ESTABLISHMENT OF PRIMARY HUMAN SKIN CELL CULTURE AND SOFT AGAR TRANSFORMATION ASSAY

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ABSTRACT

ESTABLISHMENT OF PRIMARY HUMAN SKIN CELL CULTURE AND SOFT AGAR TRANSFORMATION ASSAY

The skin, the largest organ of the human body, is a complete closed system with various functions, including physical protection from the exterior hostile environment, sensing outside signals via its complex sensory system and initiating immunologic reactions when the initial physical protection fails. These functions are possible because of the highly elaborate structure of this multilayered organ, made up of various kinds of cells which maintain the homeostasis of skin. In this study, we optimized the isolation procedures and culture conditions of primary human melanocytes and keratinocytes from the epidermis and primary human fibroblasts from the dermis. These three cell types are especially important in cancer research. Skin cancers arise mainly from melanocytes and keratinocytes. However, fibroblasts are used widely in transformation assays, which are indispensible elements of cancer research. Using the isolated primary human fibroblasts, we established the soft agar colony formation assay, an important tool for determining the transformation potential of genetically altered cells. To transform primary fibroblasts we knocked-down p53 expression using a lentiviral transduction system and in combination we expressed oncogenic forms of hRas or cMyc. The transduction efficiency of the lentiviral system was over 90 per cent, and the tumor suppressor genes p53 and Bax were knocked down successfully using shRNA based lentiviral constructs. Colonies of potentially transformed primary fibroblasts grew in attachment independent manner in soft agar upon p53 knock-down in combination with either cMyc or hRas oncogene expression, implying a successful transformation. Next, the transformation ability of the newly generated anti-ASC shRNA vector will be tested in combination with the oncogenes cMyc or hRas using primary human fibroblasts and melanocytes.

ÖZET

BİRİNCİL İNSAN DERİ HÜCRESİ KÜLTÜRÜNÜN KURULUMU VE YUMUŞAK AGAR DÖNÜŞÜM TAHLİLİ

İnsan vücudunun en büyük organı olan deri, bir çok işlevli olan kapalı bir sistemdir. İşlevleri arasında olumsuz çevre şartlarına karşı fiziksel koruma sağlanması, dışarıdan gelen uyarıları kompleks duyu sistemiyle algılanması ve fiziksel korumanın yetersiz kaldığı durumlarda immünolojik tepkilerin tetiklenmesi önde gelenlerdir. Deri, bu görevleri yerine getirebilmesini, ileri derecede ince yapılandırılmış ve çok katmanlı oluşuna borçludur. Bu katmanlardaki bir çok hücre çeşidi bu sofistike yapının devamlılığını ve homeostazını sağlar. Bu çalışmada biz, epidermisten gelen birincil insan melanositleri ve keratinositleriyle, dermisten gelen birincil insan fibroblastlarının saflaştırma prosedürlerini ve kültür koşullarını optimize ettik. Bu üç hücre çeşidi özellikle kanser araştırmalarında önem taşır. Deri kanserleri çoğunlukla melanosit ve keratinositlerdeki bozukluklardan kaynaklanırken fibroblastlar ise dönüşüm deneylerinin-ki bu deneyler kanser araştırmalarının vazgeçilmez elementlerindendir- sıklıkla kullanılan hücre çeşididir. İnsan derisinden saflaştırdığımız bu birincil fibroblastları kullanarak, yumuşak agar koloni oluşum tahlilini oturttuk. Bu tahlil genetik olarak değiştirilmiş hücrelerin dönüşüm potansiyelini belirlemekte kullanılan önemli bir araçtır. Birincil fibroblastları dönüştürmek için, p53 protein anlatımını bir lentiviral transfeksiyon sistemi kullanarak düşürdük, sonradan da hRas veya cMyc onkogenlerinden birini kombinasyon olarak ekledik. Lentiviral sistemin transdüksiyon verimliliği yüzde doksanın üzerindeydi. shRNAya dayanan lentiviral vektörler kullanarak, tümör baskılayıcı p53 ve Bax genlerini başarılı bir şekilde işlevsizleştirdik. p53 anlatımı durdurulmuş ve onkogenik cMyc veya hRas eklenmiş birincil fibroblastların çıpa bağımsız bir şekilde yumuşak agarda koloni oluşturduklarını gözlemledik. Büyük olasılıkla da dönüşüm geçirmiş oldular. Daha sonra, tasarlayıp ürettiğimiz ASC baskılayıcı shRNA vektörünü, cMyc veya hRas onkogen kombinasyonlarında kullanarak, dönüşüm yapabilme özellikleri birincil insan fibroblastlarında ve melanositlerinde araştırılacaktır.

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APS	Ammonium per sulfate
BCC	Basal Cell Carcinoma
Bcl-2	B-cell Lymphoma 2
bp	Base pair
DEJ	Dermis-Epidermis Junction
DMEM	Dulbecco Modified Eagle's Minimal Essential Medium
$DMEM^{+}$	DMEM supplemented with Penicillin/Streptomycin, L- Non Essential Amino acids
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
E. coli	Escherichia coli
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FG12	Lentiviral vector
FG12-cMYC-GFP	Lentiviral vector containing c-MYC and GFP genes with separate promoters
FG12-hRAS-GFP	Lentiviral vector containing h-RAS and GFP genes with separate promoters
FG12-GFP	Lentiviral vector containing a GFP gene
GFP	Green Fluorescent Protein
H1-p53i-GFP	Lentiviral vector containing ShDNA of p53 gene and GFP gene with separate promoters
H1-BAXi-GFP	Lentiviral vector containing ShDNA of BAX gene and GFP gene with separate promoters
HBS	HEPES Balanced Salt
HEK	Human Embryonic Kidney

HIV	Human Immunodeficiency Virus
HMGS	Human Melanocyte Growth Supplement
HKGS	Human Keratinocyte Growth Supplement
IFN	Interferon
IL	Interleukin
kb	Kilo Base
kDa	Kilo Dalton
KGM	Keratinocyte Growth Medium 154 CF
KGM^+	Medium 154CF supplemented with $CaCl_2$ with Penicillin/Streptomycin, and HKGS
LB	Luria Broth
LPS	Lipopolysaccharide
LTR	Long terminal repeats
mA	Milliamper
mM	Millimolar
ml	Milliliter
μl	Microliter
MCS	Multiple cloning site
MGM	Melanocyte Growth Medium 254 CF
MGM^+	Medium 254CF supplemented with $CaCl_2$ with Penicillin/Streptomycin, and HMGS
mRNA	Messenger ribonucleic acid
NO	Nitric Oxide
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
rpm	Revolutions per minute
pRb	Retinoblastoma protein
PVDF	Polyvinyl difluoride
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNAi	RNA interference
SCC	Squamous Cell Carcinoma

SDS	Sodium dodecylsulfate
shRNA	Short Hairpin Ribonucleic Acid
shDNA	Short Hairpin Deoxyribonucleic acid
siRNA	Small Interference Ribonucleic Acid
SV40	Simian Virus 40
TAE	Tris-Acetate-EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween 20
TEMED	N,N,N',N',-tetramethylethylenediamine
UbiC	Ubiquitin C
UV	Ultraviolet
V	Volt

1. INTRODUCTION

1.1. The Skin as an Organ

The skin covering the whole exterior area of a human being is the largest organ of the body making up approximately 15 per cent of the total body weight of the adult body. The skin is continuous with interior mucous membranes on the orifices and provides a complete closed system with various functions, including providing a physical protection from the exterior hostile environment, sensing outside signals via its complex sensory system and initiating immunologic reactions when the initial physical protection fails. Those functions are possible because of the highly elaborate structure of this multilayered organ, and the skin can interact with various other tissues such as epithelial, connective, vascular, muscular and nervous tissues. There are mainly three layers that form the skin; at the top a stratified epithelium, the epidermis, with hair follicles and sweat glands, next is the dermis enriched with extracellular matrix, and separated from the epidermis by a basal membrane with dermal-epidermal junction, and at the bottom is the hypodermis rich of adipose cells and blood vessels for nurturing the skin tissue (Kanitakis, 2002).

Embryologically, the epidermis originates from the neuroectoderm under the control of Wnt signaling pathway. On the other hand, different parts of dermis have different embryonic origins; the dermis of head and neck derives from the neural crest of ectoderm, while the rest of the dermis comes from the mesoderm, either lateral plate or somitic dermomyotome. Finally, hypodermis arises from a single germ-layer namely the mesoderm (Fuchs, 2007 and Olivera-Martinez et al., 2004).

1.1.1. Skin Architecture

The skin is a multilayered organ, whose general layered structure does not change over the surface, however significant regional variations regarding its physical features, or types and numbers of elements can be observed. The thickness of the skin can vary from one to four mm, or in some areas such as on the palms and on the soles the skin lacks hair. Also the distribution or density of cells of different constituents may vary, as in the case of the melanocytes along the epidermis (Figure 1.1).

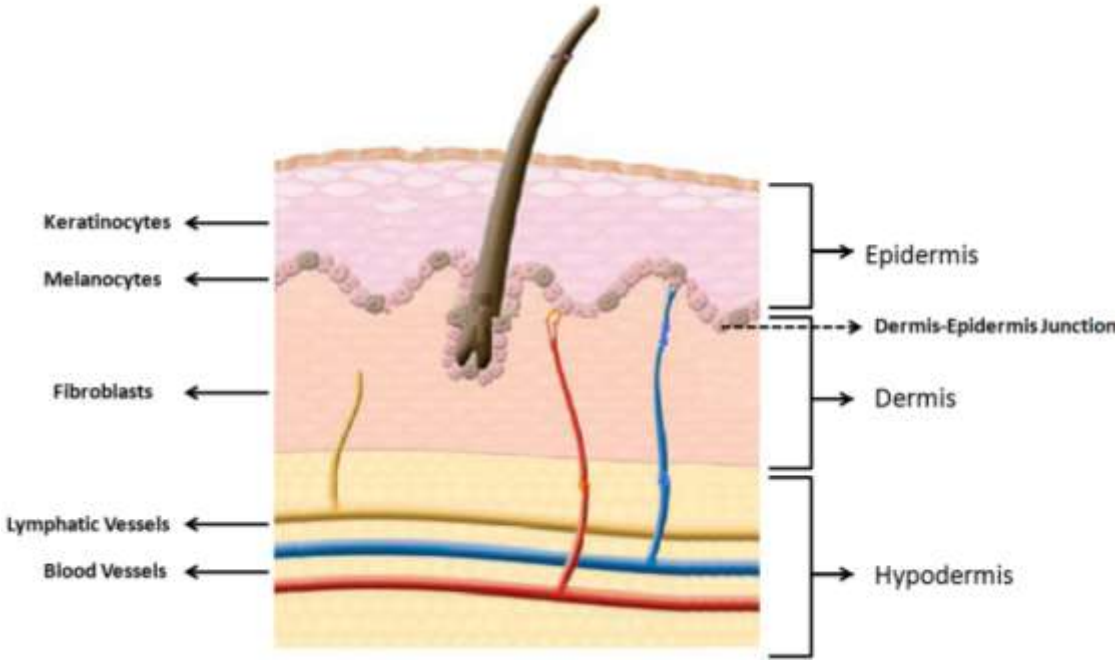


Figure 1.1. Skin Architecture. Layers of the skin and its three main cell types: melanocytes, keratinocytes and fibroblasts. (Adopted from Chudawski, 2005.)

The outermost layer of the skin, the epidermis, includes a stratified epithelium which contains various cell types, the majority (90 to 95 per cent) being keratinocytes. The rest of the cells types are Langerhans cells, melanocytes, Merkel cells, lymphocytes and Toker cells, where the latter two are scarcer than the other three. The epidermal cells are arranged in four to five distinct continuous layers. At the top are the horny layer (with five to ten cell layers) and granular layer (with one to three cell layers); next is the malpighian layer (with five to 15 cell layers), and at the bottom is the single cell layered basal layer (Kanitakis, 2002). The stem cells residing on the basal layer of the epidermis can give rise to new keratinocytes. These keratinocytes can migrate towards the upper layers near the surface of the skin and in about 30 day they undergo keratinisation and these differentiated anucleate cells shed from skin eventually.

Beside these particular cells, parts of two specific appendage structure can be observed in epidermis; the exterior part of sweat glands and hair follicles. Even if they surface of the epidermis, they are located mainly within the dermis and hypodermis. Arising among the epithelial cells these appendages are separate biological structures with their own biological regulatory systems. (Kanitakis, 2002 and Khavari, 2006).

The epidermis is connected to the dermis by an anchoring basement membrane called Dermis-Epidermis Junction (DEJ) produced collectively by epidermal keratinocytes and dermal fibroblasts. Besides reducing friction and providing mechanical support for the adhesion between the dermis and the epidermis, the DEJ also participates in the selective transport of metabolites between the two layers. Similarly, cells of other layers or tissues can migrate or invade different layers of skin through the DEJ, for instance the migration of keratinocytes (as well as proliferation, differentiation and cell polarity) is affected by DEJ components to some extent, and also lymphocytes can invade the cell layers in case of infection through the DEJ. In contrast to other basement membranes, the DEJ contains auxiliary anchoring complexes in addition to the integrin based system. The anchoring complex consists of three main parts, namely the hemidesmosomes on keratinocytes; anchoring filaments crossing the middle zone, called the lamina lucida; and anchoring fibrils enriched on sub-basal lamina. Not surprisingly, basal membrane components, such as laminin, nidogen, type IV collagen, heparan sulphate proteoglycan and type VII collagen are also present mainly concentrated at different levels in the DEJ (Burgeson, 1997).

Under the epidermis is the dermis, a protective, elastic and supportive connective tissue layer. The dermis contains cells, such as fibroblasts, dermal dendrocytes and mast cells; fibrous molecules, such as fibres majorly made of collagen types I or III; and a ECM ground substance, which biochemically contains glycoproteins and proteoglycans. The major dermal component is collagen, 98 per cent of the total dry dermal mass is collagen, which is the protein responsible of increasing the mechanical resistance of the skin.

Within the dermis there is a vertically distributed complex vascular network composed of arterial, venous and the lymphatic vessels. The cells of the epidermis and the dermis and the appendage structures (the sweet glands and hair follicles) are nurtured via this network. Also, immune reactions, regulation of interstitial fluid pressure and removal of metabolic byproducts is provided by the lymphatic vessels dispersed through the dermis. Beside the vascular and lymphatic networks, there is also complex innervation through the dermis, with members of both afferent and efferent limbs, ensuring the perception of the exterior stimuli such as touch, pressure and temperature. (Kanitakis, 2002).

The hypodermis is the deepest and thickest layer of the three. This layer is composed of the adipocytes, the fat storing cells of the skin. These cells are grouped together in lobules separated by connective tissue. This layer has an important role in thermoregulation, insulation, storing energy and buffering from mechanical impacts due to its high fat content. Moreover, the anatomical distribution of the hypodermis is highly sex related; the hypodermis generally accumulates around the belt, the abdomen and shoulder regions of men, whereas it tends to accumulate around the waist, thighs and hips of women.

1.1.2. Functions of Skin

The skin is a multifunctional organ, covering the whole body in a continuous fashion with the waterproof sublayer of epidermis. The interior of the body is adeptly separated from the environment. In addition to preventing body fluids from uncontrolled evaporation, this almost impermeable barrier provides the first line of defense against pathogenic organisms, toxic chemicals, and physical stress such as UV radiation. The hypodermis is important for efficient heat insulation as well as energy storage, and vitamin D production/storage. The regulation of the body temperature is mainly controlled through the skin with the help of the sensory network, the isolator layers and sweat glands.

Besides being a physical barrier against pathogens, the skin acts as an active immune organ. An immune response can be produced by the immune-competent cell groups of the skin. The dermis includes most of the immune system cells such as, lymphocytes, migrant leukocytes, mast cells, and tissue macrophages due to its interaction with lymphatic and blood vessels. The dermal fibroblasts are also capable of starting an immune response by producing cytokines. The epidermis deprived of the vascular network, has its local immune-competent cells. The Langerhans cells are mobile, antigen presenting cells originating from $CD34^{+}$ haemopoietic precursors of the bone marrow. They can uptake exogenous antigens, process them and presenting them to naive T-cells (Girolomoni G et al., 2002). Additionally, there are the keratinocytes, epithelial cells and melanocytes, the pigment producing cells with immune properties, as well as local and migrant lymphocytes (Salmon, 1994).

In addition to its essential immune function the skin also has the crucial task of perceiving various stimuli from the environment, so it is also an organ of communication. Nerve fibers, responsible for the somatosensory perception, have their free endings located in the epidermal skin layer. Afferent sensory neurons of the peripheral nervous system (PNS) traverse through the skin layers up to the epidermis. Their number or density can vary depending on the location. For example, they are concentrated near the tips of the fingers and are less frequent in the distal parts of the limbs (Johansson O et al., 1999). Even if the sensory neurons are spread apart in a location their signals can be detected at a fine scale by the skin. This indicates that components of skin may play a part in the reception of stimuli. Interestingly, some neurotransmitters (primarily neuropeptides) and sensory proteins are expressed by nearly all epidermal cells (keratinocytes, melanocytes, Langerhans cells and Merkel cells) suggesting that these cells can contribute to the signal transmission process (Nicholas, 2008). Sensory proteins belonging primarily to the transmembrane protein families, serve to the transformation of the received stimuli such as touch, osmotic pressure, temperature or chemical stimulations into biochemical messages (Boulais, 2007). Epidermal cells also recognize signals transmitted through neuromediators, since they express the corresponding receptors (Slominski, 2000), which points to the multidirectional connection between the brain and the skin.

1.2. Cells of Skin

The three layers of skin are composed of various cell types with diverse functions. However, we will be focusing on the three main cell types for this study, namely the melanocytes and the keratinocytes of the epidermis and the fibroblasts of the dermis.

1.2.1. Melanocytes

The upper two layers of the skin, the epidermis and dermis, are separated by a basement membrane, DEJ, and melanocytes reside on the epidermal side. They represent two to five per cent of all epidermal cells. These highly dendritic cells originate from the neural crest of the ectoderm. During the course of embryonic development the melanocyte precursor, melanoblasts, travel to various locations, so melanocytes can be found at many locations throughout the body. In the skin they are in contact with keratinocytes through

their long dendrites; one melanocyte can interact with 30 to 40 keratinocytes forming an epidermal–melanin unit.

A crucial function of melanocytes is their ability to produce and distribute melanin, a pigment that can protect the cells from the distressing effects of UV radiation. The distribution and number of melanocytes in the skin is similar regardless of ethnicity and actually the basal skin pigmentation level depends on melanogenic activity and the transfer of melanin to the keratinocytes. Melanin is produced in melanosomes, organelles originating from the endoplasmic reticulum and the size and the melanin content of the melanosomes are the determining factors of skin color. Melanosomes are transferred to neighboring keratinocytes once melanin is produced. Upon UV radiation melanin production as well as melanogenesis is increased. The dendrites of melanocytes also increase in number providing better association with keratinocytes. This process results in the tanning response of the skin upon exposure to sunlight (Tsatmali, 2002).

In addition to melanin, melanocytes can produce other signaling molecules such as cytokines (Salmon, 1994), POMC peptides, which are actually neuromediators, catecholamines (Iyengar, 1987), and nitric oxide (NO) (Graham et al., 1997) in response to UV radiation, as well as to different kinds of stimuli. These signaling molecules can in turn affect the various cell groups of the skin, such as keratinocytes, fibroblasts, mast cells, lymphocytes and epithelial cells. Some sensory neurons can receive these signals, since they have been shown to express their cognate receptors. This signifies the regulatory role of the melanocytes in the skin and thus the maintenance of the epidermal homeostasis.

One of these signaling molecules is NO, which is involved in many physiological and pathological processes. NO is produced by melanocytes upon UV radiation and bacterial lipopolysaccharide (LPS) exposure (Tsatmali et al., 2000). Although the exact role of NO production by melanocytes is not known, it is suggested that NO may act as an autocrine agent increasing melanogenesis after exposure to UV radiation (Tsatmali et al., 2002).

Cytokines are signaling molecules that can mediate inflammatory responses. Melanocytes were once thought of not having any immunologic function, but later they were found to produce various cytokines, such as interleukin-l, -3, -6 and TNF-α (Köck et al., 1992) and contribute to the immunity of the skin. Since melanocytes are located on the basal membrane of the epidermis, their location bears a strategic value affecting both the dermis and the epidermis (Zachariae et al., 1991).

The production of neuropeptides and neurotransmitters suggests a local sensor role for melanocytes. The anatomic resemblance of melanocytes to neurons and their association with the nerve endings in the epidermis (Hara et al, 1996) suggests that melanocytes can also act as neuroendocrine cells. Melanocytes are not only the pigment producing cells of skin but they can connect the sensory and immune systems together.

1.2.2. Keratinocytes

Keratinocytes are the epithelial cells of the epidermis, accounting for more than 90 per cent of all epidermal cells. There are approximately 80 billion keratinocytes in the adult skin, which makes them the most common cell type in the body's largest organ (Denning, 2004). These cells regenerate continuously maintaining a vital water proof barrier at the outermost layer of the epidermis, the stratum corneum. Beside maintaining this barrier and regenerating the epidermis, they also act as essential players during the wound healing process, highlighting their great self renewal potential. After stratification the mature keratinocytes finalize their life cycle by entering into a squamous differentiation process, they become flattened and lose their nuclei, their keratin content becomes concentrated, and eventually they are shed from the skin surface (Kanitakis, 2002). This squamous, keratin containing dead keratinocyte barrier protects the body not only from physical stress, and water loss but also acts as the primary barrier against pathogens of the outside world (Denning, 2004).

In the case of skin injury, the cutaneous barrier must be restored in a controlled and sequential manner. The inflammatory phase, if there are any intruding pathogens, is followed by a granulation phase, when the cells start to proliferate, great amounts of extracellular matrix (ECM) is produced and populated by migrant cells resulting in wound

closure. The healing process is completed by the remodeling of the scar tissue subsequent to restoration of the cutaneous barrier. This cascade of events requires strict coordination between different cell groups, two of which are keratinocytes and fibroblasts. Keratinocytes can induce the synthesis of growth factors and cytokines such as interleukin-1, which in turn induce proliferation and differentiation of keratinocytes forming a paracrine loop (Maas-Szabowski, 1999).

The synthesis of cytokines by keratinocytes ties them to the immune system, moreover they are involved both in innate and adaptive immunity (Williams and Kupper, 1996). In addition to the three primary cytokines IL-1α, IL-1β (Kupper et al., 1986) and TNF-α (Kock et al., 1990) responsible for cutaneous inflammations, a wide variety of other cytokines are produced, executing various tasks such as: chemotaxis through chemokines and promotion of suppressing immune cells.

Finally, keratinocytes also express sensory proteins similar to sensory neurons and are thus members of the somatosensory system of the skin (Boulais, 2007). They express Trp family proteins, helping the reception of thermal and noxious stimuli (Dhaka et al., 2006). Interestingly, physical contact between keratinocytes and nerve endings has been suggested by both *in vivo* and in vitro studies (Chateau, 2004). Keratinocytes present themselves as multi-functional cells of epidermis, with the ability of sensing their environment and reacting as required.

1.2.3. Fibroblasts

The fibroblasts are found in the dermal connective tissue layer. These spindle shaped cells are indeed responsible for the maintenance of the dermis as they can produce and secrete most of the ECM components, such as collagens, proteoglycans, fibronectin, tenascin, laminin and fibronectin as well as matrix metalloproteinases regulating the degradation of the ECM components. Fibroblasts achieve this by keeping their metabolic rate high and through their enhanced endoplasmic reticulum and Golgi apparatus (McAnulty, 2007). Fibroblasts from different parts of the dermis produce a diverse range of matrix molecules (Sorrel and Caplan, 2004).

Fibroblasts are the main actors of the wound healing process. The disrupted ECM is replenished by collagens and fibronectins produced by the fibroblasts, which are regulated by inflammatory cells, injured or proliferating epithelial cells and by other fibroblasts. In the granulation phase, some dermal fibroblasts acquire myofibroblast characteristics. They assemble α-smooth muscle actin containing stress fibres, crucial for the wound closure process due to their contraction ability. At the final step, remodeling of the scar tissue, the metalloproteinases and their inhibitors come to play, and the myofibroblasts are removed via apoptosis (McAnulty, 2007).

1.3. Cancer

Cancer is a group of diseases which can affect various locations of the body. It is known to have over a hundred distinct types. Under normal circumstances a regular cell can undergo only a limited number of cell divisions, after which they enter the senescence stage. Senescent cells are resistant to growth factor stimulation, and die eventually. The growth and division of the cells of higher eukaryotes are strictly controlled, since maintaining a strict balance between cell division and programmed cell death is crucial for the homeostasis of the organism. Any environmental agent and genetic disorder targeting this balance may result in cancer, characterized by uncontrolled cell division and by the acquired ability of the cells to grow in an anchorage independent manner and propagate indefinitely by metastasis. Through metastasis, cancer cells can invade the adjacent tissues, enter the bloodstream and spread to various organs, which may result in death. Indeed the major cause of cancer mortality is occurrence of metastases.

According to a report by the World Health Organization (WHO), only in 2007 7.9 million cancer related deaths have been recorded. This figure accounts for 13 per cent of all death cases worldwide, where lung, stomach, liver, colon and breast cancer have the greatest share in cancer mortality. The severity of cancer is highlighted by the low survival ratio: only about 30 per cent of cancer related deaths can be avoided, with the highest success rates coming from high-income countries. The incidence of specific cancer types is highly variable in different populations. Along with inherited genetic factors, age, sex, ethnicity and the environment are major factors that determine the susceptibility of a person to a specific type of cancer (Jemal et al., 2007).

Cancer death incidence in Turkey is similar to the worldwide distribution statistics (Figure 1.2). According to an estimation of WHO Global Database the 11.7 per cent cancer related death incidence in 2005 will rise up to 13.6 per cent in 2030. In 2005 approximately 52,000 cancer related deaths were reported in Turkey and 37,000 of these lost patients were under the age of 70.

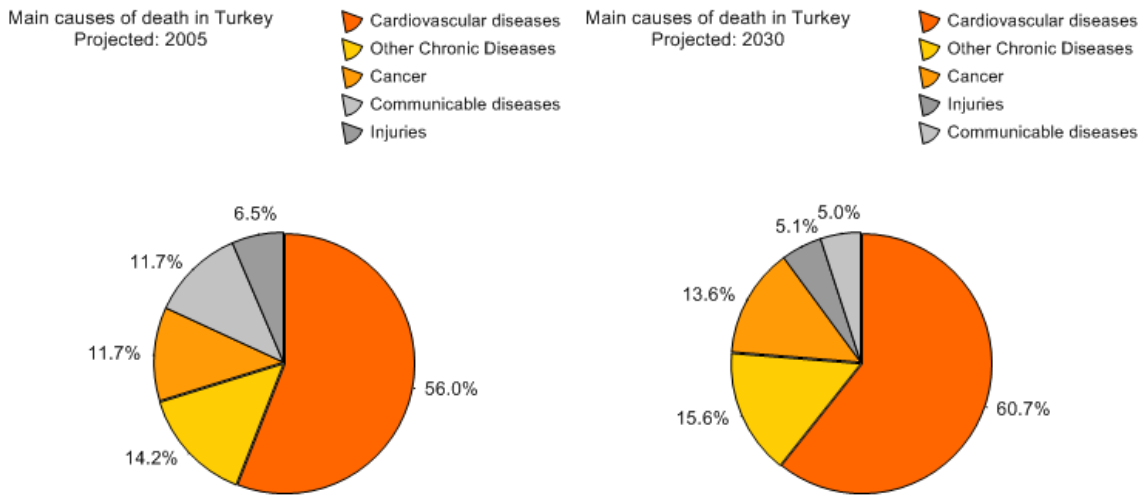


Figure 1.2. Cancer Profile of Turkey. Adopted from WHO Global Database, cancer country profiles.

The development of cancer is a multistage process: the transformation from a normal into a neoplastic cell requires at least several alterations in the genome. Once the control mechanisms of the cell are overcome, somatic mutations and genetic instability start to accumulate resulting in strengthened tumorigenic state. Genetic mutations may occur due to inherited factors, physical agents such as UV radiation, chemical agents such as tobacco constituents or biological agents such as viruses and bacteria. Consequently, in different cell populations, different subsets of malignant mutations arise, and the mutations providing selective advantage leading to increased growth rate, will become dominant among the overall population. This tendency towards aggression drives the progressive transformation of normal human cells into highly malignant derivatives. Initially they grow more rapidly locally and later can migrate to and survive in new locations.

There are two classes of genes, dearrangements in which are involved in cancer, namely the proto-oncogenes and tumor suppressor genes. Mutations in these genes can give rise to successive events which ultimately give way to carcinogenesis. Proto-oncogenes alter into oncogenes by dominant gain of function mutations, whereas recessive loss-of-function mutations are detected in both copies of tumor suppressor genes, rendering them completely inactive.

1.3.1. Oncogenes

Proto-oncogenes in certain cases acquire mutations or become abnormally activated leading to the activation of the oncogene that is associated with tumor formation. The oncogenes have different types of roles in the cell, such as being transmembrane proteins and transcription factors. Generally gain-of-function mutations bring about a change in the protein's conformation, leading to its constitutive activation or overexpression. Even if only one of the alleles is mutated, it is sufficient to affect the growth rate at a cell. Two important proto-oncogene families are the *ras* and the *myc* groups.

Members of the *myc* oncogene family are involved in the generation of various malignancies: especially three members, c-*myc*, N-*myc* and L-*myc* have been shown to be related to many types of human cancers (Ryan, 1996). Members of the *myc* family work as transcription factors. Their DNA binding leucine zipper and helix-loop-helix motifs help their interaction with DNA. Additionally, to achieve efficient sequence-specific DNA binding they interact with other proteins and form heterodimers as in the case of Max and c-Myc (Blackwood et al., 1992). c-Myc, the most extensively studied member, was discovered through its homology to one of the genes of the avian myelocytomatosis virus MC29, namely the transforming v-Myc Vennstrom et al., 1982). c-Myc via transcriptional regulation of target genes controls re-entry into and progression through cell-cycle (Eilers et al., 1991), immortalization of cells (Littlewood et al., 1990), blocking differentiation (Miner et al., 1991) and even inducing programmed cell death (Evan et al., 1992). The expression of c-Myc itself is regulated rather strictly. It is known that four major separate promoters take part in the transcriptional control of c-Myc, where some of these promoters

have regulatory domains targeted by cell-cycle-regulated transcription factors such as E2F (Stewartet al., 1984 and Hiebertet al., 1989).

The discovery of Myc proteins shifted the focus of cancer research from deregulated cell-cycle control mechanisms towards other factors contributing to tumorigenesis; the oncogenes were shown to regulate processes, such as apoptosis and metastasis through their transcriptional activity. Ability to induce apoptosis as well as cellular transformation and attachment independent growth, highlights the special character of Myc genes among the oncogene families.

Another key oncogene family is the *ras* family. These proteins act as transducers of mitogenic signals and are found to be mutated in almost 20 per cent of human cancers (Bos, 1989). The mutated forms of these proteins increase the malignancy of the cells by inducing cell growth, promoting angiogenesis, and metastasis, as well as inhibiting apoptosis (Downward, 2003). Actually the name 'ras' has its root from the cancer relation of this family; precisely form transforming genes of Rous sarcoma viruses (Barbacid, 1987). There are three major functional genes in this family, having molecular weight of 21,000 Dalton (ras p21), with very similar structures, namely H-ras, K-ras, and N-ras (Shih et al., 1979).

Ras proteins are GTPases. They serve as molecular switches; they are in the inactive state while bound to GDP molecules and they are activated when they are bound to GTP. Their enzymatic activity found to be reduced dramatically in oncogenic forms (Gibbs et al., 1984). Furthermore their biochemical function in cell growth was shown where epidermal growth factor (EGF) activation of Ras proteins indicating a strong relation between Ras pathway and mitogens (Kamata and Feramisco, 1984) such as platelet-derived growth factor (PDGF), nerve growth factor (NGF) and fibroblast growth factor (FGF) (Aaronson, 1991). As their association with mitogens were shown, their role in the cellular transformation was investigated, which in turn revealed the key role of Ras activity in transformation induced by oncogenes belonging to growth-factor receptors or plasma-membrane-associated tyrosine kinases (Smith et al., 1986). Ras proteins are associated with various kinds of effectors indicating diverse biological responses resulted by Ras

activity such as cell proliferation, growth arrest, cell senescence, differentiation, apoptosis and cell survival (Malumbres and Barbacid, 2002).

1.3.2. Tumor Suppressor Genes

The first idea that genes can suppress tumor formation or inhibit the growth of tumorigenic cells had arisen from the studies done by fusion experiments, where merging tumor forming cancerous cells with normal somatic cells. The observation that some hybrid cells lost their tumorigenic abilities led to the suggestion that normal cell contributed one or more genes that could suppress tumor formation in animals. The TS genes are lost in tumorigenic cells but can be restored by a single dominant wild-type allele, thus in cancerous cells both copies of the genes should be in a mutated state (Levine, 1993). The TS gene concept was further supported by the study of Knudson on retinoblastoma cases in infants. Even when the initial bilateral tumors were removed, new independent neoplasms were developed in these infants suggesting an inherited predisposition for retinoblastoma. Interestingly, in rare cases unilateral retinoblastomas in adults were observed leading to the combination of somatic mutation and susceptibility gene concepts. The children inherited a mutant allele of a putative TS gene, retinoblastoma susceptibility gene ‘Rb’, and later in their life these individuals acquire a second somatic mutation resulting in cancer (Knudson, 1971).

One of the most important TS genes is p53, a transcription factor with essential tumor suppressor functions. Approximately 80 per cent of all p53 mutations are missense mutations (Lozano, 2007). Besides the inactivation of the functional protein product may occur by gene deletion, degradation at protein and mRNA levels, or sequestration by viral oncogenes, which in turn increase the cancer susceptibility of a cell as a response to oncogenic stimuli and environmental stress factors promoting uncontrolled growth and malignancy. p53 becomes activated by internal and external environmental factors such as DNA damage, oncogenic, genotoxic and oxidative stress resulting in a strict response to inappropriate growth promoting or growth inhibiting conditions (Giaccia and Kastan, 1998).

Activation of p53 leads to the induction of pro-apoptotic genes such as BAX, PUMA and APAF-1, as well as cycle regulators as p21 and GADD45; also senescence genes can be activated depending on the signal and cell type (Lozano and Zambetti, 2005). p53 is found to be defective in approximately 50 per cent of human tumors resulting to the high selective pressure in tumor cells to inactivate functional copies of p53 at genetic or protein levels (Levesque, 2007). Mutations in the p53 gene, overexpression of p53 inhibitors, such as Mdm2 family members, or inactivation of upstream activators, such as $p14^{ARF}$ and $p16^{INK4a}$, result in loss of or reduced p53 activity, consequently leading to aberrations in apoptosis, uncontrolled proliferation and cellular transformation (Neil, 2008).

Another significant protein family contains the Bcl-2 (B-cell lymphoma 2) family of mitochondrial regulators. The Bcl-2 family has both proapoptotic (tumor suppressor) as well as antiapoptotic (proto-oncogene) members and the decision that apoptosis is executed from the mitochondria depends on the ratio between the proapoptotic and antiapoptotic Bcl-2 family members. The Bcl-2 protein itself was the first antiapoptotic proto-oncogene to be discovered. It blocks the activity of proapoptotic members such as Bax and Bak (Cheng et al., 2001). Bax and Bak are essential multi-domain pro-apoptotic family members whose protein products are required for cytochrome c release from mitochondria, by promoting the permeabilisation of the outer mitochondrial membrane. Additionally, by interacting with the truncated form of Bid (tBid), another Bcl-2 proapoptotic protein, Bax can self oligomerize and localize to mitochondria. Bid is cleaved by caspase 8, downstream of death receptor signaling. Thus extrinsic as well as intrinsic signals inducing apoptosis are conveyed through Bax to mitochondria (Esposti and Dive, 2003).

Due to their crucial role in apoptosis regulation, it is not unusual to find altered expression of Bcl-2 family proteins in a variety of human cancers. Escape from apoptosis provides the tumorigenic cells several selective advantages such as survival in hostile environments, regardless of incoming external death signals. This condition favors metastasis or establishes tolerance to accumulation of oncogenic factors resulting in increased malignancy. Mutant forms of Bax can be found in various human cancers such

as gastric and colorectal cancers and leukaemias. Loss of Bax is associated with increased tumorigenicity as well as enhanced transformation potential (Cory and Adams, 2002).

1.4. Skin Cancer

There are three main types of skin cancer; the most frequent ones are the non-melanoma skin cancers (NMSC) arising from keratinocytes, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) and there is the malignant melanoma (MM) arising from melanocytes which is the least frequent but most lethal. According to WHO, two to three million non-melanoma skin cancer cases and 132,000 malignant melanoma cases are diagnosed each year globally (http://www.who.int/uv/faq/skincancer/en/index1.html).

BCC arises from the cutaneous keratinocytes of the epidermis. Even though it is the most common type of skin cancer (almost 75 per cent), BCC is the least aggressive one; metastasis is rarely observed, the tumorigenic keratinocytes invade locally resulting in disfiguring of the skin. Mutations activating the hedgehog signaling pathway, which is associated with hair follicle growth and morphogenesis, are commonly observed in BCC (Khavari, 2006).

Similar to BSS, SCC also arises from keratinocytes. It is the second most common (approximately 25 per cent) and more aggressive type of skin cancer capable of forming lethal metastases. The development of SCC is a multistep process, where one of the essential risk factors is the cumulative life time exposure to UV radiation. Mutations in Ras, p53 and CDKN2A loci with protein products, $p16^{INK4}$ and $p14^{ARF}$, which are acting as cell cycle inhibitors, are associated with SCC development (Boukamp, 2005 and Khavari, 2006).

The least common type of skin cancer (less than one per cent) is malignant melanoma, which originates from melanocytes. The frequency of this most lethal skin cancer has been increasing since early the 70s. This could be partly attributed to the thinner Ozone layer. Intermittent, intense UV exposure increases the MM risk is typically associated with rather than cumulative sun exposure (Chudnovsky, 2005 and Khavari, 2006). Compared to the NMSCs, melanoma progression is more complex and

heterogeneous, thus mutations in various signaling pathways are related to melanoma development. Overactivated B-Raf (50-70 per cent mutated), N-Ras (15-30 per cent mutated), isoforms of Raf and Ras, deleted or inactivated CDKN2A and PTEN are among common genetic alterations observed in MM. Interestingly, the BRAF and NRAS mutations rarely coincide (20 per cent) in spite of the high mutation ratios in BRAF (Khavari, 2006 and Gray-Schopfer et al., 2007).

1.5. Transformation of Primary Cells

Some of the essential characteristics of malignant growth include uncontrolled growth, in the absence of growth signals or decreased sensitivity to growth arrest signals; escape from apoptotic routes; immortalization and metastasis. A primary human normal cell should go through a multistep process to become neoplastic. Malignant transformation refers to the evasion of growth control mechanisms, covering some of the key features of tumors but not all. Malignant transformation is a complex process, where genetic as well as epigenetic alterations result in a cell population, which is insensitive to internal and external growth regulatory signals, giving the transformed cell population new selective perks. Metastatic cells may even become independent, however they are not yet metastatic. Additional alterations tend to accumulate, leading to immortalization and finally metastasis (Seger et al., 2002).

The transformation process has been shown to involve activation of a several oncogenes and inactivation of several tumor suppressor genes. By mimicing the transformation processes in rodent models and in primary human cells, the genes responsible for malignant transformation could be detected (Hanahan and Weinberg 2000). These studies showed that, a single defective oncogene or a tumor suppressor gene is not sufficient for malignant transformation, as there are compensation mechanisms such as apoptosis that become engaged. The minimum requirement for transformation involves successive alterations in different tumor suppressors and/or oncogenes in combination. The combinations of oncogenes and/or tumor suppressor genes are different for every organism and cancer type. At least two genetic alterations are capable of inducing transformation in rodent cells. The same alterations do not drive malignant transformation in their human counterparts, signifying a more complex transformation mechanism for human cells (Hahn

et al., 1999). Furthermore, as each type of cancer has its own genetic network of malfunctioning genes, transformation of a specific cell type requires hits in a distinct set of genes. In the case of BCC transformation, it has been shown that a hit in one gene is sufficient to induce all the basic characteristics of BCC, whereas in more complex types of skin cancer such as SCC and malignant melanoma, hits in two or three genes are needed to transform a keratinocyte or melanocyte into malignant character cells, respectively (Khavari, 2006).

1.5.1. Molecular Biology of Human Fibroblast Transformation

Studies of tumor derived tissues lead to the identification of genetic alterations facilitating tumor formation. Initial experiments using the immortalized mouse fibroblast cell line NIH-3T3 showed that these cells could form tumors if additional viral or tumor-derived genes were introduced to these cells. However, the normal rodent cells failed to transform upon introduction of a single oncogene indicating that the genetic alterations of the immortal cells may have a critical role in malignant transformation. Such studies lead to the proposition that a combination of at least two oncogenes was required (Land et al., 1983). From this point onwards many combinations of oncogenes and tumor suppressor genes were identified, which facilitated the transformations (Hahn and Weinberg, 2002).

Hits in which the combination process of two activated oncogenes, *c*-Myc and h-Ras (Land et al., 1983) or the adenoviral E1A protein together with h-Ras (Boehm, 2005) were introduced to the rodent cells, normal mouse embryo fibroblasts can be transformed. Introduction of an oncogenic copy of Ras together with another gene having an immortalizing function, like the adenovirus E1A and E1B genes or simian virus 40 (SV40) large T antigen (LT), or human papilloma virus E6 or E7 genes (Wei et al, 2003) has a similar effect. These DNA tumor virus-encoded oncoproteins are capable of inactivating the pRB and p53 tumor suppressor proteins by binding (Hahn et al, 2002). Interestingly, the absence of serum survival factors, overexpression of Myc triggers apoptosis through $p19^{ARF}$ and p53 signaling pathways. Another important role of Myc is that it can activate the telomerase, by overexpressing the catalytic subunit of human telomerase, hTERT. This is observed in a high per centage of human cancers (Wang et al, 1998). Even if, hTERT is important for immortalization and thus transformation, primary rodent fibroblasts fail to

transform when c-Myc is replaced with hTERT, indicating that the transcription factor c-Myc has other functions besides telomere activation (Greenberg et al., 1999). These findings point to the essential roles to p53 and pRB signaling pathways, as well as the requirement for telomerase activity for malignant transformation.

Expression of an activated oncogenic Ras alone induces terminal growth arrest is similar to senescence. In murine cells, the inactivation of the p53 pathway can overcome this arrest. Similar to virus-encoded oncoproteins acting on p53 and pRB, cells lacking p53 or $p19^{ARF}$ are transformed upon introduction of oncogenic Ras. In contrast to mouse cells, inactivation of p53 together with Ras, fail to transform primary human cells (Serrano et al., 1997). Similarly, the coexpression of Myc and Ras oncogenes does not result in malignant transformation in human cells (Stevenson and Volsky, 1986) and additional genomic alterations are required. This phenomenon highlights the difference between rodent and human cells.

One of the important distinctions between human and murine cells is that primary murine cells undergo spontaneous immortalization comparatively easily. This may be attributed to differences of the telomeres; that is to say that the human telomeres are relatively shorter and telomerase enzyme activity in adult tissues is missing (Boehm, 2005). In the absence of telomerase activity human cell will become senescent. The senescence-like arrest induced by Ras which can be bypassed by the inactivation of either p53 or pRB pathways in rodents. However, inactivation of both pathways is necessary for bypassing the growth arrest in human cells. Complete transformation in human cells cannot be achieved without further alterations such as SV40 small antigen expression (Wei et al, 2003). Transformation of human fibroblasts has been shown to require the combined expression of *c*-Myc, *H*-RAS, together with inactivation of p53, RB, and PTEN tumor suppressor genes (Boehm, 2005 and Zhao et al., 2004). Transformation is tested *in vitro* using the soft agar colony formation assay.

1.5.2. Soft Agar Colony Formation Assay

Normal epithelial cells need some kind of attachment to basement membranes or other cells for growth and survival. Once they are deprived from these attachments, thus from essential signals promoting survival and proliferation, they enter a pathway of detachment-regulated apoptosis, anoikis. Therefore, even in normal tissue culture, the plasticware used for incubation of cells has modified surfaces so that the cells can attach and grow. On the other hand, the behavior of malignantly transformed cells differs from that of normal cells. Besides overcoming apoptosis, cell cycle arrest or signal dependent growth these transformed cells tend to acquire new features such as anchorage-independent growth, tumor forming ability when injected into animal hosts. When such cells are seeded into semisolid matrices lacking any kind of attachment sites, they are capable of growth, proliferation and form colonies unlike normal cells, which would in similar conditions enter apoptosis and die. So, testing anchorage-independent growth of cells in soft agar is employed frequently to check cellular transformation (Anderson et al., 2007).

Growth in soft agar has been used for as a transformation test since the connection between the tumorigenic potential of transformed cells *in vivo* and their anchorage independent growth ability in vitro was first suggested by Freedman and Shin (Freedman and Shin, 1974). This assay is relatively advantageous compared to the *in vivo* tumorigenesis assay employing animals; it is easy and fast. Nonetheless, even if this assay provides clues about cellular transformation status, still an *in vivo* tumorigenesis assay is required to determine whether the cells are malignantly transformed or not. One would keep in mind that, findings or precisely lack of findings about anchorage-independent growth capability of a supposed transformed cell can be misleading. Traditional in vitro characteristics of malignancy, such as immortalization and anchorage-independent growth, cannot be always correlated with cancer behavior in vivo. It is interesting to note that, the metastatic SCC cells isolated from tumors are not capable of growing in an attachment-independent manner in soft agar, as they cannot proliferate indefinitely (Khavari, 2006).

1.6. Gene Knock-down via shRNAs

The RNAi machinery is suggested to be related to a highly conserved defense mechanism for the genome against viruses and transposons. The process starts by shorthening of the longer dsRNAs products of either transposons, replicating viruses, or the regulatory microRNAs. These short RNAs are transported and cleaved by a cytoplasmic ribonuclease called Dicer into double stranded RNA (dsRNA) molecules into 19–25 bp long RNA duplexes. They later associate with the multiprotein complex, RNA-induced silencing complex (RISC). The helicase of the RISC unwinds and one of the single strands of the RNA duplex can recognize the target mRNA with the complementary sequence, which ultimately results in either degradation of the mRNA or translational repression in case of lower level sequence complementarity (Leung and Whittaker, 2005). In case of shRNAs, they enter the machinery from the Dicer cleavage step.

One problem of siRNAs is that their effects are not stable since the transfected siRNA duplexes are diluted in each cell division. To solve this problem, new vectors were constructed containing RNA polymerase III promoters so that shRNAs can be expressed constitutively and then are cleaved to siRNA duplexes by Dicer. The low transfection efficiency of primary cells was overcame by employing retroviral, lentiviral, and adenoviral vector systems with two to three vector components (Leung and Whittaker, 2005). Lentiviral vector systems derived from human immunodeficiency virus (HIV)-1 are especially suitable for infecting dividing as well as nondividing postmitotic cells such as neurons (Rubinson et al., 2003).

2. PURPOSE

Cancer studies, based on the use of primary cells provide crucial information about disease progression. In many ways this type of data has advantages to studies based on cell lines with various known and unknown genetic mutations. Disregarding any inherited phenotypically invisible genetic mutations, primary cells have the basic unaltered genome and represent the "normal" human cell. Thus, any phenotype resulting from an experimentally introduced new genome alteration can be attributed to the newly engineered mutation or alteration. For example, in transformation experiments using primary fibroblasts, oncogenic or tumor suppressor roles can be assigned to the genes studied. Similarly, experiments employing primary human melanocytes and keratinocytes can help the elucidation of skin cancer development, particularly since the majority of skin cancers have origins in these two cell types. Additionally, the skin is a multilayered and multifunctional organ, thus the cells of the skin can interact and regulate each other in various ways. Biological responses usually arise from cumulative effects of different types of skin cells, rarely a single skin cell type. Thus, three dimensional skin reconstructs are used more frequently nowadays instead of cultures of a particular skin cell type. In these constructs, the three major skin cells, the keratinocytes, the melanocytes and the fibroblasts, are used as the basic components of the artificial skin to mimic the most basic features of the skin in vivo. Use of such three dimensional artificial skin constructs will make possible the study of the different functions of skin, such as sensation of stimuli as well as host-pathogen interactions in immune responses.

In this study, we aimed to optimize the isolation procedures and culture conditions of primary human melanocytes and keratinocytes from the epidermis and primary human fibroblasts from the dermis for further studies.

Additionally, we performed transformation assays on the isolated primary fibroblast using a lentiviral infection system to knock-down p53 expression and express oncogenes such as hRas or cMyc. We determined their capacity to grow in an anchorage-independent manner in soft agar conditions.

Finally, with the established soft agar colony formation assay we aimed to determine the transformation potential of ASC knock-down, using the newly constructed shRNA vector, in combination with oncogenes cMyc or hRas expression using primary human fibroblasts and melanocytes.

3. MATERIALS AND METHODS

3.1. Chemicals, Plastic and Glassware

The chemicals used through the experiments were purchased from Sigma-Aldrich (USA) or Merck (Germany) with a few exceptions that are stated in the text. The cell culture media for melanocytes and keratinocytes were purchased from Cascade Biologics (Invitrogen, USA). Other cell culture media and supplements were acquired from Gibco (Invitrogen, USA). The plasticware used in the cell culture procedures were purchased from TPP (Switzerland) in sterile packages. The glassware and the laboratory-prepared buffers were sterilized by autoclaving at 121°C and/or by filter sterilization using 0.22 μm filters (Millipore, Ireland).

3.2. Cell Lines, Primary Cell Isolation and Cell Culture

The tissue samples used for the isolation of human primary skin cells were provided by Dr. Osman Oymak (from Çevre Hospital) as leftovers from plastic surgery operations with the consent of the patients. The human embryonic kidney cell lines HEK 293FT (gift from M.S. Soengas) and HEK 293T (gift from Mehmet Öztürk) and the primary human fibroblasts were grown in DMEM supplemented with 10 per cent FBS, one mM sodium pyruvate, 1X L-glutamine (Gibco, Invitrogen, USA), 50 U/ml penicillin, 50 μl/ml streptomycin and 1X MEM NNA ($DMEM^+$). Primary human melanocytes and primary human keratinocytes were grown in Melanocyte Growth Medium 254 CF (MGM) supplemented with Human Melanocyte Growth Supplement (HMGS) and in Keratinocyte Growth Medium 154 CF (KGM) supplemented with Human Keratinocyte Growth Supplement (HKGS) respectively. Cell lines and primary cells were grown at 37°C and under 5 per cent CO_2. The cells were passaged when necessary and the passage number were recorded. The cell lines and fibroblasts were stored in 7 per cent DMSO at -70°C, melanocytes and keratinocytes are stored in 38 per cent MGM, 11 per cent DMSO and 51 per cent FBS and 62 per cent KGM, 8 per cent DMSO, and 30 per cent FBS, respectively at -70°C.

3.3. Plasmids

For gene transductions a four plasmid lentiviral system was used. The pHCMV-G, pRSV-Rev and pMDLg/pRRE plasmids are part of the general mechanism of the lentiviral transduction system, whereas the FG12 and H1 plasmids are the carriers of the genes of interests (gifts from M.S. Soengas).

Table 3.1. List of plasmid vectors.

Name	Use	Important Genes
pHCMV-G*	Infection	VSV-G gene (viral entry into the cell) HCMV promoter, ampicillin resistance gene
pRSV-Rev†	Gene integration	reverse transcriptase gene
pMDLg/pRRE†	Viral packaging	gag, pol genes
FG12-cMYC-GFP	cMYC expression	Mutant cMyc
FG12-hRAS-GFP	hRAS expression	oncogenic hRas (T24 hRas)
H1-BAXi-GFP	BAX knock-down	shDNA of BAX
H1-p53i-GFP	P53 knock-down	shDNA of p53

(Dull et al., 1998†, Yee et al., 1994*)

The FG12-gene-GFP vector is shown in Figure 3.1.b represents the lentiviral plasmid vector used for transduction of the oncogenes. These FG12-cMYC-GFP and FG12-hRAS-GFP vectors have oncogenic copies of c-Myc (oncogenic c-myc from mouse, Hann et al., 1992) and h-Ras (T24 h-Ras) genes, cloned into the multiple cloning sites (MCS) downstream of a CMV promoter respectively and additionally contains the GFP gene with a human ubiquitin C promoter (UbiC). The H1-shGENE-GFP vector in figure 3.1.a is the lentiviral vector used for the knock-down of the tumor suppressor genes. H1-BAXi-GFP and H1-p53i-GFP vectors have shDNAs of BAX and p53 genes cloned into the multiple cloning sites (MCS) downstream of a H1 promoter for efficient expression of the shRNAs of the corresponding genes and additionally have the GFP gene with the human UbiC promoter.

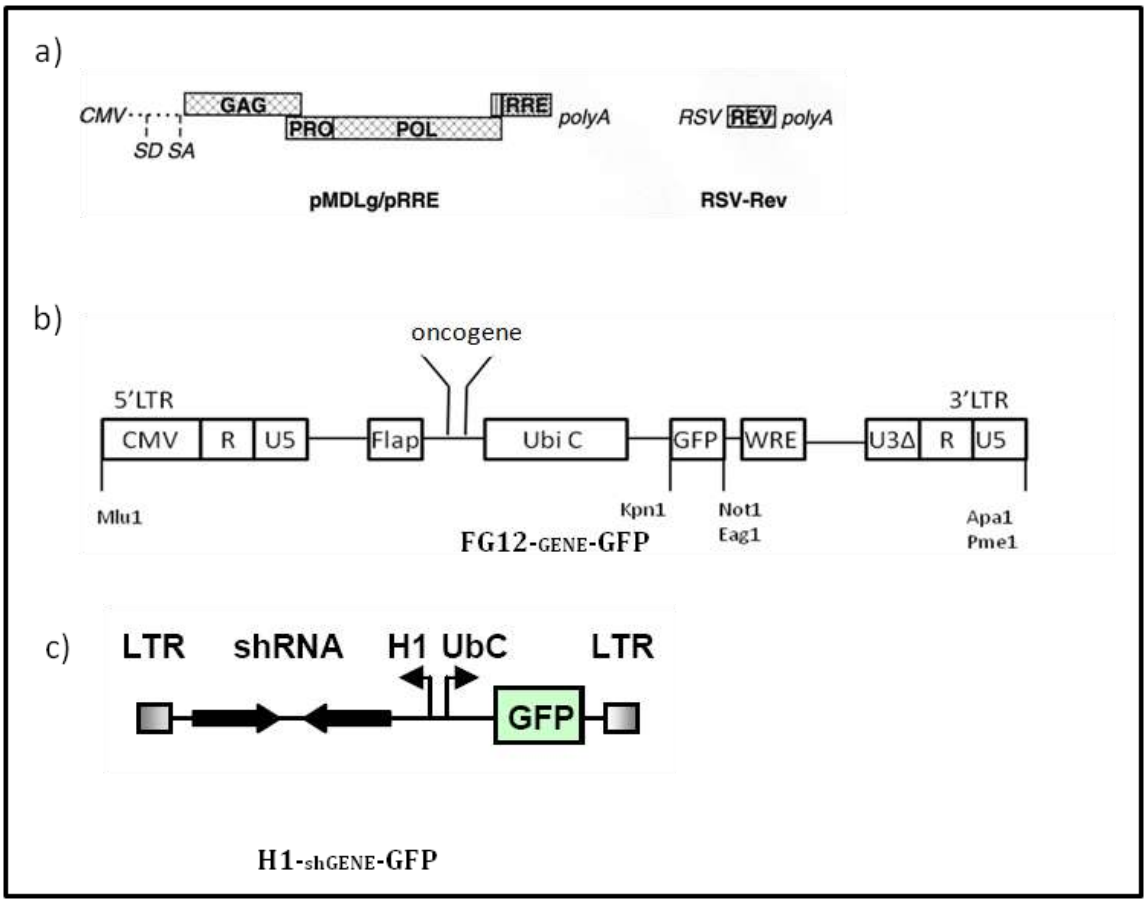


Figure 3.1. Vectors of Lentiviral Transduction System. a) pMDLg and pRSV-rev plasmids. (Adapted from Dull et al., 1998) b) FG12 vector construct. c) H1 vector construct (Adapted from Nikiforov et al., 2007)

3.3.1. Preparation of Chemically Competent Bacteria

A single colony of DH5α strain of Escherichia coli (E. coli) cells was inoculated into 10 ml LB medium (10 g/l tryptone, five g/l yeast extract and five g/l NaCl) and the LB culture was incubated overnight at 37 ^{o}C with moderate shaking. One ml of this culture was inoculated into 100 ml LB medium and the culture was grown until the OD_{595} value reaches 0.4, corresponding to the early- to mid-log growth phase. The cells were aliquoted into 50 ml pre-chilled Falcon tubes and kept on ice for 10 minutes, centrifuged at 1600 x g for 7 minutes at 4 ^{o}C. The supernatant was discarded, the bacterial pellet was dissolved in 10 ml ice-cold calcium chloride solution (60 mM $CaCl_2$, 15 per cent Glycerol, filter-sterilized using a 0.22μm filter). The cells were centrifuged again at 1100 x g for 5 minutes at 4 ^{o}C and subsequent to resuspend the pellet in 10 ml ice-cold calcium chloride

solution; the cells were incubated on ice for 30 minutes. Cells were centrifuged for a third time at 1100 x g for 5 minutes at 4 ^{o}C and the bacterial pellets were resuspended in two ml ice-cold calcium chloride solution. From these chemically competent cells 300 μl aliquots were prepared into pre-chilled 1.5 ml Eppendorf tubes. The cells were transferred to -70 ^{o}C freezer immediately.

3.3.2. Heat-shock Transformation of Bacteria

The vectors FG12-cMYC-GFP, FG12-hRAS-GFP, H1-p53i-GFP, H1-BAXi-GFP, pRSV, pHCMV-G and pMDlg/pRRE were transformed separately into chemically competent DH5α cells.

For each plasmid vector transformation, one μl of plasmid vector (500 ng/μl) was added into 100 μl competent bacteria which were thawed for 10 minutes on ice. Negative control tubes with no DNA were also prepared for each transformation experiment. The cell-plasmid mixtures were incubated for 30 minutes on ice. Next, the transformation mixture was heat-shocked in a 42 ^{o}C water bath for 45 seconds, followed by a three minutes incubation on ice. For the expression of the antibiotic resistance genes in the plasmids, one ml of SOC (0.5 per cent yeast extract, two per cent trypton, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) medium was added onto the transformation mixture and the transformant cells were incubated for one hour at 37 ^{o}C with shaking at 200 rpm without selection. To collect single colonies 50 μl, 100 μl and 200 μl of culture was spread on room temperature equilibrated LB agar plates (10 g/l tryptone, five g/l yeast extract, five g/l NaCl and 18 g/l agar) containing 100 μg/ml ampicillin and grown overnight at 37 ^{o}C.

3.3.3. Plasmid Isolation

Bacteria carrying the plasmids were spread onto selective LB agar plates containing 100 μg/ml ampicillin. From those plates single, well isolated colonies were inoculated into ten ml liquid LB medium to be grown overnight at 37 ^{o}C. Plasmids were isolated with the Endofree® Plasmid Maxi Kit or Spin Miniprep Kit (Qiagen, Germany) according to manufacturer's instructions. Plasmid DNA quantity and quality was determined via

spectroscopy by checking the absorbance values at 260 and 280 nm (NanoDrop^{TM} Thermo Scientific, USA).

3.3.4. Agarose Gel Electrophoresis

DNA samples were prepared for electrophoresis by mixing DNA samples in a 1:6 ratio with 6x Loading Dye (Fermentas, Germany) and the samples were loaded into one per cent agarose gel prepared with 1X Tris-Acetate-EDTA (TAE) Buffer (40mM Tris, 20mM acetic acid and 1 mM EDTA) and 0.5 μg/ml ethidium bromide. For gel-running times longer than one hour ethidium bromide was also added to the buffer (0.5 μg/ml). Gene Ruler 1kb (Fermentas, Germany) was used as a DNA ladder and the gel was electrophorised at 100V in 1X TAE. Bands were visualized with UV light and documented with GelDoc imaging system (BioRad,USA).

3.3.5. Preparation of KH1-ASCi-GFP Lentiviral Vector

A novel lentiviral vector for the stable expression of the shRNA against the ASC gene was designed to knock-down the ASC gene. Instead of the H1 vector backbone that was previously used in p53 and BAX, a KH1 vector (gift form Monique Verhaugen from Maria Soengas laboratory) was used, which has only one SmaI/XmaI restriction enzyme site. Two siRNAs, targeting the ASC mRNA (sequence of the transcript variant 2 was used), were designed according to the recommendations of Ambion (http://www.ambion.com/techlib/tb/tb_506.html). Two 21 bp long sequences were designed as the sense regions of the shRNAs and a scrambled sequence was designed as a negative control, having an additional HindIII restriction site. The shRNAs were designed to contain a XmaI restriction site; the sense region, targeting a complementary sequence in ASC mRNA; the loop, which is a critical sequence for the formation of the hairpin structure; the antisense region, the termination signal, TTTTT, and a the XbaI restriction site sequentially (Figure 3.2).

1) Position from start codon: 416
GC Content: 47.6%

Sense Strand1 (SS1):

XmaI RS:	5' CCGGG
sense region:	GATGCGGAAGCTCTTCAGTTT
loop:	CCACACC
antisense region:	AAACTGAAGAGCTTCCGCATC
terminator:	TTTTT
XbaI RS:	T 3'

5' CCGGGGATGCGGAAGCTCTTCAGTTTCCACACCAAACTGAAGAGCTTCCGCATCTTTTTT 3'

Antisense Strand (ASS1):

XmaI RS:	3' C
sense region :	CTACGCCTTCGAGAAGTCAAA
loop:	GGTGTGG
antisense region:	TTTGACTTCTCGAAGGCGTAG
terminator:	AAAAA
XbaI RS:	AGATC 5'

5' CTAGAAAAAAGATGCGGAAGCTCTTCAGTTTGGTGTGGAAACTGAAGAGCTTCCGCATCC 3'

2) Position from start codon: 66
GC Content: 47.62%

oligoASCi_SS2,CCGGGGTTCAAGCTGAAGCTGCTGTCCCACACCGACAGCAGCTTCAGCTTGAACT TTTTT

oligoASCi_ASS2,CTAGAAAAAAGTTCAAGCTGAAGCTGCTGTCGGTGTGGGACAGCAGCTTCAGC TTGAACC

3) Scrambled shRNA :

oligoASCi_scrS,CCGGGGTCATGGGTAAGCTTTATCGTCCACACCACGATAGAGCTTACCCATGACT TTTTT

oligoASCi_scrAS,CTAGAAAAAAGTCATGGGTAAGCTCTATCGTGGTGTGGACGATAAAGCTTACC CATGACC

Figure 3.2. shRNA constructs for ASCi. The three shRNAs for ASCi are shown, first two ASCi1 and ASCi2 are knock down shRNAs whereas the scrambled (ASCiscr) one will be used as the control. The positions from the start codon are given for the two shRNAs.

These six oligonucleotides were synthesized in the DNA Core Facility of Harvard University. The lyophylized DNA was dissolved in ddH_2O water to the stock concentration of one nmol/μl and working concentration of 100 pmol/μl. The sense and the antisense strands were initially denaturated by boiling the reaction mixture for five minutes in 1x T4 DNA ligase buffer supplemented with 0.2 M NaCl, and were left to cool down to anneal to room temperature. The now double stranded inserts were ligated into the KH1 vector, which was previously double digested digested with XmaI and XbaI (Fermentas, Germany) restriction enzymes according to the to manufacturer's instructions. In the

ligation reaction 750 ng KH1 digested vector, was ligated using T4 DNA ligase (Fermentas, Germany) with the inserts (1:10 vector insert ratio) at 16 ^{0}C over night in 10 μl final volume.

Half of the ligation mix was used directly in heat-shock transformation of chemically competent DH5α cells, cells were spread on ampicillin selective LB plates, are grown over night. 10 colonies for each insert were chosen randomly for sequencing to check if the inserts were cloned successfully. Firstly, the plasmids were isolated from those colonies using mini preps. Before sending to sequencing, restriction digestion analysis were used for the detection of the inserts. For inserts ASCi 1 and ASCi 2, ApaI and BamHI double digestions were used, normally this double digestion cuts an approximately 250 bp long piece from KH1, and if the insert is cloned successfully to this region, this piece should be observed as an approximately 310 bp band. For the scrambled ASCi insert, HindIII digestion was used, since an additional HindIII restriction site was introduced to the scrambled sequence during its design. Thus an additional band could be observed when the insert was cloned into the KH1 vector successfully. The digestion reactions were set according to the instructions of Fermentas. Mini-prep plasmids, that passed the digestion check, were sequenced by Iontek (Istanbul). The resulting chromatograms were evaluated using the Biology Workbench 3.2 program.

3.4. Primary Human Skin Cells

In the study three types of primary human cells were isolated and used. The Keratinocytes and Melanocytes were isolated from the epidermis and the fibroblasts were isolated from dermis as explained below.

3.4.1. Isolation of Primary Melanocytes and Keratinocytes from Human Skin

The tissue samples used for the isolation of melanocytes and keratinocytes were skin pieces excised during plastic surgery operations. The patients were generally middle aged women (age 40-65); and the donated pieces were form various parts of skin such as eyelids, scalp, skin from tummy and breast. The first step of the three day long protocol was separation of epidermis from dermis.

Skin pieces collected immediately after surgery were transported to the laboratory in physiological serum. Tissue pieces were washed first with sufficient amount of 95 per cent ethanol, then two times with cell culture grade PBS by immersing the tissue pieces into the solution, shaking vigorously and aspirating the used wash solutions. The cleaned tissue pieces were transferred into a clean Petri plate. The veins and adipose tissues were cleaned using sterile tweezers and scissors, as much as possible. After the yellow adipose tissue was completely cleaned, the dermis, exposed now, could be observed as a white layer below the removed adipose tissue. After the physical cleaning, the tissue was washed with PBS and transferred into a clean Petri plate. The skin sample was cut into pieces with the area of five mm to five mm. These smaller pieces were transferred to a 50 ml Falcon tube. Ten ml dispase solution (2.5U/ml Dispase grade 2, in 1M PBS) was added into the Falcon tube with the skin pieces and the pieces were incubated in the solution overnight at room temperature. On the next morning, the dispase solution was carefully aspirated and the pieces were transferred to a clean Petri plate. The pieces were positioned so that the white dermal layer of the skin was at the bottom and the epidermis layer faced the top. Using a sterile scalpel the epidermis is dragged gently over the dermis layer without touching any part of the dermis to prevent fibroblast contamination. The dermis pieces were saved for fibroblasts isolation. The epidermis pieces were put onto a clean plate, in one ml trypsin solution (0.25 per cent trypsin in PBS) and with the help of a sterile scalpel the epidermis pieces were minced until all the pieces were approximately smaller than one mm^2. The minced pieces were collected into a 15 ml Falcon tube and ten ml of trypsin solution was added onto these pieces. The trypsin solution with the epidermal pieces was incubated at 37 ^oC for 30 minutes. To break the cells apart, the solution was pipetted up and down several times. Five ml of FBS was added to stop the activity of trypsin, the cells were centrifuged at 2000 rpm for five minutes and the supernatant was aspirated. For melanocyte isolation, the cell pellet was dissolved in five ml MGM^+ (Medium 154CF supplemented with 0.2 mM $CaCl_2$, 50 U/ml penicillin, 50 µl/ml streptomycin and HMGS) and the cells were plated into a 25 cm^2 cell culture flask. For keratinocyte isolation, the cell pellet was dissolved in five ml KGM^+ (Medium 254CF supplemented with 0.1 mM $CaCl_2$, 50 U/ml penicillin, 50 µl/ml streptomycin and HKGS) and the cells were plated into a 25 cm^2 cell culture flask. The cells were kept in a humidified CO_2 incubator at 37 ^oC. After one or two weeks (the cells were attached completely) the cell media were changed and cell culture media were changed once a week or as needed.

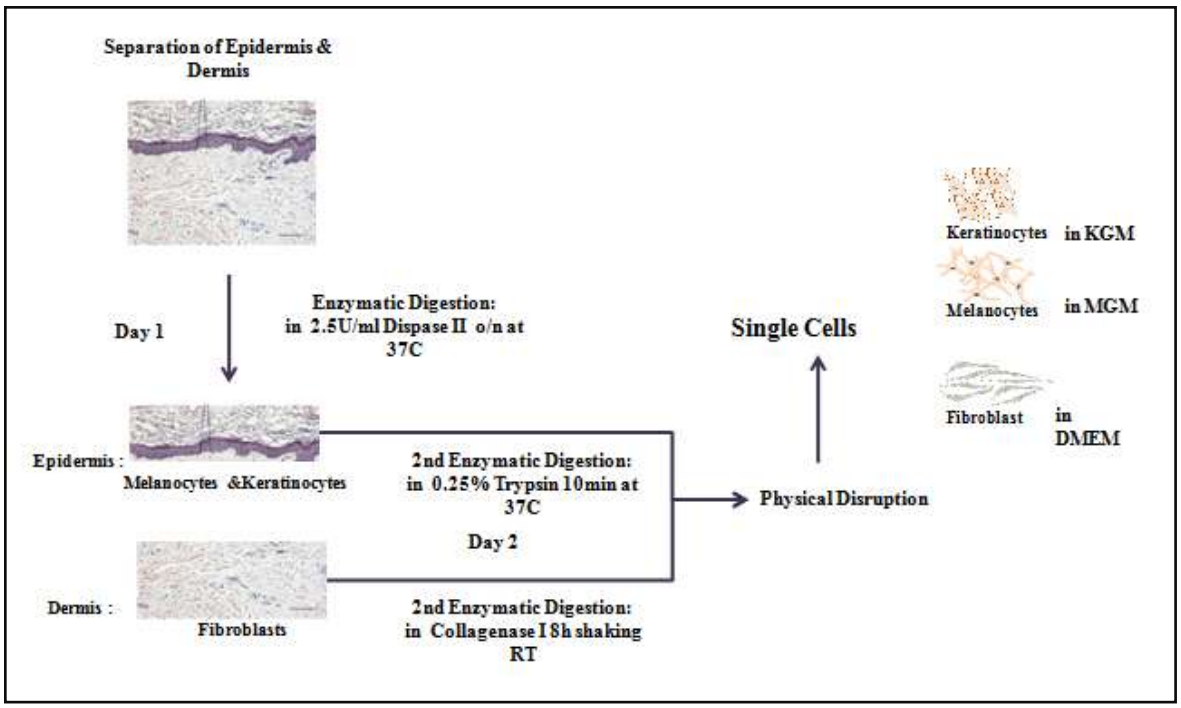


Figure 3.3. Isolation of primary skin cells. After enzymatic separation of epidermis from dermis, the dermal fibroblasts from dermis and the melanocytes as well as the keratinocytes form epidermis were reduced to single cell state by additional enzymatic digestions and physical disruption. The single cells were seeded in their specific growth media.

3.4.2. Isolation of Primary Fibroblasts from Human Skin

The saved dermis pieces (see above) were put in a 15ml Flacon tube with eight ml collagenase solution (Collagenase Type I one mg /ml, 50 U/ml penicillin, 50 µl/ml streptomycin , 25mg/ml Fungizone in sterile PBS). The collagenase solution with dermis pieces was placed on a shaker for an eight-hour incubation at room temperature with vigorous shaking. After the incubation, one ml FBS was added to the collagenase solution and the cells were centrifuged at 2000 rpm for five minutes. The supernatant was aspirated and the cell pellet was resuspended in ten ml of $DMEM^{+}$ and incubated at $37^{0}C$ under 5 per cent CO_2.

3.4.3. Unwanted Cell Purging

In case any fibroblast contamination was detected in the melanocyte or keratinocyte cultures, the fibroblasts were purged using their trypsin response differences. After the primary skin cells were attached and reached 80 to 95 per cent confluency, the flasks were checked for fibroblast contamination. The cultures with fibroblast contamination were washed with PBS and than one ml of Melanocyte trypsin (0.025 per cent trypsin-EDTA in 1X PBS) was added. The detachment of the cells was carefully observed under the light microscope; the first cell type to round up and detach form the flask were the melanocytes, followed by the fibroblasts and the keratinocytes. Four ml of MGM^+ was added to the flask when the melanocytes lifted completely and just before the fibroblasts started to lift their arms. The media with melanocytes were collected and centrifuged at 1200 rpm for five minutes. The supernatant was aspirated and the melanocyte pellet was dissolved in five ml MGM^+ and seeded into a new cell culture flask, the passage number was increased by one. In the keratinocyte culture contaminated with fibroblasts the same steps were followed but in the trypsinization step the cells detaching first were the fibroblasts. After the fibroblasts lifted, the flask was washed with PBS and five ml of KGM^+ was added to the fibroblast-free keratinocyte culture.

3.5. Lentiviral Transduction

The oncogenes and shRNAs of the tumor suppressor genes were infected into primary human fibroblasts using the two-step Lentiviral transduction system. In step one, virus production was achieved by a host packaging cell line. In the second step the viruses generated were used to infect the primary skin cells.

3.5.1. Calcium Phosphate Transfection of HEK 293T Packaging Cell Line

One day before the transfection $6x10^6$ Human embryonic kidney 293T (HEK 293T) viral packaging cells were plated into a ten cm^2 cell culture dish for each sample. On the second day, the medium of the cells was replenished with nine ml $DMEM^+$ containing 25 µM chloroquine. The plates were put into the incubator until the plasmid/calcium phosphate colloids were prepared. To prepare the plasmid/ calcium phosphate colloids

firstly a calcium chloride solution was prepared containing 250 mM calcium chloride, four µg of helper plasmids pHCMV-G, pRSV rev, pMDLg, and four µg of plasmid with the gene of interest (one of FG12-cMYC-GFP, FG12-hRAS-GFP, H1-p53i-GFP, H1-BAXi-GFP) in a final volume of 500 µl. This plasmid containing calcium chloride solution was added onto 500 µl 2X HEPES Buffered Saline (HBS) solution (280 mM NaCl, 10 mM KCl, 1.5 mM Na2HPO4 • $2H_2O$, 12 mM dextrose (D-glucose) and 50 mM HEPES with a pH adjusted to seven with 0.5 N NaOH (filter sterilized with a 0.22 µm filter) slowly. The one ml micropipette was used to produce bubbles in the HEPES solution to increase the colloidal precipitation. The solution was incubated for five minutes at room temperature and added dropwise onto the HEK 293T cells. Cells were transferred into the CO_2 incubator, their medium was refreshed after eight hours. On the fourth day, the viral particles were ready for infection. From this point on, biosafety level two rules were followed strictly while handling anything that came into contact with virus particles.

3.5.2. Lentiviral Infection of Human Primary Skin Fibroblasts

On the third day of the lentiviral transduction procedure, $5x10^6$ primary fibroblasts were plated into ten cm^2 dishes for each infection condition. On the fourth day, the supernatants of the transfected HEK 293T cells were collected using a ten ml syringe. Onto the tip of the syringe, polybreen with the final concentration of four µg/ml had been added. The supernatants was filtered through a 0.45 µm filter and four ml of the filtered supernatant was added directly onto the fibroblasts after aspirating their medium, two ml of fresh medium was added later to the fibroblasts. Six ml $DMEM^+$ was added to the HEK 293T cells and both the infected fibroblasts and the virus producing HEK 293T cells were put back into the incubator. After four hours the supernatant of the HEK 293T cells were collected again and the fibroblasts were infected for a second time following the procedure. The fibroblasts were incubated for four additional hours and after a total of eight hours the media of the fibroblast plates were refreshed to remove the polybreen. GFP production could be monitored after 48 hours. For the fibroblasts infected with shRNA plasmids, the knock-down of the particular protein could be monitored after 72 to 96 hours.

Due to biosafety level 2 regulations, plastic and glassware were bleached (ten per cent bleach in water) and the trash was autoclaved before disposal. Bleach was also used to kill the packaging HEK 293T cells and they were discarded after additional UV exposure. The surfaces of the hood were cleaned with bleach solution and 80 per cent ethanol and exposed to UV for at least for 20 minutes. Disposable coats and double gloves were used during cell culture.

For fibroblast transformation experiments infections were done in two conditions, either the infections with viruses carrying two genes were done simultaneously or sequentially. In the case of simultaneous infection, five ml supernatant collected form HEK 293 T cells transfected with oncogenic plasmids and five ml supernatant collected form HEK 293 T cells transfected with tumor suppressor knock-down plasmids were added. In the case of sequential infections the original infection procedure was followed but the transduction of the oncogene was done after two weeks from the tumor suppressor shDNA transduction. The infections are summarized in table 3.2., as the new names given to the derivative fibroblasts are also shown.

Table 3.2. Summary of primary fibroblast infections

Plasmid*	Transduced genes	Simultaneous	Sequential	Name of derivative Fibroblasts
H1-p53i-GFP FG12-cMYC-GFP	shRNA of p53, cMYC GFP	-	+	sim Fibro-p53i-cMYC-GFP
H1-p53i-GFP FG12-cMYC-GFP	shRNA of p53, cMYC GFP	+	-	seq Fibro-p53i-cMYC-GFP
H1-p53i-GFP FG12-hRAS-GFP	shRNA of p53, hRAS GFP	-	+	sim Fibro-p53i- hRAS-GFP
H1-p53i-GFP FG12-hRAS-GFP	shRNA of p53, hRAS GFP	+	-	seq Fibro-p53i- hRAS-GFP
H1-BAXi-GFP	shRNA of BAX	-	-	Fibro-BAXi-GFP
FG12- GFP	GFP	-	-	Fibro-GFP

*Helper plasmids were omitted.

3.6. SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting

The medium of the cells to be lysed were aspirated and four ml of cold 1X PBS was added. The cells were scraped with a sterile cell scraper and pelleted down at 1500 g for 5 min. 150 µl of 4X Laemmli Sample Buffer (250 mM Tris-HCl (pH 6.8), 20 per cent 2-mercaptoethanol, eight per cent SDS, 40 per cent glycerol and 0.1 per cent bromophenol blue) was added onto 10^6 cells and the harvested cells were collected in 1.5 ml Eppendorf tubes and kept at -20°C until further use.

The collected cell samples were boiled at 95°C for five minutes prior to electrophoresis and they were loaded into a 15 per cent polyacrylamide gel electrophorised at 50V initially, when the samples had reached the resolving gel and the voltage was increased to 100V.

A semi-dry transfer apparatus (BioRad,USA) was used for the transfer of proteins to the PVDF membrane (Millipore, Ireland). Thick blotting papers (Sigma-Aldrich, USA) and the membrane was cut to the size of the gel. The PVDF membrane was wetted in absolute methanol for 30 seconds and put into ice cold transfer buffer (0.293 per cent (w/V) glycine, 0.582 per cent (w/V) Tris-base, 25 per cent Methanol (pH 9.0)) together with blotting papers and the acrylamide gel. The transfer sandwich was prepared according to the manufacturer's instructions and the transfer was done at (area of polyacrylamide gel x five) mA for 45 minutes.

The resulting membrane put into the blocking solution (five per cent fat-free milk powder in 0.9 per cent (w/V) sodium chloride, 10 per cent 1M Tris-HCl (pH 7.5), one per cent Tween (TBST) and incubated for one hour with mild shaking. The membrane was transferred to a sealed bag with five ml of primary antibody solution (primary antibody diluted according to manual in five per cent bovine serum albumin (BSA), 0.02 per cent sodium azide in TBST) and incubated overnight at 4 ^{o}C. All of the primary antibodies used were purchased from Cell Signaling (Pierce, USA), the dilutions were made according to the manufacturer's instructions. On the next day, the membrane was washed in TBST for 1x2', 3x15' and incubated with the 1:10000 diluted secondary antibody (Goat anti rabbit, Sigma-Aldrich, USA) conjugated to horse radish peroxidase (HRP) in five per cent BSA in

TBST for one hour. The membrane is washed as previously. Lumi-light Western Blotting Substrate (Roche, Germany) was applied onto the membrane for five minutes and the bands were visualized using the Bio Imaging system Stella (Raytest, Germany).

3.7. Soft Agar Colony Formation Assay

The soft agar colony formation assays was performed as described with minor modifications (Clark et al, 1995). One per cent noble agar stock solution was prepared using autoclaved and filter-sterilized double distilled water as solvent. The stock solution was boiled to dissolved agar and put immediately into a 45 ^{0}C water bath before it got solidified. Fibroblasts with the concentration of $2x10^{4}$ cells/ ml were plated in 0.33% (w/v) noble agar supplemented with DMEM^{+} on top a 0.5 per cent noble agar bottom layer in six well plates. Cells were fed weekly by the addition of 400 µl DMEM^{+}. After three weeks, the colonies were counted under the inverted microscope. Uninfected normal primary fibroblasts were used as positive controls.

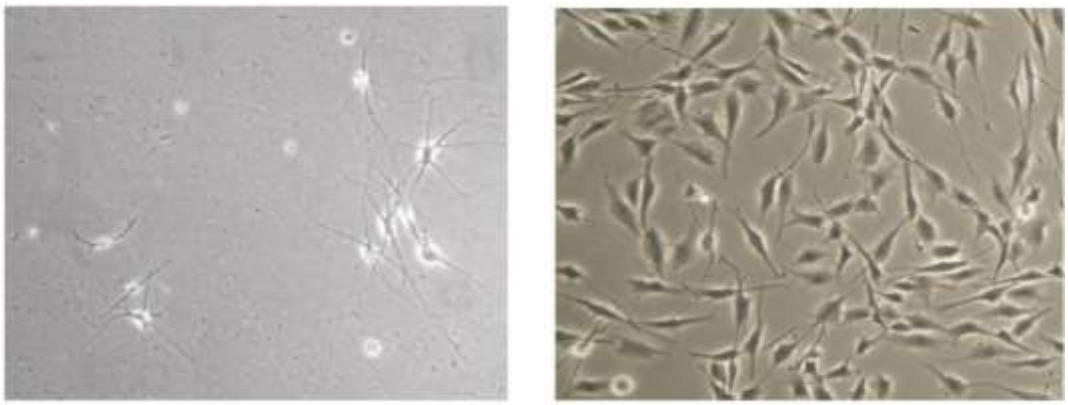
4. RESULTS

4.1. Isolation of Primary Skin Cells

We were able to successfully isolate three types of skin cells namely the melanocytes (three times) and the keratinocytes (one time) form the epidermis and the fibroblasts (three times) from the dermis. The skin samples were pieces excised during plastic surgeries. The patients were females with an age range of 40-65. The quality of the pieces varied depending on the age and condition of the patient. Another factor affecting the success of the isolation was the original location of the skin piece. It was easier to work with smaller samples such as eyelids, due to the thin the hypoderm beneath the dermis layer. On the contrary the physical cleaning step for tummy or breast pieces was time consuming. The long cleaning steps might have affected the quality of the isolated cells, because these cells were put in stress conditions. They were not in their original tissue or in their optimized medium for relatively longer period. Scalp pieces were similar to eyelids with respect to the working conditions. Due to the presence of hairs, extra sterilization steps, as well as physical cleaning steps were necessary to avoid any contamination.

4.1.1. Establishment of Primary Melanocyte Cultures

Isolation of melanocytes took the greatest effort, because of their low numbers and slow proliferation rates. Complete separation of the epidermis form the dermis was crucial for melanocytes isolation. In the case of a partial digestion, melanocytes residing on the boundary DEJ cannot be recovered fully and the isolation efficiency drops dramatically. Thus trypsin, which was initially used for enzymatic separation of dermis from epidermis, was replaced with dispase, and complete separation of the skin layer was achieved. The isolated melanocytes attached to the base of the tissue flasks in one to two weeks, after this point they grew initially slowly then fast in contrast with the fibroblasts and keratinocytes. The estimated doubling time of the melanocytes was 72 hours; later the melanocytes were passaged every 10 days. The melanocytes could be cultivated up to passage seven, and stocks were stored at -70 ^{0}C. Upon one they were freeze-thaw only 20 per cent survived.



Melanocytes (M2), passage 0 Melanocytes (M9), passage 4

Figure 4.1. Isolated Primary Human Melanocytes. The melanocytes at their 10th day of isolation corresponding to passage 0 (M2) and the melanocytes at their 50th day of isolation corresponding to passage 4 (M9) are shown (M2: patient two -age 60, M9:patient nine –age 42). Images were taken by an inverted microscope at 200X magnification.

The melanocytes in Figure 4.1. came from two different individuals. The dendritic structure of the melanocytes can be observed. Besides their minor structural differences, such as number of dendrites, their proliferation rates were also different. The relatively complex structure of M2 melanocytes indicates that these are close to their senescence stage. The M9 melanocytes were isolated from the skin sample of a relatively young patient, which might be the reason for the greater proliferation potential. The phenotypes of these melanocytes looked normal compared to the samples provided in literature as well as to the primary melanocytes isolated from baby foreskin during my two weeks training in the Laboratory of Prof. Meenhard Herlyn (Wistar Institute, Philadelphia), who is one of the pioneers in melanoma research. We were able to purify and stock primary human melanocytes from skin samples of patients successfully.

4.1.2. Establishment of Primary Keratinocyte Cultures

Similar to as melanocytes, keratinocytes also came from the epidermis and because of their high number and growth potential; the isolation of keratinocytes was comparatively easier. Keratinocytes grow faster than the melanocytes. The estimated doubling time of the keratinocytes was 24 to 36 hours. The keratinocyte cultures were passaged every week. Keratinocytes could be grown up to eight passages and stored at this

point for later use. Once they were freeze-thawed even 50 per cent survival was observed, these recovered keratinocytes acquired morphology similar to senescent cells after a while. Even though the morphologies of freshly isolated keratinocytes looked healthy, after freeze-thaw the initial healthy morphology was lost. Suggesting using unthawed melanocytes and keratinocytes in future studies would be more reliable.

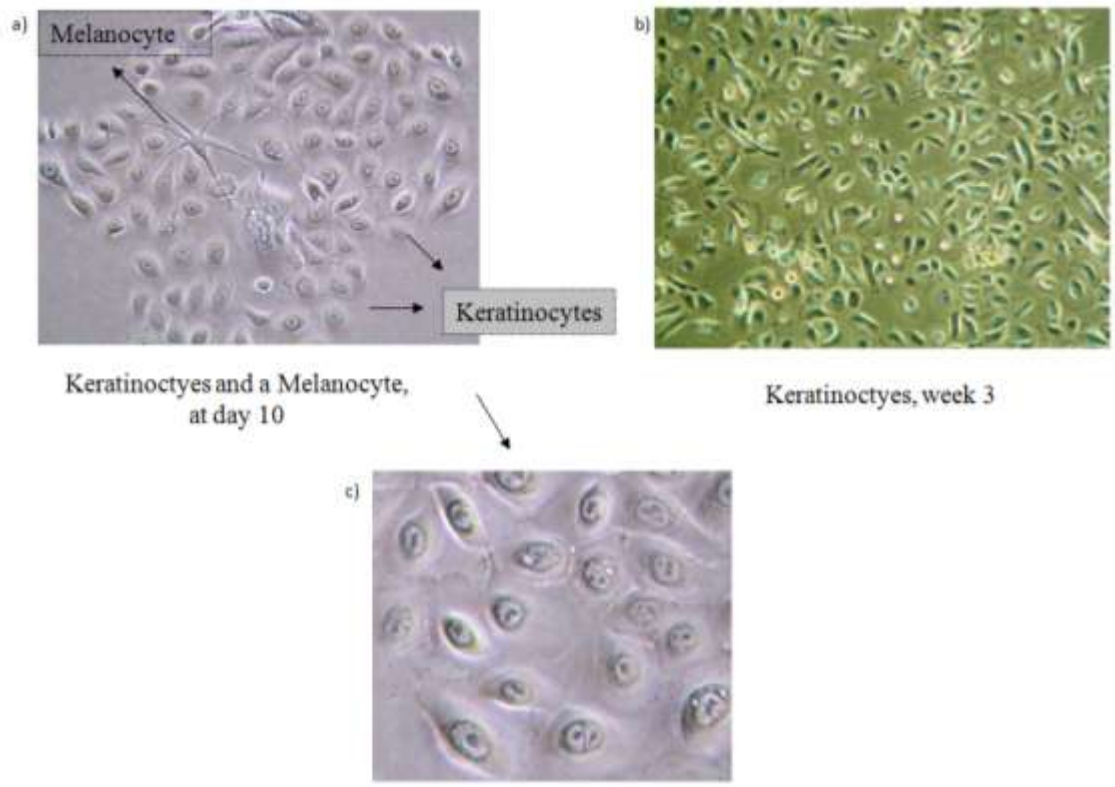


Figure 4.2. Isolated Primary Human Keratinocytes. Keratinocyte cultures can be contaminated by melanocytes shortly after their isolation, but eventually keratinocytes takes over any contaminant melanocytes. Images a) and b) were taken by an inverted microscope at 100X magnification, c) was magnified from a) to 400X.

Sometimes keratinocyte cultures were contaminated by melanocytes (Figure 4.2.), but once keratinocyte confluency reached 85 to 90 per cent any melanocyte was eliminated from the culture using purging based on different sensitivity to trypsinization (see section 3.4.3.), leaving a pure keratinocyte culture.

We were able to successfully purify and stock primary human keratinocytes from skin samples of patients.

4.1.3. Establishment of Primary Fibroblast Cultures

Primary human fibroblasts were successfully isolated from the dermis without any problem in each try. Dermis is normally crowded with fibroblasts, so contamination with other dermal cells did not take place during the isolation. These cells had great proliferation potential compared to the epidermal cells. The estimated doubling time of the fibroblasts was 24 hours. Fibroblasts could be grown to passage number 14. The fibroblast recovered fast after freeze-thaw in contrast to melanocytes and keratinocytes.

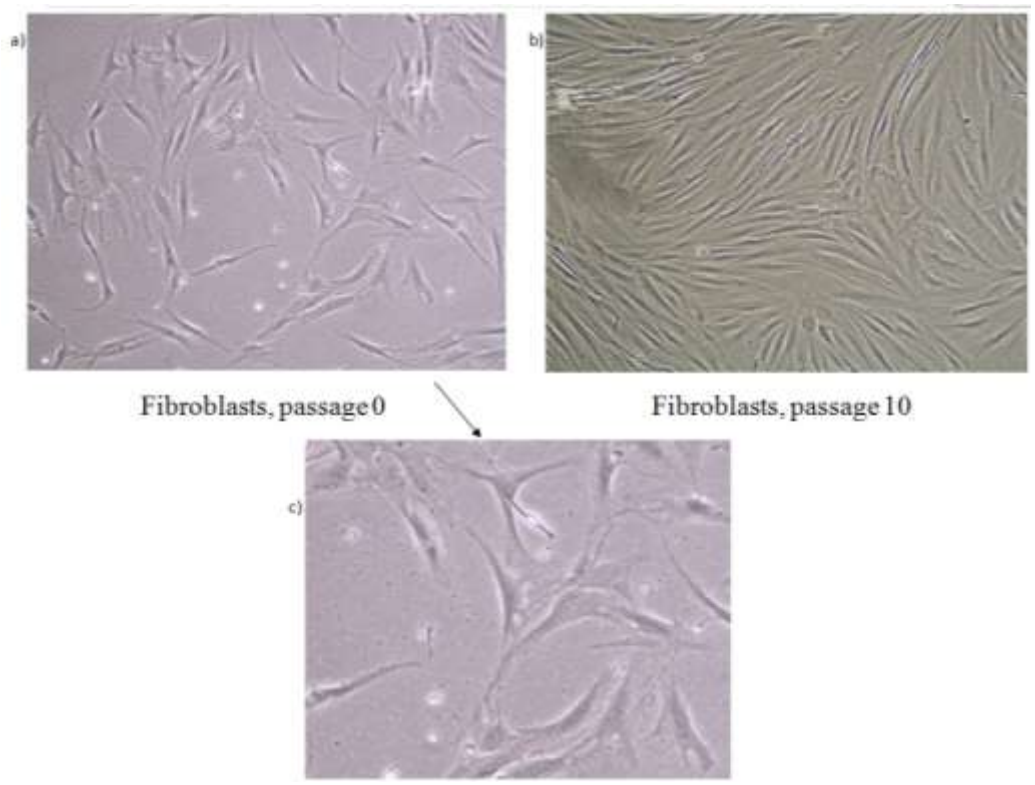


Figure 4.3. Isolated Primary Human Fibroblasts. Images a) and b) were taken by an inverted microscope at 100X magnification, c) was magnified from a) to 400X.

After switching the enzyme used for epidermis-dermis separation from trypsin to dispase, which also digests collagen, fibroblast contamination could be observed in the melanocyte cultures. Still the fibroblast contamination problem could be solved by a mild trypsinization step after the melanocytes were attached firmly to the tissue culture flask.

We were able to purify, culture and stock primary human fibroblasts from skin samples of patients. The isolated fibroblasts were used in the later experiments of this study.

4.2. Optimization of Lentiviral Transduction

Healthy, proliferating primary human fibroblasts were used before passage five for the lentiviral transduction optimization steps. This gene transfer system offers a high efficiency stable gene transduction into mammalian cells. Moreover, this four plasmid system ensures safety. Using this system cDNA copies of oncogenic copies of c-Myc or Ras are incorporated into the fibroblast genomes. Randomly, tumor suppressor genes such as p53 and BAX could be knock-down with this method. The derivative primary cells were used in the soft agar colony formation assays, assessing the anchorage-independent growth potential of those derivatives, which is one indicative of the cellular transformation. Our vectors carry GFP under a separate promoter. This allows observation of cells every day.

4.2.1. Knock-down of Bax and p53 via shRNA

A lentiviral transduction system was employed to knock down two essential tumor suppressor genes, p53 and Bax, in primary human fibroblasts, separately. This system provides stable expression of siRNAs targeting the mRNAs of these tumor suppressors for degradation. Fibroblasts isolated from two different individual were used for the production of BAXi and p53i derivative fibroblasts. Derivative tumor suppressor knock-down fibroblasts were produced by lentiviral transduction and are shown in the Figure 4.4. Stable GFP expression was first observed at day 5 post infection. The efficiency of the transduction was calculated by dividing the number of GFP expressing cell to the total cell number. The efficiency was significantly high, over 90 per cent (Figure 4.4.a) for every infection. The GFP positive fibroblasts were visualized using a florescent inverted microscope with a B-2A blue excitation filter. The indicated lentiviral plasmids used in each case and their infection efficiencies are given in the Figure. The GFP production indicated the successful incorporation of the genes into the fibroblasts genomes and the knock-down by shRNA was verified by

Western blot analysis (Figure 4.4.b). The lysates for Western blot analysis were collected three weeks after the infections. mRNAs of the tumor suppressors are effectively degraded and their translation stopped as the production of their corresponding protein products. As seen in the Figure 4.4.b. p53 protein expression was almost completely knocked down in the first fibroblast derivative, Fibro1-p53i, which was used in the further transformation experiments. Similarly, production of Bax protein was also significantly knocked down as seen in the Fibro-Baxi cell lysates.

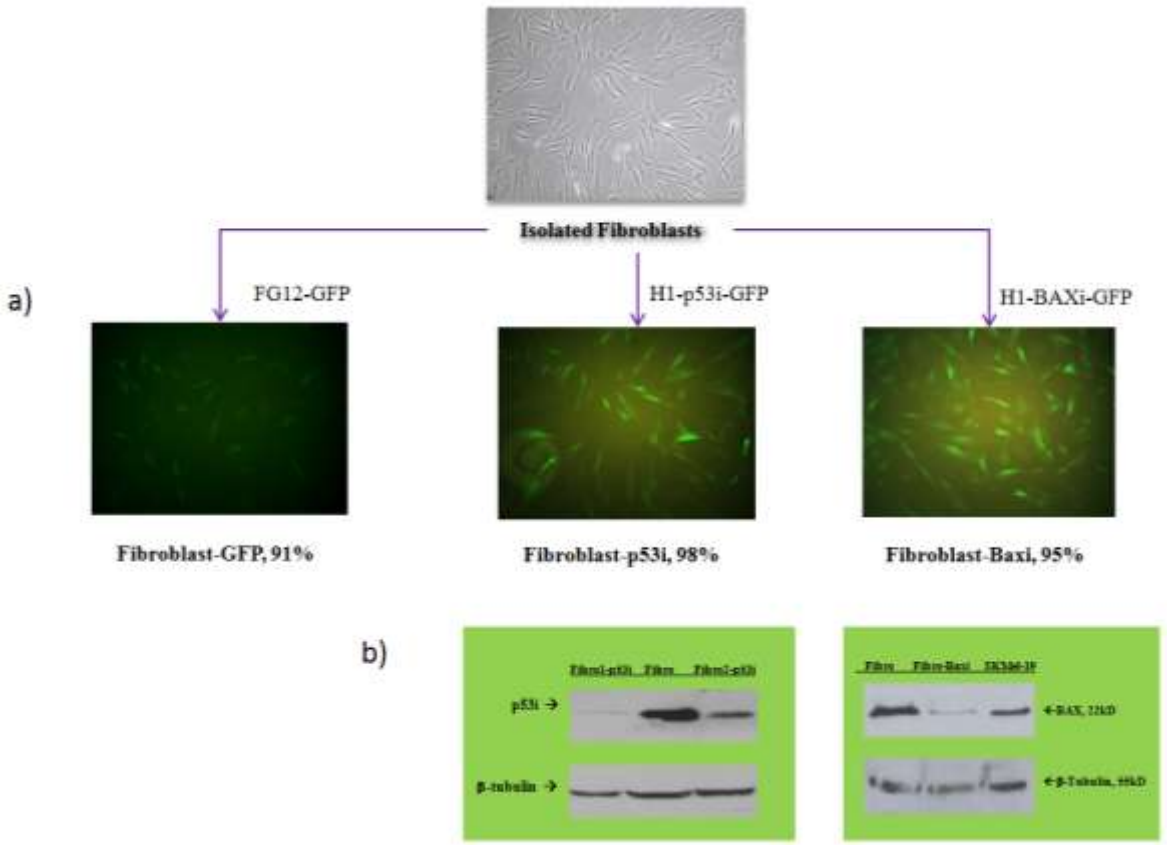


Figure 4.4. Knock-down of p53 and Bax tumor suppressor genes in primary human fibroblasts. a) The GFP positive fibroblasts at a magnification of 100X. b) p53 and Bax protein levels after knock down. Primary anti-p53 and anti-Bax rabbit and secondary goat anti-rabbit antibodies were used as detailed in Materials and Methods.

This data suggests that the lentiviral transduction system is a rather efficient system for stable expression of genes as well as knocking down genes with the help of shRNAs.

4.2.2. Transformation Assay of Primary Human Fibroblasts

We attempted to transform primary human fibroblast. It was previously shown for primary human fibroblasts that they can be transformed by inactivating both p53 and pRB tumor suppressor genes (pathways), expression of hTERT, and the additional Ras or c-Myc oncogenic activity (Boehm, 2005 and Zhao et al., 2004). As the first step of our studies, we started by knocking down a TS (p53) and adding an oncogene (c-Myc or h-Ras) (Figure 4.5.).

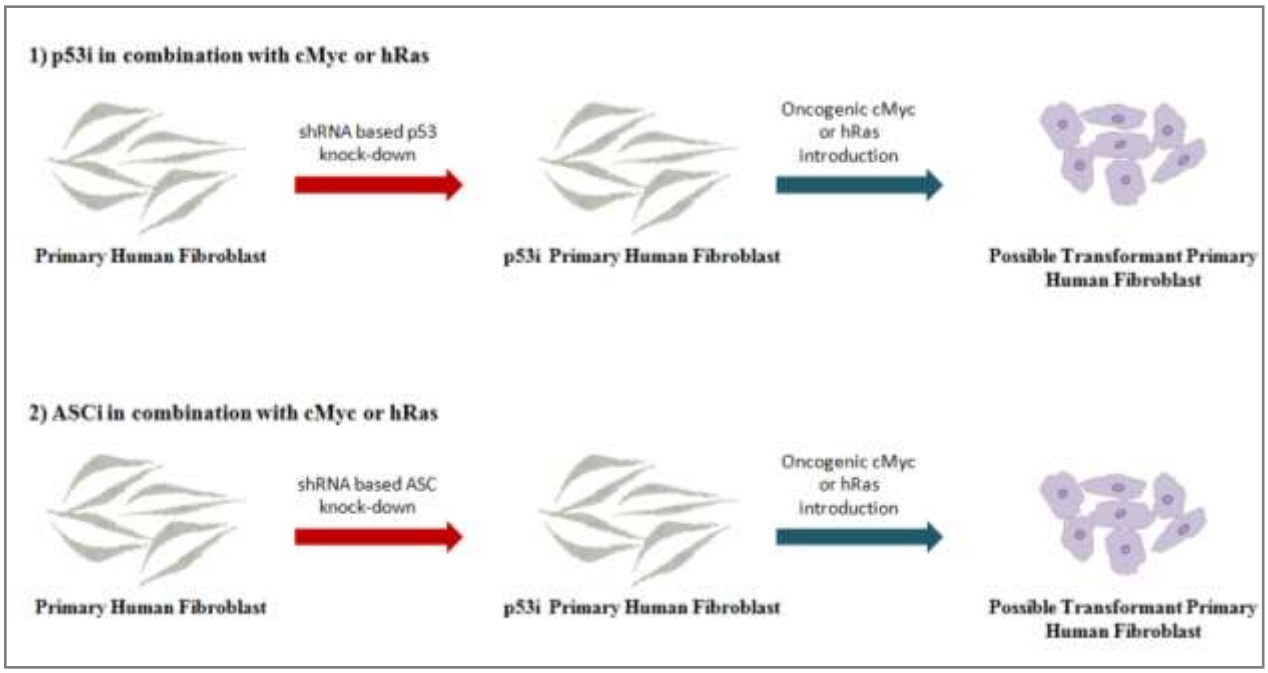


Figure 4.5. Transformation strategies for primary human fibroblasts. The first was used to determine transformation potential of p53 knock-down in combination with the oncogenic cMyc or hRas. In the second strategy, transformation ability of the newly generated anti-ASC shRNA vectors will be tested in combination with cMyc or hRas.

For stable expression of oncogenic c-Myc or oncogenic h-Ras (T24 h-Ras), the lentiviral transduction system was used. The ability to the transduced cells to stably express higher levels of c-Myc and h-Ras was analyzed by Western blotting and an increase in c-Myc and h-Ras protein levels were observed (Figure 4.6.). It has been shown that activation of Ras pathway induces c-Myc expression (Kerkhoff et al.,

1998), additionally Ras can also increase the stability of c-Myc leading to accumulation of c-Myc (Sears et al., 1999). In Fibro-Ras cells increased c-Myc level is a result of increased Ras activity. On the other hand, a positive feedback loop in the Ras pathway involving c-Myc in not known yet, thus the increase of Ras in Fibro-cMyc cells should be further investigated (Figure 4.6.).

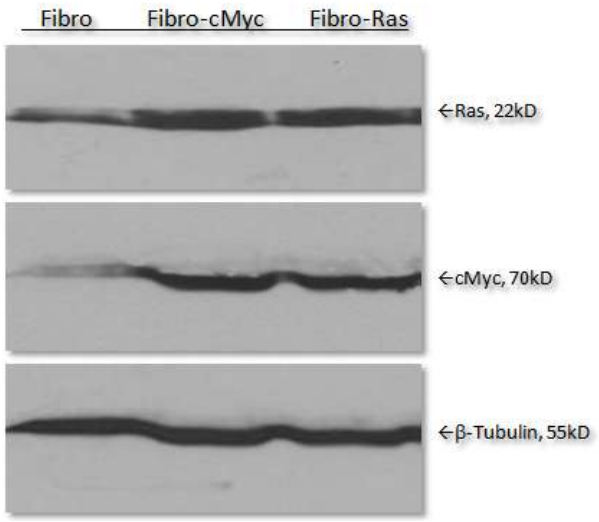
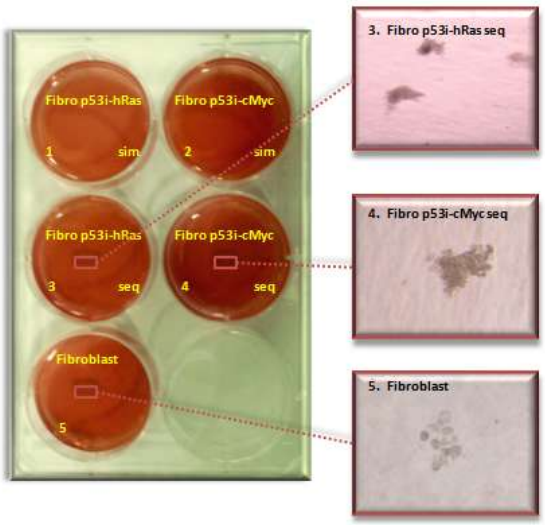


Figure 4.6. c-Myc and h-Ras expression in the derivative primary human fibroblasts. Primary anti-cMyc and anti-Ras rabbit antibodies and secondary goat anti-rabbit antibodies were used as detailed in Materials and Methods.

To determine if the order of p53 knock-down had any effect on the transformation potential, two infection strategies were applied. First, the previously produced Fibro1-p53i fibroblast were infected after two weeks with lentiviral vectors for either c-Myc or hRas and two derivative fibroblast lines were obtained, namely seq Fibro-p53i-cMYC-GFP and seq Fibro-p53i- hRAS-GFP, seq referring to the sequential manner of the infection. The second infection strategy was the simultaneous infection; here the lentiviruses carrying the p53 knock-down vector were introduced simultaneously with the lentiviruses carrying the oncogenic vectors, giving rise to sim Fibro-p53i-cMYC-GFP and sim Fibro-p53i- hRAS-GFP. These derivative fibroblasts did not show any significant differences under normal culture conditions. To determine the transformation potential of these derivative lines, their anchorage independent growth potential was examined in soft agar colony formation assay.

4.3. Anchorage-independent Growth in Soft Agar

The four derivative primary human fibroblasts were seeded into soft agar wells and colonies were left to grow for three weeks. At the end of this growth period, colonies containing more than 10 cells were counted under the inverted light microscope. The total colony numbers and size are given in Figure 4.7.



	Total Colonies	Average Colony Size
Fibro p53i-hRas sim	11	10
Fibro p53i-cMyc sim	12	10
Fibro p53i-hRas seq	**20**	**30**
Fibro p53i-cMyc seq	**162**	**50**
Fibroblast	10	10

Figure 4.7. Soft agar colony formation assay of primary human fibroblasts. a) Soft agar result at the end of three weeks for p53i+cMyc/hRas simultaneously (sim) or sequentially (seq) transduced Fibroblasts. b) The total number of colonies and the average colony size are shown in the table.

Significant colony growth in sequential infections were observed (Figure 4.7. table), in contrast to simultaneous infections. p53 knock-down fibroblasts gained the ability to grow in an attachment independent manner in soft agar. The simultaneously infected fibroblast derivatives did not show such a growth, suggesting a noteworthy role for the order of p53 knock down. Also, the observed growth in seq Fibro-p53i-cMYC-GFP colonies was major even compared to seq Fibro-p53i-hRas-GFP, implying the either the growth conditions were more suitable for the seq Fibro-p53i-cMYC-GFP derivative or introduction of c-Myc provide selective advantage at anchorage independent growth.

4.4. Preparation of Lentiviral Vector for Stable ASC shRNA Expression

As the next step of our studies were planned to test the ability of ASC knock down to cooperate with oncogenes. The highly efficient lentiviral transduction system can be used for production of stable derivative primary fibroblasts. ASC is a presumed tumor suppressor gene which has been shown to regulate apoptotic and immunologic roles in the cell (McConnell and Vertino, 2004).

The shDNA of ASC was cloned into a KH1-GFP lentiviral vector for further studies. Two targeting shRNAs and one control scrambled shRNA were designed and cloned into XbaI/XmaI double digested KH1-GFP plasmids. The shRNA inserts were successfully ligated into the double digested KH1 plasmid. The double band pattern in the first three wells in Figure 4.8. suggested that linearized KH1-GFP plasmids were recircularized due to insert ligation. The ligation reaction in ligation control is incomplete since most of the KH1 plasmid was observed still to be in linear form.

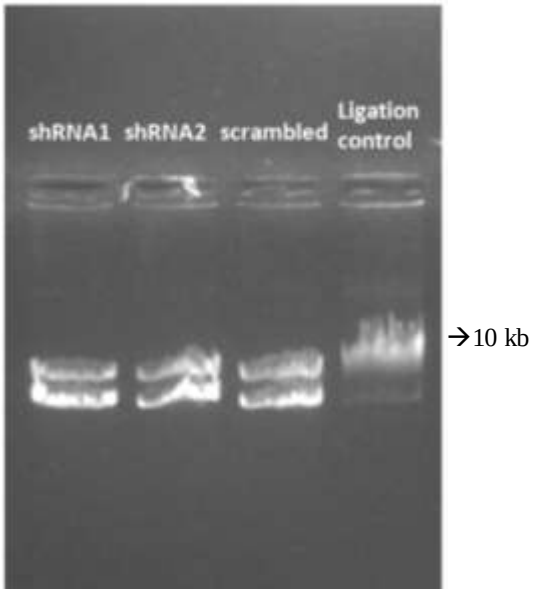


Figure 4.8. Successful ligation of shRNA inserts into XbaI/XmaI double digested KH1 vector. Ligation products were analyzed via Agarose gel electrophoresis. Recirculized plasmids show both linear and circular conformations, whereas the ligation control has only the linear form.

The plasmids isolated from the transformed bacterial colonies, were checked for the presence of inserts by restriction digestions. An extra HindIII site was introduced into the scrambled shRNA, thus the restriction profile of a KH1 plasmid with an insert gave an extra band at approximately 250bp. Selected plasmids were send to sequencing for confirmation of the exact sequences (Figure 4.9.).

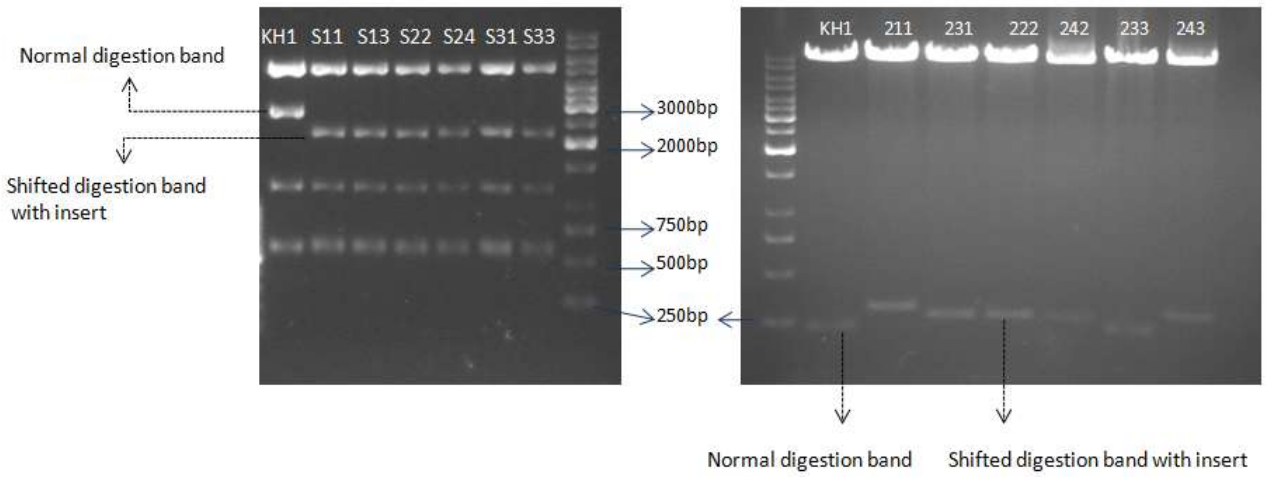


Figure 4.9. Restriction digestion control of derivative KH1 plasmids. Mini-prep plasmid, KH1-GFP-ASCi scrambled samples S11, S13, S22, S24, S31, S33 were digested with HindIII and the KH1-GFP-ASCi shRNA2 samples 211, 231, 222, 242, 233, 243 were double digested with ApaI /BamHI. The presence of shifted digestion bands indicates successful insertion.

For the actual ASC shRNAs, double digestion was employed, the two restriction enzymes, ApaI and BamHI, cut the KH1 plasmid just up- and downstream of the location that the insert was cloned, so the 250bp long restriction fragments shift to approximately 310 bp fragment when an insert is cloned. Among 10 ASCi 1, and 9 ASCi 2 plasmids send to sequencing only 1 positive insert sequence was detected, which was KH1-GFP-ASCi_222 (Figure 4.10.). The chromatograms were exported to Workbench in FASTA format and the sequences were compared to the sense strands of the designed shDNAs using BLAST. In ASCi 1 plasmids 2 cloned inserts with mutations, transition and deletions, were detected. In most of the sequences, the chromatogram was disrupted at the insert entry site, suggesting a possible problem during the sequencing reaction.

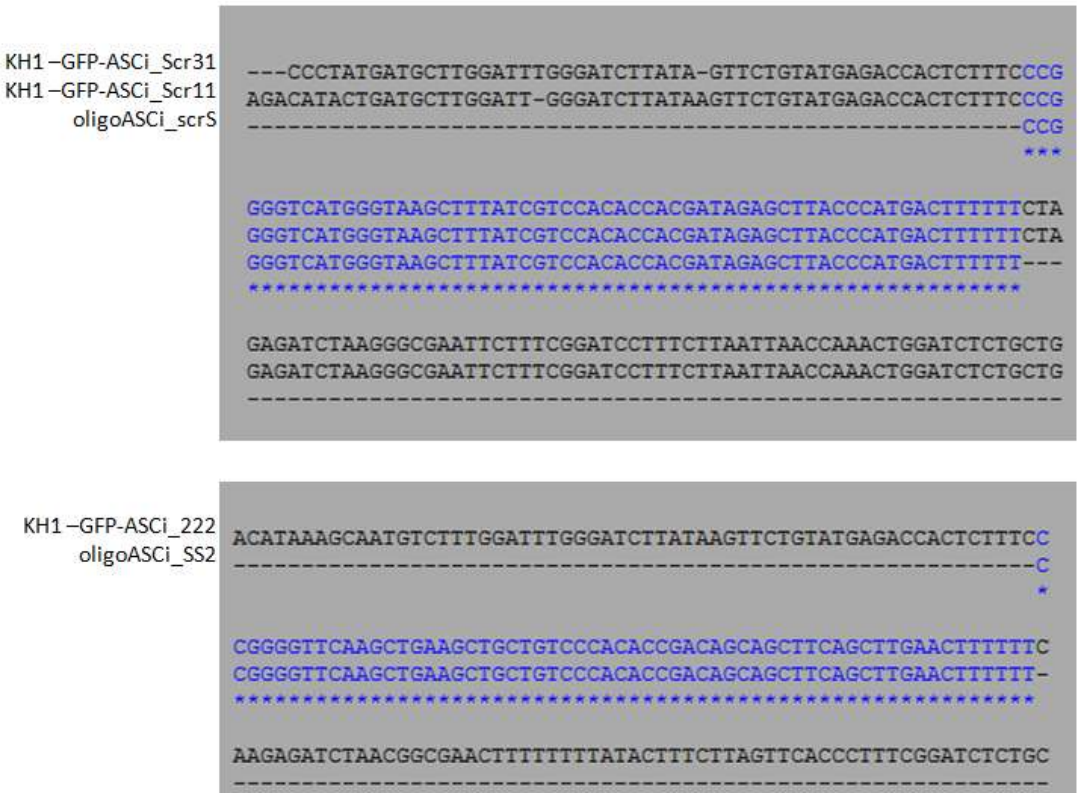


Figure 4.10. Sequence data for cloned KH1-GFP-ASCi plasmids. 3 positive results for 2 scrambled plasmids, KH1 –GFP-ASCi_Scr31 and KH1 –GFP-ASCi_Scr11, and one knock-down plasmid, KH1 –GFP-ASCi_222, was detected from sequencing data.

5. DISCUSSION

Primary human melanocytes, keratinocytes and fibroblast were successfully isolated from human skin pieces. The quality of the skin was one of the most important determinants for the purification efficiency. Skin samples from younger patients gave better results, most possibly due to their higher proliferation potential, assuming they have more time until they reach the senescence stage. We could successfully passage melanocytes to seven, keratinocytes to eight and fibroblasts more than 10 times. We attribute the shorter survival period of our cells to the fact that they did not come from baby foreskin samples which are known to possess greater plasticity and proliferation capacity.

Efficient separation of the dermis from the epidermis was crucial for melanocyte isolation, because these cells reside originally on the border between these two layers. So, the use of dispase instead of trypsin gave better results for melanocyte isolation but the fibroblast contamination possibility increased as well as a side effect.

The isolated primary human fibroblasts were then successfully manipulated using the established lentiviral transduction methodology. The efficiency of incorporation of the control GFP carrying vectors was calculated to be above 90 per cent in at least three trials. Stable expression of the GFP could be followed microscopically for at least four passages. We tested the successful expression of transduced c-Myc and h-Ras oncogenes (Figure 4.6.) as well as the knock-down of the tumor suppressors, p53 and Bax (Figure 4.4.b.) proteins via Western blot analysis.

To assess the ability of p53 knock-down fibroblasts to become transformed after the addition of an oncogene, such as Ras or c-Myc, we transduced combinations of the lentiviral vectors. We were quite intrigued to observe the appearance of colonies in the soft agar transformation assay (Figure 4.7.). In these two different trials, we saw evidence for the transformation of the primary human fibroblasts. Based on the literature, human primary cells cannot be transformed just by p53 knock-down in combination with a single oncogene expression (Boehm, 2005 and Zhao et al., 2004). This strategy was successful in murine cells, presumably because they do not require telomerase activity. On the other

hand, just by looking at the soft agar colonies it is not possible to conclude that we have transformed these fibroblasts. We plan to extend these studies in immune compromised mouse. The ability of these modified fibroblasts (Fibro-p53i-cMYC-GFP and Fibro-p53i-hRAS-GFP) to form tumors or metastases in mice will be much more solid evidence.

We should also consider that the fibroblasts used in the transformation assays were isolated from older people. Thus, genetic alterations might be accumulated in their fibroblast facilitating growth in an anchorage-independent manner in soft agar. These experiments should be repeated using fibroblasts isolated from different individuals to conclude about the transformation capability of p53 knock-down plus c-Myc or h-Ras oncogene combination.

Recently, ASC was shown as a transcriptional target of p53 (Ohtsuka et al., 2004) and it was found to be silenced via promoter methylation in various kind of cancers (Parsons and Vertino, 2006). Thus, ASC can be regarded as a putative tumor suppressor gene. As the next step of our studies were planned to test the ability of ASC knock down to cooperate with oncogenes. A lentiviral vector targeting the mRNA of the proapoptotic ASC adaptor was designed and cloned successfully.

Even though the ligation of the shRNA-ASCi1 and shRNA-ASCi2 inserts were confirmed by double digestions in many mini-prep plasmid samples, only one plasmid with the correct insert was identified by DNA sequencing. After repeating the ligation and sequencing steps several times, we realized one particular drawback of working with DNA inserts capable of forming secondary structures such as hairpins. The formation of hairpin secondary structures during the sequencing reaction might block the DNA polymerase. This actually explains the disrupted chromatograms in which the sequence stops just at the insert entry site. The stringency of the sequencing reaction should be kept high for such DNA samples with problematic secondary structures.

Finally, with the verified anti-ASC shRNA vector in hand, it will be interesting to test the transformation potential of ASC knock-down in combination with c-Myc or h-Ras oncogene using primary human skin cells.

APPENDIX A: KH1 Plasmid Data

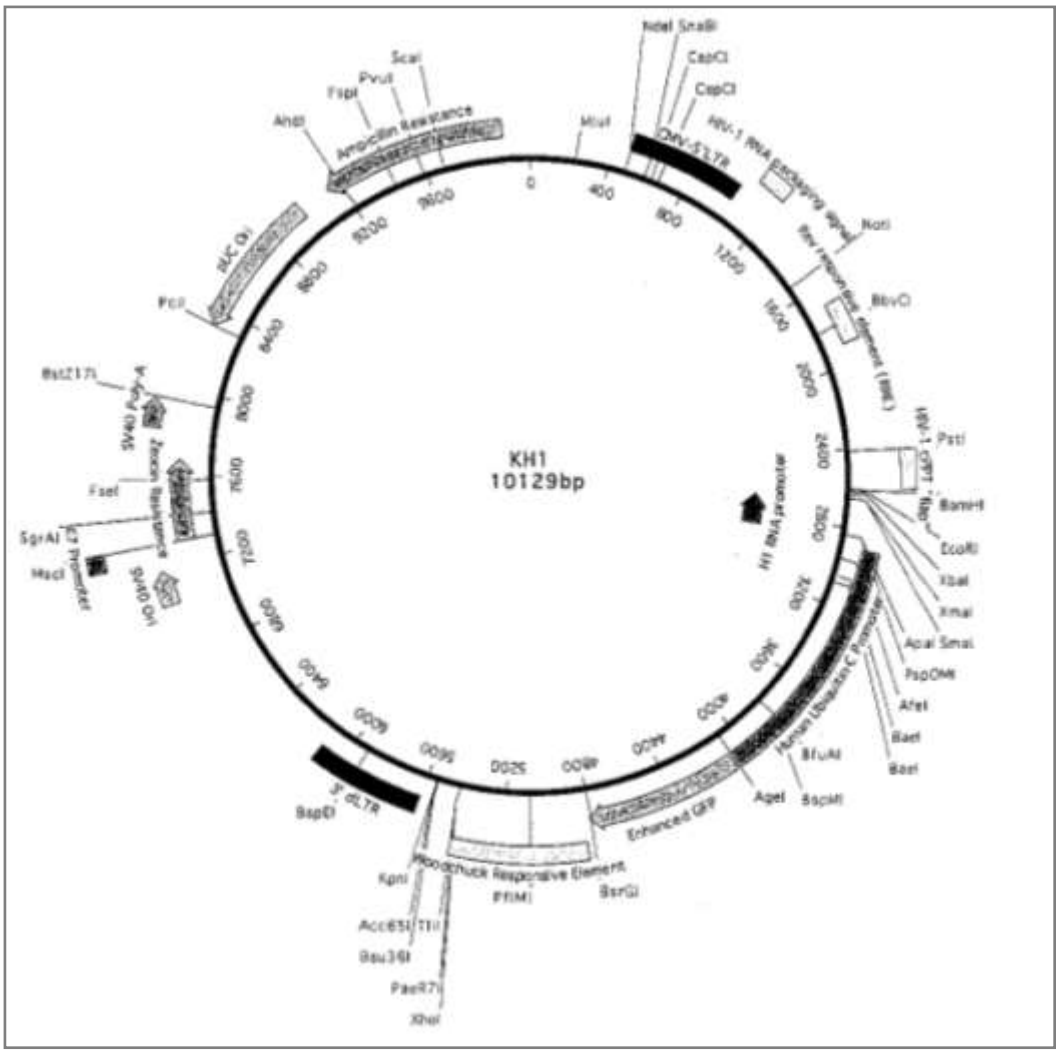


Figure A.1. KH1 plasmid.

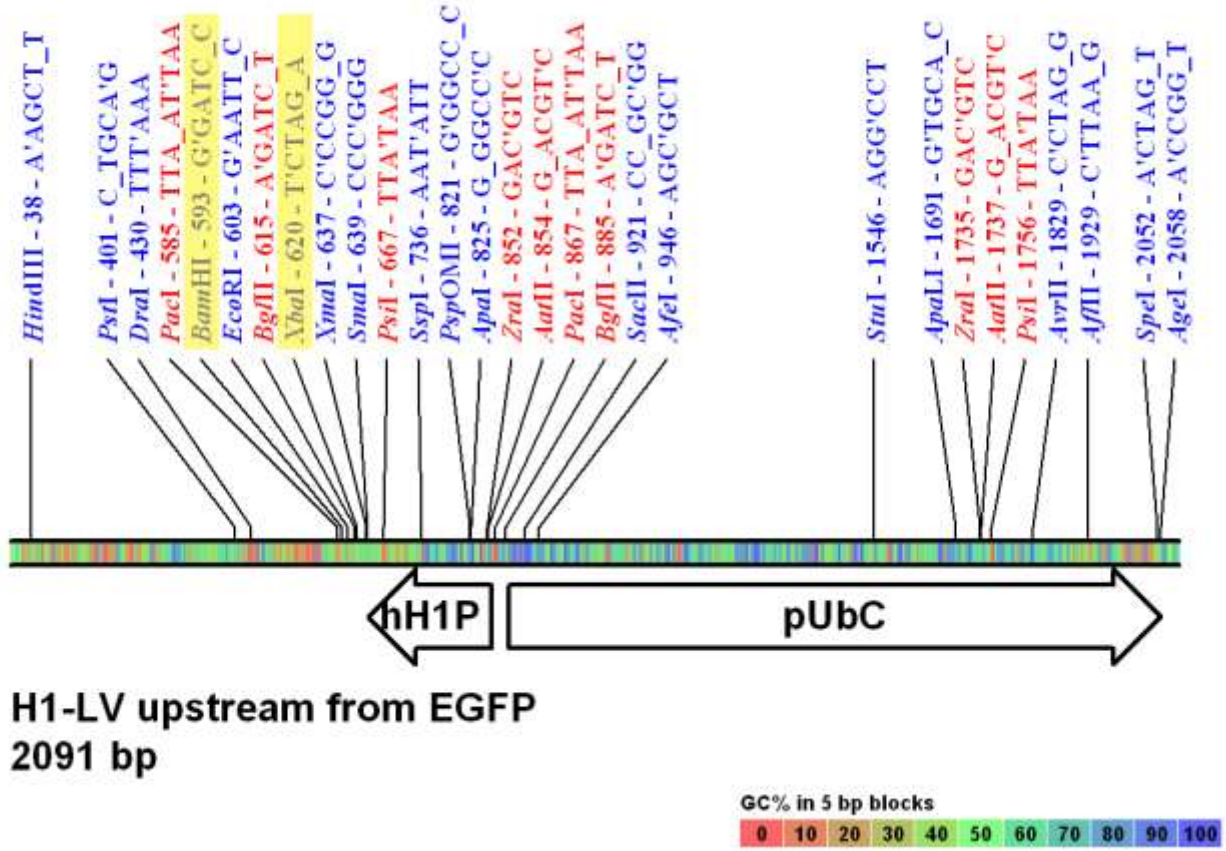


Figure A.2. Multiple Cloning site of H1 plasmid

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