma	Valproic acid sodium salt	P4543
10	StemCell	Forskolin	72114
11	Merck	IBMX	410957
12	StemCell	BDNF	78005.2
13	StemCell	GDNF	78058.2
14	Sigma	Poly (ethylene glycol)	81310
15	Sigma	Dextran from Leuconostoc spp.	31392
16	Sigma	Methanol	24229
17	ThermoScientific	Aldehyde/Sulfate Latex Beads	A37304
18	Sigma	Bovine Serum Albumin	A9418
19	Santa Cruz	CD9 Antibody	124808
20	Santa Cruz	CD63 Antibody	353004
21	Santa Cruz	HSP70 Antibody	648004
22	Gibco	Phosphate-buffered saline	14190094
23	ThermoScientific	CellTracker Red CMPTX	C34552
24	Sigma	Heparin sodium salt	H3393
25	Sigma	PKH67	PKH67GL
26	Promega	MTS	G111A
27	Sigma	D-(+)-Glucose solution	G8644
28	Sigma	Propidium Iodide	P4864
29	ThermoScientific	Hoechst 33342 Trihydrate	H3570
30	Sigma	Cresyl violet acetate salt	C5042
31	Merck	Paraformaldehyde	104005
32	Sigma	Ethanol	920.026
33	Biochemica	Nonidet P-40	A1694
34	ThermoScientific	RNase A	EN0531
35	Santa Cruz	RIPA Lysis Buffer System	24948
36	ThermoScientific	BCA Assay	23225
37	Bio-Rad	LF-PVDF	1620177
38	Bio-Rad	Blotting Grade Blocker	17006404
39	Abcam	HRP conjugated anti-mouse secondary antibody	Ab191866
40	Abcam	HRP conjugated anti-rabbit secondary antibody	Ab97040
41	ThermoScientific	SuperSignal West Femto Maximum Sensitivity Substrate	34096

2.2. MAINTENANCE OF CELL CULTURE

PDLSCs were purchased from the Celprogen and grown in tissue culture flasks (TPP, Switzerland) in order to maintain the culture. DMEM-low glucose supplemented with 10 percent FBS, 1 percent PSA (10,000 unit/ml Penicillin, 10,000 μg/ml Streptomycin, 25 μg/ml Amphotericin B) was used as a cell culture medium. Frozen cells were thawed according to the manufacturer's instruction and cultured in fresh medium in T75 flasks at 37°C and 5 percent CO_2 incubator. The culture medium was changed every 48-72 hours to maintain the cells, and they passaged at 70-80 percent confluency. The trypsinization of the cells was performed through 0.05 percent (v/v) trypsin/EDTA. Afterwards, the cells in trypsin which was inactivated by addition of serum-supplemented culture medium were pelleted by centrifugation at room temperature for 5 minutes at 200 g. Cells resuspended in the fresh medium were cultured and expanded in the incubator. Cells dissolved in the fresh medium were grown in the incubator and cultured [234]. In all experiments conducted in the thesis, cells were used in early passages (P4-6).

2.3. INDUCTION OF NEUROGENIC DIFFERENTIATION IN VITRO

In the differentiation experiments of PDLSCs, the cells were seeded at a concentration of 5000 cells/cm^2. After overnight incubation, PDLSCs were incubated for 2 days with pre-induction medium (DMEM/F12 containing 1 percent B27 supplement, 20 ng/ml bFGF, 20 ng/ml EGF) in order to induce the neurogenic differentiation. After the initial induction, the cells were incubated for 48 hours with neural inductive medium (DMEM/F12 containing 1 percent B27 supplement, 4 mM Valproic acid, 100 nM Forskolin) to arrest the cell cycle. Subsequently, the cells were incubated for 6 days, changing the medium every 48 hours, with neural maturation medium (DMEM/F12 containing 1 percent B27 supplement, 100 nM Forskolin, 200 μM IBMX, 20 ng/ml BDNF, 10 ng/ml GDNF). In the experiment, cell expanded in normal growth medium were used as a negative control. Besides, 5 μg/ml exosome was added to the neurogenic inductive medium for CSDE group.

2.4. ISOLATION OF CSDE FROM AQUEOUS EXTRACT

Citrus sinensis fruits were purchased from a local market and washed with distilled water. After the removal of fruit peel, the juice was extracted by blender for about 10 minutes at highest speed. After filtering the obtained orange juice from Whatman No 1 (GE Healthcare Bio-Sciences, UK), it was centrifuged at 5000g for 10 minutes and at 20000g for 15 minutes in order to get rid of large insoluble contaminants. The homogenous supernatant was filtered through a 0.22 μm polyethersulfone (PES) membrane prior to the application of the aqueous two-phase system (ATPS). After the exosomes were physically separated from the large particles, exosomes were purified using ATPS in order to remove non-exosomal proteins and bioactive chemicals [235]. Accordingly, filtrated juice was mixed with an equivalent volume of 2x isolation solution (7 percent Polyethylene Glycol (PEG) and 3 percent dextran in ultrapure Water) and centrifuged at 1000g for 10 minutes. After the centrifugation, the total solution was divided into two phases volumetrically. Thereafter, 80 percent of the total solution from the upper part was aspirated and centrifuged again upon adding an equal volume of washing solution (the upper phase of the centrifuged 1x isolation solution). After the washing was repeated 2 times, the lower phases were collected and combined into a new tube. Then, the remained lower phase was mixed with an equal volume of absolute methanol and centrifuged at 20000g for 20 minutes at 4°C in order to remove the dextran polymer found in the collected lower phase with exosomes. Methanol-exosome mixture in the supernatant was transferred into the new tubes and the alcohol in the solution was evaporated by vacuum centrifugation. After the evaporation of almost all alcohol in the mixture, exosomes present in the remaining solution were collected in a new tube. After sterile filtration of exosomes, they were stored at -80°C as aliquots.

2.5. CHARACTERISTICS OF EXOSOMES

2.5.1. Nanoparticle Tracking Analysis of CSDE

The nanoparticle tracking analysis (NTA) is a method that can analyze the properties of nanoparticles in terms of particle size and quantity by screening the refraction of the laser on the surface of the nanoparticles. Before starting the experiments, the quantity and the size

distributions of the vesicles were determined by Nanosight NS300 equipped with 488 nm LM14 module [236]. CSDE characterization videos were taken as 5 videos each at 60 seconds in ambient temperature and analyzed via NTA 3.3 software according to Table 2.3. The additional information about the measurement is given in the NTA data. Sterile nuclease-free water was used to dilute the concentration of exosome in the measurements.

Table 2.3. Capture and analysis settings used in the nanosight analysis.

Capture and Analysis Settings	
Camera Type	sCMOS
Laser Type	Blue488
Camera Level	14
Slider Shutter	1259
Slider Gain	366
Frame Per Second	25.0
No. of Frames	1498
Temperature	22.6 °C
Viscosity	(Water) 0.9 cP
Dilution Factor	2 x 10e1
Detect Threshold	7

2.5.2. Scanning Electron Microscopy Analysis of CSDE

Morphological characteristics of exosomes were analyzed by scanning electron microscopy [237]. Exosomes mixed with absolute methanol in 1:4 ratio were dripped on the slide and kept at -20°C for 10 minutes. Exosomes mixed with 100% methanol in 1: 4 ratio was dripped on the slide and kept at -20 ° C for 10 minutes. Thereafter, it was dried by vacuum centrifugation, then was washed with ultrapure water and dried for last time. After sample preparation, SEM analysis was performed with Zeiss EVO LS 10 microscope at 15,000x magnification under high pressure.

2.5.3. Flow Cytometry Analysis of CSDE

Flow cytometry analysis was performed by using 4 µm aldehyde/sulfate latex beads in order to detect the specific markers of exosomes [238]. The 5 µl of latex beads at a concentration

of 1.1 x 10^9 particles/ml was mixed with exosomes at a final concentration of 550 x 10^9 particles/ml. After incubation for 15 minutes on a shaker at ambient temperature, exosomes were completed to 200 µl with phosphate-buffered saline (PBS) containing 2 percent bovine serum albumin (BSA) and blocked at room temperature for 1 hour. 22 µl of 1 M Glycine solution was added into the solution. After 30 minutes of incubation, the samples were completed to 1 ml with PBS and centrifuged at 5000 g for 5 minutes and then washed once more. The exosome-beads pellet was dissolved with 500 µl and aliquoted to four different tubes as 100 µl. After separating one tube as a negative control, the remaining three tubes were given to CD9, CD63, and HSP70 fluorescent conjugated primary antibodies with 1:100 dilution, respectively. After overnight incubation, exosomes were washed with PBS under the same conditions and analyzed through flow cytometry device.

2.5.4. Uptake Analysis of CSDE

Uptake analysis is essential in terms of vesicular stability and functionality in investigating the effect of exosomes on cells [239]. PDLSCs were seeded at a concentration of 5000 cells/cm^2 onto the six-well plate with intent to investigate the effect of CSDE on cellular uptake. After overnight incubation, the cells were incubated for 40 minutes at 37°C with a serum-free medium containing 1 µM CMPTX to stain the cells. Thereafter, the heparin group was incubated with 5 U/ml heparin containing medium for 30 minutes at 37°C. At this time, exosomes were stained with PKH67 and passed through exosome spin column to remove dye particles other than vesicles. Following this, image of negative control, CSDE and heparin groups were analyzed using a fluorescence microscope after 4 hours administration to the cells.

2.6. EFFECT OF CSDE ON CELL VIABILITY OF PDLSCS

The possible toxicity of CSDE on PDLSCs was evaluated by using a water-soluble tetrazolium compound, (3-[4,5,dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4-sulfophenyl]-2H -tetrazolium, inner salt) (MTS) [240]. Experimentally, PDLSCs which were seeded at a concentration of 5000 cells per well onto 96-well plate were treated with CSDE doses at 1 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml for 24, 48 and 72 hours.

Once the incubations were complete, the MTS salt was mixed to PBS solution containing 4,5 g/L glucose in the ratio of 1:10 and transferred into the cells. After incubation at 37°C for 1 hour, the absorbance was measured at 540 nm by ELISA plate reader. The means of the two independent biological replicates by the averages of the quadruplicates in each group in the experiment was determined as the percentage compared to the control.

2.7. EFFECT OF NEUROGENIC INDUCTION MEDIUM ON CELL VIABILITY OF PDLSCS

PDLSCs were seeded at a concentration of 5000 cells/cm^2 onto the 96-well plate in order to investigate the cytotoxic effect of neurogenic differentiation medium and the MTS assay was performed on the fourth and tenth days of the protocol according to previous protocol [240]. The cells were stained with Hoechst and propidium iodide (PI) in the serum-free medium for 10 minutes to prove that the cells didn't die even though the cell cycle arrest [241]. The cells were stained with serum-free medium containing Hoechst and PI for 10 minutes to prove that the cells did not die even though the cell cycle stopped. After the staining was completed, the staining solution was replaced with PBS and fluorescence intensity was measured at wavelengths of 351/462 nm and 535/617 nm excitation/emission. The mean of the 3 independent biological replicates produced by the averages of the quadruplicates of each group in the experiment was determined as the percentage compared to the control.

2.8. CRESYL VIOLET STAINING OF DIFFERENTIATED PDLSCS

On the 10th day of the differentiation, the cells were stained with cresyl violet staining solution to analyze morphological changes and especially the nissl bodies of the cells [242]. The cresyl violet stain produces a dark blue-violet color by staining the ribosomes of the granular endoplasmic reticles which are found abundantly in the nerve cells. Non-differentiated DSCs can be detected in pale pink color. According to the protocol, the cells were washed twice with PBS subsequent to aspiration of medium and fixed with 4 percent PFA for 15 minutes at ambient temperature. After washing again with PBS, 1 ml of staining solution containing 0.5 percent cresyl Violet acetate was added and incubated at room

temperature for 30 minutes. For the last time, the cells were washed thrice with PBS and analyzed under an inverted microscope.

2.9. CELL CYCLE ANALYSIS OF DIFFERENTIATED CELLS WITH PROPIDIUM IODIDE

The DNA analysis was performed by staining with PI in flow cytometry in order to investigate the effect of the neurogenic differentiation protocol on the cell cycle and confirm the results in the previous experiments [243]. Accordingly, the cells were trypsinized and centrifuged after the completion of the differentiation process. After washing with PBS, the cells were resuspended by adding dropwise with 70 percent cold ethanol and vortexing at the lowest rate. Thereafter, the cells were fixed in this solution at least 2 hours at ambient temperature. After the incubation, the cells were washed with PBS once more by centrifugation. For the permeabilization of the cell membrane associated with cleavage of RNA molecules, the cell pellet was resuspended with 500 µl of cell cycle analysis buffer (0.1 percent Nonidet P-40, 20 µg/ml RNase) for 30 minutes at 37°C. Eventually, PI was added to the cells at a final concentration of 10 µg/ml and flow cytometry analysis was performed.

2.10. GENE EXPRESSION ANALYSIS OF DIFFERENTIATED CELL BY RT-PCR

The quantitative real-time polymerase chain reaction (RT-PCR) analysis was performed to demonstrate that neurogenically differentiated cells had a significant modulation not only in the morphology but also in the expression of neuronal genes [56]. To evaluate the gene expression, total RNA extraction of the cells collected on the end of differentiation was performed by using TRIzol reagent according to the manufacturer's instructions. The concentration and purity of purified RNAs were measured with Nanodrop 2000 and a ratio of $A_{260/280} > 1.8$ was accepted as adequately pure RNA. Therefore, cDNA templates were ensued from these RNAs by utilizing the QuantiTect Reverse Transcription Kit and used as a template in the RT-PCR via QuantiTect SYBR Green PCR Kit. The real-time reaction was prepared according to Table 2.4 and distributed to 96-well PCR plate and the reaction was performed by CFX96 Touch Real-Time PCR Detection System according to Table 2.5.

Analysis of mature neuronal marker genes and dopaminergic neuronal genes was performed. The sequences of the primers used in the experiment are given in Table 2.6.

Table 2.4. RT-PCR mixture setup per reaction.

2x Sybr Green Mix	5 μl
Forward Primer	0,5 μl
Reverse Primer	0,5 μl
Nuclease-free Water	2 μl
cDNA Template	2 μl
Total Volume	10 μl

Table 2.5. RT-PCR Protocol Preview.

Cycle	Repeat	Step	Run-down time	Set Point
1	1	1	02:00	50˚C
2	1	1	15:00	95˚C
-	40	1	00:15	95˚C
-	-	2	01:00	60˚C
-	-	3	00:30	72˚C
-	-	4		Plateread
3	1	1	01:00	95˚C
4	1	1	01:00	55˚C
5	1	1	00:30	55˚C
6	80	1	00:10	55˚C + 0.5˚C/cycle
7	-	2		Plateread
8	1	1	∞	4˚C

Table 2.6. Primer Sequences for RT-PCR.

Gene	Forward/Reverse Primer	Product Lenght	References
GAPDH	5' CGACAGTCAGCCGCATCTT 3' 5' CCAATACGACCAAATCCGTTG 3'	101 bp	[24]
MAP2	5' GGAACCAACTCTCTCTGGATTT 3' 5' GCATTCTCTCTTCAGCCTTCT 3'	126 bp	[244]
NFH	5' CAGAGCTGGAGGCACTGAAA 3' 5' CTGCTGAATGGCTTCCTGGT 3'	110 bp	[245]
NeuN	5' CCAAGCGGCTACACGTCTC 3' 5' CGTCCCATTCAGCTTCTCCC 3'	191 bp	[246]

Snap25	5' AGTTGGCTGATGAGTCGCTG 3' 5' TGAAAAGGCCACAGCATTTC 3'	207 bp	[247]
NSE	5' CTGATGCTGGAGTTGGATGG 3' 5' CCATTGATCACGTTGAAGGC 3'	188 bp	[247]
TH	5' GTCCCCTGGTTCCCAAGAAAAGT 3' 5' TCCAGCTGGGGGATATTGTCTTC 3'	332 bp	[248]
DAT	5' TCCAGTGGAGACAGCTCGG 3' 5' GCTGAAGTAGAGCAGCACGA 3'	174 bp	[23]
Nurr1	5' GGATGGTCAAAGAAGTGGTTCG 3' 5' CCTGTGGGCTCTTCGGTTT 3'	81 bp	[24]

2.11. WESTERN BLOT ANALYSIS OF DIFFERENTIATED PDLSCS

To examine the protein expression of differentiated cells which were collected on the last day of the protocol, they were resuspended with radioimmunoprecipitation assay (RIPA) buffer containing 1x protease inhibitor cocktail, 1x phenylmethylsulfonyl fluoride (PMSF), 1x sodium orthovanadate (SO) to obtain the cell lysate [249]. Thereafter, the lysate was centrifuged at top speed for 15 min at 4°C with the aim of removal of insoluble particles. After centrifugation, the supernatant was aliquoted and transferred into the new tubes and stored -80°C. The protein concentration of cell lysate was quantified by BCA assay. The gel separation of 30 ug of total protein was performed in the 4-20 percent Mini-PROTEAN gels (Bio-rad, USA) at a constant 120V for 1 hour by SDS-PAGE. After electrophoresis, the proteins in the gel were transferred to 0.45 μm methanol activated polyvinylidene fluoride (PVDF) membrane at a constant 250 mA for 1 hour. The membranes were blocked with Tris-buffered saline containing 0.1 percent Tween-20 (TBS-T) containing 5 percent non-fat powdered milk for 1 hour at room temperature. After blocking, membranes were probed with primary antibodies in various dilutions at 4°C overnight according to Table 2.7. After washing with TBS-T, the membranes were probed with HRP conjugated goat anti-mouse secondary antibody and HRP conjugated goat anti-rabbit secondary antibody in certain dilutions for 2 hours at room temperature in dark. After a final washing step, the chemiluminescence kit was used in the manufacturer's instructions for imaging the bands and they were visualized with the ChemiDoc camera system. The intensity of the band area was used as a measurement unit and the area intensity of target proteins was normalized by using GAPDH and ACTB as loading control proteins.

Table 2.7. Primary antibodies used in western blot analysis

Primary antibody	Source	Cat. No.	Dilution
GAPDH	Proteintech	60004-1-Ig	1/10000
ACTB	Proteintech	20536-1-AP	1/10000
TH	Abcam	Ab112	1/200
DAT	Abcam	Ab78068	1/1000
NeuN	Thermo	PA5-37407	1/500
FOXA2	Abcam	Ab108422	1/500
NFH	Abcam	Ab7795	1/1000
Synaptophysin	Abcam	Ab8049	1/500

2.12. IMMUNOCYTOCHEMISTRY ANALYSIS OF DIFFERENTIATED CELLS

On the last day of the differentiation protocol, cells were washed twice with pre-warmed PBS for immunocytochemistry analysis and fixed with 4 percent PFA for 10 minutes at ambient temperature [54]. After washing with PBS, the cells were blocked with a buffer solution containing 1 percent BSA for 1 hour at room temperature. NFH (13-1300, ThermoScientific) and TUBB3 (ab18207, Abcam) primary antibodies at 1/200 dilution were incubated overnight at 4°C with cells. After washing once more, the cells were incubated with Alexa Fluor 488 conjugated goat anti-mouse (ab150117) and goat anti-rabbit (ab150077) secondary antibody at 1/500 dilution for 1 hour at ambient temperature in dark. Thereafter, the cells were counterstained with Hoechst diluted 1/1000 in PBS for 5 min and washed again and analyzed through an inverted fluorescence microscope.

2.13. STATISTICAL ANALYSIS

Analysis of all the experimental data regarding thesis was performed by using GraphPad Prism 7 software [250]. Standard deviations, sample means and standard error of means were calculated through Microsoft Office Excel. One-way and two-way ANOVA analysis were also performed by GraphPad software for evaluating statistical significance. The difference among experimental groups was considered to be significant if the p-value was less than 0.05 which was accepted as the alpha value. These significant differences were indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

3. RESULTS

3.1. CHARACTERISTICS OF CSDE

The experiments associated with potential effects of CSDE purified by the modification of an earlier method [251] were performed following the completion of exosomal characterization in terms of consistency and reliability of the results. Accordingly, a variety of informative analysis was accomplished. Firstly, nanoparticle tracking analysis was performed by nanosight device. Even so, CSDE were the vast majority in solution, a small number of microvesicle contamination was observed as shown in Figure 3.1.
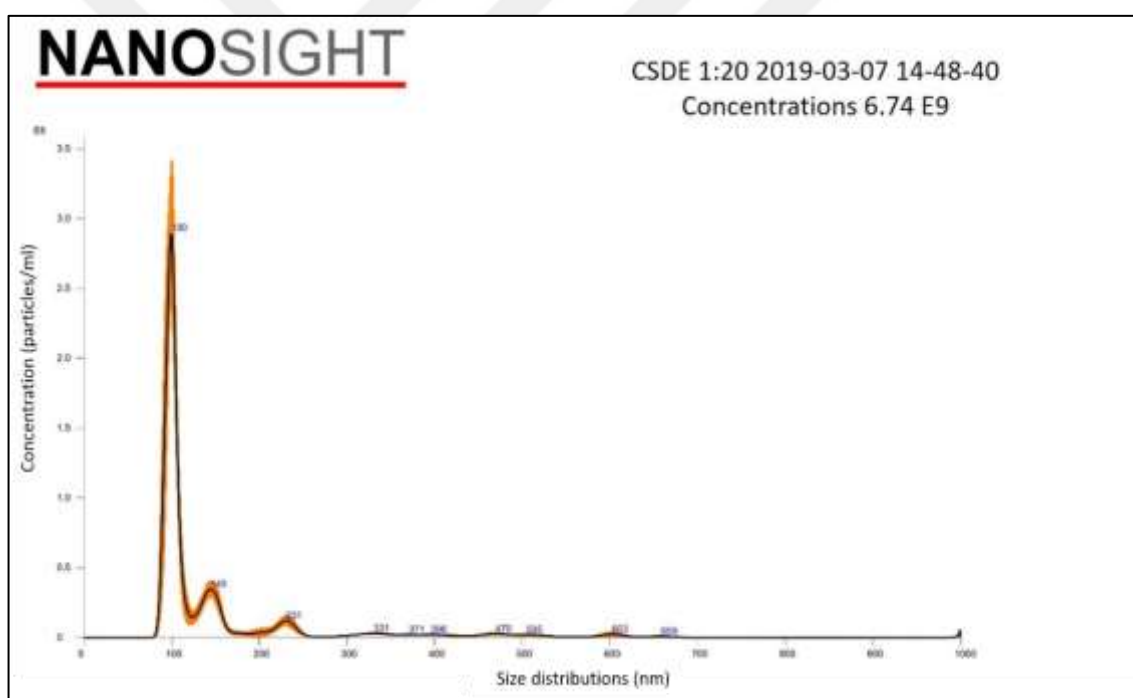

Figure 3.1. Concentration and average diameter of exosome vesicles used in all the experiments.

As a result, it was detected that the concentration of CSDE was 6.74 x 10^9 particles/ml and average vesicular size was 149 nm based on polydispersity. Moreover, the morphologic analysis of vesicles was performed by scanning electron microscopy (SEM) in order to verify the nanovesicle structures and possible contaminations which were detected in NTA. According to the SEM image in Figure 3.2, spheric and round-shaped exosomal morphology was observed as well as contaminant microvesicles.
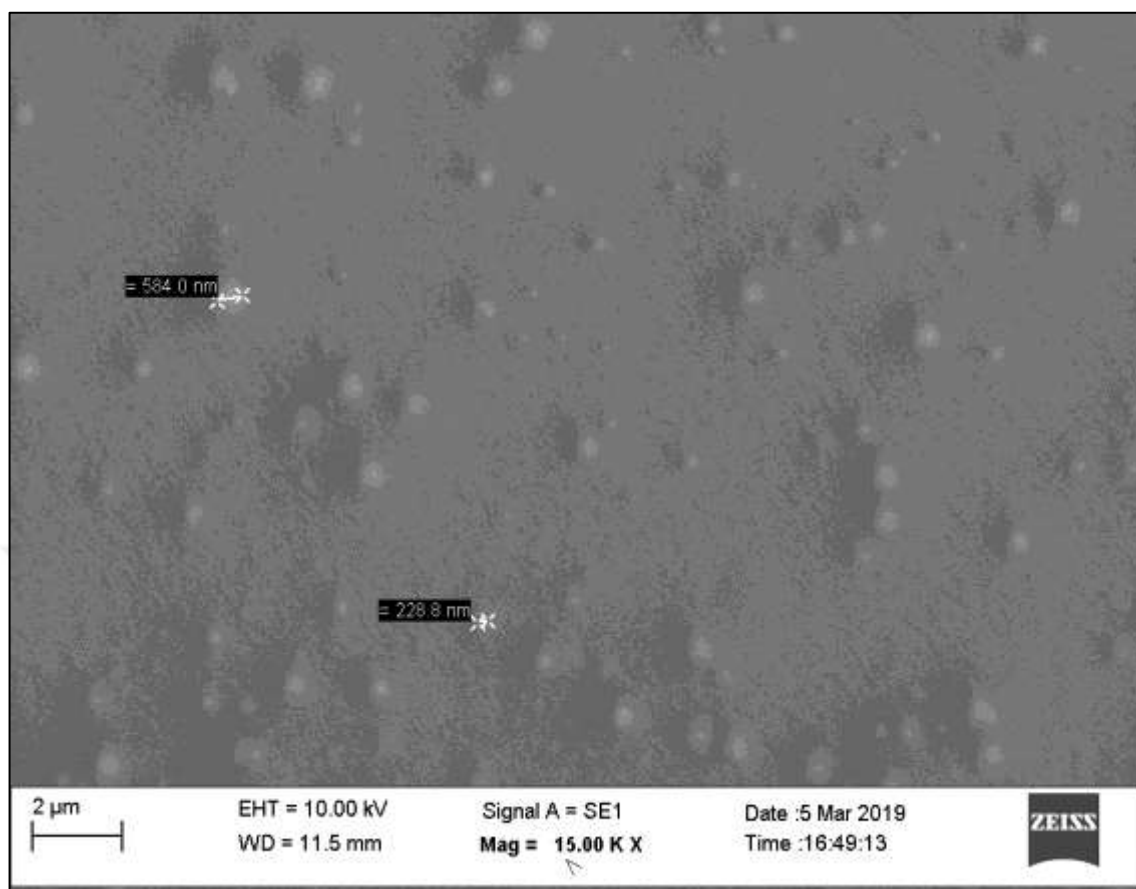
Figure 3.2. SEM images of spheric and round-shaped CSDE nanoparticles.

After morphological analysis of the vesicle structures, exosomal marker analysis was performed using specific antibodies in flow cytometry analysis in order to determine whether the CSDE carry exosomal markers. As a result in Figure 3.3, it was determined that CSDE show the CD9, CD63 and HSP70 positivity. The final experiment on the characterization of exosomes was uptake analysis. In order to monitor the uptake of exosome in a controlled manner, the internalization of fluorescently labeled exosomes was investigated by utilizing fluorescence microscopy. According to the results of the experiments in Figure 3.4, it was detected that CSDE were accumulated in the cell membrane and naturally decreased in terms of cellular uptake when CSDE was given with heparin which is an inhibitor of RME. On the contrary, the only CSDE group was found to internalize into the cells at an elevated rate. Accordingly, CSDE was fully characterized by means of the four experiments.
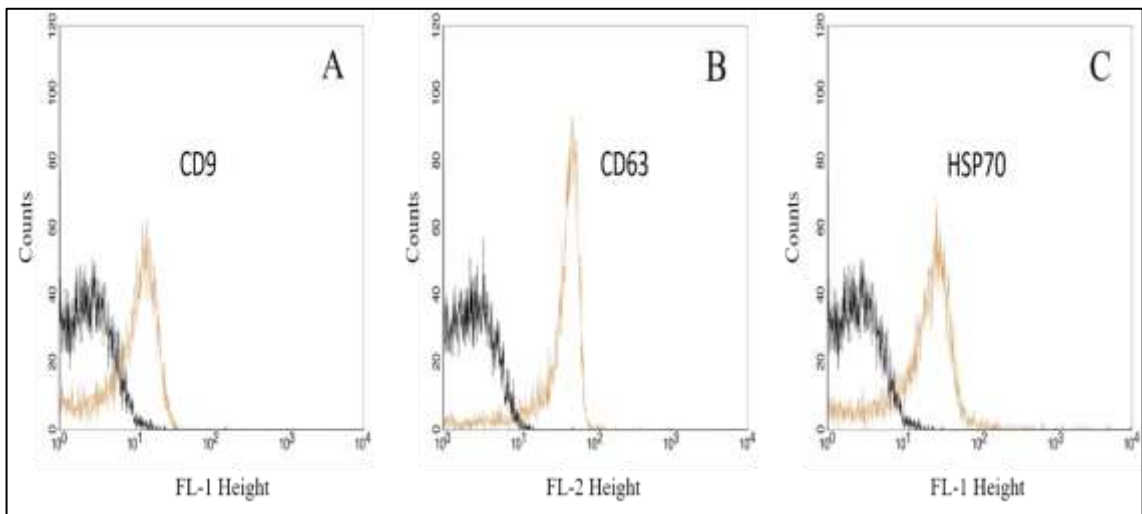
Figure 3.3. Surface phenotype analysis of exosomes by flow cytometry. Black peaks represent negative control. Data are representative of three separate experiments.
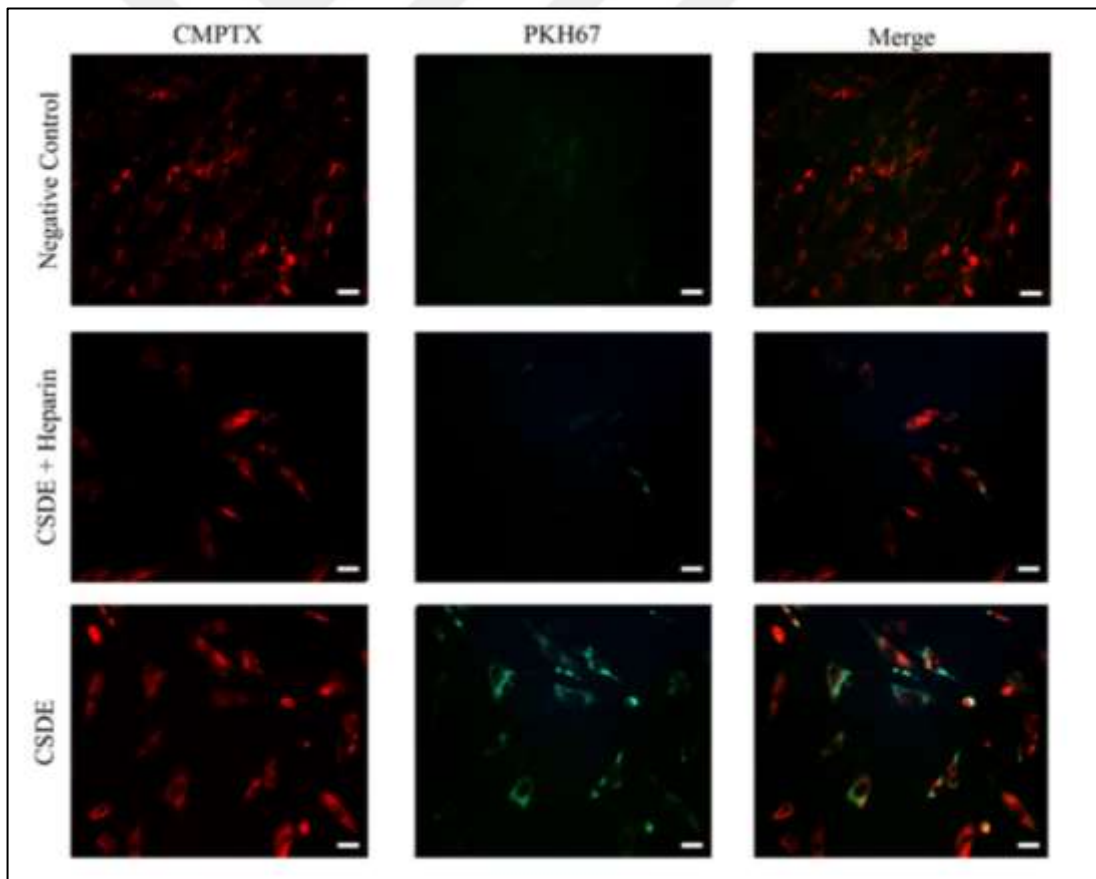

Figure 3.4. In vitro uptake analysis of CSDE. Internalization of fluorescently labeled exosomes into the PDLSCs was imaged under fluorescence microscopy. (Scale bar 20 µm)

3.2. EFFECTS OF CSDE ON CELL PROLIFERATION OF PDLSCS

Determination of the effective dose of CSDE to be used in the differentiation experiment and investigation of the possible toxicity on the cells were performed by in vitro MTS assay for 24, 48 and 72 hours. According to the result in Figure 3.5, CSDE had no effect on cell proliferation during 24 hours. After 48 hours, the cell proliferation was significantly increased at doses of 10 µg/ml, 20 µg/ml and 50 µg/ml (respectively, 27.92 percent, 27.48 percent, and 22.01 percent). After 72 hours, there was a significant increase of 26.66 percent at only 50 µg/ml dose compared to the negative control. Furthermore, it was observed that the doses of 20 µg/ml and 50 µg/ml of CSDE increased the cell proliferation 16.71 percent and 23.93 percent compared to 1 µg/ml dose. Thus, it was determined that the high doses of CSDE significantly increased cell proliferation in 48 and 72 hours.
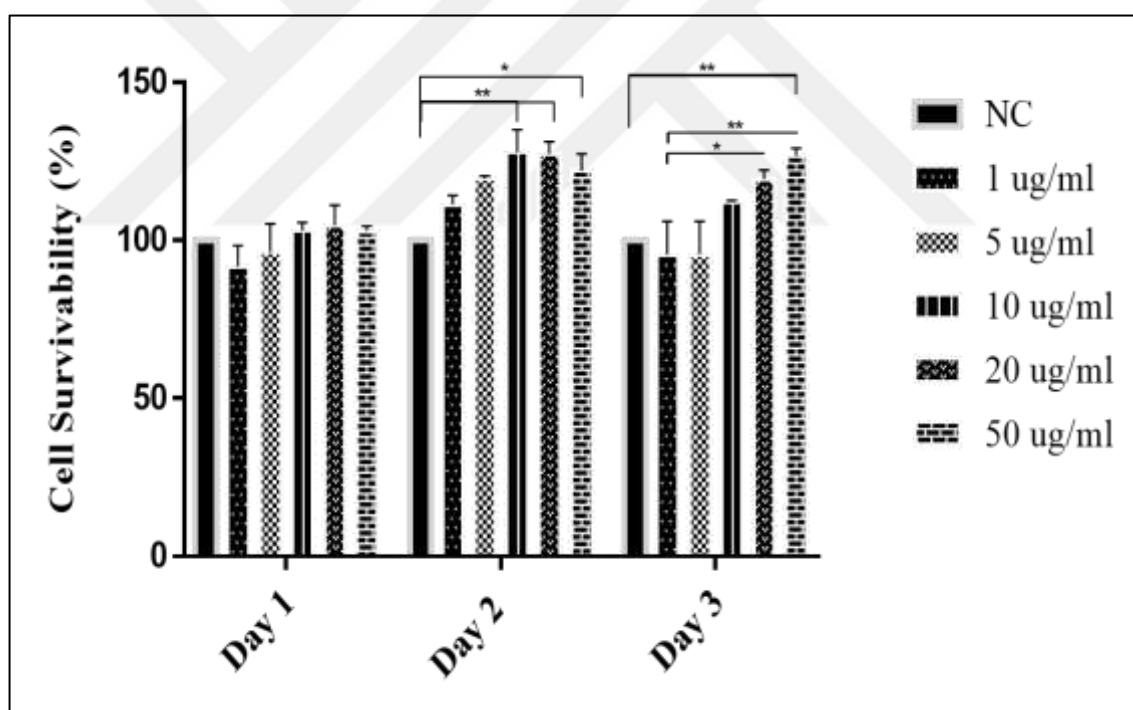
Figure 3.5. Effects of CSDE on cell proliferation on PDLSCs compared to control group in various time intervals. *p < 0.05, **p < 0.01, by two-way ANOVA for statistical significance, n=2.

3.3. EFFECTS OF NEUROGENIC INDUCTION PROTOCOLS ON CELL PROLIFERATION OF PDLSCS

The G0/G1 arrest of the cell cycle in neurogenic differentiation is an extremely essential stage. Accordingly, in order to demonstrate that the proliferation of the differentiated cells could be arrested, the cell proliferation was evaluated by MTS assay on the 4th and 10th in the differentiation protocol. According to result in Figure 3.6, it was determined that the induction medium protocols significantly reduced cell proliferation from day 4 (approx. 64 percent reduction) and this alteration was persistent until the 10th day (approx. 78 percent reduction) in the differentiated cells.

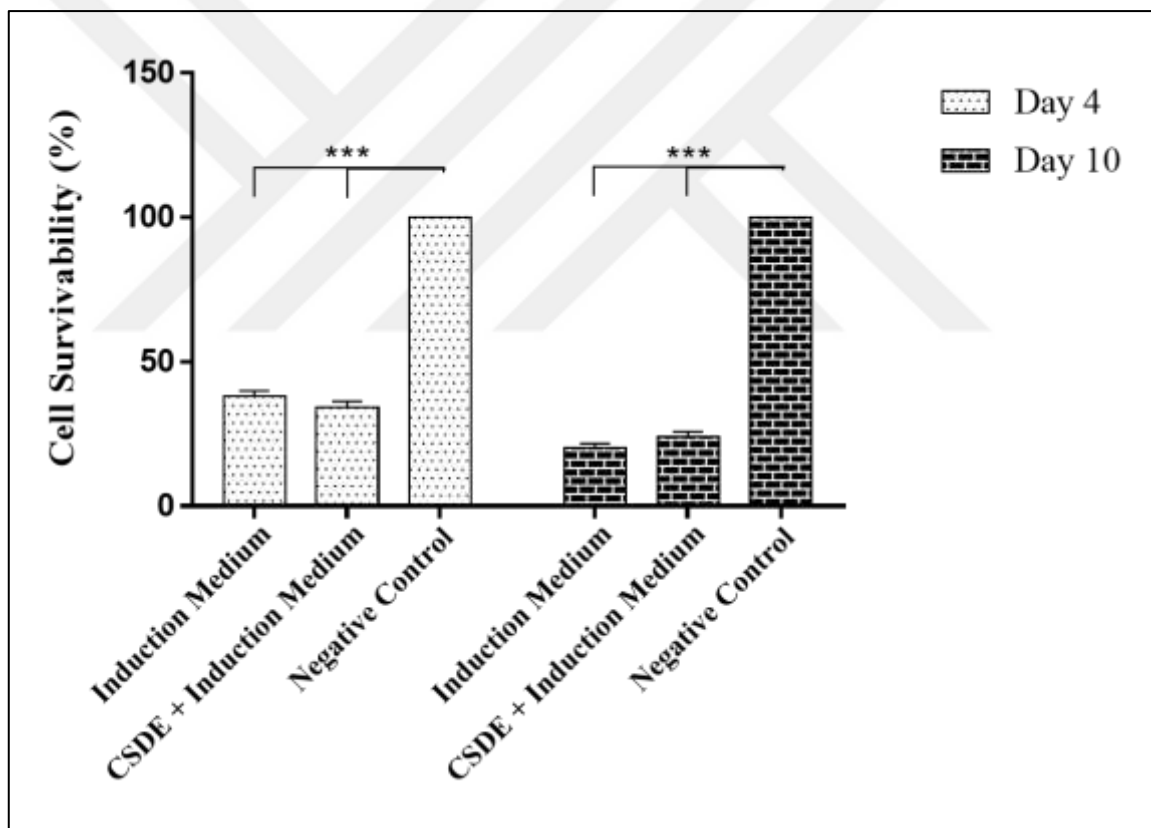Figure 3.6. Effects of neurogenic induction protocols on cell proliferation on PDLSCs compared to control group in 4th and 10th days. ***$p < 0.001$, by one-way ANOVA for statistical significance, n=3.

3.4. EFFECTS OF NEUROGENIC INDUCTION PROTOCOLS ON CELL VIABILITY OF PDLSCS

In neurogenic differentiation experiments, it must be shown that the induction medium performed the cell cycle arrest, yet doesn't induce any death signal in the cells. As a result, the differentiated cells were stained with Hoechst and PI without cell fixation and relative fluorescent intensity was measured and analyzed as shown in Figure 3.7. PI intensity associated with cell necrosis was normalized with Hoechst intensity. In the experiment, 20 percent DMSO was used as a positive control which was characterized by inducing of necrotic cell death. On the fourth day of the experiment, no significant difference was found between CSDE and negative control. A significant decreasing of the cell death, 2.43 fold increase was observed in the CSDE group in comparison with negative control as late as on the 10th day. However, the death of the cells in the CSDE group decreased significantly 3.61 and 2.86 fold compared to the only induction medium group. In the experiment, the positive control group was significantly increased compared to all groups on the 4th and 10th days.
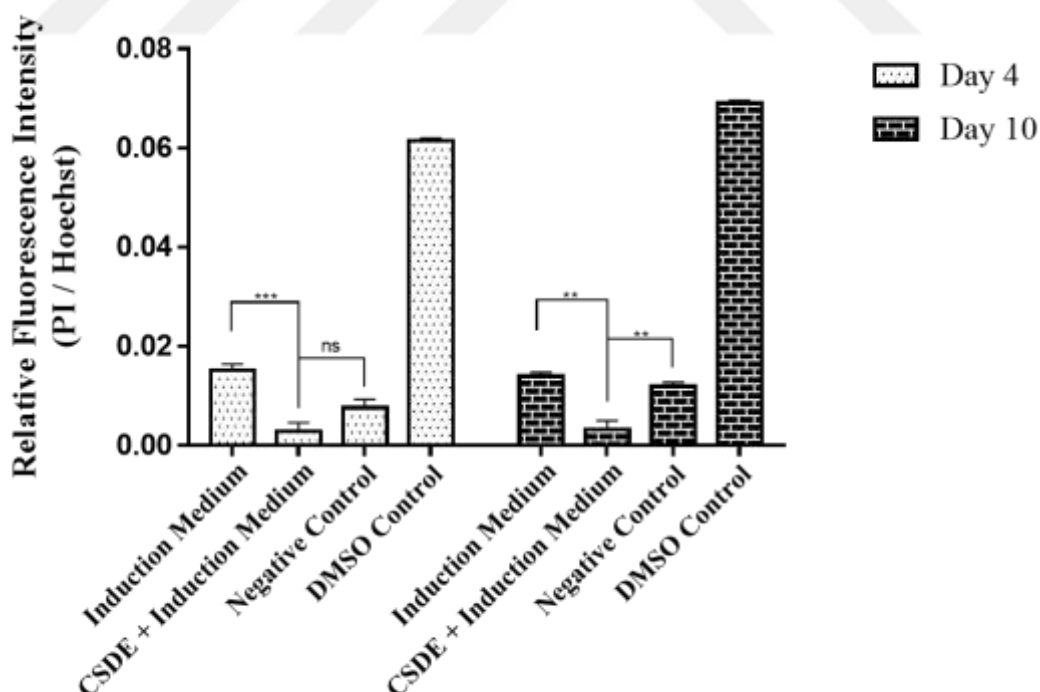
Figure 3.7. Effects of neurogenic induction protocols on cell viability on PDLSCs by measuring PI/Hoechst level of cells in 4th and 10th days. $**p < 0.01$, $***p < 0.001$, by one-way ANOVA for statistical significance, n=3.

3.5. CELL CYCLE ANALYSIS OF NEUROGENIC DIFFERENTIATION BY PI

The DNA content of differentiated cells was analyzed using PI staining via flow cytometry in order to investigate the cell cycle arrest resulted in neurogenic differentiation protocol as shown in Figure 3.8. As a result, it was observed significant modulation in the only induction medium group and CSDE group that the cell percentages in the G0/G1 phase increased 1.14 and 1.19 fold, while the cell percentages in the S phase decreased 4.52 and 5.97 fold and G2/M phase decreased 3.94 and 6.46 fold. However, there were no significant differences between differentiated PDLSCs groups. Thus, the neurogenic induction protocols have been proven to arrest the cell cycle at the G0/G1 phase without inducing death signals in the cells.
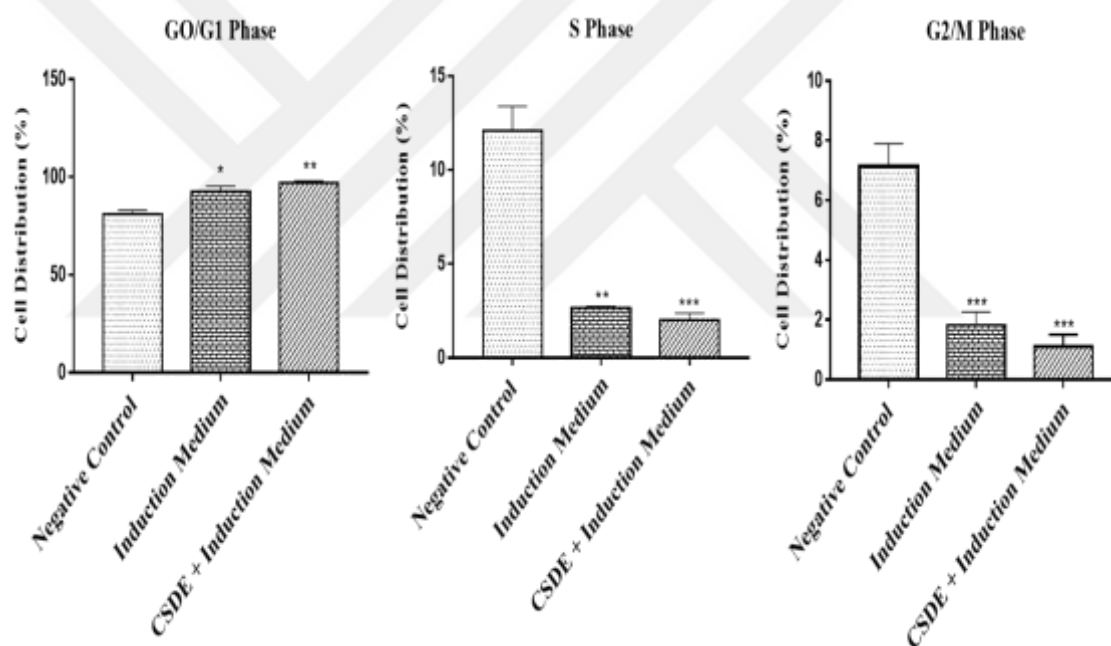
Figure 3.8. The cell cycle phases modulated by 5 µg/ml CSDE + Induction medium and only induction medium group on the 10th day. * indicates the significant difference of the experimental group compared to the negative control. *p < 0.05, **p < 0.01, ***p < 0.001, by one-way ANOVA for statistical significance, n=3.

3.6. NISSL BODIES STAINING AND ANALYSIS OF DIFFERENTIATED PDLSCS

Nissl bodies staining was performed to examine the morphological alterations and neuronal characteristics of differentiated cells. The cells of all groups were analyzed under an inverted microscope after staining the cresyl violet staining solution on the tenth day of the differentiation protocol. According to Figure 3.9, it was observed that there were differences between CSDE and only induction medium groups compared to negative control in terms of morphological modulations like retraction tendency and elongation of neural processes and increase in the density of dye in the soma based on the increment of RER-related ribosomes in the soma of the cells. In addition, the differentiated cells were observed to be relatively high in comparison with undifferentiated cells.
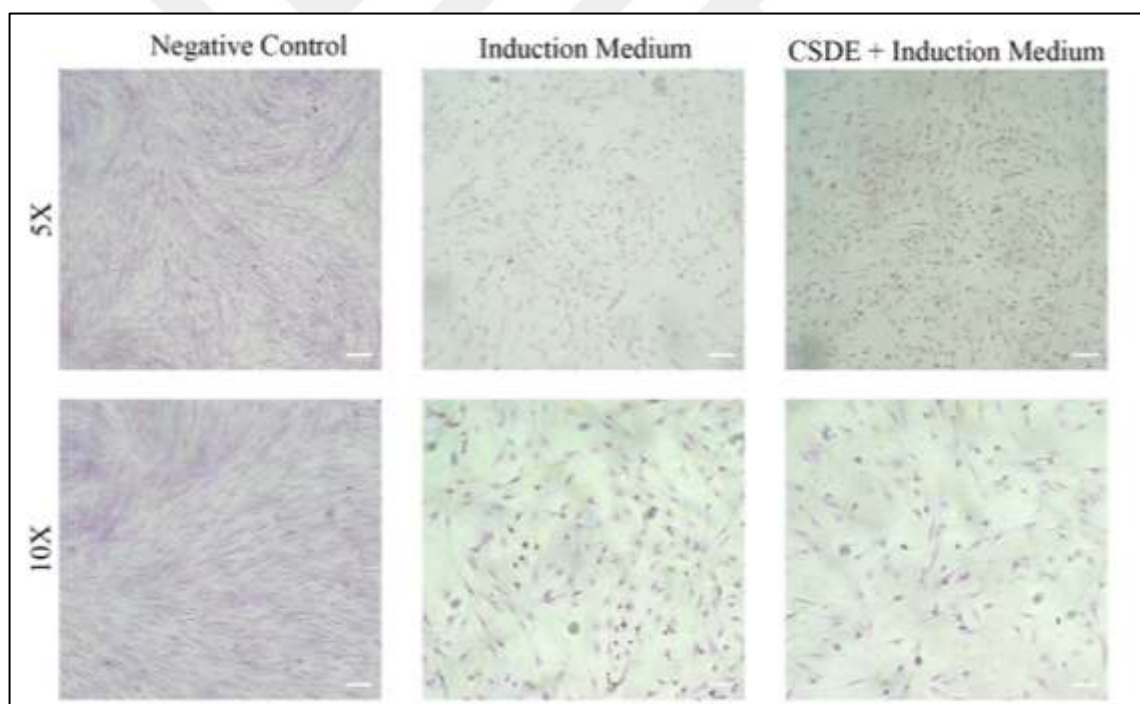
Figure 3.9. Bright field images of cresyl violet staining of differentiated and undifferentiated PDLSCs on the 10th day. (Scale bar 200 μm for 5X and 100 μm for 10X)

3.7. GENE EXPRESSION ANALYSIS OF DIFFERENTIATED PDLSCS

Neurogenic differentiation of PDLSCs needs to be analyzed with necessary molecular techniques in addition to previous morphological findings. In the experiment, expressions of NFH, NeuN, MAP2, SNAP25 and NSE genes which are essential markers of maturation of neurons were analyzed. As a result of the experiments, the fold change indication of gene expression was taken to the logarithm base and after that statistical analyses were performed. According to the results of the modulation of the gene expressions in Figure 3.10, it was determined that no significant difference was observed in the expressions of NSE and SNAP25 genes of the differentiated cells. However, NFH, NeuN and MAP2 genes in the differentiated cells were upregulated, respectively as at an approximate value of 4.6 fold, 25.8 fold, and 53.16 fold, in comparison with undifferentiated cells on the tenth day. In addition, MAP2 gene analysis showed a significant decrease of 3.5 fold in both groups on the tenth day compared to the fourth day.

Figure 3.10. Modulation of neuronal maturation genes associated with induction protocols for 10 days. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, by one-way ANOVA for statistical significance, n=3.

In addition to these genes, dopaminergic neuron markers gene such as TH, DAT and Nurr1 were also analyzed in order to investigate whether differentiated cells were going through a specific lineage during late maturation stage as shown in Figure 3.11. In both of the experimental groups, significant upregulation was detected in all 3 genes expression (TH 22.8 fold, DAT 13.5 fold, Nurr1 17.6 fold) on the last day of differentiation. Moreover, analyses performed on the 4th day showed 8.28 fold upregulation of the transcription factor Nurr1 gene expression only in the induction medium group without CSDE.

Figure 3.11. Modulation of dopaminergic neuron genes associated with induction protocols for 10 days. *p < 0.05, **p < 0.01, ***p < 0.001, by one-way ANOVA for statistical significance, n=3.

3.8. WESTERN BLOT ANALYSIS OF DIFFERENTIATED PDLSCS

The protein expressions of the differentiated cells were analyzed by immunoblotting in order to evaluate the protein expressions and consistency of the results in the gene analysis of the differentiated cells. Firstly, the analysis of neural maturation-related proteins was performed. The significant differences in the expression of NFH and Synaptophysin proteins were not observed in the experiments according to Figure 3.12. In addition, the expression of NeuN proteins was upregulated 2.2 fold compared to negative control in both groups on the tenth day of differentiation.
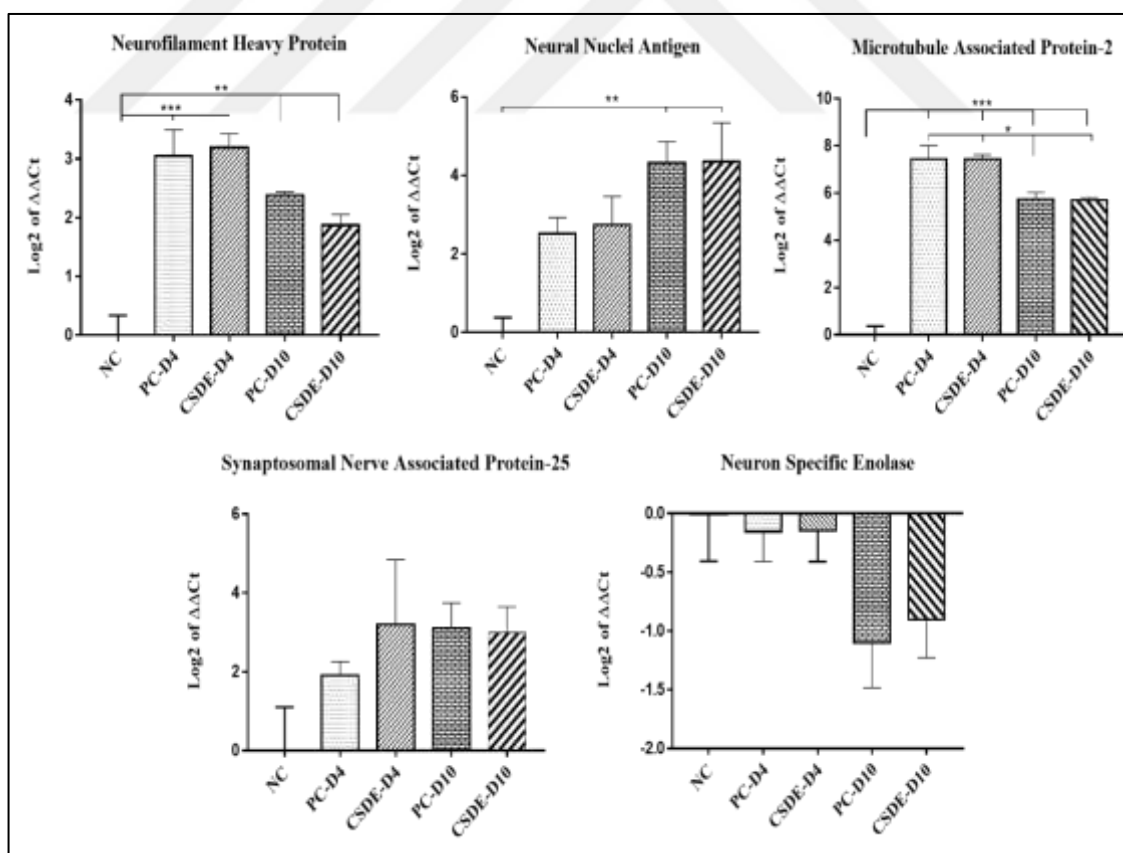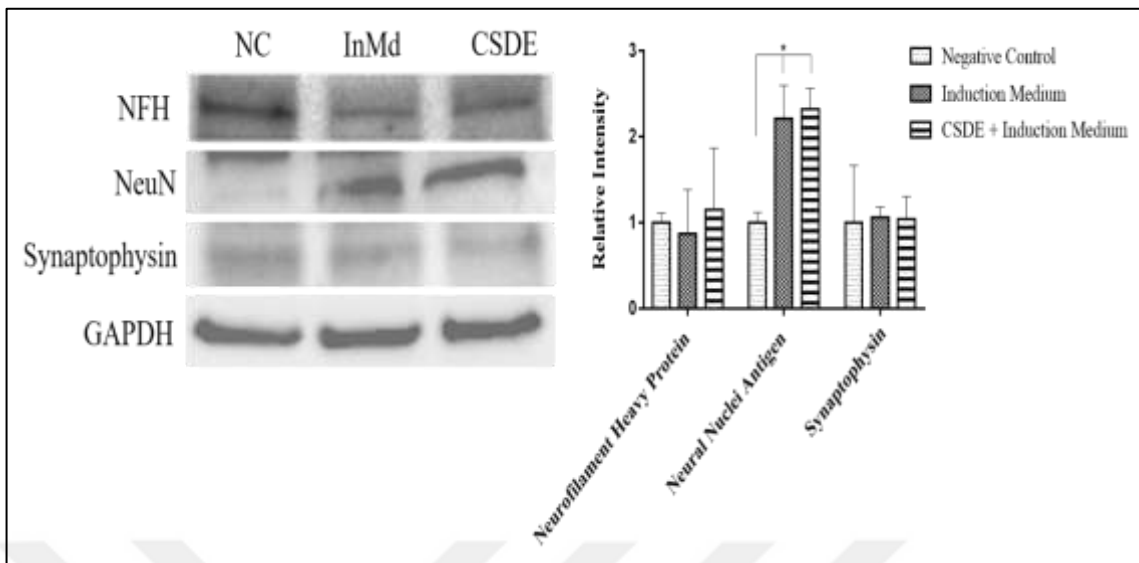
Figure 3.12. Modulation of neural maturation proteins associated with induction protocols for 10 days. *p < 0.05, **p < 0.01, ***p < 0.001, by one-way ANOVA for statistical significance, n=3.

Protein expressions of dopaminergic markers were also investigated in the protein expression of differentiated cells due to the upregulation of the expressions of dopaminergic neurons genes in PCR analysis. According to Figure 3.13, the expressions of TH and FOXA2 proteins were upregulated 1.8 fold in comparison with the negative control in both groups on the tenth day of differentiation. However, no significant differences were observed in the expression of DAT proteins.
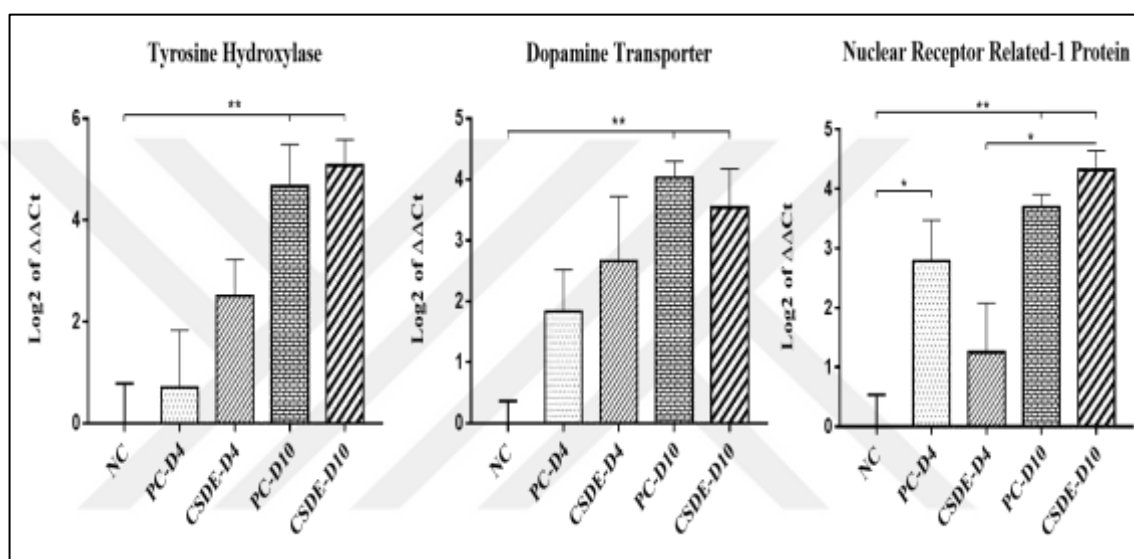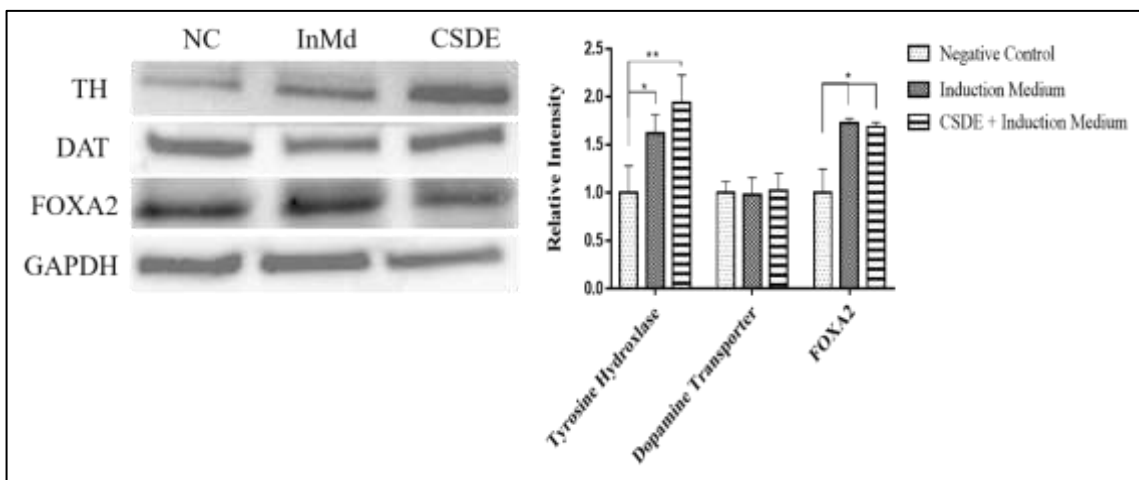

Figure 3.13. Modulation of dopaminergic neuronal proteins associated with induction protocols for 10 days. *p < 0.05, **p < 0.01, ***p < 0.001, by one-way ANOVA for statistical significance, n=3.

3.9. IMMUNOCYTOCHEMISTRY ANALYSIS OF DIFFERENTIATED PDLSCS

Phenotype analysis of differentiated PDLSCs was performed by immunofluorescence staining of neural markers. In particular, localization of TUBB3 and NFH proteins differentiated cells with neurons-like morphology was intently evaluated. Therefore, differentiated cells, as shown in the Figures 3.14 and 3.15, expressed highly NFH and TUBB3. As a result of the analyzes, differentiated PDLSCs, as shown in the Figures 3.14 and 3.15, have high levels of TUBB3 and NFH. Although these two proteins expressed also in the negative control group, the morphological modulations in differentiated PDLSCs were evident.
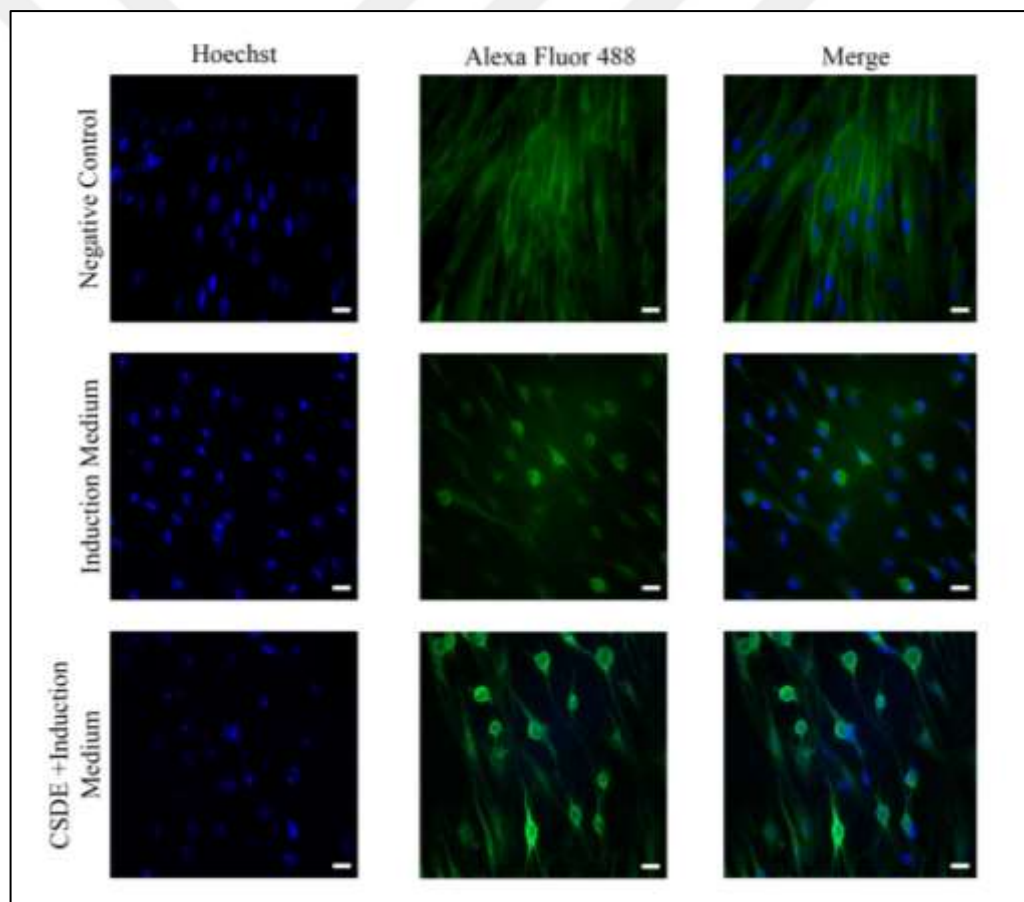
Figure 3.14. Immunofluorescence analysis images TUBB3 protein in the neuronal differentiated PDLSCs on the 10th day of differentiation. (Scale bar 20 µm).
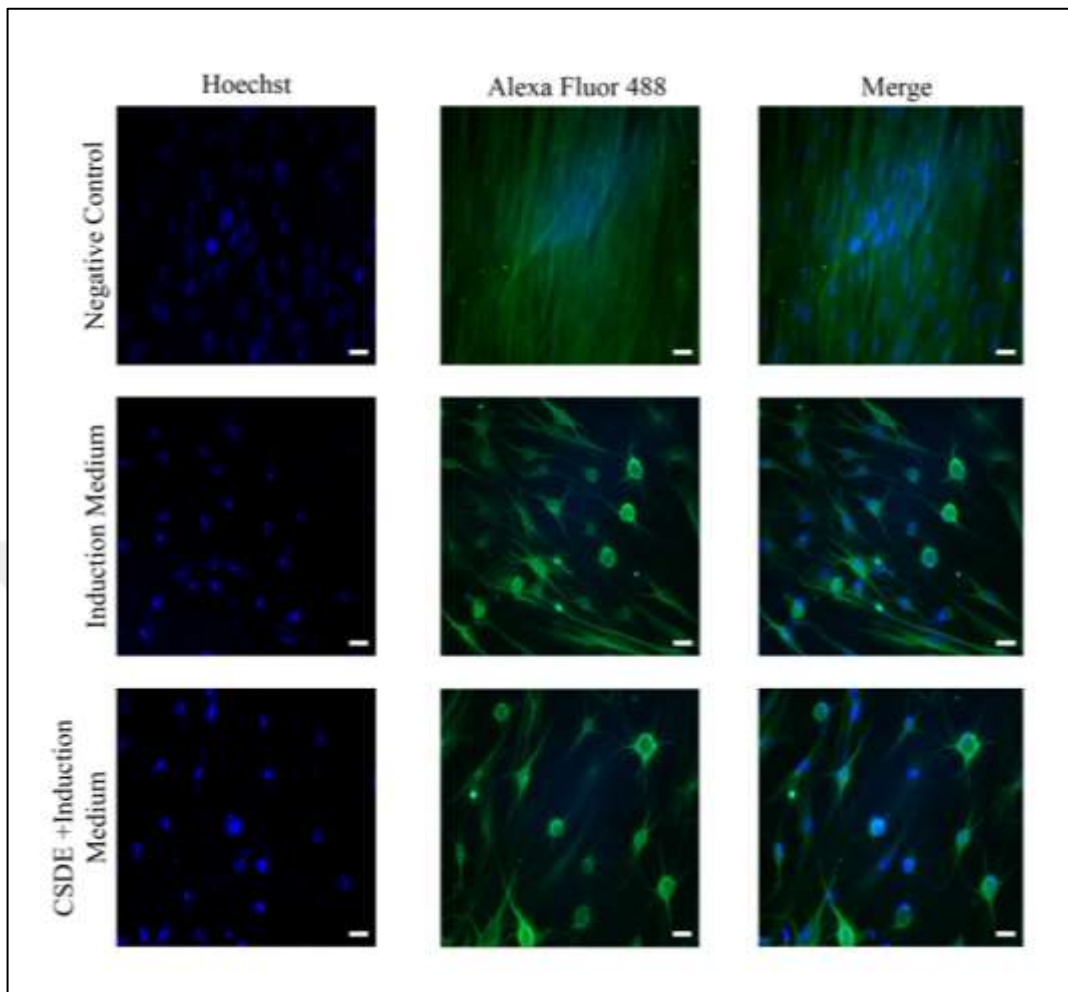
Figure 3.15. Immunofluorescence analysis images NFH protein in the neuronal differentiated PDLSCs on the 10th day of differentiation. (Scale bar 20 µm).

4. DISCUSSION

Neurodegenerative diseases are one of the diseases which are quite decisive in terms of both frequency and mortality [252,253]. As in the development of treatment for many diseases, in vitro modeling of disease, testing of potential therapeutics on these models, and even obtaining information about diseases through the cellular pathology, make significant contributions to the emergence of new approaches in therapies. Therefore, it is an important factor that the cells used in disease models can be easily cultured. In contrast, it is well known that there are difficulties encountered in vitro culture of neuronal cell lines [254-256]. If these are mentioned, lack of suitability for cell culture due to being neurons more dependent on the glial cells in discordance with most cell types, the renewability tendency of molecular relationship isn't high enough and lastly, the mature neurons aren't divided and so they don't proliferate like normal cells in cell culture. Moreover, since the practicability of dynamic relationships with microenvironment and cellular communicative models are not sufficient for modeling pathology conditions, two alternative cell sources emerge for neuronal cell culture models. One of them is the study of primary culture from in vivo and its induction into mature neuron formation [257]. Second, of them, it is applied to cultivate stem cells that are more stable in terms of self-renewal and cellular conditions and to differentiate these stem cells when necessary [258,259]. Therefore, the neural-like cellular models generated by differentiation of the stem cells are still a topic that has been carefully studied and discussed in terms of having functional neurons characteristics and their suitability and success in the use of the model. The present study suggests that neuronally differentiated stem cells are very different from the neurons studied as primary culture and that differentiated cells do not fully reflect the features of neurons and consequently it has been seen defective associated with utilizing the stem cells for the mature and functional neural model. As a result of these studies, it is one of these defects that the stem cells can differentiate neuron-like phenotypes by using various chemicals in culture conditions and this effect might be reversible after the differentiation. Because, in the differentiation of chemical-induced stem cells, the formation of a neuron-like morphology by damaging the cell skeleton, followed by the cell apoptosis [86,260-263] or by increasing the sudden concentration of cAMP in the cell by using small molecule activators, are determined that

pseudo-neurogenesis occurs and that cell return to the stem cell from depending on the effect of the substances [87].

From a very different perspective, the exosomes, whose importance has begun to be recognized for nearly 30 years, have been involved in all kinds of eukaryotes of low to high levels, and have a messenger role in cellular communication [113,264,265]. Processes for the discovery and development of exosome studies can often be said on initial reticulocytes and after immune cells [266,267]. Therefore, exosomal studies in mammalian cell culture have begun earlier than plants and have become important even if there are still issues to be explained. However, the plant-derived exosomes have been started to be investigated for almost 10 years and new findings have been started to be obtained in recent years. At present, only a handful of plant-derived exosomes have been characterized and activities of less than one of the plant exosomes, which have been discovered to have the advantages of having common biomolecules with mammalian cells as well as having plant-specific bioactives, have been investigated [128,131,226-233,268-270]. As a natural consequence, it is important to investigate the effect of plant-derived exosomes on the signalization pathways or modulation of neurogenic differentiation of the stem cells.

In addition, one of the most important issues related to plant exosomes is the isolation of all nano-vesicles structures in fruit, not only that the plant cells are excreted outside as a result of the use of the fruit as a whole during isolation. Another issue is that the fruits used as a source of nanovesicles do not have a certain standard and do not show reproducibility in term of exosomal structure and special substances. Currently, there is no solution for two important drawbacks in the literature. However, in order to reduce this effect slightly, one of the important details of the study was to pool the *Citrus sinensis* fruits before the experiments and any other source was not used for isolation. In this respect, the characterization of CSDEs was firstly completed while investigating the effects of CSDEs on neurogenic differentiation of PDLSCs. Phenotypic and mechanistic features were investigated in exosome characterization in accordance with the literature [270-273]. As shown in Figure 3.1, 3.2, 3.3 and 3.4, consistent results were obtained for characterization of CSDE. Despite the elaborate physical purification steps repeated several times in the protocol, microvesicle contamination came off even if just a smidgen. Besides, it is extremely important to show the exosomal markers positively in flow cytometry analysis and to prevent the exosome internalization by applying heparin in uptake analysis in terms

of surface properties protectin of exosomes. Moreover, it has been proven that the exosomes didn't fragmentize during removal of dextran in the lower phase. After the completion of characterization. The effect of CSDE was investigated by MTS test due to the fact that the possible effects are unknown in the literature [270]. In addition, it is important to determine in the neurogenic differentiation that the highest dose that won't induce proliferation in the cells is used in the differentiation protocol. When the results in Figure 3.5 were examined, it was determined that CSDE increase the cell proliferation at high doses compared to low doses. Following the dose selection, induction of the neurogenic differentiation without the activation of death signals is a highly important issue. For this reason, the viability of the cells was checked with both MTS and Hoechst/PI experiments on the 4th and 10th days of differentiation [241]. According to results in Figure 3.6 and 3.7, on the 4th day, the proliferation of the cells was completely arrested, but no necrosis signal was measured and continued on the 10th day. In literature, normally this control experiment is conducted by Annexin V assay [274]. Nonetheless, Hoechst/PI staining, which is only a test which gives information about necrosis, is preferred because of the low number of differentiating cells. From this point on, it is based on examining the differentiation success of protocol in the experiments. Cell cycle analysis was carried out with PI in order to confirm that the cell cycle was arrested [99,243]. However, an important point is that the G2/M phase of the negative control is 7 percent according to the results in Figure 3.8. This results in the activation of the cell contact inhibitors due to cell confluency when cells divided from the cell population are collected. In order to investigate the morphological changes of the neural characteristics of PDLSCs during differentiation, Nissl bodies were examined by staining with cresyl violet [242,275]. With the increase in the number of ribosomes on the rough endoplasmic reticulum, which is a neuron cell characteristic, a darker purple color was observed in the soma of the cells compared to normal cells (Figure 3.9). While this darkness is not observed in cellular extensions, a pale pink color should be seen in the same negative cells. As in the literature, it is important to investigate how applied protocols affect the transcriptional and translational regulation of differentiated cells. When the Figures of 3.10, 3.11, 3.12 and 3.14 were examined, it was determined that exosomal application and induction medium-differentiated cells showed mature neuron markers. However, it was found that the 4-day protocol was not sufficient for changing neuronal-related gene and protein expressions. On the 10th day of differentiation, changes in both gene level and protein levels were observed. The elements like growth factors in the differentiation protocol

are expected to lead the PDLSCs into dopaminergic according to the literature [48,56,59]. Therefore, the dopaminergic markers were also examined in the cells. At the gene-protein level, both enzymatic proteins and transcription factors are seen to be upregulated and therefore are thought to be a dopaminergic lineage during maturation.

5. CONCLUSION

A novel neurogenic differentiation protocol compromising pre-induction, cell cycle arrest and maturation as three-stage protocol lead to mature and lineage-specific neuron formation successively in vitro. According to the results of morphological, gene and protein level analysis, it was determined that PDLSCs didn't undergo to apoptosis and reverse to the fibroblastoid formation during neurogenic differentiation protocol. Thus, it is contemplated that the differentiation process doesn't pertain to pseudoneurogenesis. The CSDE didn't result in significant modulations directly in gene and protein levels on neurogenic differentiation compared to the induction medium alone. However, it was observed that the PDLSCs that differentiated by CSDE group significantly reduced the cell death compared to induction medium only. When all the experiment were considered, it is determined that the medium based induction protocol require at least 8-10 days to accomplish neurogenic differentiation.

In conclusion, this study clearly demonstrates that the induction protocol could differentiate the PDLSCs into mature neurons which contain dopaminergic markers after 10 days, but that exosomes have no direct effect on neurogenic differentiation. As a future aspect, endeavoring to obtain differentiated PDLSCs as a purer and more homogenous mature and functional neuron population and measuring membrane potentials of them by patch clamp analysis are extremely important.

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