

**THE USE OF NANOPARTICLE LABELING
IN CELLULAR TRACKING**

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ABSTRACT

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Adult stem cells (ASCs) are a population of multipotent cells which have ability of self-renewal and tissue regeneration. Due to their protective and restorative roles, ASCs become candidate for cellular therapies. Some cellular imaging methods have been developed to monitor stem cell differentiation and migration. Regrettably, none of these techniques possess the properties of an ideal imaging methods such as photostability and non-toxicity. A new type of probe, conjugated polymer based water-dispersible nanoparticles (CPN) that possess strong fluorescence light emission, non-toxicity, photosability and high brightness, has been developed to fulfill the needs of cellular tracking. The aim of this study is to show the utilization of CPN labeling in *in vitro* and *in vivo* cellular tracking. We initially focused on the monitoring of the differentiation and migration of MSCs which have been proved to be a promising therapeutic tool. First we showed 24h CPN labeling did not cause severe decrease in the cellular activity of MSCs and had no effect on their marker expression and differentiation capacity *in vitro*. In addition, 24h CPN labeled MSCs showed very intense green fluorescence emission which was still bright after 3 weeks of MSC differentiation. We also showed CPN labeled MSCs were able to migrate to the damaged site and retained their labels *in vivo*. Similar to MSCs, DPSCs were labeled intensely and with negligible decrease in their cellular activity within 24h CPN incubation and it had no effect on their differentiation capacity.

In addition, cancer cell tracking is important for the understanding of steps of metastasis and chemotherapeutic drug's mode of action. Therefore, we tested CPN labeling in Huh7 cells, and we showed that labeled cells had very intense fluorescence emission without any change in their cellular activity. Moreover, tumor xenograft model that were generated with either 24h or 72h CPN labeled Huh7 cells showed that CPN labeling retained for about 2-3 months *in vivo* and did not lose their brightness. To conclude, we aim to propose a new approach for *in vivo* cellular tracking in order to obtain unattainable information of the migration and homing behaviors of the stem cells. In addition, our approach can also be used for evaluation of cancer cell metastasis as well as the success of stem cell or anti-cancer therapy.

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Keywords: CPN, MSCs, *in vivo* cellular tracking, cell labeling, Hepatocellular carcinoma, DPSC

ÖZET

HÜCRE TAKİBİNDE NANOPARÇACIK İŞARETLENMESİNİN KULLANIMI

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Yetişkin kök hücreler farklı hücre tiplerine dönüşebilen, kendini ve bulunduğu dokuyu yenileme özelliğine sahip hücrelerdir. Bulundukları dokulardaki yenileyici ve güçlendirici etkilerinden dolayı yetişkin kök hücreler hücre tedavisi olarak kullanılmaya başlanmıştır. Kök hücreleri görüntülemek için bazı hücre işaretleme metotları geliştirilmiştir ancak bunlardan hiç biri ideal görüntüleme metotlarının özelliklerine sahip değildir. Bu metotlar kısa sürede solmanın yanı sıra bazen bulundukları hücreler için öldürücü olabilir veya hücresel faaliyetlerinin durmasına sebep olabilirler. Sentezlenen konjuge polimer bazlı suda çözünebilen nanoparçacıklar; kuvvetli floresan ışıması vermesi, güvenli olması, uzun ömürlü olması ve parlaklığından dolayı alandaki bu eksiklikleri kapatabileceği umuduyla geliştirilmiştir. Bu çalışmada, sentezlenen nanoparçacıkların *in vitro* and *in vivo* hücre takibinde kullanımının incelenmesi amaçlanmıştır. Mezenkimal Kök Hücre (MKH) 'lerin tedavisel avantajlarından dolayı öncelikli olarak bu hücrelerde nanoparçacık işaretleme yapılmış ve işaretle hücrelerin başkalaşım ve göçleri incelenmiştir. Öncelikle 24 saat nanoparçacık işaretleme MKH'lerinin hücresel aktivitelerini ciddi bir şekilde azaltmadığı, hücre yüzey belirteçlerini ve başkalaşım özelliklerini değiştirmediği *in vitro* olarak göstermiştir. Bunun yanı sıra, 24 saat nanoparçacık işaretle MKH'ler yoğun bir floresan ışıması göstermiştir ve bu ışıma 3 hafta süren MKH'lerden başkalaşım sürecinin sonunda hücrelerde aynı yoğunlukta gözlenmiştir. Bu

çalışmada nanoparçacık işaretlenmiş MKH hücrelerinin hasar görmüş dokuya göç etme özelliklerinin değişmediği gösterilmiş ve böylece bu nanoparçacıklarının *in vivo* takip için kullanıma elverişli olduğu kanıtlanmıştır. MKH'ler gibi Dental Pulpa Kök Hücre (DPKH)'ler de 24 saatte nanoparçacıklar ile kuvvetli bir şekilde işaretlenmiş ve başkalaşım özelliklerini ve yaşamsal faaliyetlerini kaybetmemişlerdir. Bunların yanı sıra, metastazın belirlenmesi ve anti-kanser tedavinin takibi uygulanan tedavinin başarısını belirlemek açısından çok önemlidir. Bu amaçla nanoparçacık ile işaretlenen Huh7 karaciğer karsinoma hücrelerinin yoğun bir florasan ışımaya sahip olduğu gözlenirken yaşamsal faaliyetlerinde değişim olmadığı gösterilmiştir. Ayrıca, 24 saat ve 72 saat nanoparçacık işaretlenmesiyle Huh7 hücrelerinin *in vivo* takibi yapılabileceği bu hücrelerin nüde farelerde oluşturduğu tümör doku kesitlerinde gösterilmiştir. Özet olarak, bu çalışmada *in vivo* hücre takibinde kullanılarak bilinmeyen yeni bilgilere ulaşılmasına yardım edecek bir yaklaşım önerilmiştir. Bu sayede kök hücrelerin göçleri ve kanser hücrelerinin metastazı takip edilirken bir yandan da kök hücre ve anti-kanser tedavilerinin başarıları değerlendirilebilecektir.

Anahtar sözcükler: Nanoparçacık, hücre işaretlenmesi, *in vivo* hücre takibi, MKH, hepatocellüler karsinom hücresi

TO MY LOVELY FAMILY...

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Abbreviations

AD	Alzheimer's Disease
ASC	Adult Stem Cell
BLI	Bioluminescence Imaging
BMMSC	Bone Marrow Mesenchymal Stem Cell
Bp	Base Pair
BSA	Bovine Serum Albumin
cDNA	Complementary deoxyribonucleic acid
CCl ₄	Carbon Tetrachloride
CNC	Cranial Neural Crest
CPN	Conjugated Polymer Nanoparticle
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic Cell
ddH ₂ O	Double Distilled Water
DEPC	Diethylpyrocarbonat
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide

DPSC	Dental Pulp Stem Cells
EB	Embryonic Body
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
ES cell	Embryonic Stem Cell
EtOH	Ethanol
FBS	Fetal Bovine Serum
FGF	Fibroblastic Growth Factor
FITC	Fluorescein isothiocyanate
GFP	Green Fluorescent Protein
GVHD	Graft Versus Host Disease
HD	Huntington's Disease
H&E	Heamatoxylin-eosin
HG	High Glucose
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HSC	Hematopoietic Stem Cells
HSC	Hepatic Stellate Cells
HSV	Herpes Simplex Virus
i.p	Intraperitoneal
iPS cell	Induced Pluripotent Stem Cell

ICM	Inner Cell Mass
LDH	Lactate dehydrogenase
LG	Low Glucose
LIF	Leukaemia Inhibitory Factor
MDB	Membrane Desalting Buffer
MEF	Murine Embryonic Fibroblast
MHC	Major Histocompatibility Complex
Min	Minutes
M-MuLV	Moloney Murine Leukemia Virus
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MSC	Mesenchymal Stem Cells
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NASH	Non-alcoholic Steatohepatitis
NCBI	National Center for Biotechnology Information
NK cell	Neural Killer Cell
NSC	Neural Stem Cell
OI	Osteogenesis Imperfecta
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PET	Positron Emission Tomography

PH	Partial Hepatectomy
QD	Quantum Dots
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Revolutions Per Minute
RT	Reverse Transcription
SD	Standard Deviation
SH	Sham
SPIO	Superparamagnetic Iron Oxide
TAE	Tris Acetate EDTA
TGF	Transforming Growth Factor Beta
UV	Ultra Violet
α -SMA	Alpha Smooth Muscle Actin
^{111}In	Indium 111

Chapter 1

1 Introduction

1.1 Cellular Tracking

Detection of a single cell in living organisms through noninvasive imaging methods provides recently a way to monitor cell homing and cell migration after therapeutic cell transplantation [1]. Many cell types are commonly used for therapeutic and delivery purposes in most of the diseases. Monitoring cell migration to the target organs is crucial to assess whether the treatment is a success or a failure. Cellular tracking is primarily required as it allows tracking of the stem cell migration to damage site, identification of any tissue rejection, detection of early cancer and tracking of neural stem cells in the case of stroke. For example, with the help of developed *in vivo* tracking methods, the administered hepatocytes can be tracked throughout the body of patients after applied gene therapy [2]. Importantly, *in vivo* tracking of the cancer cells gives information on the metastasis of cancer which is the major obstacle in the success of the cancer therapies. Therefore, newly developed methods of cell tracking assist the improvement of anticancer therapies through specifying the target step which is either the metastasis or the invaded organ [3]. Effective delivery of cells, such as circulating adult stem cells to the target organs, can be determined via *in vivo* tracking of these cells [4]. Development of non-invasive techniques will replace the need for histological analysis, in which sacrifice of the animal is required so that biopsies can be obtained. Moreover, improved cell imaging technologies have key importance in providing significant progress in cellular therapeutic strategies [1]. Since stem cells migrate to the damaged heart, they have been used to identify where the damage is in the heart [5, 6]. In addition, it is important to track transplanted neural precursor cells to damaged brain which helps to assess the progress of repair of the brain [7]. The cellular tracking is not only important in stem cell based therapies but is also used in oncology and immunology [5]. Detection

of the path of dendritic cells which are loaded with tumor antigen is vital in evaluation of the efficiency of a tumor therapy [8]. In conclusion, recent improvements in cellular tracking technologies enable scientist to monitor the primarily stem cells and cancer cells which are important for the understanding of the progress of cell based therapies.

1.2 The Techniques of Cellular Imaging

Radionuclides have been widely used in medicine for the benefits of cellular tracking for nearly 30 years. They designate the site of inflammation by tracking the leukocytes [9]. This basic radiolabeling of indium 111 (^{111}In) provides a non-specific association with cells, which can be visualized via gamma camera imaging. However, the ideal probe used in clinics requires detection of single cells and small lesions in more sensitive way. These necessities force generation of new single photon emitting radiolabels which are detected and quantified by positron emission tomography (PET). However, positron emitting radiolabels have their own limitations such as variable labeling efficiencies, short half-life (short lasting tracking) and rapid efflux [10]. The most widely used technique for *in vivo* cell tracking is magnetic resonance imaging (MRI) as it can be applied to all cell types and provides an image acquisition. It works by generating a contrast in the region based on the proton density, flow and bio-chemical structure differences which are detected as positive or negative signals. MRI takes advantage of the missing ionizing radiation which results in high resolution and image contrast. This technique usually requires a contrast agent such as superparamagnetic iron oxide (SPIO), dimeglumine, gadopentate in order to improve the quality of imaging. Since these agents are not precise in recognizing specific cell types, they do not provide very specific cell tracking. Label uptake step is the limiting step of MRI as it is variable among different cell types. Although the widely used MRI agent SPIO is easily taken by many cells, non-phagocytic and slowly dividing cells have difficulties in the uptake of these particles. In order to accomplish internalization of SPIOs in these cell types, higher concentrations are required. Different methods have been developed to solve this problem. SPIO coating with lectins, monoclonal antibodies and HIV tat peptide were shown to increase their internalization [11–14]. However these methods have their own side effects which concern technical difficulties, species specificity and biosafety. The SPIOs are also

discarded from cells after few culture passages [7]. Another negative side of MRI is its inability to separate void from agent which can create artifacts. For all of these reasons, cell tracking with these negative contrast methods are not satisfactory [15]. In addition to these techniques, bioluminescence imaging (BLI) can be used to monitor cells' differentiation, location and characteristics *in vivo*. This method takes advantage of the absence of endogenous luciferase expression in animal cells. However, the substrate of luciferase is required to be administered exogenously in intact animal [16]. In this technique, the number of cells and the signal occurred *in vivo* are correlated. This technique provides a way to make semi quantitative measurements [17]. However, the BLI does not show the exact situation of the biological processes since the results may vary according to the local niche and situations like inflammation [18]. Alternative to luciferase assay, stable expression of green fluorescence protein (GFP) in cancer cells, gives an opportunity to *in vivo* monitoring of cancer cells through histopathological experiments in very sensitive and rapid ways. By this way, the cancer metastasis to new organs or bone can be identified. The biggest advantageous of the GFP labeling is the lack of requirements of the preliminary methods, substrate or contrast agents except the blue light illumination [19–23]. Due to the specific GFP expression in only transfected cells, these cells are selectively visualized in tissues. The GFP labeling in tumor tissues could be detected with the help of simple external video recorder [24]. Also, the two-photon confocal microscopy integrated with thermoelectrically cooled color charge-coupled device camera has been used to analyze the GFP labeled cells and angiogenesis in tumor tissues of the intact animal [24, 25]. However, GFP labeling may cause genetic perturbations due to its requirement for the transfection in order to introduce the transgene [26]. In addition, tissue and skin over the tumor is a big restriction for the acquirement of the qualified image [27]. Due to above mentioned imperfections of the already existing techniques, *in vivo* cell tracking field requires a more improved, sensitive and specific techniques in advanced cell tracking.

1.3 Recent Advances in Fluorescence Cell Imaging Methods

The progress in molecular biology, organic chemistry and material science gave rise to new nanoscale types of fluorescence tags for cellular imaging, one of which is quantum dots (QDs). QDs are inorganic nanomaterials that have bright fluorescence emission in different wavelengths based on their size and extinction coefficients [28]. Due to their inorganic structure, they evade from chemical and metabolic degradation and hence they have a very long photostability, high quantum yield and signal quality [29, 30]. All together, these properties of QDs make them good candidates for biomedical uses compared to fluorophores. The most commonly used organic fluorophores (genetically encoded fluorescent proteins or chemically synthesized fluorescent dyes) had 2 restrictions; which include fading of fluorescence with time and being non-optimal for multicolor studies [31]. Recently, the usage of QDs *in vitro* and *in vivo* imaging was shown to be preferential [30, 32]. However, the cytotoxic effects of the QDs have been a controversial issue. Some researchers reported that they are non-cytotoxic whereas some imply QDs are toxic due to their physicochemical properties [20, 23, 24]. In addition to their physicochemical properties, used dosage and exposure concentrations also determine their toxicity [33, 34]. QDs were shown to be useful as labeling agents in live cell tracking in the study of labeled MSCs *in vitro*. They have provided ease to follow MSCs with bright and photostable fluorescence emission. However, the dose and the exposure time essentially need to be set to the lowest level in longer studies [29]. Indeed, QDs forced scientists to improve the imaging methods and hence unknown and inaccessible information are now being assessed both *in vivo* and *in vitro*. Furthermore, highly sensitive and high quality imaging of QD in introduced mice were shown *in vivo* [35]. Despite the advantages of QDs, there are restrictions that must be overcome before they can be used safely. Due to their large size, their translocation through the intact membrane is difficult. Therefore, a permeabilization step is required [29]. In addition, QDs are bulky materials and once they attract to a cell, they may inhibit labeling of neighboring cells. Hence, in cases of high numbers of cells, total labeling i.e. overall signal and sensitivity decreased. To sum up, the QDs work best when there are low number of molecules interested or when the molecules are dispersedly localized [36]. In addition, these

particles made aggregations when they were kept in aqueous environments for a long time and changes occurred in pH and temperature.

Cell labeling with QDs require complex methods such as microinjection, electroporation or phagocytosis [37]. These limitations force scientists to find new ways of cell labeling. Conjugated polymer nanoparticles (CPNs) are nanomaterials that are promising for the fields of bio-imaging and nanomedicine. These materials have the potential to be used as imaging agents and biosensors due to their optimized sizes and properties, biocompatibilities and non-toxic effects. Use of CPNs has advantages as they have the capability of assessing different properties based on the needs through changing their conjugated polymer or the surface composition. In addition, CPNs have advantages over the inorganic nanoparticles since they are easy to be synthesized, they are non-toxic and have adjustable properties [38]. CPNs have similar magnitudes as QDs and have brighter emission compared to them [38–40]. Similar to QDs, CPNs are resistant to bleaching and they keep their bright emission without any fade. All together, these advantages make CPN use more preferable than the QDs in cellular imaging.

1.4 Stem Cells

Stem cells are the cells that are responsible for the regeneration and repair of tissues and organs in addition to the development of the organisms. These cells are defined by their ability of self-renewal and multilineage differentiation. These cells can divide asymmetrically and give rise to more differentiated, less potent form of cells without the ability of self-renewal. In the end, the dividing progenitor cells give rise to mature cells [41]. Stem cells differentiate into different type of cells according to their level of potency. The top point of the tree of potency is the totipotency, in which cells are capable of generating the embryo and the trophoblast of placenta. The pluripotent embryonic stem (ES) cells follow the totipotent cells and they are capable of differentiating into cells from all three germ layers. The multipotent cells come after the ES cells, and they are restricted to differentiate into specific type of cells according to the location that they reside. Lastly, unipotent cells can only divide to give rise to one specific type of cell. At the end of this tree, the terminally differentiated somatic cells are generated [42] (Figure 1.1).

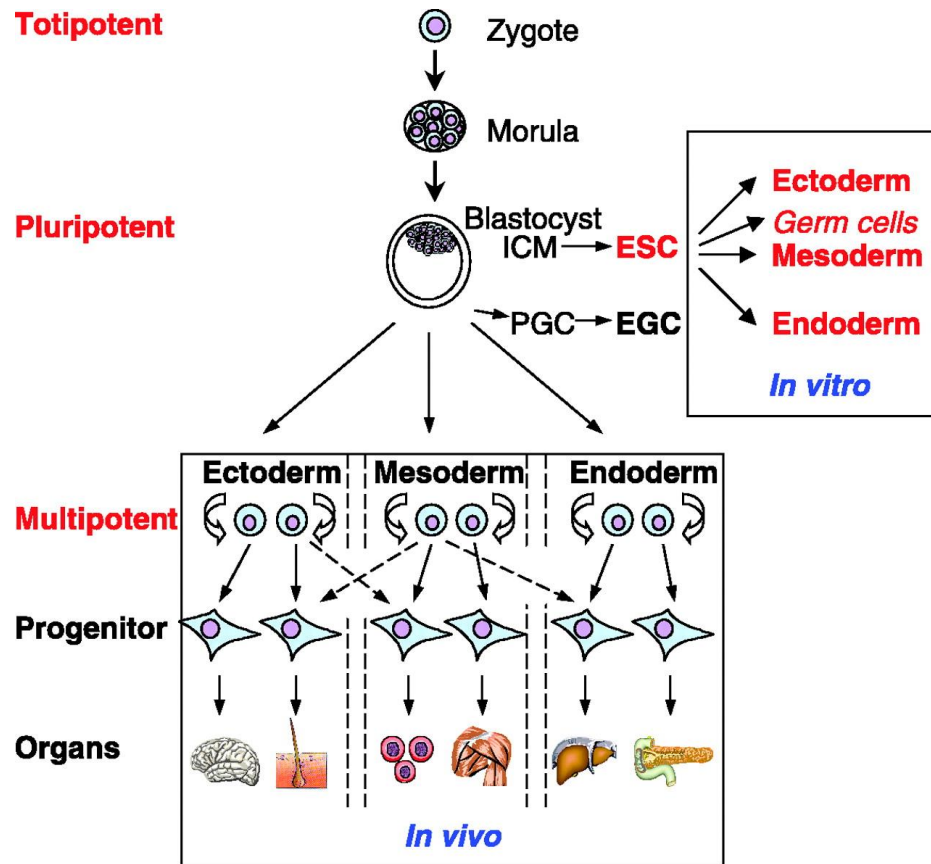


Figure 1.1 The hierarchy of stem cells

The zygote is totipotent until it differentiates into the morula stage. Totipotent cells are able to generate whole organism. Inner cell mass cells found in the blastomeres are able to generate cells from all 3 germ layer (endoderm, mesoderm and ectoderm and primordial germ cells). The multipotent stem cells which found in the adult organisms have a role in tissue regeneration of damages cells. These multipotent cells have cellular plasticity and are able to differentiate into cells from other lineages. The figure was adopted from [43]. (Copyright © 2005 the American Physiological Society)

1.4.1 Embryonic Stem (ES) Cells

Embryonic stem cells (ES) are the cells that cluster in inner cell mass (ICM) at the blastocyst stage of the embryo. These cells are capable of yielding the cells from all three germ layers (endoderm, mesoderm and ectoderm). Moreover, these cells are able to differentiate into the germ cells as well. Indeed, if ES cells are placed in the ICM of the embryo, they are capable of participating in the embryonic development [44]. ES cells are able to proliferate unlimitedly without losing their pluripotency *in vitro* [45, 46]. In addition, they do not lose their genetic integrity throughout many passages [47, 48]. ES cells are able to form precursors of nearly all cell types when they are introduced to defined signals. The transplantation of ES cells directly to the mice, without any differentiation, cause spontaneous abnormal differentiation of cells which form teratomas eventually [49].

ES cells express Oct4, Sox2 and Nanog transcription factors which are essential for keeping the ES cells in the undifferentiated state [50–53]. In addition to these transcription factors, the undifferentiated state of the murine ES cells require the presence of leukaemia inhibitory factor (LIF) and murine embryonic fibroblasts (MEFs) *in vitro* [54, 55]. On the other hand, human ES cells require MEFs feeder layer as well as basic fibroblastic growth factor (bFGF) or Matrigel containing the MEF conditioned medium [45, 46]. Without these requirements, ES cells differentiate spontaneously and form spherical aggregates which are called embryonic bodies (EB) [49]. The EBs include differentiated cells such as cardiomyocytes [56], hematopoietic cells [57], endothelial cells [58], nerves [59], skeletal muscle [60], adipocytes [61], chondrocytes [62] and liver cells [63].

ES cells have been technologically used to generate genetic alteration in mouse models due to their ability of *in vivo* differentiation into the germ cells. Through this way, the function of the gene of interest can be assessed [49]. When ES cells are transplanted into the blastocysts, they participate in the development of the animal and generate cells from 3 germ layers [64]. The studies done by using ES cells provided useful information about the biology of the genetic diseases and embryogenesis. Furthermore, since ES cells have indefinite self-renewal and differentiation potential, they become potential source of

cells to be used in biomedical studies and regenerative medicine [65]. The usage of autologous cloned human embryos overcomes the immune-rejection handicap which occurs during cellular transplantation [66].

1.4.2 Induced Pluripotent Stem (iPS) Cells

The risks of tissue rejection problems occurring during the transplantation of ES cells and the clinical utilization of ES cells derived from human embryos are controversial issues. In order to solve these kinds of problems, scientists tried to make pluripotent cells from patients' own cells. The differentiated somatic cells can be reprogrammed to undifferentiated embryonic like cells with the fusion of these cells with the ES cells [67, 68]. Yamanaka and his group hypothesized that the somatic cells can be transformed without nuclear cell fusion through administrating the ES cell transcription factors which are essential for the protection of the pluripotency state of these cells. They showed that introduced 4 transcription factors; Oct4, Klf4, Sox2 and c-Myc, were able to generate ES-like cells from human dermal fibroblast when the ES cell culturing conditions were provided. The introduction of Nanog was found to be non-essential. These newly generated cells were named as induced pluripotent stem (iPS) cells. iPS cells showed similar morphology, cell growth ability and cell marker expression as ES cells. These iPS cells showed normal karyotypes and their methylation/demethylation patterns were similar to the ES cells rather than the cells they were originated from. Introduction of iPS cells to the nude mice causes the teratoma formation which includes many types of tissues from different cell origins. In addition, iPS cells contributed to the development of the mouse embryo when they were introduced into mouse blastocysts. Also, iPS cells had high telomerase activity which gave them the capability of indefinite cell growth. The ES cells form ball-shaped cell structures called embryonic bodies. It was shown that, the generated iPS cells could also form these cell structures *in vitro*. Finally, it was proven that when they were induced with the defined differentiation medium, these cells were able to differentiate into specified cell types from all 3 germ layers such as liver, skin, heart and neurons [69].

Along with the benefits of the patient specific iPS cells, there are some side-effects that are required to be overcome before we can use them safely in clinics. Use of

retroviral vectors is inevitable for the introduction of essential transcription factors to the cells but these vectors may cause mutagenic effects. In addition, the c-Myc oncogene introduction may favor occurrence of tumors. These drawbacks of the iPS utilization should be solved for further use but at least the iPS could be used in animal models to observe cell behaviors *in vivo* [69]. Studies done in the disease animal models such as the diabetes mellitus and spinal cord injury showed that the iPS cells contributed to recovery of the disease through reducing high glucose level and favoring the neural differentiation and re-myelination, respectively [70, 71]. Moreover, the iPS cells were shown to favor the cardiovascular regeneration and recovery of the Parkinson's disease [72, 73]. Based on the benefits of iPS cell therapy shown in disease models, methodology of generation and introduction of iPS cells should be improved for their safe use in human clinical trials.

1.4.3 Adult Stem Cells

During the development of the multicellular organisms, cellular proliferation occurs under the control of genome. These sequential events cause the formation of differentiated cells and tissues/organs. However, some cells leave this sequential progress and rest as a reserve of precursor cells. These cells are called 'adult stem cells' (ASC) and are the cells that reside in specific tissues of adult organisms [74]. These cells are more specialized compared to ES cells and they differentiate into to cells that they are committed to [75]. On the other hand, ASCs are rare cells and compose nearly 1-2% of the tissue cells. ASCs have an essential role in the tissue/organ maintenance and repair during the life cycle of an organism [74]. Under continuous tissue damage, these cells self-renew and become an essential solution in the protection of tissue integrity. The asymmetric division of ASCs helps to replace the dying cells in the damaged tissue. During asymmetric division, tissue stem cell numbers remain constant through generation of daughter cells, which are exactly the same as the cells that they derived from (Figure 1.2). Without any heavy stress, these cells remain quiescent and slow cycling. By this way, stem cells are protected from the errors that may occur during DNA synthesis. One interesting mechanism that protects stem cells' DNA from replication errors is retaining the template DNA strand in the daughter stem cell and passing the newly generated strand

to the differentiated cell [76]. Undifferentiated state of stem cells is supported by the environment (niche), in which stem cells reside. A niche is composed of the tissue cells and the extracellular materials which send signals to stem cells to maintain their undifferentiated state. These signals monitor stem cells' self-renewal and differentiation [77].

Autologous transplantation of ASCs may become a potential therapy in the treatment of traumas and diseases. The benefits of using ASCs in therapies include their easy isolation from newborn to geriatric patients, and having no risk of developing immune rejections. ASCs are easily obtained from the biopsies of skeletal muscle and dermis. In addition to these benefits, due to telomerase activity, these cells can be preserved and passaged for long times without any change in their pluripotency, viability, differentiation capability and functionality [74].

Hematopoietic stem cells (HSCs) are among the most widely known ASCs. They are responsible for the continuous generation of all blood cell types. These clonogenic pluripotent cells are capable of cell renewal and differentiation into multilineage of the hematopoiesis [78]. HSCs give rise to oligolineage progenitor cells which differentiate into terminally differentiated mature blood cells [79, 80]. HSCs can give rise to not only all blood cells, but also to muscle cells, cardiomyocytes, neural and hepatic cells [81–84]. The differential differentiation of HSCs is monitored by their niche including the regulators such as extracellular matrix molecules, cytokines and growth factors. HSCs comprises 0,05-0,5% of the bone marrow [78]. HSCs can be identified by the presence of the CD34 and Sca-1 and the absence of CD38 and CD90 cell surface marker expression [78, 85, 86]. The bone marrow transplantation, which is the transplantation of allogeneic (HLA identical) HSCs, creates a hope for patients in curing the bone marrow failures and restoring the hematopoiesis. In addition, the transplanted HSCs also help restoration of hematopoiesis in patients that took chemoradiotherapy for the treatment of their malignancies [78]. However, the risk of graft versus host disease (GVHD) should be considered before HSCs are administered.

The nervous system involves cells that have high proliferation potential, multilineage differentiation ability and self-renewal, which were shown by colony forming assays of the mouse embryonic basal forebrain progenitors [87, 88]. The

existence of the neural stem cells (NSCs) were further supported by the formation of multicellular spheres (neurospheres) with the multipotent cells isolated from adult brain [89]. The formation of the neurosphere becomes an essential tool in evaluating the stem cell likeness of the cells isolated from the nervous system [90]. Similar to other adult stem cells, NSCs are regulated by factors which include Notch, Wnt, BMP and Shh found in their niche [91, 92]. According to the signal, NSCs undergo asymmetric cell division and give rise to neurons, astrocytes, oligodendrocytes and glial cells [87, 93–96]. The NSCs can be identified by the expression of the Nestin, Sox2, CD133 and Musashi [90]. The neurogenesis highly occurred in patients with Alzheimer's disease (AD), epilepsy, Huntington's disease (HD) and Parkinson's disease (PD) in order to restore the loss of the neural cells [97]. Through this way, neurogenesis is involved in the repair of the central nervous system via generating functional neural cells. Based on this information, therapeutic strategies were considered which involved the stimulation or transplantation of NSCs to the diseased patients [98]. Grafted NSCs were shown to migrate to the site of injury in the brain and restore the damaged cells [99, 100].

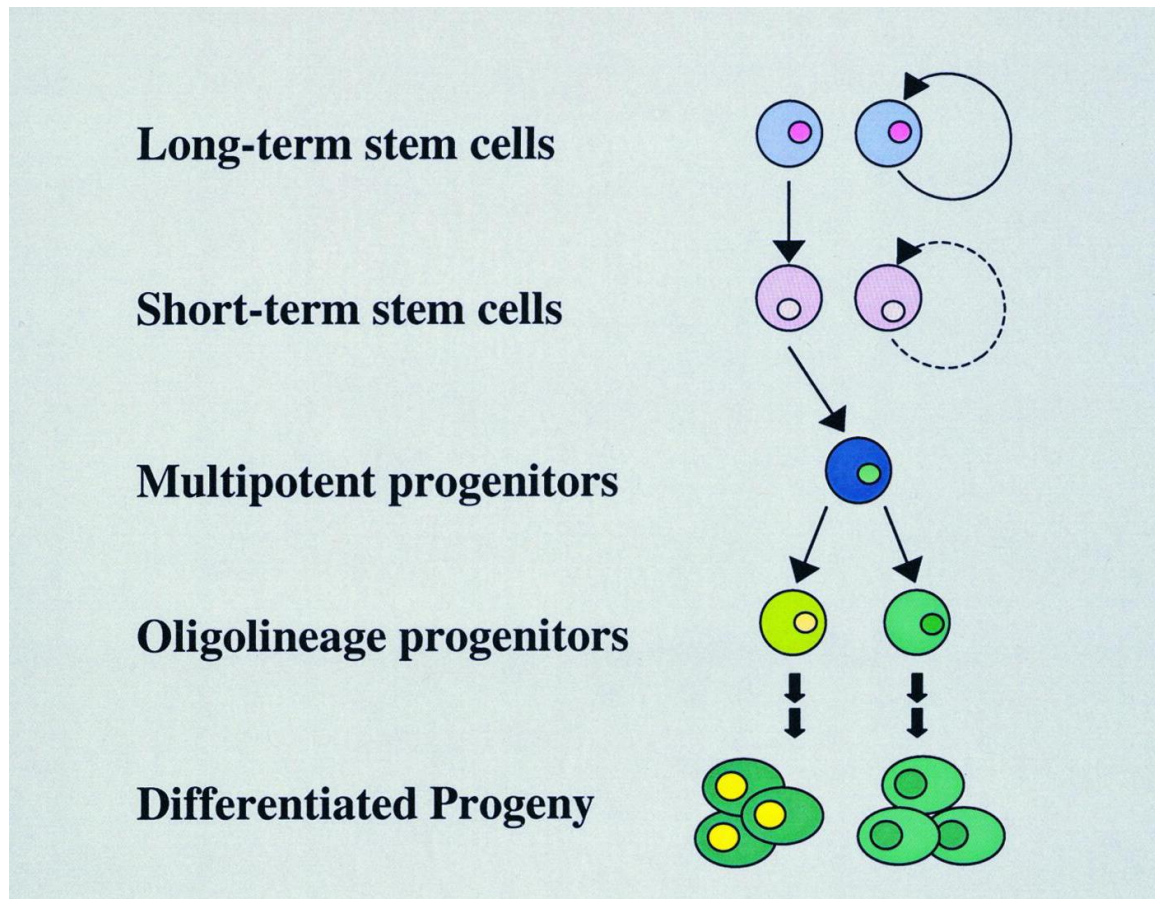


Figure 1.2 The representation of progenitor cell lineages

The figure shows the self-renewal ability and differentiation of potent stem cells into more specifically differentiated cells. Once stem cells were differentiated into more restricted progenitor, their differentiation potential and self-renewal activity are also restricted. Adopted from [41]. (Copyright © 2000 Cell Press)

1.4.3.1 Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that have the ability to differentiate into bone, muscle, cartilage, adipose, marrow stroma, tendon and ligament in addition to other connective tissues (Figure 1.3) [101]. Although MSCs mainly reside in the bone marrow, they can also be isolated from skin, muscle, placenta, umbilical cord, adipose tissue and around the blood vessels [102]. Moreover, MSCs are capable of differentiating into multiple lineages such as neural cells [103], cardiac cells

[104, 105], renal [106] and hepatic cells [82]. MSCs are essential for the maintenance of overall health of an individual, since they are involved in repair, remodeling and regeneration of tissues [102]. MSCs have the ability to migrate to the site of injury for the regeneration of tissue and promotion of angiogenesis [107, 108]. Through secreting paracrine signals they regulate migration, proliferation and survival of other cells. The intense wound healing process, which is required in case of damage such as trauma, diabetes and other conditions, is regulated by surrounding MSCs. They modulate inflammation, proliferation and remodeling that occur during wound healing process and therefore they provide a support for successful healing [109]. The utilization of MSCs in regenerative medicine is becoming a promising tool, especially in gene therapy or bone regeneration study fields. [110]. MSCs do not lose their self-renewal capacity in several ex vivo passages, they proliferate without differentiating and carrying their multilineage differentiation potential [65].

MSCs are mainly found in the bone marrow, which constitute to 0.01% to 0.001% of overall cell population [111]. Bone marrow is composed of heterogeneous population of cells which express different surface molecules. Isolation and identification of MSCs are based on these surface molecules. CD90, CD71, CD29 are the primary markers that are expressed on the surface of MSCs [110]. On the other hand, MSCs can also be identified based on the absence of hematopoietic lineage markers CD45, and CD34. Moreover, MSCs are colony forming cells which grow independent of density [112]. One of the major features of MSCs that make them good candidates for cell based therapies is their ability of repressing the T cells mediated immune response and not expressing MHC II molecules. Moreover, they inhibit the maturation and differentiation of antigen presenting dendritic cells (DC). They also shift cytokine excretion from a pro-inflammatory to an anti-inflammatory profile [113]. Therefore, MSCs do not provoke graft rejection related immune response [114, 115]. To sum up, together, these specialties of MSCs make them universal donor cells in clinical research [102].

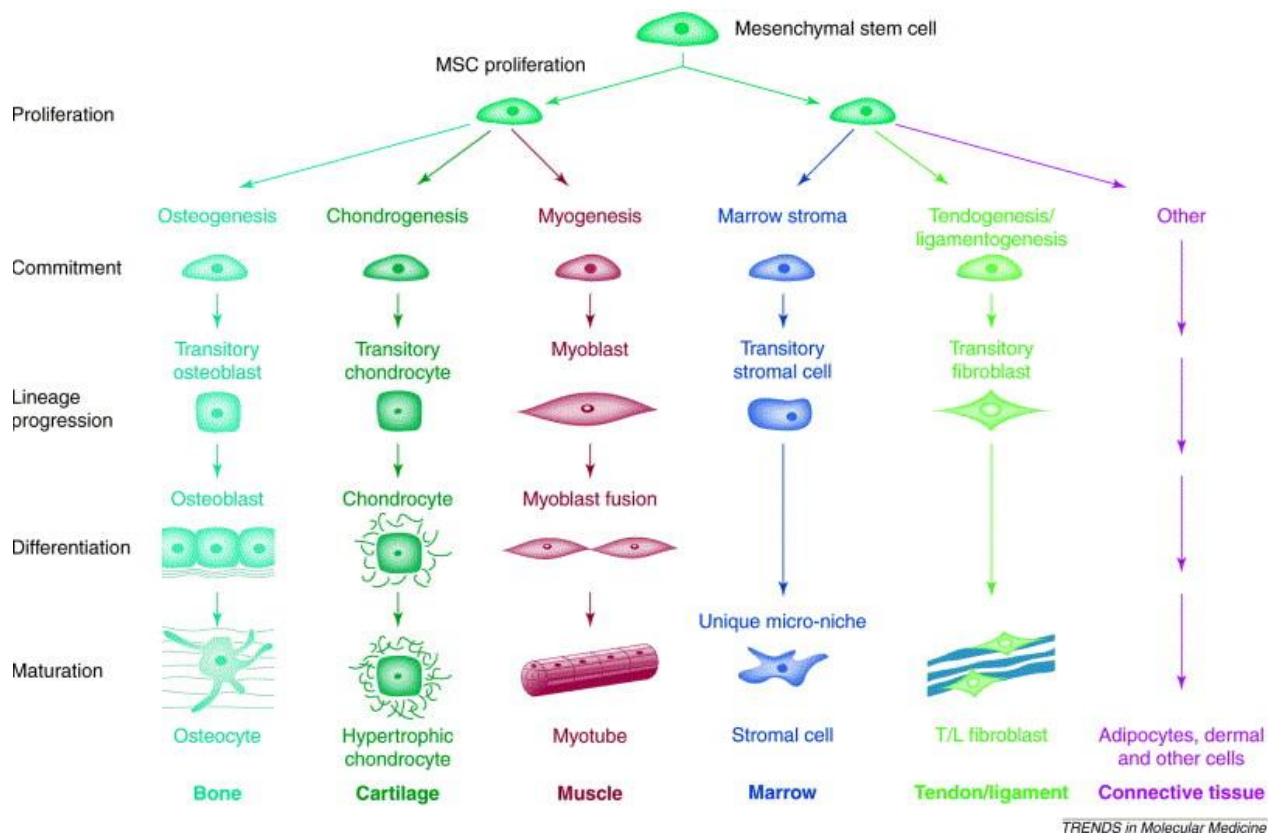


Figure 1.3 The mesengenic process.

The hierarchy of MSCs differentiation into highly differentiated cells. Adopted from [102]. (Copyright © 2001 Elsevier Science Ltd)

1.4.3.2 Dental Pulp Stem Cells (DPSC)

The multipotent dental pulp stem cells (DPSCs) that are isolated from human third molars (wisdom teeth) are valuable and easily accessible sources of mesenchymal stem cells. Obtaining DPSCs are easy since they are collected from removed wisdom teeth of the donor without any further operation. DPSCs have abilities of self-renewal and multi-lineage differentiation. These cells are able to differentiate into odontoblast, osteocyte, chondrocyte and adipocyte similar to all mesenchymal stem cells. DPSCs are located in the perivascular niche of dental pulp and are mostly isolated from pulp and periodontal ligaments of adult teeth [116, 117]. Easy accessibility of dental pulp from teeth by standard dental methods, giving high yield of stem cells after extraction and bearing

extreme ex vivo proliferation are good qualities DPSCs which make them precious sources of adult stem cells [118]. It was proven that DPSCs differentiate into osteoblast in the same biochemical way as the bone marrow MSCs (BMMSCs) do. In addition, DPSCs have similar expression patterns with BMMSCs which were shown in expression of smooth muscle and endothelial markers. Although differentially expressed genes have been found in these two cell types, they share nearly 4000 known genes of human [116, 119]. Moreover, it was shown that DPSCs are also able to inhibit the stimulation of T cells and their inhibition was even stronger than that of BMMSCs [120]. In addition to similarities in common factor regulation and similar gene expression profile, they differ in their proliferation rate and differentiation potential. DPSCs are very clonogenic types of adult stem cells, which can give yield in higher numbers compared to MSCs. In addition to that, DPSCs have higher proliferation rates than MSCs *in vitro* [121]. It was shown that, DPSCs have the ability to expand beyond Hayflicks limit (over 60 population doublings) without any changes in karyotype [118]. Due to earlier state of development of the third molar teeth compared to bone marrow, differences between DPSCs and BMMSCs in proliferation and clonogenicity may arise. Interestingly, these cells bear some markers of embryonic stem cells [121]. DPSCs are termed as ‘ectomesenchymal’ cells since cultured cells ex vivo showed some properties of neural crest [122–125]. It is hypothesized that DPSCs originated from migrating cranial neural crest (CNC) cells during the development [126, 127].

DPSCs are able to make asymmetric cell division and give rise to different cell types [116]. In addition to giving rise to osteocyte, adipocyte and odontoblast they can differentiate into myogenic and neurogenic cells [116, 128, 129]. There are differences in differentiation potentials of DPSCs and BMMSCs. DPSCs have less potential of chondrogenic and adipogenic differentiation compared to BMMSCs [120, 130]. On the other hand, BMMSCs have weaker potential to develop into neurogenic path compared to DPSCs and this may be a result of neural crest origin of DPSCs [119].

1.5 Stem Cells Utilization in Clinical Trials

Some adult stem cells from different sources remain active during whole life. These are the cells that take role in the tissue renewal and generation of cell pools in adult organism. Adult stem cells are present frequently in some tissues, which have active cell turnover such as hematopoietic bone marrow, intestine and skin. The successful transplantation of stem cells to treat some diseases created a potential way to cure diseases in clinical medicine [131]. These improvements encourage scientist to work on stem cells and emphasize their potential usage in clinical therapies. It is essential to find a tissue source which provides efficient numbers of stem cells for therapies like autologous cell therapy and individual specific cell therapy in order to reduce graft rejections. The optimal cell therapy should be identified by the researchers based on the patients' need. These investigations help to reach the goals for finding the new sources of human stem cells, controlling their potential and finding the safe ways to administer them *in vivo* [132].

1.5.1 MSC and Cell Based Treatments

MSCs are one of the best type of candidate stem cells used in preclinical and clinical trials such as bone and cartilage defects, cardiac and lung disorders, and central nervous system damages [133–135]. Easy isolation from many sources, extensive proliferation capacity, genetic stability, capability of differentiation into different cell types, ability to migrate to the site of injury and paracrine secretion of bioactive molecules to modulate immunity make MSCs excellent candidate for cell based therapies. The ability of multilineage differentiation without losing their undifferentiated state makes MSCs critical for therapeutic applications, especially in situations that require regeneration [136]. All together, these specialties of MSCs fulfill the requirements of bioengineering field [137]. First use of MSCs in clinic was for hematopoietic repair in the case of bone marrow ablation due to chemotherapy [138]. The ability of tissue repair of MSCs is accomplished by paracrine signaling molecules which monitor the surrounding cell's migration, proliferation and survival. Through this way, they regulate wound

healing and remodeling to acquire correct physiology and function through healing [139]. The bioactive molecules secreted by MSCs also play role in providing blood supply through increased angiogenesis in damaged site of tissue and protect tissue cells from apoptosis [109]. Furthermore, these paracrine signaling of MSCs have immune-regulatory action which makes these cells excellent targets for cell based therapies. Through this way, they are able to prevent T lymphocyte activation and proliferation and hence they inhibit secretion of interferons [140–142]. In addition, MSCs suppress the cytotoxic T cell activation and proliferation, but in the meanwhile increase the activity of regulatory T cells which in turn suppress the immune reactions [143–145]. Similarly, MSCs prevent activation of natural killer (NK) cells, antigen proliferating B cells and dendritic cells which all regulate the immune response [142, 146, 147]. The clinical study results support the claim that MSCs can be transplanted between different humans and no rejection occurs in transplanted organs since MSCs overcome the barriers of MHC [141, 148, 149]. Therefore, therapeutically applied MSCs suppress the acute GVHD with their immunosuppressive ability and therefore MSCs can be used in the applications of autoimmune deficiencies [150]. Another potential clinical application of MSCs was shown in wound healing process that requires complex network of growth factors and extracellular matrix proteins. MSCs recruit the attendant cells and secrete some factors that are required for tissue repair. Therefore, they help to prevent the progression of wound healing to chronic wound state. To sum up, MSCs have a role in each step of wound healing and these steps include wound repair, regulation of inflammation, epithelialization and tissue remodeling [139]. For a long time, orthopedic clinicians have been searching ways for repair and replacement of complex tissue. MSCs administration is promising for treatment of bone defects such as osteogenesis imperfecta (OI) which is shown at clinical studies in young or growing OI patients [151, 152]. The patient source of osteoblasts were all replaced with genetically healthy allogenic MSCs and it was shown that the introduced allogenic MSCs were capable of generating normal bone stock which displaced hosts' defective stock [153]. MSCs also take role in the cartilage and tendon repair. Moreover, they can be a potential approach for gene therapy if they are transduced with viral vectors bearing gene or cDNA of interest. The 74% of stable gene transfection was shown in mouse models of modified MSCs [154]. MSCs are also useful

in myocardium regeneration, which improves the activity of cardiac and ventricular wall mass [155]. These features of MSCs make them potential tools for therapies of coronary heart disease [104]. In addition to these, MSCs are promising for therapy of neuronal damages. Studies showed that administration of MSCs accelerated the repair after stroke or traumatic brain damage [156], increase re-myelination and partial regain of brain functions [157, 158]. It was also shown that the administration of MSCs into damaged brain prevented apoptosis and increased proliferation of cells. Furthermore, administration of MSCs to fibrotic animal models reduced fibrosis via secretion of anti-fibrotic molecules [159]. The fibrotic animals that were introduced to MSCs showed a decrease in the expression of fibrosis markers [160]. Also human trials of MSCs for the treatment of liver failure such as fibrosis and cirrhosis showed promising results [161]. The list of studies that favors administration of MSCs *in vivo* enlarges. MSCs are involved in the tissue repair of not only tissues mentioned above but also kidney, muscle, skin and lung [159, 162–164]. All together, these results show beneficial effects of MSCs in the cell based therapies in clinic.

1.5.1.1 Liver Regeneration and MSCs

Liver is a large vital organ which is responsible for the carbohydrate, lipid and protein metabolism, degradation of metabolic wastes (xenobiotics), serum protein production, biotransformation, glycogen, vitamin and mineral storage [165]. The major role of liver is detoxification of toxic chemicals which is primarily performed by hepatocytes. Due to its strategic location, all the circulation comes from intestine, spleen and pancreas pass through the liver via portal vein. Importantly, the liver is able to regenerate its size and function under severe damages. On the other hand, if functional excess occurred during regeneration, it is capable of adjusting itself through reducing the size or activity. The liver regeneration is orchestrated by the growth factors, cytokines and matrix protein dispositioning [166]. Liver is composed of the hepatocytes (nearly 60%), hepatic stellate cells (5-10%), Kupffer cells and endothelial cells [165]. Hepatocytes are the major cells which are responsible for the replacement of the loss of liver mass. However, when there is an end stage liver disease and hepatocytes fails to restore the liver function, liver transplantation is the only therapeutic approach. However

transplantation is challenging due to lack of organ donors, high costs and requirement for the long-term immune suppression [165, 167]. As an alternative, MSCs were shown to be potential mediators for the tissue regeneration due to their trophic activity via secreted bioactive molecules. MSCs are recruited to the site of injury and help its regeneration through differentiating into hepatocyte like cells [168, 169], decreasing the local inflammation and increasing the repair of the tissue [170]. In addition, MSCs increases the proliferation of hepatocytes and the angiogenesis while decreasing the apoptosis of hepatocytes [171, 172]. Therefore, MSCs had an important role in the regeneration of liver after a severe injury. Generally, loss of mass studies of the liver has been performed in the animals which were introduced toxic chemicals such as CCl₄. In addition, partial hepatectomy (PH) has been commonly used as a liver regeneration model in which 2/3 of the liver was surgically removed. Since rodent liver has 5 lobes, 2/3 of it is possible to be removed with easy surgery without damaging other remaining lobes. The growing liver after PH, increase the mass of tissue to the original size [166].

1.5.1.2 Liver Fibrosis and MSCs

Liver fibrosis is a worldwide severe health problem which causes mortality due to lack of validated therapy [173]. It is a kind of abnormal wound healing process occurred in damaged liver [174, 175]. The chronic injury causes accumulation and remodeling of extracellular matrix (ECM) proteins in the fibrotic liver [175, 176]. ECM accumulation disrupts the architecture of liver through generating fibrotic scar and nodules. Chronic HCV infection, alcohol consumption, chemical exposure and non-alcoholic steatohepatitis (NASH) are the causes of the liver fibrosis [177]. Hepatic stellate cell (HSC) activation is the main cause of collagen disposition in damaged liver [178]. Normally HSCs remain in quiescent state, however upon injury, HSCs become active collagen producing myofibroblast like cells and express α -SMA [178, 179]. Continuous injury results in the hepatocyte replacement with ECM and failure of regeneration [180]. Immune cells are also recruited to the parenchymal of the liver damage [181–185]. Moreover, the chronic injury in liver may promote the formation of liver cirrhosis which is the end-stage of liver fibrosis. At the end, the hepatic architecture changes and nodules are formed and, therefore, the liver functions fail [175]. During cirrhosis, the intrahepatic blood flow is

restricted which causes the hepatic inadequacy and portal hypertension [186]. Cirrhosis may result in the formation of hepatocellular carcinoma [187]. Currently, the successful therapy against liver fibrosis and cirrhosis has not been found rather than liver transplantation. Therefore, the understanding of molecular reasons of the development of liver fibrosis should be identified in order to develop an ideal therapy [188]. In order to replace the requirement of liver biopsy, a non-invasive method should be found to be used in long follow up studies of liver fibrosis [189]. The histological analysis has been the gold-standard of the disease progress [173].

Since liver fibrosis is a reversible process, unlike the irreversible cirrhosis, the optimal therapies should be identified to recover or at least slow its development [190]. It is shown that, MSCs prevent the progress of liver fibrosis by secreting some cytokines and growth factors. For instance, the hepatic growth factor secreted from MSCs has an anti-apoptotic effect on hepatocytes and, therefore, helps to liver regeneration [191, 192]. The liver fibrosis, which was generated with CCl₄ injections, was shown to be retreated in the MSC introduced mice [160, 193]. In addition, MSCs decrease the hepatocyte cell death and increase their proliferation and, therefore, restore the hepatic function [194]. Moreover, MSCs are able to differentiate into hepatocytes and revert the fibrosis by preventing the accumulation of collagens through repressing the collagen synthesis of HSCs [160, 194–197]. The decrease in the collagen level is correlated with the α -SMA activity [198]. To sum up, MSCs administration can be a potential therapy of liver fibrosis based on their ability of decrease collagen accumulation, increase hepatocyte survival and liver function.

1.5.2 DPSCs and Cell Based Treatment

The young patient's wisdom teeth are removed daily due to dental reasons and thousands of them are being thrown as they are biological waste. Due to easy isolation from removed wisdom teeth without further surgical processes, Dental pulp stem cells are becoming good stem cell candidates to be used in clinics. In addition, multilineage differentiation capability, high proliferation ability, isolation without ethical concerns and not being a oncogenic type of cells support the idea that DPSCs are usable in clinics

[132]. Since wisdom teeth are continuously obtained in many orthodontic clinics, this makes stem cells isolated from these teeth huge sources of many HLA types [199]. As they are protected from ultra violet (UV) light and other damages due to the presence of surrounding supportive tissues, they have low risks of contamination and genetic mutations [199]. Majorly, two abilities of DPSCs that make these cells valuable source of stem cells include giving high yield via high efficiency of extraction process and high proliferation capacity *ex vivo* [118]. The easy access of the donor wisdom tooth provides an extraction based on need in any time it is necessary [132]. Another advantage of DPSCs in clinics is their immunosuppressive ability through inhibition of T cell activation. Therefore, DPSCs could be used in graft versus host disease. This specialty makes DPSCs useful in autologous cell transplantation in clinical applications *in vivo*. Therefore, they are likely to be used in tissue reconstructions [120]. When compared to MSCs, DPSCs have some advantages in medical practice. They are easier to be obtained in non-invasive ways. No morbidity occurred after the isolation of pulp in the site of collection. DPSCs have higher efficiency of stem cell extraction with minimum operation [200]. They can be cryopreserved without losing the ability of multilineage differentiation for a long time [201]. In addition, they do not form teratoma *in vivo* [200]. These features make DPSCs an alternative to MSCs to be used in medical applications. Furthermore, it was shown that DPSCs are valuable source for neural crest derived ectomesenchymal stem cells [116]. These cells are likely to be used in oral defects regeneration in dentistry to repair the damaged odontoblastic cells, generation of reparative dentin and regeneration of pulp fibroblasts and collagen fibers [116, 117]. The beneficial usage of DPSCs in bioengineering of dental tissue regeneration was shown in immunocompromised animals after transplantation of DPSCs [202]. In addition to the dentistry, these ectomesenchymal cells have potential to be used in therapy of neural defects and its regeneration when required in situations like spinal cord injuries, stroke and other neural diseases [132, 203]. Since neural cells and DPSCs are coming from same embryonic origin and DPSCs bear some markers of neural cells, it can be logical to say that DPSCs are closer to nerve cells than any other adult stem cells. Therefore these cells are more likely to differentiate into functional neural and glial cells if appropriate conditions are provided [128, 203, 204]. Interestingly, these cells are potential candidates for generation of human induced

pluripotent cells (iPSC) banks with repertoire of different HLA types which can be used for human regenerative medicine [199]. In addition to all that, the beneficial effects of DPSCs in myocardial infarct through increase in anterior wall size and vessel number as well as decreasing the infarct size was shown [205]. Furthermore, the inhibition of severity of liver fibrosis and restoration of the function of liver in CCl₄ treated mice was shown in animals giving DPSCs [206]. Together these suggest that DPSCs are available to be used not only in dentistry reconstruction but also in therapeutic purposes of neural system distortions, liver diseases and myocardial diseases.

1.6 *In Vivo* Stem Cell Tracking and Imaging

Stem cell biology and regenerative medicine is currently under human clinical trials. Since stem cells have protective and regenerative roles in many diseases, they have become an alternative therapy to existing strategies like organ transplantation [207, 208]. The restorative abilities of stem cells make them good candidates for treatment of different diseases. Proved advantages of administered stem cells in human diseases are shown by the preclinical studies. The first stem cell therapy was performed with stem cells isolated from the bone marrow [209]. Lack of ideal methods for tracking stem cells in patients prevents the better understanding of stem cell biology and behavior *in vivo* [207]. Hence, ideal imaging tools are required to track stem cells to follow their migration and homing in target organ [209–211]. Due to its' need of invasive methods, histopathological determination of homing stem cells is not a preferential way for human patients. Therefore, noninvasive techniques are required to evaluate the therapeutic efficiencies of stem cells after their administration [207].

Recently, development of imaging tools such as confocal microscopy, time lapse imaging technology and reporter gene transfection provides a way to track cells *in vivo* in noninvasive ways. Among different tools of cell imaging, MRI is the most commonly used one to track stem cells *in vivo*. Stem cells should be labeled with a contrast agent in order to get signal and distinguish the cells *in vivo* in 3D resolution [212]. Cells are labeled with ferromagnetic contrast agent either in non-specific way by direct administration or in specific way by stem cell receptor mediated cell labeling [209, 213].

In addition to the benefits of using MRI, low sensitivity and toxic effects of contrast agents are the unwanted side-effects of this technique [207, 214–217].

Another commonly used method is single photo electron computed tomography imaging by using electron emitter ^{111}In oxide. ^{111}In can be internalized by stem cells nonspecifically [208, 218]. However, the possible cytotoxic effect of Indium-111 on stem cells may avert the therapeutic benefits of treatment for the patients. Similar to MRI, PET scan provide high resolution and sensitivity in stem cell tracking [219]. In addition to the advantages of this technique such as ability to track cells for months, the genetic modifications of cells and exposure of radiation maybe toxic for stem cells [5].

1.6.1 Properties of Ideal Imaging Methods for Stem Cell Tracking

The biocompatibility, safety and non-toxicity are the first and the essential properties that one tracking agent should have. The used tracking agent should not cause genetic modification. For instance, the methods that require stable transgene integration to achieve enzymatic conversion of a given substrate may cause genetic perturbations such as malignancy and abnormal growth [26]. Single cell sensitivity is important in cell tracking technologies to quantify exact number of cells in any location. This provides information about the migration pattern of stem cells which is normally unknown. The ideal imaging signal should not decrease with the cell division which affects the quantification of cell number. Since clinical trials of stem cells needs long term follow up, the ideal agents should not fade or breakdown for months to years. Finally, the tracking should not require any contrast agent.

Chapter 2

2 Aims and Strategies

An ideal cell tracking method requires a specific and sensitive detection of single cell; however, already used techniques non-specifically affiliate with the cells. In addition, they have their own restrictions which are short half-life, rapid efflux, genetic perturbations and unpredictable labeling [10]. Some solutions have been discovered to solve these problems; such as using higher concentrations of labeling agents or coating it with the materials that increase their internalization [11–14]. However, these solutions are technically difficult and have toxic effects. The obstacles of existing methods of cell tracking encourage chemists to create a new cellular imaging tool which is self-fluorescence conjugated polymer based water dispersible nanoparticles (CPN); a new class of probe. The easily applied CPNs have high brightness, photostability, high fluorescence quantum yield and non-toxicity [38, 220].

The autologous transplantation of adult stem cells was shown to be a potential therapy in human diseases and trauma. However, the lack of ideal methods for stem cell tracking hinders the understanding of stem cell therapy, biology and behaviors *in vivo*. In this thesis, we aimed to initiate *in vivo* and *in vitro* utilization of self-fluorescence CPN in the monitoring of stem cell engraftment and function which have been limited due to the weakness of the used stem cell labeling and tracking methods. Considering their potential therapeutic benefit, we primarily focused on MSCs labeling and tracking. Initially, we examined 4 different nanoparticles and determined the ideal nanoparticle based on its effect on MSCs' cellular activity, labeling intensity and differentiation capacity. Thereafter choosing the most practicable CPN, we analyze its effect on MSCs characteristic properties through surface marker analysis and differentiation assays. We further tested CPN effect on human dental pulp stem cells (DPSCs) that were isolated from the wisdom teeth as an alternative source of MSCs. DPSCs are obtained with less invasive procedures compared to MSCs which makes them more likely to be used in

human clinics. Therefore, we aimed to show the DPSCs labeling with CPNs. After the characterization of DPSCs, the cellular activity of DPSCs after CPN incubation was assessed. In order to show *in vivo* utilization of CPN in stem cell tracking, the labeled MSCs were administered to the animal models of liver regeneration and fibrosis. The migration of CPN labeled MSCs was analyzed in the liver tissues. By this way, we aimed to make a contribution to the understanding of stem cell biology, migration, homing and therapies.

Moreover, *in vitro* and *in vivo* tracking is also essential for the anti-cancer therapies in order to evaluate the success of the treatment and metastasis status of the cancer which is critical for the understanding and improving the drug's therapeutic mechanism. Therefore, we examined CPN effect on cancer cells and for this purpose the hepatocellular carcinoma cells (Huh7 cells) were used. The retaining of label *in vivo* was tested in the tumor tissues obtained from the labeled cells. These findings will provide an opportunity to analyze the unattainable information of cell biology, migration and homing. Therefore, we aim to make contributions to the understanding of the cell therapies and cancer biology.

Chapter 3

3 Materials and Methods

3.1 Materials

3.1.1 Cell Culture Reagents and Material

Dulbecco's Modified Eagle's medium (DMEM), Trypsin EDTA, Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS) and Penicilin/Streptomycin Solution were from Hyclone (GE Healthcare Life Sciences, Logan, Utah). MesenCult MSC Basal Medium and MesenCult™ Mesenchymal Stem Cell Stimulatory Supplements were from Stemcell Technologies (Canada). DMEM:F12 was from Lonza (Basel, Switzerland). The (DMSO) used for cell cryopreservation was from AppliChem (Darmstadt, Germany dimethyl sulfoxide). Cyrovials, cell culture flasks and plates, serological pipettes were from Sarstedt (Nümbrecht, Germany).

3.1.2 Cell Proliferation Kit I (MTT) and Cytotoxicity Detection Kit (LDH) Analysis Reagents

The MTT which includes the MTT labeling agent and permeabilization buffer was from Roche (Mannheim, Germany). The LDH assay which includes the Catalyst (lyophilized) and Dye Solution was from Clontech (California, USA). The both assay solution aliquots were kept in -20°C for long term storage. The assays were read with Elisa Reader from BioTek (VA, USA) and analyzed by using the KCJrWin software.

3.1.3 RNA Extraction, cDNA Synthesis and Polymerase Chain Reaction (PCR)

Reagents

Total RNA isolation kit Nucleospin RNA II was from Macherey-Nagel (Duren, Germany). DyNamo cDNA Synthesis Kit(M-MuLV RNase H⁺ reverse transcriptase (includes RNase inhibitor) ,2x RT buffer (includes dNTP mix and 10 mM MgCl₂ *), Oligo(dT) primer (100 ng/μl))and DyNAzyme II DNA Polymerase (DyNAzyme I DNA Polymerase, 10x Optimized DyNAzyme buffer (1.5 mM MgCl₂), DyNAzyme buffer, 10 mM dNTPs)were from Finnzymes (Life Technologies, Finland). The nucleic acid samples were measured with the NanoDrop ND-1000 spectrophotometer was from Thermo Fisher Scientific (Wilmington, DE, USA). Semi-quantitative PCR machine was Runic Thermal Cycler (Sacem Life Technologies).

3.1.4 Oligonucleotides

The primers that were used in this thesis were ordered from İONTEK (İstanbul, Turkey). The sequence of the primers was given at the Table 3.1.

Table 3.1 Primers used in mouse and human RT-PCR experiments.

Name of the gene	Species	Sequences of Forward (F) and Reverse (R) Primers	Size (bp)
CD 90	Rat	(F) 5'- CCAGTCATCAGCATCACTCT-3' (R) 5'- AGCTTGTCTCTGATCACATT- 3'	374
CD 34	Rat	(F) 5'- TGTCTGCTCCTTGAATCT -3' (R) 5'- CCTGTGGGACTCCAAC- 3'	281
CD 71	Rat	(F) 5'- ATGGTTCGTACAGCAGCAGA-3' (R) 5'- CGAGCAGAATACAGCCATTG-3'	182
CD 29	Rat	(F) 5'-ACTTCAGACTTCCGCATTGG -3' (R) 5'- GCTGCTGACCAACAAGTTCA-3'	190

CD 45	Rat	(F) 5'- ATGTTATTGGGAGGGTGCAA-3' (R) 5'- AAAATGTAACGCGCTTCAGG-3'	175
GAPDH	Rat	(F) 5'- CCTCCTCATTGACCTCAACTAC - 3' (R) 5' – CATGGTGGTGAAGACGCCAG – 3'	219
CD29	Human	(F) 5-TGACGGAAGAACAAAGCAAG-3 (R) 5-ACCATCATGTTTGTCTGCAGT-3	164
α-SMA	Human	(F) 5'-TATCAGGGGGCACCCTATG-3' (R) 5'-GCTGGAAGGTGGACAGAGAG-3'	165
E-cadherin	Human	(F) 5'-GACTCGTAACGACGTTGCAC-3' (R) 5'-GGTCAGTATCAGCCGCTTTC-3'	119
N-cadherin	Human	(F)5-TCCAGACCCCAATTCAATTAATATTAC-3 (R) 5-AAAATCACCATTAAGCCGAGTGA-3	118
Vimentin	Human	(F) 5'-GCAGGAGGAGATGCTTCAGA-3' (R) 5'-ATTCCACTTTGCGTTCAAGG-3'	110
Fibronectin	Human	(F) 5'-AATATCTCGGTGCCATTTGC-3' (R) 5'-CAGTAGTGCCTTCGGGACTG-3'	108
GAPDH	Human	(F) 5'-GGCTGAGAACGGGAAGCTTGTCAT-3' (R) 5'-CAGCCTTCTCCATGGTGGTGAAGA-3'	119
CD 90	Human	(F) 5'-TGACCCGTGAGACAAAGAAG-3' (R) 5'-GTGAAGGCGGATAAGTAGAG-3'	60
CD 34	Human	(F) 5'-GGCCACAACAAACATCACAG-3' (R) 5'-AACATTTCCAGGTGACAGGC-3'	60
CD 29	Human	(F) 5'-ACTTCAGACTTCCGCATTGG -3' (R) 5'- GCTGCTGACCAACAAGTTCA-3'	190

3.1.5 Agarose Gel Electrophoresis

The agarose gel was prepared with Basica Le agarose which was from Prona (Spain). GeneRuler DNA Ladder Mix and 6X loading dye were used from Fermentas (Leon-Rot, Germany). The gel electrophoresis was performed with the tank from Thermo Science (Wilmington, USA) and with the power supply from Bio Rad Laboratories (California, USA). The bands were visualized by using transilluminator form BioRad (Gel-Doc, Vilber, USA). The Chemi-Capt (Vilber Lourmat, France) software was used to obtain and analyze the gel images.

3.1.6 Antibodies

The used antibodies and their working dilutions were given in the Table 3.2. The antibodies were applied either for an hour at room temperature or overnight at +4°C.

Table 3.2 Primary and secondary antibodies used in Immunofluorescence experiments.

Antibody	Company	Catalog No	Working Dilution
α -SMA	Abcam	ab5694	1:100 in 1%BSA-TBS-T (0,1% tween 20)
Anti-rabbit IgG FITC	Sigma	F-9887	1:200 in 1%BSA-TBS-T (0,1% tween 20)

3.1.7 MSCs and DPSCs Differentiation Medium Reagents and Stains

Osteogenic differentiation and staining reagents:

Dexamethasone, Ascorbic Acid, Glycerol-2-phosphate and Alizarin Red were from Sigma (MO, USA)

Adipogenic differentiation and staining reagents:

Dexamethasone, Insulin, Indomethacin, Isobutylmethylxanthine (IBMX) and Oil Red O were Sigma (MO, USA).

Chondrogenic differentiation and staining reagents:

Dexamethasone, Ascorbic Acid, Sodium Pyruvate, Insulin /Transferrin/Selenite (ITS), BSA, Nuclear Fast Red, TGF β and Alcian Blue were from Sigma. M1 Embedding Matrix was from Thermo (IL, USA).

3.1.8 Immunofluorescence, Tissue Visualization and Hematoxylin&Eosin Staining Reagents

Slides and coverslips were from IsoLab (GmbH, Wertheim, Germany). The nucleus counterstain UltraCruz™ Mounting Medium (DAPI) was from SantaCruz (California, USA). The BSA was from Sigma (MO, USA). The hematoxylin and eosin solution was used from Sigma (MO,USA).

3.1.9 General Used Materials and Reagents

- Ethanol, Methanol, Triton X-100 was from Sigma (MO, USA)
- Paraformaldehyde, Formaldehyde, Glycerol and Ammonium was from Carlo Erba (Val de Reuil, France)
- Tween 20 was from Amresco (OH, USA)
- Fluorescence microscopy was from Leica TCS/SP5 (Japan).
- Acetic acid was from Riedel-de Haën (Fluka,Sigma Aldrich, MO,USA)

3.2 Solutions and Media

3.2.1 Cell Culture Solutions

DMEM Completed Medium: 10% Fetal bovine serum (FBS) and 1% penicillin/streptomycin. Stored at +4⁰C.

MesenCult Completed Medium: 20% MesenCult supplement and 1% penicillin/streptomycin. Stored at +4⁰C.

DMEM F12 Completed Medium:15% FBS and 1% penicillin/streptomycin. Stored at +4⁰C.

Freezing Medium: 90% FBS and 10% dimethyl sulfoxide (DMSO).

3.2.2 MSC and DPSC Differentiation Medium Solutions

Osteogenic Differentiation Medium:0.1 μ M dexamethasone, 0.2 mM ascorbic acid 2-phosphate and 10 mM glycerol-2-phosphate prepared with LG-DMEM, containing 1% penicillin/streptomycin and 10% FBS.

Adipogenic Differentiation Medium: 1 μ M dexamethasone, 10 μ g/ml insulin,100 μ M indomethacin and 0.5 mM IBMX prepared with LG-DMEM, containing 1% penicillin/streptomycin and 10% FBS.

Chondrogenic Differentiation Medium: 10 ng/ml TGF β -1 (Transforming growth factor beta 1), 100 nM dexamethasone, 50 μ g/ml ascorbic acid, 1 mM sodium pyruvate, 6.25 μ g/ml insulin, 6.25 μ g/ml transferrin, 6.25 μ g/ml selenous acid (ITS) and 1.25 mg/ml bovine serum albumin (BSA) prepared with HG DMEM, containing 1% penicillin/streptomycin and 10% FBS.

3.2.3 Differentiation Staining Solutions

- **Alizarin Red:** Prepare 40mM Alizerin Red Solution (1,4 gr Alizerin Red mixed with 100 ml distilled water). The pH was adjusted to 4.1.
- **Oil Red O:** 3x dye mixed with 2x distilled and let sit at room temperature for 20 min. Then the mixture was filtered and was ready to be used.
- **Alcian Blue:** 1g Alcian Blue (8GX) mixed with 100 ml 3% Acetic Acid (3ml Glacial acetic acid mixed with 97 ml distilled water). The pH was adjusted to 2.5 and filtered before used.

3.2.4 General Used Solutions

10x PBS

- 80 g NaCl
- 2g KCl
- 8,01g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 2g KH_2PO_4

Add to 1 liter ddH₂O pH: 7,2. The working solution was 1X and prepared by the dilution of 10X PBS to 1 to 10 with ddH₂O.

DEPC-Treated ddH₂O

1ml DEPC was added to 1lt ddH₂O and stirred for 1 hour and autoclaved for inhibition of DEPC activity.

50X TAE Buffer

2M Tris Base (242 g), 57,1 ml Glacial Acetic Acid, 50mM EDTA were added to 1 lt by ddH₂O. The working solution was 1X and prepared by the dilution of 50X TAE to 1 to 50 with ddH₂O.

1% Agarose Gel

1 g Basic Le agarose gel was added into the 100 ml 1X TAE buffer. The agarose was dissolved in TAE with the heat stirrer.

1X PBS-T

0.5 ml tween 20 was added into the 500 ml PBS.

1% BSA Block

0.1 g BSA was added into 10 ml 1X PBS-T (0,1 % tween)

4% Paraformaldehyde

4 g paraformaldehyde was added into 100 ml ddH₂O and stirred with heat about 2-3 hours in order to dissolve.

rDNase reaction buffer

10 µl rDNase mixed with the 90µl rDNase reaction buffer.

DEPC treated 70% EtOH

1ml DEPC was added to 1lt 70% EtOH and stirred for 1 hour and autoclaved for inhibition of DEPC activity.

3.3 Methods

3.3.1 Animals

The whole animals, used in all experiments, were kept in the animal holding facility of Bilkent University Molecular Biology and Genetics Department. The facility conditions were set at 22°C with 12h light and 12h dark cycles. These animals were feed with unlimited source of food and water. The all animal experiments were made with ethical methods and procedures were approved by Bilkent University Local Ethical Committee (BILHADYEK). 6-9 week old, 280-300g Spraque Dawley female rats were used for the isolation of MSCs and tracking MSCs *in vivo*. In the liver fibrosis model, the 6 week old C56BL/6 male mice were used for MSC isolation and their *in vivo* tracking. In addition, 8-16 week old CD 1 nude mice were used for the generation of the xenograft model with CPN labeled Huh7 cells.

3.3.2 Isolation and Tissue Culturing of Cells

3.3.2.1 Isolation of Mesenchymal Stem Cells (MSCs) From Rat and Mice Bone Marrow and Cell Culturing

MSCs were isolated from either 6 week old C56BL/6 male mice or 6-9 week old Spraque Dawley female rats. The animals were sacrificed by cervical dislocation. Bone marrow heterogeneous cell population were obtained from the their femur and tibia by flushing DMEM (Dulbecco's Modified Eagle's medium, Invitrogen, Paisley, UK) including 10% FBS (Fetal Bovine Serum, Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin solution (Hyclone) with 5mL syringe. The obtained heterogeneous cell populations were centrifuged at 1500 rpm for 5 min. After supernatant was removed, the cells were washed with 10 mL 1X PBS and centrifuged at 1500 rpm for 5 min. This step was repeated for 3 times. After the final centrifuge, the supernatant was removed and cell pellet was re-suspended in Mesencult Medium (StemCell Technology, Vancouver, Canada) including 20% supplement (StemCell Technology) and a 1% penicillin/streptomycin solution (Hyclone). According to the size of pellet, the isolated

cells seeded into different culture flask with equal number of medium. The cells were cultured in a 5% CO₂ incubator at 37°C. At the first day of culturing, the medium was changed with fresh medium without PBS washing. Then, the medium of cells were changed twice a week after wash the cells with 1X PBS. The mediums were warmed to 37°C prior to use. At the end of 14 days of culturing, MSCs were obtained.

3.3.2.2 Isolation of Dental Pulp Stem Cells (DPSCs) from Human Wisdom Teeth and Cell Culturing

The patients' wisdom teeth were obtained from Başkent University Dentistry Department. DPSCs must be isolated within few hours after dental surgery, otherwise, the cell yield become very rare. Firstly, the tooth was broken carefully and the pulp was taken out. The pulp was put into 2 ml Eppendorf including completed DMEM:F12 supplemented with 2mg/ml Collagenase/Dispase Solution. Initially, the pulp was physically digested with the help of a thin scissors. Afterwards, chemical digestion was done with the incubation of mixed solution of Collagenase/Dispase at 37°C for 45 min. The cell extracts were re-suspended in a period of 10 min. Then, the cells were seeded to flasks in 10ml DMEM-F:12 (Lonza) supplemented with 1% penicillin/streptomycin solution (Hyclone) and 15% FBS (Fetal Bovine Serum, Hyclone, Logan, UT, USA) and cultured in a 5% CO₂ incubator at 37°C. Next day, the medium was changed with fresh medium without washing with 1XPBS. From that day, the culture medium was changed three times a week after washing the cells with 1X PBS. The medium was warmed to 37°C prior to use. Even very few numbers of DPSCs, which attached to the flask surface, are able to generate huge colonies. 10 day culturing is optimal to obtain DPSCs, however, it can be waited until colonies formed and become confluent.

3.3.2.3 Huh7 Cell Line Culturing

Huh7 cells, one of the hepatocellular carcinoma cell lines, were used to track cancer cells in tumor xenograft model. Huh7 cells cultured in DMEM (Dulbecco's Modified Eagle's medium, Invitrogen, Paisley, UK) supplemented with 10% FBS(Fetal Bovine Serum, Hyclone, Logan, UT, USA)and 1% penicillin/streptomycin (Hyclone) solution. The medium was warmed to 37°C prior to use. Cells were kept in a 5% CO₂ incubator at 37°C.

3.3.3 Cell Passaging

DPSCs and MSCs are adherent cells which strongly associate to the surface, expand themselves onto the surface and have fibroblast like structure. Huh7 cells are also adherent cells and associate to the surface. When cells become 70%-80% confluent, they need to be passaged. In order to passage cells, initially, previous medium was aspirated with sterilized glass pipettes and cells were wash with 1XPBS. After the aspiration of PBS, the trypsin (1mL to 25 cm² flask) was added and cells were incubated for few min at 37°C in order to activate the trypsin. The cell detachment checked under microscope and cells were collected into fresh medium which includes enough FBS to inactivate the trypsin activity. In this step, re-suspension is critical to evade the formation of cell aggregates. The cells diluted in fresh medium enough to seed cells to new flasks in desired dilutions. DPSCs have higher proliferation rate compare to MSCs and quickly become confluent. Therefore, 1:4 sub-division of DPSCs was optimal. On the other hand, this ratio was changing for Huh7 to 1:4 to 1:8 based on their confluency. All solutions and the medium were warmed to 37°C before used.

The exact number of cells may be needed for some experiments. In this situation, trypsinized cells collected in fresh medium and re-suspending until single cells were obtained. 10µl from the cell suspension was put on the haemocytometer and the cells were counted. The calculated average cell number was multiplied with 10⁴ in order to assess the number of cells found in the 1ml of the cell suspension. Finally, the desired amount of cells can be taken from the cell suspension and completed to required amount with fresh medium.

3.3.4 Cryopreservation and Thawing of Cells

In order to cryopreserve the cells, they should become 60%-70% confluent. Firstly, the cells were collected with the help of trypsin and the medium including enough FBS, to inactivate the trypsin activity, was added on top of cells. Then, the cells were centrifuged at 1500 rpm for 5 min and the supernatant was removed. The obtained pellet was re-suspended in the 1mL of freezing medium which includes 90% of FBS and 10% of DMSO. The freezing cells were put into cryotubes (1ml) and freezed gradually. First the cryotubes were placed at -20°C for an hour, and then placed in -80°C for overnight. Finally, the cells were kept at liquid nitrogen for long term storage.

While thawing the cells, it is important to be quick to prevent the cell death. To acquire quick thawing, the cells taken from liquid nitrogen were placed into 37°C water bath. The thawed cells were transferred to falcon tube which contains the 9 mL of fresh medium. The cells were re-suspended in this medium and centrifuged at 1500 rpm for 3 min. Then, the medium was aspirated and cell that re-suspended in fresh medium seeded into to the cell culture flasks. After each of these steps, the thawed cells are ready for culturing at in a 5% CO₂ incubator at 37°C. It is important to change the medium with fresh medium on the next day for eliminating the death cells.

3.3.5 Conjugated Polymer Nanoparticle (CPN) Synthesize

CPNs were prepared in the Dept. of Chemistry at Bilkent University. The copolymer was synthesized using the Suzuki Coupling polymerization technique and after the synthesis of polymers, they were converted into nanoparticles by reprecipitation method [21]. The detailed explanation of CPN synthesizes can be obtained in the references [38, 221–223].

VP6—(poly[(9,9-bis(propenyl)fluorenyl-2,7-diyl))-co-(1,4-benzo-{2,1,3}-thiodiazole)] synthesis [223]:

In order to synthesize the 2, 7-dibromo-9,9-bis- (propenyl)-9H-fluorene monomer, first 2,7- dibromofluorene was incubated with with strong base to separate protons at the 9th position. Next, the excess allylbromide was added and sample was purified by column.

Polymer poly[(9,9-bis(propenyl)-9H-fluorene)-co-(benzothiadiazole)] was synthesized by coupling of 2, 7-dibromo-9,9-bis- (propenyl)-9H-fluorene monomer with 2,1,3- benzothiadiazole-4,7-bis (boronic acid pinacol ester) using Suzuki coupling method. The polymer was purified by precipitation method by mixing the THF solution with cold methanol.

poly[(9,9-bis(propenyl)fluorenyl-2,7-diyl))-co-(1,4-benzo-{2,1,3}-thiodiazole)] nanoparticle was synthesized by using the polymer poly[(9,9-bis(propenyl)-9H-fluorene)-co-(benzothiadiazole)]. After the polymer was dissolved in THF and mixed for 1h. The mixture was filtered using syringe, put into excess water and mixed for 1h. Then, THF solvent was removed with decreasing pressure to achieve the dispersion of nanoparticle in water. Finally, the solution was concentrated with decreasing pressure in to obtain a clear solution.

VP6a-Merc—(poly[(9,9-bis(carboxymethylsulfonyl-propyl)fluorenyl-2,7-diyl))-co(1,4-benzo-{2,1,3}-thiodiazole)] synthesis [223]:

The polymer poly[(9,9-bis(propenyl)fluorenyl-2,7-diyl))-co-(1,4-benzo-{2,1,3}-thiodiazole)] were functionalized to poly[(9,9- bis(carboxymethylsulfonyl-propyl)fluorenyl-2,7-diyl)-co(1,4-benzo-{2,1,3}- thiodiazole)] by using Thiol-ene click chemistry. The polymer poly[(9,9-bis(propenyl)fluorenyl-2,7-diyl))-co-(1,4-benzo-{2,1,3}-thiodiazole)] was dissolve in chloroform, was incubated with mercaptoacetic acid with Ar gas and mixed for 48h at room temperature. Then the solution was evaporated and dissolved in THF. Finally, nanoparticles were precipitated with water after the excess acid was removed.

The nanoparticle of poly[(9,9- bis(carboxymethylsulfonyl-propyl)fluorenyl-2,7-diyl)-co(1,4-benzo-{2,1,3}-thiodiazole)] was synthesized by the polymer solvation and

mixture in THF for 1h. After the solution was filtered with syringe, it was mixed with excess water for 1h. Then, the THF solvent was removed with decreasing pressure and nanoparticles were formed in water. The solution was evaporated with decreasing pressure in order to obtain a clear solution.

VP8—(poly[9,9-bis-(6-bromohexylfluorene-2,7-diyl)-alt-co-(benzene-1,4-diyl)]) [221, 222]:

The monomer 2,7-dibromo-9,9-dihexyl-9H-fluorene was coupled with phenylenediboronic acid by Suzuki coupling reaction in order to generate polymer Poly[9,9-bis-(6-bromohexylfluorene-2,7-diyl)-co-(benzene-1,4-diyl)].

The reprecipitation method was used to generate water dispersible nanoparticles. The polymer was dissolved and mixed in THF. Then, the solution was mixed with poor solvent which was excess water. When the good solvent THF was removed, the spherical shaped nanoparticles were formed.

VP9-poly[-(9,9-dihexylfluorene)-co-alt-(9,9-bis(3-azidopropyl)-fluorene)] [222, 224, 225]:

First, poly[9,9-dihexyl-9H-fluorene] was synthesized by the Suzuki coupling of 2,7-dibromo-9,9-dihexyl-9H-fluorene with 9,9-dihexylfluorene-2,7-bis(trimethyleneborate). The polymer was purified by precipitation method by mixing the THF solution with cold methanol.

Following, poly[(9,9-dihexylfluorene)-co-alt-(9,9-bis(6-bromohexyl)fluorene)] was synthesized by using Suzuki coupling reaction in which the bromide groups were changed into azide group through the substitution of nucleophiles with sodium azide in DMF to generate poly[(9,9-dihexylfluorene)-co-alt-(9,9-bis(6-azidohexyl)fluorene)].

The reprecipitation method was used to generate water dispersible nanoparticles. The polymer was dissolved and mixed in THF. Then, the solution was mixed with poor solvent which was excess water. When the good solvent THF was removed, the spherical shaped nanoparticles were formed.

3.3.6 Cell Labeling with CPNs

3.3.6.1 MSCs Labeling with CPNs

In order to assess the optimal staining time, MSCs were labeled with CPN in different time periods. The cells were cultured with MesenCult medium, involved CPN solution in 1:4 ratio (25µg/ml), for a desired time period. In these samples, which were given CPN from early days of culturing, medium was changed with fresh MesenCult medium including CPN. Continuous experiments were held with MSCs that were incubated with CPN for 24h in 5% CO₂ incubator at 37°C at their 13th day of culturing. After 24h incubation, cells were ready for further experiments.

3.3.6.2 DPSCs Labeling with CPN

After isolated DPSCs became the 70% confluent, old medium was changed with fresh completed DMEM: F12 medium including 1:4 ratio (25µg/ml) of CPN. DPSCs were incubated with CPN including medium in 5% CO₂ incubator at 37°C for 24h. After 24h incubation, cells were ready for further experiments.

3.3.6.3 Huh7 Cell Labeling with CPN

After Huh7 cells became 70% confluent, old DMEM medium was changed with fresh completed DMEM medium including 1:4 ratio (25µg/ml) of CPNs. Huh7 cells were incubated with CPN including medium in 5% CO₂ incubator at 37°C for desired times (24h- 72h). After defined time, cells were ready for further experiments.

3.3.7 CPN Labeled Cell Visualization under Fluorescence Microscope

MSCs (on the 13th day of culturing), DPSCs and Huh7 cells were collected with trypsinization and counted with the help of hemocytometer. Based on their proliferation rate, 3×10^5 MSCs, 2×10^5 DPSCs and 1.5×10^5 Huh7 cells per well were seeded to 6 well plates on to sterilized coverslips. Cells were cultured 24h with CPN included MesenCult (for MSCs), DMEM (for Huh7 cells) and DMEM:F12 (for DPSCs) in 5% CO₂ incubator at 37°C. After the incubation period, cells were ready to be visualized. First, cells were washed with 1X PBS and then fixed with ice-cold methanol for 15 min. Finally, cells were counterstained and mounted with DAPI (UltraCruz™) after washed with 1X PBS and closed on to the slides. CPN labeled cells were observed with fluorescence microscopy.

3.3.8 MTT Assay of Cellular Metabolic Activity

The effect of CPNs' on the viability of MSCs, DPSCs and Huh7 cells was detected by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (Roche Molecular Biochemicals, Indianapolis, USA). MSCs at the 13th day of culturing, and 60-70% confluent DPSCs and Huh7 cells were trypsinized, collected into falcons and counted with hemocytometer. 10^4 MSCs, 10^4 DPSCs and 5×10^3 Huh7 cells in 100 μL of medium were seeded in the wells of 96 well plates. Each group was done in triplicate. The next day, cell medium was changed with fresh ones (MesenCult for MSCs, DMEM:F12 for DPSCs and DMEM for Huh7 cells) that involved CPN (1:4 ratio). The medium of negative control group was changes with fresh medium without CPN. Cells were cultured for 24h at 37° C in 5% CO₂ incubator. After 24h, cells were incubated with 10 μL of MTT labeling reagent (at a final concentration of 0.5 mg/ mL in medium) in incubator for 4h. Then solubilizing reagent was added on top of samples and incubated overnight at 37°C in a 5% CO₂ incubator. At the end, the end product, formazan, was formed and measured with ELISA reader (BioTek, KCJrWin software) at a primary wavelength of 551 nm and a maximum wavelength of 601 nm.

3.3.9 Lactate Dehydrogenase (LDH) Assay for Cellular Toxicity

DPSCs were collected via trypsinization and counted with the help of hemocytometer. 2×10^4 cells per well were seeded in 96-well plates. The next day, old medium was changed with fresh completed DMEM:F12 medium which includes CPN in 1:4 ratio (25 μ g/ml). Negative control group was received only fresh completed DMEM:F12 medium without CPN (low control, minimum toxicity). Since 1% Triton-X is known to be toxic for cells and not interfere with LDH activity, it was used as positive control in LDH assay (high control-maximum toxicity). All groups were made in triplicates. After 24h culturing, toxicity of CPN was determined by measuring released cytoplasmic lactate dehydrogenase (LDH) from the damaged cells by using Cytotoxicity Detection Kit (LDH, Clontech) according to manufacturer's protocol. Readings were performed with ELISA reader (BioTek, KCJrWin software) at the wavelength 490 nm. For calculations, average value of background readings was subtracted from all of sample readings. Percent cytotoxicity value was calculated by the equation below:

$$\text{Cytotoxicity (\%)} = (\text{Triplicate Absorbance} - \text{Low Control}) / (\text{High Control} - \text{Low Control}) * 100$$

3.3.10 Differentiation Assays and Their Staining Protocols

3.3.10.1 Osteogenic Differentiation Of CPN Labeled MSCs and DPSCs and Alizerin Red Staining

At the 13th day of MSCs culturing (cells became nearly 80% confluent) the MesenCult medium was changed with fresh medium including CPN in 1:4 ratios. After MSCs were incubated with CPN for 24h, the MesenCult medium was changed with osteogenic differentiation medium. Also, the medium of 70% confluent DPSCs was changed with fresh DMEM:F12 medium including CPN in 1:4 ratios. DPSCs were also incubated with CPN for 24h and DMEM:F12 medium was changed with osteogenic differentiation medium. The control group was differentiated without CPN labeling. Osteogenic medium was changed twice a week and cells were not further incubated with CPNs while the differentiation period. To assess osteogenic differentiation, fresh

osteogenic medium consisting of 0.1 μ M dexamethasone (Sigma), 0.2 mM ascorbic acid 2-phosphate (Sigma) and 10 mM glycerol-2-phosphate (Sigma) to LG-DMEM (HyClone), containing 1x penicillin-streptomycin (HyClone) and 10% FBS (HyClone) was prepared. After 21 days of culturing in 5% CO₂ incubator at 37°C, cells were fixed with 10% formaldehyde for 15 min. In order to detect osteogenic cells, the cells were incubated with Alizarin Red stain (pH 6.0, Sigma) for 20 min, and then examined under bright-field microscopy. MSCs and DPSCs that differentiate into osteocytes stain intensely with Alizarin Red. CPN labeling was detected in the same sample sections under fluorescence microscopy.

3.3.10.2 Adipogenic Differentiation of CPN Labeled MSCs and DPSCs and Oil Red O Staining

At the 13th day of MSCs culturing (cells became 80% confluent) the MesenCult medium was changed with fresh medium including CPN in 1:4 ratios. After MSCs were incubated with CPN for 24h, MesenCult medium was changed with adipogenic differentiation medium. Also, the medium of 70% confluent DPSCs was changed with fresh DMEM:F12 medium including CPN in 1:4 ratios. After 24h CPN incubation, cells were washed with 1X PBS and mediums were replaced with the defined adipogenic medium. Control group was differentiated without CPN labeling. Adipogenic medium was changed twice a week and cells were not further incubated with CPNs while the differentiation period. To assess adipogenic differentiation, fresh adipogenic medium was prepared by adding 1 μ M dexamethasone (Sigma), 10 μ g/ml insulin (Sigma), 100 μ M indomethacin (Sigma) and 0.5 mM IBMX (Isobutylmethylxanthine, Sigma) to LG-DMEM (Low glucose Dulbecco's Modified Eagle's medium, HyClone), containing 1x penicillin-streptomycin (HyClone) and 10% FBS (HyClone). After cells were cultured for 21 days in 5% CO₂ incubator at 37°C, they were fixed with 4% paraformaldehyde and stained with strained Oil Red O (Sigma) for 50 min. Oil Red O was strained and mixed with distilled water (3x Oil Red O, 2x distilled water) and after 20 min, the dye was ready to be used. After the staining step, the samples were washed with tap water for nearly 4 times. At the end, 1X PBS was added onto the each well in order to avoid cells from

drying during long term sample storage at +4°C. MSCs and DPSCs that differentiated into adipocytes stain intensely with Oil Red O. CPN labeling were detected in the same sample sections under fluorescence microscopy.

3.3.10.3 Chondrogenic Differentiation of CPN Labeled MSCs and DPSCs and Alcian Blue Staining

At the 13th day of MSCs culturing (cells became 80% confluent) the MesenCult medium was changed with fresh medium including CPN in 1:4 ratios. Also, medium of 70% confluent DPSCs was changed with fresh DMEM:F12 medium including CPN in 1:4 ratios. After 24h CPN incubation, cells were washed with 1X PBS. Then, cells were collected with trypsinization and centrifuged for 5 min at 1500 rpm in round bottom tubes. Chondrogenic cell spheres were induced via specific chondrogenesis induction medium composed of 10 ng/ml TGF β (Transforming growth factor beta) 1, 100 nM dexamethasone, 50 μ g/ml ascorbic acid, 1 mM sodium pyruvate, 6.25 μ g/ml insulin, 6.25 μ g/ml transferrin, 6.25 μ g/ml selenous acid (ITS) and 1.25 mg/ml bovine serum albumin in DMEM high glucose. Control group cells were differentiated without CPN labeling. Chondrogenic medium was changed twice a week and cells were not further incubated with CPN while the differentiation period. After 28 days of culturing in 5% CO₂ incubator at 37°C, the cell nodules were fixed with M1 Embedding Matrix (Thermo). Chondrogenic spheres were sectioned, placed on to the slides and stained with Alcian Blue (Sigma) for 30 min. Slides were washed with tap water and mount with DAPI (nucleus counterstain). The chondrogenesis was examined under bright-field microscopy. For the detection of CPN labeling, same sections were examined under fluorescence microscopy.

3.3.11 Total RNA Isolation from Cell Culture of MSCs

CPN labeled MSCs (labeled 24h with CPN at 13th day of culturing) and MSCs (non-labeled) on the 14th day of culturing were collected with trypsinization. Same as MSCs, DPSCs were collected with trypsinization when they became 70% confluent. The

cells were collected into the falcons and total cell pellets were obtained by the 1500 rpm 5 min centrifugation. Total RNA was isolated from these pellets by using the Nucleospin RNA II (Macherey-Nagel, Duren, Germany) according to the manufacturer's protocol. First, cells were lyzed with 350 μ L of RA1 buffer including 1% beta-mercaptoethanol and vortexed rigorously. Then, cell lysates were passed through 0.9mm needled syringe for more than 5 times for further lyzation. The cell lysates was transferred to microcentrifuge tubes and 350 μ L of DEPC treated 70% EtOH was added on top of them and homogenized by pipetting 5 times. The mixture were loaded to NucleoSpin RNA column placed in a collection tube (2mL) and centrifuged for 30 seconds at 13.000 rpm at room temperature. After NucleoSpin RNA column placed in a new collection tube, 350 μ L of MDB(membrane desalting buffer) was added and centrifuge at 13.000 rpm for 1 min. DNA digestion was done by adding 95 μ L rDNase reaction mixture (10 μ L rDNase and 90 μ L rDNase reaction buffer) on the center of silica membrane of the NucleoSpin RNA column and incubate for 15 min at RT. rDNase activity was inactivated by adding 200 μ L of RAW2 buffer and centrifuge at 13.000rpm for 30 seconds. After place the column into a new collection tube, 600 μ L of RAW3 buffer was added to center of the silica membrane of the column to wash the sample and centrifuge at 13.000rpm 30 seconds. Flow through were discarded and column were place back into same collection tube. 250 μ L of RAW3 buffer were added and samples were centrifuge at 13.000rpm 2 min centrifuge was done to dry the membrane. The column was placed to nuclease free collection tube. To elute RNA 40 μ L (30-60 μ L according to the concentration of RNA sample) of RNase-free H₂O was added to center of membrane of the column and centrifuged at 13.000rpm for 1 min. Purity and concentration of eluted RNAs was measured by Nanodrop 2000. A260/A280 values were checked in order to assess the quality and purity of isolated RNAs. RNA samples were stored at -80°C for long term storage.

3.3.12 First Strand cDNA Synthesis

cDNAs were synthesized from total RNA with the DyNAmo cDNA synthesis kit (Finnzymes, Espoo, Finland) according to the manufacturer's protocol. The amount that would be taken from RNA sample was calculated according to NanoDrop concentration results. 1 µg of total RNA was needed in the cDNA mixture which was calculated based on the concentration of RNA samples. 1 µL of Oligo(dT) primers were added and mixture completed to 12 µL with RNase free water. The samples were incubated at 65°C for 5 min for breaking the secondary structure of RNAs and then quickly centrifuged to spin down the solution. The samples were incubated on ice for 5 min. Afterwards 10 µL of 10X Reaction Buffer and 2 µL of M-MuLV RNase H⁺ reverse transcriptase were added to mixture. The mixture was incubated sequentially at 25°C for 10 min for primer extension, 42°C for 60 min for cDNA synthesis and 80°C for 10 min for reaction termination. cDNAs were stored in -20°C for further experiments.

3.3.13 Primer Design for Expression Analysis

Primers were design according to interested gene sequences which are acquired from NCBI (National Center for Biotechnology Information- <http://www.ncbi.nlm.nih.gov/>) by using the Primer Blast tool of NCBI. Primers were chosen in a length of 18-25bp which is long enough to being specific for the gene of interest and short enough to associate quickly to the template. In addition, GC content tried to be kept between 40-60% and melting temperature around the 55°C-65°C. The differences in melting temperature of reversed primers were tried to be tune around 1°C. The continuous repeats may cause the hairpin formation; therefore, the repeat sequences were avoided while choosing the correct primer pairs. Coherence of designed primers was checked with Ensemble (<http://www.ensembl.org/index.html>) and Blast (NCBI) tools which are available online. Primers list for all genes were listed in Table 3.1.

3.3.14 Expression Analysis RT-PCR

cDNAs generated for the amplification of interested genes were used in RT-PCR. DyNAzyme II (Finnzymes) PCR kit was used in this study. Preparation of sample mix was done according to the Table 3.3. PCR conditions for the genes are listed in Table 3.4

Table 3.3 Reaction mixture used in RT-PCR experiments

Component	Volume
10x Tag Reaction Buffer	5 μ l
10 mM dNTPs	1 μ l
50 mM MgCl ₂	1.5 μ l
10 μ M Primer forward	1 μ l
10 μ M Primer reverse	1 μ l
Template DNA (cDNA)	1 μ l
5 U/ μ l Tag Polymerase	0.25-1 μ l
ddH ₂ O	Add to 25 μ l

Table 3.4 Annealing temperatures and cycle numbers for each primer set used in RT-PCR experiments.

Genes	Initial denaturation	Denaturation	Annealing	Extension	Final extension
<i>CD 90 (rat)</i>	95°C, 5 min	94°C, 30 sec	55°C,30 sec	72°C,30 sec	72°C, 5 min
<i>CD 34 (rat)</i>	95°C, 5 min	94°C, 30 sec	55°C,30 sec	72°C,30 sec	72°C, 5 min
<i>CD 71 (rat)</i>	95°C, 5 min	94°C, 45 sec	66°C,60 sec	72°C,45 sec	72°C, 5 min
<i>CD 29 (rat)</i>	95°C , 5 min	94°C, 30 sec	60°C,30 sec	72°C,30 sec	72°C, 5 min
<i>CD 45 (rat)</i>	95°C, 5 min	94°C, 30 sec	60°C,30 sec	72°C,30 sec	72°C, 5 min
<i>GAPDH (rat)</i>	95°C , 5 min	94°C, 30 sec	59°C,30 sec	72°C,30 sec	72°C, 5 min
<i>CD90 (human)</i>	95°C , 5 min	94°C, 30 sec	60°C,30 sec	72°C,30 sec	72°C, 5 min
<i>CD 29 (human)</i>	95°C , 5 min	94°C, 30 sec	60°C,30 sec	72°C,30 sec	72°C, 5 min
<i>CD 34 (human)</i>	95°C , 5 min	94°C, 30 sec	60°C,30 sec	72°C,30 sec	72°C, 5 min
<i>Vimentin (human)</i>	95°C , 5 min	94°C, 30 sec	60°C,30 sec	72°C,30 sec	72°C, 5 min
<i>Fibronectin (human)</i>	95°C , 5 min	94°C, 30 sec	60°C,30 sec	72°C,30 sec	72°C, 5 min
<i>α-SMA (human)</i>	95°C , 5 min	94°C, 30 sec	60°C,30 sec	72°C,30 sec	72°C, 5 min
<i>N-cadherin (human)</i>	95°C , 5 min	94°C, 30 sec	61°C,30 sec	72°C,30 sec	72°C, 5 min
<i>E-cadherin (human)</i>	95°C , 5 min	94°C, 30 sec	60°C,30 sec	72°C,30 sec	72°C, 5 min

3.3.15 Agarose Gel Electrophoresis

After RT-PCR, amplified DNAs were loaded to agarose gel to be visualized. According to the size of amplicon, agarose gel concentration was calculated. For bigger amplicons the less concentrated agarose gel (1%) were prepared. For smaller amplicons concentration of agarose can be increased up to 3%. Agarose gel was prepared in 1X TAE Buffer. First the solution was heated to let the agarose dissolve in buffer. Then, ethidium bromide solution was added in amount of 5% of the buffer solution. Ethidium bromide helps to visualize DNA amplicons under UV light. The gel was left to become rigid and then, samples were loaded to gel in equal amounts after mixed with 10X agarose Gel Loading Dye (2.5µl of dye into 25 µl of PCR products). 5µl of Gene Ruler DNA Ladder Mix (Fermentas, #SM0331) was loaded to the gel to be used as a marker. The gel was run at 80-100V for 45 min(time can be changed according to the band separation) in tanks including 1XTAE buffer. Finally, gel was visualized with the transilluminator (Gel-Doc Bio-Rad, USA or Vilber Lourmat, France). Photo of the gel was taken with Chemicapture (Vilber Lourmat, France) software.

3.3.16 *In Vivo* Tracking of CPN Labeled Cells

3.3.16.1 MSC Tracking in Damaged Liver

Liver injury was generated by partial hepatectomy (PH) operation in 6-9 week old male Sprague Dawley rats. PH operation resected 70% of the liver mass. Control sham (SH) groups, which were underwent the identical surgical procedures but without resection, were generated. 3 animals per group were used in the experiments. Rats were anesthetized during these operations. On the 13th day of culturing, MSCs were labeled with CPN for 24h. On the 14th day of culturing, CPN labeled and non-labeled MSCs collected by trypsin and counted with hemocytometer. 5×10^6 MSCs (non-labeled) and CPN-MSCs (24h CPN incubated) were obtained, centrifuged at 1500 rpm for 3 min and re-suspended in 500µl of sterile 1XPBS. The cells suspensions were injected to the PH and SH group of rats through tail vein with the help of insulin syringe. After 3 days of

injection, animals were sacrificed and their livers were embedded to paraffin blocks or frozen with liquid nitrogen.

In order to visualize migrated CPN-MSCs, paraffin embedded livers were sectioned (5 μ m) and placed onto slides. The samples were de-paraffinized with xylene for 20 min and decreasing amount of ethanol solutions (respectively put into 100%, 75%, 50% and distilled water-5min each) were applied. Slides were then mounted with UltraCruz mounting medium DAPI (SantaCruz Biotechnologies, California, USA) for nuclear counterstaining. Slides were observed with a fluorescent microscope (Leica TCS/SP5, Japan). Excitation wavelength for CPN and DAPI were 490nm and at 359nm, respectively.

3.3.16.2 MSC Tracking in Liver Fibrosis

Liver fibrosis model was generated by intraperitoneal (i.p) injection of CCl₄ at 8 μ l/g of body weight in the C57BL/6 male mice. CCl₄ was administered twice a week to the mice for 4 weeks. The CCl₄ was prepared in mineral oil, so that, the control groups were given mineral oil as the same way that experimental group were given CCl₄. After 4 weeks, experimental and control groups were given 5x10⁶CPN-MSCs (24h) from tail vein. 3 days later, mice were sacrificed and their liver was taken. The half of liver was frozen with liquid nitrogen to be used in immunostainings. The other half was embedded to paraffin blocks to visualize labeled MSCs and perform H&E (haematoxylin-eosin) staining.

3.3.16.3 The Huh7 Tracking in Xenograft Model

The 70-80% confluent Huh7 cells were incubated with CPN either for 24h or 72h. CPN labeled Huh7 cells were collected with trypsinization and counted with hemocytometer. 5x10⁶ cells were suspended in 250 μ l of 1XPBS and were injected subcutaneously on one side of the CD1 nude mice. The same number of non-labeled Huh7 cells was injected to the other side of the same mouse. After administration of

cancer cells, tumor occurrences were monitored for almost 2 months. The tumor growth was measured twice a week. When tumor tissues became visible (after 2-3 months), the tissues were taken and freeze with liquid nitrogen.

3.3.17 Hematoxylin and Eosin (H&E) Staining

Paraffin embedded tissues was sectioned in 7 μ m pieces and placed on to the slides. Firstly, samples were de-paraffinized with xylene for 20min. The samples were hydrate with decreasing amount of ethanol starting from 100% to 75%, 50%, to distilled water (5 min each). The samples were washed with 1X PBS-T (0,1% tween 20). Hematoxylin was applied to the samples for 4 min. The samples were rinsed with tap water. Then, non-specific hematoxylin staining was eliminated with 1% acid alcohol. After rinsed with tap water, blueing up was done with 1% of ammonium solution applied for few seconds. Before and after the cytoplasmic staining with eosin (for 1 min), samples were rinsed with tap water. The samples were de-hydrated with increasing concentration of ethanol starting from distilled water to 100% ethanol (5 min each). Finally, the samples were mounted with glycerol. The slides were stored in +4°C.

3.3.18 Immunofluorescence Staining

Frozen tissue samples were sectioned in 7 μ m pieces and placed on to the slides. The 4%paraformaldehyde was prepared in distilled water and put onto stirrer with heatin order to be solved. After cooling the paraformaldehyde, tissue slides were fixed with 4% paraformaldehyde for 30 min. Before the blocking step, samples were washed with 1X PBS-T (0,1% tween 20). BSA blocking solution was prepared in 1X PBS-T. 1% BSA blocking was done in humidified chambers for 1h at room temperature. a-SMA (Abcam) primary antibody is specifically recognizes actin that is accumulated upon fibrosis. Primary antibody incubation was done in humidified chambers at +4°C for overnight. Before secondary antibody incubation, the samples were washed with 1X PBS-T to remove unspecific binding of primary antibody. Secondary antibody Anti-rabbit IgG FITC (Sigma) was applied in humidified chambers for 1h at room temperature. Finally,

the nucleus counter staining and mounting was done with DAPI (UltraCruz Mounting Medium, Santa Cruz). The slides were stored in +4°C after visualized with the fluorescence microscopy. Excitation wavelength for FITC and DAPI were at 490nm and at 359nm, respectively.

3.3.19 FACS Analysis

MSCs were collected with trypsinization and centrifuged at 1500 rpm for 5 min at +4°C. MSCs were washed with 10ml 1X PBS and centrifuged again at 1500 rpm for 5 min at +4°C. Cell pellets were re-suspended and 10^6 cells were collected in 0.5mL 1X PBS. Cells were analyzed by BD Accuri C6 Flow Cytometer. For each sample, 10.000 cells were acquired. To get rid of debris, contaminating particles and apoptotic bodies, P2 gate was used. CPN positivity of MSCs was analyzed by using FL-1 channel of flow cytometer.

3.3.20 Statistical Analysis

All data are expressed as means $\pm$ SD (Standart deviation). The two sample significance test was performed with non-paired t-test using ANOVA. The multi sample significance test was performed with multiple comparison test of ANOVA using the Tukey's Multiple Comparison Test. A value of $P < 0.05$ was considered to be statistically significant. The values above the 0,05 ($p > 0,05$) were considered to be statistically non – significant.

Chapter 4

4 Results

4.1 Comparison of 4 Different Nanoparticles

Initially, we used 4 different nanoparticles synthesized at Chemistry Department of Bilkent University. These nanoparticles bear a significant auto-fluorescence. These 4 nanoparticles were taken by cells without any preference of cell type and showed very intense fluorescence inside the cells. These nanoparticles could be used as a cell tracking agent due to their small (nanometric) size and easy uptake by the cells. In order to use these nanoparticles in cellular tracking experiments, first it is important to be certain about the labeled cells had significant levels of cellular activity after labeling. MTT assay was performed to test how these nanoparticles affected the cellular activity of MSCs and Huh7 cells. It was shown that 70% of VP6 (CPN) labeled MSCs had cellular activity which was the least decrease among 4 different nanoparticles (Figure 4.1). On the other hand, these 4 nanoparticles did not significantly change the cellular activity of Huh7 cells. Nevertheless, VP6 labeled Huh7 cells had very similar cellular activity as the non-labeled Huh7 cells (Figure 4.2). Collectively, among 4 different nanoparticles, VP6 labeled cells were shown to have most similar activity to control groups.

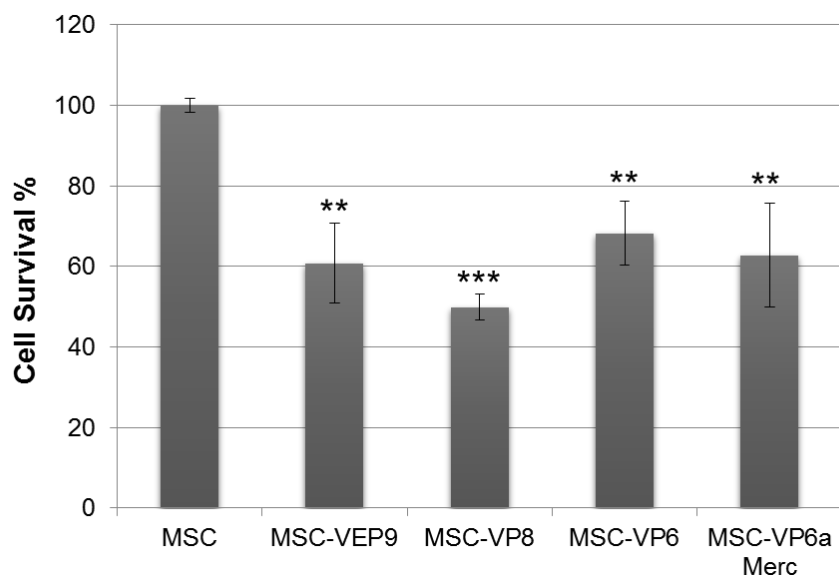


Figure 4.1. MTT assay of MSCs which were incubated with 4 different nanoparticles.

All MSCs that were incubated with different nanoparticles (VEP9, VP8, VP6 and VP6a Merc) had comparable level of cellular activity; however, the VP6 had the least decrease on MSCs cellular activity (70%). The cellular activity of non-labeled MSCs was assumed as 100% and percentage of nanoparticle labeled MSCs' cellular activities were normalized based on the control group. MSCs that were labeled with other three nanoparticles had cellular activity below 70%. (Statistical analysis were done with ANOVA and Tukey's Multiple Comparison Test ** $p < 0,01$, *** $p < 0,001$)

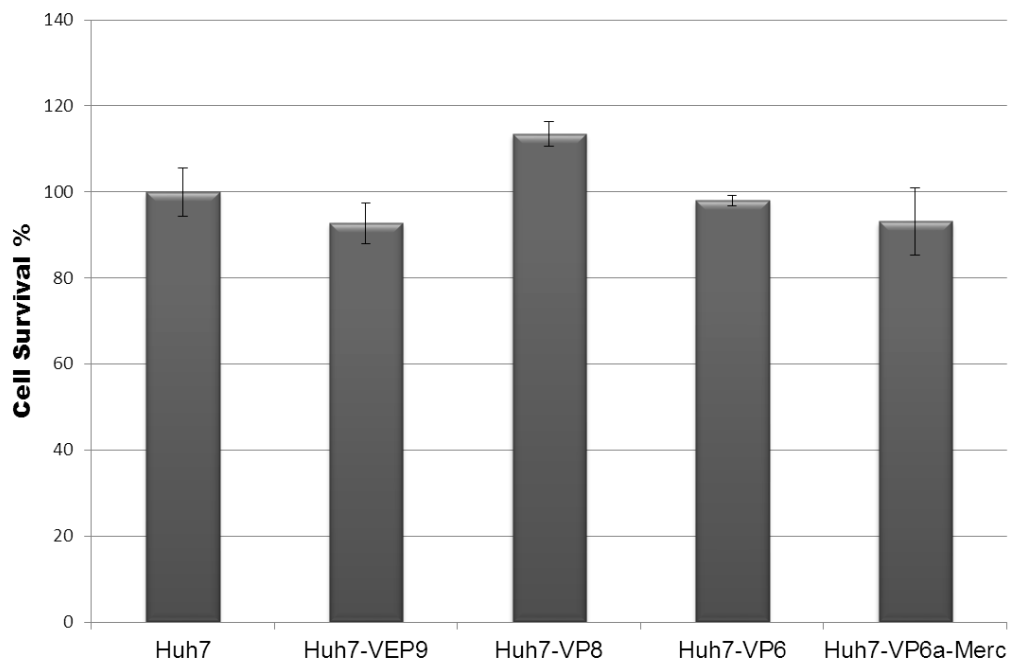


Figure 4.2. MTT assay of Huh7 cells which were incubated with 4 different nanoparticles.

All Huh7 cells that were incubated with different nanoparticles (VEP9, VP8, VP6 and VP6a-Merc) had comparable level of cellular activity. The cellular activity of non-labeled Huh7 cells was assumed as 100% and percentage of nanoparticle labeled Huh7 cells' cellular activities were normalized based on the control group. The differences between experimental group and control group were not significant (Statistical analysis were done with ANOVA and Tukey's Multiple Comparison Test $p > 0,05$)

After testing the effect of CPN on cellular activity, cells which were incubated with these newly synthesized nanoparticles for 24h were fixed and observed under fluorescence microscope according to their UV and PL data (Figure 4.3). VEP9 and VP8 nanoparticles emitted blue fluorescence light at 359nm. This may create a conflict when DAPI counterstain should be used to detect the nucleus of the labeled cells. On the other hand, VP6 and VP6a-Merc emitted green fluorescence light at 490nm, therefore, could be used together with DAPI counterstain. Compare to VP6a-Merc, VP6 labeled MSCs and Huh7 cells more intensely. Based on these advantages, we concluded that VP6 was the most suitable nanoparticle to be used in cellular tracking.

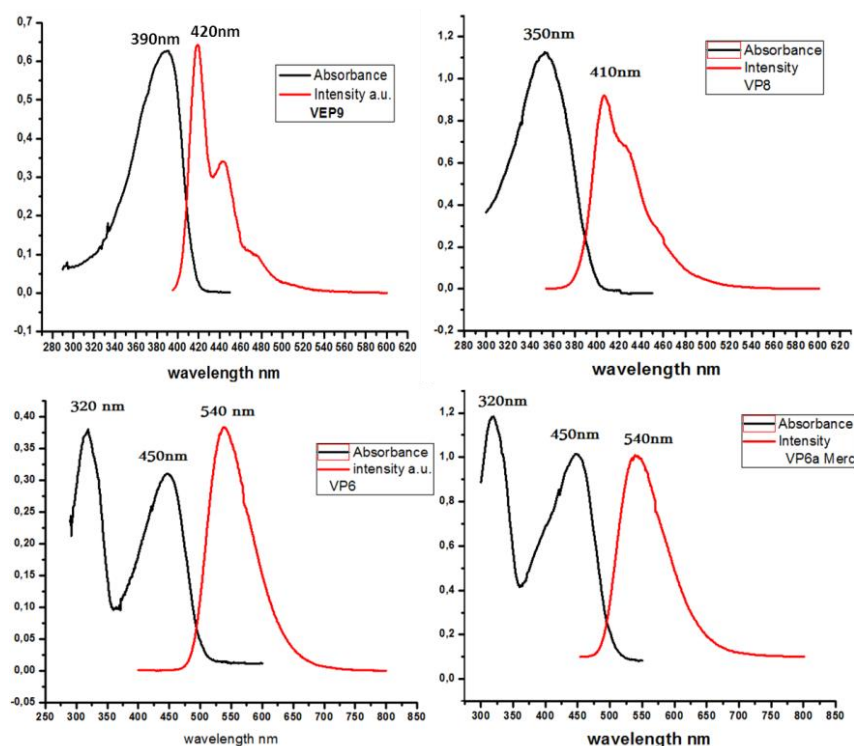


Figure 4.3. The wavelength (nm) data of nanoparticles.

Each nanoparticle could be observed with fluorescence microscopy according to their UV and PL data.

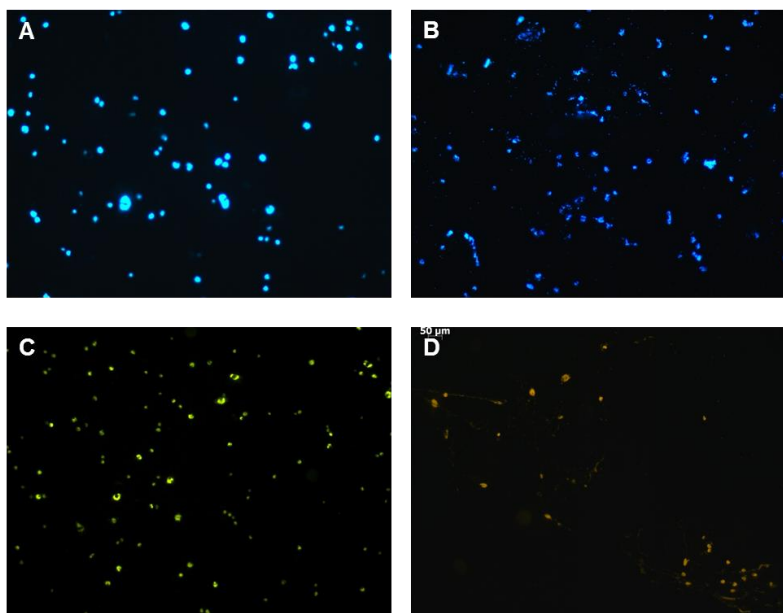


Figure 4.4. *In vitro* MSC labeling with 4 different nanoparticles.

The isolated MSCs were incubated with nanoparticles; VEP9 (A), VP8 (B), VP6 (C) and VP6a-Merc (D) separately at the 13th day of their culturing. After 24h incubation with nanoparticles, MSCs were fixed and visualized under fluorescence microscopy with 10X magnification according to UV and PL data of nanoparticles (Figure 4.3).

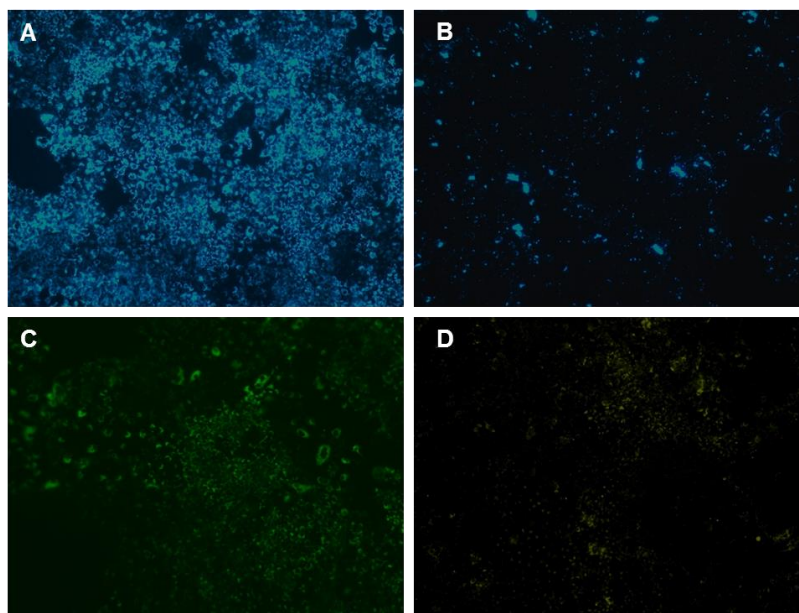


Figure 4.5. *In vitro* Huh7 cell labeling with 4 different nanoparticles.

Huh7 cells were incubated with nanoparticles; VEP9 (A), VP8 (B), VP6 (C) and VP6a-Merc (D) separately for 24h. Then, Huh7 cells were fixed and visualized under fluorescence microscopy with 10X magnification according to UV and PL data (Figure 4.3).

Finally, in order to show and compare the effects of these nanoparticles on MSCs differentiation capacity, MSCs cells were isolated from bone marrow and labeled with nanoparticles at the 13th day of culturing. After 24h incubation with nanoparticles (at the 14th day of MSCs culturing), MSCs were differentiated into adipocytes and osteocytes with defined differentiation medium without any further nanoparticle incubation. After 3 weeks of differentiation period, adipocytes were stained with Oil Red O and osteocytes were stained with Alizerin Red Staining. According to results, VP6 labeled MSCs had strong adipogenic differentiation ability compare to other MSCs labeled with other nanoparticles. On the other hand, VEP9 and VP8 significantly decrease the ability of differentiation of MSCs into adipocytes (Figure 4.6). The osteogenic differentiation of MSCs showed that VEP9, VP8 and VP6 labeled MSCs had comparable levels of differentiation capacity. However, VP6a-Merc labeled MSCs showed a decrease in potential of osteogenic differentiation in labeled cells (Figure 4.7). Based on these results, we concluded that VP6 nanoparticle had the least side effect on MSC differentiation

potency and, therefore, could be a potential tool to be used in *in vivo* and *in vitro* tracking of MSCs.

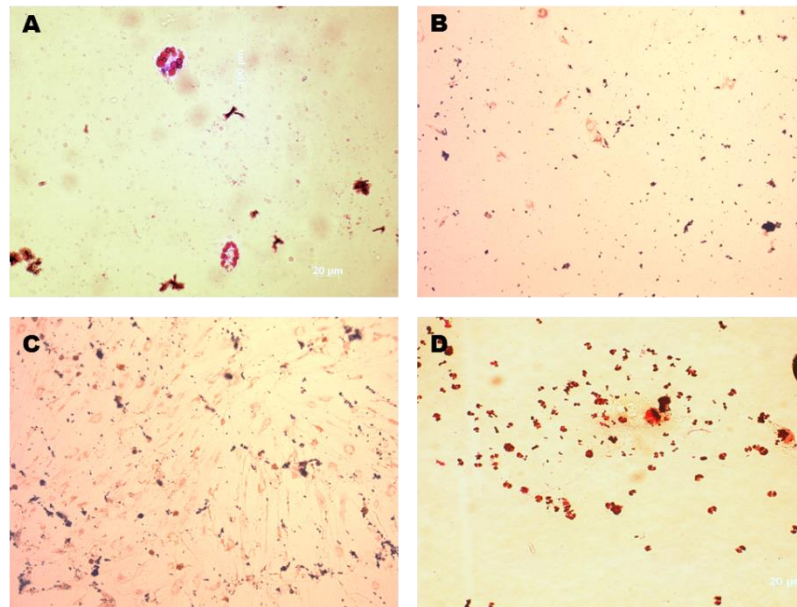


Figure 4.6. Adipogenic differentiation of 4 different nanoparticles labeled MSCs.

The isolated MSCs had been labeled with nanoparticle; VEP9 (A), VP8 (B), VP6 (C) and VP6a-Merc (D) at their 13th day of culturing. After 24h nanoparticle incubation (at 14th day of MSCs culturing), cells were induced to differentiate into adipocyte with defined differentiation medium without any further nanoparticle incubation. After 3 weeks of differentiation period, adipocytes were detected with Oil Red O staining.

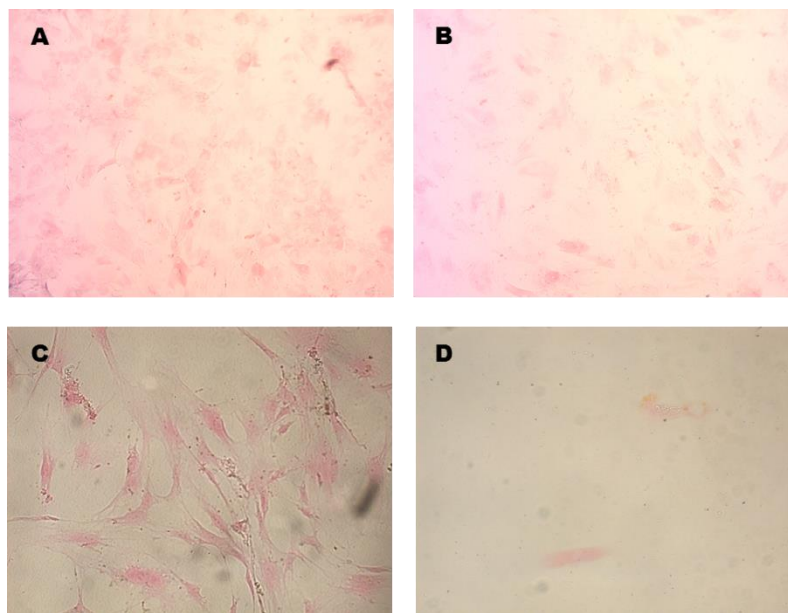


Figure 4.7. Osteogenic differentiation of 4 different nanoparticles labeled MSCs.

The isolated MSCs had been labeled with nanoparticle; VEP9 (A), VP8 (B), VP6 (C) and VP6a-Merc (D) at their 13th day of culturing. After 24h nanoparticle incubation (at 14th day of MSCs culturing), cells were induced to differentiate into osteocytes with defined differentiation medium without any further nanoparticle incubation. After 3 weeks of differentiation period, osteocytes were detected with Alizerin Red Staining.

4.2 Properties of Used CPN

In this study, we aim to propose an approach which can potentially be used in *in vitro* and *in vivo* cellular trafficking. The experiments which were performed with 4 different nanoparticles lead us to continue with the nanoparticle VP6 (called CPN from now on) since it did not result in a severe decrease in cellular activity and it was efficient to label cells intensity. Moreover, compare VP6 nanoparticle did not change the differentiation capacity of labeled MSCs. In addition, the differences between 4 nanoparticles could also be explained by the differences in their functional groups. The other nanoparticles have toxic and reactive functional groups whereas the VP6 nanoparticle is neutral and non-reactive (Figure 4.8). Therefore VP6 showed the least effect on cellular survival and characteristics. In addition, VP6 nanoparticle had green

emission at 540 nm unlike the VEP9 and VP8, and, therefore, could be used together with DAPI or Hoechst nucleus staining. Together these properties make the VP6 (CPN) proper probe to be used in cellular tracking.

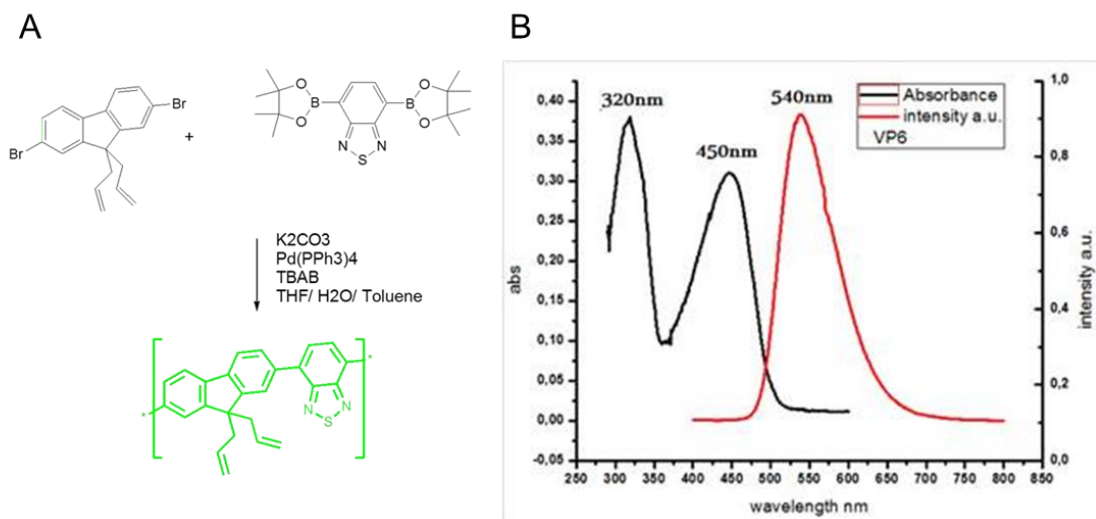


Figure 4.8. Chemical properties of the chosen nanoparticle-VP6 (CPN).

A) Chemical properties of CPN. B) VP6 excitation is at 450 nm and emission is at 540 nm. The decrease in the fluorescence intensity of VP6 nanoparticle was very low after it had been irradiated with 254 UV light for 5h. The concentration of VP6 after evaporation was 1 mg mL⁻¹ ($2,64 \times 10^{-3}$ mmol mL⁻¹) [223, 226].

4.3 Optimization of Incubation Time

The optimal incubation time of CPN were analyzed with MTT assay of MSCs that were labeled with CPN in different time periods. CPNs were introduced either at the 1st, 4th, 7th, 10th and 13th day of culturing of the cells isolated from bone marrow. The bone marrow heterogeneous population was cultured with CPN in defined times until MSCs were obtained which takes 14 day after bone marrow isolation. After MSCs were incubated with CPNs in defined time periods, we performed MTT assay with labeled cells to analyze if CPN had any cellular proliferation inhibition in MSCs based on the time of incubation. Our results showed that, there were no significant changes occurred in cellular activity of MSCs that were labeled with CPN in different time periods (Figure 4.9A). Afterwards, FACS analysis was performed with MSCs that were incubated with the same

concentration of CPN but in different time points. According to FACS results, optimum condition in terms of MSC viability and labeling were obtained in the cells that were incubated with CPN for 24h (Figure 4.9B). In other incubation periods, lower cellular vitality was obtained even though staining levels were comparable. Finally, *in vitro* CPN labeling of different time periods were analyzed with fluorescence microscopy. All time periods, except 6h incubation, had comparable levels of labeled MSCs which had strong fluorescence emission (Figure 4.10). At the end, we conclude to continue with 24h CPN incubation since it did not show adverse effects on MSCs cellular activity and was enough to label cells intensely.

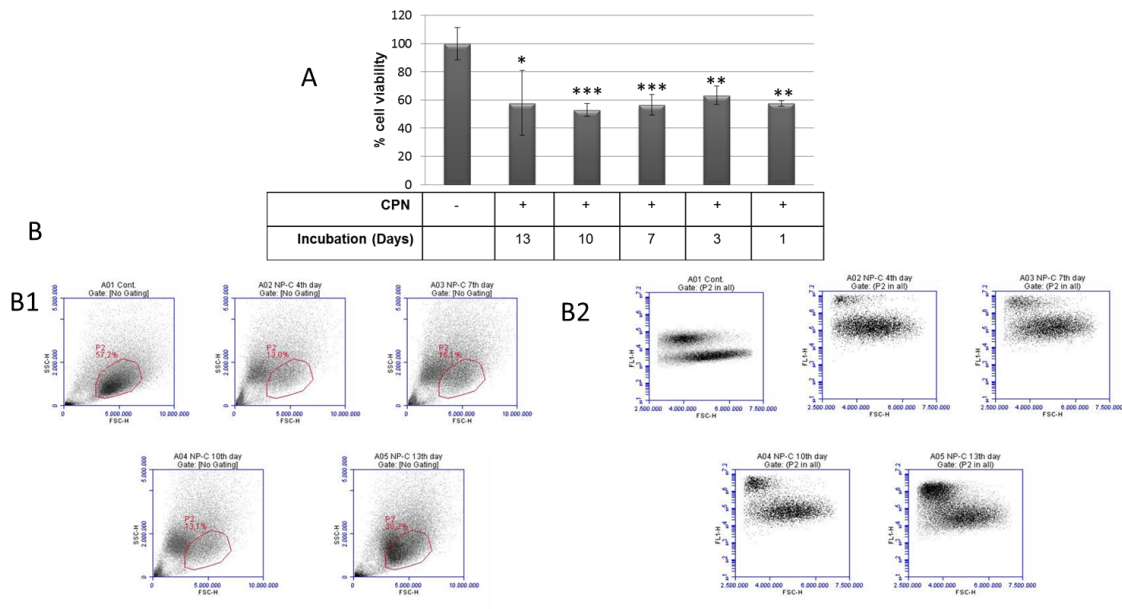


Figure 4.9. Cellular activity and labeling intensity of MSCs that were incubated with CPN in different time periods.

CPNs were introduced to MSCs at their 1st, 4th, 7th, 10th and 13th day of culturing. 14 days after the bone marrow isolation, MSCs were obtained. The cells were incubated with nanoparticles during this period with defined times. A) MTT assay was performed with CPN labeled MSCs. (Statistical analysis were done with ANOVA and Tukey's Multiple Comparison Test. * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$) B) FACS analysis was performed with MSCs labeled with CPN in different time points, the cellular activity and labeling intensity were assessed.

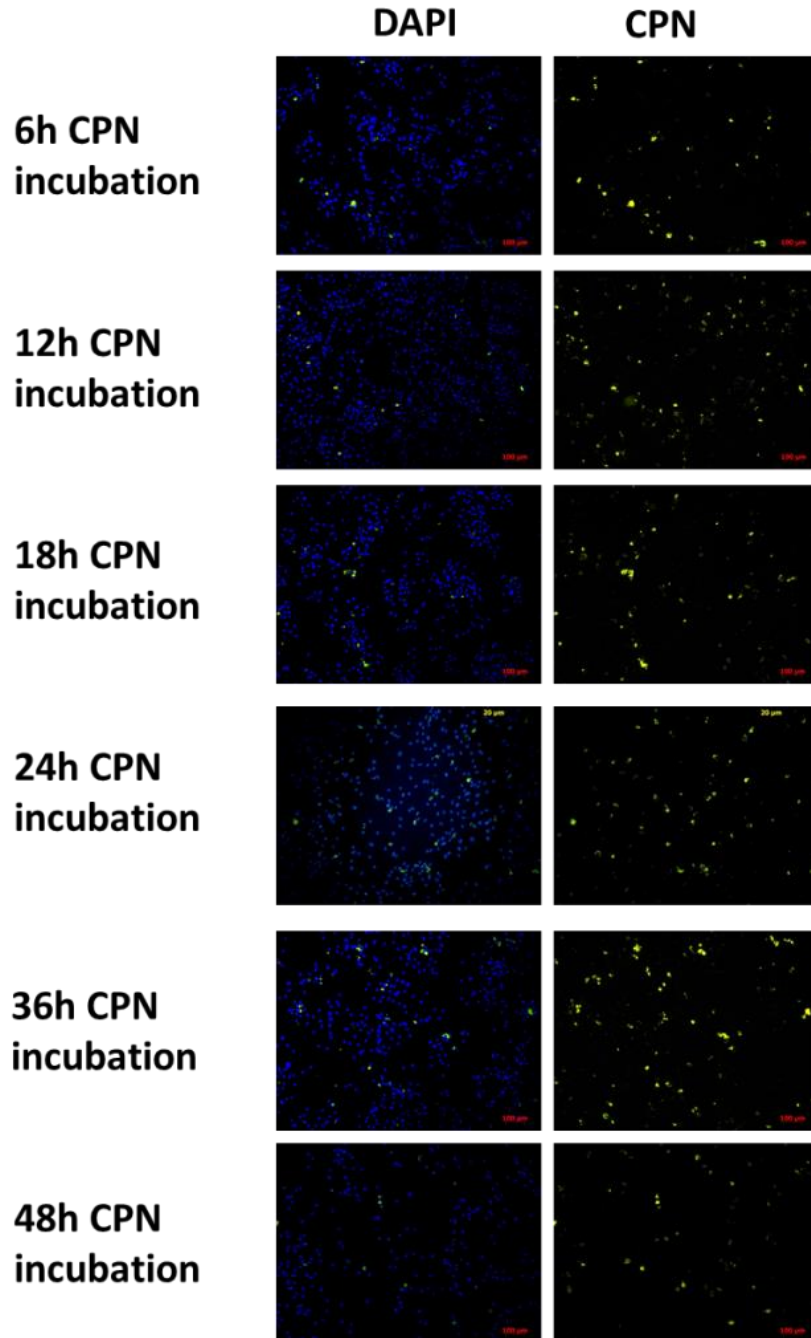


Figure 4.10. *In vitro* analysis of labeling intensities of labeled MSCs in different time periods.

The isolated MSCs were incubated with CPN in defined time periods; afterwards the cells were fixed and visualized under fluorescence microscopy. DAPI was used in nucleus staining. CPN was visualized at 540nm with fluorescence microscopy. CPN positive cell percentage were 62%, 68%, 67%, 70%, 73% and 64%, respectively.

4.4 Cellular Activity after CPN Labeling

In order to assess the proliferation inhibition of CPN in different cell types, MTT assay was performed with MSCs, DPSCs and Huh7 cells. The bone marrow heterogeneous cell population was cultured until MSCs were obtained which takes 14 day after bone marrow isolation. At the last day of culturing, MSCs were incubated with CPN for 24h and MTT assay was performed with labeled cells to analyze if CPN had any cellular proliferation inhibition. The DPSCs and Huh7 cells can be cryopreserved and, therefore these cells were thawed based on our need. DPSCs and Huh7 cells were labeled with CPN for 24h when they reach 70% confluency. According to MTT results, 70% of MSCs (Figure 4.11 A) and DPSCs (Figure 4.11 B) had cellular activity after 24h incubation of CPN whereas 99% of Huh7 cells (Figure 4.11 C) had cellular activity. Since MSCs and DPSCs are kind of sensitive cells, the 70% cellular activity of CPN incubated cells could be accepted. To further evaluate the cytotoxicity of CPN on DPSCs, LDH assay was performed. According to LDH results, compare with high (Triton-X) and low toxicity (non-labeled cell) control group results, CPN labeling did not show any toxic effects on DPSCs. Collectively, these assays proved that 24h CPN incubation can be used for MSCs, DPSCs and Huh7 cells since it did not show significant proliferation inhibition in labeled cells.

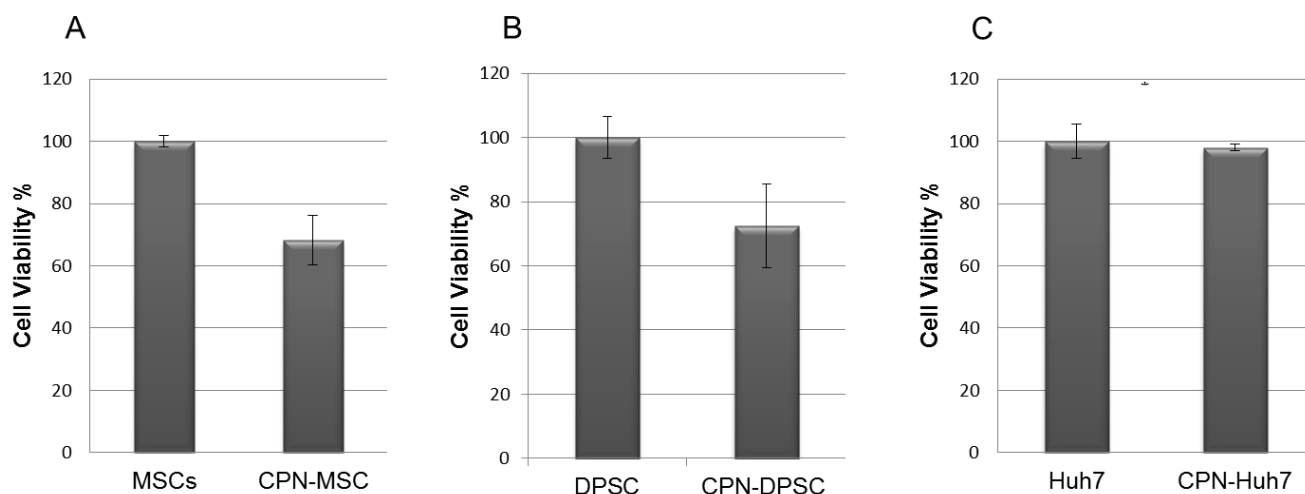


Figure 4.11. Cellular activity of CPN labeled cells.

MTT assay was performed with 24h CPN labeled MSCs (A), DPSCs (B) and Huh7 cells (C). Cellular viability was measured. The differences of cellular activity of control and CPN labeled cells were non-significant. (Statistical analysis were done with ANOVA unpaired, two tailed t-test $p > 0,05$).

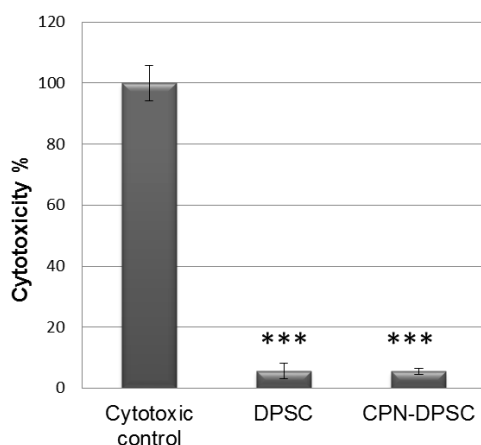


Figure 4.12. Cytotoxicity test of CPN in DPSCs.

Cytotoxicity of CPN in DPSCs was assessed with LDH assay. Non- labeled DPSCs (0% toxic) were used as negative control of LDH whereas 1% Triton treated cells (100% toxic) used as positive control. (Statistical analysis were done with ANOVA and Tukey's Multiple Comparison Test. *** $p < 0,001$. The differences between non-labeled and labeled DPSCs was non-significant $p > 0,05$)

4.5 Characterization of MSCs after CPN Incubation

4.5.1 Cell Surface Marker Analysis

Prior to use CPN labeled MSCs *in vivo*, it is important to be certain that CPN did not change characteristics of them. Cell surface marker analysis of MSCs was initially performed to assess if CPN administration changed the expression of known MSC surface markers. Hence, RT-PCR was performed with the primers of MSC surface markers which are CD90, CD71 and CD29 [227] with the RNAs isolated from MSCs which were labeled with nanoparticles for 24h at their 13th day of culturing. In addition, the absence of hematopoietic cell surface markers which are CD45 and CD34 [227] were also analyzed with the same RNAs by RT-PCR. RT-PCR results showed that CPN labeled MSCs had exactly the same expression profiles with non-labeled MSCs (Figure 4.13). Consequently, it was shown that CPN labeling did not change the MSCs literally known characteristics demonstrated by stable surface marker expression.

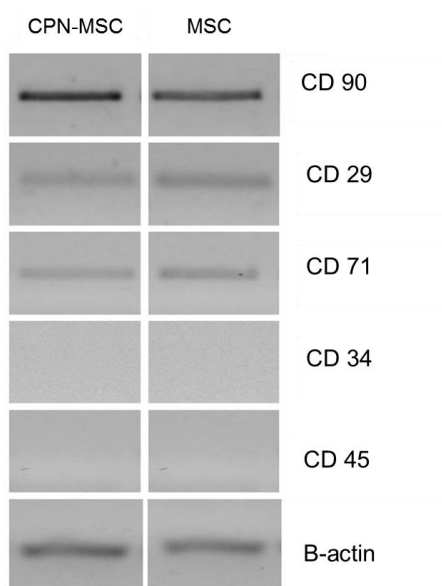


Figure 4.13. Cell surface marker analysis of MSCs with RT-PCR.

The comparison of surface marker expression using the RNAs isolated from 24h CPN incubated MSCs on the 13th day of culturing (CPN-MSCs) and non-labeled MSCs (MSCs). β -Actin was used for as loading control.

4.5.2 Differentiation Potential of CPN Labeled MSCs

Multilineage differentiation ability is another characteristic of MSCs which should be analyzed to demonstrate that CPN did not change the characteristics of MSCs. In order to assess the differentiation potential of CPN labeled MSCs, the isolated MSCs were labeled with CPN for 24h at the 13th day of culturing. On the 14th day culturing, MSCs were obtained and they were incubated with defined differentiation mediums which induced them to differentiate into adipocyte, chondrocyte or osteocytes without any further CPN incubation. After 3 weeks of differentiation period, differentiation potentials of CPN labeled and non-labeled MSCs were analyzed with Oil Red O staining for adipocytes, Alizarin Red Staining for osteocytes and Alcian Blue Staining for chondrocytes. Our results showed that differentiation potential of MSCs into adipocyte, osteocytes and chondrocytes was not affected from CPN labeling and comparable with the non-labeled MSCs (Figure 4.14). Due to lack of change in their potential of differentiation, we concluded that CPN did not change the characteristics of MSCs. Together with stable cell surface marker expression; significant differentiation capability of CPN labeled MSCs pointed the potential utilization of CPN in MSCs tracking *in vivo* since it did not change the literally known characteristics of MSCs.

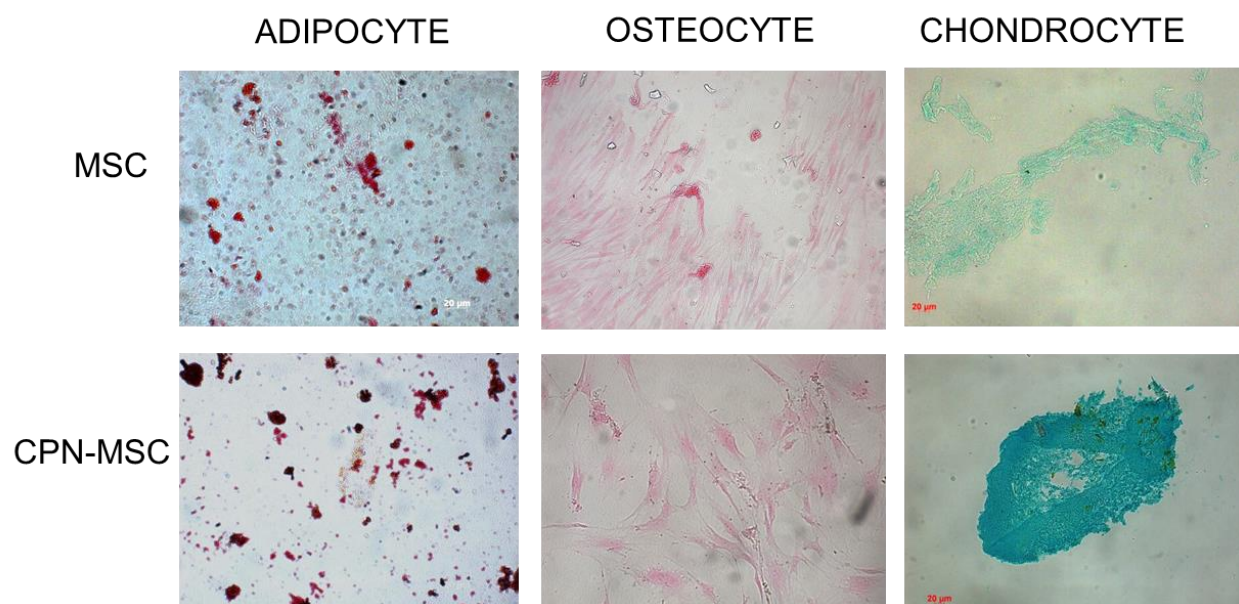


Figure 4.14. Differentiation capacity of CPN labeled MSCs.

The isolated MSCs had been labeled with CPN at their 13th day of culturing. After 24h nanoparticle incubation (at 14th day of MSCs culturing), cells were induced to differentiate into adipocyte, osteocyte and chondrocyte. Oil Red O Staining indicated adipogenic differentiation, Alizarin Red Staining indicated osteogenic differentiation and Alcian Blue Staining indicated chondrogenic differentiation.

4.6 Characterization of DPSCs

4.6.1 Cell Surface Marker Analysis of DPSC

In order to evaluate CPN effect on different MSC types such as DPSC (an alternative source for human MSCs), first we proved that used DPSCs showed the literally known characteristics of DPSCs. We used 3 different patients' wisdom teeth to investigate whether DPSCs isolated from different patients had any different characteristics. Basically, cell surface marker analysis was performed with RT-PCR in DPSCs. The marker analysis showed that isolated DPSCs showed similar cell surface marker expression with MSCs. DPSCs were positive for MSCs cell surface markers which are CD90 and CD29. On the other hand, the absence of hematopoietic cell surface marker which is CD34 also proved the similarity of these cells with MSCs (Figure 4.15).

The results of the RT-PCR were also indicated that there were no differences found in DPSCs which were isolated from different patients.

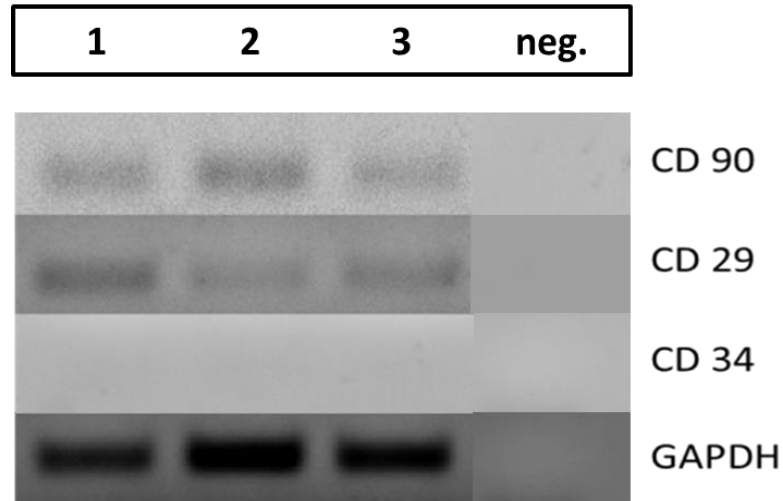


Figure 4.15. Cell surface marker analysis of DPSCs with RT-PCR.

Wisdom teeth of three patients (1, 2 and 3) were used as different sources of DPSCs. These different DPSCs were compared in terms of the MSC cell surface marker expression (CD90 and CD29) with RT-PCR. The absence of hematopoietic cell marker (CD34) was assessed with RT-PCR. GAPDH was used as a loading control.

In addition to cell surface marker analysis of DPSCs, other mesenchymal cell markers, α -SMA, Fibronectin, Vimentin, N-cadherin, were further analyzed by RT-PCR. DPSCs from three different patients were all positive for the mesenchymal cell markers. Additionally, the absence of epithelial cell marker (E-cadherin) further supports the mesenchymal cell characteristics of DPSCs. Similarly, DPSCs isolated from 3 different patients had similar expression pattern of the markers (Figure 4.15). Together these results demonstrated that, human DPSCs showed the similar characteristics with MSCs and, therefore, they can be a potential alternative source to human MSCs.

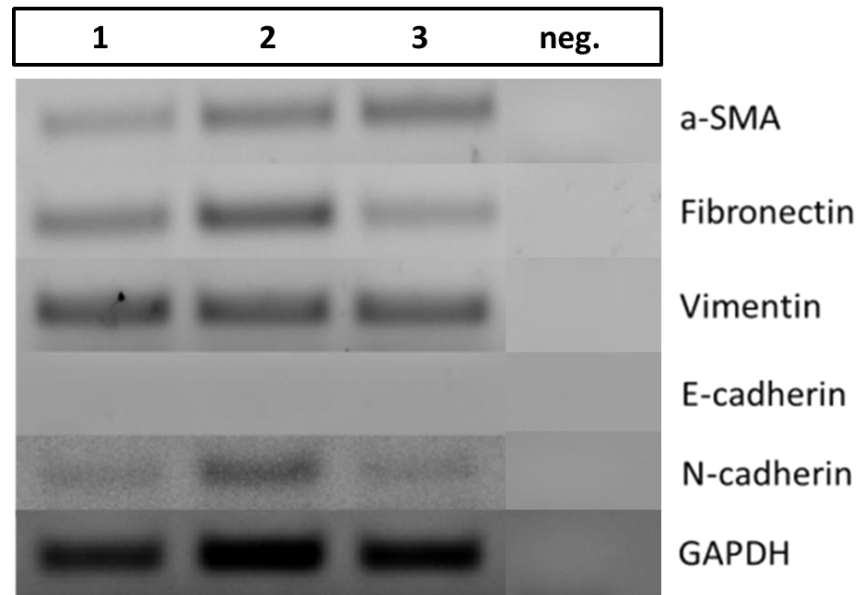


Figure 4.16. Mesenchymal cell marker analysis of DPSCs from 3 different patients with RT-PCR.

Mesenchymal cell features of DPSCs (Patient 1, 2 and 3) were analyzed with the RT-PCR using primers of mesenchymal cell specific markers (α -SMA, Fibronectin, Vimentin, and N cadherin). Additionally, the absence of epithelial cell marker (E-cadherin) further supports mesenchymal cell characteristics of DPSCs. GAPD was used as loading control.

4.6.2 The Differentiation Potential of Used DPSCs

Similar to MSCs, DPSCs are able to differentiate into adipocyte, osteocyte and chondrocyte. In order to utilize DPSCs as an alternative source for MSCs, it is important to show that these two cell types showed similar characteristics. Differentiation potential of isolated DPSCs was assessed to indicate their similarities with MSCs. After isolation of DPSCs from wisdom teeth, they were induced to differentiate into adipocyte, osteocyte and chondrocyte when they reached 70% confluency. After 3 weeks of differentiation period, DPSC differentiation was assessed with Oil Red O Staining for adipocytes, Alizarin Red Staining for osteocytes and Alcian Blue Staining for chondrocytes. DPSCs were able to differentiate into adipocyte, osteocytes and chondrocytes and their differentiation potential was shown to be comparable to MSCs (Figure 4.17). Collectively, based on the similarities in cell surface expressions and differentiation potential, DPSCs were asserted as an alternative source of mesenchymal like stem cell source that may supersede MSCs' therapeutic strategies.

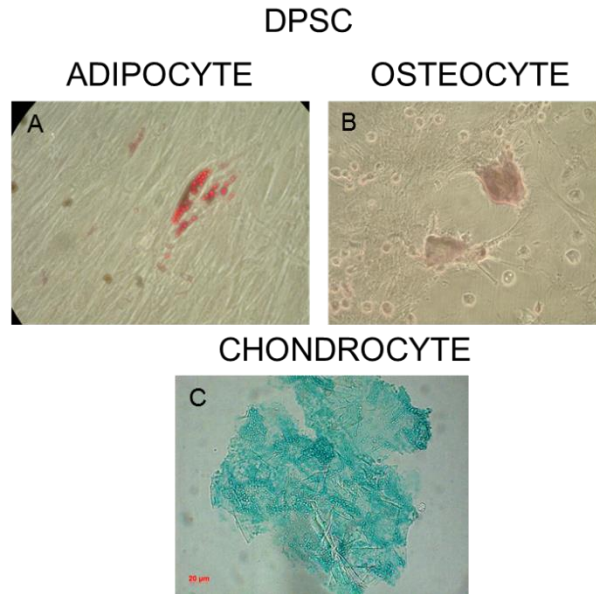


Figure 4.17. Differentiation potential of isolated DPSCs.

The DPSCs isolated from wisdom tooth were differentiated into adipocytes, osteocytes and chondrocytes when they became 70% confluent. Adipogenic, osteogenic and chondrogenic differentiation of DPSCs were obtained with incubation of DPSCs with defined differentiation mediums. Oil Red O Staining indicated adipogenic differentiation (A) and Alizarin Red Staining indicated osteogenic differentiation (B) whereas the Alcian Blue Staining showed chondrogenic differentiation (C).

4.7 Visualization of CPN Labeled Cells *in vitro*

Thereafter showing CPN did not have serious adverse effects on cellular activities and characteristics of cells, we analyzed CPN labeled cells with fluorescence microscopy in order to assess their potential utilization *in vitro*. At the 13th day of MSCs culturing, CPN were administered. After 24h CPN incubation, at the 14th day of culturing MSCs were obtained and visualized under fluorescence microscope. DPSCs and Huh7 cells could be cryopreserved. Thawed DPSCs and Huh7 cells were incubated with CPN for 24h when they reach 70% confluency. After CPN incubation, cells were fixed, counterstained and mounted with DAPI to be visualized under fluorescence microscope. According to emission-excitation data of CPN, labeled cells could be observable at 540nm wavelength of fluorescence microscope. Strong green fluorescence light was observed in CPN labeled cells. The CPN labeling together with DAPI nucleus staining indicated the specific cell labeling with CPNs. Importantly, CPN luminescence did not fade during fluorescence light excitation. 70% MSCs and DPSCs labeled with CPN (Figure 4.18, 4.19) whereas 90% Huh7 were labeled with CPN (Figure 4.20). Indeed, CPNs were taken by MSCs, DPSCs and Huh7 cells without any preference which indicated that CPN did not label a particular cell type but many different cell types. Strong fluorescence light emission without a risk of fading makes CPN a potential tool for cellular tracking *in vitro*.

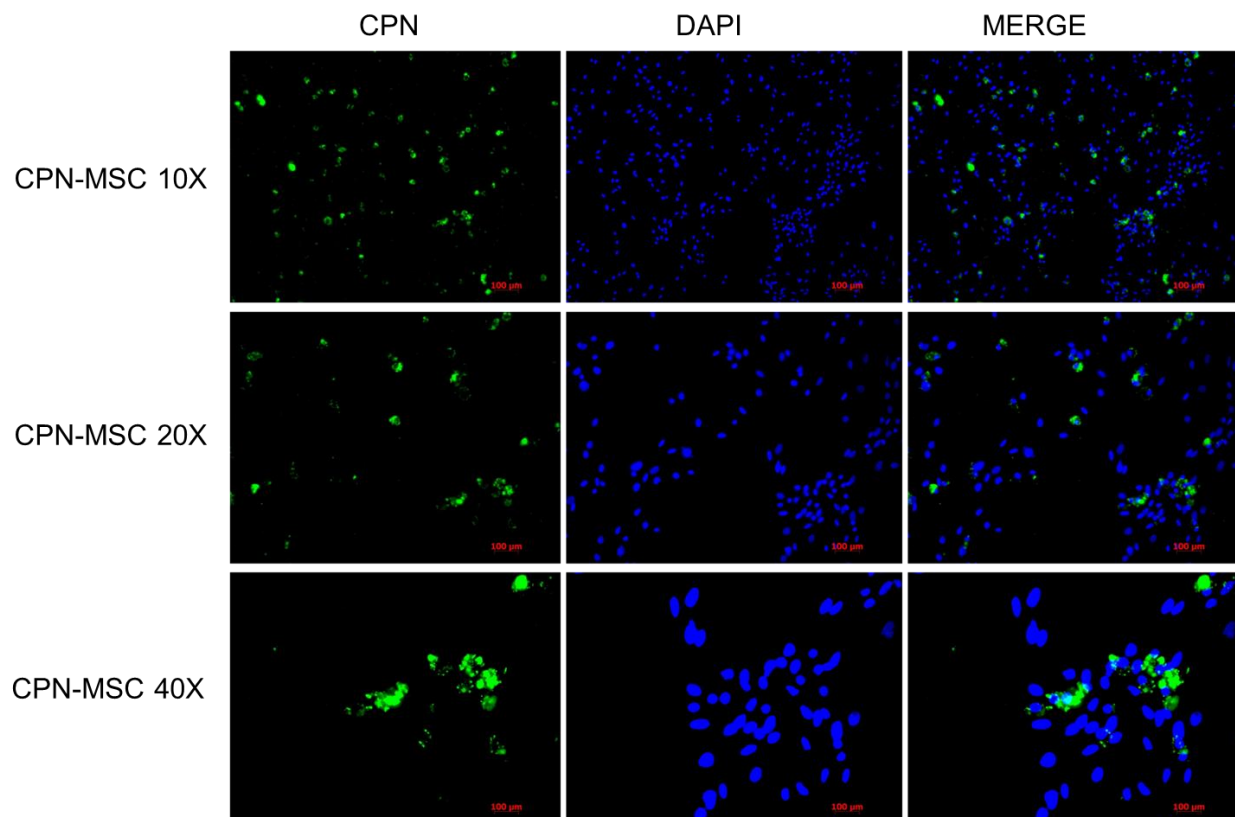


Figure 4.18. *In vitro* visualization of CPN labeled MSCs.

The isolated MSCs were incubated with CPN on their 13th day of culturing. After 24h CPN treatment, at the 14th day of culturing the incubated MSCs were fixed, counterstained and mounted with DAPI to be visualized with fluorescence microscopy. CPN labeled MSCs were observed at 540nm and nucleus DAPI staining were observed at 460nm with fluorescence microscope. Cell photos were taken with 10X, 20X or 40X magnifications.

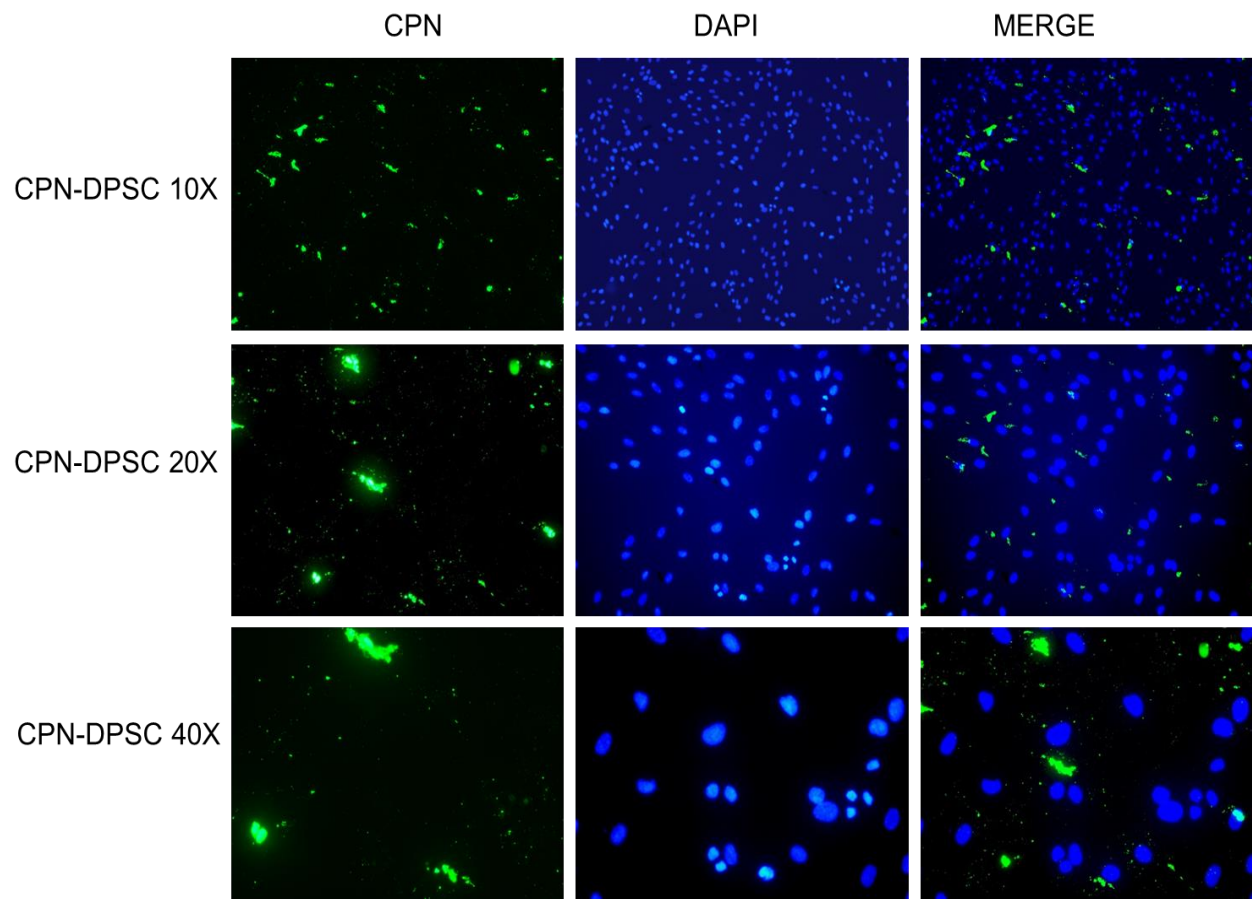


Figure 4.19. *In vitro* visualization of CPN labeled DPSCs.

24h CPN incubated 70% confluent DPSCs were fixed, counterstained and mounted with DAPI to be visualized with fluorescence microscopy. CPN labeled MSCs were observed at 540nm and nucleus DAPI staining were observed at 460nm with fluorescence microscope. Cell photos were taken with 10X, 20X or 40X magnifications.

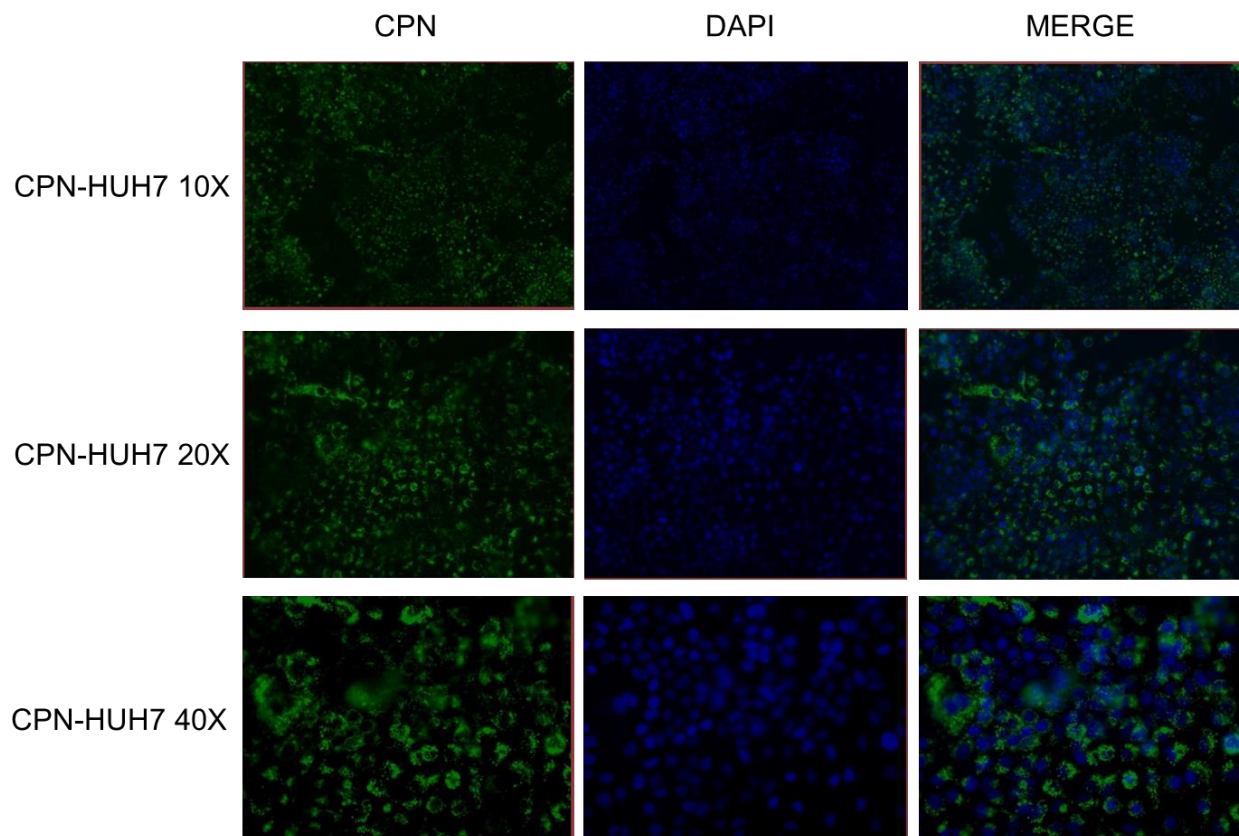


Figure 4.20. *In vitro* visualization of CPN labeled Huh7 cells.

24h CPN incubated 75% confluent Huh7 cells were fixed, counterstained and mounted with nucleus staining DAPI. CPN labeled MSCs were observed at 540nm and nucleus DAPI staining were observed at 460nm with fluorescence microscope. Cell photos were taken with 10X, 20X or 40X magnifications.

4.8 *In vitro* Differentiation of CPN Labeled Cells

The effect of CPN on cellular differentiation and its activity in differentiated cells were analyzed with differentiation assay of the labeled cells. For this purpose, the isolated MSCs were labeled with CPN for 24h at the 13th day of culturing. At the 14th day culturing, MSCs were obtained and they were incubated with defined differentiation mediums which induced them to differentiate into adipocyte, chondrocyte or osteocytes without any further CPN incubation. Adipocytes, osteocytes and chondrocytes were detected with Oil Red O, Alizerin Red and Alcian Blue, respectively. In addition, we examined CPN activity in other type of differentiated cells which were DPSCs. Hence, when DPSCs reach 70% confluency after their thawing, cells were incubated with CPN. After 24h incubation, DPSCs were differentiated into chondrocytes without any further CPN incubation. Alcian Blue staining was performed for the detection of CPN labeled DPSCs chondrogenic differentiation. According to results, CPN labeling retained its' sharp luminescence for 3 weeks in both types of cells which were differentiated. Therefore, we proved that CPN remained in the labeled cell while the cell was differentiating (Figure 4.21). CPN labeling was not only proper to track differentiated MSCs but also could be used for tracking of DPSCs differentiation proved by sharp CPN luminescence in chondrocytes of CPN-DPSCs (Figure 4.22). Importantly, differentiated cells did not have background fluorescence emission which was demonstrated in differentiation of non-labeled MSCs (Figure 4.23). Together these indicated that CPN labeling remain in stem cells even if they were differentiated into cells that they were committed to. Therefore, here we propose a potential approach to monitor cellular differentiation. In addition, CPN labeling did not fade during this period and had very intense fluorescence which is essential in long term follow up studies.

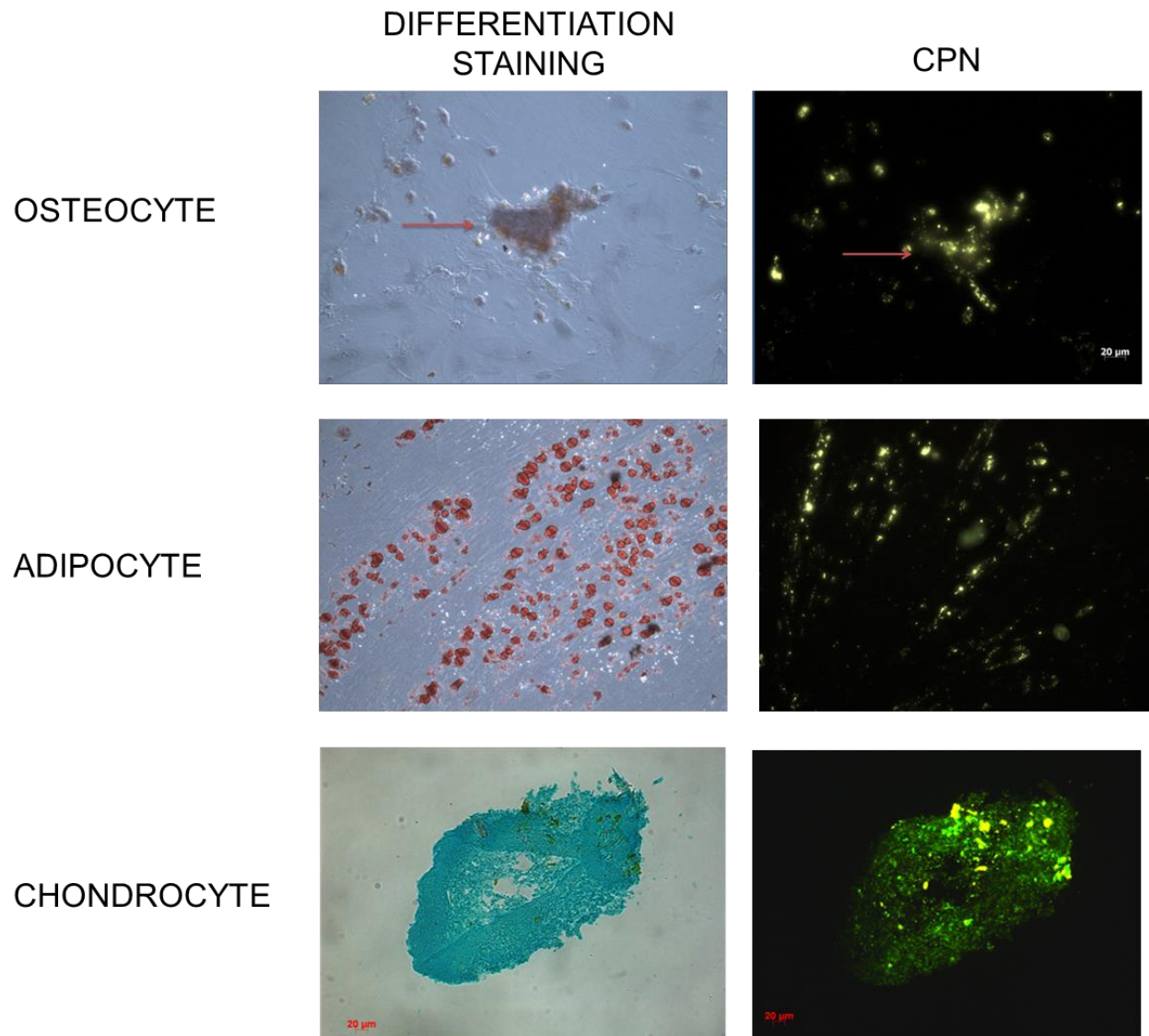


Figure 4.21. Visualization of CPN in differentiated MSCs.

The isolated MSCs were labeled with CPN for 24h at the 13th day of their culturing. At the 14th day of culturing, MSCs were obtained and induced to differentiate into adipocyte, osteocyte and chondrocyte with defined differentiation mediums without further CPN incubation. Afterwards, differentiated cells were analyzed with Oil Red O staining for adipocytes, Alizarin Red staining for osteocytes and Alcian Blue staining for chondrocytes. CPN labeling was shown in differentiated cells with fluorescence microscopy at 540nm.

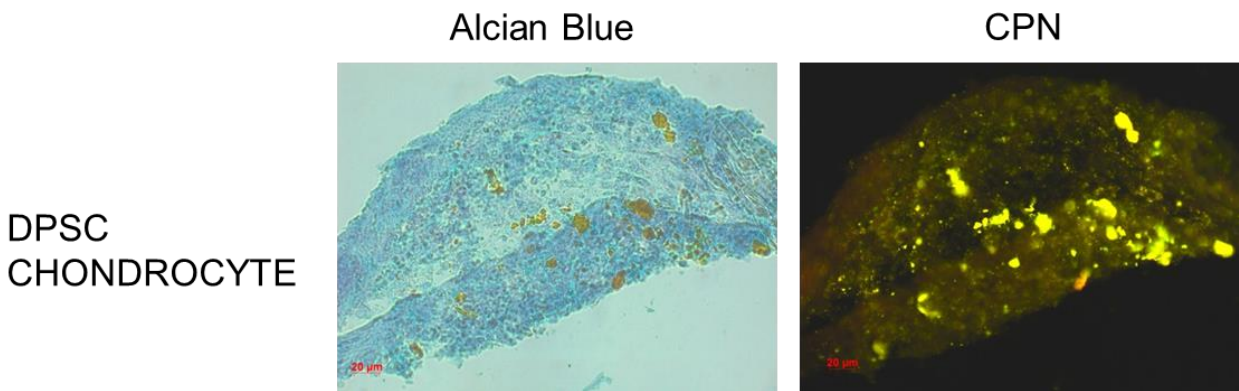


Figure 4.22. Visualization of CPN labeling in chondrocytes of DPSCs.

24h CPN incubated DPSCs (70% confluent) were differentiate into chondrocyte with defined differentiation medium. DPSCs were not further incubated with CPN during differentiation period. Afterwards, chondrocytes were detected with Alcian Blue Staining. CPN labeling was shown in differentiated cells with the fluorescence microscopy at 540nm.

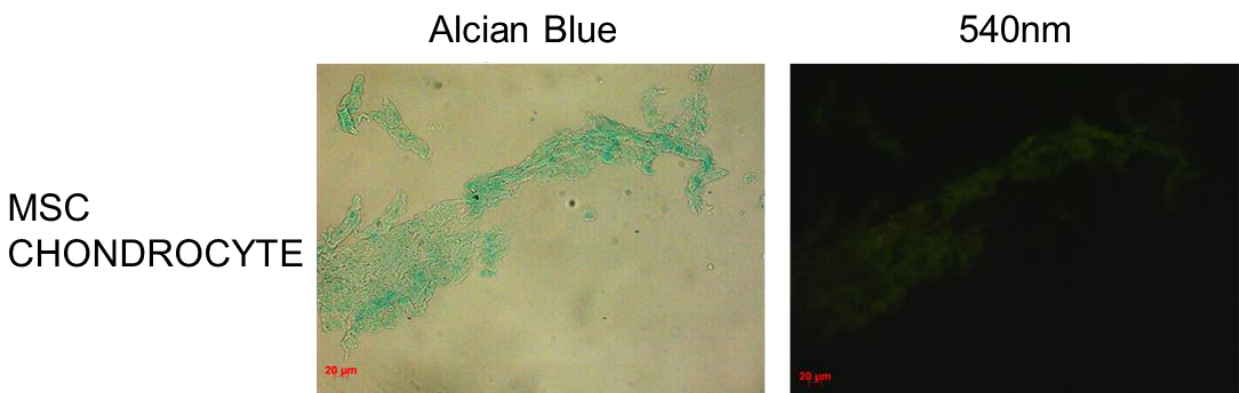


Figure 4.23. Monitoring auto-fluorescence of non-labeled MSC chondrocytes.

In order to assess background fluorescence emission of stained chondrocytes, non CPN labeled and Alcian Blue stained differentiated cells were visualized with fluorescence microscopy at 540nm. Chondrogenic differentiation was analyzed with Alcian Blue staining which was detected with bright field microscopy.

4.9 Labeled Cell Tracking *in vivo*

4.9.1 CPN labeled MSC tracking *in vivo*

4.9.1.1 CPN Labeled MSC Tracking in Liver Regeneration Model

MSCs are capable of migrating to the site of injury and help tissue regeneration. Based on these properties of MSCs, many cellular therapies have been developed [228]. While using MSCs for a therapeutic purpose, it is important to track these cells *in vivo* to assess if delivered MSCs migrate and reside in the target tissue. Here we propose a new approach to track MSCs *in vivo* in liver regeneration model. Bone marrow isolated MSCs were incubated with CPN at the 13th day of culturing. After 24h CPN incubation (at the 14th day of MSC culturing), the labeled cells were collected and administered from tail vein of the PH rats (3/5 of the liver was taken prior to injection) and SH rats (operation without liver resection). PH rats which were given non-labeled MSCs from tail vein used as negative control. 3 days after cell administration, the rats were sacrificed, their liver were extracted, sectioned and fixed in order to monitor labeled cells. Promisingly, administered CPN-MSCs were observable in the liver of PH rats and CPN-MSCs were co-localized with DAPI nucleus staining which proved the presence of the CPN labeled cells. On the other hand, CPN-MSCs were not found in the intact liver tissues of sham groups. Likewise, non-labeled MSCs could not be observed in the livers tissues of PH rats (Figure 4.24). By this way, we proved that CPN labeling did not change the migration capability of labeled MSCs which is one of the most important properties of MSCs to be used in clinics. On the hand, we proposed a potential *in vivo* tracking method for MSCs in liver regeneration model.

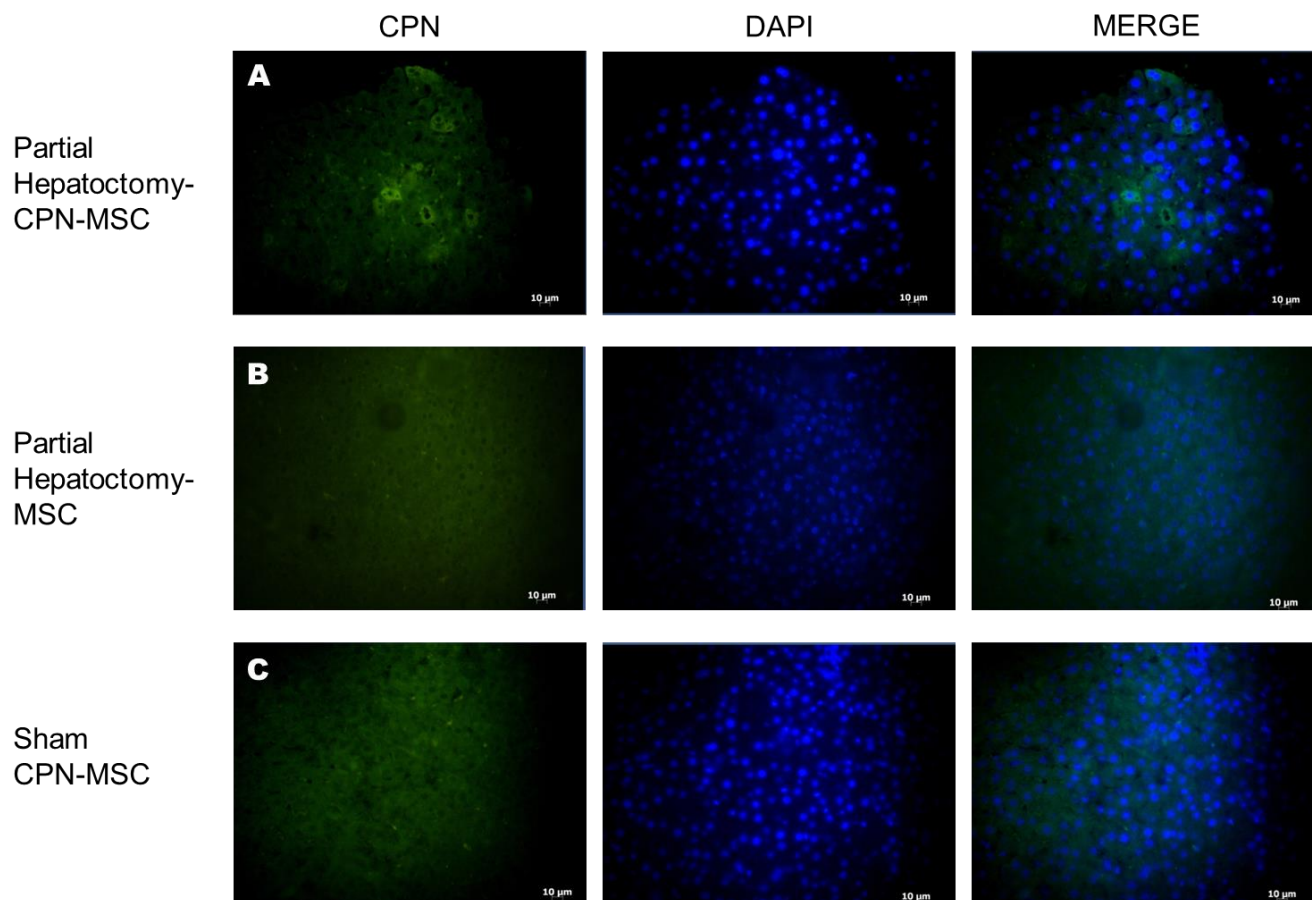


Figure 4.24. *In vivo* tracking of CPN labeled MSCs in liver regeneration model.

CPN labeling was performed at the last day of MSCs culturing for 24h hours. At the 14th day of culturing labeled and non-labeled MSCs were collected and administered to animal groups. A) CPN-MSCs were given from tail vein of the rats which had partial hepatectomy (PH) operation. B) Non-labeled MSCs were given from tail vein of the rats which had PH operation. C) 24h CPN incubated MSCs were given to the rats which had sham (SH) operation. After cells were administered, the rats were let for 3 days. At the end of 3 days, rat livers were extracted and embedded in paraffin blocks. The livers were sectioned, de-paraffinized and visualized under fluorescence microscopy after the counterstained and mounted with DAPI. CPN labeled MSCs were observed at 540 nm. The photos were taken with same exposure.

4.9.1.2 CPN labeled MSC Tracking in Liver Fibrosis Model

In addition to liver regeneration model, we tested tracking of CPN-MSC in a different liver injury model which was liver fibrosis. Liver fibrosis was induced in mice via intraperitoneal administration of CCl₄ twice a week for 4 weeks. Since CCl₄ was prepared in mineral oil, control groups were given mineral oil in the same way. Bone marrow isolated MSCs were incubated with CPN at the 13th day of culturing. After 24h CPN incubation (at the 14th day of MSC culturing), the labeled cells were collected and administered from tail vein to fibrotic and non-fibrotic mice. After mice were sacrificed, their livers were extracted, fixed and observed under fluorescence microscope. Primarily, fibrosis in CCl₄ introduced mice was detected by H&E staining and α -SMA expression analysis. According to the results of these assays, CCl₄ introduced mice had fibrotic properties showed with increased expression of α -SMA and liver damage detected with H&E staining (Figure 4.25 & 26). On the other hand, mineral oil given mice had intact liver tissue and were negative for α -SMA. Foremost, our results showed that the CPN labeled MSCs migrate and reside in the liver of fibrotic mice which were absent in non-fibrotic liver (Figure 4.27). These results further supported that CPN labeling provides an approach to follow MSCs migration to the damage area without changing their migration ability.

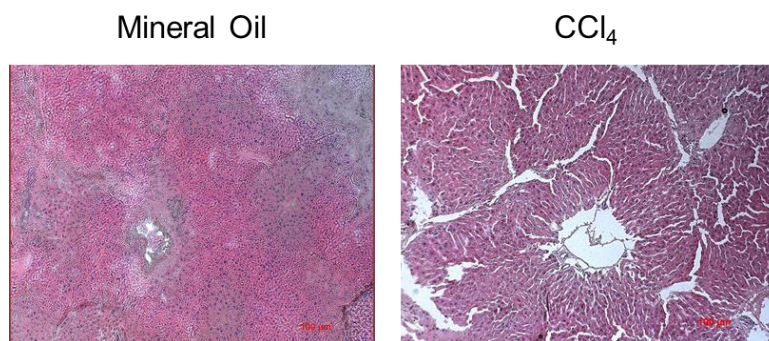


Figure 4.25. H&E staining of the liver sections.

H&E staining was done in the liver sections of mice that were induced liver fibrosis through administrating CCl₄. The control groups were administered mineral oil (non-fibrotic). Photos were taken with 10X magnification of bright field microscope.

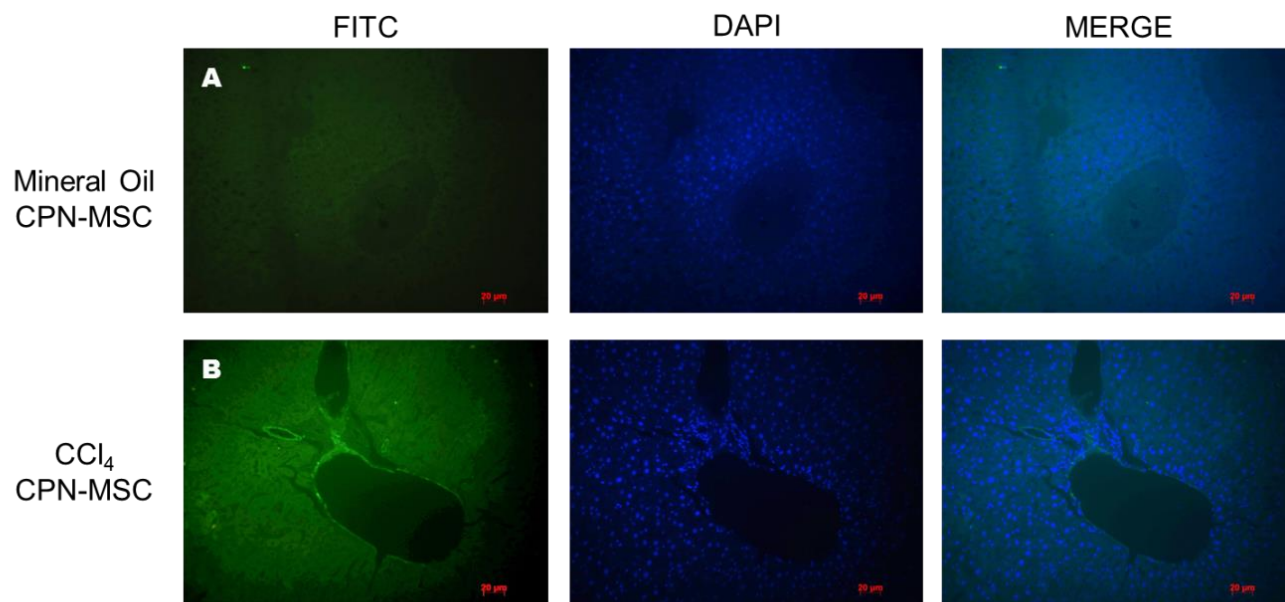


Figure 4.26. Analysis of the expression of α -SMA in liver tissues.

A) Mice were given mineral oil for 4 weeks by i.p injection (non-fibrotic) B) Mice were given CCl_4 for 4 weeks by i.p injection (fibrotic). MSCs were labeled with CPN for 24h on the last day of their culturing. At the 14th day of culturing, MSCs were collected and administered to fibrotic and non-fibrotic mice from tail vein. After 3 days, mice's livers were extracted and half of the livers were frozen in liquid nitrogen. The severity of liver fibrosis was assessed with immunofluorescence staining of liver fibrosis marker; α -SMA.

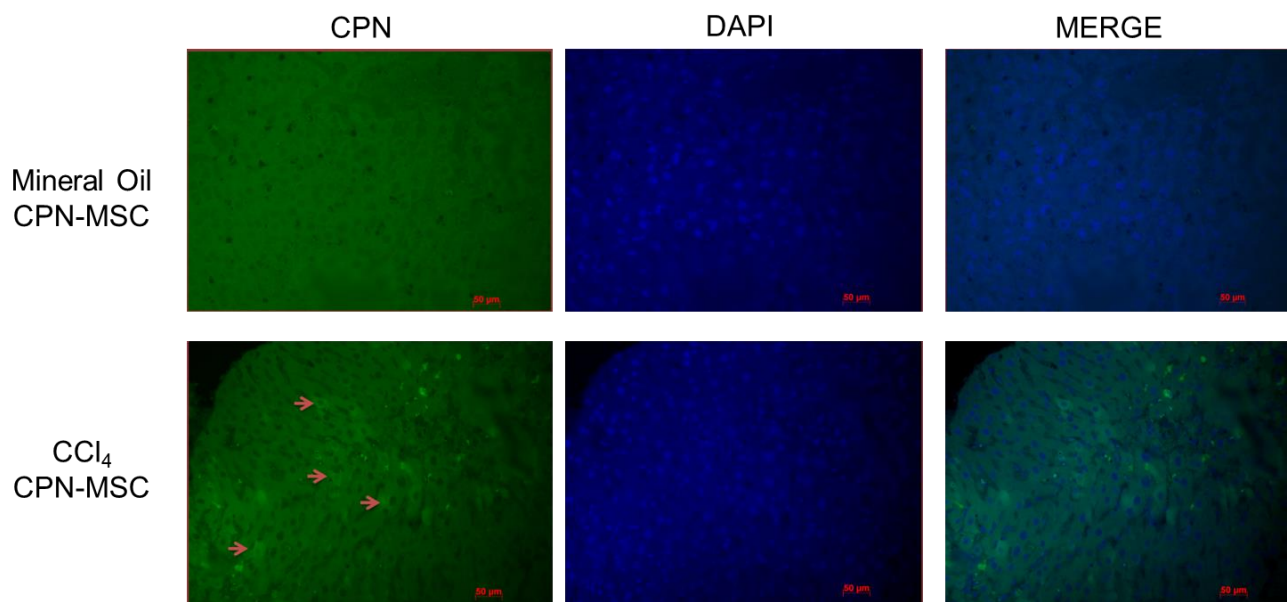


Figure 4.27. *In vivo* tracking of CPN labeled MSCs in the liver fibrosis model.

MSCs were labeled with CPN for 24h on the last day of their culturing. At the 14th day of culturing, MSCs were collected and administered to either non-fibrotic (mineral oil) or liver fibrotic (induced by CCl₄) mice from tail vein. After 3 days of cell administration, the livers were extracted and half of the livers were embedded in paraffin blocks. Then, the livers were sectioned, de-paraffinized and visualized under fluorescence microscopy after counterstain and mounted with DAPI. CPN labeled MSCs were observed at 540nm. Photos were taken with 20X magnification with same exposure.

4.9.2 CPN labeled Huh7 Tracking *in vivo*

We further investigated *in vivo* tracking of CPN labeled different cell types. In order to test CPN efficiency in tracking of cancer cells, we used CPN-labeled hepatocellular carcinoma cells; Huh7. Tumor xenograft model was generated with subcutaneous injection of CPN labeled Huh7 (24h or 72h) to nude mice. Non-labeled Huh7 cells were used as negative control and administered to nude mice subcutaneously. Thereafter tumor occurrence (after 2 months), the tumor tissues were extracted and analyzed with fluorescence microscopy. Different incubation periods of CPN, which were 24h and 72h, were compared. CPN labeling was shown to retain in tumor cells during the period of tumor occurrence. Permanent and significant *in vivo* fluorescence intensity of CPN supported the photostability of CPN labeling. Comparing different time periods, 72h CPN incubated-Huh7 cells had the highest fluorescence intensity in tumor tissues. On the other hand, 24h CPN incubated Huh7 cells had also adequate levels of fluorescence intensity (Figure 4.28). Together these results showed that CPN did not only specific for MSCs but can also be used for other cell types such as cancer cells. Another promising result was stable fluorescence intensity of CPN which could be observable even after months. Collectively, these advantages of CPN make them a good candidate to be used in cellular tracking in the analysis of metastasis and evaluation of the-anti cancer therapies.

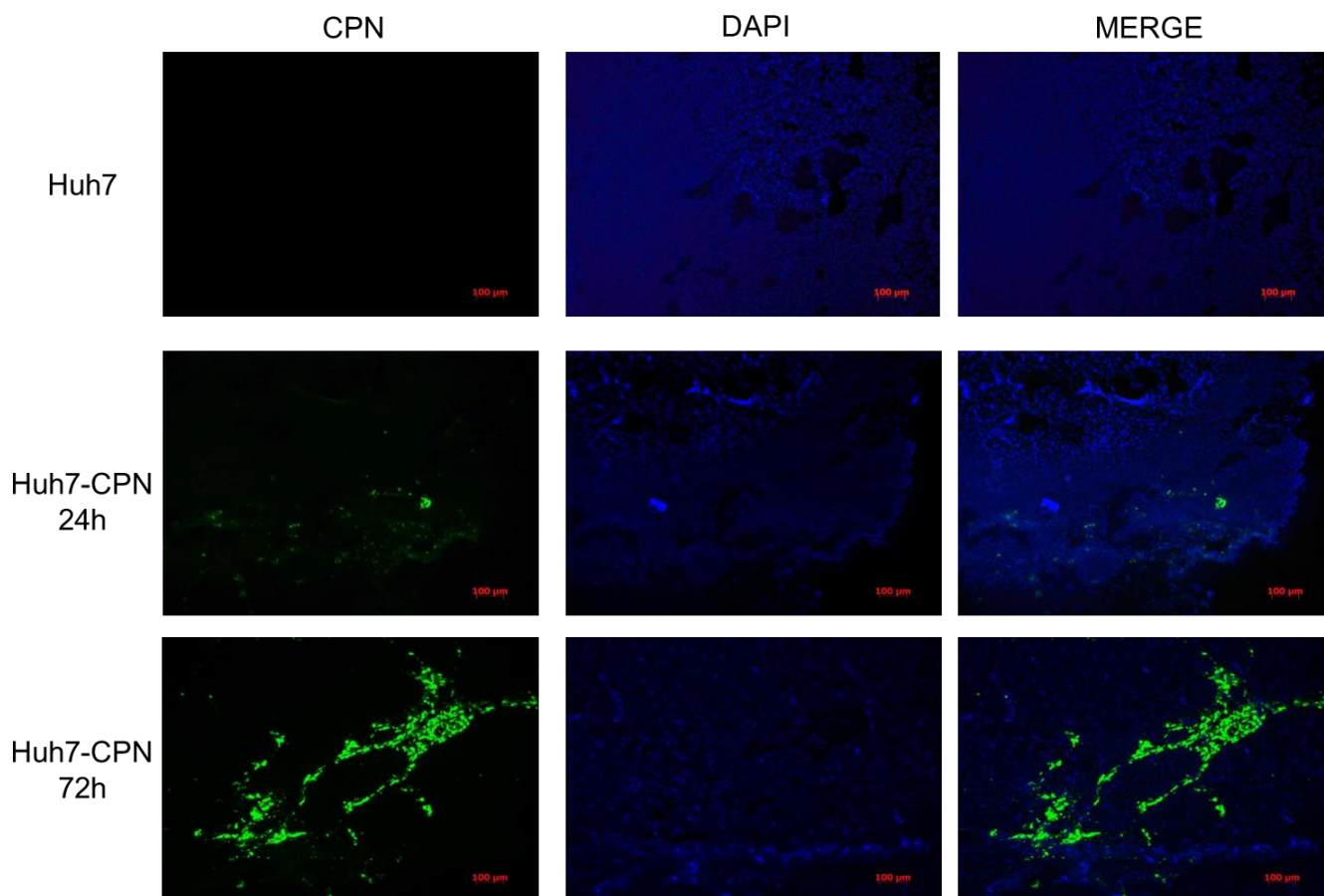


Figure 4.28. *In vivo* tracking of CPN labeled Huh7 in xenograft model.

Non-labeled (negative control), 24h CPN incubated and 72h CPN incubated Huh7 cells were injected subcutaneously to the nude mice. After tumor occurred, the tissues were taken, sectioned. CPN labeling had been visualized under fluorescence microscope at 540nm after samples were counterstained and mounted with DAPI. The photos were taken with same exposure.

Chapter 5

5 Discussion

Targeted cells are being used either as therapeutic agents or delivery cargos in the fields of immunology, oncology, transplantation and stem cell therapies. Indeed, these cellular therapeutic applications require cellular tracking in order to assess the treatment success. The methods of cellular tracking assist scientists to follow the migration and homing of administered cells [1]. One of the most commonly used cellular therapies is stem cell transplantation. It is essential to monitor stem cell migration to the site of injury in order to assess whether the cellular therapy served its purpose. For instance, it is important to follow migration of the integrated neural stem cells to the damaged brain as this allows the evaluation of the progress of regeneration [7]. In addition to beneficial effects obtained in regeneration studies, cellular tracking also has a vital role in tracking of cancer cells to analyze cancer occurrence as well as detection of metastasis. In addition to providing an opportunity to evaluate the success of the cellular therapies, cellular tracking is also important for the improvement of cellular understandings and cancer therapies. The progressive technologies of cellular imaging assist improvements in strategies of cellular therapeutics.

Radionuclides (^{111}In) that are widely used in the detection of site of inflammation were analyzed with the help of gamma camera imaging [9]. The lack of sensitive recognition of a single cell forced scientist to search for new methods of cellular tracking. Positron emitting radiolabels had been started to be used to fulfill the needs of cellular tracking. Regrettably, scientists decided that positron emitting radiolabels were also far away from being ideal imaging tools. It had certain limitations which included having a short half-life, varying efficiencies of labeling and quick outflow from the cells [10]. In response to these failures, MRI offered a solution for cellular tracking *in vivo*. Even today, the MRI still outcompetes with new strategies of cell tracking and continues to be used in

many assays of cellular tracking. Due to lack of missing ionizing radiation, MRI provides a high resolution image acquisition. However, this method requires an extra labeling agent administration to generate a contrast and improve the image quality. Label uptake efficiencies are limiting step of MRI due to variations occurring in different cell types. For example, slow cycling and non-phagocytic cells were hardly uptaking the labeling agents of MRI. In order to overcome this problem, the labeling agents were applied in higher concentrations or were coated with proteins [11, 12, 14]. However, these solutions are technically difficult and lack specificity and biosafety [7]. Additionally, MRI may give false positive results due to consisted artifacts. Moreover, transfection of cells with GFP provides a stable and heritable labeling which enable scientist to monitor cells long terms *in vivo* [229]. The whole body fluorescence imaging with blue light illumination allows to the visualization of GFP labeled cells in intact animal [230]. However, this method may result in the genetic abnormalities in transfected cells due to requirement of stable transfection. Unfortunately, all of these techniques that have been used in cellular tracking still haven't fulfilled the requirements of an imaging tool which include specificity, high quality and non-toxicity.

Demand for a more advanced tracking tool combined with developed biomaterial science provoked development of nanoscale fluorescence probes; quantum dots (QDs), which could be used in cellular imaging. QDs had adjustable bright fluorescence emission in different wavelengths [28]. Inorganic structure of QDs protect them from chemical and metabolic degradation and hence the fluorescence intensity of probes are protected for a long time without any risk of fade [29, 30] unlike the fluorophore proteins [31]. Toxicity of recently used QDs *in vitro* and *in vivo* imaging was controversial [30, 32]. The results of studies involving QDs showed that their applied concentration and duration of application are critical factors for their clinical usage. The utilization of QDs in the *in vitro* and *in vivo* was shown [29, 35]. On the other hand, QDs have their own limitations; one of which is having a difficulty in translocation into the cells due to their large size. Therefore, permeabilization is necessary [29]. In addition, the bulky structure of QDs may prevent the efficiency of labeling and sensitivity in studies that involve high number of cells. QDs work efficiently in small numbers of cells or in cases where cells are

distributed dispersedly. Bulky structure of QDs also causes formation of aggregates during long term storage [36].

In an ideal imaging method, first rule is to use a material which is biocompatible and non-cytotoxic. It is critical that a tracking method should not cause any genetic defect as this may be a problem in the studies that require transgene expression for cellular labeling [26]. In addition, a labeling agent should sensitively detect the single cells and should not fade or be effluxed from the cells. In an attempt to improve already found labeling applications, conjugated polymer nanoparticles (CPNs) had been developed. These nano-materials are proper to be used in bio-imaging and nanomedicine as imaging agents or biosensors. The advantages of CPNs over other methods include their adjustable sizes and properties, biocompatibility and non-cytotoxicity. Consequently, CPNs hold many properties of an ideal cellular imaging tool. Compared to QDs, CPNs have adjustable properties and sizes which are possible as their conjugated polymers and surface molecules can be changed. In addition, CPNs have even brighter emission compared to QDs and can be more easily synthesized and uptaken by cells [38–40]. In addition to these advanced properties, CPNs are photostable and resistant to bleaching. In conclusion, all these advantages of CPNs which have been missing in other methods make CPNs more suitable to be used in cellular tracking studies.

The utilization of CPNs in the drug delivery and cellular imaging was shown previously in cancer studies [226]. Here, we provide an evidence of potential utilization of CPNs not only *in vitro* but also *in vivo* cellular tracking of stem cells and cancer cells. Due to their potential utilization in clinics, initially, MSCs were used to analyze CPN labeling in *in vitro* and *in vivo* cellular tracking. MSCs are essential in clinical therapies of bone and cartilage defects, cardiac diseases and nervous system damages [133–135]. Easy isolation, high proliferation capacity and ability to differentiate into many cell types make MSCs potential targets for cellular therapies. In addition to these, MSCs have ability to migrate to the site of injury and help tissue regeneration through modulating the wound healing process. More importantly, studies showed that MSCs have immune suppressive ability [125, 231–234]. Therefore, MSCs prevent occurrence of graft versus host diseases since they can escape from MHC barriers. Hence, no rejections occurred when MSCs

were transplanted among different humans [141, 148–150]. Administered MSCs had recuperative effects which were shown in the studies of myocardium regeneration, osteogenesis imperfecta, trauma, liver fibrosis, heart failures and neural defects [104, 133–135, 151, 152, 155–161]. Collectively, due to these advantageous properties of MSCs, they become a good candidate for cellular therapeutics. The follow up studies of administered MSCs are important in order to be certain about MSCs migration and homing in the target organ. Therefore, tracking of administered MSCs is critical to evaluate the success of applied therapies. In order to propose a method for *in vivo* cellular tracking, we monitored migration of CPN labeled MSCs *in vivo* in animal models. But first, we showed the potential utilization of CPN labeling in *in vitro* assays. In the beginning, we had started with 4 different polymer conjugated nanoparticles (VEP9, VP8, VP6 and VP6a-Merc), two of which emitted green (VP6 and VP6a-Merc) or blue (VEP9 and VP8) fluorescence. The bone marrow heterogeneous cell population was cultured 14 days to obtain MSCs. The nanoparticles were introduced at the 13th day of MSCs culturing. After 24h nanoparticle labeling, MTT assay was performed to assess if these nanoparticles showed any proliferation inhibition in MSCs. According to MTT results, all MSCs which were labeled with these nanoparticles had comparable levels of cellular activity. When compare to others, VP-6 nanoparticle labeled MSCs showed 70% of cellular activity which was the highest among 4 nanoparticles. MSCs are sensitive cells, therefore, 70% cellular activity can be accepted in normal range for these cells [235]. Following the MTT assay, *in vitro* cellular labeling intensities of nanoparticles was assessed under fluorescence microscopy. Compared to others, VP8 and VP6 were uptaken more frequently and had the highest labeling intensities in MSCs. However, VP8 was indistinguishable from the DAPI staining of the nucleus due its emission in blue. Therefore, VP6 was more preferable as it had green fluorescence emission which provided an opportunity for double staining with DAPI. While comparing the nanoparticles, the effects of nanoparticles on the MSCs differentiation were also critical to be analyzed. The 24h nanoparticle labeled MSCs (at the 13th day) were induced to differentiate into either adipocytes or osteocytes. After differentiation period, Oil Red O staining was done to assess adipogenic differentiation of MSCs. According to Oil Red O staining, the nanoparticles, except VP6, had negative effects in MSC adipogenic

differentiation. On the other hand, VP6 incubated MSCs were stained intensely with Oil Red O which indicated that VP6 labeling did not affect adipogenic differentiation of MSCs. Additionally, Alizerin Red staining was done to assess osteogenic differentiation of labeled MSCs. According to the Alizerin Red staining results, all nanoparticles, except for VP6a-Merc, had no effect in potency of osteogenic differentiation of MSCs. However, only the VP6 labeled MSCs showed complete physiology of osteogenic cells. Collectively, based on the *in vitro* analysis of 4 different nanoparticles, VP6 was the most suitable probe for MSC tracking as it had the least decrease in cellular activity and differentiation potential of MSCs. The differences between these 4 nanoparticles were occurred due to their different functional groups. Only the VP6 was neutral and non-reactive. On the other hand, other nanoparticles had reactive and toxic functional groups such as azide group which may be the reason of showing a more severe effect on the cellular activity and characteristics. The VP6a Merc was functionalized from VP6; however, it had acidic property which may make the VP6a Merc more reactive for MSCs. Therefore, our further studies concentrated on the VP6 labeled MSCs (called CPN in thesis).

After we decided to continue with VP6, the optimal incubation period was determined. Bone marrow isolated MSCs were incubated with CPN from 1st, 4th, 7th, 10th and 13th day of culturing. At the 14th day of culturing, MSCs were obtained and cytotoxic effects of different CPN incubation times were assessed by MTT assay. There were no significant changes occurred in the cellular activities of MSCs that were labeled with CPN in different time periods. 13 day CPN incubated MSCs had similar levels of cellular activity compared to 1day CPN incubated MSCs. These results indicated that increased exposure of CPN did not inhibit cell growth of MSCs. Therefore, CPNs became more advantageous to be used compared to QDs whose longer incubation time had toxic effects on the cells [35]. The frequency of CPN labeling in MSCs with time of incubation were assessed with FACS analysis. According to the results, 24h CPN incubation found to be optimal among other time points since lower viability was observed in other time points even though labeling frequencies were similar. Then we analyze the labeling intensities of MSCs that were labeled with CPN in different time periods with fluorescence microscopy. The incubation period above 12h was shown to be enough to obtain intense

and frequent labeling *in vitro*. Based on these results, we set incubation time to 24h as we considered it to be short for an incubation time but enough to label cells intensely. Continuous experiments were performed with 24h CPN incubated cells.

Afterwards, *in vitro* labeling assay of MSCs was repeated with the set conditions of incubation. Repeatedly, bone marrow isolated MSCs were labeled with CPN for 24h at their 13th day of culturing. The cellular activity of CPN labeled and non-labeled MSCs were analyzed with MTT assay at the 14th day of culturing when MSCs were obtained. The MTT results showed that the cellular activity is 70% in 24h CPN incubated MSCs. After being certain about non adverse effects of 24h CPN incubation on MSCs' cellular activity, we analyzed the CPN labeling effects on MSCs characteristics. Initially, we analyze the cell surface markers of MSCs that were incubated with CPN for 24h at the last day of culturing. RT-PCR results indicated that CPN labeled MSCs had the same cell surface marker expression with non-labeled MSCs. In addition, the ability of multilineage differentiation is another well-known characteristic of MSCs which should be analyzed after CPN incubation. At the 13th day of culturing, MSCs were incubated with CPN for 24h. At the 14th day of culturing, MSCs were obtained and induced to differentiate into adipocyte, osteocytes and chondrocytes which were detected by Oil Red O, Alizerin Red staining and Alcian Blue, respectively. Differentiation assays indicated that, 24h CPN labeling did not change the differentiation potential of MSCs. Together these results indicated that CPN labeling did not change the known characteristics and differentiation potential of MSCs. Moreover, we showed 70% of MSCs was labeled with CPN and had strong green fluorescence light emission. The cell specific CPN labeling was assured with the co-localization of CPN labeling with nucleus DAPI staining. The fluorescence emitting particles without DAPI staining indicated the non-specific association of CPNs. Subsequently, we analyze CPN effects and labeling in differentiated cells. For this purpose, 24h CPN incubated MSCs (on the last day of culturing) were differentiated into adipocytes, chondrocytes and osteocytes without any further CPN incubation. After differentiation period (3 weeks), the differentiated cells were analyzed with both bright field microscope and fluorescence microscope to identify co-localization of the lineage specific differentiation staining with CPN labeling. Promisingly, microscopy results indicated that CPN labeling had been retained in differentiated cells without any loss in

their fluorescence intensity. In addition, together with these results, we also proved long term photostability of CPNs.

After verifying the efficiency of *in vitro* CPN labeling in MSCs, we developed liver damage models to follow labeled MSCs migration *in vivo*. Initially, CPN labeled MSCs migration were investigated in the liver regeneration model generated by partial hepatectomy (PH). 24h CPN incubated MSCs were injected from the tail vein to the rats which had either PH or sham (SH) operation, and then cells were allowed to migrate to the site of injury for 3 days. Promisingly, CPN labeled MSCs were observable in tissue sections of damaged liver which were detected under fluorescence microscopy. The CPN labeled MSCs were co-localized with DAPI nucleus staining which assured the cell specific CPN labeling. The labeled MSCs were missing in the intact liver. Since there was no damage in the sham groups' liver, CPN labeled MSCs did not migrate to these livers. The CPN labeled MSCs specificity was also tested with the PH group of rats which were introduced non-labeled MSCs. The lack of CPN labeled cells in the PH rats which were given non-labeled MSCs were supported the presence of CPN labeled MSCs in the experimental liver tissues. These results were further supported by another liver damage model; liver fibrosis. Prior to analysis of labeled MSCs in damaged liver, the occurrence of liver fibrosis was demonstrated through H&E staining and α -SMA immunofluorescence staining. The CCl₄ administered mice was shown to lose its intact liver architecture and increase α -SMA expression. On the other hand, mineral oil administered control group protected its intact liver architecture and slightly express α -SMA. CPN labeled MSCs were observable in the liver sections of fibrotic animals which were missing in the non-fibrotic livers. Again, the CPN co-localization with DAPI nucleus staining demonstrated the cell specific staining of CPNs. Since there was no damage in the control liver tissue, CPN labeled MSCs did not migrate to these non-fibrotic liver. Collectively, animal model of liver fibrosis further supported the results of previous liver damaged model and supported the possibility of tracking labeled MSCs *in vivo*. Importantly, these results also demonstrated that CPN incubation did not change migration ability of MSCs to the site of injury. In conclusion, all these studies proved the potential usage of CPN as a cellular tracking agent for MSCs tracking *in vivo*. Therefore, we hope that we have made a contribution to the understanding of MSCs biology,

migration and homing mechanism. Additionally, we offered a potential approach to assess treatment efficiencies of stem cell therapies.

Isolation of bone marrow MSCs required invasive ways which may be painful for the studies with human subjects. Therefore, utilization of easily accessible dental pulp stem cells which are another sources of MSC type of cells, may be preferable compared to bone marrow MSCs. Due to their easy isolation from patients' wisdom teeth, which may otherwise end up being biological waste, DPSCs studies evade from the ethical concerns. DPSCs are easily accessed and collected from removed wisdom teeth without any additional surgery other than dental operations. Isolated DPSCs could be cryopreserved for a long time without losing their clonality [205]. Although DPSCs show similar properties with MSCs such as self-renewal, cells surface marker expression and multilineage differentiation capacity; they have advantages over MSCs as they have higher activities of proliferation, immune suppression and percentages of yield of stem cells [204]. Due to their superiorities over bone marrow MSCs, DPSCs may replace clinical utilization of MSCs in the future. DPSCs have been shown to have a therapeutic potential in the neural damage, liver fibrosis and myocardial infection [136, 207, 209, 210]. In this study, we aimed to obtain DPSCs from patients' wisdom teeth in order to analyze CPNs effects on a different source of MSC. Initially, we made characterization of isolated DPSCs that were isolated from three different patients' wisdom teeth. MSC specific marker analysis with RT-PCR showed us that DPSCs had expressed all characteristic markers of MSCs. In addition, we showed DPSCs isolated from different patients had similar expression profiles. Same as MSCs, the isolated DPSCs attempted to differentiate into the adipocyte, osteocyte and chondrocyte, which were detected by Oil Red O, Alizerin Red and Alcian Blue, respectively. According to our results, isolated DPSCs had exactly the same potential of differentiation with MSCs. Thereafter we demonstrated the successful isolation and culture of DPSCs, we investigated the effects of CPN labeling on DPSCs. In order to utilize CPNs in DPSC tracking, it is essential to demonstrate non-adverse effects of CPNs on DPSC cellular activity. MTT assay results showed 75% of DPSCs had cellular activity after 24h CPN incubation. This result was further supported by LDH assay, which showed the cytostatic effects of CPNs on DPSCs. Due to the fact that DPSCs are sensitive cells like bone marrow MSCs, 75% survival rate

indicated that CPN labeling could be applicable to these cells [220]. Similar to MSCs, 24h CPN incubated DPSCs showed very intense and specific labeling which was detected with fluorescence microscopy. Additionally, DPSCs were incubated with CPN for 24h when they reached 70% confluency after isolated from wisdom tooth and were differentiated into chondrocytes to analyze CPN effect on their differentiation capacity. It was shown with intense Alcian Blue staining that CPNs did not affect differentiation potential of DPSCs. Promisingly, after differentiation period, CPNs resided in the differentiated cells and had very intense fluorescence emission. Through this way, we showed intense and specific labeling of MSCs *in vitro* which did not differ in patients that they were isolated from.

In vivo tracking of cancer cells are important in the assessment of tumor occurrence and success of anticancer therapies as well as evaluating metastasis. In addition, *in vivo* observation of cancer cells allows a better understanding of mechanism of drug's mode of action and, therefore, improves the existing methods of cancer treatments. Here we aimed to show *in vitro* labeling and *in vivo* tracking of hepatocellular carcinoma cell (HCC); Huh7. Initially, 70% confluent Huh7 cells were incubated with 4 different nanoparticles (VEP9, VP8, VP6 and VP6a-Merc) in order to find an optimal nanoparticle to be used in cancer cells. According to MTT results, which were performed with Huh7 cells incubated with these nanoparticles for 24h, there were no significant differences in cellular activity of Huh7 cells. However, VP6 incubated Huh7 cells showed the most similar cellular activity (nearly the 100 %) with the non-treated Huh7 cells. On the other hand, VP8 showed an increase in cellular activity of Huh7 cells, which may be considered as dangerous to be used as it also causes an increase in the tumorigenic activity of Huh7 cells. Altogether, none of these nanoparticles made a significant change in the cellular activity of Huh7 cells. Afterwards, 24h nanoparticle incubated Huh7 cells were analyzed *in vitro* with fluorescence microscopy. When compared to VP8 and VP6-Merc, VEP9 and VP6 were more frequently uptaken by Huh7 cells and had very intense fluorescence emission. However, the utilization of VP9 may create a conflict when DAPI nucleus staining is performed. Therefore, based on these results and non-toxic chemical properties we decided to continue with VP6 nanoparticle in same conditions for Huh7 cell labeling. The detailed *in vitro* analysis of CPN (VP6) labeled Huh7 cells revealed that

almost all cells had uptaken CPN and had emitted very bright green fluorescence light. The CPN labeling together with DAPI nucleus staining indicated the cell specific staining of CPNs. The fluorescence emission without DAPI co-localization showed the non-specific association of CPNs to the surface. After proving intense and significant *in vitro* labeling of Huh7 cells with VP6, we planned to monitor CPN labeled Huh7 cells *in vivo*. 24h and 72h CPN incubated Huh7 cells were administered subcutaneously to the nude mice. Thereafter tumors had been occurred; the tumor tissues were taken and tissue slides were analyzed under fluorescence microscopy to identify the presence of CPN incubated cells. Promisingly, CPN labeled Huh7 cells were visible in tumor tissue that was generated with CPN labeled cells and had very strong fluorescence emission. Although 24h CPN incubation was enough to track labeled cells *in vivo*, 72h incubation of CPN had more significant results. There was no background fluorescence emission in tumor tissue which was proven in tumor sections occurred by non-labeled Huh7 cells. Since tumor occurrence took nearly 2 months, we also demonstrated the photostability of CPN labeling which retains strongly after this long period without any bleaching. Therefore, photostability of CPN offers a way for long term tracking of cancer cells *in vivo* and *in vitro*. Additionally, these results indicated that CPN was not only specific for MSCs but also for the cancer cells. The tumor xenograft model of CPN labeled Huh7 cells also gave an idea about the CPN distribution during cellular division. If CPN was uptaken equally by the newly generated cells, then we would expect to see homogeneous staining of CPN in tumor tissues. However, the CPN labeled cells dispersed heterogeneously in the restricted areas. These results may indicate that, the CPN labeling retained in the cells that they were initially integrated.

In the close future, CPNs are expected to replace the already existing strategies of cellular tracking. Our results provided *in vitro* and *in vivo* experimental evidence for potential usage of CPNs in cellular tracking. Here, we proved non-adverse effect of CPN on cellular activities and their photostability. In addition to these advantages, being easily applicable without any need for complex methods make CPNs a candidate tool to be used in cellular tracking studies. By this way, we hope to increase the understanding of stem cell biology through identifying their migration and homing pattern. Finally, we proved the potential utilization of CPN in long follow up cancer studies. By this way, we propose

a new approach for monitoring tumor occurrence and metastasis as well as analyzing the success of cancer therapies. Therefore, we aim to enhance the understanding of cell biology and treatments through revealing the unattainable information about stem cells and cancer cells.

Chapter 6

6 Future Perspectives

In vitro and *in vivo* cell tracking provides a great understanding for cell biology in terms of cellular survival, migration, immunogenicity and tumorigenic activity in animals. Among all cell imaging methods, fluorescence imaging is one of the most preferable method due to its inexpensiveness, non-toxicity, easy and quick labeling. Risk of photobleaching limits the usage of organic fluorophores for long time studies and, therefore, highly photostable conjugated polymer nanoparticles take advantage over the existing tools. Here we offered highly efficient, non-cytotoxic and photostable *in vitro* and *in vivo* imaging method for tracking of MSCs and Huh7 cells. In addition to MSCs and Huh7 cells, CPN utilization in cellular tracking should be investigated on other types of adult stem cells and cancer cells. By this way, differential activity and specificity of CPN labeling in different cell types could be assessed. Here we used a single concentration of CPN; the different concentrations should be investigated in *in vitro* and *in vivo* assays. By this way, more significant CPN labeling could be found. In addition, we concentrated on the VP6 labeling of MSCs and Huh7 cells. The other three nanoparticles (VEP9, VP8 and VP6a-Merc) did not used in *in vivo* assays of the cells. The optimized concentration for the utilization of any of these three nanoparticles may give promising results and, therefore, should be investigated. Together these new experiments may reveal a more specific cell labeling agent without any inhibition of cellular growth or changes in cellular characteristics.

There was a big handicap in the representation of the co-localization of CPN labeling with MSCs due to the conflict in CPN labeling and the antibody used as a MSC marker. In addition, labeled MSCs were visible in paraffin embedded tissues. However, MSC immunofluorescence should be performed in frozen tissues where labeled MSCs are hard to be observed. Therefore, an optimal method to show co-localization of MSC marker with CPN labeling should be investigated in the future.

Here we showed quick and efficient uptake of CPN by MSCs, DPSCs and Huh7 cells. However, the mechanism of cellular translocation of CPN is unknown. In order to predict the behavior of CPN in different types of cells such as non-phagocytic or slowly dividing cells, the mechanism of cellular uptake should be revealed. In addition, subcellular localization of CPNs in labeled cells should be analyzed in details. Moreover, detailed exploration about the behavior of CPNs in dividing cell should be analyzed in the future studies to reveal if CPNs are distributed into daughter cells or they retain in the cell that they were initially uptaken. In order to answer this question, CPN distribution during cellular division should be followed up during mitosis with time laps fluorescence microscopy. This will reveal CPN distribution in dividing cells. The non-homogenous CPN labeled cells in tumor tissues may indicate that CPN labeling remain in the cells that they were initially uptaken and were not equally distributed to newly generated cells. Therefore, this kind staining may suggest that CPN labeled cells could be cancer stem cells. Keeping the CPN staining for an extended time period (2 months) also straighten the idea of cancer stem cells. The cancer stem cell markers should also be monitored and their potential co-localization with CPN labeling may indicate the specific labeling of cancer stem cells with CPNs.

Huh7 cells were administrated subcutaneously to the nude mice. The CPN labeled cells were analyzed only in tumor tissues which were established from CPN labeled Huh7 cells. Since we used liver hepatocellular carcinoma cells, it was likely that labeled cell invade to liver tissue and, therefore, should be analyzed to show utilization of CPN labeling in detection of cancer cell metastasis.

Cellular tracking of CPN labeled stem cells and cancer cells should be analyzed in intact animal by using a real time whole body imaging methods [24]. By this way, migration of cells and steps of cancer cell metastasis could be monitored in real time.

CPN labeling could also assist the improvements in cancer treatments and their utilization as an anticancer drug carrier was shown [226]. CPNs which were conjugated with anti-cancer drugs could potentially be used for chemotherapeutic drug screening. The opportunity of cancer cell imaging together with drug loading capability provides an all in one system which will offer a better therapeutic approach in the future. The detection of

the presence of drug loaded CPNs in tumor tissues will provide a potential approach to evaluate the success of anti-cancer treatments. Therefore, anti-cancer treatments will be improved with expanding utilization of CPN in cellular tracking which ameliorates the understating of cancer cell biology as well as behaviors and therapeutic mechanism of drugs. In addition to providing an opportunity to monitor drug bio-distribution; the increased drug efflux, target specificity and immune suppression could be achieved by using CPN due to their adjustable chemical properties.

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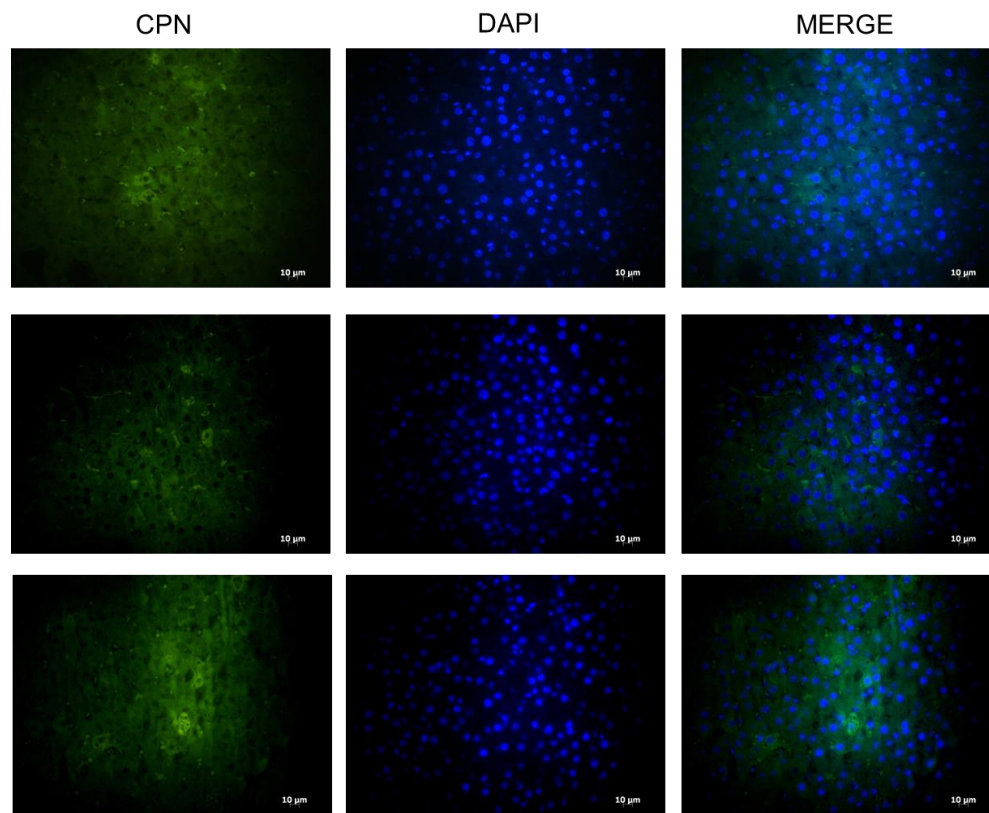
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Appendix

Supplementary Figures

In vivo MSCs tracking supplementary figures

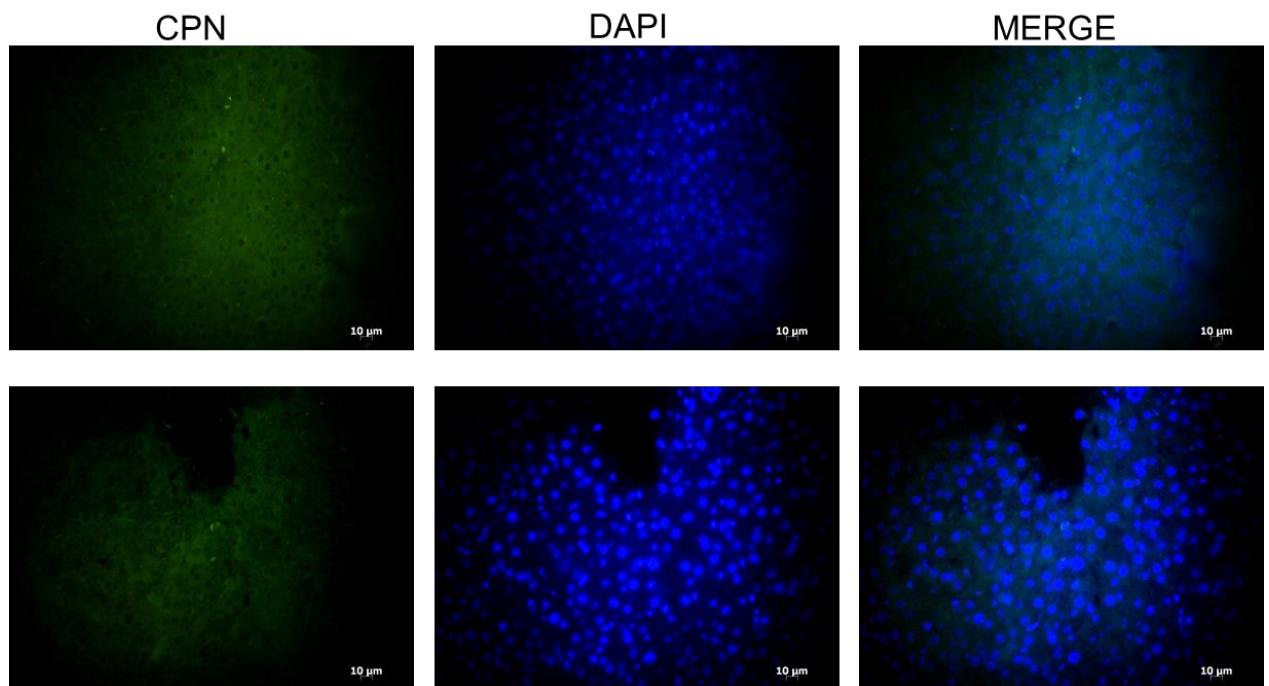
PH-CPN-MSCs



Supplementary Figure 1: *In vivo* CPN labeled MSC tracking in liver regeneration

CPN labeling was performed at the last day of MSCs culturing for 24h hours. On the 14th day of culturing labeled and non-labeled MSCs were collected and administered to animal groups. CPN-MSCs were given from tail vain of the rats which had partial hepatectomy (PH) operation. After cells were administered, the rats were let for 3 days. At the end of 3 days, rat livers were extracted and embedded in paraffin blocks. The livers were sectioned, de-paraffinized and visualized under fluorescence microscope after samples were counterstained and mounted with DAPI. CPN labeled MSCs were observed at 540 nm.

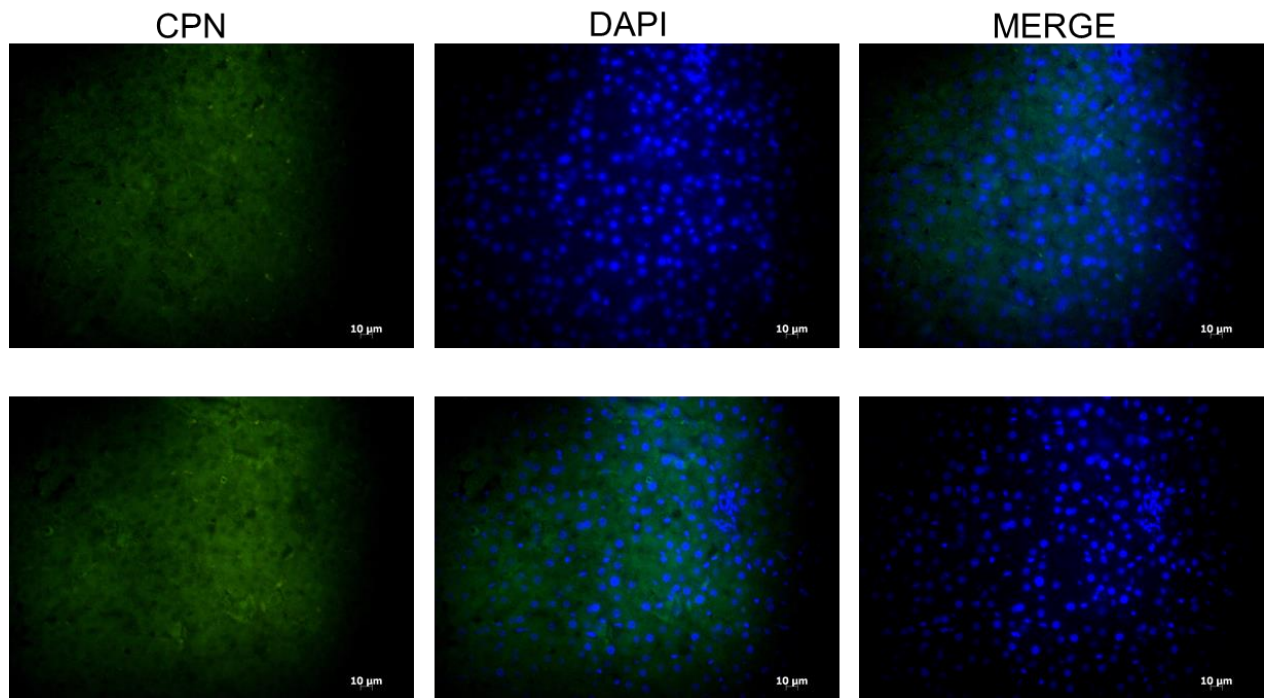
PH-MS



Supplementary Figure 2: *In vivo* MSC tracking in Liver Regeneration

Non-labeled MSCs were given from tail vein of the rats which had PH operation. After cells were administered, the rats were let for 3 days. At the end of 3 days, rat livers were extracted and embedded in paraffin blocks. The livers were sectioned, de-paraffinized and visualized under fluorescence microscope after samples were counterstained and mounted with DAPI.

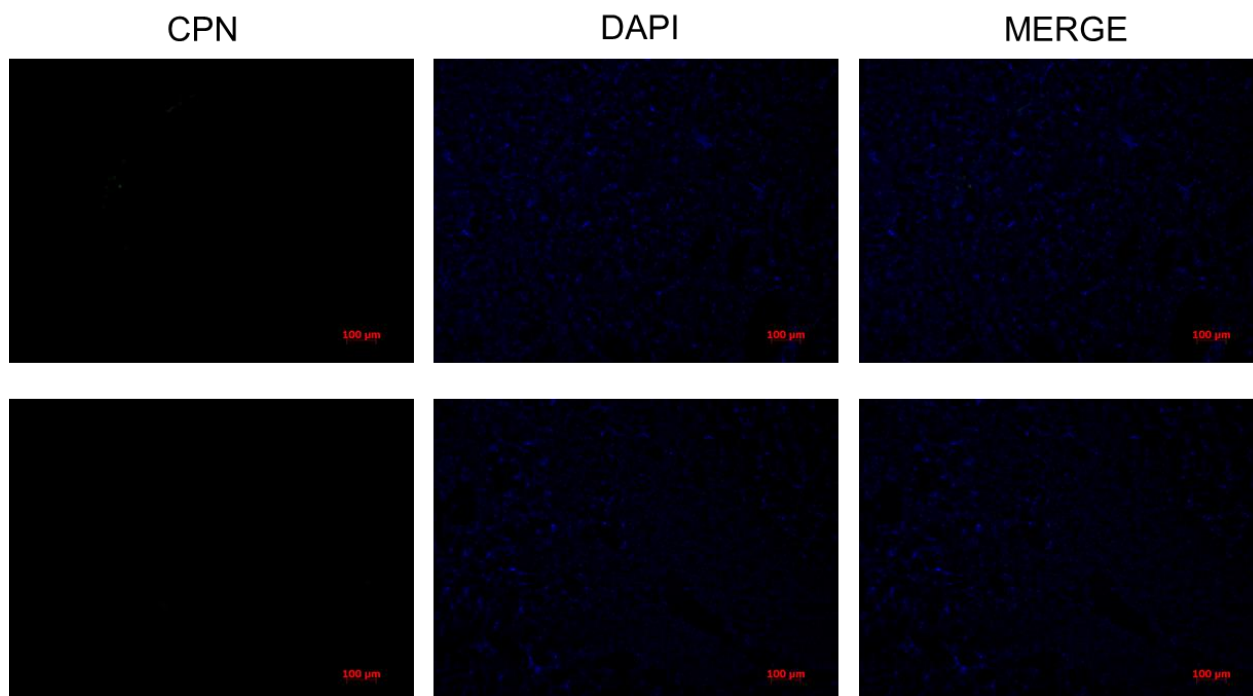
SH-CPN-MSC



Supplementary Figure 3: *In vivo* CPN labeled MSC tracking in Sham Group

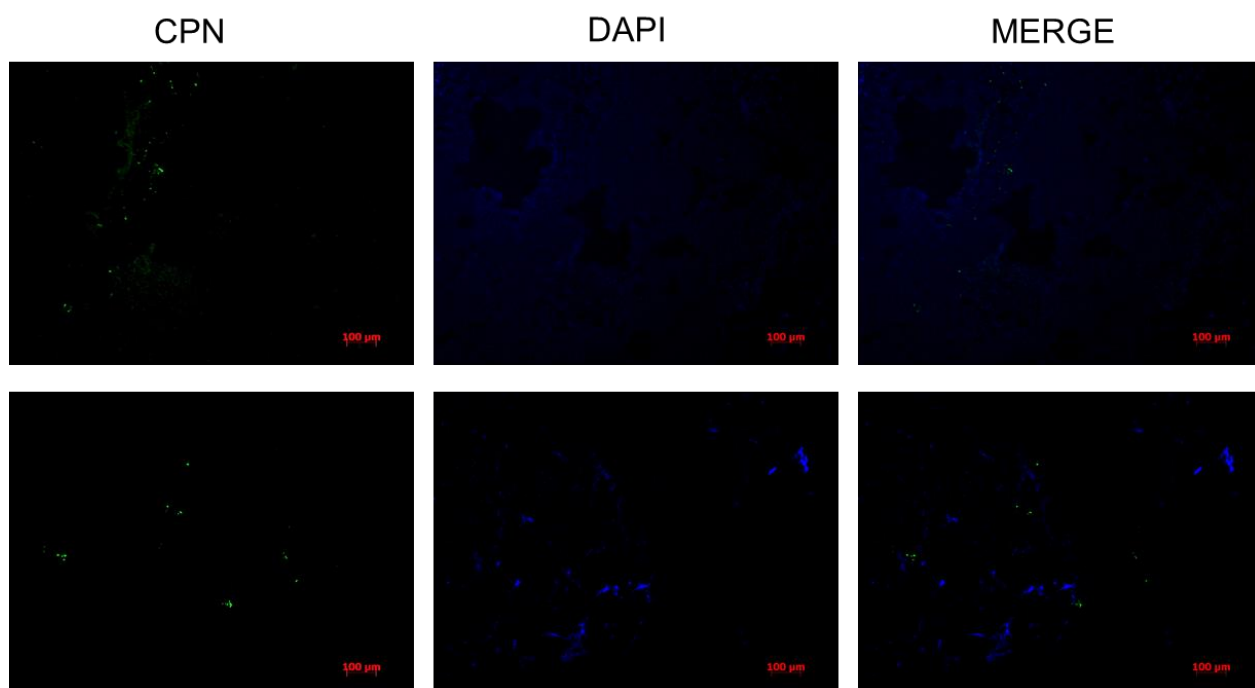
CPN labeling was performed at the last day of MSCs culturing for 24h hours. On the 14th day of culturing labeled and non-labeled MSCs were collected and administered to animal groups. CPN-MSCs were given from tail vein of the rats which had sham (SH) operation. After cells were administered, the rats were let for 3 days. At the end of 3 days, rat livers were extracted and embedded in paraffin blocks. The livers were sectioned, de-paraffinized and visualized under fluorescence microscope after samples were counterstained and mounted with DAPI. CPN labeled MSCs were observed at 540 nm

***In vivo* Huh7 cells tracking supplementary figures**



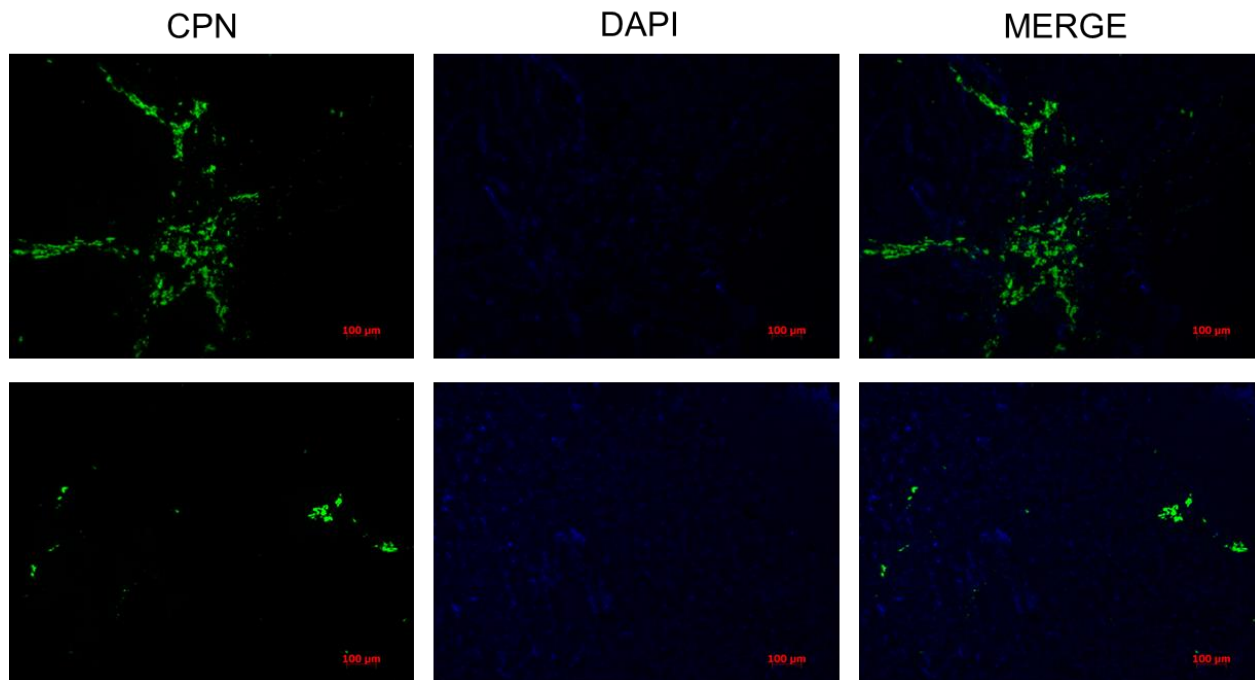
Supplementary Figure 4: *In vivo* tracking of Huh7 in xenograft model.

Non-labeled (negative control) Huh7 cells were injected subcutaneously to the nude mice. After tumor occurred, the tissues were taken and sectioned. Background staining was analyzed under fluorescence microscope at 540nm after samples were counterstained and mounted with DAPI.



Supplementary Figure 5: *In vivo* tracking of 24h CPN labeled Huh7 in xenograft model.

24h CPN incubated Huh7 cells were injected subcutaneously to the nude mice. After tumor occurred, the tissues were taken and sectioned. CPN labeling was analyzed under fluorescence microscope at 540nm after samples were counterstained and mounted with DAPI.



Supplementary Figure 6: *In vivo* tracking of 72h CPN labeled Huh7 in xenograft model.

72h CPN incubated Huh7 cells were injected subcutaneously to the nude mice. After tumor occurred, the tissues were taken and sectioned. CPN labeling was analyzed under fluorescence microscope at 540nm after samples were counterstained and mounted with DAPI.