INVESTIGATION OF CANCER DEVELOPMENT ON HUMAN NEWBORN FORESKIN CELLS AND HUMAN MELANOCYTES VIA EXOSOMES CONTAINING RAB27A AND MET ONCOGENE ISOLATED FROM HUMAN MALIGNANT MELANOMA CANCER CELL LINES

by

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

Yeditepe University
2017

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ACKNOWLEDGEMENTS

Primarily I want to thank my supervisor Prof. Fikrettin ŞAHİN. It has been an honor to be her first Ph.D. student. Following my thesis in his supervision has been an experience that widens the awareness and presents an unlimited source of learning. I appreciate all his helps of funding to make my Ph.D. experience creative and inspiring.

I am also appreciative for the outstanding model he has delivered as a successful scientist and professor. The associates of the molecular diagnostics laboratory have contributed hugely to my professional time at Yeditepe University. I am especially grateful to Dr. Pakize Neslihan TAŞLI and Dr. Selami DEMİRCİ for their aids and contributions throughout my thesis experimentation process.

I thank Burçin KESKİN for her technical support. I specially thank to Professor Salih SOMUNCU for being my role model as a scientist, parent, mentor and teacher.

Finally yet importantly, I would like to thank my mum Minire SOMUNCU, my dad Salih SOMUNCU and my brother Doruk SOMUNCU for their endless love and support, which made everything more beautiful. I also would like to thank to my fiancé Aykut EKICI for his motivating support and for always being there to help me.

ABSTRACT

INVESTIGATION OF CANCER DEVELOPMENT ON HUMAN NEWBORN FORESKIN CELLS AND HUMAN MELANOCYTES VIA EXOSOMES CONTAINING RAB27A AND MET ONCOGENE ISOLATED FROM HUMAN MALIGNANT MELANOMA CANCER CELL LINES

Exosomes are small membrane derived vesicles originating from endocytic core with 40-100 nm. Exosomes transmit DNA constituents, mRNAs, microRNAs and proteins that are relocated from a donor cells to a receiver cell. Many divergent cells comprising mesenchymal cells, immune cells and cancer cells discharge exosomes. Studies show that cancer cell exosomes create the entry and reprogramming of essentials connected to tumor environment. A reference study established that melanoma-derived exosomes convey diverse proteins such as c-Met and Rab27a, which indicate a melanoma mark. Increased Met expressions in serum exosomes have thought to be a predictor of disease progression. Meanwhile, Rab27a has identified to be important in exosome discharge. Decreased expressions of Rab27a in human melanoma cells determined to diminish exosome release. This project aims to examine the effects of downregulation and upregulation of Rab27a and c-Met in human melanocyte and recently identified stem cells isolated from human newborn foreskin tissue by utilizing the isolated exosomes from malignant melanoma cell lineages. Throughout the analysis of cancer-like formation; different protocols covering gene transfections, flow cytometry analysis of the transfection efficiency, Annexin-V apoptosis assays, cell migration assays, tube formation assays, ELISA assays and gene expression profiling were performed. According to the results, exosomes derived from cancer cells conveyed information to healthy fibroblastic like stem cells from human newborn prepuce and healthy melanocytes/keratinocyte and induce cellular reaction with Met and Rab27a overexpression and silencing may be beneficial approaches for future treatment possibilities. The developing molecular contextual of melanoma exosomes and their implications for improved management of melanoma patients can be an astonishing therapeutic methodology for future actions.

ÖZET

İNSAN MELANOMA HÜCRLERİNDEN SALINAN RAB27A VE MET GENLERİNİ TAŞIYAN EKZOZOMLARININ İNSAN YENİDOĞAN HÜCRELERİ VE İNSAN MELANOSİTLERİ ÜZERİNDEKİ KANSER OLUŞTURUCU ETKİLERİNİN İNCELENMESİ

Ekzozomlar endositik merkezden köken alan 40-100 nm büyüklüğündeki küçük veziküllerdir. Verici hücreden alıcı hücreye taşınması amacıyla içlerinde DNA parçaları, mRNA parçaları, mikroRNA ve proteinleri taşırlar. Mezankimal kök hücreler, immün sistem hücreleri, kanser hücreleri vb. ekzozom salınımı yaparlar. Çalışmalar kanser kök hücrelerinden salınan şekillenen ekzozomların tömör oluşumuna özgü çevresel düzenlemeyi sağlayacak bir çok yeniden programlama faktörlerini oluşturduklarını göstermektedir. Referans niteliğindeki çalışmaların başında melanoma kanser hücrelerinden tomurcuklanan ekzozomların c-Met ve Rab27a gibi melanomaya spesifik işaretleyici genlerin ve proteinlerin iletimini sağladıklarına dair bilimsel yayın gelmektedir. Serum ekzozomlarında artan c-Met ekspresyonu hastalığın gelişmesinde önemli bir etken olarak düşünülmektedir. Rab27a ise ekzozomların hücrelerden salınımında önemli bir gen olarak belirlenmiştir. Deri kanseri hücrelerinde azalan Rab27a ekspresyonunun ekzozom salınımı sekteye uğrattığı yapılan çalışmalar ile gösterilmiştir. Bu projede Rab27a ve c-Met ekspresyonlarının deri kanseri hücreleri ve dolayısıyla ekzozomlarında arttırılıp ve azaltılıp, sağlıklı insan yenidoğan sünnet derisi kök hücreleri ve insan fibroblastlarına uygulandıklarında nasıl bir etkilerinin olduğunun anlaşılması amaçlanmıştır. Bu süreçte hücrelere gen transfeksiyonları, akış sitometrisi ile transfeksiyonların etkilerinin bakılması, Annexin-V ile kontrollü hücre ölümü analizi, hücre göçünün miktarı, ELISA testleri ve gen anlatımı düzeylerinin analizi yapılmıştır. Deri kanseri hücreleri ekzozomlarının insan fibroblast görünümlü kök hücreler ve insan deri yetişkin hücreleri üzerinde kanser hücreline benzeyen davranışları arttırdığı ve azalttığı c-Met ve Rab27a ekspresyonlarının artışı ve azaltılışı üzerinden bu çalışma ile gösterilmiştir. Melanoma kanser hücrelerinden salınan ekzozomların içeriğinin belirlenmesi ve moleküler uygulamalar üzerinden gelecekteki çalışmalar için incelenmesi çok önemli bir terapi yöntemi olarak düşünülmektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

BMDCs	Bone Marrow Derived Cells
B-Raf	v-Raf Murine Sarcoma Viral Oncogene Homolog B
c-Met	Hepatocyte Growth Factor Receptor
CMV	Cytomegalovirus
CO_2	Carbon Dioxide
Dexosomes	Dendritic Cell-Derived Exosomes
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
ECM	Extracellular matrix
EGFR	Epidermal Growth Factor Receptor
EtOH	Ethanol
FBS	Fetal bovine serum
FGF	Fibroblastic Growth Factor
GAB1	GRB2-associated-binding protein 1
GFP	Green Fluorescent Protein
GRB2	Growth factor receptor-bound protein 2
GTP	Guanine Nucleotide-Binding Proteins
EV	Extracellular Vesicles
HDF	Human Dermal Fibroblasts
HGF	Hepatocyte Growth Factor
HGFR	Hepatocyte Growth Factor Receptor
hnFSSCs	Human Newborn Foreskin Stem Cells
HRPAvidin	Horseradish Peroxidase Avidin
Hsp	Heat Shock Protein
ISD	Information Services Division
LBPA	Lactoferrin-Binding Protein
Lenti	Lentivirus
mRNA	Messenger Ribonucleic Acid
miRNA	MicroRNA
MSCs	Mesenchymal stem cells

MVBs	Multivesicular Bodies
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-Kinase
PLC-γ	Phosphoinositide Phospholipase C
PM	Plasma Membrane
Puro	Puromycin
Rab	G-Protein
RFP	Red Fluorescent Protein
SCs	Stem cells
SF	Scatter Factor
SHP2	Tyrosine-Protein Phosphatase Non-Receptor Type 11
Slp	Synaptotagmin-Like Protein
STAT3	Signal transducer and activator of transcription 3
Texosomes	T Cell-Derived Exosomes
Tsg	Tumor Susceptibility Gene
Tyr	Tyrosine
VEGF	Vascular endothelial growth factor
LNs	Lymph nodes
ROS	Reactive oxygen species
UV	Ultra-violet

1. INTRODUCTION

1.1. EXOSOMES

Exocytosis is essential for numerous functions such as intercellular signaling and plasma membrane growth. It has been explained as the fusion of an intracellular trafficking vesicle with the plasma membrane [1]. Integration of multivesicular bodies (MVBs) with the plasma membrane and following release of their cargo signify an additional mechanism of exocytosis. MVBs may also interact with the plasma membrane triggering the discharge of internal vesicles into the extracellular space. Once they are released into the extracellular matrix, then can hit their way into blood, urine, lymph and other body liquids from where they may be isolated and studied. Those excluded packages has named as exosomes [2].

Exosomes are tiny vesicles released through a cell that cover different RNA and proteins, mostly specific to the cell and the cell state. Specific proteins, RNA or other materials may be collected and packed up in small packages and can be transferred to the surface of the cell [2]. Cargo molecules in another words the excluded packages also carry extracellular matrix (ECM) components, neurotransmitters and peptide growth factors which can be emancipated either constitutively or regarding to a particular stimuli [3].

Many cell types release exosomes via exocytosis covering hematopoietic cells, B and T lymphocytes, mast cells, reticulocytes, dendritic cells, intestinal epithelial cells, neurons, astrocytes, platelets and tumor cells. Regarding to their source, they have called as dexosomes (dendritic cell derived exosomes) or texosomes (T-cell exosomes or tumor exosomes) [2].

Exosome secretion is an organization to eliminate undesirable proteins for some cells. Exosomal discharge as an alternative to lysosomal dispensation can be of benefit to cells, which carry reduced degradative capabilities or positioned to a drainage arrangement like the tubules of kidney and the gut [4].

Exosomes have elaborated in lots of practices, both advantageous and pathological. Primarily, exosomes have categorized as a mean for the developing reticulocyte to eliminate superfluous proteins. Readings have revealed that exosomes are covered in removal of

redundant proteins or undesirable molecules from the cell, conversation of constituents among cells, intercellular communication, dissemination of pathogens, utilities of the immune system (both stimulatory and inhibitory) antigen demonstration and several more. Through witnessing the changing protein substances, it is easy to recognize how exosomes, which originate from dissimilar cell, sorts may have distinct utilities and evidence to be extremely beneficial molecules in many biological purposes [5].

1.1.1. Exosome Structures

Intercellular rudiments, which can be expressed in some manner moves towards the outside of the cell. Eukaryotic cells discharge materials into the extracellular surroundings by the exocytic trail in which the membranes of Golgi derivative vesicles, tubules and integrate with the plasma membrane [3].

Exosomes have defined as 40-100 nm diameter extracellular vesicles, which carry significant cup, shaped morphology once negatively stained under electron microscope. They circulate in the extracellular space neighboring the position of release that they can be crashed in a few minutes. Meanwhile, some of the exosomes may move far by diffusion and can appear in biological fluids such as blood, amniotic fluid, breast milk, semen, saliva and lung surfactant. Exosomes have explained as more stable than soluble proteins regarding to their double layer membrane made of a peculiar lipid composition along with protective proteins [3].

Proteomic studies showed that exosomes cover a particular subgroup of proteins from endosomes, the plasma membrane and the cytosol, nevertheless very limited from other intercellular organelles such as golgi, mitochondria and nucleus. Those studies established that exosomes signify a precise subcellular section, as they do not comprise a random set of proteins [6]. Extracellular vesicle array results from previous studies have shown that exosomes or other extracellular vesicles can be utilized to determine an authentic reflection of both phenotype as well as enumeration of circulating vesicles. Capture of exosomes have identified as carrying CD9, CD63 and CD81 on the surface [7].

1.1.2. Exosomes and Signalling

The discharge of membrane surrounded vesicles from tumor cells and platelets, probably clarifying explanations of extracellular vesicles (EVs) inside of the tissues or body fluids have acknowledged at least 40 years before [6].

Firstly, EVs were supposed to bud straight from the plasma membrane (PM). At the time of 1980s, two group revising reticulocyte maturation pronounced a more multifarious manner of EV secretion. The small vesicles molded by interior budding within the intercellular endosome, bringing the formation of a multivesicular body (MVB), that could then interact with the plasma membrane and discharge outside its internal vesicles. The uncommon EV secretion pathway has established later in antigen introducing cells, epithelial cells and tumor cells [6].

Regarding to double layer lipid structure of the exosomes, they may show a more effective instrument of signal distribution in comparison with the soluble molecules. They should indeed,

i. Offer more steady conformational conditions of the protein substance.
ii. Upsurge bioactivity of the proteins expressed in their context.
iii. Increase molecule bio-distribution (for the capability of microvesicles to recirculate in body fluids and spread into distant organs).
iv. Stimulate a more efficient interface with target cells (owing to the highly fusogenic features of exosomes) [8].

1.1.3. Extracellular trafficking and exosomes

There are some unexplained vagueness concerning the mechanisms modifiable exosome traffic between the outside and the inside of cells covering the internalization of exosomes in target cells. It has suggested that the communication of exosomes and recipient cells might happen through receptor ligand binding. On the other hand, exosomes pass into normal dendritic cells through an endocytic pathway or they can fuse with platelets [9].

Additionally, exosomes comprehend a quantity of mutual proteins, covering Tsg101, Hsc70 and various tetraspanins the proteins that contribute in vesicle formation and trafficking,

such as the LBPA-binding protein, Alix [10]. Meanwhile, other studies have concentrated on exosomal RNA; the kinds of RNA and the nucleotide arrangement of them, their capability to be transmitted amongst cells, their occupation whrn transported and the mechanism of how they are operated to MVBs and into exosomes. Innovative studies of Valadi and associates presented that exosomes are enriched in mRNA and miRNA [11]. Most recent results have recognized additional non-coding RNAs in exosomes, nonetheless restricted quantities of DNA or ribosomal RNA [12].

1.2. CANCER

Cancer is a foremost community health concept in the world. The likelihood of being spotted with an aggressive cancer is advanced for men (43%) than for women. The explanations for augmented vulnerability is men are not well unstated, but to some scope likely imitate alterations in environmental acquaintances, endogenous hormones and multifaceted connections amongst these effects. Current revisions propose that tallness may also be an influence. For adults younger than 50, caner prevalence is enhanced for women than for man related to the genital, thyroid and breast cancers in women. The assessed possibility of emerging cancer is founded on the typical experience of the overall population and can undervalue individual risk owing to variances in exposure (e.g. smoking antiquity), medicinal past and genetic defenselessness [13].

Cancer cells vary from normal cells in numerous means, which permit them to nurture unbridledly and turn out to be invasive. One vital dissimilarity is that normal cells are more specialized than the cancer cells whereas normal cells develop into very different cell kinds with precise utilities. Regarding to this fact, unlike normal cells, cancer cells last to split deprived of discontinuing [13]. Furthermore, cancer cells are able to disregard signals, which generally direct cells to end dividing, or that start a progression named as programmed cell death, or apoptosis, which the body utilizes to discard unwanted cells [13, 14].

Cancer cells are able to effect the normal cells, particles, and blood vessels that frame and nourish a tumor with an expanse called the microenvironment. For example, cancer cells may persuade neighboring normal cells to create blood vessels that fund tumors with oxygen and nutrients, which they must grow. These blood vessels also eliminate unwanted products from tumors [13].

Cancer cells also frequently avoid the immune system, a system of organs, tissues, and specialized cells that defends the body from infections and other circumstances. Even though the immune system usually eliminates injured or irregular cells from the body, some cancer cells can “hide” from the immune system [13, 14].

The enlargement of human cancers is a multistep progression in which normal cells gain features that eventually trigger their alteration into cancer cells. Several difficulties should be experienced for this process to happen; of these problems, is the capability to endure an inhospitable microenvironment. It is acknowledged that the intercommunication among tumor cells and their neighboring microenvironment is important for eliminating this problem, for the tumor to development, metastasize, and establish itself at reserved places in the body [15].

“Tumor” definition is originated from Latin, which funds a compulsive distension, whether of inflammatory, cystic, edematous or additional environment. With the intention of evading misperceptions, the definition tumor development is utilized up until the natural surroundings of the pathological swelling is proven. The word neoplasm is found to contain the abbreviation neo- (new) and plasis (formation). Carcinogenesis identifies the instigation of a tumor, and oncogenesis designates the conservation and the following progression of the tumor. Neoplasms can grow in the vicinity; they can attack or weaken neighboring tissues. Cells are separated from original tumors and they can be transported via blood or lymph in the direction of other organs, where they will mature subordinate tumors, a progression named metastasis. Metastasis happens with the help of imbedding, contiguity, tissue steadiness or canalicular route (tracheobronchial tree, digestive tract, etc.). Initial neoplasms signify the presence and expansion of a tumor cell clone, creating a nodule with infiltrative evolution, subsequent to metastasis and death. Manifold primary neoplasms are characterized by numerous tumor developments of several kinds in the same organ scheme or in dissimilar organ organizations. These neoplasms have found in more than a few animal types, with an improved incidence in dogs. The existence of nonthreatening tumors designates a disposition to malignant neoplasms. The Boxer breed is exposed to a significantly increased risk for the development of multiple primary neoplasms, and 6-8 years old children presents an advanced probability of neoplasm formation. Hormonal factors have shown to be menace aspects for manifold primary neoplasms [15].

Benign tumors are unhurried tissue progressions, native spreads, deprived of the attack to nearby tissues, without the manufacture of metastasis and short of reappearances after surgical operations. These physiognomies have directed the name respectable environment tumor. Malignant tumors are identified via fast spread, the assault of the adjacent structures, repetitions next to surgery and metastasis, ensuing most commonly in the demise of the pretentious individual. These features have resulted the utilization of the definition depraved environment tumor [15].

Once the post-mitotic offspring cells do not improve, the distinguished tumor is categorized as immature such as the immature squamous cell carcinoma or malpighian carcinoma, explained with feeble or no keratinization. Anaplastic tumors are homogenous organizations progressively misplace their structural properties that makes them less parallel to the initial tissue. Solid tumors is the identification of neoplasms shaped by thick, undeviating tissue, with no variation propensity. Endophytic tumors on the other hand are established by growth on the way to the penetration of the tumors such as the surface epithelia tumors that expanse via piercing the subjacent tissues. Exophytic tumors are defined with progression through the outside to papillae. Tubular tumors are considered via assemblies underneath the tubules which covers neoplastic cells and are detached by an interactive stroma. Meanwhile cystic tumors are neoplasms including one or more holes with fluid, necrosed tissue or keratinized structures. Simple tumor is a definition utilized for single tumors covering epithelial cells. Complex tumors are explained with the existence of vascular connective tissue amongst epithelial structures. Teratomas are congenital tumors created via embryonic tissue related tissue types. Examples to teratomas may be the malignant teratomas or teratocarcinomas like dermoid cysts are benevolent teratomas [15].

Recent studies have indicated that tumor cells initiate to shape the host environment starting at early stages of the neoplastic development, to help their survival and growth. When host components like the immune system may primarily try to restrict disease formation, barricades gradually round via the motivation of suppressive pathways or even revolved into tumor-promoting elements [9].

From a therapeutic perspective, cancer has confronted as a disease originating from modifications of the cellular genome and changing expression and occupation of oncogenes and tumour suppressor genes. Though, it is currently extensively acknowledged that tumor microenvironment carries an essential part in cancer growth and evolution. Certainly, tumor

cells start to alter the host environs, initiating at primary stages of the neoplastic progression, to promote their endurance and enlargement. Once the immune system firstly tries to limit cancer headway, defense mechanisms of the body increasingly dulls via the suppressive pathways. Changes of dysfunctional dendritic cells (DCs), absence of normal immune response or apoptosis in anti-tumor effector T cells, have defined to happen in cancer patients, while the definite involvement of these paths in supporting *in vivo* development requires to be completely identified in the human background. Clearly, the difficulty of the setup recognized amongst tumor cells and their microenvironment turns the examination of possible interferences for unsettling these harmful connections, into a difficult task [8].

Notwithstanding the self-origin of most tumor antigens, the manifestation of unplanned cancer immunity in cancer is a well-recognized concept. This progression, defined as cancer immune-oversight, signifies the venture of the immune system to cover cancer development in the initial stages of expansion. Nonetheless, this complaint of steadiness frequently miscarries the disease evolution and outflow appliances embraced by tumor cells to silence their immunogenic property and endure via triggering immune-suppressive pathways. Although the fine alterations occurring in cancer cells, which are in control for the shift from immune acquaintance to immune suppression, are partly defined, transformed cells are irrefutably a kind of pool of damaging particles intensely meddling with host occupations [8].

The eventual properties of these pathways can actually been observed at a tumor location, in demanding lymph nodes (LNs) and in the peripheral flow of cancer patients as deficiencies practically altering the immune system. Regarding to their main function in training precise immune responses, dendritic cells require demonstrating the front line of immune response. Additionally, dendritic cells are effected in case of cancer mostly on their capability to submit exogenous antigens over their antigen dispensation mechanism [8].

1.2.1. Melanoma Cancer

Malignant melanoma is an aggressive type of skin cancer whose occurrence last to enhance worldwide. Even though a huge number of primary melanomas may be cured through surgery, treatment of metastatic melanomas stay puzzling. Mostly, melanoma kind nevus

which turns into dysplastic lesions before developing into a primary melanoma that can conquer into the dermis and local lymph nodes and from there migrate to reserved metastatic sites [16].

The frequency of malignant melanoma has amplified quickly throughout the past several decades (Figure 1). A study distributed in 2006 assessed that; there would be 62,190 novel cases of melanoma and 7,910 deaths that year, making it the most public of all skin malignancies. The mainstream of these deaths are regarding to distant metastasis from the primary site since melanoma is notorious for its partiality to metastasize [17].

Melanoma is poorly open to cytotoxic chemotherapy and the continued existence of patients is founded on screening, early detection and extensive elimination of the primary lesion. The inclusive endurance for patients with metastatic melanoma changes from only 4.7 to 11 months, with an average survival of 8.5 months [17].

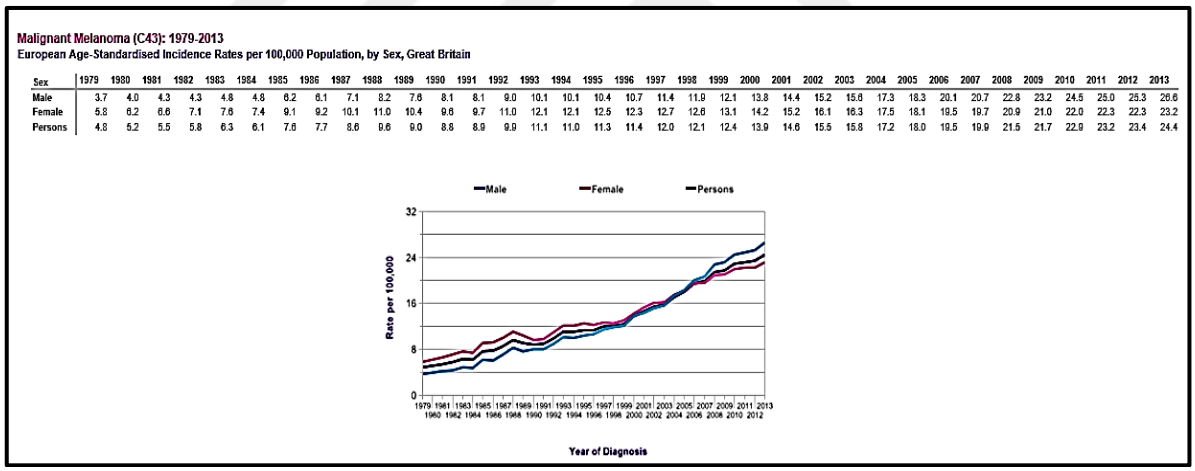

Figure 1.1. The frequency of malignant melanoma.

Data has provided by the Office for National Statistics on request, July 2015, ISD Scotland on request, April 2015, ISD Scotland on request, April 2015 and Welsh Cancer Intelligence and Surveillance Unit on request, February 2015.

Numerous aspects should be noted once one cures a patient with metastatic melanoma. These aspects cover the complete illness and age of the patient, the location and amount of metastasis the step of the illness and the patients' willing for treatment. There are different treatments for melanoma including single agent chemotherapy (decarbazine, taxanes, temozolomide), combinational chemotherapy with or without tamoxifen (Dartmouth

regimen, cisplatin, decarbazine), immunotherapy (interferon-alpha, interleukin-2), biochemotherapy, radiation therapy and surgery [17].

UV endorses malignant alteration in the skin via carrying straight mutagenic influences on DNA, by encouraging the cellular elements of the skin to generate growth factors, through decreasing cutaneous immune defense, and via stimulating ROS of melanin that cause DNA damage and suppress apoptosis. Melanoma grows with the reasons of accrued deformities in genetic paths inside of the melanocyte. These deficiencies stimulate cell spread and inhibit normal apoptosis. The changed melanocyte is thus inclined to accumulative DNA impairment causing genetic alterations, which let a creation of malignant-like phenotype. Even though the appliances of distinctive cancer cell assassination are not well understood, assortment of cells that are robust to apoptotic progression might donate to opposition of melanoma cells to the cytotoxic properties of chemotherapy, radiotherapy and immunotherapy particularly over the expression of apoptosis inhibitors like Bcl-2 and Bcl-xL. Growth factors like stem cell factor, fibroblast growth factor and transforming growth factor α are manufactured via the exploit of solar radiation on melanocytes and neighboring keratinocytes and fibroblasts. Subsequent signals are transduced via the Ras/RAF paths, eventually activating the transcription of a group of genes covered in cellular expansion and motility. Solar radiation also arouses creation of melanocortin that gestures with cyclic AMP to persuade manufacture of sun-defensive pigment eumelanin. Alterations in melanocortin receptor causes a generatin of pheomelanin that is anti-sundefensive. In other words, melanin harvests may trigger oxidative stress and subsidize a malignant modification [17].

SK-MEL-1 has proven as the first of a group of melanoma cell lines identified from patient-derived tumor samples. This cell lineage can be grown in suspension culture. They have detected to express mutant B-Raf (V600E) and wildtype N-Ras. This cell line has isolated in 1966 from a metastatic site (thoracic lymph duct) in a 29-year-old Caucasian male with malignant melanoma [18].

SK-MEL-3 is one of a series of melanoma cell lineages that has obtained from patient derived tumor samples. This cell line has recognized to generate tumors in immunocompromised mice. This cell line has been isolated in 1972 from a metastatic site (lymph node) in a 42-year-old Caucasian female with malignant melanoma [19].

A continuous tissue culture line (SH-4) isolated from pleural effusion cells of a patient with metastatic melanoma has described in 1975. Pigment deposition in the cells has identified as light and unfinished. However, more noticeable than in cells of the original pleural effusion which has been demonstrated by electron microscopy [20].

Among 1995–2010 several readings stated the existence of a precise sub-population of cancer cells described by self-renewal and the ability to initiate, restock and enlarge human tumors. These cancer cells with stem cells properties and labelled as cancer stem cells or tumor-initiating cells have been insulated from dissimilar human solid tumors such as melanoma. Cancer stem cells have been testified to express a diversity of markers covering CD34, ALDH1, CD271, CD44, ALDH1 and JARID1. Therefore none of them has been publicized to be actually CSC-specific [18].

This philosophy infers that therapeutic obliteration of CSCs may damage tumor development. Meanwhile, one should contemplate the capability that diverse subpopulations of CSCs can present inside single tumors (intra-tumoral heterogeneity) counting melanoma and between dissimilar tumors (inter-tumoral heterogeneity). In the nonexistence of consistent markers that may describe such subpopulations it will be problematic, if not difficult, to operate and eradicate tumorigenic CSCs and to utilize them as a target for therapeutics [18].

A related difficulty of the theory of CSCs deceits in the frequency and ambiguity of CSCs in each human solid tumor that in the majority of situations studied have been specified to be low, so interrogating the skilled pro-tumorigenic jobs of these cells. A distinguished exclusion is melanoma where the incidence and development of single human melanoma CSCs as measured via xenotransplantation in immune-deficient gene altered mice, could reach 27% [18].

In order to be operative in tumor-endorsing action, CSCs require to eliminate the antitumor immune-related responses of the patient. Melanoma CSCs have revealed to express a diversity of antigens acknowledged to be recognized via T cells such as MelanA/Mart1, HMB45, tyrosinase, gp100, NYESO1. Nevertheless, variation and cancer testis antigens regularly provoke a feeble answer even in purposely-immunized melanoma patients that infrequently is linked with a scientific comeback [18].

To clarify this, studies established that melanoma CSCs may trigger numerous appliances, which let them to endure in an antagonistic microenvironment and eliminate immune reactions as it can happen with other tumors like glioblastoma and colorectal cancer. Furthermore, human tumors CSCs have revealed to be recognized and demolished via autologous NK cells related to the differential expression of specific markers such as CD133, CD117, CD271 [18].

1.3. EXOSOMES & CANCER

Cancer cells have thought to pattern microenvironment constituents and affect immune system utility mostly by pathways containing cell-to-cell interaction and the discharge of suppressive soluble elements. Conversely, a different innovative mechanism that is currently developing includes the dynamic discharge via tumor cells of immune suppressive membrane microvesicles, defined as exosomes [9].

Recent studies have established that exosomes may carry a bimodal part in cancer. Exosomes have detected to control the local and systemic environment to assistance the cancer growth and dissemination [21]. Therefore, they have presented to be able to carry oncogenic signal receptors and beta-amyloid peptides from one cell to another, along with the precise subsection of mRNAs and microRNAs [22].

To metastasize, tumor cells require to change their microenvironment to adjust circumstances for deposition and development mutually locally and at a distance. Regarding to the “seed and soil” hypothesis for instance cancer stem cells or metastatic cells work as “seeds” and a specific organ niche assists as the “soil”. Exosomes discharged from tumor cells alter the resident tumor microenvironment, remodeling extracellular matrix, and endorsing vascularization and tumor cell proliferation. Exosomes move to aloof places to encourage the production of the pre-metastatic niche. Vascularization is amplified and endothelial and stromal cell differentiation is persuaded, foremost to a pro-tumor environment. Possible locations for distant tumor imbedding may be arranged onward to real metastasis. For certain cancers like metastatic melanoma, the progression of metastasis includes lymphatic distribution even though the exact role of lymph nodes perform in helping this process is not identified yet [23].

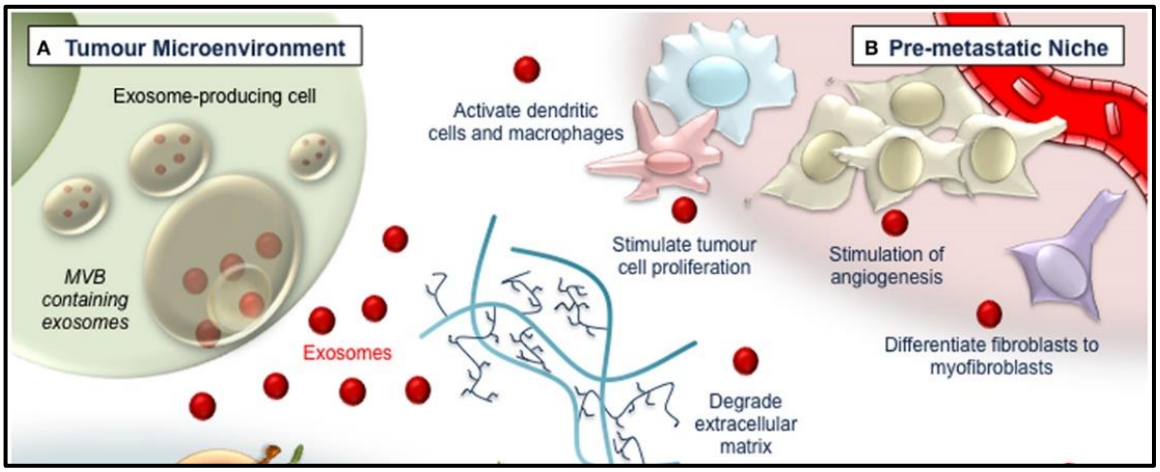

Figure 1.2. Exosomes help promoting the "seed and soil hyposthesis" [23].

Another hypothesis suggests that melanoma cells experience concurrent hematogenous and lymphatic spread and the existence of tumor cells in sentinel or local nodes is just revealing to metastasis. On the other hand, sentinel or regional nodes carry a vigorous role in the development of melanoma metastasis. The results that regional lymph nodes downstream of melanomas endure responsive lymphangiogenesis important to metastasis propose that melanoma metastasis is aided via the arrangement of a pre-metastatic niche inside of the lymph nodes. This progression is thought to be facilitated by tumor secretion of paracrine angiogenic growth factors [2].

Exosomes are naturally occurring biological nanovesicles (~ 40-100 nm) that are formed by the inward budding of multivesicular bodies (MVBs), as a component of the endocytic pathway. They are generated constitutively and released into the tumor microenvironment and circulation via fusion of multivesicular bodies with the tumor cell plasma membrane. The nanoscale size of exosomes facilitates their penetration and interaction with local tumor cells as well as with cell types that are distant to an advancing tumor cell front. This may result in tumor immune evasion by direct suppression of T cell activation and induction of apoptosis, suppression of the antitumor activity of natural killer cells and other mechanisms [24].

Exosomes released through normal and cancer cells transmit and deliver a diversity of molecules. Up to now, mechanisms stating the tumor exosome trafficking comprising release and cell transmission have not defined. Exosomes may be vigorously discharged inside of

the tumor tissues or directly dropped into the bloodstream but basically deprived of any commitment to tissue targeting [9].

A clinical subtype of glioblastoma has detected as EGFRvIII mutant/variant. Circulating serum exosomes have distinguished as positive for this mutant/variant EGFRvIII while the parental glioblastoma cells have also expressed this mutant/variant [21].

1.4. RAB27A GENE

1.4.1. GTPase Superfamily, Rab Family

Protein transport is controlled via different effectors. The word "controlled secretion" usually has been utilized for the latest stage of the exocytic pathway stimulus. Throughout the progression, neuropeptides and hormones are deposited in secretory granules and discharged after prompt of an exterior initiate. Therefore, "fundamental" transport, which does not need exterior stimuli, is also delimited, and its arrangement is significant for permitting protein and membrane conveyance during preserving cellular association. Initially, instruction is obligatory for discriminative categorization of cargo into vesicles and for directing the track and specificity of next vesicle training and merging. Secondly, studies proposed that organization among separate secretory steps can be vital for the precarious stable- position conservation of fragment morphology. Thirdly, regarding to the importance of protein transport in cell interaction with their neighboring environment, this transportation may be controlled via additional cellular routes over cell signaling. Small GTPases which comes from the Rab family are key regulators of protein trafficking in entire stages [25].

The compartmentalization of eukaryotic cells need the transportation of lipids and proteins among separate membrane confined organelles. This carriage is strongly controlled and usually happened through transport vesicles that bud from a donor section and fuse with an acceptor part. The Rab family is derived from the Ras superfamily of small GTPases have defined as central regulators of vesicle maturing, motility and fusion [26].

Rab GTPases regulate trafficking in the endocytic and secretory paths via transferring particular effector proteins onto membrane surfaces to provide either cargo collection, organelle motility or vesicle docking at target membranes [22].

1.4.2. Exosome and Rab27a

Trafficking of biological substantial through membranes is an evolutionary preserved mechanism and is part of any normal cell homeostasis. Such transport is composed of active, passive, export through microparticles and vesicular transport (exosomes) that collectively maintain proper compartmentalization of important micro and macromolecules [27].

Rab27a is a participant of small GTPase Rab27 subfamily that regulates carriage and exocytosis of lysosome associated organelles in particular cell lineages. Rab27a in its active GTP bound form positions to the membrane of melanosomes [28].

The participation of Rab27a in vesicle secretion was long-established in many tumor cell lineages, such as murine melanoma and mammary carcinoma and human squamous cell carcinoma cells. It is significant to understand that Rab27a regulates not only exosome secretion but also the discharge of a subgroup of soluble factors. For example, inhibition of Rab27a reduced secretion of the soluble prometastatic factor metalloproteinase 9 in 4TI mammary carcinoma cells, along with the pro-angiogenic placental growth factor 2, platelet derived growth factor A and osteopontin in the B16-F10 melanoma cells [6].

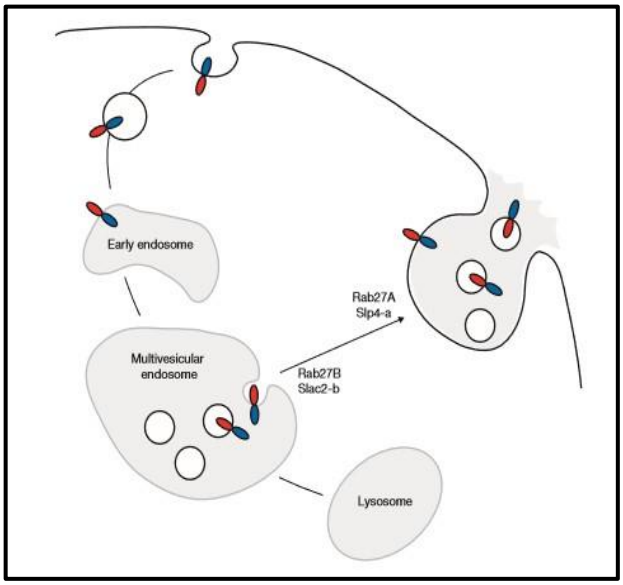

Figure 1.3. The part of Rab27 proteins and their helpers in the discharge of exosomes.

Receptors of the cell surface are endocytosed and transported to early endosomes, in a place that ubiquitylated receptors are received into tiny intra-lumenal vesicles, thus creating multivesicular endosomes (MVEs). Loads of receptors packed into the tiny vesicles are brought to lysosomes for a breakdown; in less common occasions, exosomes are molded via undeviating fusion of MVEs by the plasma membrane. Ostrowski et al. display the title role of Rab27B and Rab27A and their particular helpers, Slac_2b and Slp4, in exosome discharge. It is not acknowledged if Rabs work over a shared path or whether they trigger similar ways so far. Slp4 and Rab27A are similarly operate close by to membrane fusion; Slac_2b and Rab27B maybe interrelate with an undefined molecular motor which controls movement of a subgroup of MVEs (Figure 3) [29].

1.5. C-MET ONCOGENE PROTEIN

A receptor tyrosine kinase called as c-Met elaborates in invasion, cell proliferation, angiogenesis and metastasis. c-Met has been recognized to encourage the aggressive development of cancer cells, enhance their metastasis capacity and also acknowledged to be secreted and genetically altered in a diversity of solid tumors [17].

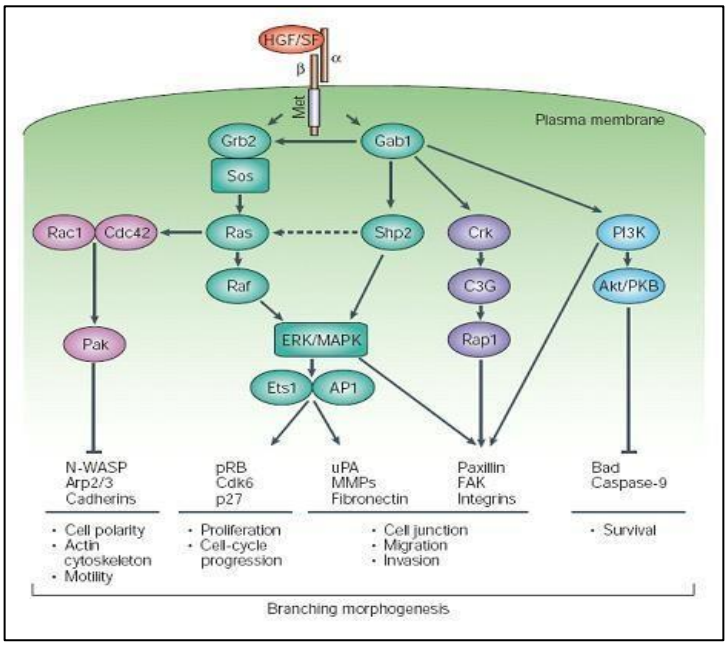

Figure 1.4. Scheme of c-MET stimulation with its ligand HGF (Hepatocyte growth factor) [30].

This stimulation encourages c-MET kinase catalytic action that activates the tyrosine Tyr 1234 and Tyr 1235 transphosphorylation. Two tyrosines involve numerous signal transducers, therefore introducing a huge range of biological actions determined via c-MET, jointly distinguishes as the aggressive development program. These transducers act together with the intracellular multi-substrate c-MET docking portion either straightly, like GRB2 and the phosphatidylinositol-3 kinase (PI3K) regulatory subunite p85, or circuitously over the scaffolding protein Gab1. Tyr 1356 and Tyr 1349 of the multi-substrate docking sites are equally covered in the interface with SRC, SHC and GAB1, where only Tyr 1356 is elaborated in the enrolment of p85, SHP2 and GRB2. GAB1 is a crucial controller of the cellular reactions to c-MET and integrates to the c-MET intracellular area with elevated efficiency, but decreased affinity. Right after the interface with c-MET, GAB1 turns into phosphorylated on different tyrosine deposits, which, collect a quantity of signaling effectors, containing PLC-γ, SHP2 and PI3K. Phosphorylation of GAB1 via c-MET outcomes in a continued signal, which facilitates most of the downstream signaling paths (Figure 3) [30].

1.5.1. C-Met oncoprotein and Melanoma Cancer

Most missense alterations of c-Met are stated in a diversity of cancers, by means of them acknowledged in the cytoplasmic stimulation loop tyrosine kinase domain. On healthy skin, c-Met is existing on melanocytes and epithelial cells, where HGF is created largely via mesenchymal cells and so, interrelates through its receptor in a paracrine way. HGF is a human melanocyte mitogen and c-Met overexpression relates with the aggressive development stage of melanoma cells [17].

Hepatocyte growth factor is indiscernible from scatter factor (SF), formerly acknowledged as an influential polypeptide factor encouraging epithelial cell movement. HGF/SF has publicized to employ a pleiotropic action on numerous cells mostly coming from epithelial root. It is an important mitogen for hepatocytes mutually in vitro and in vivo and induces the growing in vitro of some extra epithelial cells covering keratinocytes, endothelial cells and kidney tubular cells [31].

Remarkably, HGF/SF and its receptor in stimulating evolution of tumor cells and in prompting the metastatic actions has suggested. It has lately revealed that the c-Met coded

HGF/SF receptor is highly expressed in the gastrointestinal tract carcinomas and in thyroid carcinomas derived from clinically and histologically progressive subtypes. HGF/SF has established to be mitogenic for melanocytes, generally in attendance of synergistic influences and its receptor has spotted in melanomas grown and melanocytes *in vitro* [31].

A former study has focused on the narration of c-Met/HGF receptor in the regular antiquity of human melanocytic lesions, via investigating primary melanomas, benign nevi and metastasis. They have given away that the expression of noticeable c-Met/HGF receptor in an immunological manner upsurges in metastatic lesions, signifying an association with the development of melanoma [31].

2. AIM OF THE STUDY

Exosomes associated with the tumor cells endorse metastatic niche formation via training BMDCs (bone marrow derived cells a pro-metastatic and pro-vasculogenic phenotype via upregulation of c-MET oncoprotein. Studies indicate that a particular expression form of Ras associated (Rab) proteins are linked with exosome generation in melanoma [32]. Therefore, the project aims to investigate the effects of downregulation and upregulation of c-Met and Rab27a in human melanocyte and recently identified stem cells isolated from human newborn foreskin tissue via using the isolated exosomes from malignant melanoma cell lineages. According to those literature knowledge, this study aims to define the melanoma development from healthy melanocyte cell lines (human dermal fibroblasts) and chosen fibroblastic shaped stem cells.

3. MATERIALS

3.1. CELL CULTURE MEDIA

Diverse diversity of cell culture media and their supplements are delivered from Invitrogen, Gibco, UK Company. The variations that have ordered are listed on the Table 3.1

Table 3.1. Cell culture medium and the supplement varieties of GIBCO, UK Company and ATCC, USA Company.

Variety	Catalog Number#
Dulbecco's Modified Eagle's Medium (DMEM) low glucose	31885-049
Dulbecco's Modified Eagle's Medium (DMEM) high glucose	41966-052
Fetal Bovine Serum, qualified , heat inactivated, Canada origin	12484-010
Antibiotic-Antimycotic (100X)	15240-062
Dermal Cell Basal Medium (ATCC, USA)	PCS-200-030
Melanocyte Growth Kit (ATCC, USA)	PCS-200-041

3.2. CELL LINEAGES

Every single cell line beside the human newborn foreskin cells are sent from ATCC,USA Biotechnology Company. List of the cell lineages are shown on the Table 3.2

Table 3.2. Cell lineages that were used and their related companies/catalog numbers which mostly ordered from ATCC, USA.

Variety	Catalog Number#
SK-Mel 3 (Malignant Melanoma)	HTB-69
SH-4 (Malignant Melanoma)	CRL-7724
SK-Mel 1 (Malignant Melanoma)	HTB-67
Human Newborn Foreskin Cells	Isolated by Yeditepe University Biotechnology Laboratories
Human Primary Epithelial Melanocytes (Human Dermal Fibroblasts)	Isolated by Yeditepe University Biotechnology Laboratories

3.3. PLASMIDS AND LENTIVIRAL PARTICLES

Rab27a and Met expressions of the cells either induced or inhibited via plasmids or lentiviral particles. List of those are indicated on Table 3.3.

Table 3.3. Plasmids that were utilized for the transfections and their related companies/catalog numbers which mostly ordered from Santa Cruz, USA.

Variety	Catalog Number#
Rab27a siRNA (Human, Santa Cruz)	sc-41834
Met siRNA (Human, Santa Cruz)	sc-29397
Met shRNA Lentiviral Particle (Human, Santa Cruz)	sc-29397-V
pLenti-GIII-CMV-hRAB27A-RFP-2A-Puro Lentiviral Vector (ABM)	LV280228
pLenti-MetGFP (AddGene)	37560

3.4. QUANTITATIVE REAL TIME PRIMERS

Specific genes expression patters that examined via the quantitative real time PCR are listed below. Primer sequences for marker genes illustrated in Table 3.4.

Table 3.4. Primers that utilized for the real time PCR that mostly ordered from Life technologies, USA.

Primers	Sequences
Rab 27a Primers	F,5'GAAGCCATAGCACTCGCAGAG3' R5'ATGACCATTTGATCGCACCA3' [33]
c-Met Primers	F5'CATGCCGACAAGTGCAGTA3' R5'TCTTGCCATCATTGTCCAAC3' [34]
STAT3 Primers	F5'GAGGACTGAGCATCGAGCA3' R5'CATGTGATCTGACACCCTGAA3' [35]
Caspase-7 Primers	F5'GGAGAAAGCCAGGCTGTGT3' R5'TCCCCTTGGCTGTGTTTTG3' [36]
Bcl-2 Primers	F5'CCTGTGCACCAAGGTGCCGGAACT3' R5'CCACCCTGGTCTTGGATCCAGCC3' [37]
BAX Primers	F5'TGGAGCTGCAGAGGATGATTG3' R5'GAAGTTGCCGTCAGAAAACATG3' [38]

3.5. EXOSOME PURIFICATION

The process will be performed via using an optimized centrifuge and total exosome isolation kit (Invitrogen, Life Technologies, USA). Materials that applied for the process are listed on 3.5.

Table 3.5.Exosome Isolation and expansion materials.

Exosome Purification and Characterization Chemicals	**Catalog Number#**
Total Exosome Isolation Kit From Cell Culture Media (Invitrogen, Life Technologies)	4478359
Exosome-Depleted FBS (GIBCO, Life Technologies)	A25904DJ

3.6. ELISA KITS

ELISA Kits were used in order to analyze the expressions of each and individual genes that have been selected. ELISA Kits that employed are listed on Table 3.6.

Table 3.6. ELISA Kits and their catalog numbers/companies.

ELISA Kits	**Catalog Number#**
Human Epidermal growth factor,EGF ELISA Kit (Bioassay Technology Laboratory)	E0144Hu
Human basic fibroblast growth factor,VEGF ELISA Kit (Bioassay Technology Laboratory)	E228013
Human Ras-related protein Rab-27A(RAB27A) ELISA kit (Cusabio)	CSB-EL019177HU
Human hepatocyte growth factor receptor(cMET/HGFR) ELISA Kit (Cusabio)	CSB-E13490h

3.7. CLONOGENIC ASSAY, ANGIOGENESIS ASSAY, CELL MIGRATION ASSAY AND ANNEXIN V-FITC APOPTOSIS DETECTION ASSAYS

Every single assay that was mentioned at the title will be ordered from different biotechnology companies. List of the kits and their related companies are specified below (Table 3.7.)

Table 3.7 Different Kits for specialized functions and their catalog numbers/companies.

Specific Kits	**Catalog Number#**	**Clinical Use**
Clonogenic Assay Kit (BioPioneer, USA)	CA-001	Clonogenic Assay
Cultrex Cell Migration Assay (R&D Systems)	3465-096-K	Cell Migration, Cell Invasion Assay
Annexin V-FITC Apoptosis Detection Kit (abcam, Turkey)	ab14085	Apoptosis Assay
BD Matrigel Matrix Growth Factor Reduced (BD Sciences, Turkey)	354230	Angiogenesis Assay

4. METHODS

4.1. CELL CULTURE OF ALL THE CELL LINES

Human newborn foreskin cells were composed from the newborn foreskin (prepuce) tissue in Yeditepe University/TURKEY Biotechnology Laboratories. The newborn foreskin cells were grew to confluency in Dulbecco's Modified Eagle's Medium (DMEM) low glucose supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) PSA (10.000 units/mL penicillin, 10.000 µg/mL streptomycin, 25 µg/mL amphotericin B) (Invitrogen, Gibco, UK). Human dermal fibroblast cells were ordered from a biotechnology company (ATCC, USA). Human primary epithelial melanocytes were also ordered from ATCC, USA with the code of PCS200-013. The human dermal fibroblast cells were grown in Dermal Cell Basal Medium (ATCC PCS200-030, USA) supplemented with 1% (v/v) PSA (10.000 units/mL penicillin, 10.000 µg/mL streptomycin, 25 µg/mL amphotericin B) (Invitrogen, Gibco, UK). Human malignant melanoma cell lines which are SK-Mel 1 and SK-Mel 3 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) PSA (10.000 units/mL penicillin, 10.000 µg/mL streptomycin, 25 µg/mL amphotericin B) (Invitrogen, Gibco, UK). Once the cells were seeded on T-150 flasks (Zelkultur Flaschen, Switzerland), they were preserved at 37 ^{o}C and 5% CO_2 in a humidified incubator.

4.2. PLASMID INTRODUCTION TO THE CELLS

Lenti-GIII-CMV-Mouse-RAB27A-RFP-2A-Puro Lentivirus were used in order to overexpress the Rab27a gene of host cells. Lentiviruses carry the ability to participate into the host genome and produce a stable cell line expressing the selected gene. The vector size is 8834bp, the tag of ir is RFP and the selection marker is puromycin. Cells were plated to 24-well plate, 24 hours before the viral infection at a density of 0.5×10^5 cells per well. 0.5 ml of complete optimal medium (with serum and antibiotics if required) was added and to the wells and the plate was incubated the cells at 37°C with 5% CO_2 overnight. A combination of complete media with polybrene was arranged at a concentration of 8 µg/ml.

The growth media then was removed from the wells and was exchanged with 0.5 ml of the polybrenemedia-mix per well. One well was maintained left of uninfected as an additional standard control. After the infection, cells were incubated at 37°C with 5% CO_2 overnight. The culture medium was removed and was replaced with 1 ml of complete medium. Cells were incubated at 37°C with 5% CO_2 overnight. The next day, cells were passaged as 1:3 or 1:5 and will be endured incubating for 48 hours in complete media. The infected cells could then be carefully chosen for stable expression by appropriate antibiotic selection at a minimum concentration, as found via a killing curve. Downstream expression was then examined by a number of techniques, covering RT-PCR. The other plasmid that was used to enhance the Met expression is pLenti-MetGFP. The vector size is 4170bp, the tag of it is GFP and the selection marker is also puromycin. Therefore, the same protocol for that was used for LentiGIII-CMV-Mouse-RAB27A-RFP-2A-Puro Lentivirus applied to it as well.

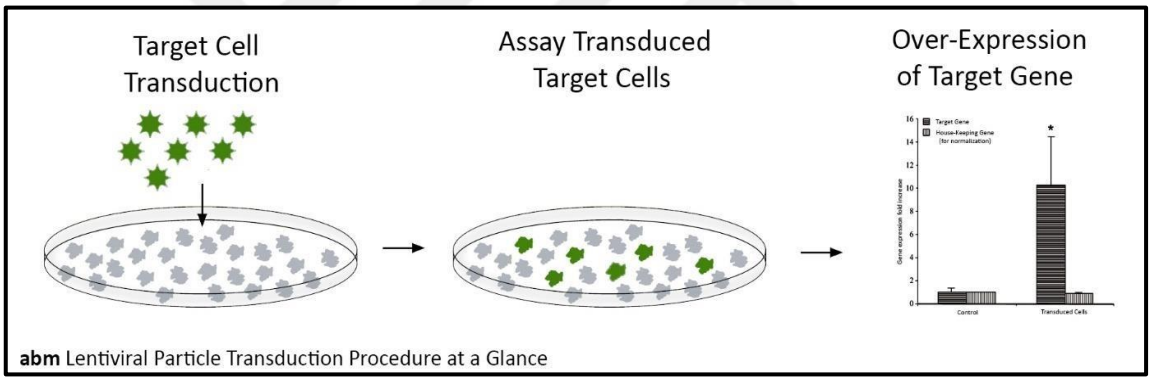

Figure 4.1. Aimed over-expressions and gene silencing processes after the gene introductions.

4.1. EXOSOME PURIFICATION

Cells were cultured in media accompanied with 10% exosome-free FBS (FBS, Hyclone). Cell culture mediums were harvested. Then the cell culture media was centrifuged at 2000x g for 30 minutes for removing cells and debris. The supernatants were transferred covering the cell-free culture media to a new tube deprived of disturbing the pellet.

The essential volume of cell-free culture media was transferred into a new tube and 0, 5 volumes of the total isolation reagent (Invitrogen by Life Technologies) was also

supplemented. Cell culture media and the reagent mixture then mixed properly via vortexing until there was a homogenous solution. Samples were incubated at 4 ^{0}C overnight. Afterwards, the supernatants were discarded. Exosomes seemed to be contained in the pellet at the bottom of the tube. The pellet was suspended in a convenient volume of 1xPBS. Once the pellet was resuspended, the exosomes were ready for further analysis.

4.2. TRANSFECTION EFFICIENY ANALYSIS WITH FLOW CYTOMETRY

All cell types including untransfected hnFSSCs,HDF cells, SK-Mel-1 cells, SK-Mel-3 cells; c-Met transfected hnFSSCs,HDF cells, SK-Mel-1 cells, SK-Mel-3 cells; shMet lentiviral particle applied hnFSSCs,HDF cells, SK-Mel-1 cells, SK-Mel-3 cells; shRab27a lentiviral particle applied hnFSSCs,HDF cells, SK-Mel-1 cells, SK-Mel-3 cells were fixed with 4% PFA at 4 ^{0}C for 30 minutes. Cells then sent to centrifuge at 1500 rpm for 5 minutes. Centrifuged cells were washed in PBS two times and were dissolved in sterile PBS. Afterwards, cells all sent to flow cytometry analysis. RFP related and GFP related samoles then were analysed in correct gating procedure.

4.3. EXOSOME EXAMINATION BY ELECTRON MICROSCOPY

ESEM Quanta 400 were used for the scanning electron microscopy analysis.the exosomes. Samples were fixed by using 3.7% glutaraldehyde (Sigma–Aldrich GmbH, Taufkirchen, Germany) in PBS 15 min long. Upon PBS washing for two times, exosomes which were fixed left dehydrated via ethanol. Once ethanol evaporated, the samples were left to dry at room temperature for a day long on a glass, afterwards will be examined by SEM later on gold–palladium popping.

4.4. CLONOGENIC ASSAY ANALYSIS

Colony formation assay or clonogenic assay or was used for examining the *in vitro* cell survival based on the ability of a single cell to grow into a colony. Cells were harvested and were plated in an appropriate number on 6 well plates. Seeded cells were incubated for a few hours at 37 ^{0}C. Then the cells were treated with the specific kit chemicals (BioPioneer, USA).

Cells were incubated at 37 ^{0}C for 1-3 weeks until cells in control plates generate colonies. Plates were fixed via specific fixation solution of the kit. Cell culture medium was removed and cells will be washed with PBS. 0,5% crystal violet solution was added and the plates were incubated at room temperature for 2 hours. Afterwards, 10 ml of cell culture media was added this way cells will be detached. Plates were left to dry on a table at room temperature for a few days. At the end, colony numbers were counted via microscope with the same procedure established in a reference study [39]. Data calculation was proceeded by using the formula of ;

Plating competence = number of formed colonies/ number of seeded cells x 100%

Surviving Portion = number of formed colonies after treatment/ number of seeded cells x Plating competence

4.1. ENDOTHELIAL TUBE FORMATION ASSAY

Endothelial Tube Formation Assay (In Vitro Angiogenesis Assay) Kit was used for the determination (Cell BioLabs, San Diogo) of the endothelial formation. The ECM gel was thawed. 50 µL of thawed ECM gel solution was added into every single well of a 96-well sterile plate. They were incubated for 30 minutes to 1 hr at 37ºC for letting the ECM solution to generate a gel. Endothelial cells were harvested and they were resuspended in wanted culture medium including 0.5-10% serum and the anticipated angiogenesis helpers at 1-2 x 10^5 cells/mL. 150 uL of cell suspension (1.5-3 x 10^4 cells) per well were supplemented onto the solidified ECM gel. Then, the assay plate was incubated at 37ºC for 12 hours. The endothelial tubes were observed under light microscope in 4X magnification.

4.2. CELL MIGRATION ANALYSIS

Cultrex Cell Migration Assay, 96 well (Cultrex, Helgerman) was utilized for this examination. Culturing cells were examined to not greater than 80% confluence. 24 hours earlier to the assay, cells required to be serum ravenous to express unbound receptors on their plasma membranes. Cells were assayed for standard curving. Afterwards, cells were harvested and they were diluted to operating concentration (1 x 10^6 cells/ml) in a serum free

medium. 50 µl of cells were added into every chamber. 150 µl of medium per well will be added to the bottom chamber. Cells were incubated at 37 ^{0}C in CO_2 incubator, incubation times may diverse in 44 hours to 48 hours. After the incubation process, top chamber was aspirated without destroying the membrane. The bottom chamber was aspirated and was washed with 200 µl 1X Wash Buffer. 12 µl of Calcein solution was added into 10 mL of 1X Cell Dissociation Solution. 100 µl of Cell Dissociation Solution/Calcein-AM was supplemented into each well of bottom chamber, and they were incubated at 37 0C in CO_2 incubator for 30 minutes. Kit's own device was tapped 10 times on the side and they were incubated at 37 ^{0}C in CO_2 incubator for an additional 30 minutes. Cell migration device was dissembled and it was read at 485 nm excitation, 520 nm emission utilizing the same parameters as standard curve or controls. The data was compared to standard curve to regulate the amount of the migrated cells, in addition to percent cell migration.

4.3. ELISA ANALYSIS FOR OBTAINING RELATIVE EXPRESSION LEVELS

Cusabio Elisa Kits (China) were utilized for the ELISA analysis for EGF (Human epidermal growth factor), c-MET/HGFR (Human hepatocyte growth factor receptor), VEGF (Human vascular endothelial cell growth factor) and Rab-27a (Human Ras-related protein Rab-27a) kits individually. Each kit was covering specific 96 well plates coated with those antibodies.

Reagents were equipped and samples were prepared with respect to the protocols defined in each and every ELISA kits (Bioassay Technology Laboratory, Cusabio). 100 µl samples were added into each well and they were incubated for 2 hours. The liquid of each well was withdrawn and discarded. 100 µl of Biotin antibody (1X) was supplemented into each well and was incubated for 1 hour at 37^{0}C. Cells were aspirated and washed three times. 100 µl of HRPAvidin (1X) was incubated for 1 hour at 37^{0}C. Cells were aspirated and washed five times. 90 µl TMB Substrate was added into every single well and was incubated at 37^{0}C for 15-30 minutes in the dark. 50 µl of stop solution was added into each well. Results were obtained at 450 nm within 5 minutes.

4.4. ANNEXIN V-FITC APOPTOSIS DETECTION KIT

Annexin V-FITC Apoptosis Detection Kit (Abcam, Turkey) 1-5 x 10^5 cells were collected via centrifugation. Cells were suspended in 500 µl of 1X Binding Buffer. 5 µl of Annexin V-FITC and 5 µl of propidium iodide were added. They maintained at room temperature for 5 minutes in the dark. Quantification was performed by flow cytometry. Annexin V-FITC binding was analysed via flow cytometry, utilizing FITC signal detector and PI staining via the phycoerythrin discharge signal detector. For the adherent melanoma lineages, cells were trypsinized and washed for one time with serum-including media afore interaction with Annexin V-FITC.

4.5. QUANTITATIVE REAL TIME ANALYSIS FOR DETECTING THE RELATIVE MRNA EXPRESSION LEVELS FOR DIFFERENT PRIMARIES

Isolation of total RNA from transfected human newborn foreskin cells, human melanocyte cells and human malignant melanoma cell lines whereas the non-transfected human newborn foreskin cells, human dermal fibroblast cells and human malignant melanoma cells were completed by using High Pure RNA Isolation Kit (Roche, Germany) regarding to the manufacturer's instructions. cDNA (complementary DNA) synthesis from isolated RNA samples was utilized in High Fidelity cDNA Synthesis Kit (Roche, Germany). Real time PCR was applied with Maxima SYBR Green/ROX (Fermentas, USA) for the investigation of expression levels of marker genes after exosomal experimental applications. Isolated cDNAs were employed as template and were mixed with primers and Maxima SYBR Green/ROX qPCR Master Mix (2X). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-Actin were used as the housekeeping gene for the optimization of the data. The results of real time PCR data were stabilized by the mRNA level of GAPDH. Primer sequences for marker genes were illustrated in Table 3.4.

4.6. STATISTICAL ANALYSIS

The statistics were analyzed by one-way analysis of adjustment and Turkey post hoc test. The values of $P<0.05$ were measured statistically significant.

5. RESULTS

5.1. MET AND RAB27A TRANSFECTIONS INTO HUMAN DERMAL FIBROBLAST CELLS, HUMAN NEWBORN FORESKIN STEM CELLS AND MALIGNANT MELANOMA CELL LINES SK-MEL-1 AND SK-MEL-3

Lenti-GIII-CMV-Mouse-RAB27A-RFP-2A-Puro Lentivirus was used in order to examine the overexpression Rab27a gene of not only for SK-Mel-1 cell lines and also for SK-Mel-3, Human dermal fibroblasts (human melanocyte like cells) and hnFSSCs. The vector size was 8834bp, the tag was chosen as RFP and the selection marker was selected as puromycin. The other plasmid that has been selected was pLenti-MetGFP for enhancing the c-Met expression. This time, the vector size was selected as 4170bp, the tag of it was chosen as GFP and the selection marker was designated as puromycin as well.

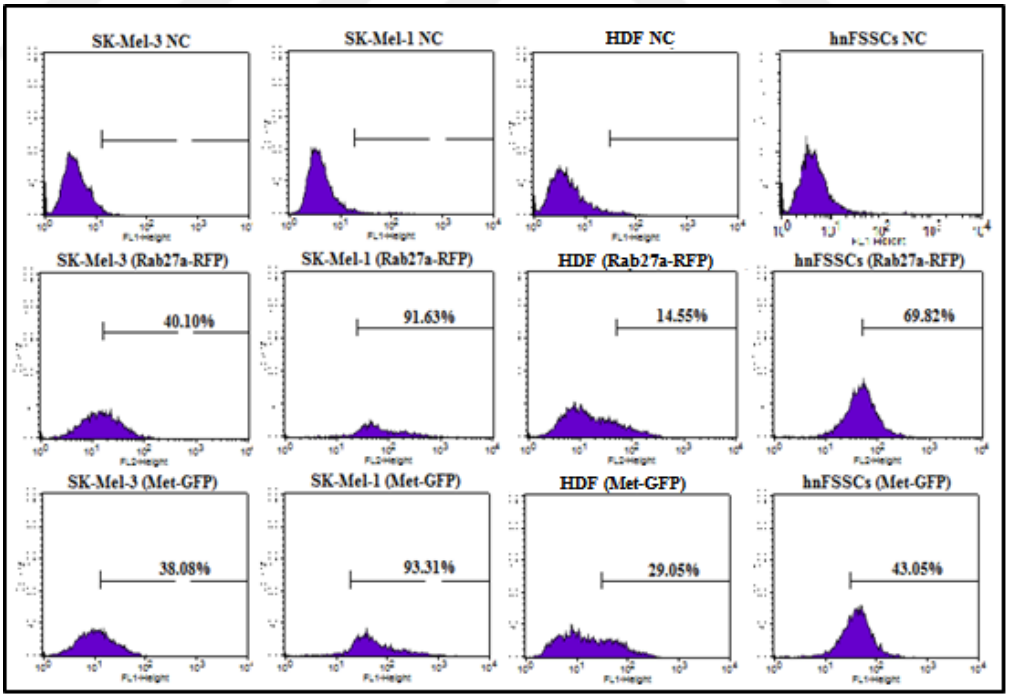

Figure 5.1. Rab27a and c-Met gene transfection results obtained from flow cytometry for human malignant melanoma SK-Mel-1, SK-mel-3 and healthy human melanocyte and hnFSSCs cell lines. Abbrevations: hnFSSCs; Human newborn foreskin stem cells, HDF; Human dermal fibroblasts.

Transfection results were determined by the flow cytometry that was shown at Figure 5.1. SK-Mel-3 transfections showed 40% positive transfection rate for Rab27a gene and 38% positive transfection for c-Met gene. SK-Mel-1 transfections have already completed for the first committee report, whereas the applications were repeated again for freezing the cells for future applications. 91% Rab27a gene transfection and 93% c-Met gene transfections was achieved for SK-Mel-1 malignant melanoma cell lines. Same transfection protocols were applied for the healthy cell lineages as well. Human dermal fibroblast outcomes indicated as 15% positive for Rab27a gene and 29% positive for c-Met gene. Lastly, the human newborn foreskin stem cells (hnFSSCs) transfections were found as 70% for Rab27a and 43% for c-Met transfections. Related to the fact that cell were needed almost 100% transfected, all of the cell were sent to sorting. As a result, all cell colonies were obtained in a pure 100 % transfected manner for the next applications.

5.2. EXOSOME DETECTION AND OBSERVATION VIA ELECTRON MICROSCOPY

ESEM Quanta 400 was utilized for the scanning electron microscopy analysis of the exosomes. Samples were immobilized with 3.7% glutaraldehyde (Sigma–Aldrich GmbH, Taufkirchen, Germany) in PBS 15 minutes. After PBS washing for two times, exosomes were dehydrated with 70% ethanol. When ethanol was faded, the samples were left to desiccate at room temperature for a day long on a glass made slide. Samples then sent to SEM and covered with gold–palladium popping for the observation.

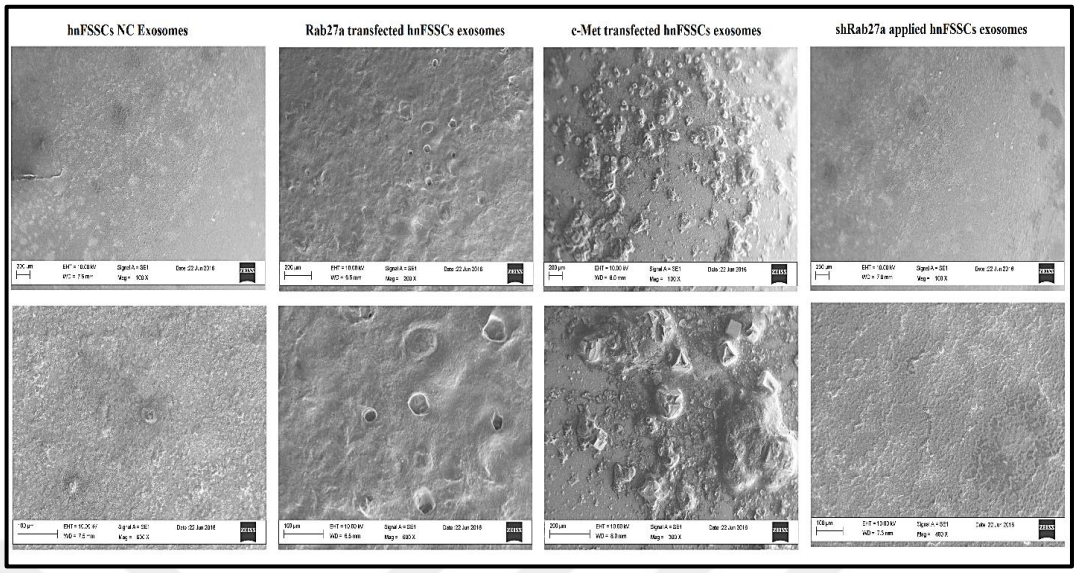

Figure 5.2. SEM images of hnFSSCs exosomes coming from untransfected, Rab27a transfected, c-Met transfected and shRab27a lentiviral particle added cells respectively. Abbreviations: hnFSSCs; Human newborn foreskin stem cells.

SEM images indicated that once the Rab27a gene were transfected to hnFSSCs, the exosome release increased and the number of exosomes were found higher than the untransfected exosome number, which was correlated with the literature [29]. Therefore, when Rab27a was silenced, the number of secreted exosomes from hnFSSCs found almost vanished. c-Met transfections to hnFSSCs cell line brought the highest number of secreted exosomes (Figure 5.2).

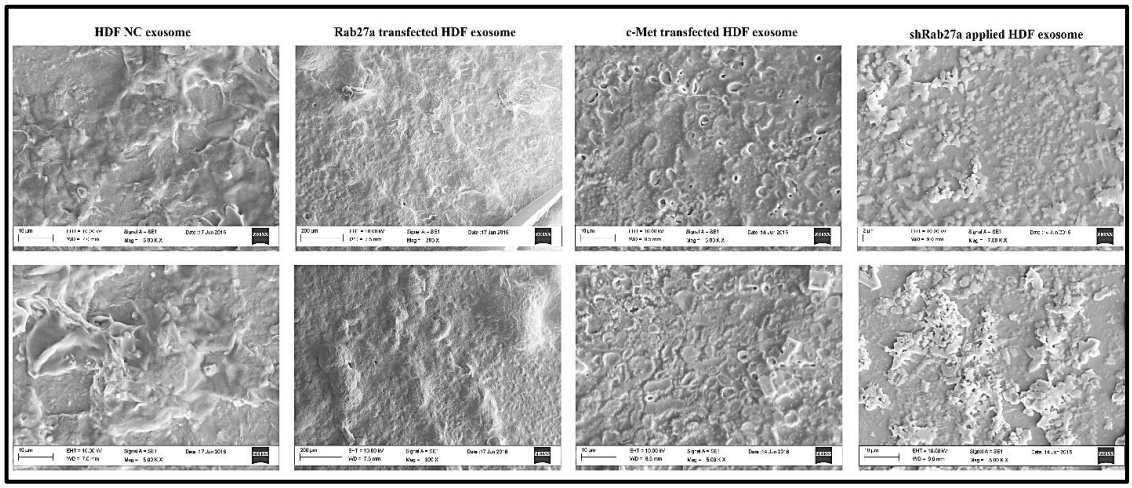

Figure 5.3. SEM images of HDF (Human dermal fibroblast) exosomes coming from no-transfected, Rab27a transfected, c-Met transfected and shRab27a lentiviral particle added cells respectively. Abbreviations: HDF; Human dermal fibroblasts.

SEM imaging results of adult healthy cell line HDF cells demonstrated that gene transfections of both Rab27a changed exosome number in a minimum manner. c-Met transfection to HDF cells group again showed higher numbers of exosome correlating with the hnFSSCs. Therefore, neither Rab27a overexpression nor Rab27a down regulation changed the exosome release and exosome numbers (Figure 5.3).

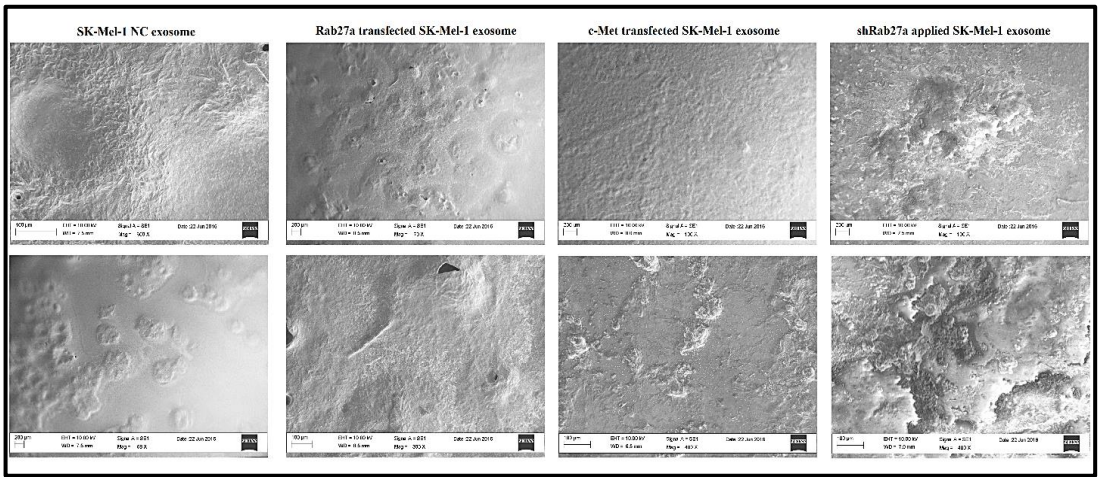

Figure 5.4. SEM images of SK-Mel-1 malignant melanoma exosomes coming from no-transfected, Rab27a transfected, c-Met transfected and shRab27a lentiviral particle added cells respectively.

SEM images of non-adherent SK-Mel-1 malignant melanoma cells displayed that once Rab27a was transfected to cells, exosome release were heightened and the number of exosomes were found greater than the untransfected exosomes. Silencing of Rab27a seemed to trigger decreased release of exosomes. The exosomes of silenced Rab27a cells were detected bigger than the other exosomes size wise. Meanwhile, c-Met transfection to the cell results indicated almost unchanged results with the untransfected ones (Figure 5.4).

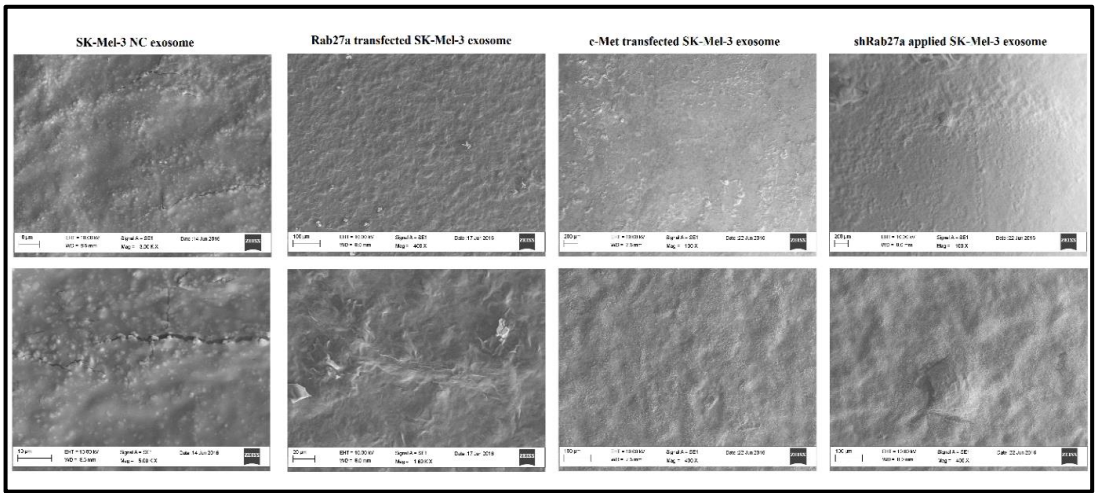

Figure 5.5. SEM images of SK-Mel-3 malignant melanoma exosomes coming from no-transfected, Rab27a transfected, c-Met transfected and shRab27a lentiviral particle added cells respectively.

SEM images of adherent malignant melanoma cells SK-Mel-3 exosomes indicated that Rab27a transfection to the cells enhanced the exosome released and the number of exosomes was found the highest when compared with the other groups. Therefore, c-Met transfection to the cells did not seem to have an efficient effect on the number of exosomes released. Once Rab27a was silenced in cells, exosome numbers decreased significantly (Figure 5.5).

5.3. CELL VIABILITY

Cell viability test was performed with the aim of examining the cytotoxic effects of human malignant melanoma cell exosomes on human newborn foreskin stem cells (hnFSSCs). This protocol was run via the 3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS)-assay with respect to reference studies [40].

5.3.1. Cytotoxicity analysis of hnFSSCs after hnFSSCs exosome applications

MTS assay were done for day1 and day 3 individually. Five different hnFSSCs exosome concentrations (1%, 2.5%, 5%, 7.5%,10%) were examined on hnFSSCs in order to check the toxicity for all hnFSSCs NC exosome, hnFSSCs c-Met exosomes, hnFSSCs Rab27a exosomes and hnFSSCs shRab27a exosomes.

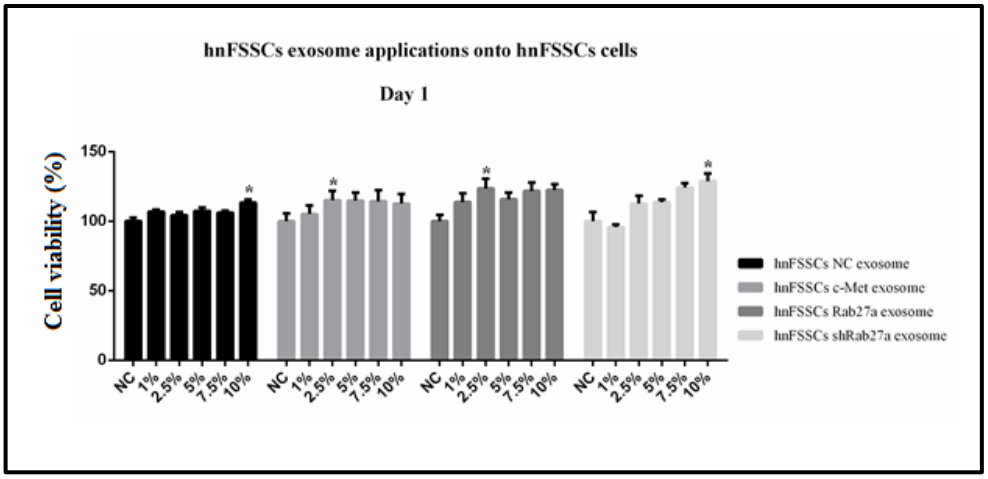

Figure 5.6. Cytotoxicity analysis of hnFSSCs exosomes on hnFSSCs in different concentrations at day 1. Abbreviations: hnFSSCs: Human foreskin stem cells. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Cytotoxicity analysis of hnFSSCs exosomes on hnFSSCs at day 1 indicated that 10% of untransfected hnFSSCs exosome, 2.5% of c-Met transfected hnFSSCs exosome, 2.5% of Rab27a transfected hnFSSCs exosomes and 10% of shRab27a carrying hnFSSCs exosomes were the optimum dosages of exosomes that should have been applied for next studies (Figure 5.6).

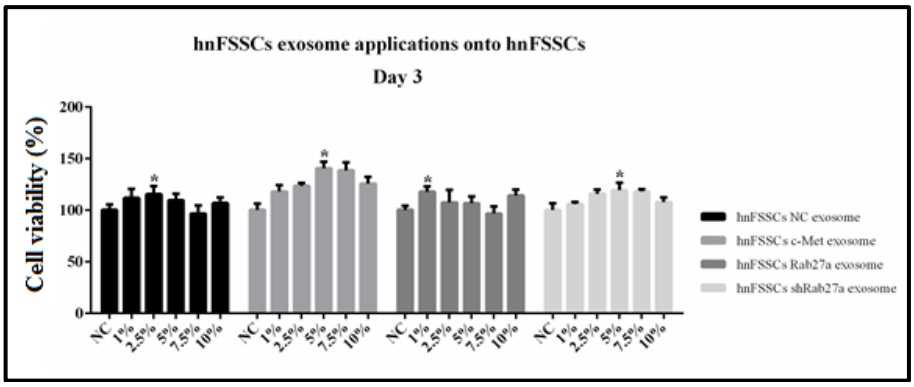

Figure 5.7. Cytotoxicity analysis of hnFSSCs exosomes on hnFSSCs in different concentrations at day 3. Abbreviations: hnFSSCs: Human foreskin stem cells. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Cytotoxicity analysis of hnFSSCs exosomes on hnFSSCs at day 3 designated that 2.5% of untransfected hnFSSCs exosome, 5% of c-Met transfected hnFSSCs exosome, 1% of

Rab27a transfected hnFSSCs exosomes and 5% of shRab27a carrying hnFSSCs exosomes were the optimum dosages of exosomes that should have been applied for next studies (Figure 5.6).

For the next applications, optimum day 3 concentrations were selected but with the cytotoxicity assay results it was shown that none of these exosome concentrations were highly toxic to hnFSSCs.

5.3.2. Cytotoxicity analysis of HDF cells after HDF exosome applications

MTS assay were completed for day1 and day 3 separately. Five different HDF exosome concentrations (1%, 2.5%, 5%, 7.5%,10%) were examined on HDF cells in order to check the toxicity for all HDF NC exosome, HDF c-Met exosomes, HDF Rab27a exosomes and HDF shRab27a exosomes.

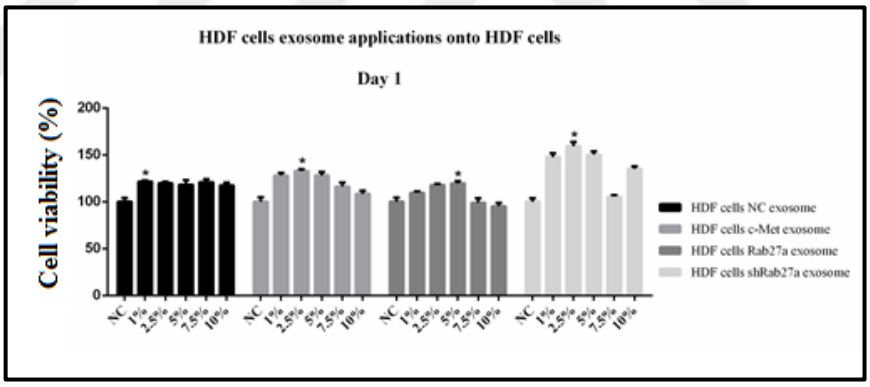

Figure 5.8. Cytotoxicity analysis of HDF exosomes on HDF cells in different concentrations at day 1. Abbreviations: HDF: Human dermal fibroblasts. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Cytotoxicity analysis of HDF exosomes on HDF cells at day 1 specified that 1% of untransfected HDF exosomes, 2.5% of c-Met transfected HDF exosomes, 5% of Rab27a transfected HDF exosomes and 2.5% of shRab27a carrying HDF exosomes were the optimal doses of exosomes that should have been practiced for next studies (Figure 5.8).

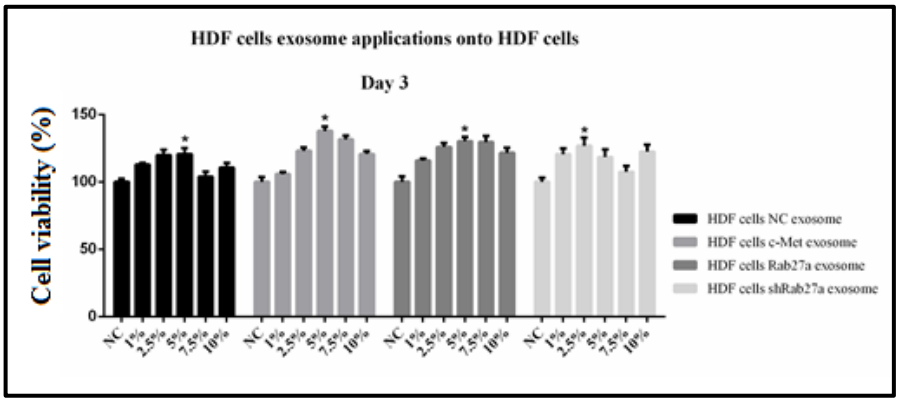

Figure 5.9. Cytotoxicity analysis of HDF exosomes on HDF cells in different concentrations at day 3. Abbreviations: HDF: Human dermal fibroblasts. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Cytotoxicity analysis of HDF exosomes on HDF cells at day 3 stated that 5% of untransfected HDF exosomes, 5% of c-Met transfected HDF exosomes, 5% of Rab27a transfected HDF exosomes and 2.5% of shRab27a carrying HDF exosomes were the optimal doses of exosomes that should have been practiced for next studies (Figure 5.9). For the further experimentations, optimum day 3 concentrations were selected but with these cytotoxicity assay results it was shown that none of these exosome concentrations were highly toxic to HDF cells.

5.3.3. Cytotoxicity analysis of hnFSSCs cells after SK-Mel-1 exosome applications

MTS assay were done for day1 and day 3 individually. Five different SK-Mel-1 malignant melanoma cell exosome concentrations (1%, 2.5%, 5%, 7.5%,10%) were examined on hnFSSCs in order to check the toxicity for all SK-Mel-1 NC exosome, SK-Mel-1 c-Met exosomes, SK-Mel-1 Rab27a exosomes and SK-Mel-1 shRab27a exosomes.

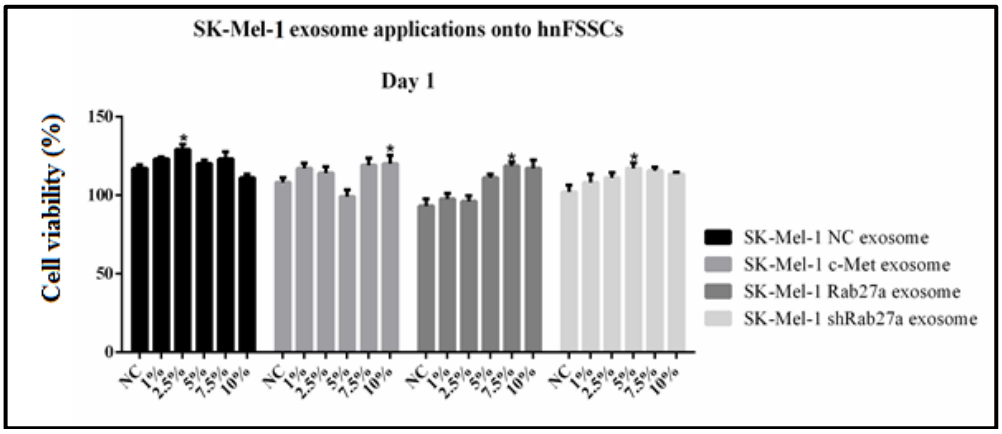

Figure 5.10. Cytotoxicity analysis of SK-Mel-1 exosomes on hnFSSCs in different concentrations at day 1. Abbreviations: hnFSSCs: Human foreskin stem cells. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Cytotoxicity analysis of SK-Mel-1 exosomes on hnFSSCs at day 1 specified that 2.5% of untransfected SK-Mel-1 exosomes, 10% of c-Met transfected SK-Mel-1 exosomes, 7.5% of Rab27a transfected SK-Mel-1 cell exosomes and 5% of shRab27a carrying SK-Mel-1 cell exosomes were the optimal doses of exosomes that should have been practiced for next studies (Figure 5.10).

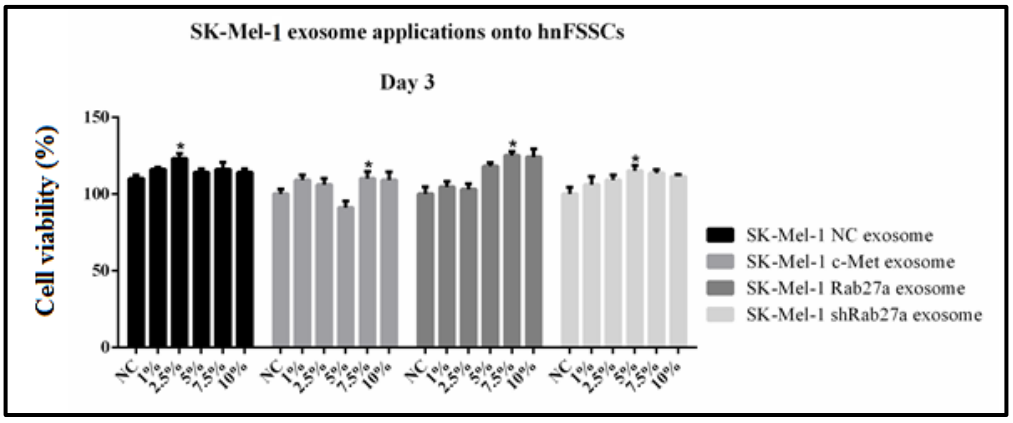

Figure 5.11. Cytotoxicity analysis of SK-Mel-1 exosomes on hnFSSCs in different concentrations at day 3. Abbreviations: hnFSSCs: Human foreskin stem cells. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Cytotoxicity analysis of SK-Mel-1 exosomes on hnFSSCs at day 3 stated that 2.5% of untransfected SK-Mel-1 cell exosomes, 7.5% of c-Met transfected SK-Mel-1 cell exosomes,

7.5% of Rab27a transfected SK-Mel-1 cell exosomes and 5% of shRab27a carrying SK-Mel-1 cell exosomes were the optimal doses of exosomes that should have been practiced for next studies (Figure 5.11). For the additional investigations, optimum day 3 concentrations were designated but with these cytotoxicity assay results, it was revealed that none of these exosome concentrations were extremely toxic to hnFSSCs cells.

5.3.4. Cytotoxicity analysis of HDF cells after SK-Mel-1 exosome applications

MTS assay were completed for day1 and day 3 individually. Five dissimilar SK-Mel-1 malignant melanoma cell exosome concentrations (1%, 2.5%, 5%, 7.5%,10%) were studied on HDF cells in order to check the toxicity for all SK-Mel-1 NC exosome, SK-Mel-1 c-Met exosomes, SK-Mel-1 Rab27a exosomes and SK-Mel-1 shRab27a exosomes.

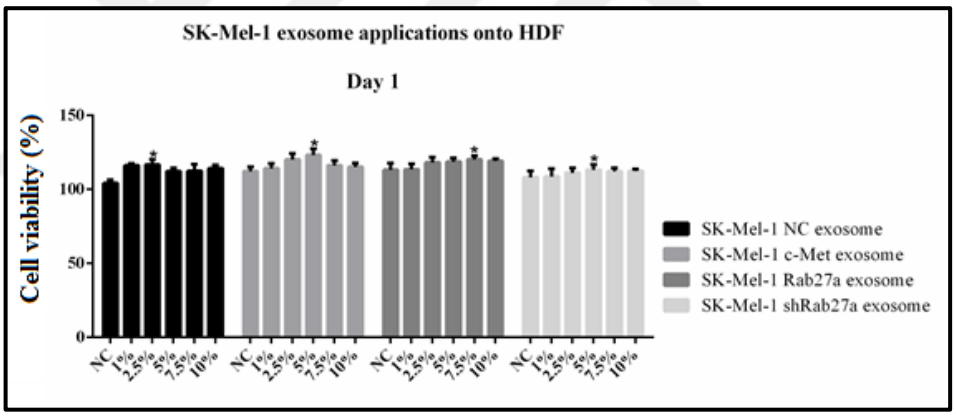

Figure 5.12. Cytotoxicity analysis of SK-Mel-1 exosomes on HDF cells in different concentrations at day 1. Abbreviations: hnFSSCs: Human foreskin stem cells, HDF: Human dermal fibroblasts. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Cytotoxicity analysis of SK-Mel-1 exosomes on HDF cells at day 1 specified that 2.5% of untransfected SK-Mel-1 exosomes, 5% of c-Met transfected SK-Mel-1 exosomes, 7.5% of Rab27a transfected SK-Mel-1 cell exosomes and 5% of shRab27a carrying SK-Mel-1 cell exosomes were the optimal doses of exosomes that should have been practiced for next studies (Figure 5.12).

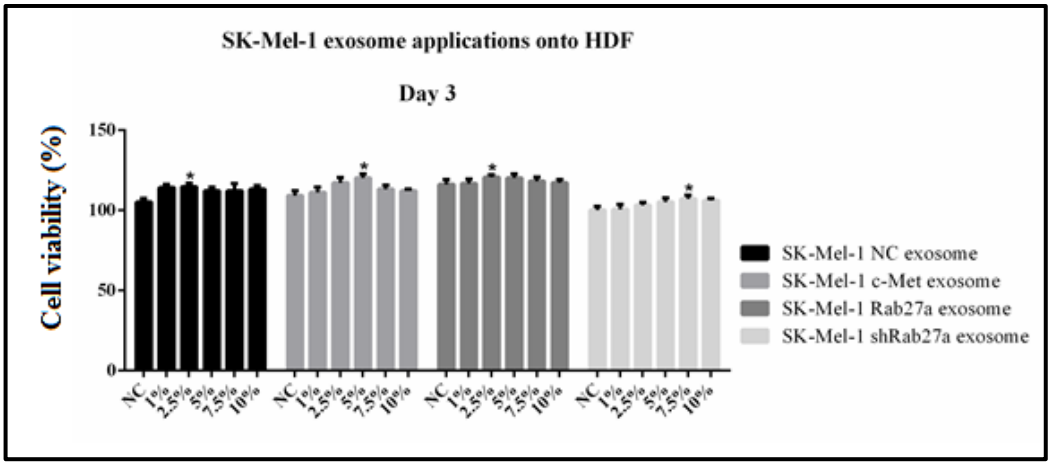

Figure 5.13. Cytotoxicity analysis of SK-Mel-1 exosomes on HDF cells in different concentrations at day 3. Abbreviations: HDF: Human dermal fibroblasts. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Cytotoxicity analysis of SK-Mel-1 exosomes on hnFSSCs at day 3 stated that 2.5% of untransfected SK-Mel-1 cell exosomes, 5% of c-Met transfected SK-Mel-1 cell exosomes, 2.5% of Rab27a transfected SK-Mel-1 cell exosomes and 7.5% of shRab27a carrying SK-Mel-1 cell exosomes were the optimal doses of exosomes that should have been practiced for next studies (Figure 5.13). For the additional investigations, optimum day 3 concentrations were designated but with these cytotoxicity assay results, it was revealed that none of these exosome concentrations were extremely toxic to HDF cells.

5.3.5. Cytotoxicity analysis of hnFSSCs after SK-Mel-3 exosome applications

MTS assay were done for day1 and day 3 individually. Five different SK-Mel-3 malignant melanoma cell exosome concentrations (1%, 2.5%, 5%, 7.5%, 10%) were examined on hnFSSCs in order to check the toxicity for all SK-Mel-3 NC exosome, SK-Mel-3 c-Met exosomes, SK-Mel-3 Rab27a exosomes and SK-Mel3-1 shRab27a exosomes.

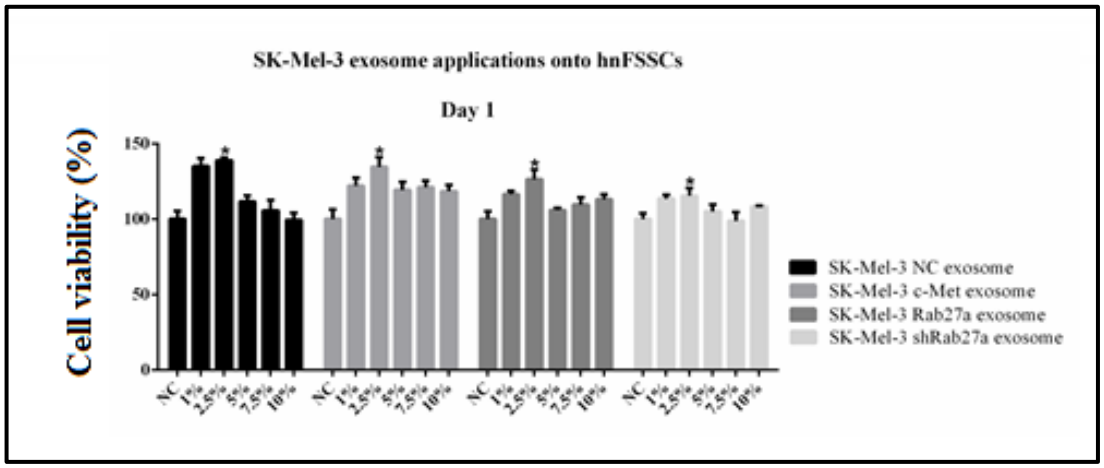

Figure 5.14. Cytotoxicity analysis of SK-Mel-3 exosomes on hnFSSCs cells in different concentrations at day 1. Abbreviations: hnFSSCs: Human foreskin stem cells. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Cytotoxicity analysis of SK-Mel-3 exosomes on hnFSSCs at day 1 specified that 2.5% of untransfected SK-Mel-3 exosomes, 2.5% of c-Met transfected SK-Mel-3 exosomes, 2.5% of Rab27a transfected SK-Mel-3 cell exosomes and 2.5% of shRab27a carrying SK-Mel-3 cell exosomes were the optimal doses of exosomes that should have been practiced for next studies (Figure 5.14).

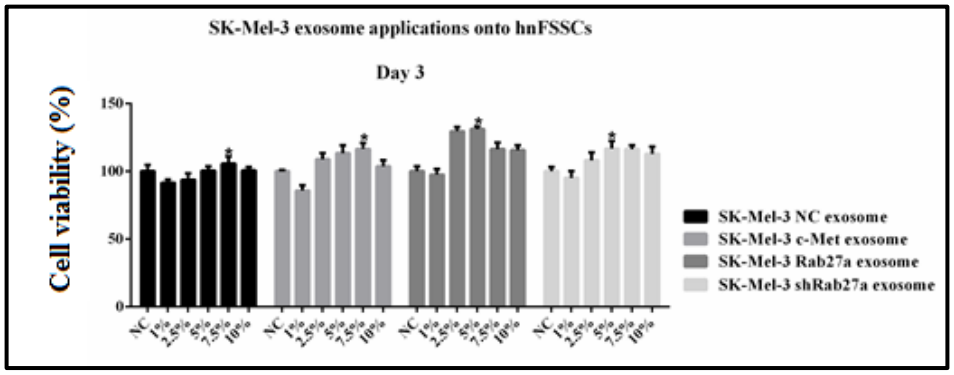

Figure 5.15. Cytotoxicity analysis of SK-Mel-3 exosomes on hnFSSCs cells in different concentrations at day 3. Abbreviations: hnFSSCs: Human foreskin stem cells. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Cytotoxicity analysis of SK-Mel-3 exosomes on hnFSSCs at day 3 stated that 7.5% of untransfected SK-Mel-3 cell exosomes, 7.5% of c-Met transfected SK-Mel-3 cell exosomes,

5% of Rab27a transfected SK-Mel-3 cell exosomes and 5% of shRab27a carrying SK-Mel-3 cell exosomes were the optimal doses of exosomes that should have been practiced for next studies **(Figure 5.15).** For the extra inquiries, optimum day 3 concentrations were chosen but with these cytotoxicity assay results it was discovered that none of these exosome concentrations were extremely toxic to hnFSSCs cells.

5.3.6. Cytotoxicity analysis of HDF cells after SK-Mel-3 exosome applications

MTS assay were completed for day1 and day 3 individually. Five dissimilar SK-Mel-3 malignant melanoma cell exosome concentrations (1%, 2.5%, 5%, 7.5%,10%) were studied on HDF cells in order to check the toxicity for all SK-Mel-3 NC exosome, SK-Mel-3 c-Met exosomes, SK-Mel-3 Rab27a exosomes and SK-Mel-3 shRab27a exosomes.

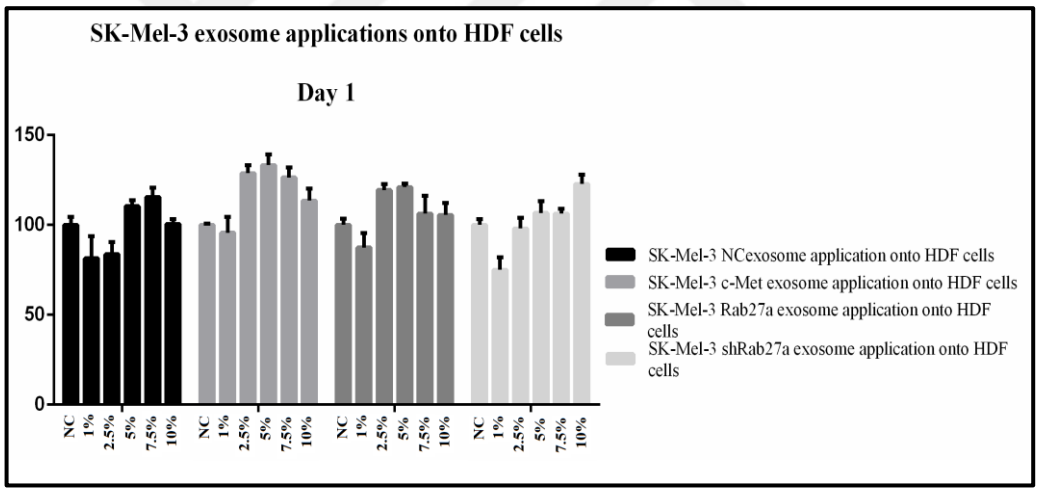

Figure 5.16. Cytotoxicity analysis of SK-Mel-3 exosomes on HDF cells in different concentrations at day 1. Abbreviations: HDF: Human dermal fibroblasts. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Cytotoxicity analysis of SK-Mel-3 exosomes on HDF cells at day 1 specified that 7.5% of untransfected SK-Mel-3 exosomes, 5% of c-Met transfected SK-Mel-3 exosomes, 5% of Rab27a transfected SK-Mel-3 cell exosomes and 10% of shRab27a carrying SK-Mel-3 cell exosomes were the optimal doses of exosomes that should have been applied for following studies (Figure 5.16).

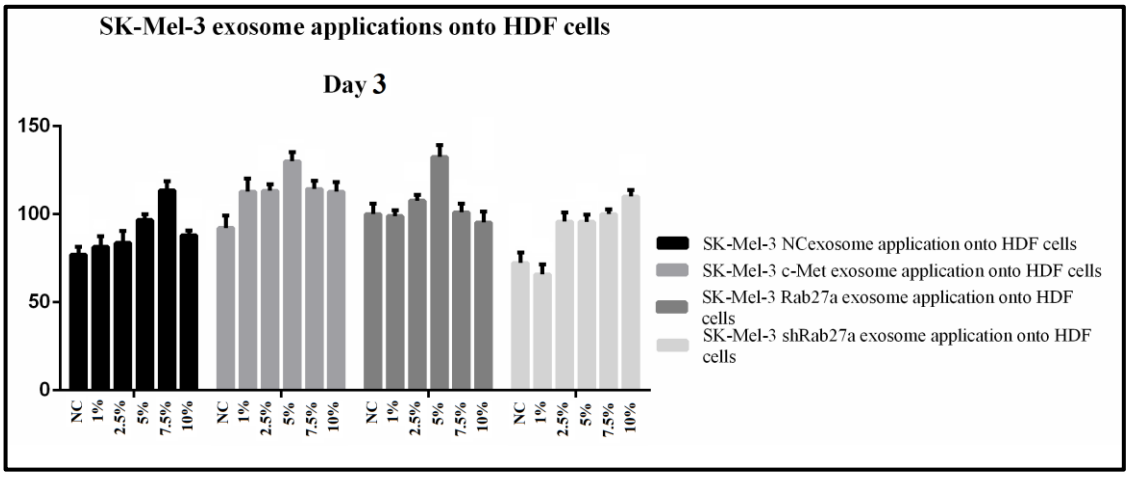

Figure 5.17. Cytotoxicity analysis of SK-Mel-3 exosomes on HDF cells in different concentrations at day 3. Abbreviations: HDF: Human dermal fibroblasts. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Cytotoxicity analysis of SK-Mel-3 exosomes on HDF cells at day 3 identified that 7.5% of untransfected SK-Mel-3 cell exosomes, 5% of c-Met transfected SK-Mel-3 cell exosomes, 5% of Rab27a transfected SK-Mel-3 cell exosomes and 10% of shRab27a carrying SK-Mel-3 cell exosomes were the optimal doses of exosomes that should have been practiced for next studies (Figure 5.17). For the extra inquiries, optimum day 3 concentrations were chosen but with these cytotoxicity assay results, it was discovered that none of these exosome concentrations were extremely toxic to hnFSSCs cells.

5.3.7. Cytotoxicity analysis of hnFSSCs and HDF cells after shMet carrying exosome applications

MTS assay were completed for day1 and day 3 individually. Five dissimilar shMet carrying exosome concentrations (1%, 2.5%, 5%, 7.5%,10%) were studied on both hnFSSCs and HDF cells in order to check the toxicity for all hnFSSCs shMet exosome, HDF shMet exosomes, SK-Mel-1 shMet exosomes, SK-Mel-3 shMet exosomes.

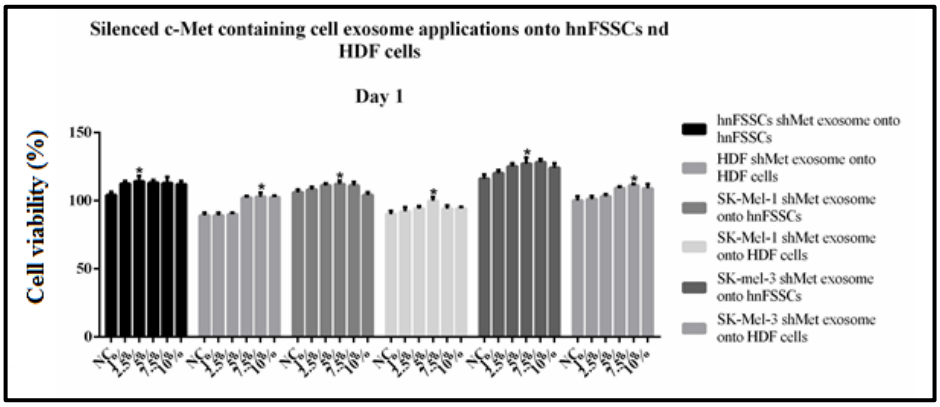

Figure 5.18. Cytotoxicity analysis of shMet carrying exosomes on both hnFSSCs and HDF cells in different concentrations at day 1. Abbreviations: hnFSSCs: Human foreskin stem cells, HDF: Human dermal fibroblasts. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

hnFSSCs shMet exosome application onto hnFSSCs optimum concentration was selected as 2.5%. HDF shMet exosome application onto HDF cells optimal dosage was chosen as 7.5%. SK-Mel-1 shMet exosome presentation onto hnFSSCs appropriate dose was designated as 5%. SK-Mel-1 shMet exosome presentation onto HDF cells was picked as 5% as well. SK-Mel-3 shMet exosome submission onto hnFSSCs optimum dosage was selected as 5% and SK-Mel-3 shMet exosome submission onto HDF cell presentation concentration was chosen as 7.5% for day 1 cytotoxicity assay results (Figure 5.18).

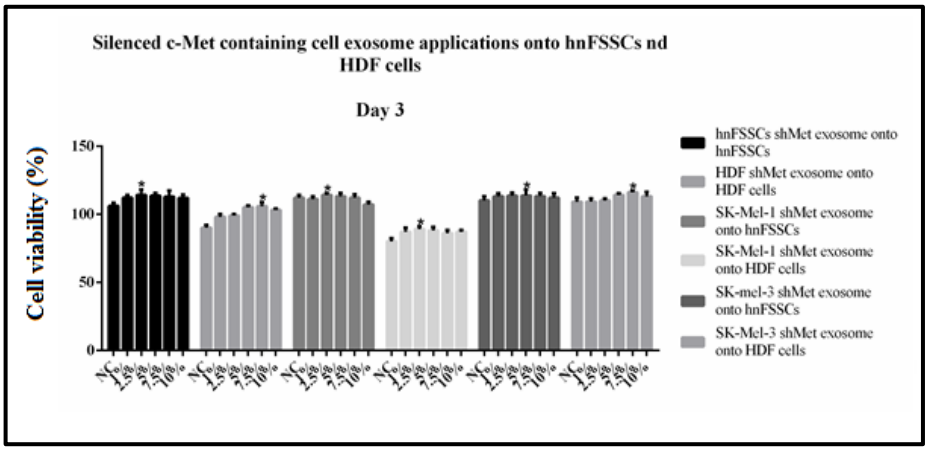

Figure 5.19. Cytotoxicity analysis of shMet carrying exosomes on both hnFSSCs and HDF cells in different concentrations at day 3. Abbreviations: hnFSSCs: Human foreskin stem cells, HDF: Human dermal fibroblasts. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

hnFSSCs shMet exosome application onto hnFSSCs optimum concentration was selected as 2.5%. HDF shMet exosome application onto HDF cells optimal dosage was chosen as 7.5% which were relevant to the day 1 analysis. SK-Mel-1 shMet exosome presentation onto hnFSSCs appropriate dose was designated as 5%. SK-Mel-1 shMet exosome presentation onto HDF cells was picked as 2.5% as well. SK-Mel-3 shMet exosome submission onto hnFSSCs optimum dosage was selected as 5% and SK-Mel-3 shMet exosome submission onto HDF cell presentation concentration was chosen as 7.5% for day 3 cytotoxicity assay results (Figure 5.19). For the next examinations, optimum day 3 concentrations were chosen but with these cytotoxicity assay results it was exposed that none of these exosome dosages were particularly toxic to hnFSSCs cells.

5.4. COLONY FORMING ASSAY

Clonogenic assay or colony formation assay was used for determining the in vitro cell survival related to the capability of a single cell growth into a colony formation. Cells then harvested and cells with suitable numbers were plated on 6 well plates. Seeded cells were incubated for ten days at 37 ^{0}C until cells in control plates generate colonies. Plates then fixed via 2% paraformaldehyde. The fixative agent was detached and cells were washed with

PBS. 0,5% crystal violet solution was added and maintained at room temperature for 2 hours. Subsequently, 6 well plates were left to arid on a table at room temperature for a two hours. Finally, colony numbers were calculated by light microscope [41].

Data calculation was performed via utilizing the formula of;

Plating efficiency = no. of colonies formed/ no. of cells seeded x 100%

Surviving Fraction = no. of colonies formed after treatment/ no. of cells seeded x Plating efficiency

As results, surviving fractions were calculated. Results are listed below;

Table 5.1. Plating efficiency and surviving fraction percentages of the exosome applied HDF cells

Name of the cells	Plating efficiency	Surviving fraction
HDF NC exosome application onto HDF cells	20%	4%
HDF Rab27a exosome application onto HDF cells	20%	4%
HDF c-Met exosome application onto HDF cells	32,5%	10,5%
SK-Mel-1 NC exosome application onto HDF cells	22,5	5,06%
SK-Mel-1 Rab27a exosome application onto HDF cells	15%	2,25%
SK-Mel-1 c-Met exosome application onto HDF cells	35%	12,25%
SK-Mel-3 NC exosome application onto HDF cells	20%	4%
SK-Mel-3 Rab27a exosome application onto HDF cells	10%	2%
SK-Mel-3 c-Met exosome application onto HDF cells	10%	2%

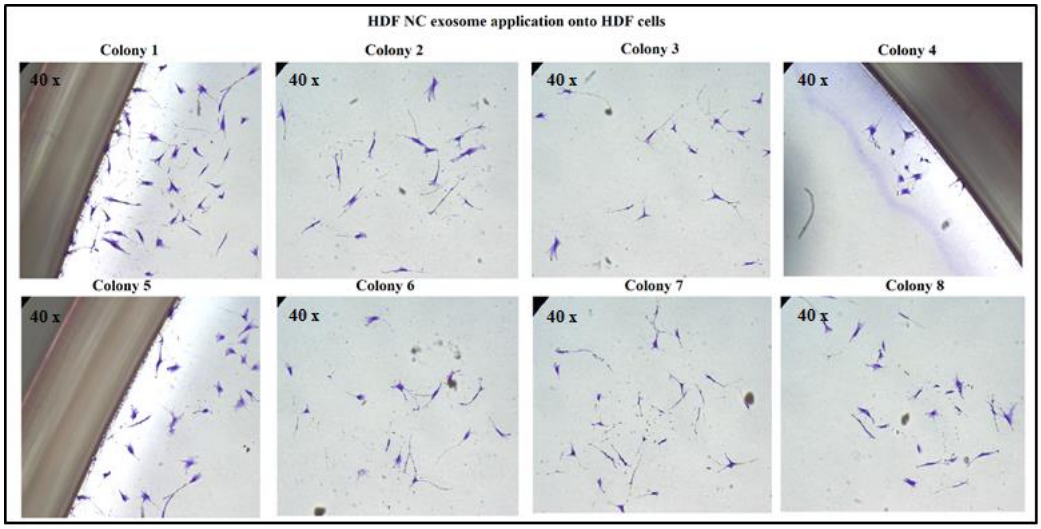

Figure 5.20. Colony formation images of the HDF NC exosome applied HDF cell lines. Abbreviations HDF; Human dermal fibroblasts.

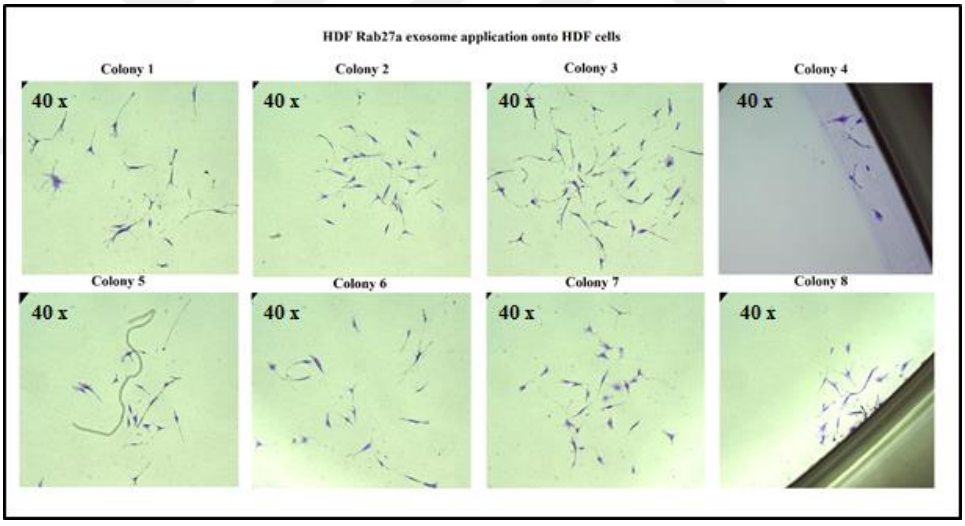

Figure 5.21. Colony formation images of the Rab27a transfected HDF cell exosome applied HDF cell lines. Abbreviations HDF; Human dermal fibroblasts.

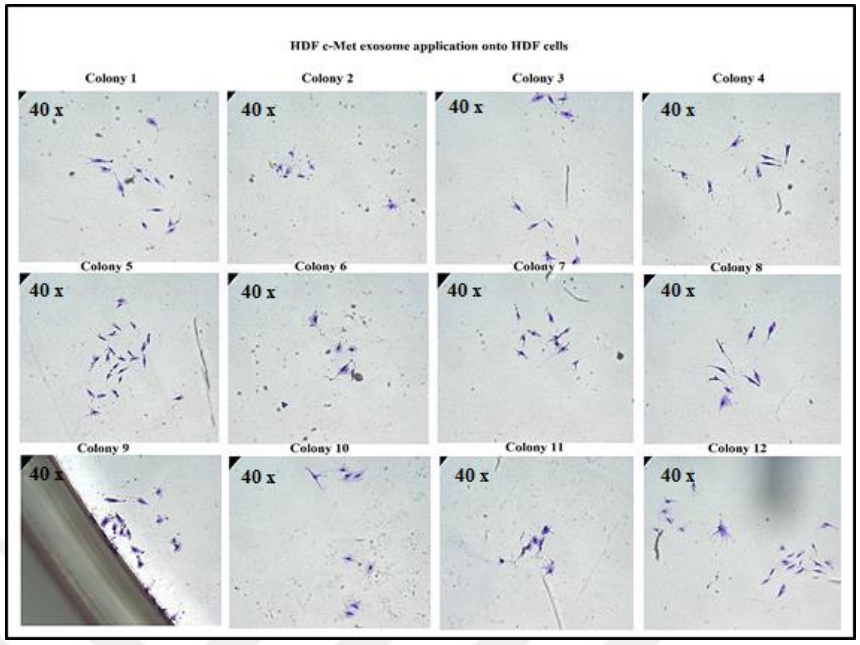

Figure 5.22. Colony formation images of the c-Met transfected HDF cell exosome applied HDF cell lines. Abbreviations HDF; Human dermal fibroblasts.

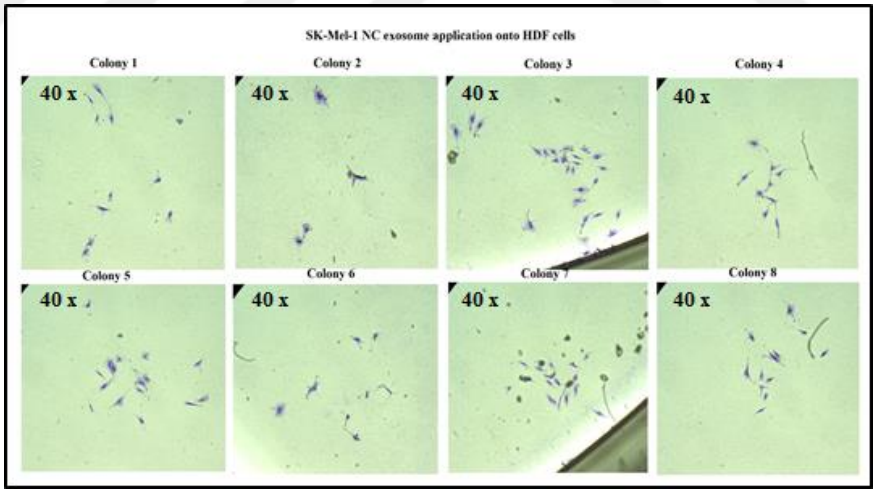

Figure 5.23. Colony formation images of the non-transfected SK-Mel-1 cell exosome applied HDF cell lines. Abbreviations HDF; Human dermal fibroblasts.

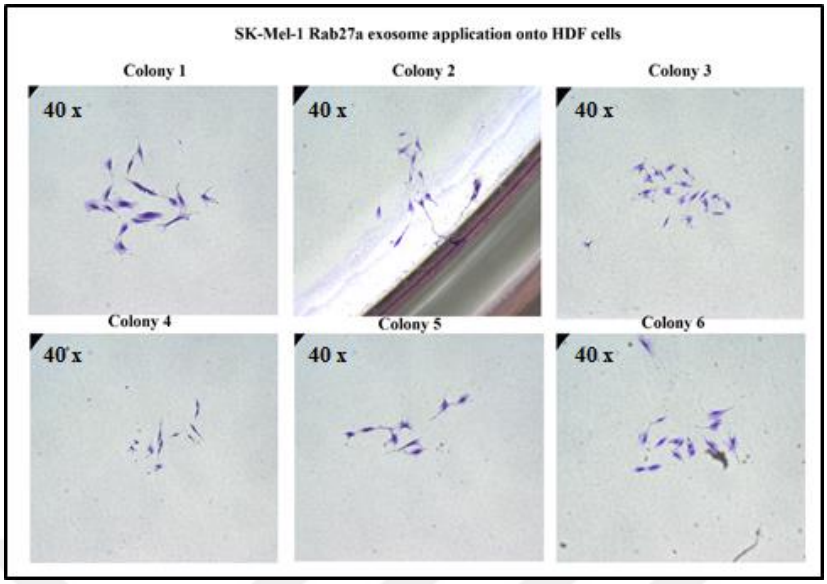

Figure 5.24. Colony formation images of the Rab27a transfected SK-Mel-1 cell exosome applied HDF cell lines. Abbreviations: HDF; Human dermal fibroblasts.

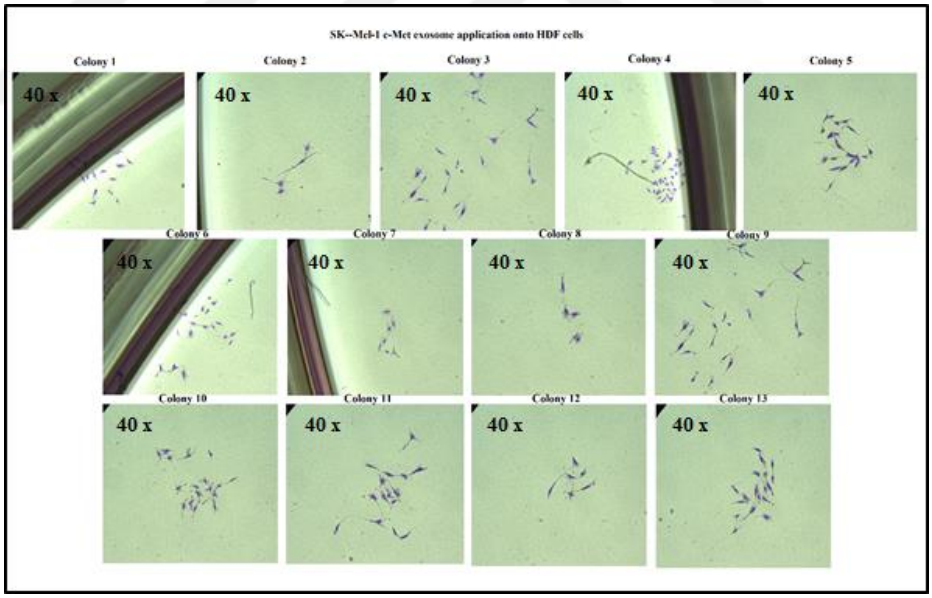

Figure 5.25. Colony formation images of the c-Met transfected SK-Mel-1 cell exosome applied HDF cell lines. Abbreviations HDF; Human dermal fibroblasts.

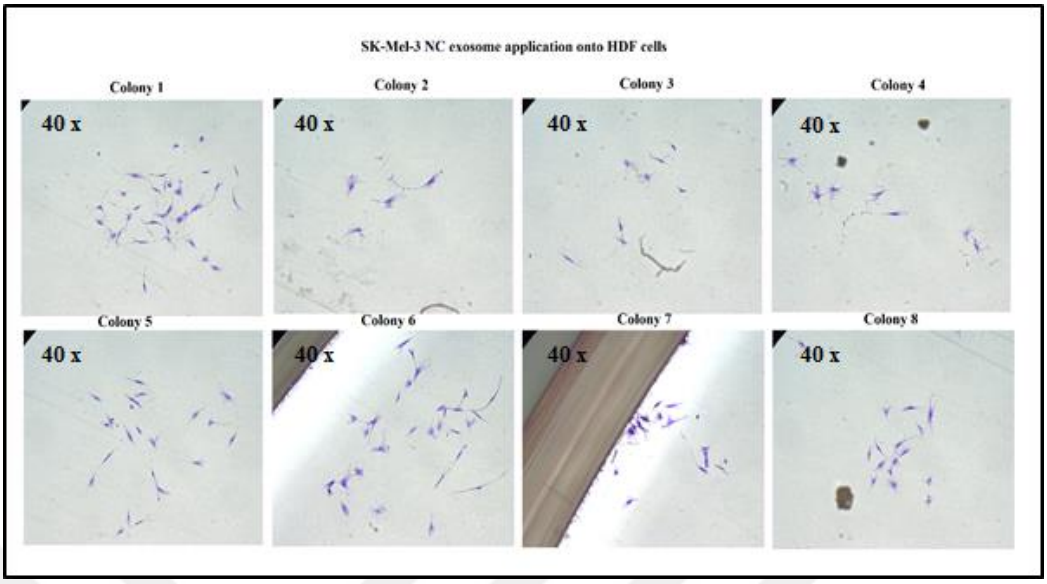

Figure 5.26. Colony formation images of the non-transfected SK-Mel-3 cell exosome applied HDF cell lines. Abbreviations: HDF; Human dermal fibroblasts.

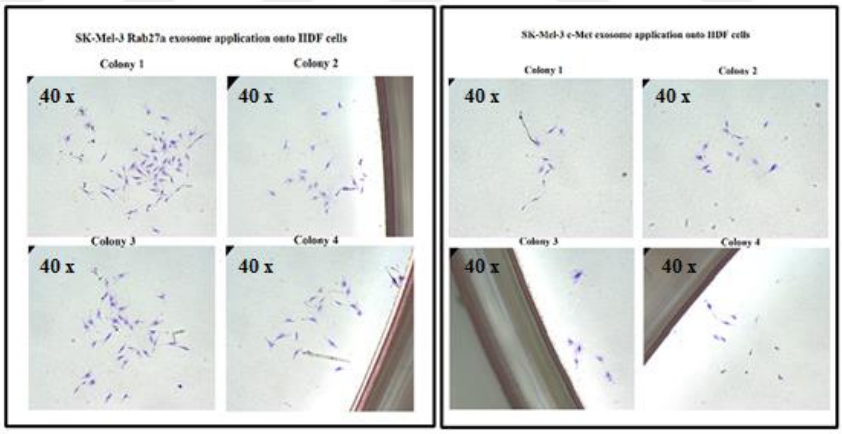

Figure 5.27. Colony formation images of the Rab27a transfected and c-Met transfected SK-Mel-3 cell exosome applied HDF cell lines. Abbreviations: HDF; Human dermal fibroblasts.

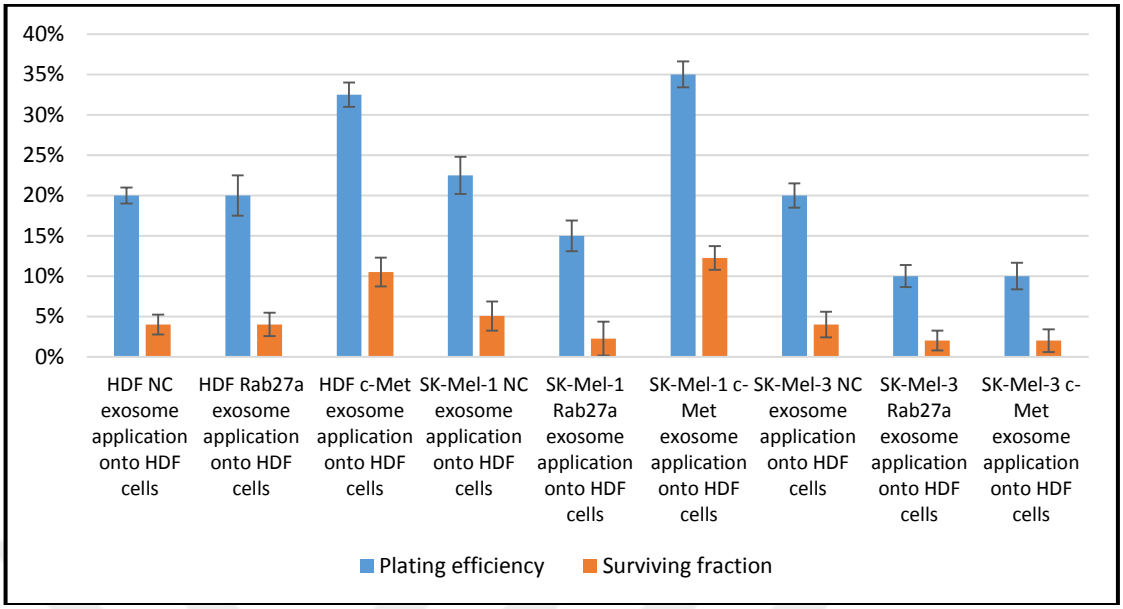

Figure 5.28. Plating efficiency and surviving fraction quantities of untransfected and transfected exosome applications onto both hnFSSCs and HDF cells. Abbreviations: hnFSSCs: human newborn foreskin stem cells, HDF: Human dermal fibroblasts.*P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Colony forming assay results indicated that; c-Met transfected HDF cell exosome applications onto HDF cells showed higher plating efficiency than untransfected HDF cell exosome and Rab27a transfected HDF cell exosome applications (Figure 5.20, Figure 5.28). Rab27a transfected exosomes showed equal results with the untransfected exosome applications (Figure 5.21, Figure 5.28). It suggested that metastatic c-Met gene transfections may increase metastasis and also the same gene can be found in the exosomes as well and may induce healthy cells to show cancerous behaviors. Meanwhile, c-Met transfected applications also showed higher surviving fractions (Figure 5.22, Figure 5.28).

Untransfected SK-Mel-1 cell exosome applications showed almost four times higher plating efficient than Rab27a transfected and c-Met transfected SK-Mel-1 cell exosome applications onto HDF cells (Figure 5.23, Figure 5.28). As indicated, cancer cell exosome applications increase colony formations of healthy cells. c-Met transfected group showed doubled plating efficiency than Rab27a transfected exosome applications (Figure 5.24, Figure 5.28). Therefore, surviving fractions was the highest in the c-Met transfected SK-Mel-1 exosome onto HDF application group, which also showed high metastatic potential of the healthy HDF cells in this group (Figure 5.25, Figure 5.28).

Untransfected SK-Mel-3 cell exosome applications showed two times higher plating efficient than Rab27a transfected and c-Met transfected SK-Mel-3 cell exosome applications onto HDF cells (Figure 5.26, Figure 5.28). As indicated, cancer cell exosome applications increase colony formations of healthy cells. c-Met transfected group showed same plating efficiency with Rab27a transfected exosome applications. Consequently, surviving fractions was also the highest in the non-transfected SK-Mel-3 exosome onto HDF application group which also displayed high metastatic capacity of the healthy HDF cells in this group (Figure 5.27, Figure 5.28).

5.5. ANNEXIN-V APOPTOSIS ASSAY

Annexin V-FITC Apoptosis Detection Kit (Abcam, Turkey) 1-5 x 10^5 cells were collected by centrifugation. Cells then suspended in 500 µl of 1X Binding Buffer. 5 µl of Annexin V-FITC and 5 µl of propidium iodide were applied. They stayed at room temperature for 5 minutes in the dark. Quantification was completed with flow cytometry. Annexin V-FITC binding may be examined with flow cytometry, using FITC signal detector and PI staining by the phycoerythrin release signal detector. For the adherent SK-Mel-3, HDF and hnFSSCs cells were trypsinized and washed again with serum added media afore interface with Annexin V-FITC [42].

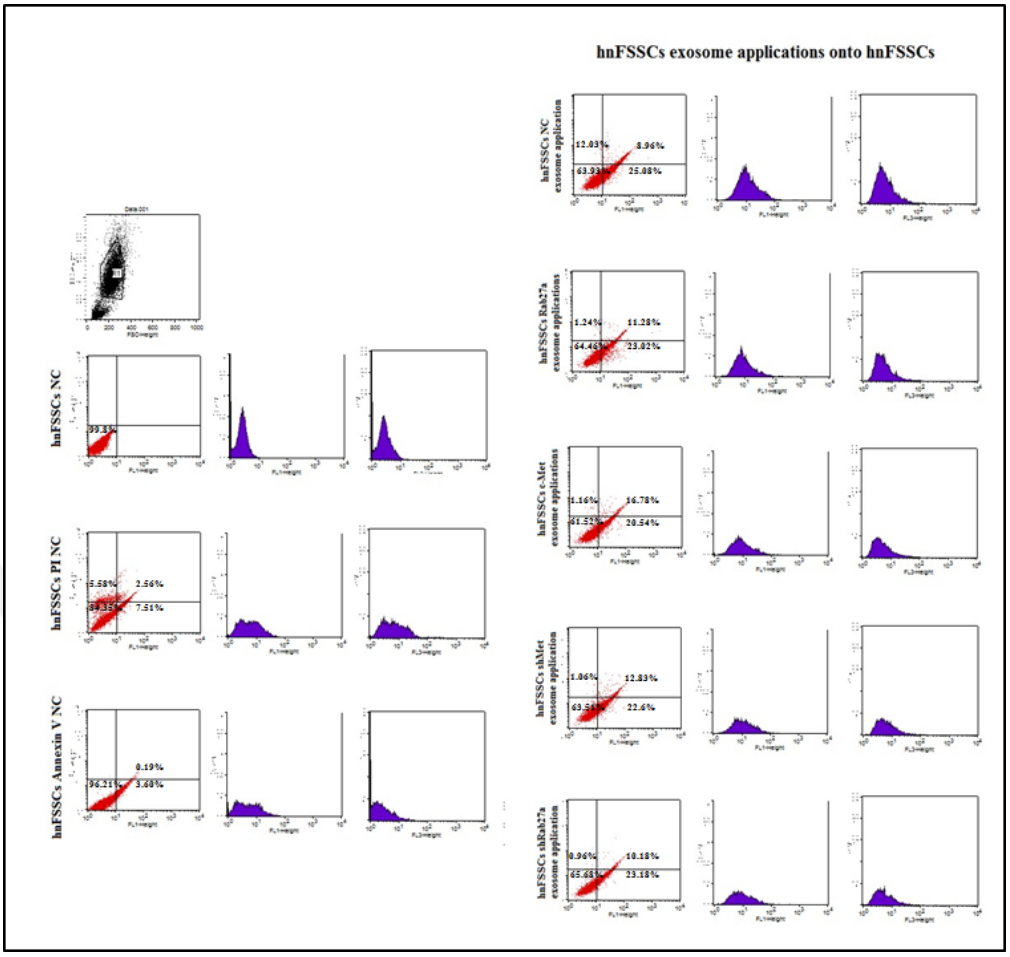

Figure 5.29. Annexin V-FITC Apoptosis Detection analysis for hnFSSCs after hnFSSCs exosome applications. Abbreviations: hnFSSCs; human newborn foreskin stem cells.

Annexin-V FITC apoptosis detection was performed in order to examine the apoptotic behavior of healthy cells after they have interacted with their own transfected cells. hnFSSCs exosome application to hnFSSCs results indicated that once Rab27a transfected hnFSSCs exosomes were given, apoptosis decreased. When Rab27a transfected ones compared with the shRab27a added ones, apoptosis was higher in Rab27a transfected ones respectively. C-Met transfected hnFSSCs exosome application results indicated significant outcomes. C-Met transfected exosome applications decreased cell death in both apoptosis and necrosis when compared with untransfected exosome application and shMet carrying exosome application (Figure 5.29).

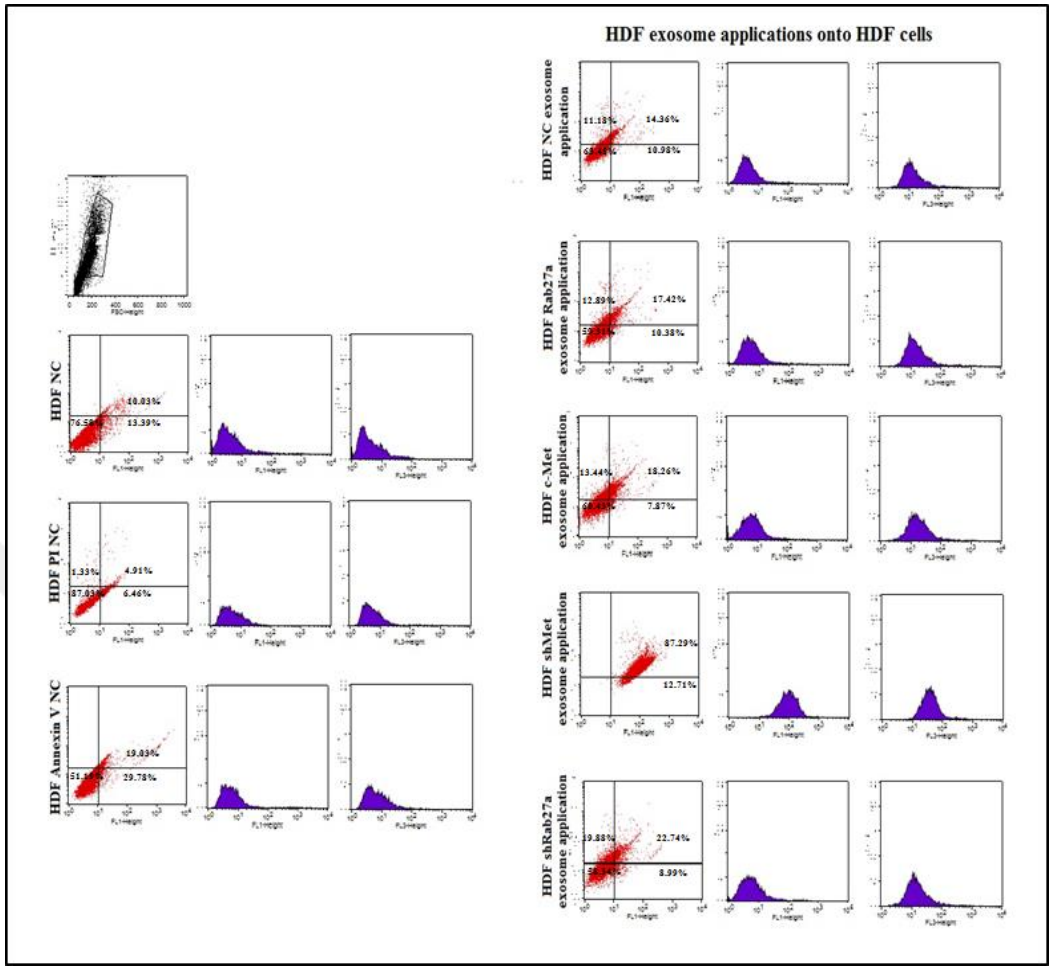

Figure 5.30. Annexin V-FITC Apoptosis Detection analysis for HDF after HDF exosome applications. Abbreviations: HDF; Human dermal fibroblasts.

Annexin V-FITC apoptosis assay was also performed for HDF exosome applications onto HDF cells. Rab27a transfected HDF cell exosome application increased both necrosis and apoptosis when compared with untransfected HDF exosome application group. Rab27a silencing in HDF cells and therefore in exosomes increases late apoptosis in comparison with the Rab27a transfected cell exosome application group. C-Met transfected HDF cell exosome application to HDF cells increased late apoptosis. Silencing c-Met in HDF cells and exosomes vanished life in cells and HDF cells were found in late apoptotic phase once exosomes presented (Figure 5.30).

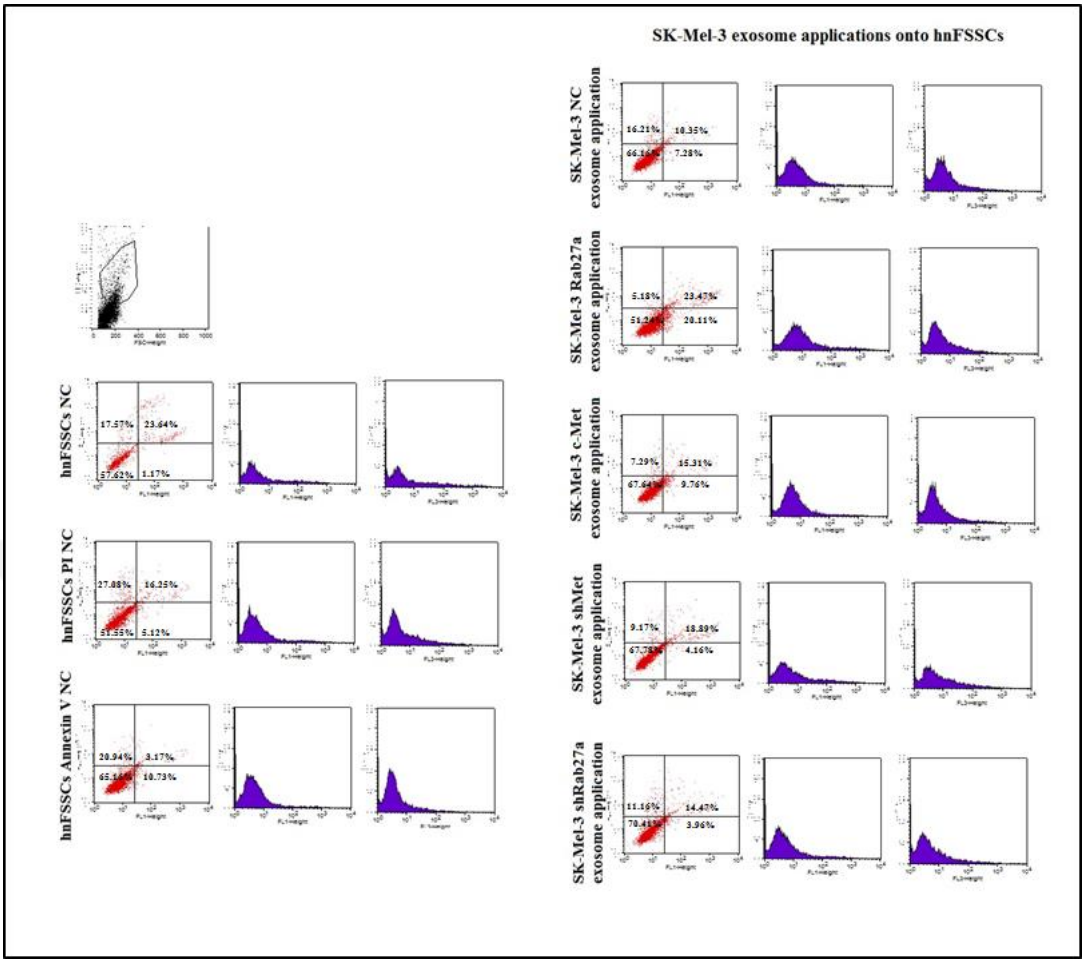

Figure 5.31. Annexin V-FITC Apoptosis Detection analysis for hnFSSCs after SK-Mel-3 exosome applications. Abbreviations: hnFSSCs; human newborn foreskin stem cells.

Adharent SK-Mel-3 malignant melanoma exosomes were given to hnFSSCs. Rab27a transfected SK-Mel-3 cell exosome application reduced necrosis and increased late apoptosis visibly when compared with the untransfected melanoma exosome application group. C-Met transfected melanoma exosome application decreased necrosis significantly once compared with untransfected melanoma exosome application group, therefore increased early apoptosis slightly .Silencing c-Met in melanoma exosomes increased late apoptosis when added to hnFSSCs more than c-Met transfected melanoma exosome added ones. Silencing Rab27a in melanoma exosomes brought reduced early and late apoptosis once presented to hnFSSCs when compared with Rab27a transfected exosome application ones (Figure 5.31).

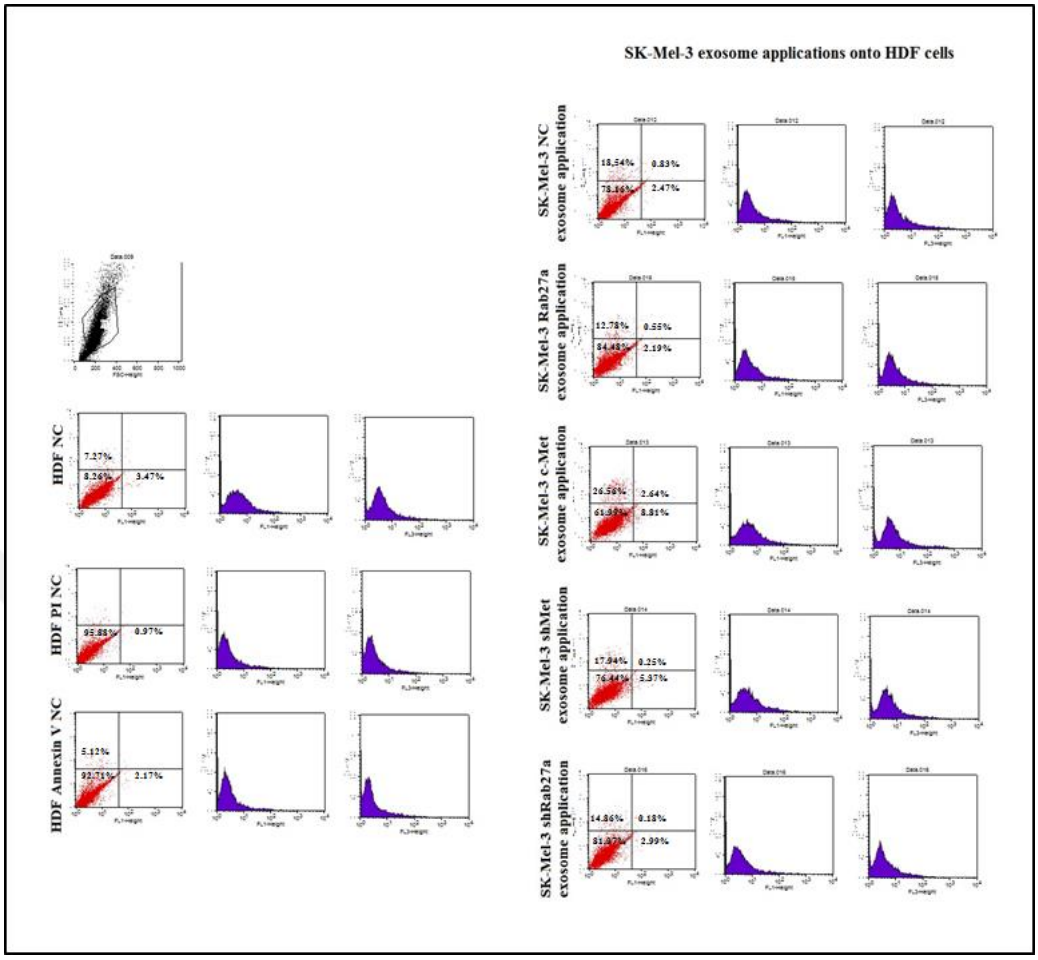

Figure 5.32. Annexin V-FITC Apoptosis Detection analysis for HDF after SK-Mel-3 exosome applications. Abbreviations: HDF; Human dermal fibroblasts.

Adherent SK-Mel-3 malignant melanoma exosomes were given to adult healthy human cell line HDF as well. Annexin V-FITC analysis results indicated that Rab27a transfected SK-Mel-3 melanoma cell exosome application decreased necrosis effectively when compared with untransfected melanoma cell exosome presentation group. Once c-Met was transfected to SK-Mel-3 cells and exosomes, HDF necrosis increased after application when compared with the untransfected melanoma cell exosome application ones. shMet given melanoma cell exosome application decreased both early and late apoptosis visibly though. Silencing Rab27a in cancer cells on the other hand, reduced necrosis of HDF cells after the application (Figure 5.32).

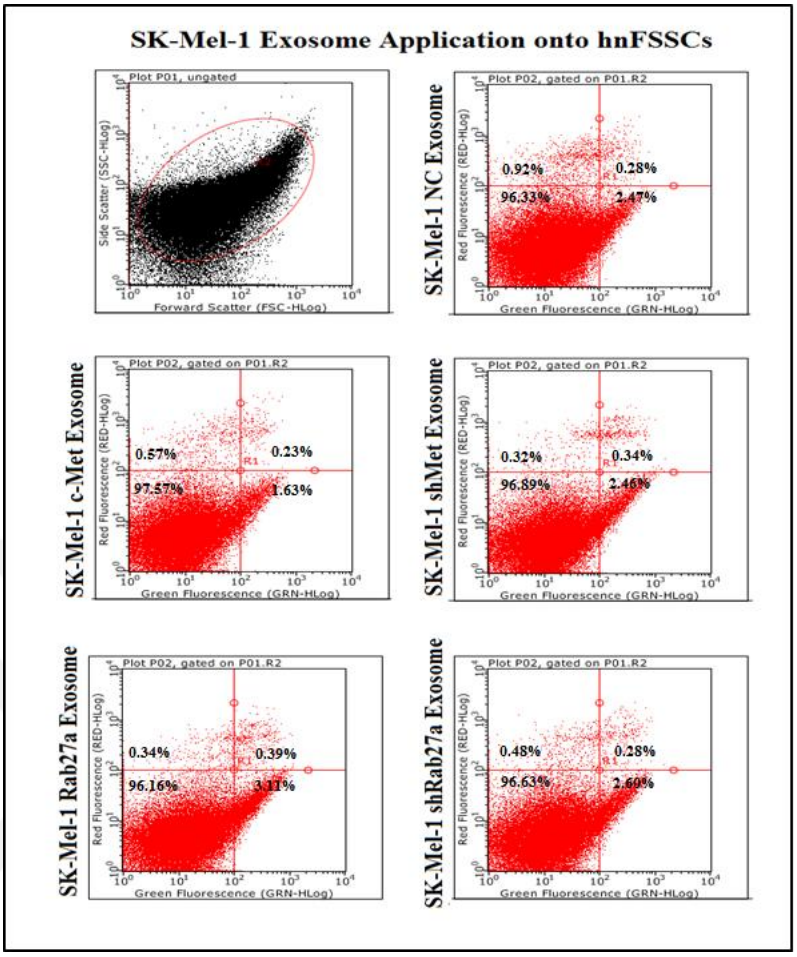

Figure 5.33. Annexin V-FITC Apoptosis Detection analysis for hnFSSCs after SK-Mel-1 exosome applications. Abbreviations: hnFSSCs; human newborn foreskin stem cells.

Non-adherent SK-Mel-1 exosome applications onto hnFSSCs were also performed for investigating both necrosis and apoptosis. C-Met transfection to SK-Mel-1 melanoma cell exosomes caused reduced levels of late apoptosis and decreased levels of necrosis when compared with untransfected SK-Mel-1 cell exosome application to hnFSSCs. Therefore once c-Met was silenced in melanoma exosomes, hnFSSCs necrosis decreased after exosome applications. Rab27a transfection to melanoma cells on the other hand stimulated decreased necrosis and relatively increased late apoptosis of hnFSSCs in comparison with the untransfected melanoma exosome applied hnFSSCs group. As expected, Rab27a silencing in cancer cell exosomes triggered enhanced necrosis of hnFSSCs after presentation (Figure 5.33).

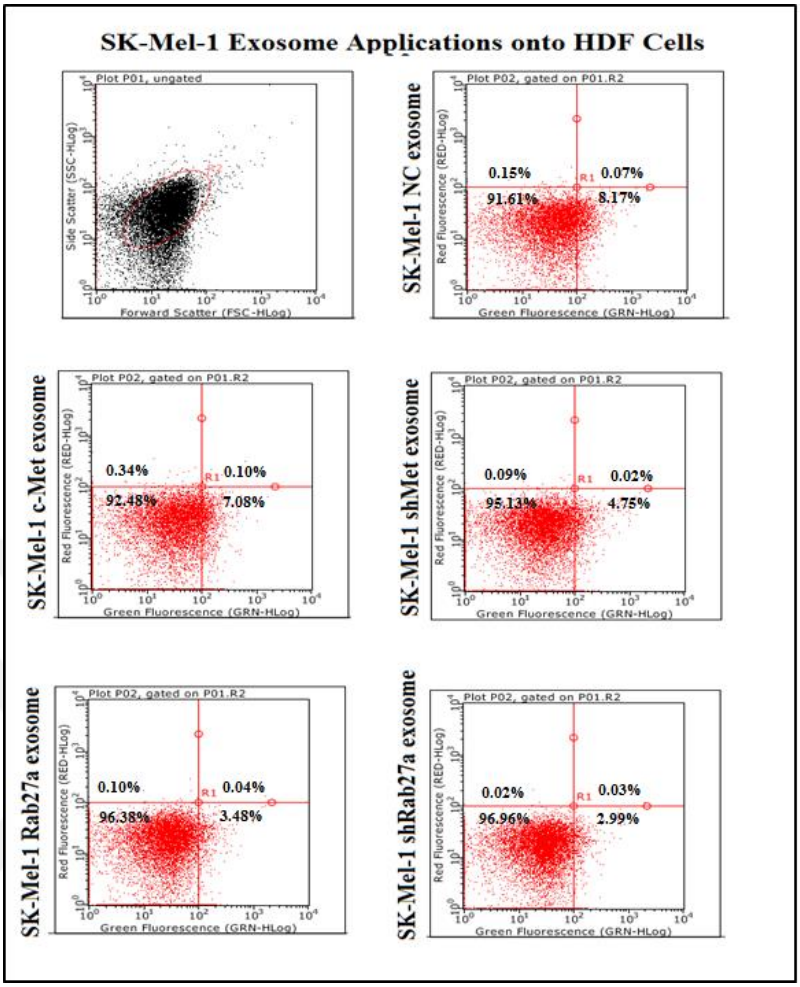

Figure 5.34. Annexin V-FITC Apoptosis Detection analysis for HDF cells after SK-Mel-1 exosome applications. Abbreviations: HDF; Human dermal fibroblasts.

Non-adherent SK-Mel-1 malignant melanoma exosomes were given to adult healthy human cell line HDF as well. Annexin V-FITC analysis results specified that c-Met transfected SK_Mel-1 cell exosome application increased late apoptosis of hnFSSCs and silencing c-Met triggered reduced late apoptosis as expected. Rab27a transfection to melanoma cell exosomes also decreased late apoptosis therefore silencing c-Met in exosomes demonstrated almost no effect on hnFSSCs after exosomal applications (Figure 5.34).

5.6. CELL MIGRATION ASSAY

Publications related to cancer cell exosomes opened a unique characteristic of exosomes coming from cancer cells, representing that the substances of exosomes can encourage

migration. One recent study demonstrated that exosomes of cancer cells cover numerous migration related factors. Subsequently, the exosomes of lung cancer cells amplified the migration of endothelial cells and cancer cells [43].

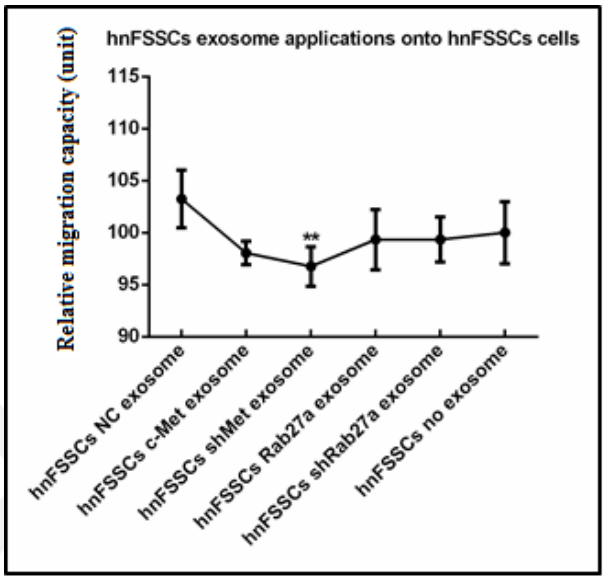

Figure 5.35. Cell migration levels of hnFSSCs after the application of hnFSSCs exosomes. Abbreviations: hnFSSCs: Human newborn foreskin stem cells. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Previous studies inspected the effects of exosomes on cell migration that is a main mechanism in metastasis. Recent results displayed that exosomes stimulate cell migration [44]. Furthermore, exosomes induce migration proportional to the metastatic potential of the cell from which the exosomes originated. On the other hand, different experimentations indicate that the c-met axis is covered in the targeted relocation of stem cells, and this sort of HGF related chemo-attraction can direct stem cells into the HGF rich surroundings of injured sites (e.g., infarcted myocardium) [45].

Cell migration analysis of this study outcome underlined that c-Met transfection to hnFSSCs exosomes decreased the motility in comparison with the untansfected exosome application to hnFSSCs. Therefore, silencing c-Met in exosomes also caused decreased migration when added to hnFSSCs environment when compared with c-Met transfected exosome applications. Rab27a transfection to hnFSSCs cells and exosomes found to increase migration in small levels. However, silencing of Rab27a in cells caused limited exosome

release and of course triggered less migration once added to hnFSSCs environment **(**Figure 5.35).

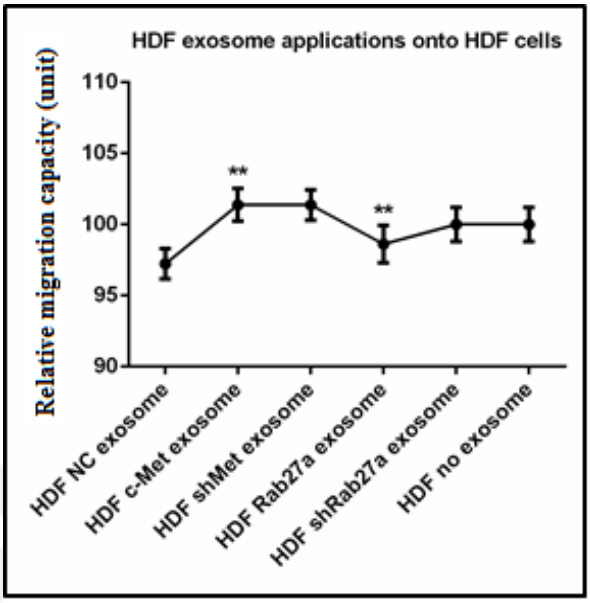

Figure 5.36. Cell migration levels of HDF after the application of HDF exosomes. Abbreviations: HDF: Human dermal fibroblasts. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Adult healthy fibroblastic cell line HDF exosomes were also presented in order to check their effect on HDF migration itself. Outcomes were found almost totally different than the stem cell results. Once untransfected HDF cell exosomes were given to HDF cells itself, the migration decreased. Subsequently, c-Met transfections to HDF cells and exosomes increased migration significantly. Silencing of c-Met did not cause relative decrease though. In contrast to other results, Rab27a transfection to cells and exosomes seemed to have opposite effect on HDF cell migration. Once silenced, cell migration increased visibly (Figure 5.36).

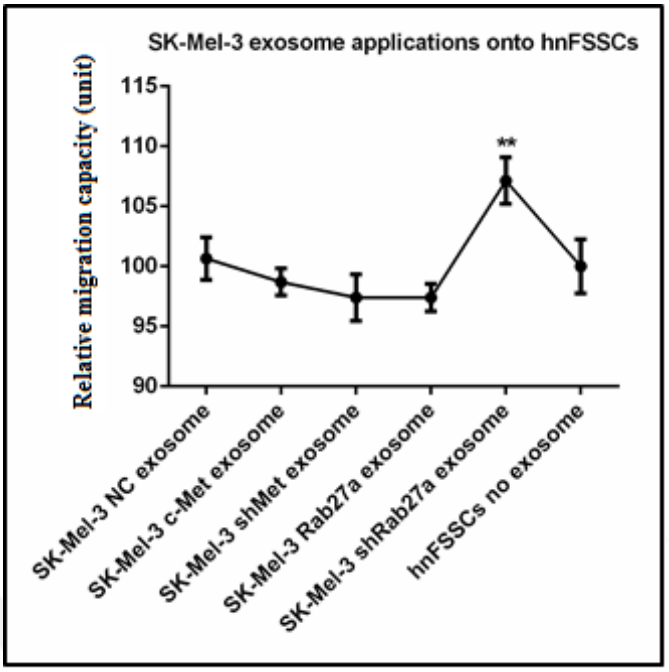

Figure 5.37. Cell migration levels of hnFSSCs after the application of SK-Mel-3 exosomes. Abbreviations: hnFSSCs: Human foreskin stem cells. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

A recent study revealed that gastric cancer exosomes promote stem cell migration [46]. In order to check whether cancer cell exosomes carry an effect on healthy cell migration, we analyzed SK-Mel-3 adherent malignant melanoma cell exosomes and their effects on hnFSSCs. Untransfected melanoma exosome presentation found to have almost no effect on hnFSSCs migration. Besides, c-Met transfections to melanoma cells and turning them highly overexpress c-Met seemed to cause decreased effect on hnFSSCs. Silencing c-Met in melanoma cells and their exosomes triggered decreased cell migration of hnFSSCs after pexosome presentation. Relevant to the data, Rab27a silencing in melanoma cells and exosomes cause decreased exosome release and decreased cancer cell-stem cell communication. It was found that once Rab27a silenced in melanoma exosomes, hnFSSCs migration increased after melanoma cancer exosome presentation to the environment (Figure 5.37).

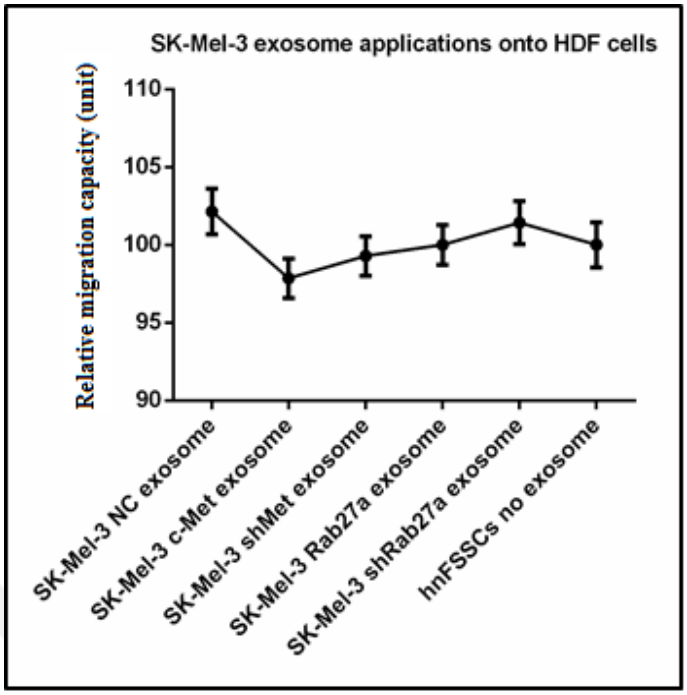

Figure 5.38. Cell migration levels of HDF after the application of SK-Mel-3 exosomes. Abbreviations: HDF: Human dermal fibroblasts. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

As mentioned in previous studies, cancer exosomes revealed to enhance fibroblast to myofibroblast differentiation and also tumor development [47]. Untransfected SK-Mel-3 exosomes application reasoned increased hnFSSCs migration. C-Met transfections to already metastatic melanoma cells, increased the c-Met level in the cells and exosomes enormously and triggered reduced cell migration of HDF when applied. As expected, c-Met silencing in cancer exosomes caused heightened HDF migration after exosome application. Similar to other data, Rab27a silencing of melanoma cells and exosomes, increased HDF migration when exosomes added to the environment (Figure 5.38).

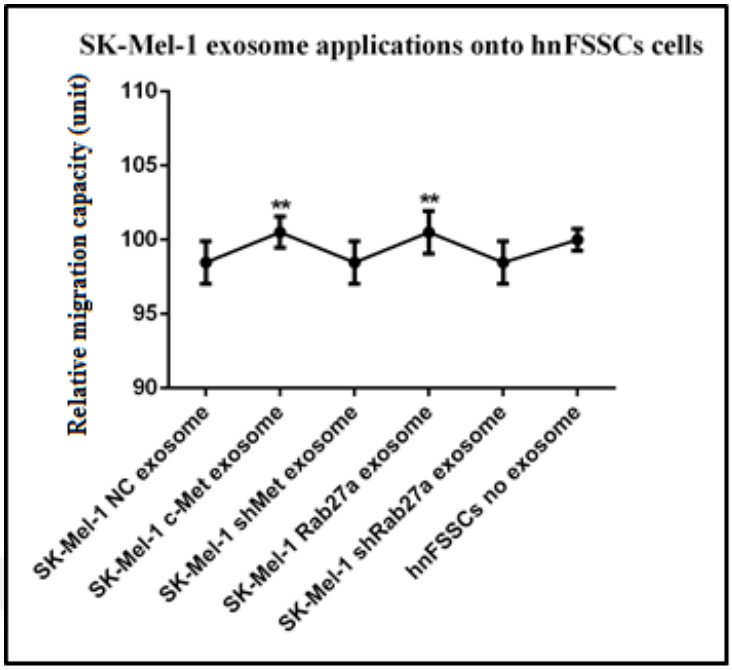

Figure 5.39. Cell migration levels of hnFSSCs after the application of SK-Mel-1 exosomes. Abbreviations: hnFSSCs: Human foreskin stem cells. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Non-adherent malignant melanoma exosome applications to stem cells displayed different results. C-Met transfections to melanoma cells found important in enhancing hnFSSCs migration after exosome submission. Meanwhile, Rab27a transfections to cancer cells and relatively to their exosomes also increased hnFSSCs migration after presentation. Besides, both of Rab27a and c-Met silencing seemed to cause decreased hnFSSCs migration (Figure 5.39).

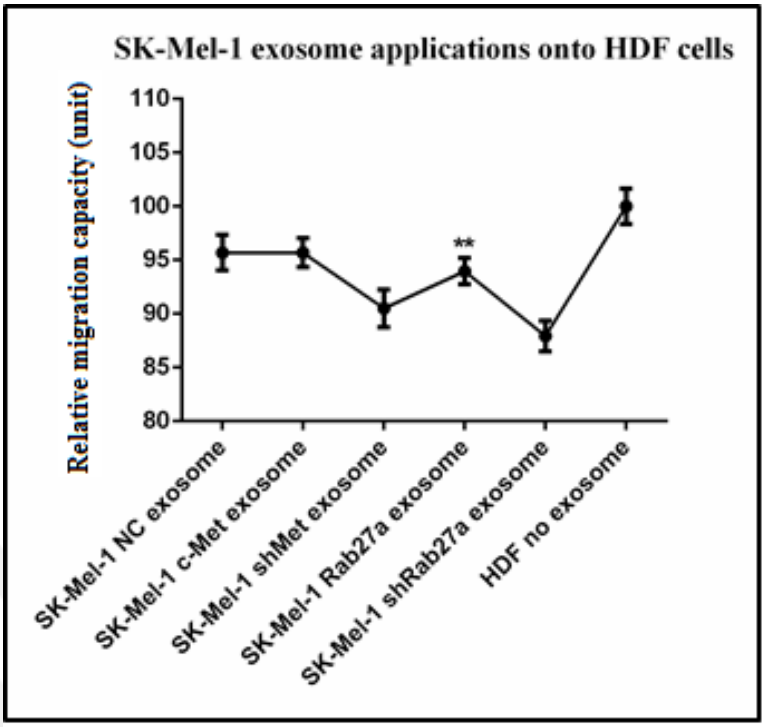

Figure 5.40. Cell migration levels of HDF after the application of SK-Mel-1 exosomes. Abbreviations: HDF: Human dermal fibroblasts. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

SK-Mel-1 exosome effects on cells were also examined for HDF cells. They showed relatively same results with their exosome onto stem cells. Only difference was established as the fact that every single one of the cancer exosome applications caused decreased cell migration of HDF cells. Overexpression of both c-Met and Rab27a in melanoma exosomes triggered enhanced HDF migration, whereas silencing of them brought decreased HDF migration once exosomes applied (Figure 5.40).

5.7. ENDOTHELIAL CELL TUBE FORMATION ASSAY

Endothelial tube formation assays were performed with exosomes derived from hnFSSCs, HDF, SK-Mel-1 and SK-Mel-3 cells following 24 hours normoxic conditions. No exosome application onto HUVEC cells showed longer tube formations when compared with hnFSSCs NC exosome applications onto HUVEC cells. Meanwhile, hnFSSCs shMet exosome presence triggered almost one and a half times enhanced tube formation in

comparison with hnFSSCs c-Met exosome presence. Correlating with the c-Met expressions silenced Rab27a expressed exosomes of hnFSSCs illustrated two times higher tube formation than Rab27a transfected hnFSSCs exosomes. Besides, silencing c-Met seemed more relevant to endothelial tube formation than silencing Rab27a as in hnFSSCs exosomes (Figure 5.41).

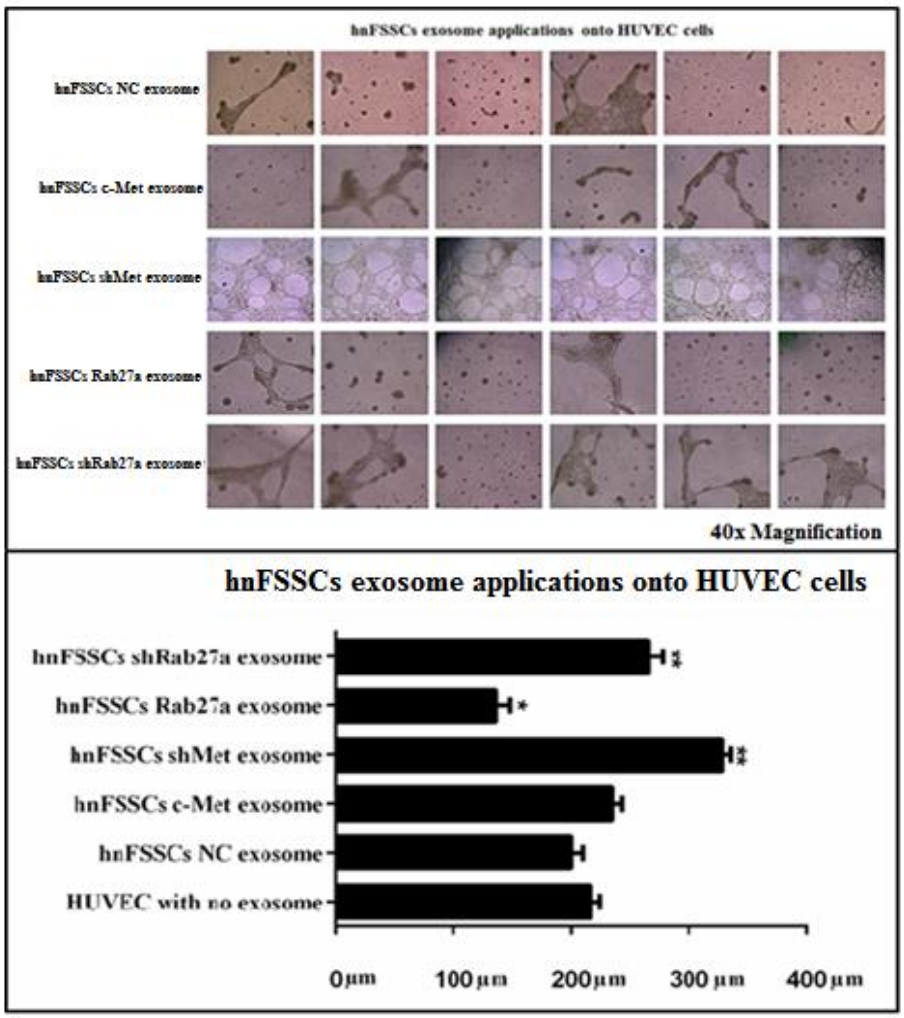

Figure 5.41. Effects of hnFSSCs exosomes on endothelial tube formation. Abbreviations: HUVEC: Human umbilical vein endothelial cells, hnFSSCs: Human newborn foreskin stem cells. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

HUVECs were seeded onto Matrigel and cultured for 24 h under no supplementation with or without pre-treatment of optimum hnFSSCs exosomes. Capillary tube formation on Matrigel was visualized with an inverted ZEISS microscope at a magnification of 40X.

HDF exosome applications were also tested for tube formation assays. Similar to the hnFSSCs no exosome application onto HUVEC cells were providing more formation than NC exosomes of HDF cells. As healthy cells lines, HDF Met exosome applications also indicated lower tube formation than silenced c-Met carrying HDF exosome ones which was almost two times lower. As distinct from the hnFSSCs results, Rab27a carrying HDF exosomes showed relatively advanced tube formations in comparison with shRab27a carrying HDF cells (Figure 5.42).

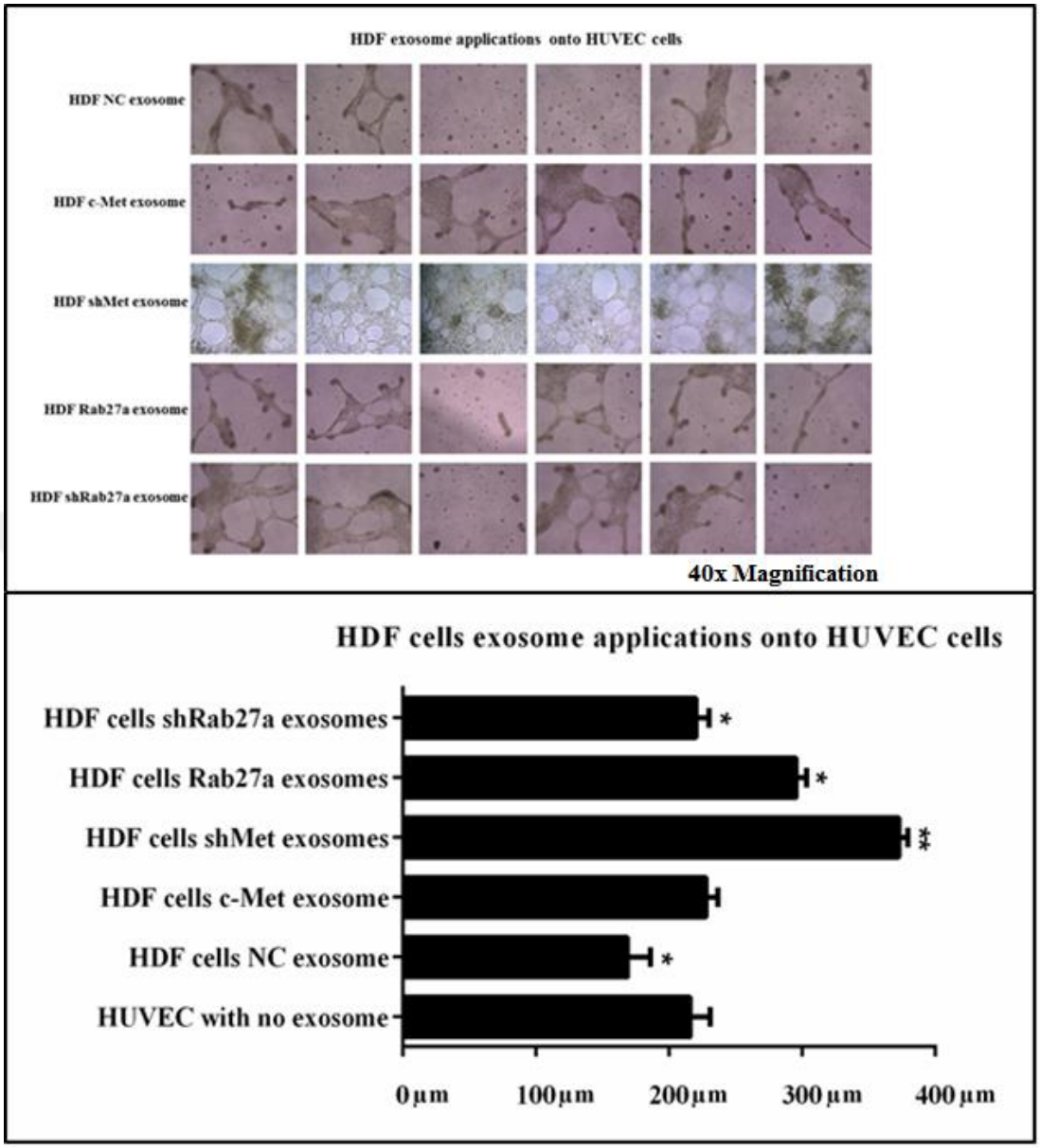

Figure 5.42. Effects of HDF cell exosomes on endothelial tube formation. HUVEC: Human umbilical vein endothelial cells, HDF: Human dermal fibroblasts. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

HUVECs were seeded onto Matrigel and cultured for 24 h under no supplementation with or without pre-treatment of optimum HDF cell exosomes. Capillary tube formation on Matrigel was visualized with an inverted ZEISS microscope at a magnification of 40X.

Suspension malignant SK-Mel-1 melanoma cells were also tested in tube formation manner. In harmony with the healthy cell line exosome applications, no exosome provided higher tube formation than Sk-Mel-1 NC exosome applications. Beside that c-Met transfected SK-Mel-1 exosome applied HUVEC cells showed significantly higher tube formations than SK-Mel-1 shMet exosome applied ones. Likewise, SK-Mel-1 Rab27a exosome applied HUVEC cells verified not much higher formations compared to shRab27a exosome applied HUVEC cells (Figure 5.43).

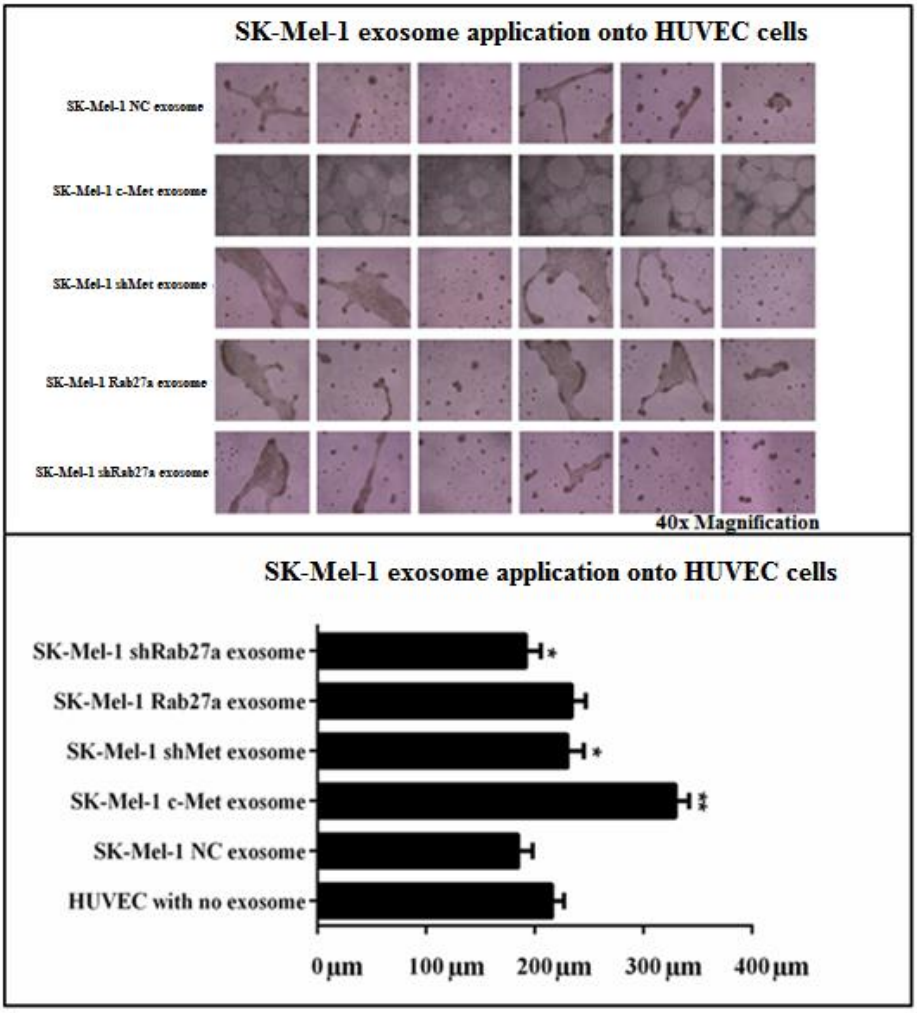

Figure 5.43. Effects of non-adherent SK-Mel-1 exosomes on endothelial tube formation. HUVEC: Human umbilical vein endothelial cells.*P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

HUVECs were seeded onto Matrigel and cultured for 24 h under no supplementation with or without pretreatment of optimum SK-Mel-1 exosomes. Capillary tube formation on Matrigel was visualized with an inverted ZEISS microscope at a magnification of 40X.

Adherent malignant melanoma cell line SK-Mel-3 exosome applications were also analyzed under this perspective. Therefore, existence of SK-Mel-3 NC exosomes delivered moderately greater tube formations of HUVEC cells than any other SK-Mel-3 exosome applied groups and no exosome applied group. Similar to SK-Mel-1 results, SK-Mel-2 c-Met exosome presentation onto HUVEC cells group displayed significantly better tube formations than silenced c-Met exosome ones. Interestingly, SK-Mel-3 shRab exosome applied HUVEC cells showed higher formation compared to Rab27a transfected exosome application group. Then again, c-Met transfected SK-Mel-3 exosome group indicated almost equal HUVEC tube formation with the SK-Mel-3 NC exosome applied group (Figure 5.44).

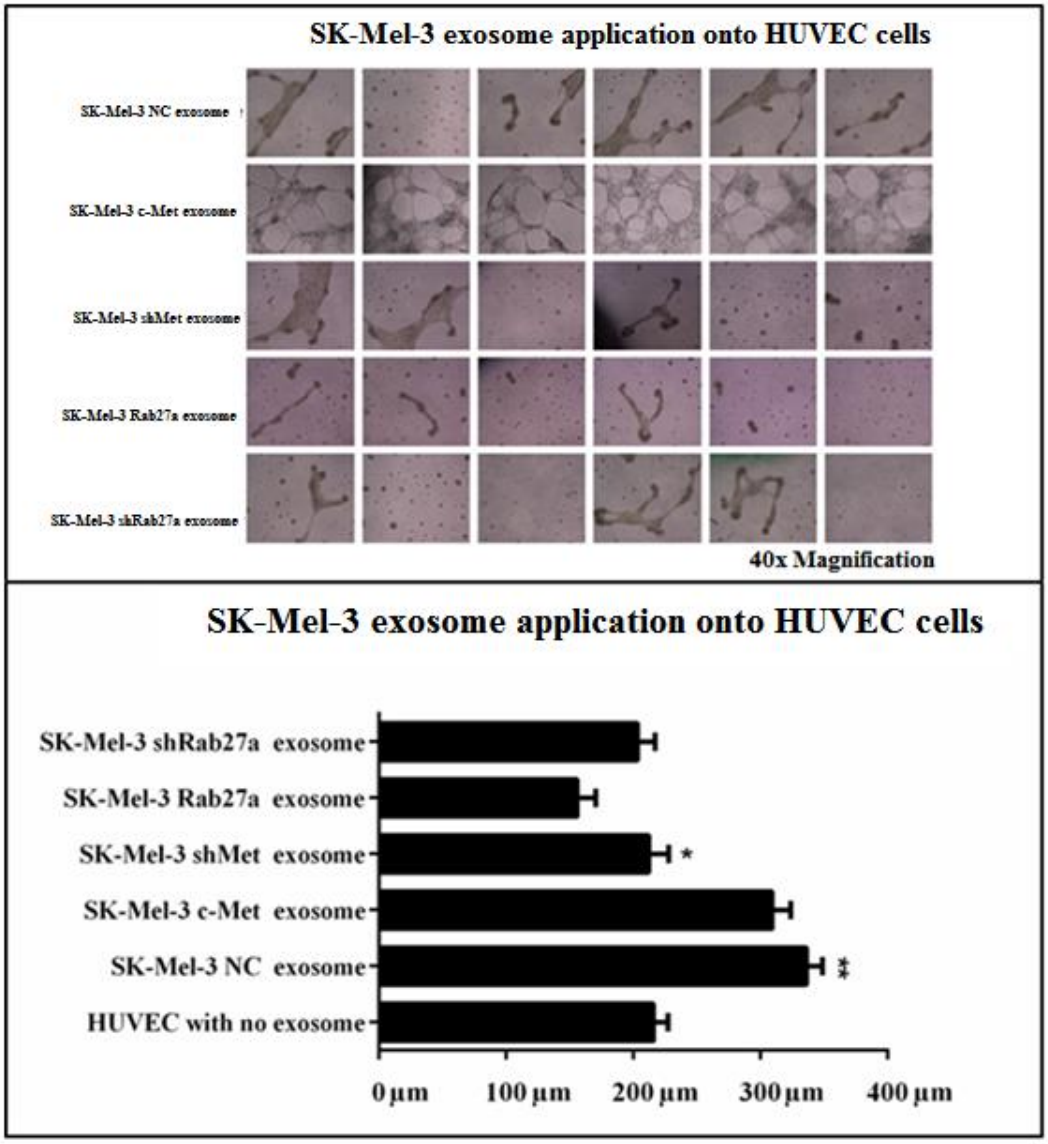

Figure 5.44. Effects of adherent malignant SK-Mel-3 cell exosomes on endothelial tube formation. Abbreviations: HUVEC: Human umbilical vein endothelial cells.*P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

HUVECs were seeded onto Matrigel and cultured for 24 h under no supplementation with or without pre-treatment of optimum SK-Mel-3 exosomes. Capillary tube formation on Matrigel was visualized with an inverted ZEISS microscope at a magnification of 40X.

5.8. ELISA ASSAYS

5.8.1. EGF Expression Results

Epidermal growth factor receptor (EGFR) is the main dynamic in epithelial malignancies. Regarding to this situation, enhanced action of EGFR brings tumor development, invasion and metastasis. It is a participant of the ErbB family of tyrosine kinase receptors which convey a development-tempting signal to cells that have been enthused via EGFR ligants such as epidermal growth factor (EGF) [48].

In healthy tissues, the accessibility of EGFR ligands is strongly controlled to guarantee that the mechanisms of cell proliferation exactly counterpart the tissue necessities for homeostasis. Therefore, in cancer, EGFR is frequently continually stimulated related to the continued generation of EGFR ligands in the tumor microenvironment or as an outcome of an EGFR mutation, which restricts the receptor in a level of persistent stimulation. Abnormal TGFα or EGFR expression in tumors usually deliberates a more destructive phenotype and is consequently mostly prognostic of poor forecast. Expectedly, EGFR has arisen as a major objective for beneficial interference. In EGF-high melanoma cells, EGF knockdown triggered an important reduction of lymph node metastasis and primary tumor lymphangiogenesis in vivo, same as the impairment of tumor cell migration in vitro [49]. EGFR moderates HGF/c-Met action via persuading c-Met ectodomain shedding and HGF/c-Met trans-activates EGFR, directing to an improved activation of downstream signaling pathways. Crosstalk amongst EGFR and c-Met play a key role in regulating healthy cell migration, proliferation, and wound healing [50].

Alterations in Rab expression intensities may affect cell development and proliferation over their impact on growth factor receptor trafficking and signaling. This concept is mainly relevant to those Rabs regulating the endocytic routes of the several membrane receptors of mitogenic ligands like the epidermal growth factor receptor (EGFR) [51].

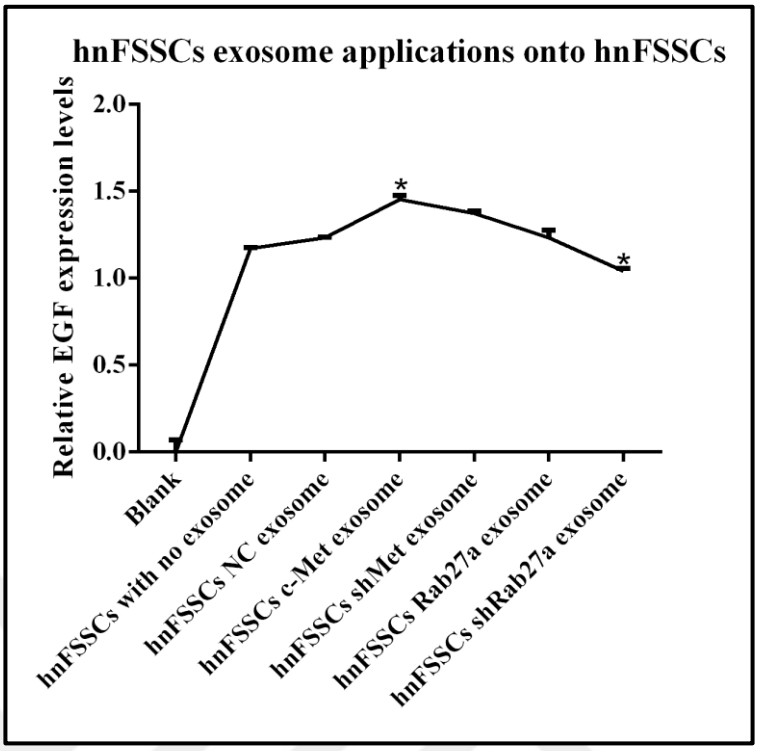

Figure 5.45. Relative EGF expression analysis of hnFSSCs after the application of hnFSSCs exosomes with ELISA technique. Abbreviations: hnFSSCs: human newborn foreskin stem cells, EGF: Epithelial Growth Factor. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

EGF expressions were examined for hnFSSCs in order to understand the crosstalk between exosomes carrying c-Met and Rab27a discharged from hnFSSCs itself. Normally, hnFSSCs NC exosome applications to hnFSSCs showed higher EGF expressions than no exosome application group. Most importantly, exosome presentations carrying c-Met indicated the highest EGF expression in comparison with the other groups. hnFSSCs Rab27a exosome applied hnFSSCs cells also showed more enhanced expressions than hnFSSCs NC exosome applied ones. These data suggested that both Rab27a and c-Met may be cross talking with EGF. Meanwhile, exosome-cell interactions may alter the relative expressions of EGF. Thus, presented shMet and shRab27a carrying hnFSSCs exosomes decreased EGF expressions in hnFSSCs after submission (Figure 5.45).

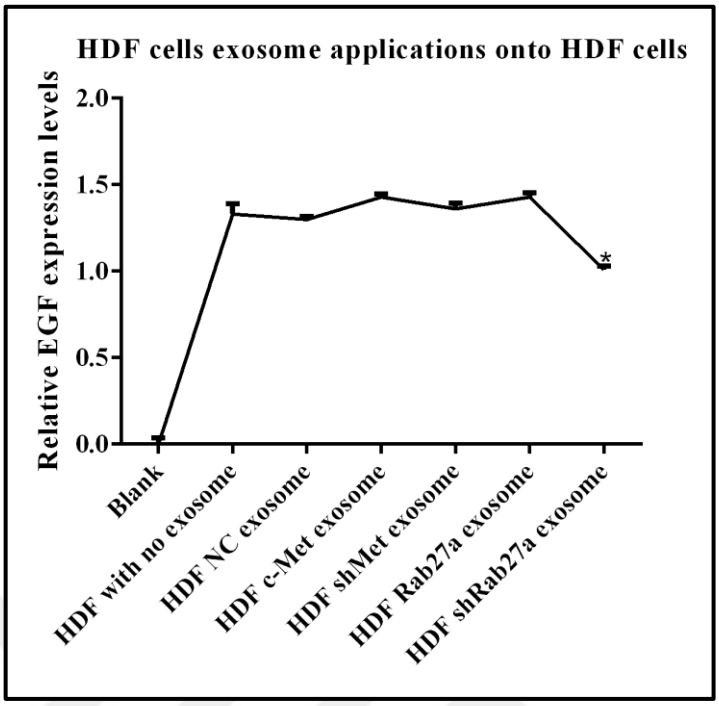

Figure 5.46. Relative EGF expression analysis of HDF after the application of HDF exosomes with ELISA technique. Abbreviations: HDF: Human dermal fibroblasts, EGF: Epithelial Growth Factor.*P<0.05. Notes: Results were examined by oneway ANOVA and Turkey's posttest.

Relative expression levels of HDF cells were analyzed after the exosome applications of the same cell line. Human dermal fibroblasts normally express EGF in order to induce wound healing and for normal cell proliferation. C-Met transfected HDF cell exosome application to HDF cells itself indicated higher expressions of EGF when compared with no exosome application and untransfected HDF cell exosome application. Meanwhile, silencing c-Met in cells and the presentation of silenced c-Met carrying exosomes showed reduced EGF expressions. Rab27a carrying exosome applications seemed to express higher EGF than both NC exosome application and no exosome application groups. Yet, silencing Rab27a in HDF cells and in automatically in their exosomes found to cause relatively decreased EGF expressions after the application *in vitro* (Figure 5.46).

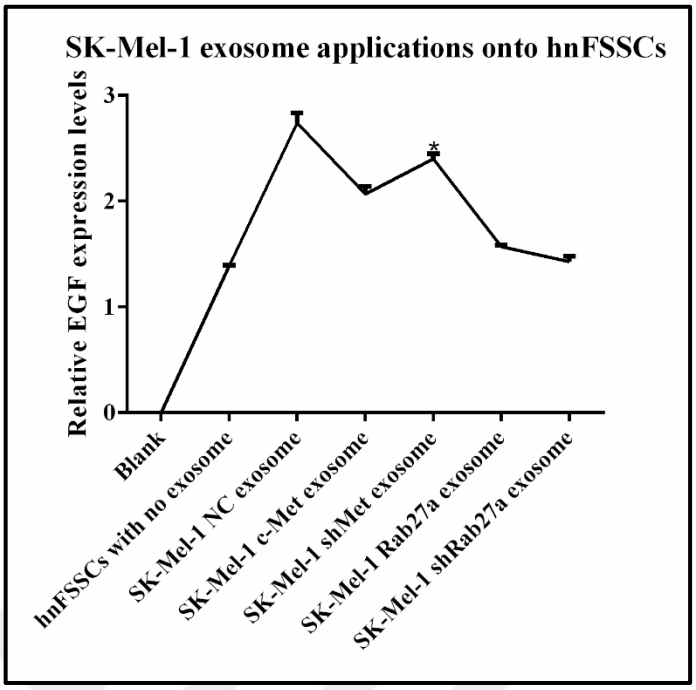

Figure 5.47. Relative EGF expression analysis of hnFSSCs after the application of SK-Mel-1 exosomes with ELISA technique. Abbreviations: hnFSSCs: human newborn foreskin stem cells, EGF: Epithelial Growth Factor. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

c-Met and EGFR receptors are extensively expressed on cancer cells; they are associated with the growth and evolution of cancer over a overabundance of acts on cell cycle development, apoptosis, motility and metastasis and are possible targets for combined treatment. Recent studies have demonstrated that c-Met is overexpressed in 90% of cancers [52]. SK-Mel-1 malignant melanoma cells can be defined as non-adherent cells and they may be categorized as highly metastatic. Exosomes derived from transfected and untransfected SK-Mel-1 exosomes were also applied to both HDF and hnFSSCs healthy cell lines in optimum concantrations. C-Met gene were transfected to the already highly metastatic SK-Mel-1 melanoma cells. Therefore, very much overexpressed c-Met carrying exosomes were discharged by those cells and applied to hnFSSCs. Results indicated that once c-Met is highly overexpressed, EGF levels decreases in hnFSSCs. However, when silenced c-Met carrying exosomes applied to hnFSSCs, the EGF expression increases. Meanwhile, untransfected SK-Mel-1 cell exosome application, in other words normal metastatic c-Met expressing exosome applications, indicated advanced EGF expressions in hnFSSCs. On the other hand, overexpressed Rab27a carrying exosome presentation can not

be identified as much effective as c-Met groups. Yet, silenced Rab27a carrying melanoma exosome submission, demonstrated lower levels of EGF expression in hnFSSCs (Figure 5.47).

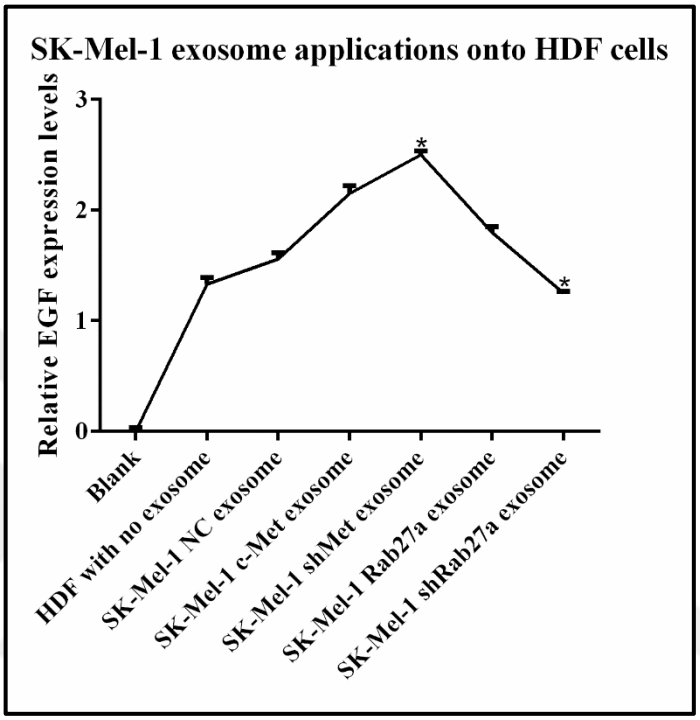

Figure 5.48. Relative EGF expression analysis of HDF after the application of SK-Mel-1 exosomes with ELISA technique. Abbreviations: HDF: Human dermal fibroblasts, EGF: Epithelial Growth Factor. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Nevertheless, same SK-Mel-1 exosomes were applied to HDF healthy cells as well. Results displayed similar effects with the hnFSSCs in EGF expression manner. Therefore, highly overexpressed c-Met exosome presentation showed higher EGF expression than untransfected SK-Mel-1 exosome application ones and Rab27a silencing in cells of melanoma seemed to have effects that are more significant in healthy adult HDF cells. Once silenced in melanoma exosomes, EGF expression decreased in HDF cells (Figure 5.48).

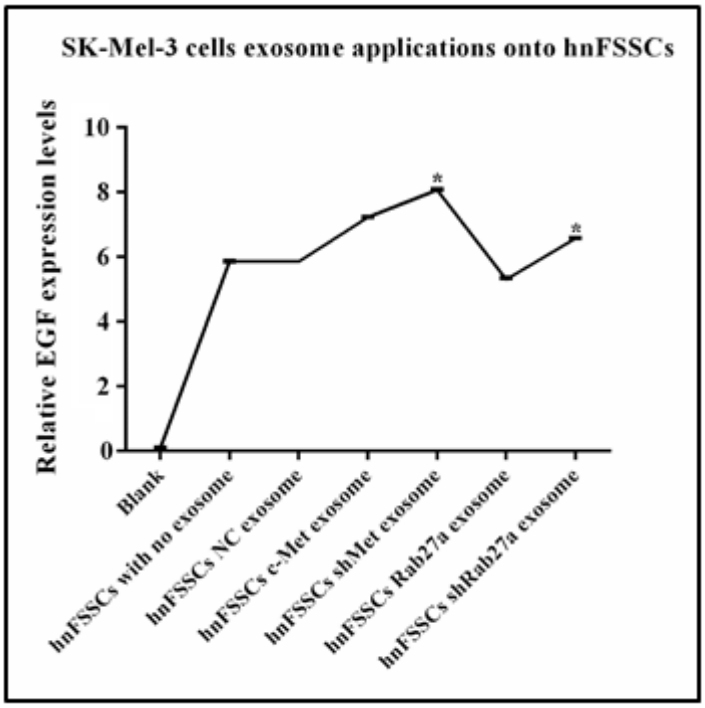

Figure 5.49. Relative EGF expression analysis of hnFSSCs after the application of SK-Mel-3 exosomes with ELISA technique. Abbreviations: hnFSSCs: human newborn foreskin stem cells, EGF: Epithelial Growth Factor. *P<0.05. Notes: Results were inspected by one-way ANOVA and Turkey's posttest.

SK-Mel-3 malignant melanoma cells are adherent cells. EGF expressions of hnFSSCs were mainly similar to the SK-mel-1 exosome demonstrated ones. In these groups, highly advanced overexpression of c-Met in melanoma exosome applications displayed lower EGF expression than silenced c-Met carrying ones in hnFSSCs similar to previous SK-Mel-1 exosome presentation results. Nonetheless, Rab27a carrying exosome application results were different from the previous ones. Once Rab27a transfected SK-Mel-3 exosomes applied to hnFSSCs, EGF expressions of hnFSSCs enhanced. When silenced, the EGF expressions increased (Figure 5.49).

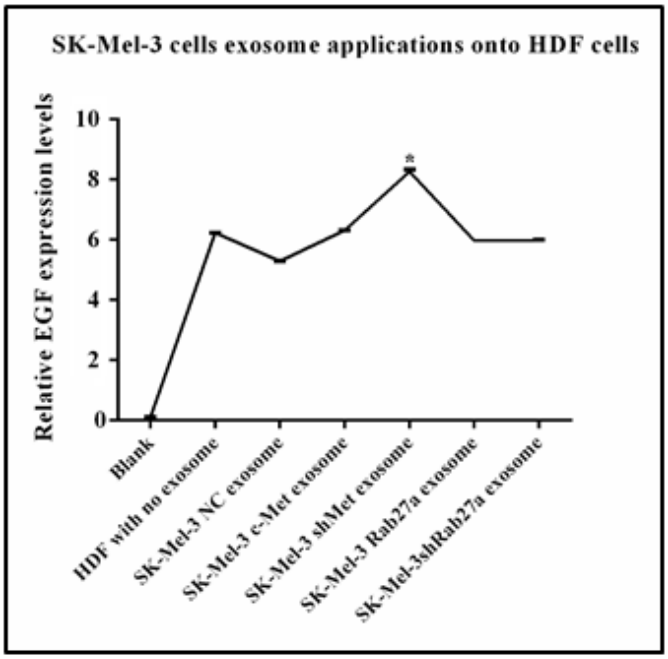

Figure 5.50. Relative EGF expression analysis of HDF cells after the application of SK-Mel-3 exosomes with ELISA technique. Abbreviations: HDF: Human dermal fibroblasts, EGF: Epithelial Growth Factor. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

The results were found similar for HDF adult healthy cells as well. Therefore, Rab27a transfection to adherent melanoma cells and their applications to HDF cells seemed to have no effect whether when they are overexpressed or silenced individually (Figure 5.50).

5.8.2. VEGF Expression Results

The appliances that give rise to the development of dysplastic nevus to malignant melanoma is not identified. Therefore, recent studies have revealed that raised levels of VEGF in malignant melanoma is more associated to its amplified generation via transmuted melanocytes instead of manufacture ensued from tumor development persuaded hypoxia. Results also disclosed that VEGF serum levels were advanced in melanoma patients in comparison with the healthy people. Additionally in melanoma group, the serum levels were increased in grander tumor widths. Nominal levels of VEGF mRNA have also been discovered in the normal skin. Outcomes of accumulation of VEGF on the vascular

endothelium in healthy dermis proposes that a basic low level VEGF expression can control skin vessel utility in normal biological circumstances. Overexpression of VEGF has been stated in skin illnesses which are categorized by angiogenesis and amplified vascular permeability [53].

It has publicized that c-MET rates associate with VEGF-A and VEGF-C expression in different carcinomas and the inhibition of c-MET decreases tumor development and angiogenesis in human cancer models [54]. Regarding to the important role of c-MET signaling in relocation, invasion, angiogenesis and bone marrow cell deployment it has been theorized that exosomes could deliver c-MET from melanoma to bone marrow stem cells and that this transfer could be a formerly unknown instrument for endorsing metastatic progression. Results designated that after 28 d of exosome application, c-Met expression was amplified in bone marrow stem cells from mice injected with B16-F10 (mouse melanoma cell) exosomes [32]. Overexpression of Rab27a lessens the calcium action in retinal epithelium cells that then decreases the VEGF excretion levels [55].

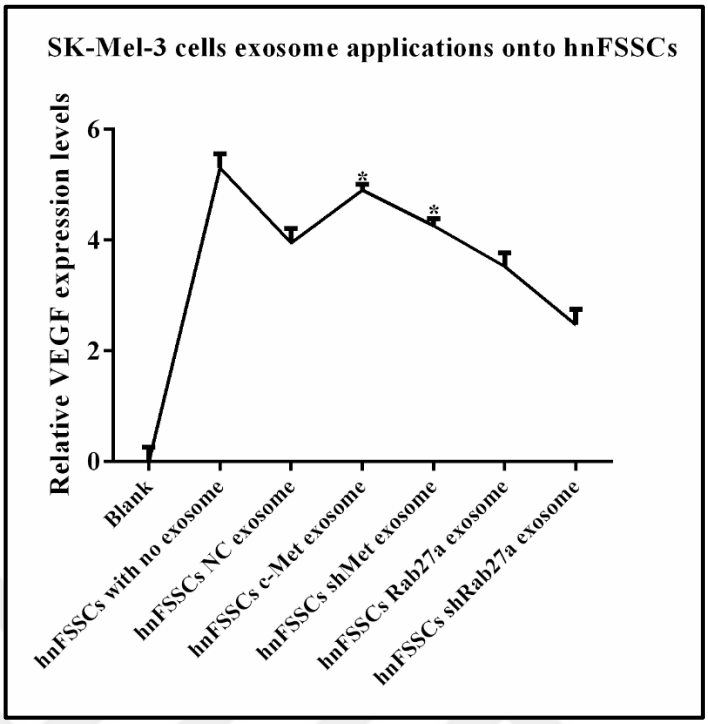

Figure 5.51. Relative VEGF expression analysis of hnFSSCs after the application of SK-Mel-3 exosomes with ELISA technique. Abbreviations: hnFSSCs: human newborn foreskin stem cells, VEGF: Vascular Endothelial Growth Factor. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Adherent human malignant melanoma cell exosomes were given to healthy stem cell line hnFSSCs in optimum concentrations. C-Met transfected melanoma cell exosomes showed higher VEGF levels in hnFSSCs cells after exosome presentation than untransfected malignant melanoma exosome applications. Silencing of c-Met in melanoma cells and exosomes automatically, brought decreased levels of VEGF in hnFSSCs after exosome applications. Interestingly, no exosome applied hnFSSCs group indicated higher VEGF levels than untransfected melanoma exosome applied hnFSSCs. In the Rab27a transfected melanoma exosome applied group, VEGF expression found to be higher than untransfected exosome applications (Figure 5.51).

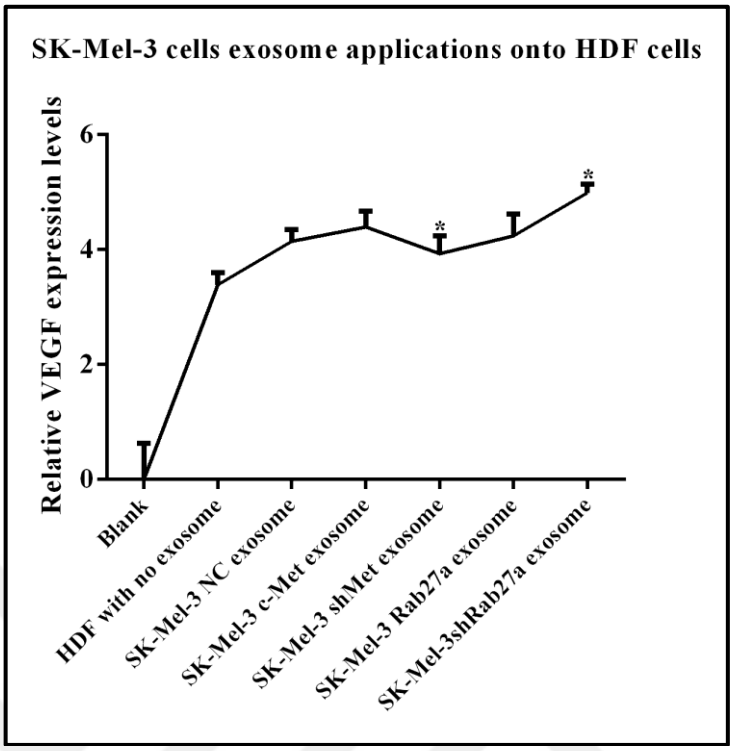

Figure 5.52. Relative VEGF expression analysis of HDF cells after the application of SK-Mel-3 exosomes with ELISA technique. Abbreviations: HDF: Human dermal fibroblasts, VEGF: Vascular Endothelial Growth Factor. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Recent publications revealed that cancer exosomes may activate fibroblast to myofibroblast differentiation. This was found to be related to exosomal TGFb1 and the heparan sulphate proteoglycan, betaglycan. Likewise, the biological significance of exosomal TGFb1 in moderating cancer stroma has not been well defined. Scientists have hypothesized that cancer-related exosomes are constituent of tumour to stromal cell association needed for producing a tumour-supporting stromal phenotype [48].

SK-Mel-3 exosome applications to HDF adult healthy cells demonstrated similar c-Met expression profile with the hnFSSCs expressions. Down-regulation of c-Met in melnaoma exosomes also generated lower VEGF expressions in HDF cells. Therefore, Rab27a transfected melanoma exosome education seemed to deliver very different effects on VEGF expression profiles in HDF cells. Very interestingly, both ELISA and tube formation assay

results indicated same outcomes which was found to be that once Rab27a expression is decreased in melanoma cells, the angiogenesis increases (Figure 5.52).

5.8.3. HGFR Expression Results

C-Met (HGFR) and its ligand, hepatocyte growth factor (HGF), have found upregulated in metastatic melanoma in different studies and have been associated with the invasion and disease progression. Therefore, former readings of c-Met and HGF in melanoma are incomplete in scope. Preliminary experimentations propose that inhibition of HGF or c-Met may decrease melanoma progression [56]. Meanwhile, other studies indicated that Rab27a inhibition reduces exosome release and therefore tumor growth and metastasis. So that, Rab27a expression may be associated with c-Met expression [31].

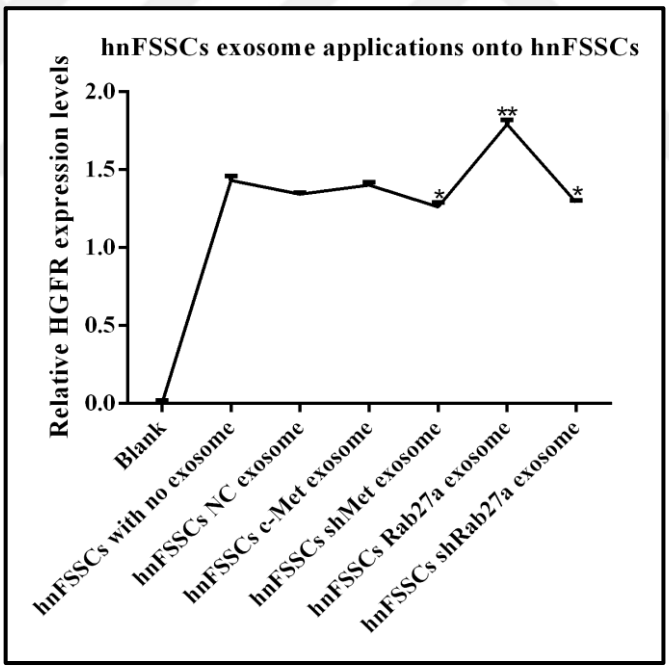

Figure 5.53. Relative HGFR expression analysis of hnFSSCs after the application of hnFSSCs exosomes with ELISA technique. Abbreviations: hnFSSCs: human newborn foreskin stem cells, HGFR: Hepatocyte Growth Factor Receptor. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Transfecting c-Met metastatic oncogene to a healthy stem cell line hnFSSCs seemed to have limited effect on c-Met expression itself, therefore once the transfected exosome was given

to hnFSSCs, c- Met expression increases even if not much. Besides, once the c-Met expression was silenced in hnFSSCS exosomes, hnFSSCs c-Met expression decreases in hnFSSCs itself after presentation. As a proof of correlation between c-Met and Rab27a, once Rab27a transfection occurs to the cells so as exosomes, HGFR expression increases in hnFSSCs after application significantly. Likewise, when silenced in exosomes, hnFSSCs HGFR expression decreases distinctly in hnFSSCs after exosome addition to the environment (Figure 5.53).

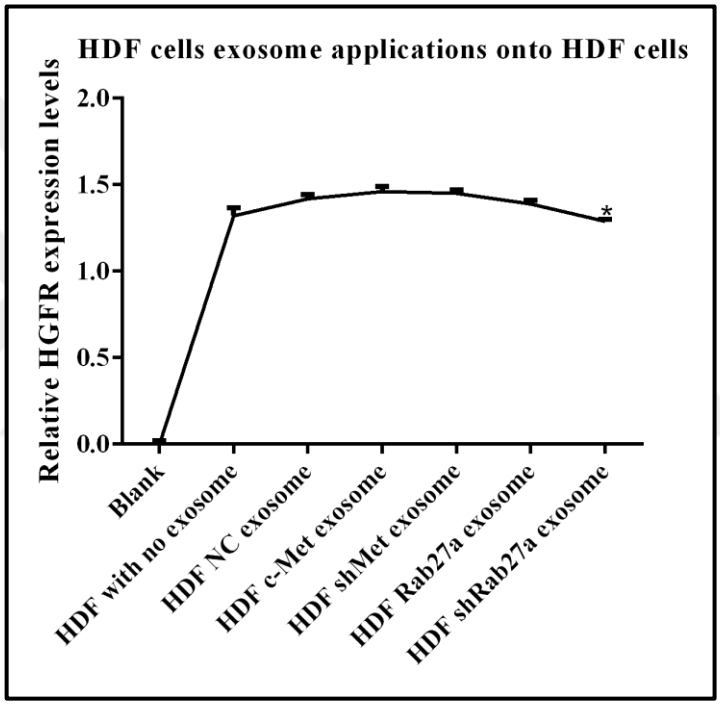

Figure 5.54. Relative HGFR expression analysis of HDF after the application of HDF exosomes with ELISA technique. Abbreviations: HDF: Human dermal fibroblasts, HGFR: Hepatocyte Growth Factor Receptor. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Results of this study also displayed similar outcomes when HDF exosomes were applied to HDF cells itself. Once c-Met transfected exosomes were presented to HDF cells, HGFR expression increases. Likewise, when silenced in exosomes, the expression decreases in HDF cells after presentation. Differently, Rab27a transfection did not seem to carry enormous effects on HGFR expression as much as hnFSSCs. However, it also was found to

be effective and Rab27a silencing exosomes triggered reduced levels of HGFR in HDF cells after exosome application (Figure 5.54).

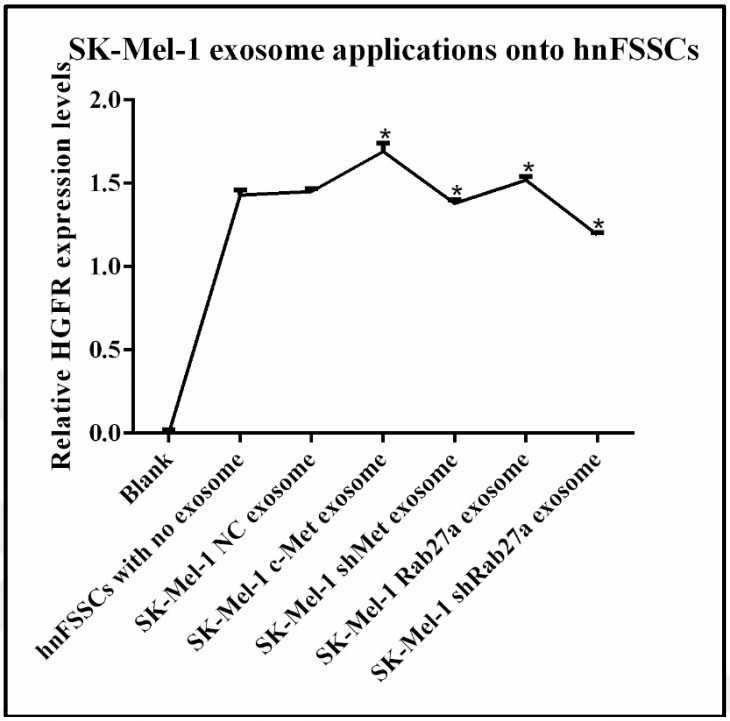

Figure 5.55. Relative HGFR expression analysis of hnFSSCs after the application of SK-Mel-1 exosomes with ELISA technique. Abbreviations: hnFSSCs: human newborn foreskin stem cells, HGFR: Hepatocyte Growth Factor Receptor. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

As indicated before, c-Met transfection to already malignant and c-Met expressing melanoma cells and by implication to exosomes suggested to carry clear effects in HGFR expressions. Once the transfected melanoma cell exosomes presented to healthy hnFSSCs cells, c-Met expression increases exceedingly. So as, once silenced, the expression reduces in hnFSSCs after exosome addition. In case of Rab27a transfection to malignant melanoma cells, exosome secretion increases and metastatic oncogene expression enhances in hnFSSCs after exosome supplementation. When silenced, exosome discharge reduces and HGFR expression lessens in hnFSSCs after cancer exosome addition (Figure 5.55).

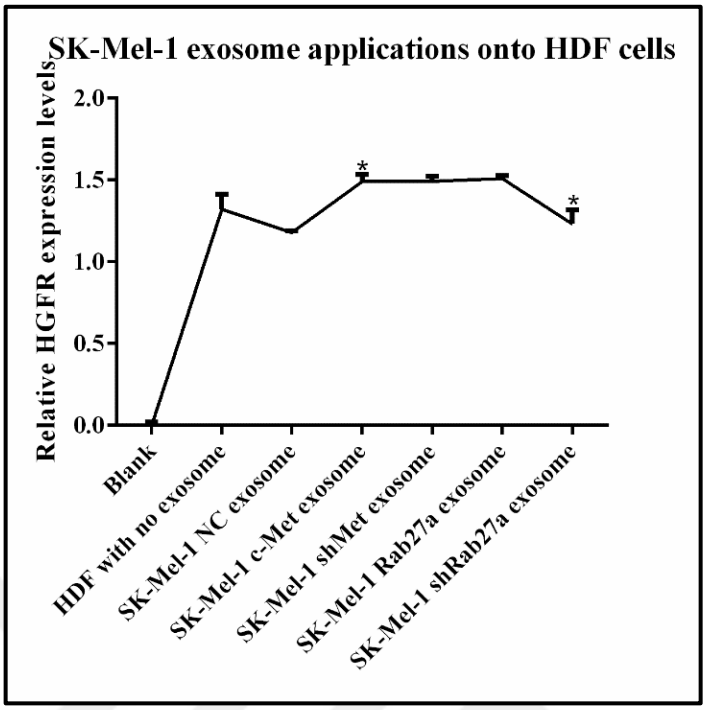

Figure 5.56. Relative HGFR expression analysis of HDF after the application of SK-Mel-1 exosomes with ELISA technique. Abbreviations: HDF: Human dermal fibroblasts, HGFR: Hepatocyte Growth Factor Receptor. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Unlike to the results, neither c-Met nor Rab27a transfections to the malignant melanoma cells found to have very low effects in HGFR expressions after their exosomes were given to HDF cells. In fact, hnFSSCs seemed to express higher HGFR than the untransfected melanoma exosome applied group. Only significant result was that silencing of Rab27a in melanoma exosomes decreased the HGFR expressions of HDF cells once the exosomes added to the environment (Figure 5.56).

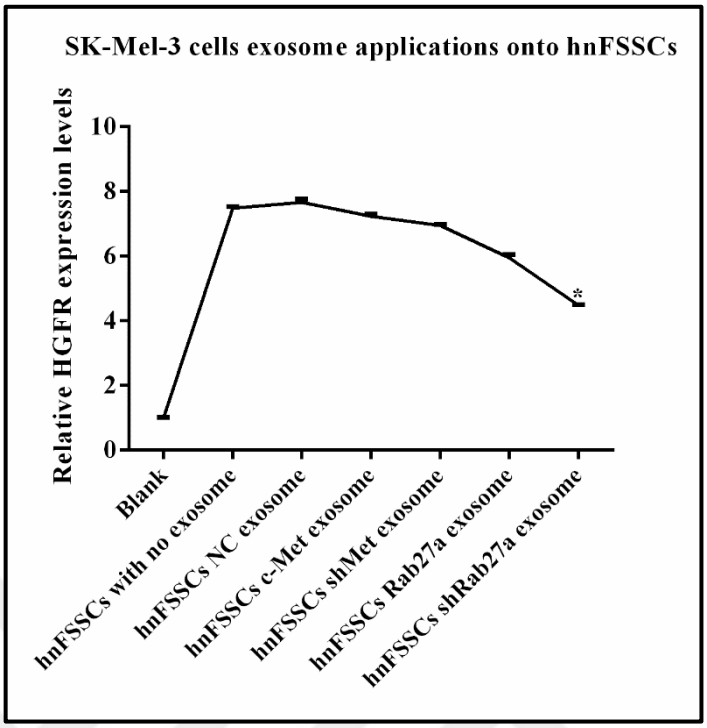

Figure 5.57. Relative HGFR expression analysis of hnFSSCs after the application of SK-Mel-3 exosomes with ELISA technique. Abbreviations: hnFSSCs: human newborn foreskin stem cells, HGFR: Hepatocyte Growth Factor Receptor. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Adharent malignant melanoma cell exosome were applied to hnFSSCs and c-Met transfected melanoma exosome application group displayed lower expressions of HGFR than untansfected melanoma exosome application group in hnFSSCs. Once c-Met was silenced in melanoma exosomes, hnFSSCs demonstrated reduced HGFR expressions upon exosome application. Similar to the other results, Rab27a silencing seemed to carry the most significant effect on HGFR expression of hnFSSCs after melanoma exosome applications and it dropped significantly (Figure 5.57).

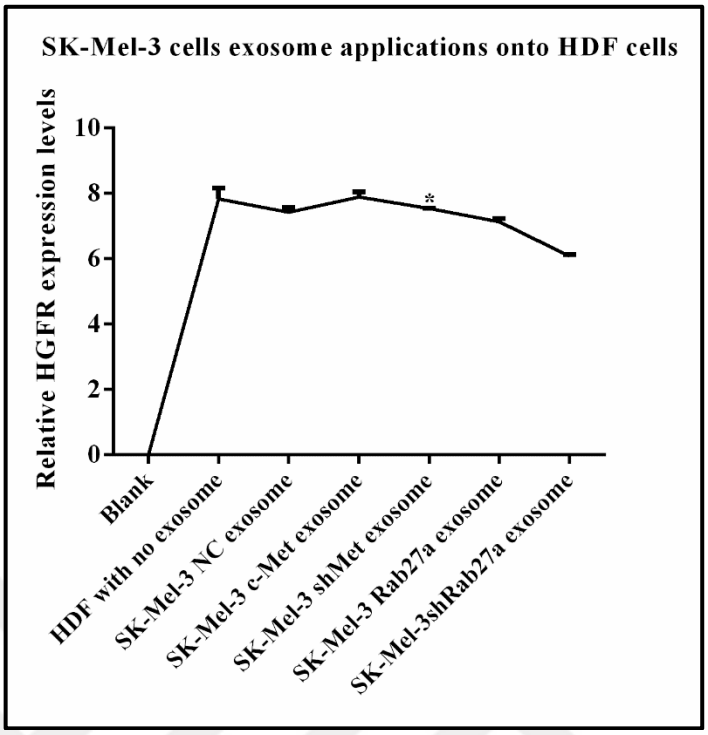

Figure 5.58. Relative HGFR expression analysis of HDF after the application of SK-Mel-3 exosomes with ELISA technique. Abbreviations: HDF: Human dermal fibroblasts, HGFR: Hepatocyte Growth Factor Receptor. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Adherent malignant melanoma exosome applications to HDF cells displayed almost similar effects except the untransfected exosome application group and no exosome applied group. In this one, untansfected exosome application seemed to have lower HGFR expression than no exosome one. Correlated with the other results, Rab27a silencing triggered lower HGFR expression in HDF after exosome presentation. In this one c-Met transfection was found significant as well and silencing brought lower HGFR expressions in HDF after cancer exosome submission (Figure 5.58).

5.8.4. Rab27a Expression Results

Different studies have displayed that; the augmentation of RAB27A expression would be consistent with the elevated melanin synthesis in melanocytes for effectively dispensing melanosomes to keratinocytes and so covering the epidermis for cutaneous photo-protection.

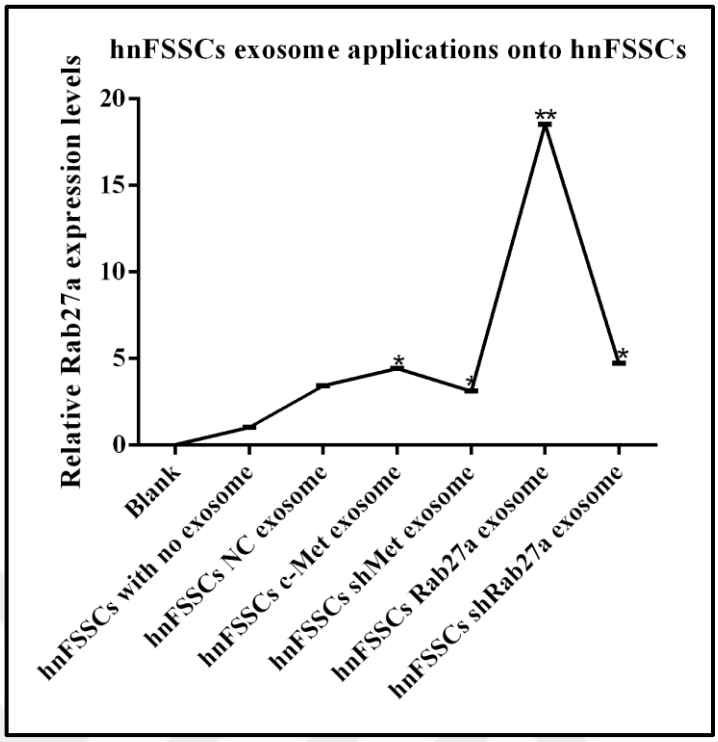

Figure 5.59. Relative Rab27a expression analysis of hnFSSCs after the application of hnFSSCs exosomes with ELISA technique. Abbreviations: hnFSSCs: human newborn foreskin stem cells. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Rab27a expression of hnFSSCs found to be enhanced after c-Met transfected hnFSSCs exosomes added to the environment. The expression also found decreased in exosome-applied hnFSSCs once c-Met was silenced in hnFSSCs exosomes. When Rab27a was transfected to hnFSSCs, Rab27a expression was thought to increase in exosomes. As thought, after the exosome application, Rab27a expression also increased in hnFSSCs. Meanwhile, once Rab27a was silenced in cells/exosomes, exosome applied hnFSSCs showed significantly lower expressions (Figure 5.59).

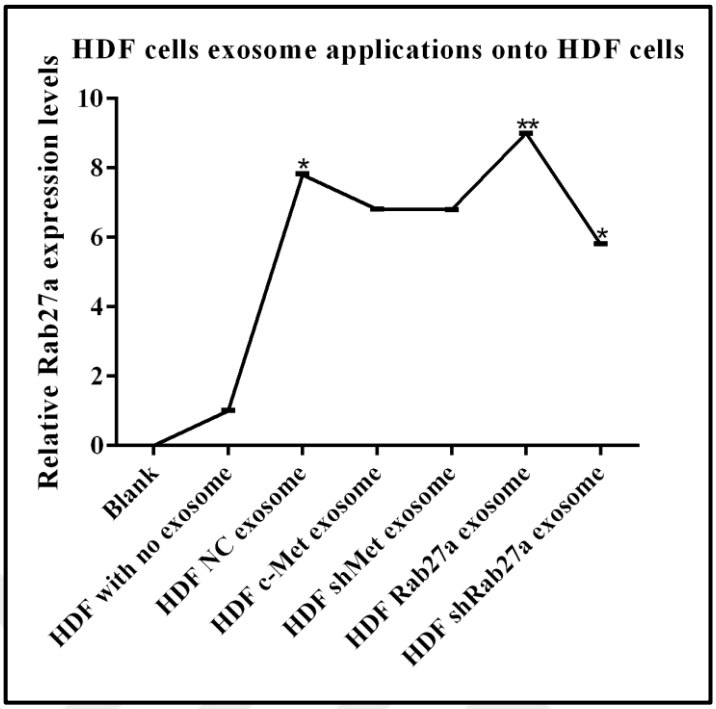

Figure 5.60. Relative Rab27a expression analysis of HDF after the application of HDF exosomes with ELISA technique. Abbreviations: HDF: Human dermal fibroblasts. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Relative Rab27a expressions were also analyzed after un-transfected and transfected HDF exosomes added to HDF cell environment. Results indicated that even the untransfected HDF exosome application increased the relative Rab27a expression almost nine times when compared with no exosome applied HDF cells. Another result was demonstrated as ineffective c-Met transfection to the cells. Once c-Met was silenced in HDF exosomes, HDF Rab27a expression was not found significantly changed. As expected, Rab27a transfected HDF exosome applications enhanced Rab27a expression in HDF cells sufficiently. When silenced in exosomes, it reduced importantly after application (Figure 5.60).

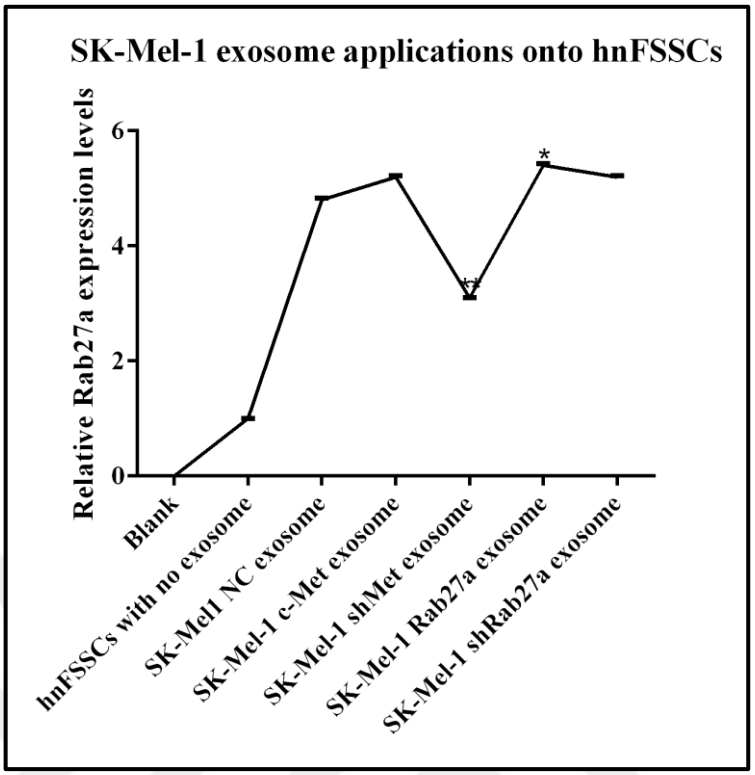

Figure 5.61. Relative Rab27a expression analysis of hnFSSCs after the application of SK-Mel-1 exosomes with ELISA technique. Abbreviations: hnFSSCs: human newborn foreskin stem cells. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

In case of non-adherent SK-Mel-1 malignant melanoma exosome applications onto hnFFSCs, even untransfected cell exosome applications increased the Rab27a expression in hnFSSCs after application almost 6 times higher than no exosome applied ones. C-Met transfections to already metastatic melanoma cells triggered overexpression of c-Met in melanoma exosomes. The presentation of these exosomes to hnFSSCs increased Rab27a expression in hnFSSCs but not significantly. Therefore, silencing of c-Met in melanoma cells, effected Rab27a expression of hnFSSCs cells after exosome application and the expression lowered visibly. As anticipated, Rab27a expression heightened after Rab27a transfected melanoma exosomes supplemented to hnFSSCs. However, even silencing of Rab27a in melanoma exosomes did not cause relevant Rab27a expression drop after the presentation to HDF cells *in vitro* (Figure 5.61).

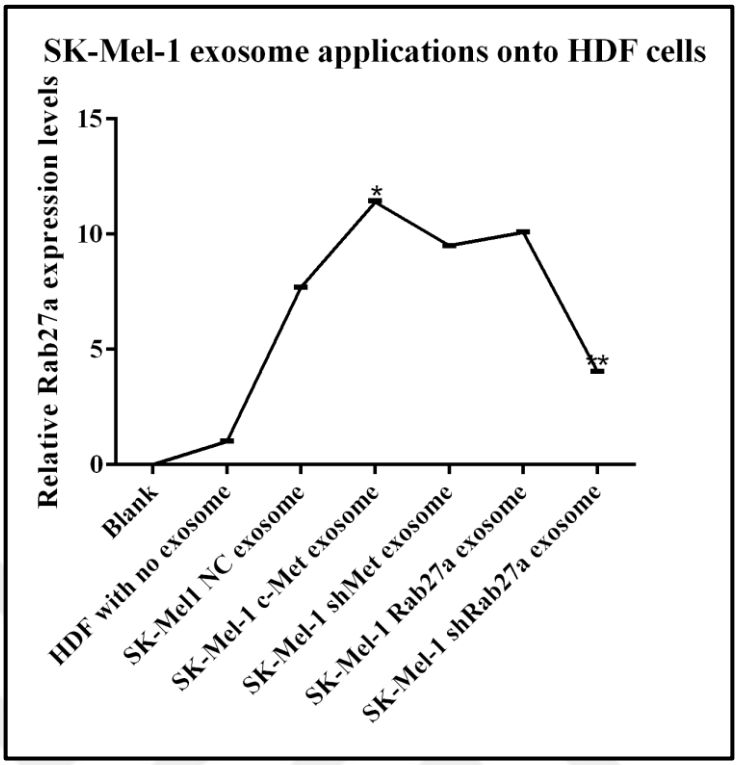

Figure 5.62. Relative Rab27a expression analysis of HDF cells after the application of SK-Mel-1 exosomes with ELISA technique. Abbreviations: HDF: Human dermal fibroblasts. *P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Results found similar with the SK-Mel-1 exosome application onto hnFSSCs in HDF cells. C-Met transfection to melanoma cell exosomes increased Rab27a expression after the presentation to HDF cells and silencing of it caused down-regulation. Only significant outcome was found as Rab27a silenced exosome application group. The relevant Rab27a expression dropped almost to its half once those exosomes were given to HDF cells (Figure 5.62).

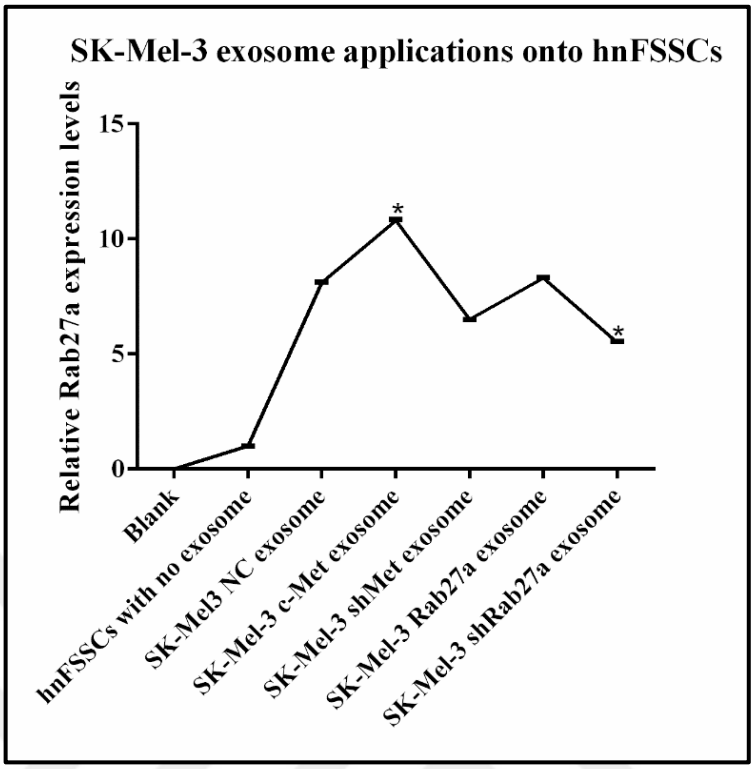

Figure 5.63. Relative Rab27a expression analysis of hnFSSCs after the application of SK-Mel-3 exosomes with ELISA technique. Abbreviations: hnFSSCs: human newborn foreskin stem cells.*P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

Adherent SK-Mel-3 melanoma exosome presentation to hnFSSCs results were found similar to SK-Mel-1 exosomes presented hnFSSCs and HDF cells. Only difference was that c-Met transfections to melanoma exosomes triggered significantly higher Rab27a expression once given to hnFSSCs (Figure 5.63).

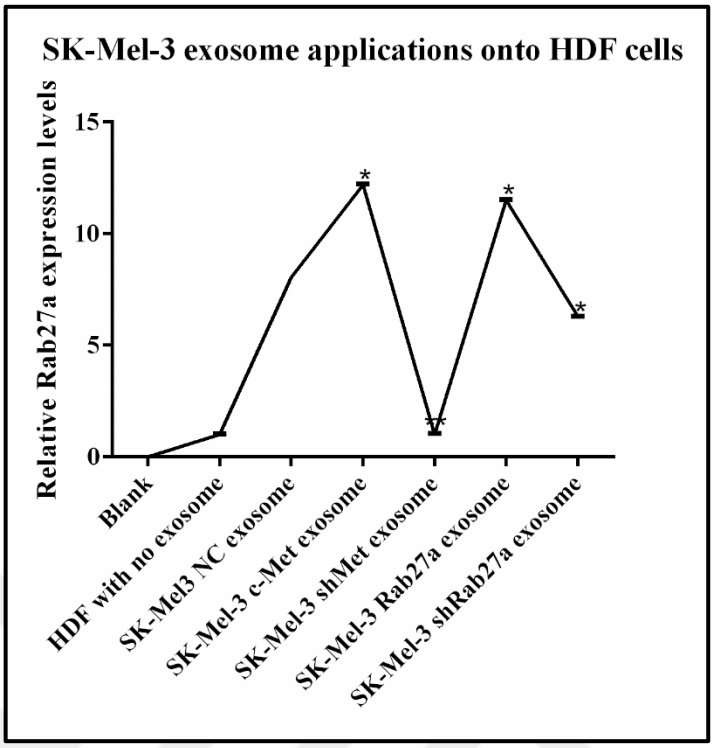

Figure 5.64. Relative Rab27a expression analysis of HDF cells after the application of SK-Mel-3 exosomes with ELISA technique. Abbreviations: HDF: Human dermal fibroblasts.*P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

SK-Mel-3 exosome application onto HDF cells indicated importantly significant results when compared with the other ones. c-Met transfection to melanoma cells and so exosomes increases the RAB27a expression once given to HDF cells relatively. Meanwhile, once silenced, Rab27a dropped to almost 15 times lower expression in HDF cells. Rab27a transfection to melanoma exosomes triggered enhanced Rab27a expression in HDF cells once exosomes applied and silencing caused significantly half of the expression level in HDF cells (Figure 5.64).

5.9. REAL TIME PCR ANALYSIS

Relative mRNA expression levels were examined for six different genes including Rab27a, c-Met, Caspase-7, STAT 3, BAX and Bcl-2 [57]. Caspase 7 (CASP7) expression has significant utility throughout the cell cycle evolution and cell development in specific cancer cells. Caspase 7 induces the apoptosis [57, 58]. Stat3 is a transcription factor, which is triggered in many human cancers where it functions as an important intermediary of oncogenic signaling through transcriptional activation of genes encoding apoptosis inhibitors (eg Bcl-xL) [58]. BAX expression is upregulated via the tumor suppressor protein p53, and BAX has been revealed to be covered in p53-related apoptosis [59, 60]. Bcl-2 is mainly deliberated a significant anti-apoptotic protein therefore it is not thought as a proto-oncogene regarding to the fact that it is not a development signal transducer [61].

5.9.1. Gene Expression Analysis of hnFSSCs Exosome Applied hnFSSCs

Real time analysis of hnFSSCs exosome applied hnFSSCs mRNA expression results were underlined individually as it was stated as; Rab27a expression was detected four times higher in c-Met transfected hnFSSCs exosome application group when compare with the untransfected hnFSSCs exosome application group. According to the results, Rab27a expression was almost twelve times bigger than the untransfected exosome application group in Rab27a transfected hnFSSCs exosome applied ones. Silencing of c-Met in hnFSSCs exosomes made hnFSSCs to express almost the same level of Rab27a with untransfected exosome applied ones, so that the expression decreased tremendously. Untransfected hnFSSCs exosomes hightened the expression four times more than the untreated hnFSSCs group. With the addition of shRab27a to the cells exosomes, Rab27a expression reduced to the same level with the Rab27a transfected exosome application group (Figure 5.65).

C-Met transfections to hnFSSCs cells and to their exosomes increased the c-Met expression of hnFSSCs slightly. Once silenced, the c-Met expression of hnFSSCs reduced to the half of the expression of untransfected exosome added group. Rab27a transfected exosome presentation doubled the c-Met expression in hnFSSCs. Once Rab27a was silenced, the c-Met expression decreased to its half (Figure 5.65).

Caspase-7 expression of hnFSSCs decreased once c-Met and Rab27a transfected hnFSSCs exosomes added to the environment. Therefore, silencing of these two have found non-effective in hnFSSCs Caspase 7 expression manner (Figure 5.65).

STAT3 expression decreased when c-Met transfected exosomes were given. However, when silenced c-Met carrying exosomes were added, the expression of STAT3 increased. Untransfected hnFSSCs exosome presence also hightened the STAT3 expression. Rab27a transfected exosome application on the other hand, decreased the expression to its half level (Figure 5.65).

BAX expressions of hnFSSCs lowered to the half expression of negative control group. Similarly, Rab27a transfection also triggered decreased BAX expressions in hnFSSCs once exosomes were added (Figure 5.65).

Bcl-2 expression doubled when c-Met transfected cell exosomes were presented to hnFSSCs. Meanwhile, the level of Bcl-2 expression enhanced more than two times in comparison with the negative control (Figure 5.65).

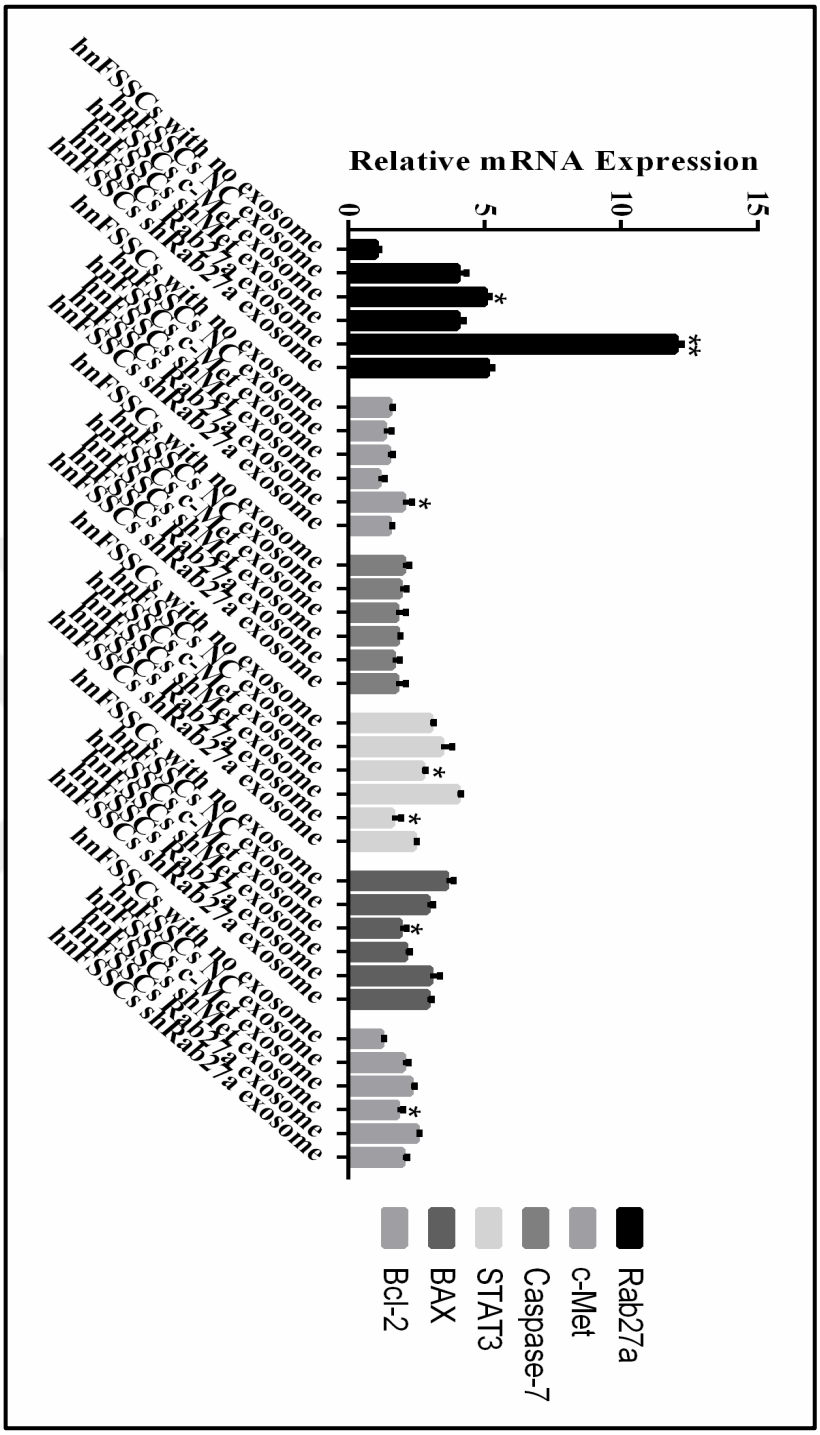

Figure 5.65.Relative mRNA expression analysis of hnFSSCs after the presentation of hnFSSCs exosomes. Abbreviations: hnFSSCs: human newborn foreskin stem cells, HDF: Human dermal fibroblasts, STAT3: Signal transducer and activator of transcription 3, BAX: Bcl-2-associated X protein, Bcl-2: B-cell lymphoma 2.*P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

5.9.2. Gene Expression Analysis of HDF Exosome Applied HDF cells

Relative mRNA expression levels were almost similar to the hnFSSCs exosome applied hnFSSCs. The only difference was found as, the presentation of Rab27a transfected hnFSSCs exosomes improved the Rab27a expression in hnFSSCs but not as much as the hnFSSCs Rab27a carrying exosome applications (Figure 5.66).

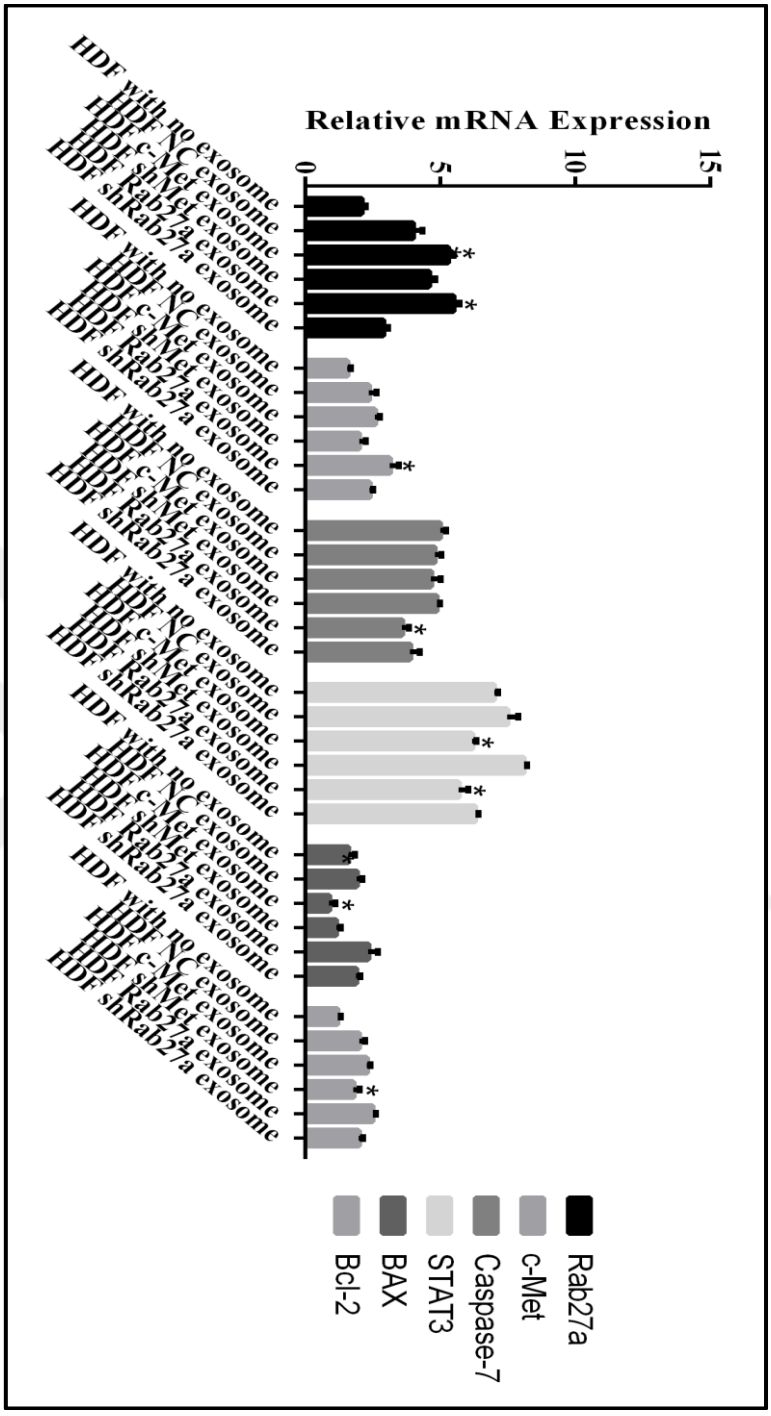

Figure 5.66. Relative mRNA expression analysis of HDF after the presentation of HDF exosomes. Abbreviations: hnFSSCs: human newborn foreskin stem cells, HDF: Human dermal fibroblasts, STAT3: Signal transducer and activator of transcription 3, BAX: Bcl-2-associated X protein, Bcl-2: B-cell lymphoma 2.*P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

5.9.3. Gene Expression Analysis of SK-Mel-1 Exosome Applied hnFSSCs

Real time analysis of SK-Mel-1 exosome applied hnFSSCs mRNA expression results were underlined individually as it was stated as; c-Met transfected SK-Mel-1 cell exosome application generated ten times higher expressions of Rab27a when compared with the negative control. Even the untransfected SK-Mel-1 cell exosome application increased the Rab27 expression four times more. Silencing c-Met in melanoma cell exosomes dropped the expression to more than c-Met transfected exosome application group is half of the expression. Once Rab27a transfected to the cells, Rab27a expression increased to five five times more after exosome application. shRab27a introduction to melanoma cell exosomes caused decreased Rab27a expression in hnFSSCs when those exosomes were added (Figure 5.67).

C-Met expression of hnFSSCs increased ten times when c-Met trnasfected SK-Mel-1 exosomes were applied. The expression dropped to its half when c-Met was silenced. Rab27a transfected exosome addition provided enhanced c-Met expression in hnFSSCs. Silenced Rab27a carrying exosome application decreased c-Met expression in hnFSSCs (Figure 5.67).

C-Met transfected exosome application decreased Caspase-7 expression in hnFSSCs significantly. Therefore, Rab27a transfected melanoma exosome application seemed to have limit effect on Caspase 7 expression of hnFSSCs (Figure 5.67).

C-Met carrying melanoma exosome presentation put down the expression to almost zero. Rab27a transfected melanoma exosome application on the other hand decreased the expression relevantly. Once shRab27a presentation happened to melanoma cells, STAT 3 expression doubled in hnFSSCs after exosome addition (Figure 5.67).

BAX expression decreased significantly in hnFSSCs once c-Met and Rab27a transfected melanoma exosomes were supplemented (Figure 5.67).

Bcl-2 expression more than doubled in hnFSSCs when c-Met transfected melanoma cell exosomes were added. Rab27a transfected exosome application also increased the Bcl-2 expression expressively in hnFSSCs (Figure 5.67).

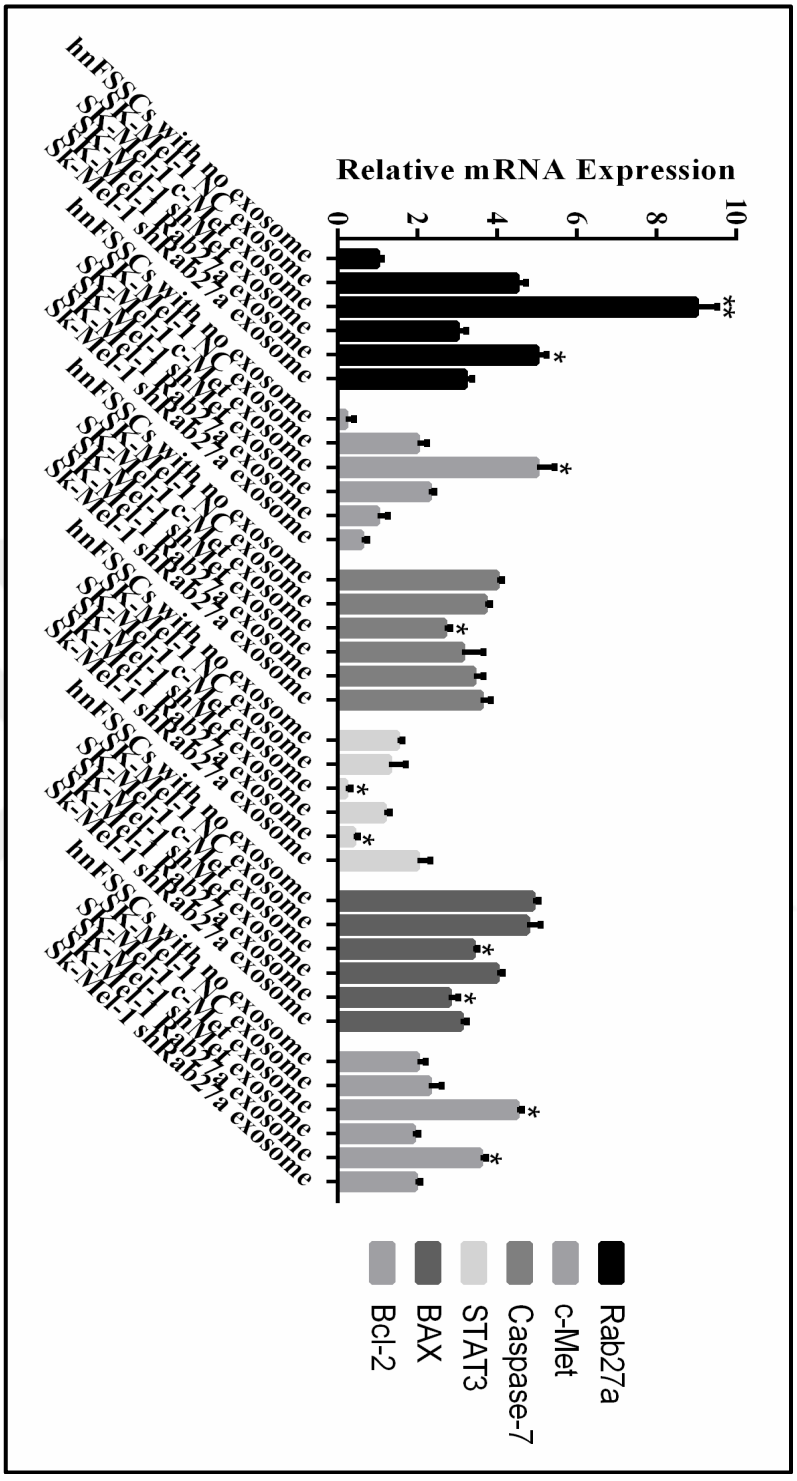

Figure 5.67. Relative mRNA expression analysis of hnFSSCs after the presentation of SK-Mel-1 exosomes. Abbreviations: hnFSSCs: human newborn foreskin stem cells, HDF: Human dermal fibroblasts, STAT3: Signal transducer and activator of transcription 3, BAX: Bcl-2-associated X protein, Bcl-2: B-cell lymphoma 2 .*P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

5.9.4. Gene Expression Analysis of SK-Mel-1 Exosome Applied HDF cells

Relative mRNA expression levels were almost similar to the SK-Mel-1 exosome applied hnFSSCs. Only difference was established as, c-Met transfected melanoma exosome application onto HDF cells increased BCl-2 expression in HDF cells more than it did in hnFSSCs (Figure 5.68) .

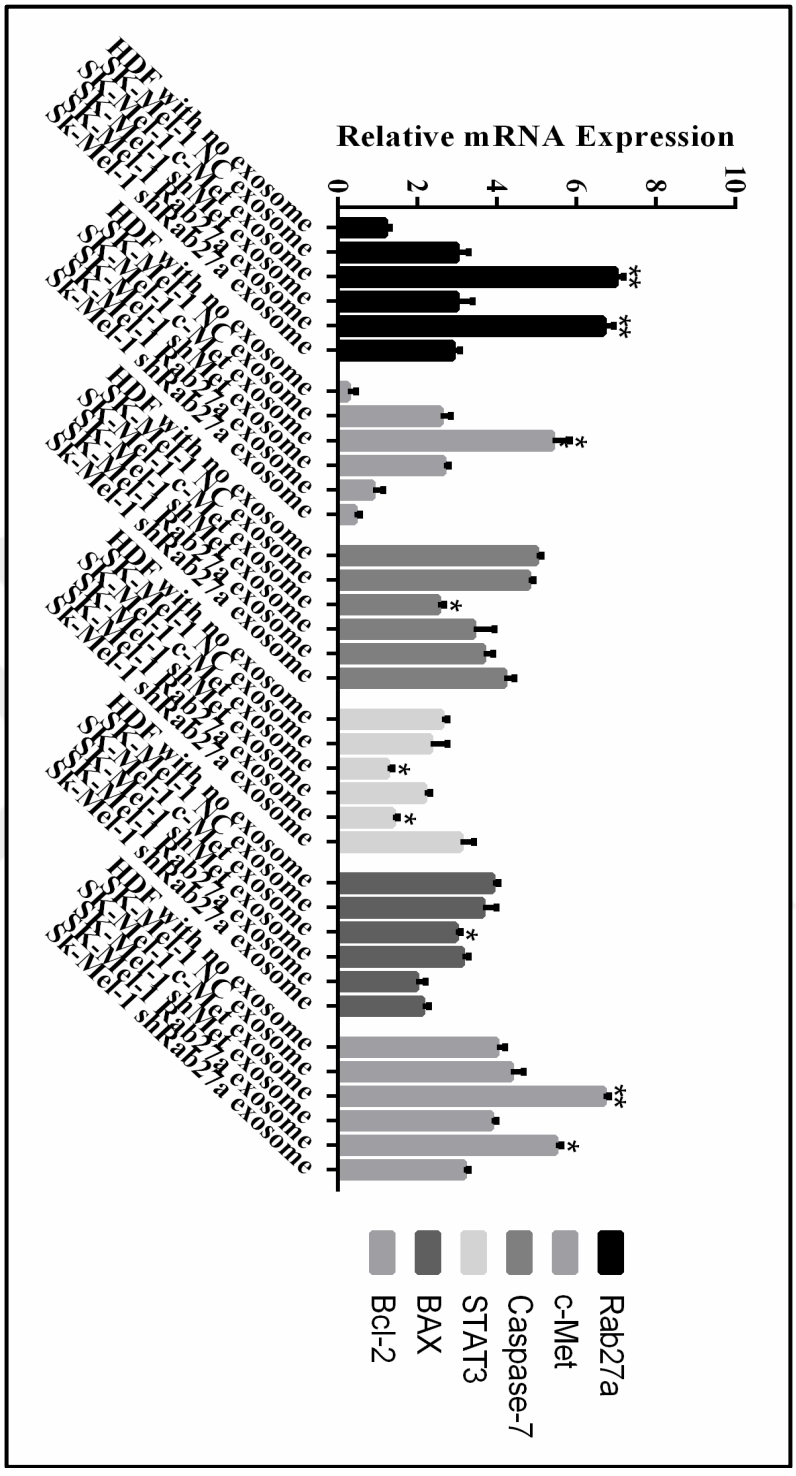

Figure 5.68. Relative mRNA expression analysis of HDF cells after the presentation of SK-Mel-1 exosomes. Abbreviations: hnFSSCs: human newborn foreskin stem cells, HDF: Human dermal fibroblasts, STAT3: Signal transducer and activator of transcription 3, BAX: Bcl-2-associated X protein, Bcl-2: B-cell lymphoma 2.*P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

5.9.5. Gene Expression Analysis of SK-Mel-3 Exosome Applied hnFSSCs

SK-Mel-3 exosome application onto hnFSSCs results were similar to SK-Mel-1 exosome applications onto hnFSSCs. hnFSSCs c-Met, Rab27a and Bcl-2 expressions increased when Rab27a and c-Met were transfected to melanoma exosomes and those exosomes added to the environment. Nevertheless, BAX, Caspase-7 and STAT 3 expressions of hnFSSCs decreased with the application of silenced Rab27a and silenced c-Met carrying melanoma exosome presentations (Figure 5.69).

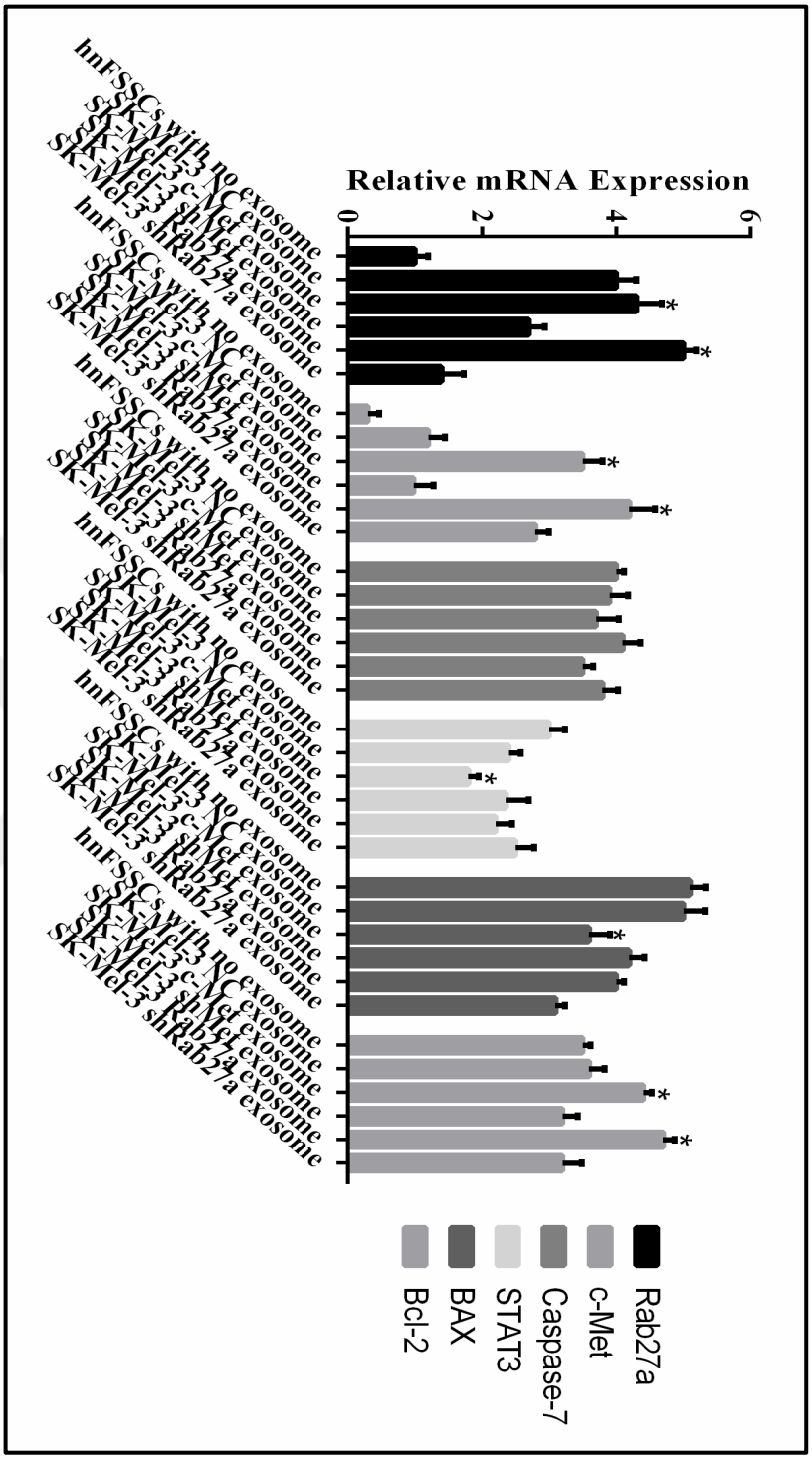

Figure 5.69. . Relative mRNA expression analysis of hnFSSCs after the presentation of SK-Mel-3 exosomes. Abbreviations: hnFSSCs: human newborn foreskin stem cells, HDF: Human dermal fibroblasts, STAT3: Signal transducer and activator of transcription 3, BAX: Bcl-2-associated X protein, Bcl-2: B-cell lymphoma 2.*P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

5.9.6. Gene Expression Analysis of SK-Mel-3 Exosome Applied HDF cells

SK-Mel-3 exosome application onto HDF results were related to SK-Mel-1 exosome applications onto HDF. HDF c-Met, Rab27a and Bcl-2 expressions amplified when Rab27a and c-Met were transfected to melanoma exosomes and those exosomes supplemented to surroundings. Nevertheless, BAX, Caspase-7 and STAT 3 expressions of HDF lessened with the submission of silenced Rab27a and silenced c-Met carrying melanoma exosome additions (Figure 5.70).

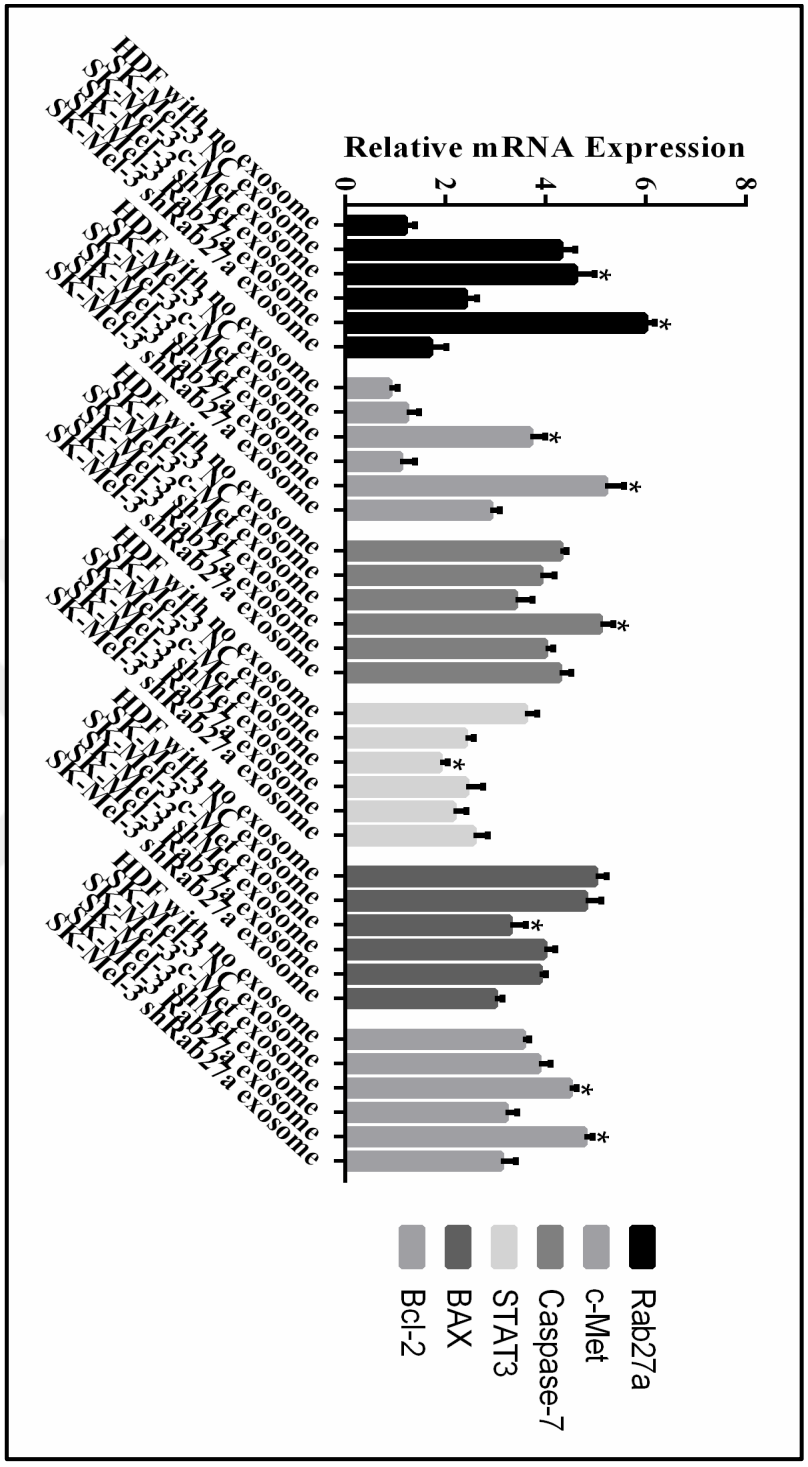

Figure 5.70. . Relative mRNA expression analysis of HDF cells after the presentation of SK-Mel-3 exosomes. Abbreviations: hnFSSCs: human newborn foresin stem cells, HDF: Human dermal fibroblasts, STAT3: Signal transducer and activator of transcription 3, BAX: Bcl-2-associated X protein, Bcl-2: B-cell lymphoma 2.*P<0.05. Notes: Results were examined by one-way ANOVA and Turkey's posttest.

6. DISCUSSION

Collection of evidences demonstrates that exosomes are intermediaries of metastasis [62]. Different cell types covering endothelial cells, fibroblastic cells and bone marrow progenitor cells donate to the creation of metastatic niche [63] and they can be impressed through the transference of molecules. Therefore, the mechanism providing this interaction have not established yet [32]. This study showed that exosomes may carry a potential in cancer development and metastatic environment production besides the effect of tumor-related growth factors, chemokines and extracellular matrix proteins.

Cancer related exosomes may endorse the training and deployment of progenitor cells and stem cells which then carry tumor angiogenesis, migration and invasion [32]. The c-Met oncogene facilitates cellular alteration and tumor cell proliferation, endurance, migration, assault and metastasis [64]. In this study, we investigated the effect of exosome-packaged c-Met in metastatic melanoma cells SK-Mel-1 and SK-Mel-3 cells. The cargo of exosomes from melanoma cells can train different cell populations such as HDF cells and hnFSSCs. The molecular mechanisms of exosome biogenesis and secretion are still not described. Therefore, studies indicate specific RAB genes covered in exosome secretion such as Rab27a [65].

Flow cytometry analysis of transfection efficiencies demonstrated low transfection rates in SK-Mel-3 and HDF individually. Rab27a transfection efficiency to SK-Mel-3 cells was detected as 40% and c-Met transfection efficiency was detected as 38%. In HDF cells, Rab27a transfection efficiency was only 14, 55% and c-Met transfection efficiency was 29,05% (Figure 5.1). This low efficiency may be coming from the reason of high sized plasmids. Transfection technique was selected as lentiviral gene transfer. Therefore, electroporation would have been a better option for obtaining better transfection efficiency. Related to the fact of infection, cells also got infected several times and the protocol needed to be repeated several times. Once transfections were completed, cell sorting was performed in order to acquire pure transfected colonies.

SEM electron microscopy results indicated that Rab27a transfections to all hnFSSCs, HDF, SK-Mel-1 and SK-Mel-3 cells triggered enhanced exosome release. Therefore, exosome numbers were higher than the other groups in every cell type. It was of course expected

related to the Rab27a utility in exosome discharge [32]. Interestingly, c-Met transfected hnFSSCs and HDF cell exosome numbers were the highest. Both hnFSSCs and HDF cells are fibroblastic cells and therefore they express basic fibroblastic growth factor. FGF and c-Met crosstalk may increase cell-cell communication and therefore they may be using exosomes in order to interact with each other [66]. This may the reason why the exosome numbers were higher in c-Met transfected hnFSSCs and HDF cells than the other transfected and untransfected hnFSSCs and HDF cells (Figure 5.2. and Figure 5.3). In SK-Mel-1 melanoma cells, Rab27a transfections increased the exosome release ant the number was known as the highest. Rab27a silencing on the other hand brought decreased exosome release. Unlike the other results, shRab27a SK-Mel-1 exosome numbers were lower but the sizes of exosomes were bigger (Figure 5.4). This situation may be because of the Rab27a disruption.

Once Rab27a was silenced, exosome release should have been reduced. Exosomes that were prepared by the cell cannot be secreted. Therefore, small vesicles may have grown by integration inside of the cell. Suitable with the previous studies, Rab27a transfection to SK-Mel-3 exosomes increased Rab27a release and silencing decreased the exosome release tremendously (Figure 5.5). SK-Mel-1 and SK-Mel-3 are highly metastatic cancer cells and they already express c-Met. Transfecting c-Met to those cells may have triggered high over expression of c-Met. Elevated overexpression of c-Met also promotes cell death [32], therefore this may be the reason of reduced amounts of exosome in case of c-Met exosome to already metastatic cells (Figure 5.4. and Figure 5.5).

Cytotoxicity assay results indicated the optimum concentrations of usage for the next studies. Main outcome was that none of the exosome dosages between 1%, 2,5%, 5%, 7,5% and 10% caused toxicity to both hnFSSCs and HDF cells (Figure 5.6, Figure 5.8, Figure 5.10, Figure 5.12, Figure 5.14, Figure 5.16 and Figure 5.18). This may be related to the exosomal regulation of cytotoxicity. Recent studies indicate that even in cell culture, exosomes may control the cytotoxic activity of cells [67].

Colony forming assay analysis publications demonstrated that cancer exosome applications onto healthy cell carried no effects in colony formation [68]. Therefore, the colony forming assay result of this study established the opposite Untransfected SK-Mel-3 melanoma exosome applications showed the highest plating efficiency and surviving fraction. SK-Mel-3 is a highly metastatic melanoma cell line, c-Met transfection might have triggered cell

death, and this may be the reason to low surviving fraction. As expected, c-Met transfected HDF exosome applications might have brought metastatic behavior even in the healthy cells and cell death may have decreased**.** The increased surviving fraction once c-Met transfected exosomes added may be explained by this (Figure 5.28).

Rab27a transfected hnFSSCs and HDF cell exosome applications onto both hnFSSCs and hnFSSCs increased apoptosis. It can be expected due to the fact that exosomes were coming from healthy cells (Figure 5.29 and Figure 5.30). Once c-Met was transfected to the exosomes, both necrosis and apoptosis decreased after exosome applications. C-Met transfection to healthy exosome might have brought metastatic behavior even to the healthy cells and cell death may have been decreased. However, c-Met transfection to SK-Mel-1 cell and the exosome applications seemed to increase late apoptosis in both HDF cells and hnFSSCs (Figure 5.33 and Figure 5.34). In melanoma cancer cell exosome applications, the Rab27a transfected exosome application triggered reduced necrosis in both hnFSSCs and HDF cells (Figure 5.30, Figure 5.33 and Figure 5.34). As known, Rab27a and c-Met transfection might have improved the exosome release and cancer cell exosome- healthy cell interaction might have enhanced. Therefore, healthy cells may have escaped from the cell death like cancer cells. As mentioned before, SK-Mel-3 is a highly metastatic melanoma cell line and c-Met transfection might have triggered cell death signaling in the environment and this may be the reason for enhanced necrosis and apoptosis in HDF cells when c-Met transfected SK-Mel-3 exosomes were given (Figure 5.32).

Addition of c-Met transfected hnFSSCs exosomes found to have limited effects on motility. This can be related to converting them into a cell type expressing tremendous amounts of c-Met like c-Met transfected SK-Mel-3 cells. This may be the reason of observing deceased migration in both c-Met transfected hnFSSCs exosome application group and c-Met transfected SK-Mel-3 exosome application group (Figure 5.35, Figure 5.37 and Figure 5.38). Silencing of Rab27a in cells caused limited exosome release and of course triggered less migration once added to hnFSSCs environment (Figure 5.35). Metastatic but not as much as SK-Mel-3 cell line SK-Mel-1 exosome application results were similar to HDF exosome application results in motility manner. As expected, increased c-Met expression in exosomes triggered enhanced cell motility after exosome applications in both. Rab27a on the other hand found to have decreasing effect on motility in both (Figure 5.36, Figure 5.39 and Figure 5.40).

Tube formation assay results were found as expected. In healthy cell lines c-Met transfected exosome application results were lower than the silenced ones (Figure 5.41 and Figure 5.42). This situation was explained as a control mechanism that fibroblastic cells carry via utilizing p53 regulation in the cells [68]. Transfected SK-Mel-1 malignant melanoma exosome applications seemed to enhance the tube formation as expected (Figure 5.43) , regarding to the fact of cancer cells induce angiogenesis [69]. Because of the fact that SK-Mel-3 is a highly metastatic melanoma cell line and c-Met transfection might have triggered cell death signaling in the environment, it was expected to see lower tube formations in c-Met transfected group (Figure 5.44).

EGFR moderates HGF/c-Met action via persuading c-Met ectodomain shedding and HGF/c-Met transactivates EGFR, directing to an improved activation of downstream signaling pathways. Crosstalk amongst EGFR and c-Met play a key role in regulating healthy cell migration, proliferation, and wound healing [44]. Modifications in Rab expression amounts may affect cell expansion and proliferation over their impact on growth factor receptor trafficking and signaling. This idea is mainly significant to those Rabs controlling the endocytic routes of the several membrane receptors of mitogenic ligands like the epidermal growth factor receptor (EGFR) [45].

In both hnFSSCs and HDF application groups to hnFSSCs and HDF cells, EGF was found increased in c-Met transfected application ones (Figure 5.45 and Figure 5.46). This may be explained by maintaining the halthy cell proliferation in the cell. Rab transfected cell exosome application groups also showed increased EGF expression which may be defined with preserving the EGFR pathways (Figure 5.45 and Figure 5.46). In SK-Mel-1 exosome applied groups, EGF expression was decreased in c-Met transfected exosome application ones in both HDF and hnFSSCs. It was also expected because most probably exosome addition triggered cancer cell like behavior in healthy cells. A recent study indicated that Rab proteins enhance β1 integrin recycling in EGF-induced cancer invasion [70], therefore it was expected that Rab27a transfected cancer cell exosomes would increase EGF expression. (Figure 5.47 and Figure 5.48). Respected to the same reason, SK-Mel-3 exosome c-Met transfection might have triggered cell death signaling in the environment, it was expected to see lower EGF expressions when added to both HDF cells and hnFSSCs (Figure 5.49 and Figure 5.50). VEGF expressions of SK-Mel-3 exosome applied hnFSSCs and HDF

cells were found similar to tube formation assay results and the reasons were established as the same (Figure 5.51 and Figure 5.52).

c-Met (HGFR) and its ligand, Hepatocyte Growth Factor (HGF), have established enhanced in metastatic melanoma in various studies and have been linked to the invasion and disease progression [71]. Consequently, previous readings of c-Met and HGF in melanoma are unfinished in field. Initial experimentations suggest that inhibition of HGF or c-Met may decrease melanoma progression [50]. In the meantime, other readings designated that Rab27a inhibition reduces exosome discharge and so tumor growth and metastasis. Hence, Rab27a expression may be connected to c-Met expression [32].

Relative HGFR expression in all cell types after exosome presentation once cell exosomes were transfected with c-Met and Rab27a. It was relevant to the data, and c-Met transfection may have carried a interactive communication with Rab27a signaling pathway (Figure 5.53, Figure 5.54, Figure 5.55, Figure 5.56, Figure 5.57 and Figure 5.58). Relative Rab27a expression results were similar to the relative HGFR expression results, which was logical because of Rab27a and c-Met correlation. Only difference was, silencing both Rab27a and c-Met in exosomes caused highly decreased Rab27a expressions after the exosomal application. Meanwhile, overexpression of these two genes also regulated highly increased Rab27a expression in hnFSSCs and HDF after exosomal addition (Figure 5.59, Figure 5.60, Figure 5.61, Figure 5.62, Figure 5.63 and Figure 5.64).

As mentioned before, Caspase 7 expression covers significant utility in the cell cycle progression and cell expansion of cancer cells. Caspase 7 induces the apoptosis [53 [71] . STAT3 is a transcription factor which is prompted in most of the human cancers where it works as a vital intercessor of oncogenic gesturing over transcriptional activation of genes coding apoptosis inhibitors (eg. Bcl-xL) [54, [72]. BAX expression is upregulated through the tumor suppressor protein p53, and BAX has been publicized to be included in p53-related apoptosis [55, 56]. Bcl-2 is mainly deliberated a significant anti-apoptotic protein therefore it is not thought as a proto-oncogene regarding to the fact that it is not a development signal transducer [57].

Cancer exosome applications onto HDF cells and hnFSSCs results were obtained ant the results indicated improved c-Met, Rab27a and Bcl-2 expressions when Rab27a and c-Met were transfected to melanoma exosomes and those exosomes supplemented to surroundings.

Nevertheless, BAX, Caspase-7 and STAT 3 expressions of HDF cells and hnFSSCs lessened with the submission of silenced Rab27a and silenced c-Met carrying melanoma exosome additions (Figure 5.67, Figure 5.68, Figure 5.69 and Figure 5.70). In the meantime, healthy cell exosome application results were found similar to cancer exosome application groups as well (Figure 5.65, Figure 5.66). This situation can be defined as appropriate. Because, c-Met covering exosome applied group may have shown cancer-like phenotypic behaviors and inhibit apoptotic mechanism with Caspase-7 pathways. STAT3 donates to tumor cell evolution via modifying the expression of genes that are convoluted in cell survival and proliferation [73, 74]. In contrast to what was expected, STAT3 expression was found decreased in c-Met transfected exosome applied hnFSSCs and HDF cells. Normally, if the cancer development would occur, then STAT3 expression would have been enhanced. This situation may suggest that cancer exosome/healthy cell interaction carry no effects on HGFR/STAT3 constitutive activation.

Finally yet importantly, exosomes obtained from c-Met transfected and Rab27a transfected cells have thought to carry c-Met r Rab27a hypothetically. Therefore, the examination were performed through this prospective. However, there is always a probability for the exosomes to be untransfected. That is why; the future analysis may be started with the proof of the existence of both Rab27a and c-Met in the exosomes.

7. CONCLUSION

Granting the cancer exosomes have proposed to attend to the spread of oncoproteins and genetic material (RNA) our study is one of the few, to our knowledge, to display that transfer of the c-MET oncoprotein by exosomes to human dermal fibroblasts and human newborn foreskin stem cells endorses the metastatic progression *in* vitro. Particularly, we displayed that exosomes may modify the hnFSSCs and HDF in a long-lasting manner. Here we recommended a novel appliance that regulates metastatic development over the crosstalk among cancer derived exosomes and healthy cells. Together, our data classified exosome related transmission of the oncoprotein c-MET and Rab27a as crucial regulators of melanoma cancer development, mobilization and metastatic progression hypothetically.

The predicted results in melanoma could carry significant medical effects for the forecast and deterrence of metastasis. This present study may propose that the MET oncoprotein detection in serum exosomes can be utilized as an analytical pointer for patients of primary melanoma. Metastatic actions in melanoma patients who have acknowledged to have exosomes with elevated MET expressions could be disallowed via planning therapeutic strategies to aim MET or Rab27a. These kinds of interventions to lessen or prevent the metastatic burden are expected to significantly advance the likelihood of survival of melanoma sufferers. Then again, exosomes were thought to carry c-Met and Rab27a hypothetically.

This study aimed to open a perspective of melanoma diagnosis without any requirements of tissue biopsy and directly with the utilization of exosomal markers isolated from body liquids. This way, melanoma patients are expected to suffer less and cancer development levels would be examined easily. Therefore, effortlessly accessible exosomal kit productions for the diagnosis may be a future based target. Meanwhile, examining the effects of melanoma exosome interactions with healthy cells might also be studied *in vivo* in order to obtain deeper information.

As a conclusion, overall results suggested potential genetic markers for the early diagnosis of melanoma cancer. Together with the relevant gene over-expressions or down-regulations, there may be a potential to diagnose the disease without biopsy operations and only with the body liquids in precise amounts. Another perspective can be the probability to produce a

vaccine including very less numbers of cancer exosomes in order to immunize the healthy body for the antibody production and prevent the future disease progression in theory.

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