DUAL ROLE OF HUMAN TOOTH GERM DERIVED MESENCHYMAL STEM CELLS ON CANCER CELLS

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ABSTRACT

DUAL ROLE OF HUMAN TOOTH GERM DERIVED MESENCHYMAL STEM CELLS ON CANCER CELLS

Mesencyhmal stem cells (MSCs), important components of the tumor microenvironment, could modulate tumor growth and development in many ways. MSCs have been used as targeted-delivery vehicles for cancer gene therapy because of their potential of tumor tropism. In this study, we investigated the effects of Human Tooth Germ (HTG) derived MSCs on MCF-7 (Human breast cancer cell line) and SH-SY5Y (Human metastatic neuroblastoma cell line) cells treated with doxorubicin and paclitaxel, anticancer drugs. We also investigated the *in vitro* effect of genetically modified MSCs secreting apoptosis inducers (Tumor necrosis factors related apoptosis inducing ligand (TRAIL), Dickkopf-related protein-1(Dkk-1)) on SH-SY5Y cells.

In the first part, conditioned medium (CM) of hTGSCs was collected when the cells reached to 70% confluency. Effects of this CM on MCF-7 and SH-SY5Y treated doxorubicin and paclitaxel were measured by MTS (cell viability assay) assay and real time PCR analysis of apoptotic markers caspase3 and p53.

In the second part, TRAIL and Dkk-1 genes tagged with pkh67 (Green Fluorescent Cell Linker Kit) were transferred into MSCs by electroporation either alone or in combination. A GFP gene transfected group was used as a control. Genetically modified MSCs were co-cultured with SH-SY5Y cells for 24 hours. Thereafter the cell survival of SH-SY5Y cells was determined by using flow cytometry. The expression of Bcl-2, Bax, Stat3 (proliferation genes), FADD, caspase3 (apoptotic genes) in SH-SY5Y cells were assessed by real time PCR.

The results showed CM of hTGSCs increased the survival of MCF-7 and SH-SY5Y cells treated with doxorubicin and paclitaxel by 30% and also CM reduced doxorubicin and paclitaxel induced apoptosis. On the other side, genetically modified MSCs secreting

TRAIL and Dkk-1 reduced the cell survival of cancer cells by inducing apoptosis and inhibiting the proliferation. Our findings demonstrated that MSCs play roles in growth of tumor cells by decreasing the effect of anti-cancer drugs on cancer cells. On the other hand genetically modified HTG derived MSCs expressing Trail and Dkk-1 might be a potential gene and cell therapy approach to treat certain types of cancer.

ÖZET

İNSAN DİŞ GERM KÖK HÜCRELERİNİN KANSER HÜCRELERİ ÜZERİDEKİ ÇİFT YÖNLÜ ROLÜ

Tümör mikro çevresinin önemli bileşenleri olan mezenkimal kök hücreler tümor gelişimi ve büyümesini pek çok şekilde kontrol edebilir. Mezenkimal kök hücreler (MKH), tümor tropizma potansiyelleri sebebiyle hedefli kanser gen tedavisi araçları olarak kullanılmışlardır. Çalışmada insan diş jermlerinden elde edilen mezenkimal kök hücrelerin antikanser ilaçlar olan doksorubisin ve paklitaksel ile muamele edilmiş MKH ve SH-SY5Y hücreleri üzerine olan etkilerini araştırdık. Ayrıca bu çalışmada apoptoz tetikleyiciler olan TRAIL ve Dkk-1 üretmek için genetik olarak modifiye edilmiş mezenkimal kök hücrelerin *in vitro* etkilerini inceledik.

Çalışmanın ilk kısmında %70 yoğunluğa ulaşan insan diş jerm kök hücrelerinden besiyeri toplandı. Elde edilen bu besiyerinin doksorubisin ve paklitaksel ile muamele edilmiş MCF-7 ve SH-SY5Y hücreleri üzerindeki etkisi MTS deneyi ve kaspaz3 ile p53 gibi apoptotik markörlerin gerçek zamanlı PZR ile analiz edilmesi ile belirlendi.

Çalışmanın ikinci kısmında TRAIL ve Dkk-1 genleri tek başına veya kombine edilerek pkh67 (yeşil floresans boya) ile boyanan mezenkimal kök hücrelere transfekte edildi. GFP ile transfekte edilmiş bir grup kontrol olarak kullanıldı. Genetik olarak modifiye edilmiş mezenkimal kök hücreler SH-SY5Y hücreleri ile 24 saat boyunca birlikte kültüre edildi. SH-SY5Y hücrelerinin canlılığı flow sitometri kullanılarak belirlendi. Bcl-2, Bax, Stat3 (hücre çoğalma genleri), FADD, caspase3 (apoptotik genler) genlerinin anlatım düzeyi gerçek zamanlı PZR ile belirlendi.

Sonuçlar insan diş jerm kök hücrelerinden elde edilen besiyerinin doksorubisin ve paklitaksel ile muamele edilmiş MCF-7 ve SH-SY5Y hücrelerinin canlılığını % 30 oranında artırdığını ve ayrıca doksorubisin ve paklitakselin neden olduğu apoptozu azalttığını göstermiştir. Diğer yandan TRAIL ve Dkk-1 üretmek üzere genetik olarak

modifiye edilmiş mezenkimal kök hücreler apoptozu indükleyerek ve hücre çoğalmasını inhibe ederek kanser hücrelerinin canlılığını azalttı. Bulgularımız mezenkimal kök hücrelerin, antikanser ilaçların kanser hücreleri üzerindeki etkisini azaltarak tümor hücrelerinin büyümesinde rolü olduğunu göstermiştir. Başka bir deyişle TRAIL ve Dkk-1 ekspresyonu yapabilen genetik olarak değiştirilmiş insan diş jermleri kaynaklı mezenkimal kök hücreler bazı kanser türlerinin tedavisi için potansiyel bir gen ve hücre terapisi yaklaşımı olabilir.

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LIST OF SYMBOLS / ABBREVIATIONS

Ang	Angiopoietin
APC	Adenomatous Polyposis Coli
Bax	Bcl-2 Associated X Protein
Bcl-2	B-cell Lymphoma
BMMSCs	Bone Marrow Mesenchymal Stem Cells
CD	Cluster of Differentiation
CM	Conditioned Medium
DAPI	4',6-diamidino-2-phenylindole
Dkk-1	Dickkopf-related Protein-1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ECM	Extra Cellular Matrix
EGF	Epidermal Growth Factor
FADD	Fas-Associated Protein with Death Domain
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FITC	Fluorescein-iso-thio-cyanate
GAPDH	Glycer-aldehyde-3-phosphate-de-hydrogenase
GFP	Green Fluorescent Protein
HGF	Human Growth Factor
HLA	Human Leukocyte Antigens
HTG	Human Tooth Germ
ICM	Inner Cell Mass
IL	Interleukin
MCF-7	Human Breast Cancer Cell Line
MSCs	Mesenchymal Stem Cells
MTS	3-(4, 5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium
NK-cell	Natural Killer Cell

PCR	Polymerase Chain Reaction
PDGF	Platelet-derived Growth Factor
PDLSCs	Periodontal Ligament Stem Cells
PSA	Penicilin/Streptomycin/Amphotericin
RT-PCR	Real Time Polymerase Chain Reaction
SCAP	Stem Cells from Apical Papilla
SHED	Stem Cells from Human Exfoliated Deciduous Teeth
SH-SY5Y	Human Metastatic Neuroblastoma Cell Line
Stat3	Signal Transducer and Activator of Transcription 3
TAF	Tumor-associated Fibroblasts
TGF- β	Transforming Growth Factor
TNF- α	Tumor Necrosis Factor Alfa
TRAIL	Tumor Necrosis Factors Related Apoptosis Inducing Ligand
VEGF	Vascular Endothelial Growth Factor

1. INTRODUCTION

1.1. GENERAL ASPECTS OF STEM CELLS

Stem cells are unspecialized cells that have the ability to self-renew, proliferate, regenerate and become specialized cells with specific characteristics and function by differentiating under proper conditions. The best example of a stem cell is the bone marrow stem cell that is able to give rise to different specialized blood cells (white or red blood cells) with special functions, such as antibody production or gas transport [1].

Stem cells are classified into two major categories based on their source: embryonic stem cells and adult stem cells. Embryonic stem cells have a greater capacity of self-renewal and differentiation compared with the others. They appear in the early stages of embryonic development before blastocyst formation and are called totipotent embryonic stem cells with the potential to form an entire living organism (Table1) [2]. The inner cell mass (ICM) of the 5- to 6-day old human blastocyst is the source for the pluripotent embryonic stem cells and these cells are able to differentiate into tissues derived from all 3 germ layers (ectoderm, mesoderm and endoderm) but can not form the embryonic structures such as placenta and umbilical cord. Multipotent adult stem cells have less differentiation capacity and known to be specialized cells with restricted differentiation and regeneration potential, however they are able to form a specific number of cell types. Unipotent cells can produce only a single type of cell, which distinguishes them from non–stem cells and are found in different tissues [3-4].

Table 1.1. Types of stem Cells [2]

Type of cell	Definition	Traditional examples
Totipotent	Stem cells that can become an entire human being	Stem cells from a fertilized human egg
Pluripotent	Stem cells that can develop into any body cell type but cannot become an entire human being	Stem cells from a 7-day-old embryo or blastocyst
Multipotent	Stem cells that can only differentiate into the same tissue type	A bone marrow stem cell can differentiate into another type of bone marrow cell, but not into kidney, heart muscle, or brain
Unipotent	Cells that can produce only one cell type, but have the property of self-renewal, which distinguishes them from non–stem cells	

The second and more detailed classification of stem cells is based on their origin; embryonic stem cells, fetal stem cells, umbilicial cord stem cells and adult stem cells was shown in Figure 1.1. As described previously, embryonic stem cells obtained from the inner mass or blastocysts are characterized by a high proliferative potency and can give rise not only to all cell types derived from the three germ layers, but also to embryonic structures, such as placenta and umbilical cord. Fetal tissue is potential source for the stem cells with differentiation and self-renewal abilities. These stem cells are primitive cell types in the fetus that develop into the various tissues and organs of the body, such as blood, liver, and lung and have similar characteristics to their counterparts in adult tissues [5].

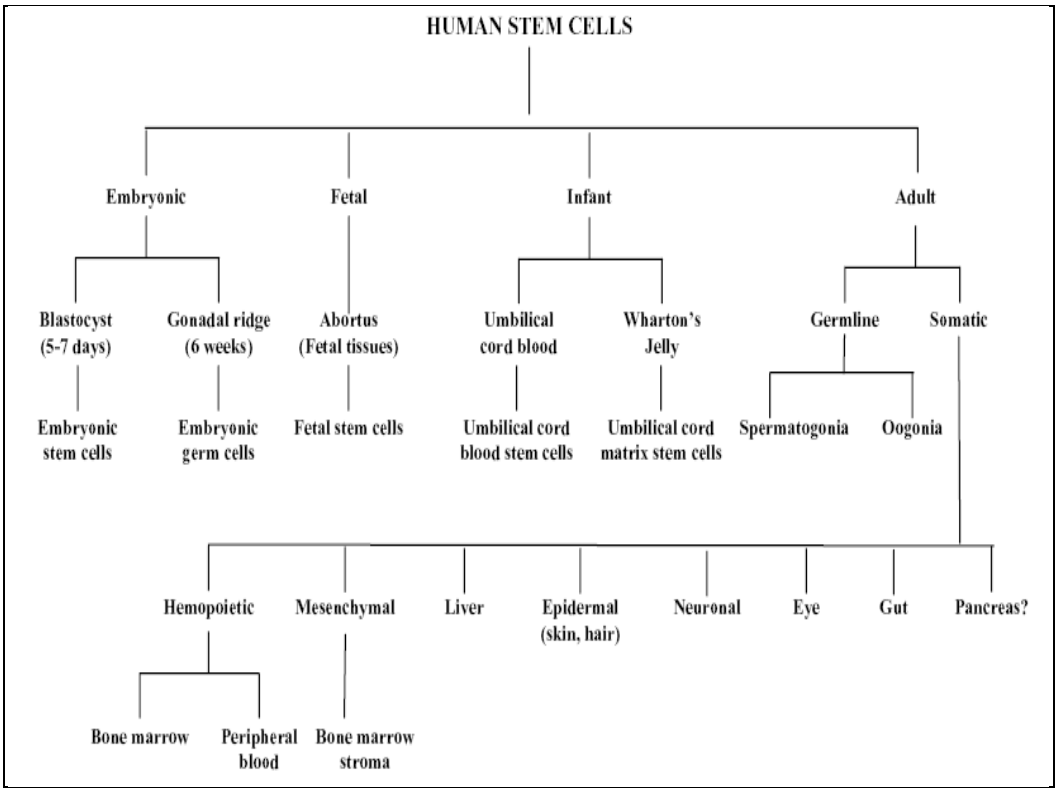

Figure 1.1. Classification of Stem Cells [6]

Cord Blood stem cells show similarities with bone marrow stem cells in terms of their potential to differentiate into other tissue types but are much more primitive than the bone marrow stem cells and do not cause a severe immune response. The decreased rejection of the cord blood stem cells due to the decreased expression of beta-2-microglobulin [7] and long-term storage (cryopreservation) without significant loss in their properties makes these cells useful in the clinical applications. Cord blood is a rich source of hematopoietic stem cells and mesenchymal stem cells (MSCs). Finally, adult stem cells are precursor cells residing in different tissues and organs of adults and are able to differentiate into special cells of the tissue where they are located, such as the generation of neurons, oligodentrocytes by the central nervous stem cells. Adult stem cells have been defined to be found in many tissues; bone marrow, trabecular bone, periosteum, synovium, muscle, adipose tissue, breast gland, gastrointestinal tract, central nervous system, lung, peripheral blood, dermis, hair follicle, corneal limbus, etc. [8]. Adult stem cells are responsible for the regeneration of damaged tissue and maintanence of tissue homeostasis,

for example physiological replenishment of skin and blood cells. MSCs, as one type of adult stem cells, will be discussed in the following part in details.

1.2. MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) were first identified by Friedenstein and colleagues in 1976, who described an adherent fibroblast-like population with the potential to differentiate into osteogenic precursor cells [9]. Subsequently, it has been demonstrated that these cells are capable of differentiating into three lineages; chondrocyte, osteoblast, and adipocyte due to their ability of multilineage differentiation and self-renewal potential [10]. Although MSCs were originally isolated from bone marrow, similar populations have been identified in virtually other tissues including umbilical cord blood, muscle, bone, cartilage, and adipose tissue [11].

MSCs constitute a heterogeneous population of cells and no single specific marker has been identified. Recently, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed minimal criteria to define human MSCs [12]. They must express CD 29, CD73, CD90, CD 105 and lack the expression of CD34, CD45, CD11b and HLA-DR or CD14, CD19 or CD79α. MSCs must also be plastic-adherent under the standard culture conditions and also should differentiate into osteocytes, adipocytes, and chondrocytes *in vitro* [13].

MSCs secrete a variety of cytokines and growth factors such as monocyte chemotactic protein-1, VEGF- A, epidermal growth factor (EGF), fibroblast growth factor-2, interleukin-6 and so they both function in an autocrine or paracrine manner [14]. MSCs themselves do not differentiate and influence the regeneration of cells or tissues by a bioactive factor effect. This property of MSCs will be introduced in the following part, entitled as ‘cross-talk between MSCs and cancer cells’.

1.3. DENTAL STEM CELLS

Stem cell biology has gained a big attention in the recent years as an important area of regenerative medicine. Dental derived MSCs are promising candidates for therapeutic

applications among many other type of stem cells which are located in the different tissues of the body. They were first isolated by Gronthos *et al* in 2000 from the human pulp tissue and termed as ‘postnatal dental stem cells’ [15].

Afterwards other 3 types of mesenchymal like stem cell populations were isolated from different sources and characterized as exfoliated deciduous teeth (SHED) [16], periodontal ligament stem cells (PDLSCs) [17], and stem cells from apical papilla (SCAP) [18-19]. Newly identified dental stem cells have compared with the bone marrow MSCs through a characterization process. Dental mesenchyme was named as ectomesenchyme due to its interaction with the neural crest. Therefore dental stem cells are able to differentiate into at least three distinct cell types including osteo/odontogenic, adipogenic, and neurogenic.

A number of studies have concluded that multipotent MSCs reside in the human tooth germs have the capacity to give rise to different cell types [20]. Human tooth germ stem cells (hTGSCs) are obtained from the third molar human tooth germs of young adults generally at the ages between 10 and 16. They were reported to have mesenchymal stem cell characteristics. The tooth germs contain progenitor cells that are able to produce a whole tooth. It comprises the dental papilla, the dental follicle and the enamel organ.

Dental pulp is the soft connective tissue in the center of the tooth germ containing nerves and blood vessels. Dental pulp tissue is a combination of ectodermic and mesenchymal components [21]. Dental pulp has four parts and the first part contains dentin producing odontoblasts. The second part is rich in extracellular matrix and named as “cell free zone”. On the other hand the third part is “cell rich zone” that contains pluripotent progenitor cells. The inner part contains the vascula area and nervous plexus [22]. Post natal dental pulp stem cells produces dentin. Dental pulp stem cells are able to differentiate into osteogenic-odontogenic, chondrogenic and myogenic cells [23-24]. Dental follicle cells are obtained from the dental follicle which is an ectomesenchymal tissue surrounding the dental papilla of the tooth germ and the enamel organ. Cells in different parts of the dental follicle differentiate into different type of cells and this differentiation is controlled by the growth factors and cytokines. Third molar tooth germs stay undifferentiated until the age of six. This proves that tooth germ stem cells are highly proliferative and

multipotent. Tooth germ stem cells were reported to differentiate into distinct cell types that are originated from the different germ layers: ecto-, meso- and endo-derm.

To date dental derived MSCs have been used for several applications including tissue engineering studies in order to increase their potential usage in clinical studies [18-25]. In this study we tested the potential effect of hTGSCs on cancer progression.

1.4. CROSSTALK BETWEEN MSCs AND CANCER CELLS

Solid tumors are combination of tumor cells and promoter non-tumor components referred as tumor stroma that sustain tumor viability. Tumor cells interact with their microenvironment during their development, and metastasis. The stromal cells supply a physical environment providing (extra cellular matrix, ECM), cytokines, growth factors and eliminating the metabolic wastes [26]. These stromal cells composed of immune system cells, endothelial cells, fibroblasts, ECM blood vessels, tumor-associated fibroblasts (TAF). It was shown in Figure1.2. [27-30].

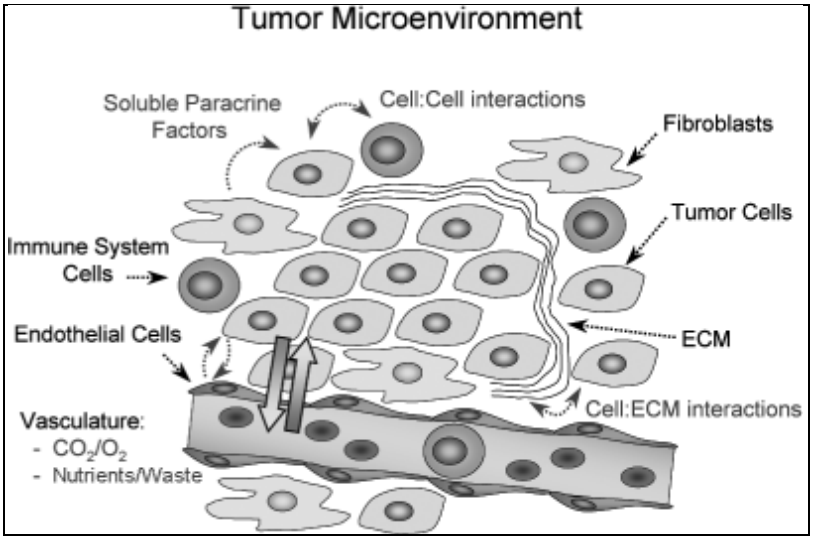

Figure 1.2. Synergy of tumor and stroma [31]

Fibroblasts are responsible for providing the structural support of ECM and also production of many paracrine growth factors that regulate cell morphology, proliferation, survival, and death. Immune cells including monocytes/macrophages, natural killer cells,

dentritic cells, T cells etc. support cancer development via releasing soluble factors that are regulating several processes such as migration, angiogenesis, cell proliferation and metabolism [32]. TAFs are derived from organ fibroblasts but circulating MSCs can be precursor of TAFs [33]. It is supported that MSCs have potential to differentiate into fibroblasts, pericytes, myofibroblasts in response to TGF- β, growth factor secreted by tumor cells within the tumor mass and can became TAF [34]. TAFs affect the tumor cells via producing growth factors cytokines, chemokines etc. and also providing arrangement of tumor stroma by generating the ECM components.

MSCs display homing at injury sites and repair or replace the damaged tissue by differentiating into multiple cell types including osteocytes, adipocytes, myocytes and condrocytes [35]. Additionally they have the ability of migration towards tumor site in response to tumor-secreted factors and differentiate to TAFs that support tumor growth (Figure 1.3.). Besides this, MSCs secrete a variety proteins and cytokines which can enhance the proliferation and encourage angiogenesis such as Vascular Endothelial Growth Factor (VEGF) and Platelet-derived Growth Factor (PDGF) [36].

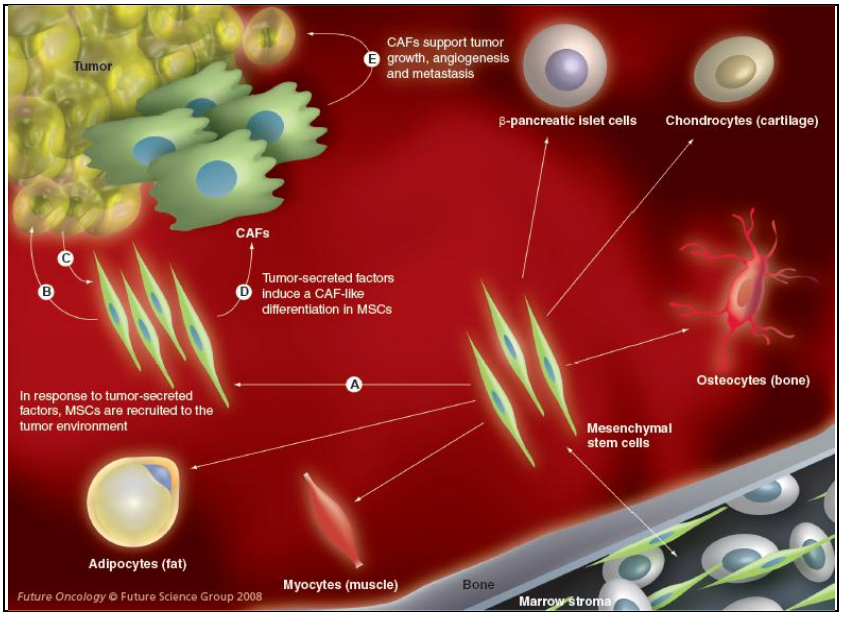

Figure 1.3. MSC Promotion of Tumor Growth [37]

MCSs help the tumor growth via suppressing the immune response and cause the malignant cell proliferation. They create an immunosuppressive microenvironment by secreting anti-inflammatory molecules [38]. They can reversibly inhibit the differentiation of dentritic cells into $CD14^{+}$ monocytes and hinder their maturation via the downregulating of Adenomatous polyposis coli (APC)-related molecules. Furthermore, MSCs change the secretion of cytokines profile such as TNF- α, inhibit the proliferation of T-lymphocyte response to IL-10, IL-4, HGF, TGF- β and suppress the NK-cell activation [39].

Angiogenesis which involves new vessel formation is one of the most important activities of the tumor stroma. As described previously MSCs secretes proangiogenic factors such as, VEGF, PDGF, FGF-2, FGF-7, angiopoietin-1 (Ang-1), and cytokines (IL-6, TNF-α) and these molecules induce angiogenesis and vasculogenesis with cooperation with endothelial cells [40]. The studies about MSCs promotion of tumor growth are shown in Table 1.2.

Table 1.2. Studies reporting that MSC promote Tumor Growth [41]

Isolation	**Tumor model**	**MSC: Tumor cell ratio**	**Findings**	**Proposed mechanism**
Human BDM-MSC	Breast (MFC/Ras, MDA-MB-231, MDA-MB-435, and HMLER)	3:1 coinjected	İncreased size in one cell line (MCF/Ras) and increased metastasis	Chemokine secretion (CCL5)
Fetal and adult BMD-MSCs	Colon cancer cell line (SW480 and F6)	10:1, 1:1 coinjected	Increased incidence	Enhanced proliferation and angiogenesis
Mouse BMD-MSC	Melonoma(B16)	1:1 coinjected	Increased incidence	Immunologic
Human and Mouse ASCs	Breast (4T1 and MDA231)	10:1 coinjected i.v. 24 hours later	Increased size	Paracrine factor(SDF-1/CXCR secretion)
Human ASC	Lung or glioma (H460 or U87MG)	1:1, 2:1, 1:10 coinjected	Increased size	Reduced apoptosis
Mouse ASC	Breast (BB1)	1:1	Increased incidence and size	vasculogenic
Human ASC	Prostate (PC3)	1:2 injected in contralateral flank after 7 days	Increased incidence and size	Vasculogenic and modulation f tumoral CXCR4
Human ASC	Melanoma (A375 and M4Beu) and glioblastoma multiforme (8MGBA)	1:10-1:5 coinjected or i.v. synchronous with tumor injection	Decreased latency and increased size of melanoma xenografts	VEGF and SDF-la/CXCR4
Human ASC	Prostate	1:10 coinjected subcutaneously	Increased size	Vasculogenic with differentiation into endothelial cells
Human MSC	Colon(KM12SM)	1:2 coinjected into cecum	Increased size and metastasis	Increased angiogenesis and reduced apoptosis

Many studies have shown that MSCs promote tumor progression and metastasis while other studies report that MSCs suppress the tumor growth. Hongliang Jiao *et al.* 2011 showed that human umbilical cord blood-derived MSCs decrease the cyclin D1 protein expression and inhibit glioma growth [42]. Additionally MSCs can upregulate the mRNA expression of cell-cycle negative regulator p21 and apoptosis-associated protease caspase-3, resulting in a G0/G1 phase arrest and apoptotic cell death of tumor cells [43].

MSCs have the ability of migration towards the inflammatory site and they can be used as a vehicle for gene and drug delivery. They are suitable for cancer therapy not only, due to their migration capability but also; their ease of isolation, anti-inflammatory properties, ability to differentiate to multiple cell types and the capability of providing immunosupression. MSC migration and homing to the inflamed sites are mediated by receptors, chemokines and cell membrane molecules. The chemokine receptors on the surface of MSCs support their sensivity to chemokines within the sites of injury. It was shown in Figure 1.4.

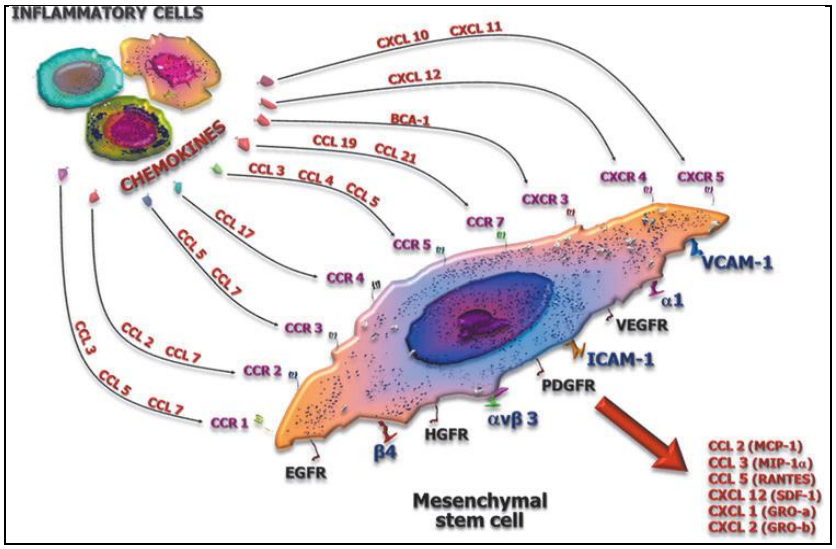

Figure 1.4. Migration of MSCs to Inflammatory Site. Purple: Chemokine receptors, Blue: adhesion molecules, Black: growth factor receptors, Red: chemoattractive molecules [44]

Recent findings led to a great excitement about the modification of MSCs to express anti-cancer molecules and in order to use them as vectors in the cancer therapy. Cytokines, such as IL-2, IL-12 and IFN-β induce the activation of T cells, which mediate an immune

response to eliminate tumors. To date, a number of anticancer genes have been engineered into MSCs and successfully resulted in anticancer effects on various carcinoma models as listed in Table 1.3.

Table 1.3. In vivo studies of cancer treatment by using engineered MSCs [44]

Tumor types	**Source of MSCs**	**Therapeutic molecules**	**Administration routes**	**Tumor hosts**
Melanoma lung metastasis	BM-MSCs	INF-alpha	systemic	mouse
Prostate lung metastasis	BM-MSCs	INF-beta	systemic	mouse
Melanoma	BM-MSCs	INF-beta	systemic	mouse
Breast cancer	BM-MSCs	INF-beta	systemic	mouse
Glioma	BM-MSCs	INF-beta	I.t. and systemic	mouse
Glioblastoma	AD-MSCs	CD-UPRT	systemic	mouse
Glioblastoma	AD-MSCs	TK	I.t. and systemic	mouse
Melanoma	AD-MSCs	CD-UPRT	systemic	mouse
Colon cancer	AD-MSCs	CD-UPRT	systemic	mouse
Pancreatic cancer	BM-MSCs	TK	systemic	mouse
Gastric cancer	BM-MSCs	CD-UPRT	systemic	mouse
Renal cancer	BM-MSCs	IL-12	systemic	mouse
Ewing sarcoma	BM-MSCs	IL-12	systemic	mouse
Melanoma	BM-MSCs	IL-12	systemic	mouse
Glioma	BM-MSCs	IL-18	I.t	Rat
Glioma	BM-MSCs	IL-23	systemic	mouse
Glioma	BM-MSCs	IL-2	I.t	mouse
Glioma	BM-MSCs	TRAIL	I.t	mouse
Glioma	BM-MSCs	TRAIL	I.t	mouse
Glioma	BM-MSCs	TRAIL	I.t	mouse
Lung cancer	BM-MSCs	TRAIL	I.t	mouse
Lung cancer	BM-MSCs	TRAIL	systemic	mouse
Glioma	BM-MSCs	TRAIL	systemic	mouse
Glioma	UCb-MSCs	TRAIL	I.t	mouse
Cervical carcinoma	AD-MSCs	TRAIL	systemic	mouse

1.5. APOPTOTIC SIGNALLING PATHWAYS

The p53 tumor-suppressor protein is a key regulator of apoptosis and cancerogenesis. p53 stimulates two major apoptotic pathways by a wide network of signals. The extrinsic, death receptor pathway activate caspase cascade, and the intrinsic, mitochondrial pathway alter the balance in the Bcl-2 family and consequently caspase-mediated apoptosis [45]. p53 is activated by external and internal signals and induces either viable cell growth arrest or apoptosis. It is also best known tumor suppressor gene in cancer and it has the ability to prevent cells from becoming malignant.

The extrinsic apoptotic pathway is triggered by three transmembrane proteins: TNFα, FAS/CD95 ligand (FASL) and APO2/TRAIL ligand. The cell-surface receptor FAS is a important component of the extrinsic pathway [46]. FAS links with a protein FADD (Fas associated death domain) to form a complex called DISC (Death-inducing signaling complex), then DISC activates the procaspase 8 to caspase 8 that induces the activation of caspase 3 and caspase7 resulting apoptosis. Induced TNFR1 and APO2 also promote cell death through caspase 8 [47].

The intrinsic apoptotic pathway is directed by the Bcl-2 family of proteins, which regulate the release of cytochrome c from the mitochondria [48]. The Bcl-2 family is consist of anti-apoptotic (prosurvival) and pro-apoptotic members. Activated proapoptotic members of Bcl-2 family neutralize the antiapoptotic members of the same family which otherwise inhibits the cell death by preventing the release of cytochrom-c from mitochondria [49]. Antiapoptotic members are Bcl-2, Bcl-XL, Bcl-w and the recentşy identified Mcl-1 and A1. Proapoptotic members are subdivided to BAX subfamily (which includes BAX, BAK and BOK) and BH3-only subfamily (which includes BID, BIM, BAD, BIK, BMF, PUMA, NOXA and HRK) [50]. Proapoptotic BH3-only proteins inhibit the activity of Bcl-2 during apoptosis. BAX and BAK play a key role in the activation of the intrinsic pathway of apoptosis. In response to stress activation, Bax forms a homodimer and releases cytochrome c from the mitochondria which results in the caspase-9 activation [51].

Caspases can be subdivided to two types: the initiator caspases and the executioner caspases. The initiator caspases (e.g. caspase 8, caspase 9 and others) play a role in activating the other procaspases which become the executioner caspases (e.g. procaspase 3 and 7 can be activated by caspases 8, 9 and others). In extrinsic apoptotic pathway caspase 8 activate the executioner caspases 3 and 7 [52]. p53 signalling pathway is shown in Figure 1.6.

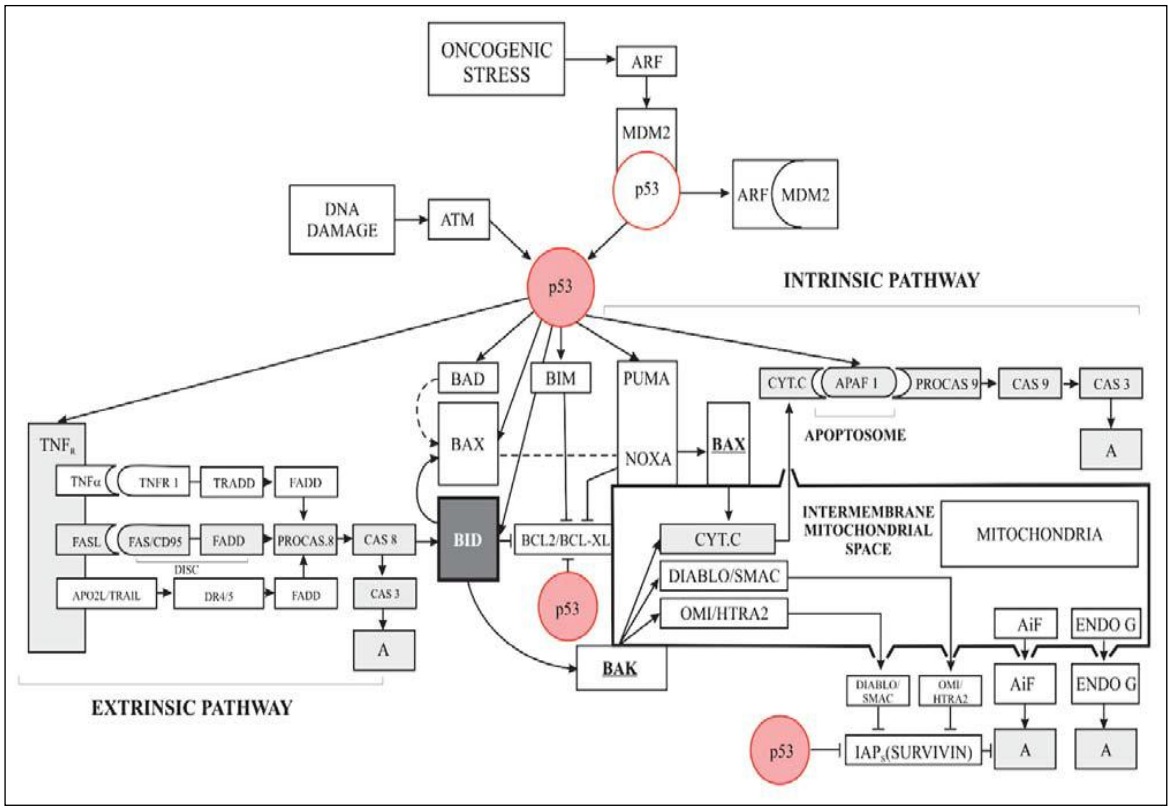

Figure 1.5. p53 signalling pathway [53]

1.5. AIM OF THE STUDY

In this research, it was aimed to demonstrate the effect of hTGSCs on the cancer cells SH-SY5Y and MCF-7 treated with the anti-cancer drugs, doxorubicin and paclitaxel. We have also investigated the effects of genetically modified hTGSCs secreting apoptosis inducing factors on cancer cells. By examining of the cross-talk between HTG derived MSCs and cancer cells we aimed to suggest a new anti-cancer therapy strategy.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Human Tooth Germ Stem Cells (hTGSCs)

Human tooth germ was isolated from the wisdom teeth of 14 years old patient. The wisdom tooth was surgically removed with orthodontic procedure in Istanbul University School of Dental Medicine and were sent to our facility in a sterile 15ml plastic tube containing DMEM with 10% (v/v) FBS and 1%PSA (v/v).

2.1.2. Cell Lines

The human neuro-blastoma cell line SH-SY5Y and the human breast cancer cell line MCF-7 were obtained commercially from ATCC – American Type Culture Collection, Manassas, VA, USA.

2.1.3. Cell Culture

Dulbecco's Modified Eagle Medium, DMEM (Invitrogen, Gibco, UK, cat # 31885), Fetal bovine serum, FBS (Invitrogen, Gibco, UK, cat # 10270-106), Penicilin/Streptomycin/ Amphotericin, PSA (Invitrogen,Gibco, UK, cat # 15240-062), 0.25% (w/v) Tyrpsin-EDTA solution (Invitrogen, Gibco, UK, cat # 25200), T75 Cell Culture Flasks (Zelkultur Flaschen, Switzerland,cat # 90075), T150 Cell Culture Flasks (Zelkultur Flaschen, Switzerland,cat # 90150), 6-well culture plate (Zelkultur Flaschen, Switzerland,cat # 92006), 96-well culture plate (Zelkultur Flaschen, Switzerland, cat # 92096), Serological pipets; 5 ml, 10 ml, 25 ml (LP ITALIANA SPA), Centrifuge tubes, 15 ml (Isolab), Incubator (Thermo, US, model no:3131).

2.1.4. Anti- Cancer Chemotherapy Drugs

Paclitaxel (Sigma Aldrich, cat # T1912), Doxorubicin hydrochloride (Sigma Aldrich, cat # D1515).

2.1.5. Cell Viability Assay

MTS-assay (3-(4, 5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2- (4-sulfo-phenyl)-2H-tetrazolium) (CellTiter96 Aqueous One Solution, Promega, UK, cat # 2587530), 96-well culture plate (Zelkultur Flaschen, Switzerland, cat # 92096), ELISA (Biotek, model no: EL800).

2.1.6. Real Time PCR

High Pure RNA Isolation Kit (Roche Applied Science, cat # 11828665001), Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, cat # 05081955001), SYBR® Premix Ex Taq (Takara, cat# RR041A), Primers (Invitrogen), iCycler RT-PCR detection system (Bio-Rad, Hercules, CA, USA, icycler iQ Optical Module).

2.1.7. Cell Labelling

PKH67 Green Fluorescent Cell Linker Kit (Sigma Aldrich, cat # MINI67), Fluorescence microscope (Nikon Eclipse TE200, Germany, Model no: CCD1300B).

2.1.8. Flow Cytometry (FACs) Analysis

Primary anti-bodies against CD29 (cat # BD556049), CD34 (cat # SC-51540), CD45 (cat # SC-70686), CD90 (cat # SC-53456), CD105 (cat #SC-71043), CD133 (cat #SC-65278), CD166 (cat # SC-53551) (SantaCruz Biotechnology Inc., Santa Cruz, CA, USA) and CD73 (cat # BD550256) (Zymed, San Francisco, CA, USA), Fluorescein-iso-thio-cyanate (FITC)-conjugated chicken antimouse secondary antibodies (cat # SC-2989)

(SantaCruz Biotechnology Inc., Santa Cruz, CA, USA), The Becton Dickinson FACS Calibur flow-cytometry system (Becton Dickinson, San Jose, CA, USA).

2.1.9. Gene Clonning

2.1.9.1. Plasmid Production

PureLink™ HiPure Plasmid Kits (Invitrogen, cat # K2100-06, Germany)

2.1.9.2. Electroporation

RPMI Medium 1640 (*Invitrogen* Corporation, *Gibco 21875*-034), DMEM (Invitrogen, Gibco, UK, cat # 31885), The plasmids expressing TRAIL, Dkk-1 and GFP (Mr. Mehmet Emir Yalvac, Yeditepe Univesity, Department of Genetic and Molecular Biology), Electroporation cuvettes (Bio-Rad, cat # 165-2086), Gene Pulser® II Electroporation System (Bio-Rad, cat # 165-2110)

2.1.9.3. Co-culture

6-well Cell Culture Insert Companion Plate (BD Falcon, cat # 353502), Cell culture inserts for 6-well plates. 0.4 μm pores, Transparent PET Membrane. (6/sp, 48/ca) (BD Falcon, cat # 353090).

2.1.9.4. Immunocytochemistry Analysis

Triton-X 100 (Bio Basic Inc, cat# 9002-93-01), Paraformaldehyde (Sigma-Aldich, cat# P6148), 2% goat serum (Sigma, US cat # G9023), Primary antibodies : TRAIL (SantaCruz Biotechnology Inc. #A0708, Santa Cruz, CA, USA), Dkk-1 (SantaCruz Biotechnology Inc. #C2310, Santa Cruz, CA, USA), Goat anti rabbit IgG Alea Fluor 488 (Invitrogen, USA, cat # A11008), DAPI (4',6-diamidino-2-phenylindole) (Applichem, Germany, cat # A40990010), Fluorescence microscope (Nicon Eclipse TE200, Germany, Model no: CCD1300B).

2.2. METHODS

2.2.1. Cell Culture

2.2.1.1. Culture of hTGSCs

Human impacted third molar tooth germs obtained from 14 years old patient were surgically removed due to orthodontic reasons. Isolated human tooth germ was minced small pieces and placed into 6-well plates containing DMEM supplemented with %10 (v/v) FBS, 2 mM L-Glutamine and %1(v/v) PSA. The cell reached to 80% confluency after 8-10 days of incubation at 37 °C with 5% CO2 and 95% humidity in the incubator. The medium was changed every day. And the cells were passed by using 0.025 (w/v) tyrpsin-EDTA solution.

2.2.1.2. Culture of SH-SY5Y and MCF-7 Cells

SH-SY5Y and MCF-7 cells were maintained in DMEM with %10 (v/v) FBS, 2 mM L-Glutamine and %1 (v/v) PSA. Cells were cultivated in T-75 flasks at 37 °C with 5% CO_2 and 95% humidity in the incubator until they reach to confluency. They were usually passaged every 2-3 days.

2.2.2. Characterization of hTGSCs

The specific surface antigens of hTGSCs were characterized by flow cytometry analyses. Cells were removed by trypsinization and were incubated with anti-CD29, CD34, CD45, CD90, CD105, CD133, CD73 and CD166 primary antibodies prepared in PBS with 1:100 dilution for 1 hour. After removing the primary antibodies by washing with PBS stem cells were incubated with the fluorescein-iso-thio-cyanate (FITC)-conjugated chicken antimouse secondary antibodies in dark at 4°C for 1 hour except for CD29; the antibody against phyco-erythrin (PE) and CD29 was tagged with red light-harvesting protein containing chromophore. Afterwards, cells were analyzed using Becton Dickinson FACS Calibur flow cytometry system.

2.2.3. Preparation of Doxorubicin and Paclitaxel

Doxorubicin and paclitaxel powders were dissolved in PBS to get final the concentrations of 100µM and 10µM respectively. They were diluted to different concentration in order to test the toxic effects on SH-SY5Y and MCF-7 cells.

2.2.4. Collection of Conditioned Medium of hTGSCs

Conditioned medium (CM) was taken from the passage # 3 healty hTGSCs that were reached to %70 confluency. Before the day of the collection of CM, culture medium of hTGSCs was changed with the FBS free DMEM. CM was filtered with 0.2 µm ministart filters (Sigma-Aldrich, cat # 16534K) and stored in a sterile 2ml plastic tube at -20°C.

2.2.5. Cell Viability Assay of SH-SY5Y and MCF-7 Cells Treated with Doxorubicin, Paclitaxel and CM

SH-SY5Y and MCF-7 cells were seeded on 96-well plates at a concentration of 5000 cells/well. Six concentrations (0.5µM, 1µM, 3µM, 5µM, 10µM) of doxorubicin- paclitaxel were combined with four concentrations (%20, %30, %40, %50) of CM in DMEM with %1 PSA, without FBS and then applied on the cells for 24 hours Cell viability was measured by the MTS-assay (CellTiter96 Aqueous One Solution, Promega, UK, cat # 2587530) according to the manufacturer's instructions. MTS (3-(4, 5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium) is a tetrazolium-salt based colori-metric assay for measuring the activity of enzymes that reduce MTS to formazan dyes, giving a purple color [46]. Briefly, 10µl MTS reagent with 100µl growth medium was added to each well incubated for two to three hours followed by reading absorbance at 490 nm with an ELISA plate reader.

2.2.6. Primer Designing and RT-PCR Analysis

Primers of caspase3, p53, Bcl2, Bax, FADD, STAT3 genes were designed by using Primer BLAST online software of The National Center for Biotechnology (NCBI) and ordered from Invitrogen company to be synthesized at 50 nmoles. The other primers

(GAPDH, Beta-actin) sequences were used as previously described in the literature. Table 2.1 shows primers that were used in this study.

Table 2.1. Primers that were used in this study

Primers	**Sense (5'-3')**	**Antisense(5'-3')**	**Base pair**
Caspase3	GAGGCGGTTGTAGAAGAGTTTCGTG	TGGGGGAAGAGGCAGGTGCA	177
P53	ACGCTTCCCTGGATTGGCAGCC	CCATTGCTTGGGACGGCAAGGG	166
Bcl-2	AACGGAGGCTGGGATGCCTTTGTG	ACCAGGGCCAAACTGAGCAGAGT	104
Bax	TGCAGAGGATGATTGCCGCCG	ACCCAACCACCCTGGTCTTGG	250
FADD	TCGAGCAGCGAGCTGACCGA	CACACAGGTCTTCTTCCCCAGGCG	250
STAT3	TCCCAAGGAGGAGGCATTCGG	TGCATCAATGAATGGTGTCACACAGA	127
GAPDH	TGGTATCGTGGAAGGACTCA	GCAGGGATGATGTTCTGGA	123 [54]
B-actin	GACAGGATGCAGAAGGAGATTACT	TGATCCACATCTGCTGGAAGGT	141 [55]

SH-SY5Y and MCF-7 were seeded into 6-well plates at a concentration of 130.000cells/well. The cells treated with the best effective doses of drugs and CM (5µM of doxorubicin -3µM of paclitaxel for MCF-7 cells and 2µM of doxorubicin-1µM of paclitaxel for SH-SY5Ycells, in both cases %20(v/v) CM was used) based on the MTS results. Total RNA from SH-SY5Y and MCF-7 cells treated with drugs and CM were isolated using High Pure RNA isolation kit according to the manufacturer's instructions after 24 hours of the treatment. cDNA synthesized using cDNA High Fidelity cDNA synthesis kit. SYBRgreen real time PCR method was used to detect the gene levels. *cDNAs* were mixed with primers and SYBR Premix Ex Taq in a final volume of 20µL. Table 2.2 shows the procedure of RT-PCR. GAPDH (glycer-aldehyde-3-phosphate-de-hydrogenase) gene was used as the house-keeping gene for normalization of the data. All RT-PCR experiments were done using iCycler RT-PCR detection system. PCR conditions were shown in Table 2.3.

Table 2.2. Regeants in PCR

Reagents	**Volume**
SYBRGreen	10µl
Primer Forward (10µM)	0.4µl
Primer Reverse (10µM)	0.4µl
Distilled water	4.2 µl
Template (100ng/ml)	5 µl

Table 2.3. PCR conditions

cycle	repeats	step	dwell time	Set point
1	1	1	3 min	93°C
2	40	1	30 sec	93°C
-	-	2	40 sec	61°C
-	-	3	45 sec	72°C
3	1	1	10 min	72°C
4	110	1	12 sec	40°C
5	1	1	-	4°C

2.2.7. Co-Culture Experiments

2.2.7.1. Cell Labeling

hTGSCs were labeled using PKH67 (green) fluorescent cell linker kit for general cell membrane labeling, according to the manufacturer's instructions. Briefly, the cells pellet were resuspended in diluent-C buffer and mixed with 2 x dye working solution, consisting of diluent-C and fluorescent dye (PKH67). Cell suspension was mixed immediately by gentle pipetting and incubated for 5 min at room temperature. Labeling was blocked by the addition of FBS. Labeled cells were co-cultured with non-labeled SH-SY5Y cells and observed under fluorescence microscope.

2.2.7.2. Flow Cytometry Analysis of Co-Cultures

Labeled SH-SY5Y cells and non-labeled SH-SY5Y cells were co-cultured together as a control group. Green labeled hTGSCs and non-labeled SH-SY5Y were successfully separated and identified using the flow cytometry. After co-cultured in 6-well plates for 24 hours at a concentration of 50.000/50.000cells in the presence and absensence of paclitaxel, doxorubicin. Cell survival non-labeled SH-SY5Y cells were analyzed by flow cytometry in all co-culture experiments (Figure 2.1.).

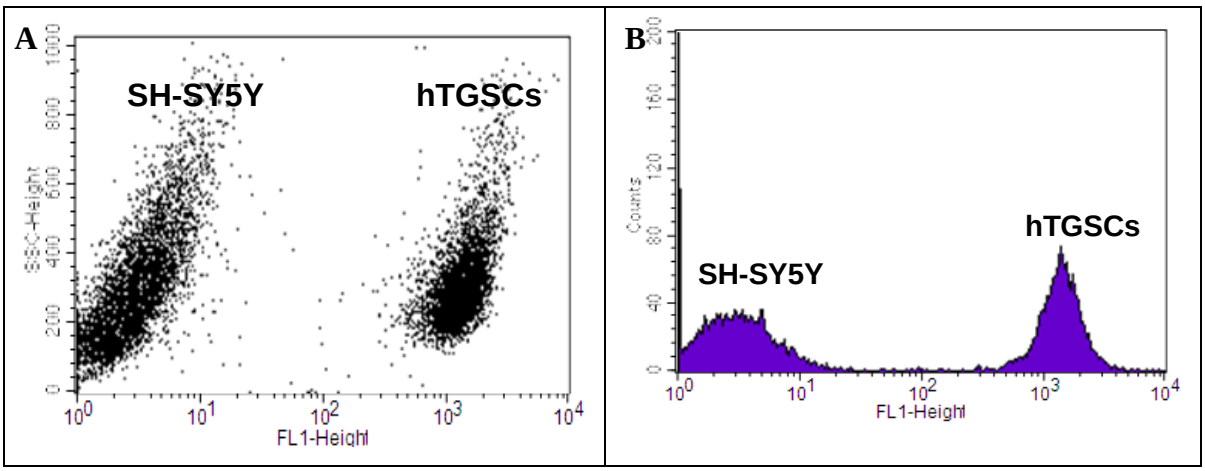

Figure 2.1. Cell viability non-labeled SH-SY5Y cells co-cultured with green hTGSCs by separation with Flow Cytometry. A: Dot Blot view of two cell lines B: Histogram Blot view of two cell lines

2.2.8. Production of Genetically Modified MSCs

hTGSCs that are passage 3 were trypsinized, and collected by centrifugation at 1,500 rpm for 5 min and re-suspended in RPMI-1640 medium without serum and antibiotic at 1 x 10^6 cells/ml. A total of 500 µL of hTGSCs were mixed with 20 µg of plasmid DNAs (Trail, Dkk-1 and GFP carrying plasmids kindly provided by Mr. Mehmet Emir Yalvac) in 0.4 mm electroporation cuvvettes, and electro-porated using the exponential wave electrical impulse at 250µV, 500µF using Gene Pulser II electro-poration system. A total of 1000 µl recovery medium (low glucose DMEM supplemented with 20% of FBS and 2% of PSA solution) was added to cuvvettes immediately following electroporation, and the cell suspension was transferred into 6-well plates. Genetically modified hTGSCs were seeded

either in 6 well plate for direct co-culture with SH-SY5Y cells for flow cytometry or transferred on Cell Culture Inserts for co-culture with SH-SY5Y cells in 24 well plates for real time PCR analysis. It is shown in Figure 2.2.

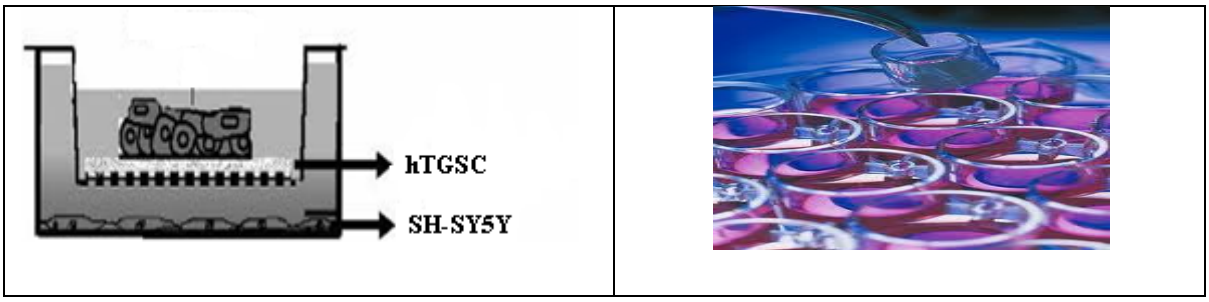

Figure 2.2. Co-culture of hTGSCs and SHSY5Y cells

2.2.9. Immunocytochemistry Analysis

hTGSCs electroporated with TRAIL and Dkk-1 were fixed in 2% (w/v) paraformaldehyde by incubating for 30 mins at 4ºC. The cells were washed three times for 5 min with PBS (7.4) by gentle shaking on the plate shaker. Permeabilization of cells was done by incubating the cells with 0.1% (v/v) Triton-X 100 diluted in PBS for 5 minutes at room temperature. The cells washed again with PBS three times for 5 min. Then they were incubated with 2% goat serum diluted in PBS for 20 min at 4ºC in order to block the non-specific binding of antibodies followed by washing with PBS three times for 5 minutes. The cells were incubated with primary antibodies (rabbit anti-TRAIL and rabbit-Dkk-1) dissolved in 1:100 blocking buffer overnight at 4ºC. After incubation with antibodies the cells were washed with PBS three times for 5 min to remove the unbound primary antibodies. Goat anti rabbit IgG-TRITC Tetramethylrhodamine isothiocyanate secondary antibodies were added to the samples and incubated for 1 hour at 4ºC. The cells were rinsed with PBS three times for 5 minutes. The nuclei of the cells were labeled with DAPI (4',6-diamidino-2-phenylindole) by another 3 sets of wash with PBS for 20 min at 4ºC followed by rinsing with PBS for three times. The labeled samples were observed under the fluorescence microscope.

2.2.10. Statistical Analysis

Graphics were drawn using Microsoft Office Excel and GraphPad Prism5 softwares and Standard errors and t-test and one-way anova values were calculated using GraphPad Prism5 software. For the statistical analysis student t test and one-way anova test were applied and p value less than 0.05 was considered statistically significant.

3. RESULTS

3.1. CHARACTERIZATION of hTGSCs

Flow cytometry analysis showed that cells isolated from the impacted third molar tooth germs were positive for CD105, CD90, CD73, CD166, but negative for CD34, CD45 and CD133. Results were shown in figure 3.1. This data demonstrated that hTGSCs are positive for MSCs markers and negative for HSC markers. It was shown in Figure 3.1.

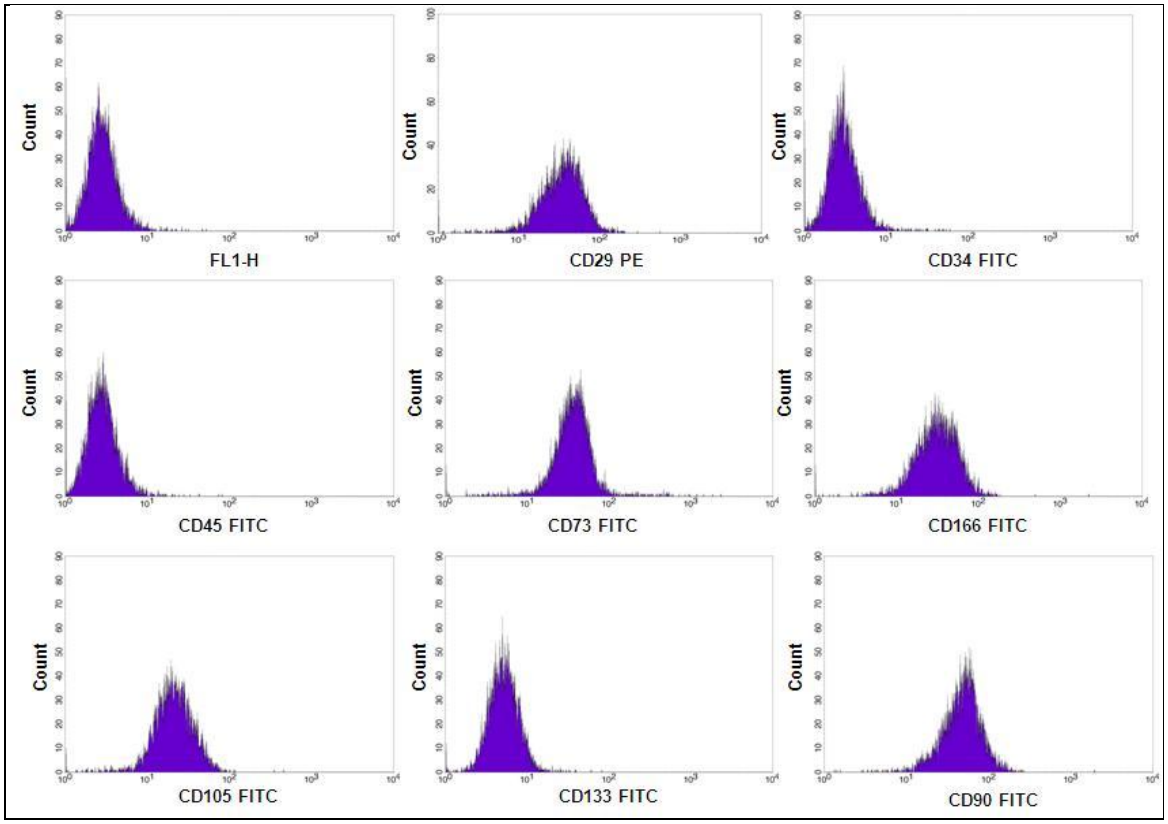

Figure 3.1 Flow cytometry analysis of hTGSCs

3.2. CELL VIABILITY ASSAY OF SH-SY5Y AND MCF-7 CELLS TREATED WITH DRUGS AND CM

Different doses of the doxorubicin and paclitaxel with 20% CM or without CM were applicated to the SHSY5Y and MCF7 cells and their toxicities were checked by MTS assay. MTS results showed that CM of hTGSCs increased the survival of doxorubicin and paclitaxel treated MCF-7 and SH-SY5Y cells by 30% approximately. It was shown in Figure 3.2. and 3.3.

The cell viability of negative control was assumed 100% and the experimental groups normalized with negative control. We have significant results in the experimental groups that are SH-SY5Y cells treated with 1.5μM, 2μM and 2.5μM doxorubicin with 20%CM or without 20%CM. In this group, the cell viability of SH-SY5Y cells was almost 28% but it reached to 53% when the cells were exposed to 20%CM. We did not observed any significant differences in the doses of 1μM and 3μM. We also have significant results in all of the doses of paclitaxel (1μM, 3μM and 5μM), the survival rate of SH-SY5Y cells with CM was increased to 103% from 80%.

The results for MCF-7 cells were similar to SH-SY5Y cells. CM increased the cell survival of MCF-7 treated with 0.5μM, 1μM, 5μM and 10μM doxorubicin to 110% from 67% and also the cell viability of MCF-7 cells treated with 1μM, 3μM and 5μM paclitaxel reached to 99% from 70% when they were cultured with 20%CM. All results are statistically significant.

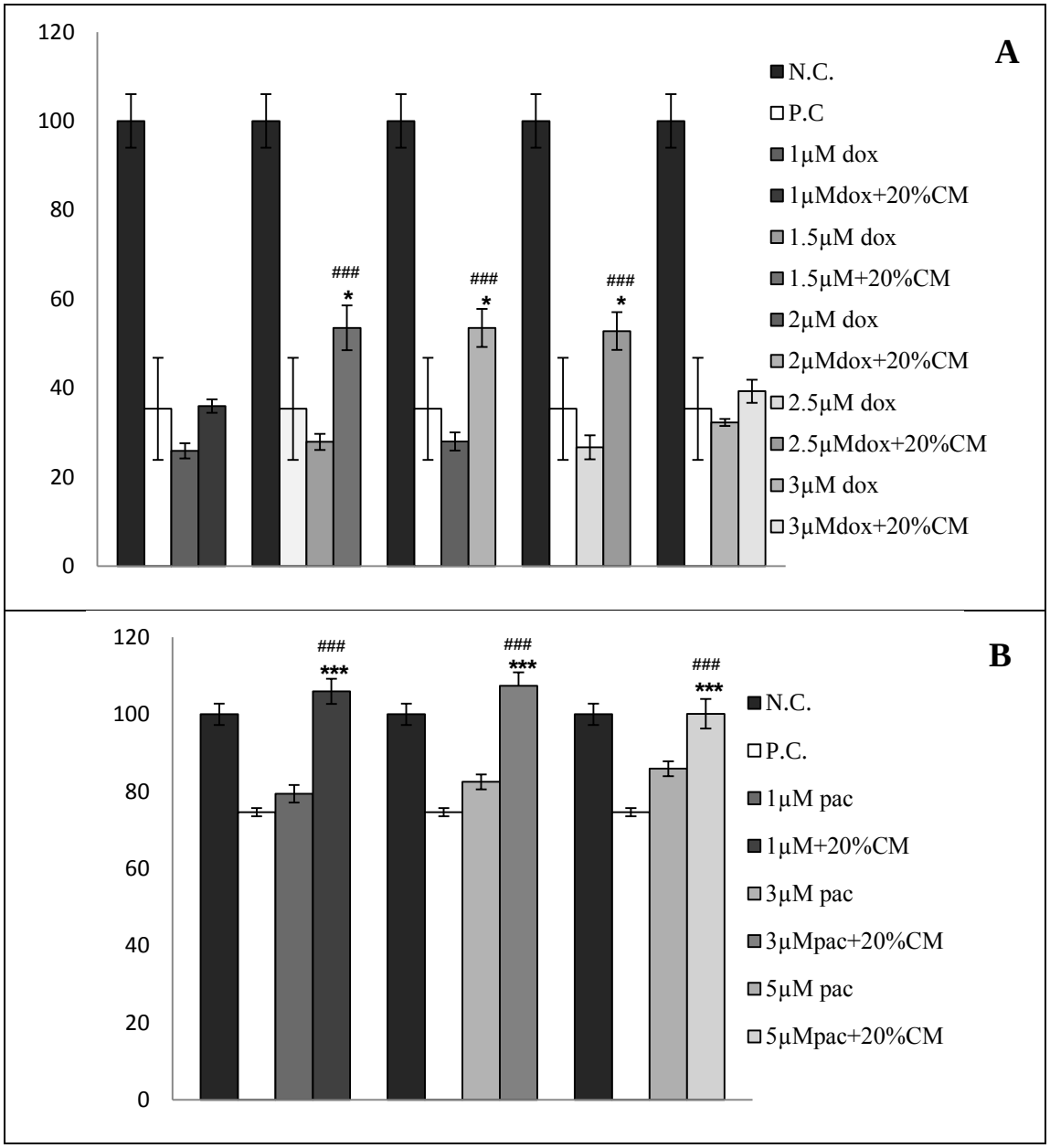

Figure 3.2. The survival rate of SH-SY5Y cells treated doxorubicin and paclitaxel. A: Doxorubicin treated SH-SY5Y cells. B: Paclitaxel treated SH-SY5Y cells. N.C: Negative control, P.C: %10 DMSO treated cells, CM: Conditioned medium. *$p < 0.05$/***$p < 0.001$ compared with P.C. ###$p < 0.001$ compared with the groups treated with paclitaxel and doxorubicin without CM

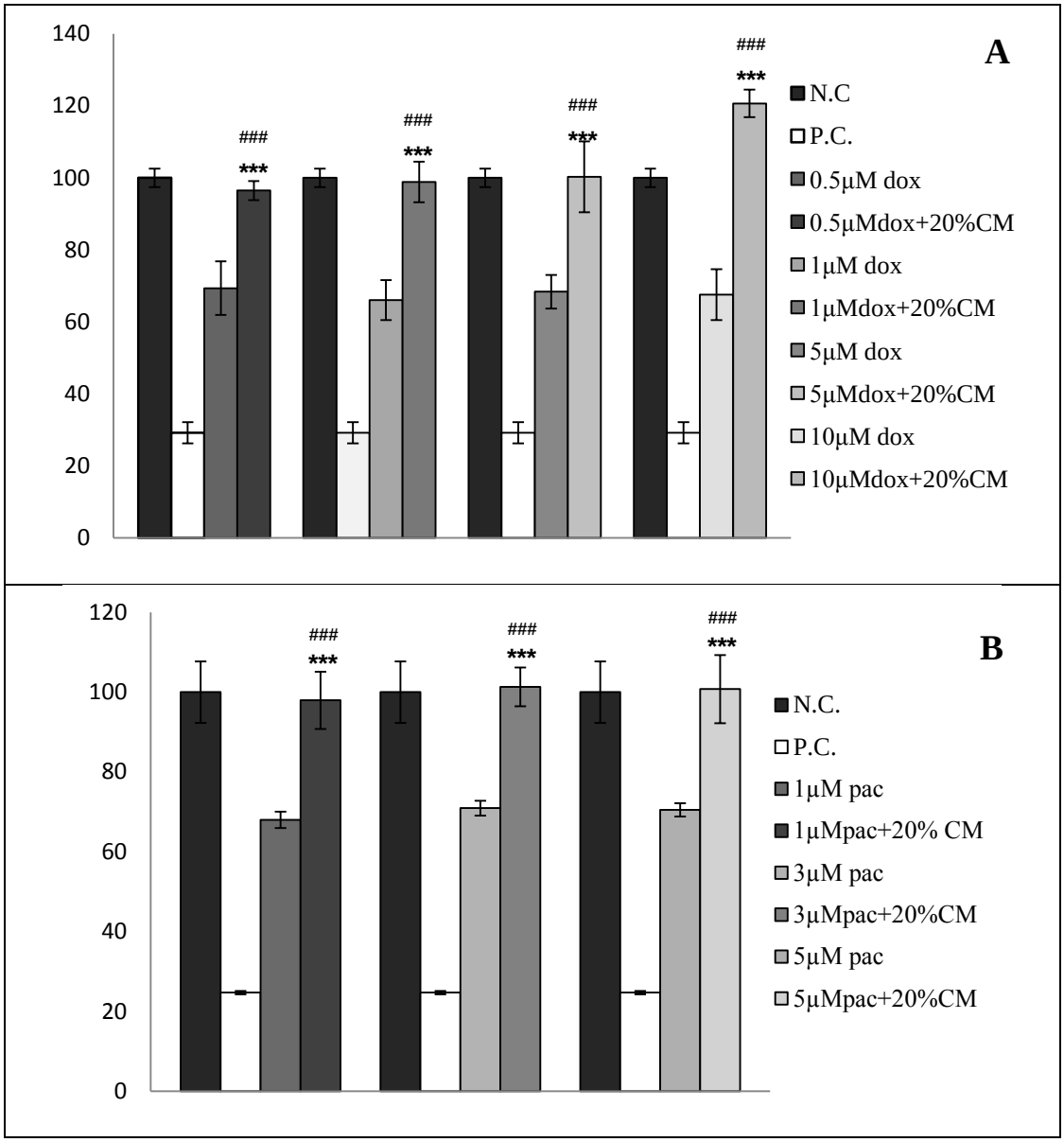

Figure 3.3. The survival rate of MCF7 cells treated doxorubicin and paclitaxel. A: Doxorubucin treated MCF7 cells. B: Paclitaxel treated MCF7 cells. N.C: Negative control, P.C: %10 DMSO treated cells, CM: Conditioned medium. $*p < 0.05$ compared with P.C. $###p < 0.001$ compared with the groups treated with paclitaxel and doxorubicin without CM

3.3. REAL TIME PCR ANALYSIS OF SH-SY5Y AND MCF-7 CELLS TREATED WITH THE DRUGS AND CM

Caspase3 and p53 gene expression levels were determined by real time PCR method. GAPDH was used as a house keeping gene to normalize results. Real time PCR analysis demonstrated that CM of hTGSCs has protective effect on MCF7 and SH-SY5Y cells against doxorubicine and paclitaxel- lowering the expression of p53 and the caspase3 genes. Figures 3.4. and 3.5. demonstrate the results.

The caspase3 and p53 gene expression are lower in MCF-7 and SH-SY5Y cells treated with doxorubicin, paclitaxel (5μM dox, 3μM pac are for MCF-7- 2μM dox, 1μM pac are for SH-SY5Y) and 20%CM in comparison with the cells treated with only doxorubicin and paclitaxel.

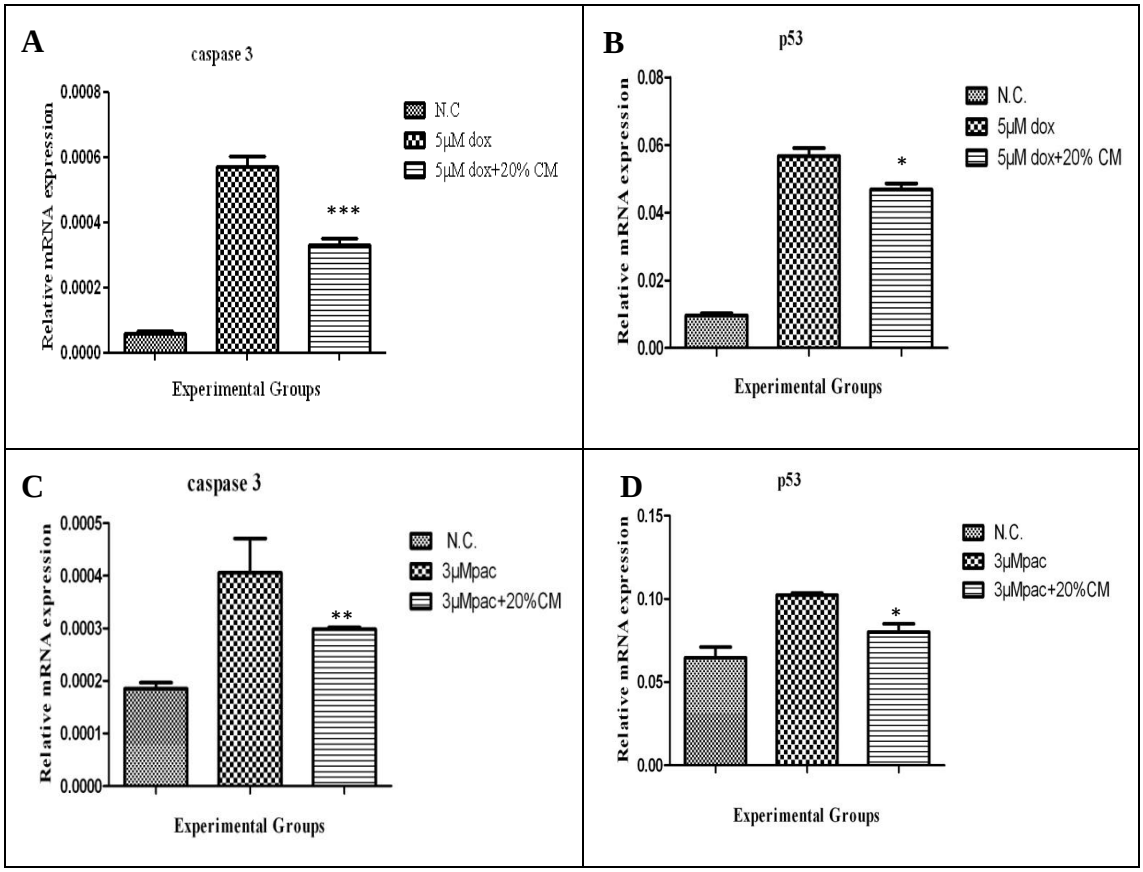

Figure 3.4. The caspase3 and p53 gene expression of MCF7 cell. A: The caspase3 expression of MCF7 cells treated with doxorubicin. B: The p53 expressions of MCF7 cells treated with doxorubicin. C: The caspase3 expressions of MCF7 cells treated with paclitaxel. D: The p53 expressions of MCF7 cells treated with paclitaxel. $*p < 0.05$/$*p < 0.01$/$***p < 0.001$ compared with the groups treated with paclitaxel and doxorubicin without CM

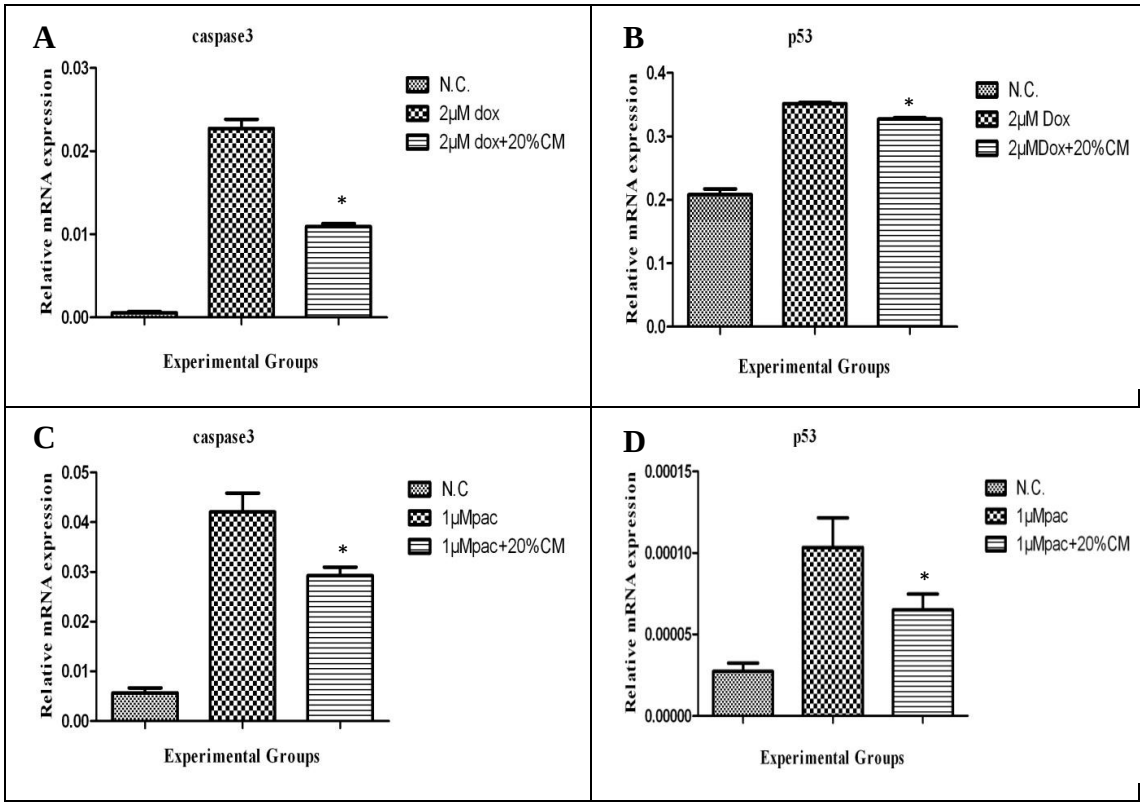

Figure 3.5. The caspase3 and p53 gene expressions of SH-SY5Y cells. A: The caspase3 expressions of SH-SY5Y cells treated with doxorubicin. B: The p53 expressions of SH-SY5Y cells treated with doxorubicin. C: The caspase3 expressions of SH-SY5Y cells treated with paclitaxel. D: The p53 expressions of SH-SY5Y cells treated with paclitaxel. *$p < 0.05$ compared with the groups treated with paclitaxel and doxorubicin without CM

3.4. CO-CULTURE OF SH-SY5Y CELLS AND hTGSCs

3.4.1. Fluorescence Microscopy of Co-cultured SH-SY5Y and hTGSCs

Labeled hTGSCs and non-labeled SH-SY5Y cells were co-cultured for 3 days and observed under fluorescence microscope. It was shown in Figure 3.6.

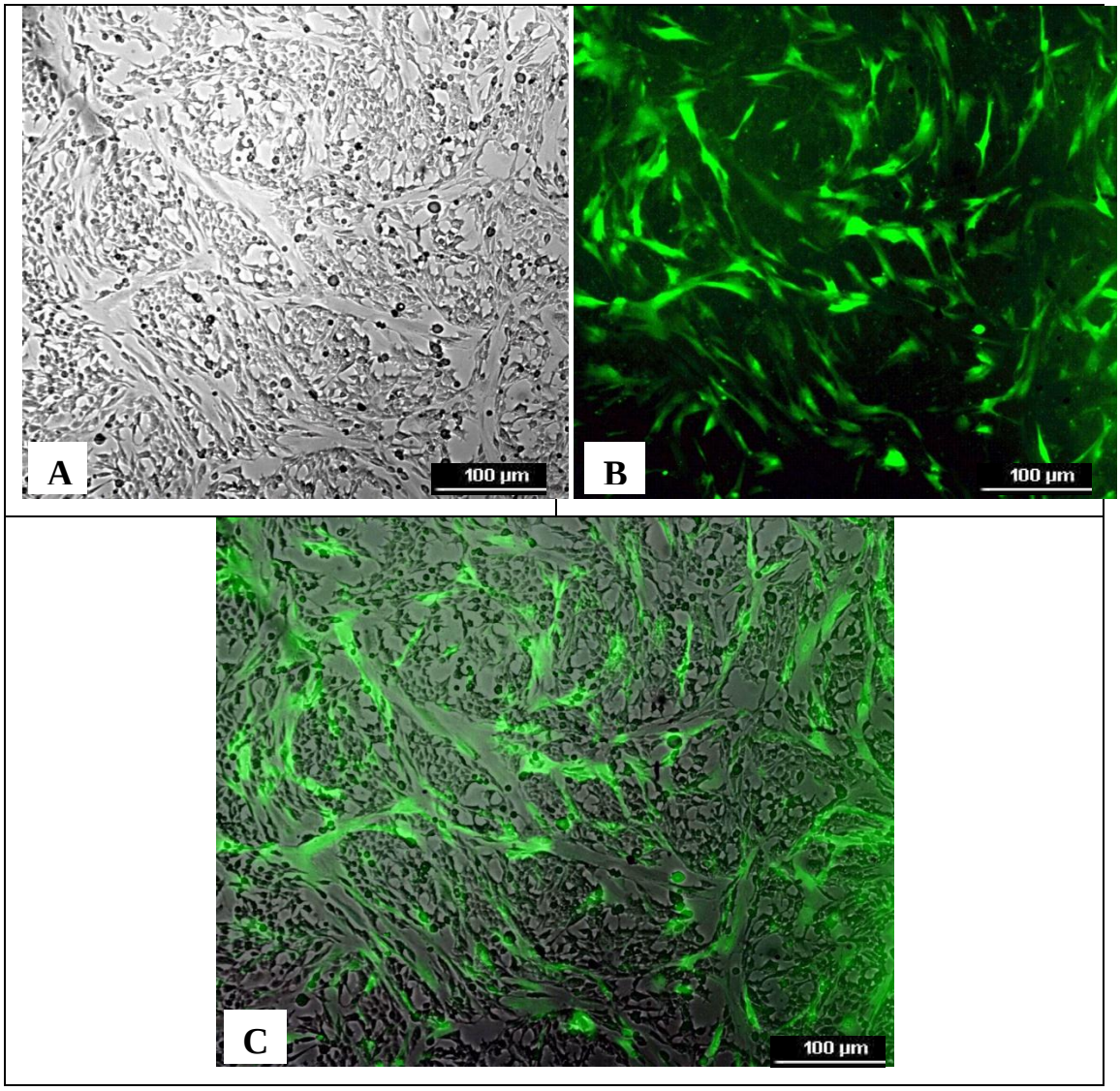

Figure 3.6. hTGSCs and SH-SY5Y cells after 3 days co-culture. Scale bar: 100 µm. A: Bright field image. B: hTGSCs labeled with Pkh67 (green fluorescence). C: Merged image of panels A&B

3.4.2. Flow Cytometry Analysis

To determine the effect of hTGSCs (labeled with Pkh67 green fluorescent dye) on the viability of SH-SY5Y cells (non-labeled), they were co-cultured for 24 hours with paclitaxel and without paclitaxel. Co-culture of non-labeled SH-SY5Y cells and labeled SH-SY5Y cells was used as a negative control. Cell viability of individual cell populations was assayed by flow-cytometry. This allowed monitoring the seperation of these two cell populations according to their green fluorescence intensity. These percentages, as shown in

Figure 3.7. and 3.8. are the cell viabilities of SH-SY5Y cells. The number of alive SH-SY5Y cells increased to 74.14 from 53.04 when they were co-cultured with hTGSCs. Also the results of the cells treated with paclitaxel were similar. The number of alive SH-SY5Y cells with hTGSCs were 75.38. Flow cytometry analysis showed that hTGSCs increased cell survival of both SH-SY5Y cells and SH-SY5Y cells treated with paclitaxel by 20%. The results are statistically significant.

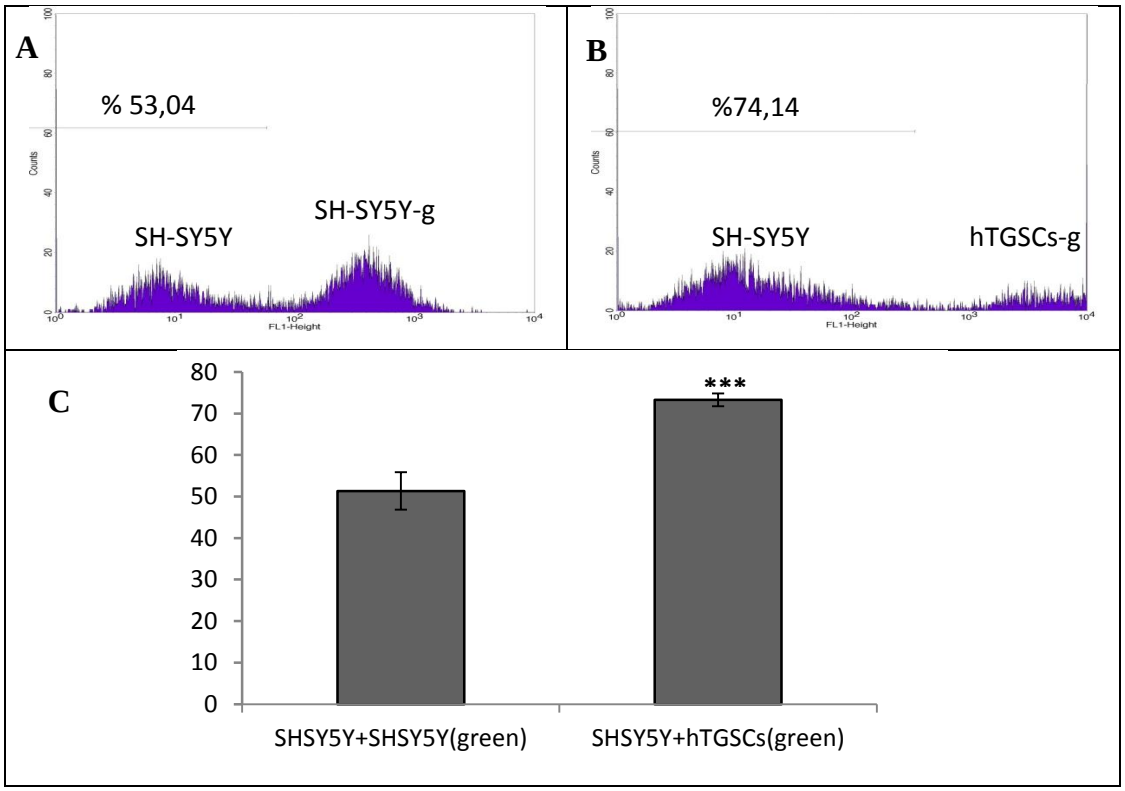

Figure 3.7. Cell viability analysis of SHSH5Y cells by flow cytometry. (***$p < 0.001$) A: Flow cytometry analysis of SH-SY5Y cells co-cultured with SHSH5Y cells labeled with pkh67. B: Flow cytometry analysis of SH-SY5Y cells co-cultured with hTGSCs labeled with pkh67. C: Cell viability of SH-SY5Y cells co-cultured with hTGSCs

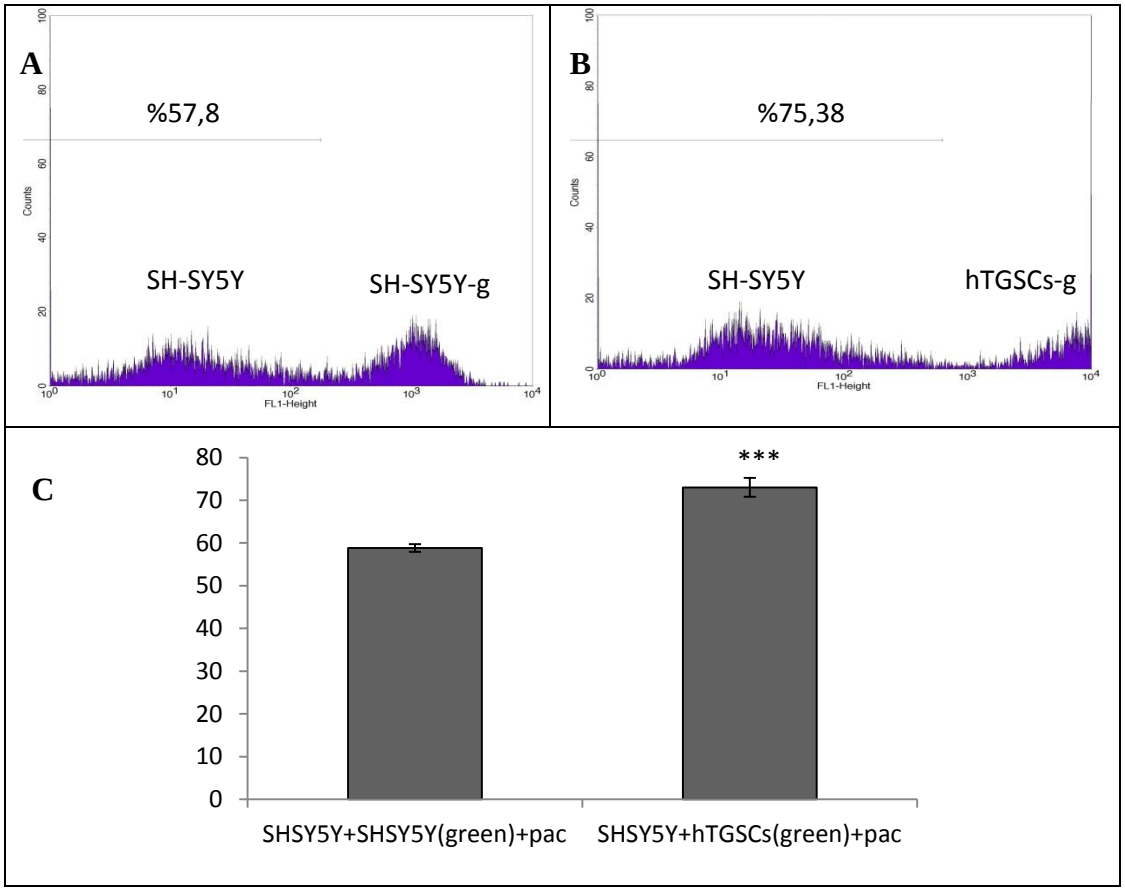

Figure 3.8. Cell viability analysis of SHSH5Y cells were co-cultured with hTGSCs and SH-SY5Y cells labeled with pkh67 by flow cytometry, *(****p*<0.001). A: Flow cytometry analysis of SH-SY5Y cells co-cultured with SHSH5Y cells labeled with pkh67. B: Flow cytometry analysis of SH-SY5Y cells co-cultured with hTGSCs labeled with pkh67. C: Cell viability of SH-SY5Y cells were co-cultured with hTGSCs and treated with paclitaxel

3.4.3. Immunocytochemistry Analysis of GM hTGSCs

TRAIL and Dkk-1 expressions were shown by immunocytochemistry confirming the succesfull transfection of hTGSCs. The nuclei of cells labeled with DAPI for counter staining. It was shown in Figure 3.9.

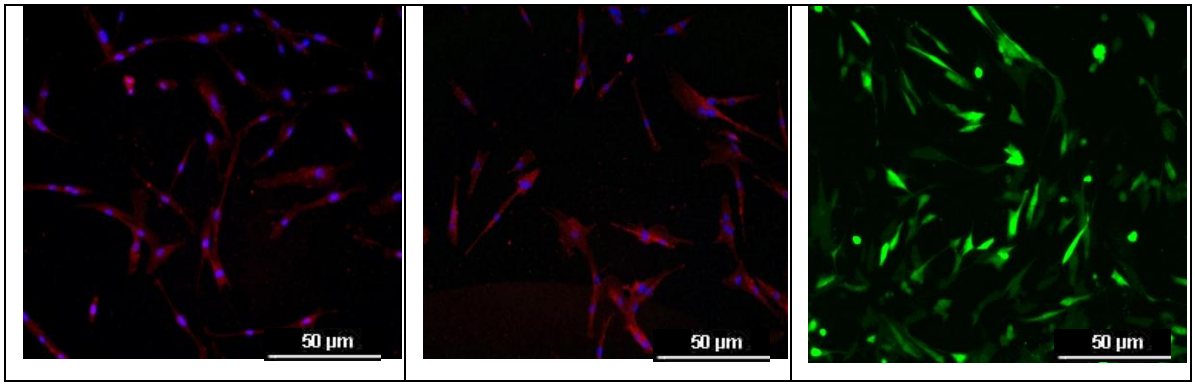

Figure 3.9. Immunostaining of TRAIL and Dkk-1. Scale bar: 50 µm. a: TRAIL transfected hTGSCs, b: Dkk-1 transfected hTGSCs, c: GFP transfected hTGSCs, negative control

3.4.4. Cell Viability of SH-SY5Y Cells Co-cultured with GM- hTGSCs

The cell viability of SH-SY5Y cells 55% in TRAIL group, 73% in Dkk-1 group and 45% TRAIL+Dkk-1 group and flow cytometry analysis demonstrated that hTGSCs transfected with TRAIL and Dkk-1 decreased the cell viability of SH-SY5Y cells in comparison with the negative control group where GFP transfected hTGSCs were co-cultured with SH-SY5Y cells. Figure 3.10. shows the results.

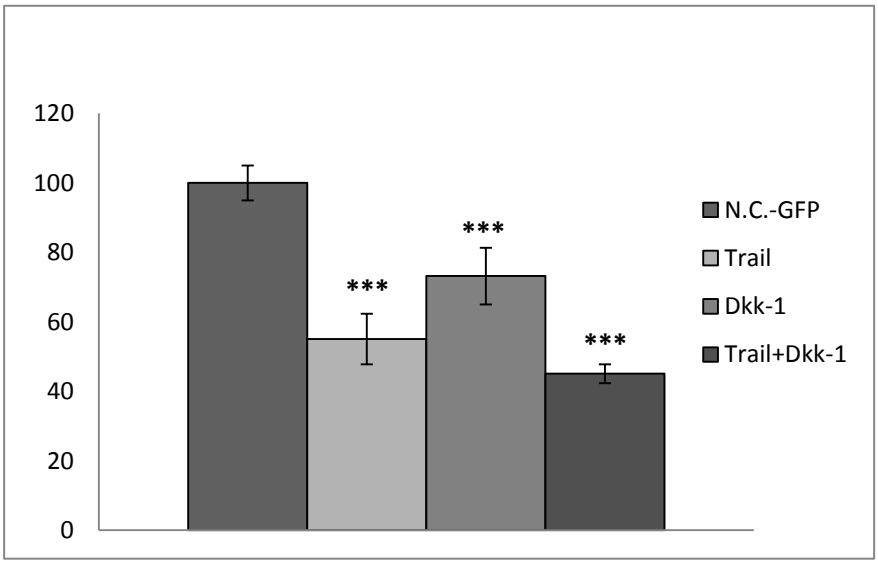

Figure 3.10. Cell viability analysis of SHSH5Y cells co-cultured with genetically modified hTGSCs by flow cytometry. N.C: Negative Control, GFP gene transfected group. ***p < 0.001 compared with N.C.-GFP

3.4.5. Real Time Analysis of SH-SY5Y Cells co-cultured with transfected GM-hTGSCs

Real-time analysis showed that GM- hTGSCs secreting TRAIL and Dkk-1 induce the apoptotic genes (Bax, caspase3, Fadd) and inhibit the proliferation genes (Bcl-2, Stat3) comprared to control group. Beta-actin was used as a house keeping gene to normalize results. The results was shown in Figure 3.11. and 3.12.

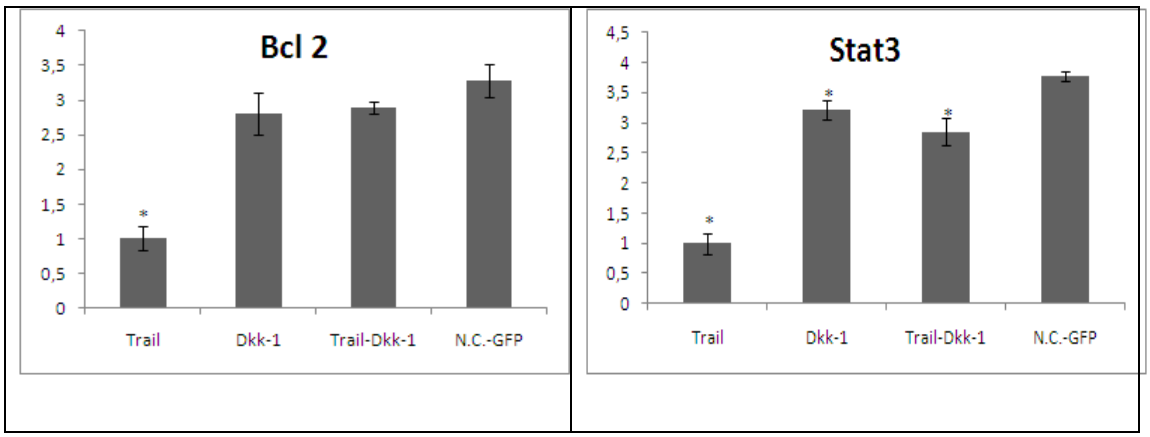

Figure 3.11. The proliferation genes expressions of SH-SY5Y cells co-cultured with hTGSCs secreting TRAIL, Dkk-1 or GFP. *$p < 0.05$ compared with N.C.-GFP

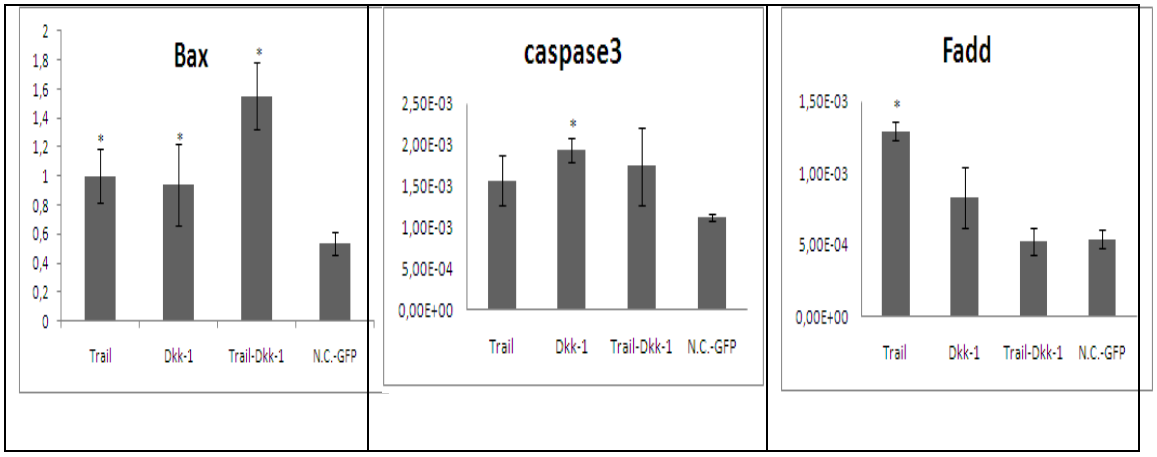

Figure 3.12. The expression of apoptotic genes of SH-SY5Y cells co-cultured with hTGSCs secreting TRAIL, Dkk-1 or GFP. *$p < 0.05$ compared with N.C.-GFP

4. DISCUSSION

In the recent years, tumor microenvironment has gained a great attention due to its contribution to the tumourigenesis and metastasis [56]. MSCs are important components of the tumor microenvironment in cancer progression. They have been the theme of an increased interest due to their ability to differentiate into multiple cell lines; adipocytes, chondrocyte, osteoblast, the fact that they can migrate towards inflammation site and their potential use as tissue regenerative cells and tools for gene delivery [57]. MSCs also secrete several paracrine factors for endothelial lineage cells, monocytes and macrophages as well as inflammatory factors including various chemokines and interleukins [58].

The complex role of MSCs in cancer progression is not clearly understood The relationship between MSCs and cancer cells have two horizons. The first one is MSCs can promote angiogenesis and increase tumor survival by becoming TAFs and secreting growth factors and cytokines. The second one is they can be used to inhibit the tumor growth directly after being genetically modified to secrete some genes such as Dkk-1, a negative regulator of Wnt signaling pathway or TRAIL [59].

In vitro systems to mimic tumor microenvironment are important for anti-cancer drug treatment and the establishment of model that mimic the relationship between cancer cells and stem cells. It helps to know the mechanisms underlying the tumor development and drug screening. Albert A. Rizvanov et al. 2010 have proven that cancer and stem cells interactions play important roles in the cellular behavior of cancer cells. To determine the MSCs effect on SH-SY5Y cells MSCs and SH-SY5Y cells wereco-cultured and it was observed that direct cell-cell interaction between MSCs and SH-SY5Y cells lead SH-SY5Y cells to form colony like structure as observed in the tumor biopsies. [60]. Lin Li Hui Tian et al. 2011 have investigated the effect of MSCs on lung cancer cell line A549 and esophageal cancer cell line Eca-109 in vitro and in vivo. This study suggested that MSCs have an ambivalent role in the tumor growth [61]. Karnoub et al. 2007 reported that weakly metastatic human breast cancer cells stimulated MSCs to oversecrete the chemokines, via a cell-to-cell contact, which then acted in a paracrine fashion on the cancer cells and enhanced their invasion, and metastasis [62].

In this study we investigated the effects of Human tooth germ (HTG) derived MSCs on SH-SY5Y cells and MCF-7 cells treated with doxorubicin and paclitaxel in vitro aiming to understand the specific roles of HTG derived MSCs in anti-cancer treatments.

Because of ethical problems concerning embryonic stem cell research, adult stem cells have gained a lot of interest for stem cell based investigations. In adult, there are several sources for stem cell and the main source of MSCs is the bone marrow [63]. Bone marrow MSCs (BM-MSCs) can be easily isolated and expanded in vitro but there are some risks and difficulties such as; surgical process, contamination, the limited amount of isolated cells [64]. Thus, establishment of a new stem cell sources has been gained a great attention. However, MSCs from other tissues have been described based on their properties; adipose tissue [65], umbilical cord blood [66], amniotic fluid [67], peripheral blood [68] and dental tissues [69]. Efforts to find the most suitable stem cell source and to develop most practical isolation procedures are still ongoing.

Tooth germs that is, pulp and surrounding tissues have been shown to be an alternative stem cell source. Dental stem cells (DSCs) are easily isolated after routin relativaley easy surgical operations from waste materials. Furthermore, DSCs do not cause any ethical argument [70]. Isolation procedure is extremely efficient and after the operation dental tissues show very low injury in the operated site. Therefore they seem to give a promise for solution of the problems related with the stem cell isolation and cultivation.

In this research, hTGSCs were successfully isolated from impacted third molar tooth germs and characterized for MSCs surface markers. They were cultured and when they reach the %70 confluency, conditioned medium was collected. The reason of chosen this confluency is to provide CM from healthy and efficient hTGSCs. %20 CM was applied to the SH-SY5Y cells and MCF-7 cells because our previous range determination studies showed that %20 is the most efficient concentration. Additionally, the suitable doses of doxorubicin and paclitaxel were assigned by the analysis of cytotoxicity with range studies. MTS results showed that CM of hTGSCs increased the survival of doxorubicin and paclitaxel treated MCF-7 and SH-SY5Y cells by approximately 30%. The cause of increased cell survival against doxorubicin and paclitaxel could be the secretion of several growth factors by hTGSCs. It has already been suggested that MSCs release some growth

factors (VEGF, PDGF, FGF-2, FGF-7) and cytokines (IL-6, TNF-α) [71-72]. But this is the first study demonstrated that CM of hTGSCs has the protective effect on MCF7 and SH-SY5Y cells treated with doxorubicin and paclitaxel reducing the effect of anticancer drug. Caspase-3 has been identified as being a key mediator of apoptosis of mammalian cells and it is activated in the apoptotic cells [73]. p53 is a tumor suppressor protein has many mechanisms of anticancer function, and plays a role in apoptosis, genomic stability, and the inhibition of angiogenesis [74]. The expression of caspase3 and p53 were analyzed and it has been revealed that CM reduced doxorubicin and paclitaxel induced apoptosis through downregulation of these two proteins. Several studies have shown that MSCs have the ability to inhibit apoptosis of various types of host cells through paracrine mechanisms. Kortesidis et al. 2005 also demonstrated that BM-MSCs express factors that support the cell survival and avoid apoptosis thereby preserving cells [75]. Timo Schinköthe et al. 2008 indicate that MSCs secreted angiogenic, immunosuppressive, anti-apoptotic, and proliferation-stimulating factors [76]. Supporting the data in the literature we have shown that the expression of caspase3 and p53 was lower in CM added SH-SY5Y cells than the control group upon addition of anti cancer drugs

When the cells were co-cultured enabaling physical cell to cell contact between cells and it was shown in Figure 3.4. MSCs increased the cell survival of SH-SY5Y treated with paclitaxel. Previously our findings have proved that the fusion occurs between HTG derived MSCs and SH-SY5Y cells under co culture conditions resulting in oncogenic resistance and a special self-organization pattern in SH-SY5Y cells like the ones observed in the tumor biopsies. They also observed that MSCs protect the SH-SY5Y cells from the oxidative stress and increase the survival of SH-SY5Y cells [59-77].

Although MSCs have properties related with promoting the tumor growth promotion, they can be modified to express anti-cancer molecule and used as vectors in cancer therapy. As part of this study; hTGSCs were transfected with apoptotic genes (TRAIL, Dkk-1) and in vitro effects of GM-hTGSCs on SH-SY5Y cells was investigated. The efficiency of engineered MSC to deliver cytotoxic drugs or cytokines with anti-cancer activity to the site of primary tumors has been recently tested in the preclinical models. For instance; MSCs modified to express IFN-β were able to inhibit tumor growth [78-79]. MSCs infected with adeno- or retrovirus encoding IL-12 showed inhibitory effects on the

tumor growth through the activation of NK cells and CD8+ T cells [80]. Laura S. Sasportas et al. 2009 showed that MSC-transfected recombinant TRAIL has profound anti-tumor effects in vivo by inducing the caspase-mediated apoptosis in established glioma cell lines [81].

TRAIL is apoptosis inducer protein in cancer cells while sparing normal cells, thus representing a new ideal candidate for tumor therapy. Although, because of its short pharmacokinetic half-life, the recombinant soluble protein needs high dose and repeated infusions to be effective. Loebinger et al. 2009 transduced BM-MSCs producing high quantities of TRAIL and showed that these cells exert high in vitro cytotoxic activity against lung, breast, squamous, and cervical cancers [82].

Dkk-1 is a negative regulator of Wnt signaling pathway and an inhibitory factor on tumor cell proliferation. Dkk-1 secreted by MSCs suppresses the Wnt signal activity in inhibiting cell proliferation [83-84]. Therefore, proteins that block Wnt signaling cascade, especially the soluble DKK-1, could be used in the tumor therapy [85].

Our findings also demonstrated that that GM-hTGSCs expressing TRAIL or Dkk-1 decreased the cell viability of SH-SY5Y cells when co-cultured, in comparison to GFP expressing hTGSCs. In addition we tested if GM-hTGSCs alter the gene expression of Bcl-2, Stat3 (proliferation genes), FADD, caspase3, Bax (apoptotic genes) in SH-SY5Y cells which was assessed by real-time PCR.

FADD, Fas-associated death domain, is the main signal transducing intermediate adaptor molecule of several death receptors including Fas, TNF-R1 (tumor necrosis factor receptor 1), DR3 (death receptor 3), TRAIL-R1 (TNF-related apoptosis-inducing ligand, DR4), and TRAIL-R2 (DR5) [86-87]. We have shown that, the expression of FADD was increased in SH-SY5Y cells co-cultured with TRAIL expressing GM- hTGSCs. We also found that Caspase3 expression was higher in SH-SY5Y cells co-cultured with Dkk-1 expressing GM-hTGSCs. Our data suggested that genetically modified MSCs derived from HTGs might be used as an anti cancer gene delivery vehicle.

Bcl-2 is a proto-oncogene and can suppress apoptosis. Bax is a protein of the Bcl-2 gene family. It promotes apoptosis by competing with bcl-2 proper [88]. Youxin Zhou et al. demonstrated that Dkk-1 has a pro-apoptotic function of in glioma and the expression of bax and caspase-3 increased, whereas the expression of bcl-2 decreased [89]. Fulda S. et al. 2002 has proved that the bcl-2 overexpression caused the inhibition of TRAIL-induced apoptosis [90]. Our results also showed that the expression of bcl-2 is the lowest in cancer cells co-cultured with TRAIL expressing GM-hTGSCs On the other hand GM-hTGSCs caused the increase of Bax expression in SH-SY5Y cells.

The activation of STAT3 has been reported to be sufficient to induce the tumor formation in several human cancers [91-92]. It has demonstrated that activated STAT3 plays an important role in the cross-talk between cancer cells and immune cells and the activated STAT3 inhibits the expression of mediators necessary for immune activation against tumor cells [93]. Many recent studies showed that inhibition of STAT3 caused immunosuppression in the tumor microenvironment [94]. In our study, the expression of STAT3 was shown to be reduced in SH-SY5Y cells which were cultured together with GM-hTGSCs expressing TRAIL or Dkk-1. This results suggest that GM-hTGSCs might reduce the suppression capacity of cancer cells making them more vulnerable to anti-cancer agents.

In conclusion; this study has shown that CM of hTGSCs increased the survival of MCF-7 and SH-SY5Y cells treated with doxorubicin and paclitaxel by 30% and also CM reduced doxorubicin and paclitaxel induced apoptosis. On the other side, genetically modified MSCs secreting TRAIL and Dkk-1 reduce the cell survival of cancer cells by inducing apoptosis and inhibiting the proliferation. Our findings demonstrated that HTG derived MSCs play roles in growth of tumor cells by secreting some cytokines or chemokines and they could be a promising candidate for the cancer gene therapy. For future, the effects of hTGSCs on different cancer cell line such as; PC3 (prostate cancer cell line), A549 (Human lung adenocarcinoma epithelial cell line), Y79 (Human retinoblastoma cell line) need to be investigated. To develop the clinic application of cancer gene therapy in vivo studies are set to be done.

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