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HRAS G12V transformation effect on MCF10A cells harboring different p53 variants

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Thesis Approval

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Dedication

To my mother and father...

To my brothers and sisters...

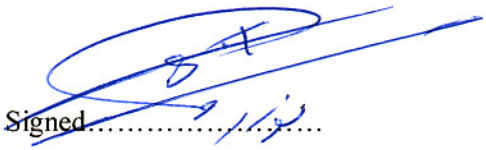
To my friends...

To all beloved people who supported, assisted and encouraged me.

Nooraldeen Yousef Mousa Tarade

Declaration

I certify that this thesis submitted for the degree of master is the result of my own research, except where otherwise acknowledges, and that this thesis (or any part of the same) has not been submitted for the higher degree to any other university or institute.



Signed.....

Nooraldeen Y. Tarade

Date: 30 /01 / 2019

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List of abbreviations

°C	Degree Celsius
Bax	Bacl-2 associated X protein
BCL-2	B-cell lymphoma 2
cDNA	Complementary DNA
cm	centimeter
CGS	Cancer related Gene Signature
COSMIC	Catalogue of somatic mutations in cancer
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EMT	Epithelial mesenchymal transition
EMC	Extracellular matrix
ER	Estrogen receptor
ERBB2	v-erb-b2 avian erythroblastic leukemia oncogene homolog 2
G	gram
H	hours
HER2	Human epidermal receptor 2
KO	Knockout
kDa	Kilodalton
L	liter
M	Molar
MDM2	Mouse double minute 2 homolog

mg	Milligram
min	Minute
mL	Milliliter
mRNA	Messenger RNA
Ng	Nanogram
nm	Nanometer
nM	Nanomolar
Nt	Nucleotide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
rpm	Round per minute
Rb	Retinoblastoma
RNA	Ribonucleic acid
SFM	Serum free media
Sec	Second
TCGA	The cancer genome atlas
TP53	Tumor protein 53
U	Units
Wt	Wilde-type
Wtres	Wild-type rescue
µL	Microliter
µM	Micromolar

Abstract

Breast cancer is the most frequent cancer type between women, which influences more than 2 million women every year. It represents 15% of total cancer deaths among women. Genomic alternations are the main cause of breast cancer transformation. The differences in gene expression patterns among breast cancer subtypes are likely to reflect basic differences in the cell biology of each type and that gives the apparent phenotypes. The imbalance between the activities of tumor suppressors and oncogenes is a common event that leads to cancer transformation. Our preliminary data shows that different p53 variants lead to differential gene expression. Thus we hypothesized that different gene alteration combinations would result in differential gene expression pattern and consequently to different phenotypes. We aimed to create different breast cancer transformation models by transforming the normal mammary epithelial cell lines, MCF10A cells, that contain different *TP53* gene variants with *HRAS G12V*. Afterwards, we tested the differences in distinct cellular phenotypes (cancer hallmarks) caused by this combination. The *HRAS* overexpression increased cell proliferation, migration, invasion and resistance to apoptosis. Moreover, *HRAS* overexpression in combination with different *TP53* mutations lead to different phenotypes and gene expression patterns. This study demonstrates a critical role of two hit system in induction of cancer transformation. Altogether, we successfully generated models for studying breast cancer transformation that might help in understanding the differential behavior of different breast cancer tumors, which could be used to improve breast cancer detection, diagnosis and treatment.

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Chapter 1

1. Introduction

1.1. Cancer transformation

Cancer is a genetic disease. Cancer cells are an out of controlled growth cells resulting from genetic mutations and epigenetic alternations, cellular signaling and hemostasis misregulation (Hanahan & Weinberg, 2011). Frequent pathological genetic changes could modify the actions of oncogenes and tumor suppressor genes. The oncogenes are group of genes that promote carcinogenesis. They result from abnormal activation of normal proto-oncogenes like *RAS* and *Her2* (Reuter, Morgan, & Bergmann, 2000; Spandidos, 1985). Tumor suppressor genes are a set of genes involved in inhibiting cell growth and cancer development like *TP53* and *Rb* (Imran *et al*., 2017). Both activation of oncogenes and inactivation of tumor suppressor genes, abrogates cell hemostasis and induces different mutations. The accumulation of mutations forces normal cell to transform into cancer cell in a multistep process called cancer transformation by altering various pathways till tumor formation takes place (Johnson, Warmoes, Shen, & Locasale, 2015; Northcott et al., 2014).

Different genetic alternations affect driver pathways during the multistep transformation process. Consequently, different cancer cell hallmarks result (Figure 1.1). These hallmarks include; continuous proliferation, resistance to apoptosis, evading tumor suppressors, immortality, enhanced angiogenesis, invasion and metastasis, inflammation, metabolism changes and immune evasion (Hanahan & Weinberg, 2011).

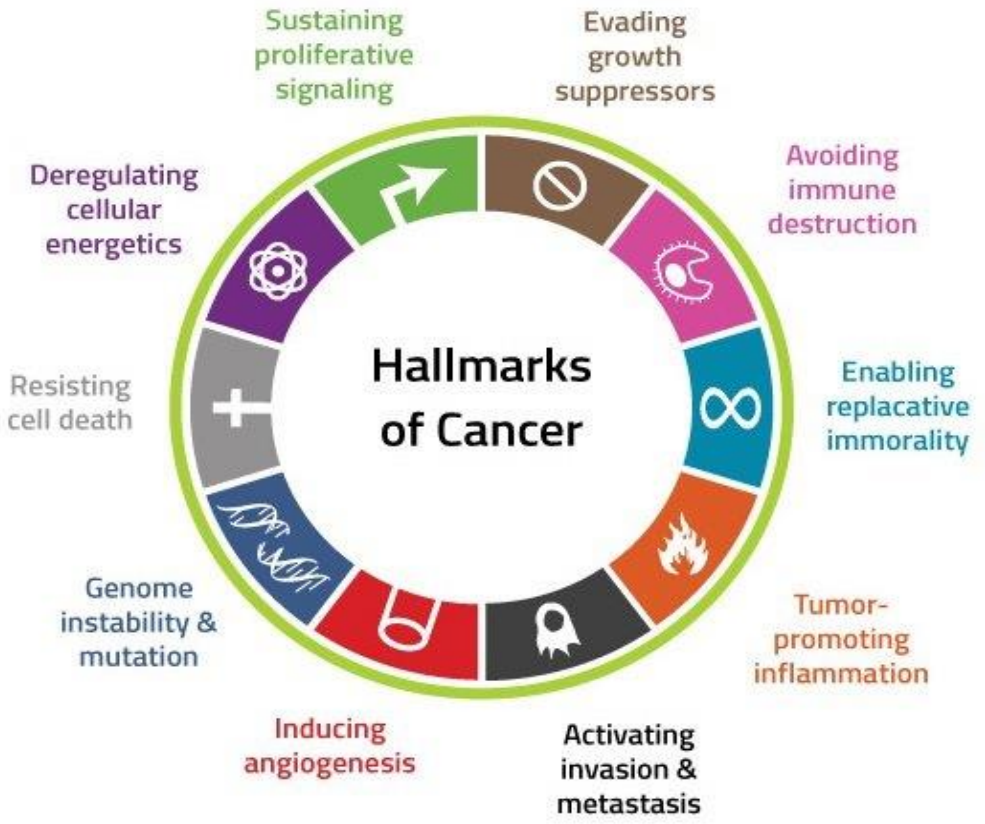


Figure 1.1: Cancer hallmarks. The hallmarks of cancer as described by Hanahan and Weinberg (modified from (Hanahan & Weinberg, 2011)).

1.2. Breast Cancer

Breast cancer is recognized as the most frequent cancer type among women, which influences more than 2 million women every year. It is the most cause of mortality among women worldwide. In 2018 627,000 women died from breast cancer worldwide, which makes up to nearly 15% of total cancer deaths among women (WHO, 2018). Breast cancer is a very heterogeneous cancer type and can be classified into different categorizes depending on different parameters. Based on clinical features, breast cancer is divided into 3 groups: triple negative breast cancer (TNBC), estrogen receptor positive (ER+), and ERBB2-enriched (Cancer Genome Atlas, 2012).

The majority of molecular findings of breast cancer studies have concentrated on one or two genetic profiles. Most common are mRNA expression or DNA copy number analysis, and

more recently enormously deep sequencing. Directed data of mRNA expression clustering has consistently established that breast cancer includes several different disease entities, often mentioned as the built-in subtypes of breast cancer. The current development of supplementary high information content assays concentrated on aberrations in microRNA expression, DNA methylation and protein expression(Ciriello *et al.*, 2015).Based on gene expression profiles, breast cancer is categorized into 4 main subgroups: luminal A, luminal B, HER2-enriched and basal-like (Wang, Liu, Ying, Lin, & Zhou, 2016).

The differences in gene expression patterns among breast cancer subtypes are likely to reflect basic differences in the cell biology of each type and that gives the apparent phenotypes (Sorlie et al., 2003).

1.3. The Oncogen-RAS

RAS family of proteins belongs to GTPases, which have the ability to switch between the inactive GDP-bound form and the active GTP-bound form. This conversion is controlled by guanine nucleotide exchange factors (GEFs)(Karnoub & Weinberg, 2008).

There are 3 isoforms of RAS including KRAS, HRAS and NRAS. They are very similar in their structure with 85% of amino acids sequence similarity and in their function (Downward, 2003). Active RAS proteins activate different signaling pathways that regulate different cellular functions, such as cellular growth, differentiation, proliferation and others (Figure 1.2) (Karnoub & Weinberg, 2008; Myers et al., 2016; Reuter et al., 2000). Furthermore, RAS pathway is from the highest frequent altered pathway in cancer (Maertens & Cichowski, 2014). It was established that 30 % of cancers harbor a somatic mutation in one of RAS superfamily (Prior, Lewis, & Mattos, 2012). These oncogenic mutated RAS proteins become unresponsive to GTPase-activating proteins (GAPs) and constitutively active. Furthermore, *RAS* mutations were found in high percentage in different type of cancer such as pancreas, lung, colorectal and others ranging from 30% to 90% (Downward, 2003).

HRAS mutations are common among cancer patients in salivary gland 15%, urinary tract 11%, cervical cancers 9% upper and aerodigestive tract 9%. According to the COSMIC database, mutations in *HRAS* is (~1%) in breast cancer (Myers et al., 2016). Nevertheless, low frequent measurement of RAS subfamily individuals in breast cancer is still of attention because they play a critical role in breast signaling pathways involving P13K

and MAPK pathways (Myers et al., 2016). The most common frequent *HRAS* somatic point mutations take place in codons 12, 13, 61 and 146 and lead to the constitutively active RAS (Fernandez-Medarde & Santos, 2011; Myers et al., 2016).*G12V* is a hotspot mutation arises from a single nucleotide replacement (c.35 G>T) that results in the exchange from Glycine (G) to Valine (V) and dominates in *HRAS* (57%) (Hobbs, Der, & Rossman, 2016). *HRAS G12V* is located at position 12 of the GTP nucleotide binding domain of HRAS protein. This mutation leads to the reduction of GTPase activity of HRAS protein, loss GAPs binding sensitivity and continuous HRAS activation. Altogether, these events induce cell transformation (Figueiredo, Stein, Jochem, & Sandgren, 2012; Hobbs et al., 2016).

Several studies have described mutant *HRAS G12V* ability and role in transforming normal cells to cancer cells *in vitro* and *in vivo.* A study has shown that the combination between *HRAS G12V* and *Ink4a/Arf*$^{-/-}$, a tumor suppressor locus, in mammary cells causes cancer transformation and 100% tumorigenesis when injected into syngeneic mice mammary fat pads. The resulting tumors were triple negative (Kai et al., 2014). Likewise, another *in vitro* study described that MCF10A cell lines harboring *HRAS* overexpression alone or with mutant *P13KA* or *TP53* wild-type shows high oncogenic features(Geyer et al., 2018).

In a further study, MCF10A cell lines with *HRAS G12V* and *LBX1* were transformed to cancer cells. They expressed mesenchymal markers and developed tumors when injected into nude mice (Yu et al., 2009). Moreover, MCF10A cell lines containing *HRAS G12V* co-expressed with *Bim-1* show high epithelial to mesenchymal transition ability and form tumor masses when injected into mammary fat pad mice(Datta et al., 2007).

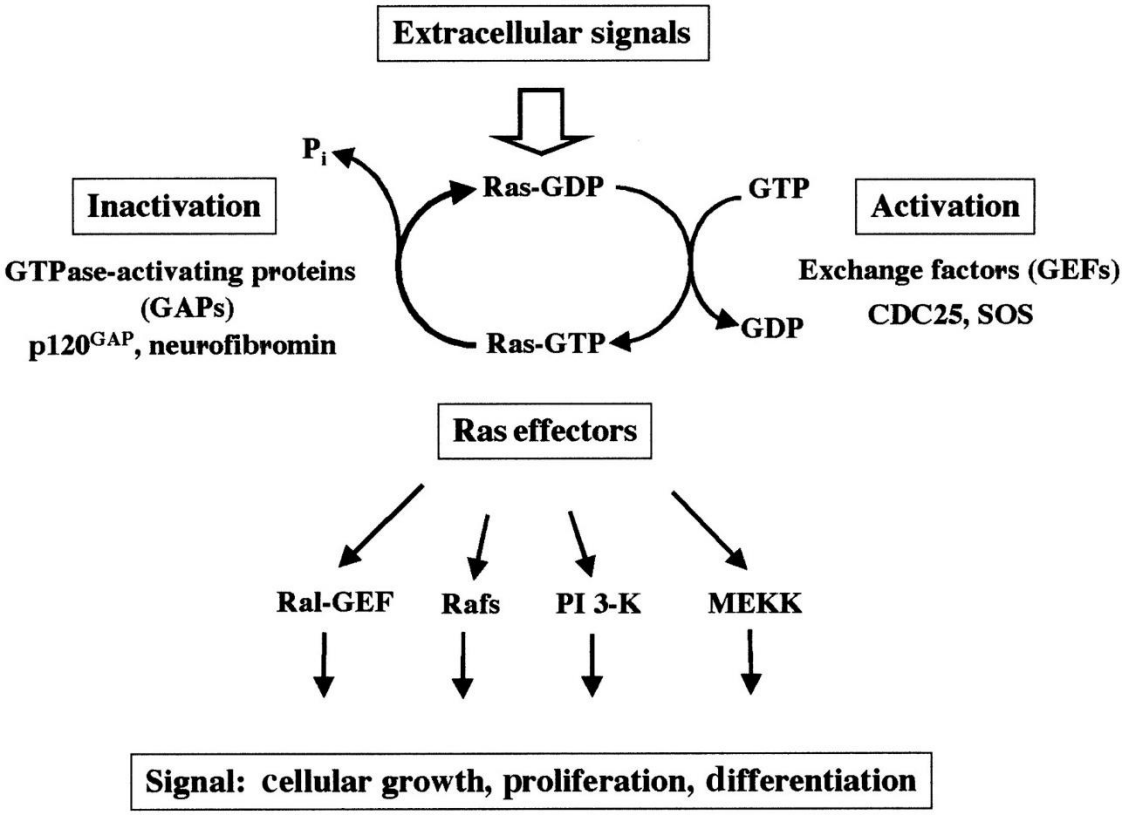


Figure 1.2: The activation (GTP-bound) and inactivation (GDP-bound) cycle of RAS protein in response to extracellular signals (Reuter et al., 2000).

1.4. The tumor suppresser- p53

TP53 gene is positioned on the short arm of 17p13.1 chromosome and encodes p53 protein (Matlashewski et al., 1984). p53 was named back to 1979 after its molecular weight which is 53 kailo dalton (kDa)(Ziemer, Mason, & Carlson, 1982).

p53 is recognized as a transcription factor and a multifunctional protein that regulates different cellular mechanisms including apoptosis, oncogenes expression deregulation, cell cycle and DNA repair (Ford, 2005; T. Li et al., 2012). p53 critical role in the cell comes from its function as a transcription factor that controls more than 100 genes in the cell by its ability to bind to p53 specific DNA-binding sequences (Ljungman, 2000). Moreover, p53 has been called the guardian of the genome(Lane, 1992) due to its role in maintaining the genome stability by avoiding mutations (Figure 1.3)(Levine & Oren, 2009; Strachan & Read, 1999).

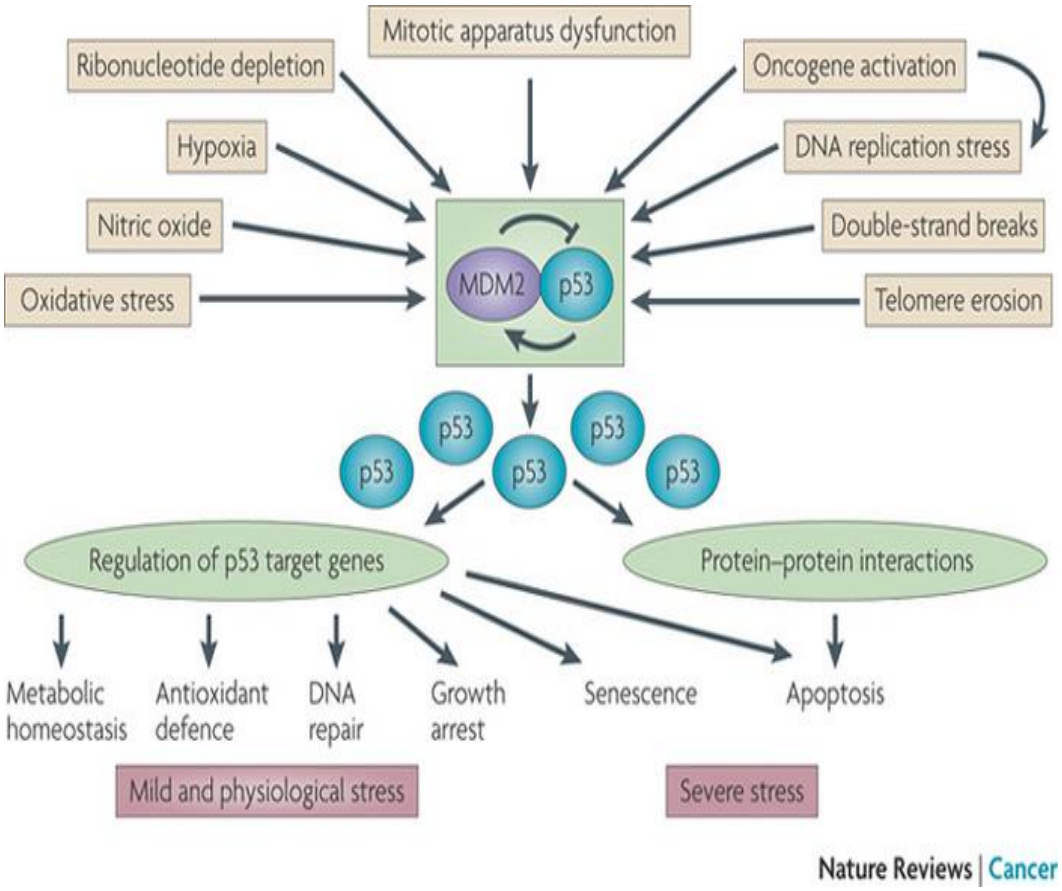


Figure 1.3: Scheme for p53 functions and different p53 activating signals. A verity of stress signals including oxidative stress, hypoxia, ribonucleotide depletion, mitotic apparatus dysfunction, oncogene activation, DNA replication stress, double-strand breaks and others lead to p53 liberation from MDM2 then to activate different p53 target genes or protein- protein interactions. Afterwards, cell response depends on stress severity (Levine & Oren, 2009).

Many of p53 target genes have been determined. Numerous of them encode proteins that are induced in cell cycle and apoptosis control. From the major p53 direct effectors that we study here are p21 and BAX. p21 is a cyclin-dependent kinase inhibitor, which when induced, promotes cell growth arrest or death and represents an important regulator for transition from G1 to S phase in the cell cycle(Tang et al., 1998). *Bax* is a proapoptotic gene. Both are transactivated directly by p53(Levine & Oren, 2009). In addition to its genomic function, p53 has non-transcriptional biochemical activities that are induced in part by interacting with other proteins in the cytoplasm and in the nucleus of the cell. The apoptosis-regulatory Bcl2 protein family is an example for cytoplasmic proteins that are inhibited by

p53 to increase the mitochondrial outer membrane permeabilization then to release of cytochrome C and apoptosis (Vaseva & Moll, 2009).

p53 protein level in the cell is tightly regulated by MDM2. p53-MDM2 interaction is from the most important protein- protein interaction in the cell (Levine & Oren, 2009). MDM2 is recognized as p53 gatekeeper because of its ability to inhibit p53 activity and keeping p53 at the basal level in normal cells in order to prevent apoptosis. That could be accomplished by different ways. First, by blocking p53 transactivation function, and second, by promoting the ubiquitylation and proteosomal degradation of p53, which is catalyzed by E3 ubiquitin ligase activity of MDM2 (Momand, Zambetti, Olson, George, & Levine, 1992; Oliner et al., 1993). Moreover, MDM2 gene expression is regulated by p53 in a negative feedback loop for p53 protein at the activity level and in a positive feedback loop for MDM2 at the transcription level (Wu, Bayle, Olson, & Levine, 1993).

A huge body of research has published that *TP53* gene is mutated or deleted in about half of all human cancer types and that *TP53* is the most studied gene in several cancer specimens (Espinosa & Emerson, 2001; Levine & Oren, 2009; Vogelstein, Lane, & Levine, 2000). Moreover, *TP53* mutation occurrence represents the most between all tumor suppressor genes and from the highly important drivers for carcinogenesis in different cancer types(Forbes et al., 2011; Myers et al., 2016). Of the frequent widespread tumors related to *TP53* mutations are breast, colon, lung, head and neck cancers (Petitjean et al., 2007). In breast cancer, *TP53* gene is mutated in nearly 34% of breast cancer patient's samples based on the cBioPortal database tool (Cerami et al., 2012; Gao et al., 2013; The cBioPortal for Cancer Genomics, 2016). Moreover, patients with breast cancer expected to have low survival rate when they have *TP53* mutations (Olivier et al., 2006). Noteworthy, *TP53* mutations can be inherited in the germlines and causes tumor formation in childhood such as in Li-Fraumeni syndrome. Which, also known as the sarcoma, breast, leukemia and adrenal gland (SBLA) syndrome (F. P. Li & Fraumeni, 1969).

The majority of *TP53* mutations in breast cancer are somatic mutations (Walerych, Napoli, Collavin, & Del Sal, 2012). These mutations are mostly missense substitutions (Figure 1.4), which include contact mutations that affect p53 DNA-binding ability with low influence on p53 conformation and structural mutations that intensively interrupt p53 conformation(D. P. Liu, Song, & Xu, 2010). There are different *TP53* hotspot mutations at different codon positions such as R175H/G, R248Q/W/G and R273H/C/G/L/P which all are in the p53

DNA-binding domain (Figure 1.5) (Cerami et al., 2012; Gao et al., 2013; The cBioPortal for Cancer Genomics, 2016).

TP53 mutations can lead either to loss of tumor suppression activity or gain of a new function that enhances cancer development (D. P. Liu et al., 2010) and causes different functional oncogenic phenotypes (Bullock, Henckel, & Fersht, 2000). Gain of oncogenic functions was identified in the very popular *TP53* contact mutations such as *R273H* and *R248W*, and structural mutations like *R175H* and *R248Q* (D. P. Liu et al., 2010).

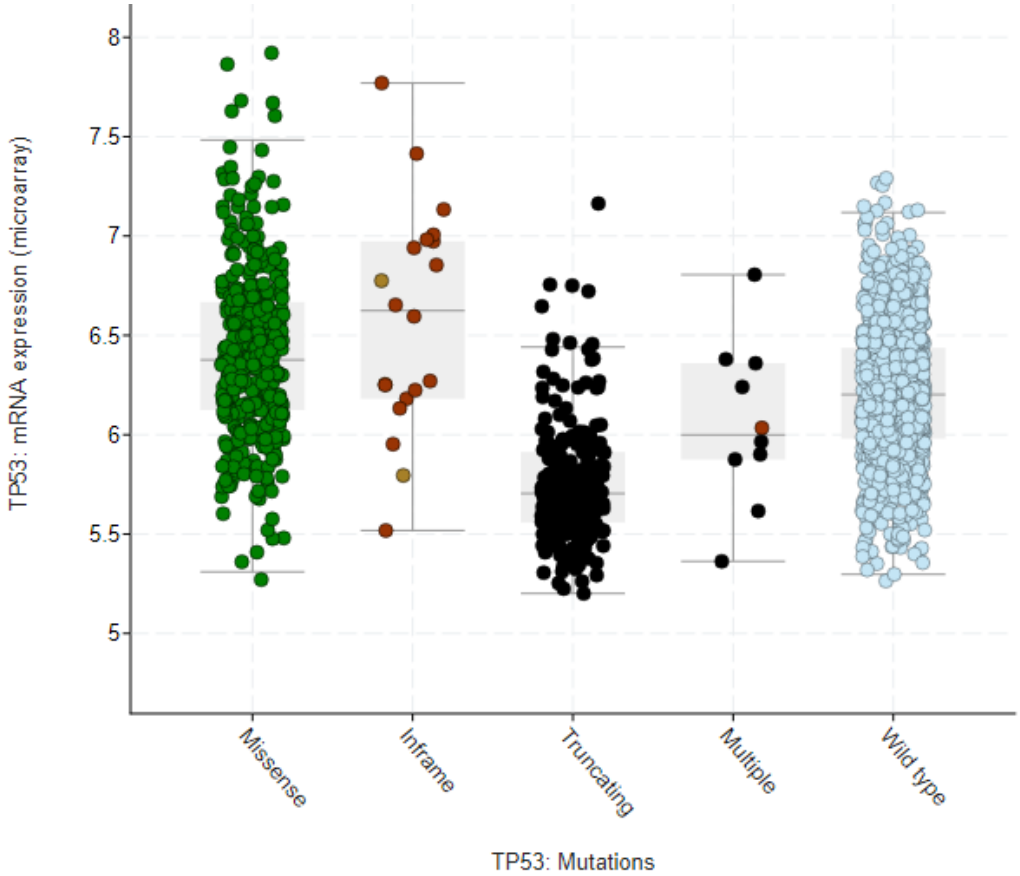


Figure 1.4: *TP53* mutation types and distribution in breast cancer samples. Microarray results showing the expression level of different *TP53* mutation expression among 2173 breast cancer samples as indicated on the y-axis. The y-axis shows the relative expression of the different types of mutations relative to *TP53* mRNA expression (Cerami et al., 2012; Gao et al., 2013; The cBioPortal for Cancer Genomics, 2016).

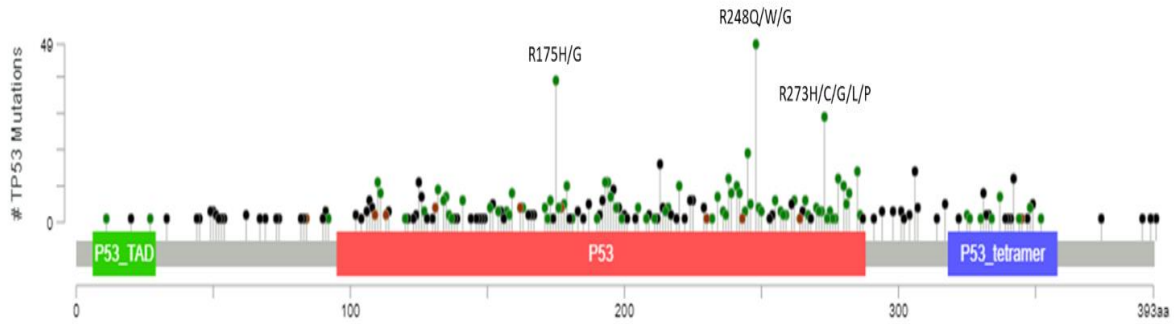


Figure 1.5: The distribution of hotspot mutations on *TP53* in breast cancer. The y-axis indicates the number of *TP53* mutations among 2173 breast cancer samples. Green, black, and red colors of single dots refer to missense, truncating (Nonsense, Nonstop, Frameshift deletion, Frameshift insertion, Splice site) and Inframe (deletion and insertion) mutations, respectively. Blue color represents wild-type p53. The x-axis shows the position of the mutations on the respective domains in p53 protein (Cerami et al., 2012; Gao et al., 2013; The cBioPortal for Cancer Genomics, 2016).

1.5. *HRAS G12V* and *TP53* variants combination role in cancer transformation

A range of studies showed that when mutated *TP53* is transfected into normal cell lines, it could collaborate proficiently with several oncogenes, remarkably *HRAS*, and transform primary cells *in vitro*. In addition, when mutant *TP53* is overexpressed, it leads to cell immortalization (Eliyahu, Raz, Gruss, Givol, & Oren, 1984; Jenkins, Rudge, & Currie, 1984; Parada, Land, Weinberg, Wolf, & Rotter, 1984). Besides, further studies described that, the combination between the *HRAS G12V* and inhibition of *TP53* gene is highly coupled with cancer transformation (Buganim et al., 2010). Noteworthy, that combination between oncogenic *RAS* and inactive/or mutated *TP53* can promote tumorigenesis in lung and pancreatic tumors (Solomon, Brosh, Buganim, & Rotter, 2010) by affecting virtually all the hallmark processes that are associated with tumorigenesis, including inflammation, proliferation, motility, metastasis, and angiogenesis (Hanahan & Weinberg, 2000).

Previously, in an *in vivo* study, a comb 4ination of constitutively active *HRAS* and inactivated p53 was shown to lead to G2 arrest bypass, moving to cancer transformation,

activation of epithelial to mesenchymal transition and to a rise in stemness in urothelial cells (He, Melamed, Tang, Huang, & Wu, 2015). In another study, a mouse model was used to express *R172H TP53* hotspot mutation and activated *KRAS G12D* in the pancreas. This combination led to high level of invasion and metastatic carcinoma (Hingorani et al., 2005).

p53 and RAS are master proteins that regulate many downstream targets. The molecular link between these two proteins was the focus of different studies (Buganim et al., 2010). For example, the crosstalk between them was shown to control the expression of some specific gene cluster that regulate precancerous secreted molecules, which are called Cancer related Gene Signature (CGS) (Buganim et al., 2010). From the secreted molecules that were shown to be affected by active HRAS and to have pro-cancerous activity, present in the CGS, are some chemokines like CXCL1, CXCL2 and CXCL3, interleukins such as IL-1b, IL-6 and CSF2 and extracellular matrix related proteins as MMP-3, CLECSF2 and TREM-1(Solomon et al., 2012). Also, *ATF3* and *BTG2*, which are p53 downstream target genes, were found to produce inhibitors for RAS-induced transformation(Solomon et al., 2010). In a previous research, wild-type p53 was shown to suppress the expression and activity of CGS genes through different mechanisms. One of these mechanisms is that p53 activates BTG2, then BTG2 binds with HRAS *G12V* and deactivates it. Moreover, p53 stabilization leads to ATF3 activation, which binds with CGS genes and repress their activity (Solomon et al., 2012). Another evidence that links p53 and RAS is the fact that p53 protein is stabilized in case of RAS activation via diverse molecular loops that involve ARF, DMP1, PML, and PRAK proteins (Buganim et al., 2010).

1.6. Problem statement and motivation of the study

Breast cancer is a heterogeneous disease. Many researchers are trying to unravel the genetic changes and mechanisms that contribute to breast epithelial cell transformations. The imbalance between the activities of tumor suppressors and oncogenes is the common event that leads to cancer transformation (Solomon et al., 2010). Also, two or more genetic alterations are required for tumorigenesis. Our project aimed to create breast cancer transformation model that could be employed to study breast cancer in two hit system context.

Studying particular cancer mutations would help in understanding how do different types of mutations could affect cancer prognosis and treatment regimens (Myers et al., 2016). In fact, several clinical trials were based on targeting RAS or p53 pathway (Levine & Oren, 2009). Therefore, studying the effect of *HRAS* function in the context of different p53 variants in the context of breast cancer might help in understanding the differential behavior of different breast cancer tumors.

1.7. Hypothesis

This project is based on the assumption that various gene transcription reprogramming lead to different changes in oncogenic cellular phenotypes. We hypothesize that in conjunction with *HRAS G12V* overexpression, cells harboring different *TP53* variants will behave different and thus the expression of different cell phenotypes will appear.

1.8. Aims of study

1.8.1. The main goal of this research is to create different breast cancer transformation models by transforming normal mammary epithelial cell lines, MCF10A cells, that contain different *TP53* gene variants with *HRAS G12V*.

1.8.2. Testing the differences in distinct cellular phenotypes (cancer hallmarks) caused by the combination between *HRAS G12V* and different *TP53* variants.

Chapter 2

2. Materials and Methods

2.1. Materials

Table 2.1: Equipment and tools

#	Name	Company
1	Analytical Balance	METLER TOLEDO AB104
2	Autoclave	HIRAYAMA HV-110
3	Vortexes: SA6, Genie 2, Reax top	Stuart Scientific, neoLab, Heidolph
4	Biological hood (HERA guard)	Heraeus
5	Biofuge Stratos Reconditioned	Heraeus 75005289R
6	Biofuge Fresco	Heraeus 75005521
7	Digital dry bath	Labnet
8	Elisa reader	BioTek EL-X800
9	Hera cell 150 CO2 Incubator	Heraeus
10	Inverted microscope	Olympus ck40-SLP
11	Labofuge 200 centrifuge	Heraeus
12	RT-PCR	(Applied Bio-systems 7500 Real Time PCR
13	GlobMax discover reader	Promega
14	Axiovert 40 CFL microscope with built-in camera Axiocam MRC	Carl Zeiss
15	Benchtop centrifuges: Labofuge 200, Biofuge fresco	Therom Fisher Scientific Heraeus
16	Cell counter CASY	Casy , Innvatis
17	Cell culture hood – HERAsafe Safety Cabinet KS12	Thermo Fisher Scientific Heraeus
18	Cell culture incubator (37 ℃ , 5% CO2)	Binder
19	Computers	hp, Fujitsu, Siemens
20	Floor centrifuge – Heraeus Multifuge 4KR	Thermo Fisher Scientific
21	Heraeus Sepatech Verifuge 3.0 R	Heraeus
22	Freezer -20 ℃	Libherr
23	Freezer -80 ℃ ,VIP	Sanyo
24	Freezing container	Sigma-Aldrich
25	Fridge 4℃	Liebgerr

26	Horizontal roller shaker ,RM5	neoLab
27	Ice machine	Hoshizaki
28	Image Xpress microscope	Molecular Device
29	Light microscope Wilovert S	Hund Wetzlar
30	Liquid nitrogen storage system	Cryotherm
31	LSM cell Observer	Carl Zeiss
32	MilliQ Biocel Water Purification System	Millipore
33	Multichannel pipette	Eppendorf
34	Multistep pipette Biohit (5-100 µl)	Biohit
35	Multistep pipette Ripette (200 µl – 5 ml)	Ritter Medical
36	Thermomixer comfort	Eppendorf
37	Olympus Scanning microscope	MJ Research
38	Pipettes (0.5 – 1000 µl)	Eppendorf , Gilson
39	Safety hood	Waldner
40	Scanner	Epson
41	Suction device for cell culture	neoLab
42	SPIN-micropipette site	Nano Spinreact
43	Ultracentrifuge	BECKMAN COULTER optima LE80H
44	Water Bath Orbital Shaking	Grant OLS200
45	Water Baths	Grant LTD6G, Julabo TW20
46	PCR machine 96 well	Applied Biosystem #9902
47	Neubauer count chamber (0.1 mm depth)	BLAUBRAND

Table 2.2: Reagents and Chemicals

#	**Name**	**Company**
1	DMEM-F12 medium	Biological industries , Gibco
	DMEM medium	Invitrogen
2	RPMI (1640) medium	Gibco
3	Horse serum	Biological industry
4	Fetal bovine serum (FBS)	Gibco
5	Hydrocortisone	Sigma-Aldrich
6	Insulin	Sigma -Aldrich
7	Epidermal growth factor (EGF)	Sigma -Aldrich
8	Cholera toxin	Sigma -Aldrich
9	L-Glutamine	Biological industries
10	Penicillin/streptomycin	Biological industries
11	Dimethyl sulfoxide	Sigma -Aldrich
12	Sterile phosphate buffer saline PBS	Biological industries
13	Puromycin ,	Sigma -Aldrich
14	XTT kite	Biological industries

15	Trypane blue	Biological industries
16	Isopropanol biological gradient	Sigma -Aldrich
17	Ethanol biological gradient	Sigma-Aldrich
18	Chloroform biological gradient	Sigma -Aldrich
19	qScript™cDNA synthesis kit	Quanta Biosciences
20	SYBR® Green	Applied Biosystems
21	TRIZOL	Sigma -Aldrich
22	Diff-Quick System	Sigma -Aldrich
23	0.5% and 0.05% Trypsin-EDTA	Invitrogen
24	Agrose	Sigma-Aldrich
25	BCA protein assay kit	Thermo Fisher Scientific
26	Choroform	Merck
27	Deoxynucleoside triphosphates (dNTPS)	New England Biolab
28	DNA Loading dye (6x)	Thermo Fisher Scientific
29	GeneRuler 1 kb DNA Ladder	Thermo Fisher Scientific
30	GeneRuler 100 bp DNA Ladder	Thermo Fisher Scientific
31	Geneticin G148 (Neomycin)	Gibco
32	Methanol	Sigma-Aldrich
33	Nuclease Free Water	Ambion
34	Phosphate buffered saline (PBS)	Gibco
35	OptiMEM	Invitrogen
36	Lipofectamine	Invitrogen
37	Matrigel matrix	Corning
38	Anti-p53	Santa Cruz Biotechnology
39	Anti-B-Actin	Santa Cruz Biotechnology

Table 2.3: Cell lines

#	**Name**	**Origin**
1	MCF10A *TP53*KO	Sigma-Aldrich
2	MCF10A Wtres	DKFZ Genomic and Proteomics core facility
3	MCF10A R248Q	
4	MCF10A R175H	
5	MCF10A R273H	
6	HEK293FT	Invitrogen
7	MCF10A parental	ATCC

Table 2.4: Plasmids

#	Name	Origin
1	rwpLX305_hRAS_G35T_IRES_Puro K 1	DKFZ, Genomic and Proteomics core facility. Kindly provided by Dr. Rainer Will
2	rwpLX305_MCS_HA_IRES_Puro K1	
3	rwpLX305_RedFF_HA_inakt_IRES_Puro K1	
4	PsPAX2	Addgene
5	PMD2.G	Addgene

2.2. Methods

2.2.1. Cell culture

Stable MCF10A cell lines expressing different *TP53* mutations were established by the Genomics and Proteomics Core Facility at the DKFZ- Germany. These lines were generated by overexpressing different p53 constructs in MCF10A *TP53*KO cells from Sigma-Aldrich (catalogue number CLLS1049). Overexpression was achieved by using the lentiviral vector pLx305 that expresses either FLAG-tagged *TP53 R175H* or *TP53 R248Q* or wild-type *TP53*.

All MCF10A cell lines were cultured in DMEM-F12 supplemented with 5% horse serum, 20 ng/mL EGF, 0.5µg/mL hydrocortisone, 1% L-glutamine, 100ng/mL cholera toxin, 1% penicillin/streptomycin (50 U/µL penicillin and 50µg/mL streptomycin as final concentration) and 10µg/mL insulin. DMEM medium supplemented with high glucose and 10% FBS was used to culture HEK293FT cells. Cells were passed every 3-4 days. For this purpose, the medium was eliminated and cells were washed with 5 mL PBS X1 one time. Afterwards, 3 mL trypsin (0.05% concertation) was used followed by incubation for 15-20 minutes in 37 °C, 5% CO_2 incubator. Then, 5 mL full growth medium was added to detach the cells. Cells were counted by using Neubauer counting chamber (0.1 mm depth) and $0.5x10^6$ cells were seeded in $75cm^2$ flasks.

2.2.2. Cells cryopreservation

To prepare the freezing medium, full growth DMEM-F12 medium supplemented with 20% horse serum and 10% DMSO was used. Trypsinized cells were collected and then counted with Neubauer counting chamber and spun down. The preferred quantity of the cells ($1 – 2 \times 10^6$ cells/mL) was resuspended in the freezing medium and was distributed into cryo-vials. To obtain a gradually cooling rate of the cells (1°C /min), cryo-vials were placed inside freezing container with isopropanol and left into a -80°C deep freezer. Finally, cells were moved to liquid nitrogen tank for long term storage. To restore and thaw the cells from freezing, a cryo-vial holding cells were resuspended in 1 mL full growth medium and transferred into 75 cm^2 flask having 15 mL full growth medium.

2.2.3. Generation of stable cell lines pools

Viral particles production and Transduction

$2x10^5$ of HEK293FT cells were seeded on poly-lysine-coated 100 mm dish with full growth medium. Next day, cells were transfected using the following procedure. First, to prepare transfection mixture, 1450 µL OptiMEM and 50 µL lipofectamine were mixed and incubated for 5 minutes at room temperature. To prepare viral particles, we mixed 7 ug of psPAX2 packaging vector with 6 ug of retroviral transfer vectors (rwpLX305_ RedFF _HA_ inakt_ IRES_ Puro K1 or rwpLX305 _MCS _HA _IRES _Puro K1 as controls, or rwpLX305_ *hRAS*_G35T _IRES _Puro K 1), and 3 ug pMD2.G envelope vector. Then this plasmid mixture was mixed with transfection mixture and incubated for 30 minutes at room temperature. Afterwards, this mixture was added to HEK293FT cells and incubated for 5 hours at 37 °C, 5% CO_2 incubator. Later, the transfection solution was replaced with 10 mL DMEM full growth medium. 48 hours following transfection, the media containing the viral particles was collected and frozen until being used for cell infection.

For infection, $5X10^5$ cells / well were seeded in 6 well plate in full growth medium. Next day, DMEM-F12 medium that was collected from HEK293T cell plates was

centrifuged, and the supernatant was collected. 2ml of this medium was afterwards mixed with polybrene and added to cells for two days at 37 ℃, 5% CO_2 incubation.

2.2.4. Selection

To select for clone pools, the transduction medium was changed with fresh full growth medium containing the appropriate selection antibiotics, Puromycin (stock = 10 mg/mL, diluted 1:5000, final concentration 1μg/ml) and Geneticin (Neomycin, stock = 50 mg/mL, diluted 1:100, final concentration 500 μg/ml). The selection antibiotic was kept on cells until control non infected cells totally died.

2.2.5. Cell count using fluorescent microscope

1000 cells were seeded in 96 well plates in full growth medium. After 7 hours of incubation, the medium was aspirated and replaced with DMEM-F12 medium supplemented with 0.9% horse serum, 1 or 0 ng/mL EGF, 0.5 μg/mL hydrocortisone,100 ng/mL vibrio cholera toxin and 10 μg/mL insulin. For the two EGF concentrations, ten replicates were used. The number of the cells in the seeding control plate was measured after 24 hours' of incubation, and 72 hours' incubation for the assay plate by adding 1:5000 dilutions of Hoechst staining. The cells count refers to the nuclei count by using the Molecular Devices microscope.

2.2.6. Cell count using Neubauer counting chamber

Triplicates of $3x10^4$ cells were seeded in 6 well plates and incubated at 37 ℃ , 5% CO_2 for 24 hours. Next day, the attached cells were collected in 15 ml tubes and centrifuged at 1200 RPM for 10 min. Then, the supernatant was eliminated and cells were resuspended in full growth medium. Lastly, cells were counted by using Neubauer counting chamber.

2.2.7. XTT test

Triplicate of $2x10^3$ cells/well were seeded in 96 well plate and incubated at 37°C , 5% CO_2 for 24, 48, 72 hours and cell proliferation was assessed by using XTT stain according to manufacturer.

2.2.8. Wound healing assay

Triplicates of $2x10^5$ cells/well were seeded in 12 well plates in full growth medium and incubated at 37 °C, 5% CO_2 until having 100% growth confluence. To generate wound, cell monolayer was scratched using 10µl pipet tip. Afterwards, floating cells were washed out using PBSX1. Finally, cells were supplied with starvation medium that lacks serum and wound was photographed over 24 hrs.

2.2.9. Survival assay

200 and 400 cells were seeded in 6 well plates in full growth medium. Every 3-4 days the medium was changed until visible colonies were seen. Later, the growth medium was removed and the plates were washed using PBS x1. Next, cells were fixed for 15 minutes by using absolute methanol and then left to air dry. After fixation, colonies were stained using Coomassie blue, washed and counted using naked eye.

2.2.10. Anchorage-independent growth assay

$4X10^4$ cells were seeded in 10 cm cell culture plates and ultra-low attachment plates in full growth medium and incubated at 37 °C and 5% CO_2 for 14 days. The spheroids that formed were monitored, photographed, and counted.

2.2.11. Soft agar assay

The assay medium is composed of a lower and upper agar layers. The lower layer was prepared by using RPMI 1640 medium containing 1% Agar. 2 mL of this mixture was poured in each well of 6 well plates and kept at 4 °C for at least one hour. Then,

$2X10^4$ cells were mixed with 1.2 mL of the upper layer that contained 0.7% agar. This cell mixture was then added on the top of the lower layer and put back in CO2 incubator until colonies were visible to naked eye.

2.2.12. Matrigel 3D culture assay

First each well of 8 well chamber slide was coated with 40ul of growth factor reduced Matrigel and left for at least 15 minutes in the incubator at 37°C until it became solid. Then 3000 cells were mixed with 400ul growth medium containing 4% Matrigel and added to the Matrigel bottom layer. The assay chamber slide was then incubated in the incubator for two weeks. Forming mammospheres were photographed using inverted microscope.

2.2.13. RNA Extraction

Cells were seeded in 10 cm cell culture plates in full growth medium and incubated at 37 ℃ and 5% CO_2 for 24 hours. Next, the medium was aspirated 1 mL Trizol was added to the plates and incubated on ice for 5 minutes. Then, the cells collected in RNase free tubes and incubated on ice. Afterwards, the collected cells mixed thoroughly with 200 µL chloroform using vortex and incubated for 15 minutes on ice. The tubes were centrifuged for 15 minutes at 12000 RPM at 4 ℃, then the supernatant was transferred to new RNase free tubes and 500 µL pre-coold isopropanol was added, mixed and incubated for 15 minutes on ice. Subsequently, the tubes were centrifuged at 12000 RPM for 15 min at 4 ℃. Next, the supernatant was discarded and 500 µL 70% Ethyl alcohol was added. Thereafter, the tubes were centrifuged at 12000 RPM for 10 min at 4 ℃ and the pellet left to dry for 1 minute. Later, the pellet was resuspended with DEPC treated water and incubated at 60 ℃ for 5 minutes in dry bath. Lastly, the concentration of the extracted RNA was measured and run by gel electrophoresis.

2.2.14. cDNA synthesis

In PCR tubes, 1 µg of extracted RNA was mixed with 4 µL RT buffer, 1µl enzyme and ultra-pure water added to complete the mix volume to 20 µL. Later, the tubes were put in PCR machine according to manufacturer recommendations.

2.2.15. Real time PCR

In 96 q-PCR well plate, 3 µL of diluted cDNA (1:10), 1 µL of primer (10 µM), 10 µL SYBR® Green and 6 µL ultra-pure water were added per well. Then, the q-PCR plates were sealed and centrifuged for 5 minutes. Lastly, the plate was put in q-PCR machine with pre-conditioning at 50 °C for 2 minutes, activation at 95 °C for 10 minutes, denaturation at 95 °C for 15 seconds and annealing and extension at 60 °C for 1 minute.

2.2.16. Western blot

Cells were lysed by using RIPA lysis buffer containing 1x Complete Mini Protease Inhibitor Cocktail, 1x PhosSTOP phosphatase inhibitor. All the steps were done on ice. Cell scraper was used to detach cells, and the cell lysates were incubated at 4 °C for 10 minutes. The lysates were collected and centrifuged at 13000 rpm at 4 °C. The concentration of protein in the supernatant was measured using BCA Protein Assay kit (Thermo). 40ug protein was used for gel loading after mixing the sample with sample buffer. Before loading samples mixed with sample buffer were boiled for 10min in a dry path. Polyacrylamide gels with the concentration of the running gels 12.5% and stacking gels 4% were prepared. A prestained protein ladder was used as molecular weight marker. The separation was done at 100 V/cm for 1.5 hours in the 1x SDS-PAGE running buffer 250 mM Tris base and 1.92 M Glycine. Proteins on the gel were transferred to Millipore`s PVDF membrane Immobilon-FL using the semi-dry method. The transfer was done for 60 minutes at 25V. Before adding antibodies, membranes were blocked by using 5% skim milk in 1XTTBS. After blocking, the membrane was incubated with the primary antibody (P53 Santa Cruz

Biotechnology) which was diluted in the blocking solution for overnight at 4 ℃. The next day, the membrane was washed 3 times, 10 minutes each, with TBST and then incubated with secondary antibody anti-mouse conjugated to DyLight 680 in 1:10000 dilutions in TBST. The membrane was washed 3 times each10 minutes with TBST and scanned with infrared scanner at 700 and 800 nm. B-Actin housekeeping gene was used as a loading control.

2.2.17. Primer design

The primers that were used in this study are listed in the table below and was designed by using Primer 3 software (http://primer3.ut.ee/).

Table 2.5: List of the designed primers

No	Gene	Primers	Reference
1	*HRAS*	F.P_5'- tgccatcaacaacaccaagt-3' R.P-5'- agccaggtcacacttgttcc-3'	NM_001130442.2
2	*Bax*	F.P_5'- ggttgtcgccctttctact-3' R.P-5'- aagtccaatgtccagcccat-3'	NM_001291428.1
3	*BCL2*	F.P_5'- gccctgtggatgactgagta-3' R.P-5'- gaaatcaaacagaggccgca-3'	NM_000633.2
4	*Cyclin B1*	F.P_5'- gtcaccaggaactcgaaaat -3' R.P-5'-ttaccaatgtccccaagagc-3'	NM_031966.3
5	*Slug*	F.P_5'- atacagtgattatttccccg -3' R.P-5'- agcggtagtccacacagtga-3'	NM_003068.4
6	*Snail1*	F.P_5'- acactggcgagaagccctt -3' R.P-5'- gcctggcactggtacttctt -3'	NM_005985.3
7	*Twist1*	F.P_5'- cggagacctagatgtcattg-3' R.P-5'- cgccctgtttctttgaattt-3'	NM_000474.3
8	*Wnt5A*	F.P_5'- atgaagaagtccattggaat -3' R.P-5'- ctgggcgaaggagaaaaata -3'	NM_003392.4
9	*Cyr61*	F.P_5'- atctgcagagctcagtcaga-3' R.P-5'- tcttggggacacagaggaat-3'	NM_001554.4
10	*Bad*	F.P_5'- ctcctttaagaagggacttc-3' R.P-5'- gatgtggagcgaaggtca-3'	NM_004322.3
11	*GLUT 1*	F.P_5'- agaaggtgatcgaggagttc-3' R.P-5'- agagaaggagccaatcatgc-3'	NM_006516.3
12	*IDH 1*	F.P_5'- ctacatagctatgatttaggc -3' R.P-5'- ctcaaccctcttctcatcagg-3'	NM_001282386.1
13	*p21*	F.P 5'- cgtcaaatcctccccttcct-3' R.P 5'- atgggttctgacggacatcc -3'	NM_001291549.1
14	*HUBC*	F.P 5'- gtcgcagttcttgtttgtgg-3' R.P 5'-gatggtgtcactgggctcaa-3'	NM_021009.6

Chapter 3

3. Results

3.1. Generation of *HRAS G12V* MCF10A clones harboring different *TP53* variants

To investigate in what way *HRAS G12V* overexpression influences cellular phenotypes in MCF10A cells expressing different *TP53* variations, the first goal of this project was to create different MCF10A *HRAS G12V* clones harboring different *TP53* variants. To do so, either *HRAS G12V* expressing or empty retroviral viral vectors were used to stably transduce MCF10A cells that express different *TP53* constructs. Here, we expressed *HRAS G12V* in *TP53* knockout cells MCF10A cells expressing either wild-type *TP53* (control cells) or *TP53* with *R175H* mutation or in cells lacking *TP53* (*TP53* knockout (KO) cells). To validate the generation of the clones, we used real time PCR to test the expression of *HRAS* and *TP53* using *HRAS* and *TP53* specific primers described in table 1. Based on our Real time PCR results we were able to generate the relevant clones. For example, *HRAS* expression was higher in MCF10A cells that were transduced with *HRAS G12V* construct compared to its expression in MCF10A cells that transduced with empty vector construct (Figure.3.1). The same was obtained with the different *TP53* clones where *TP53* expression is high in cells overexpressing either Wt or *R175H TP53* and low in *TP53* KO cells (Figure.3.1). Altogether, our results show that we were able to generate different MCF10A clones overexpressing *HRAS G12V* in combination with different *TP53* variations.

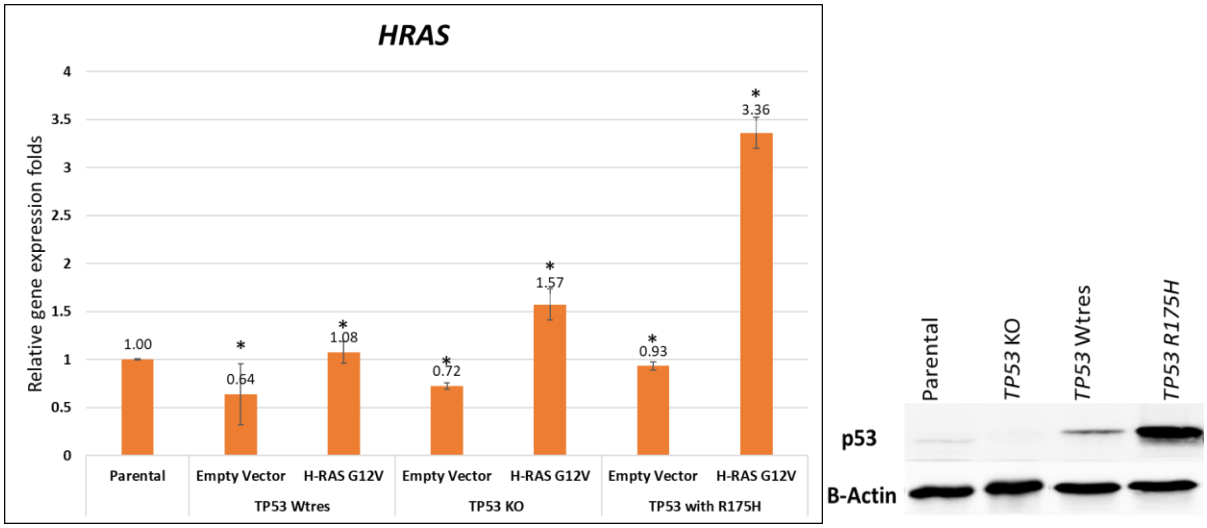


Figure 3.1: *HRAS* and *TP53* expression levels in transduced MCF10A cells. (A) Real time PCR results showing the relative expression level of *HRAS* gene relative to the house keeping gene UBC. All gene expression folds were calculated relative to the expression level in parental cells. Bars indicate the standard deviation of the mean of three biological replicates. (B) Western blot for p53 protein. B-Actin was used as a protein loading control. The statistical significance of the results was determined by measuring the p value. (** means that p-value>0.05 and * means that p-value <0.05).

3.2. Phenotypic change characterization of *HRAS* transduced MCF10A cells

To examine the effects of *HRAS* overexpression in various *TP53* transduced MCF10A cells, several functional assays were performed. Different cancer hallmarks were tested by a couple of functional assays, such as cell nuclei counting assay, XTT assay, total cell count assay, migration assay, anchorage-independent growth assay, survival assay, soft ager assay and Matrigel 3D culture assay.

3.2.1.1. Effect of *HRAS* overexpression in combination of different *TP53* variants on MCF10A cell proliferation

Cancer cells are characterized by uncontrolled growth and proliferation which is an important cancer hallmark (Hanahan & Weinberg, 2011). *HRAS* is recognized to enable cell growth and proliferation (Karnoub & Weinberg, 2008), whereas p53 protein stops cell proliferation(Levine & Oren, 2009). Hence, the combination of *HRAS* overexpression with *TP53*KO or Wt *TP53* or *TP53 R175H* mutation was hypothesized to affect MCF10A cells proliferation in different manners. To test the

previous hypothesis, several cell proliferation assays, i.e. nuclei counting, total cell counting and XTT were performed.

Nuclei counting assay was carried out by using fluorescent microscope (MoleDev), which counts nuclei of the cells that trap Hochest stain (Figure 3.2). However, because *HRAS G12V* clones grow in clumps (Figure 3.2), the microscope was not able to count all cell nuclei in a reliable manner. As shown in Figure 3.2, there is a discrepancy between the number of nuclei that were captured (Blue color) and the number of nuclei that the microscope had counted (red color) and thus we didn't present the results of this experiment. As it appears from these figures, the microscope failed to count all cells that were in clumps (indicated by arrows) (Figure 3.2).

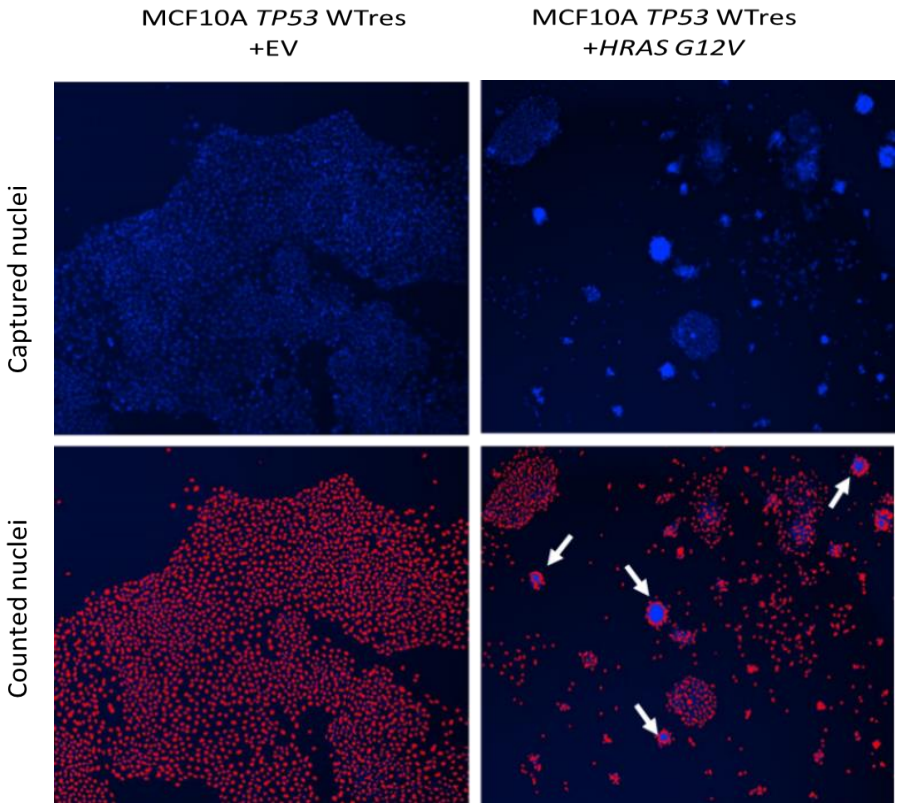


Figure 3.2: Automatic nuclei counting assay using fluorescent microscope. Representative micrographs showing how does fluorescent microscope capture figures and count the cells. Captured cell nuclei appear in blue color and the counted nuclei appear with the red color. Each of the above pictures represent a quarter of the field after 2 days of cell culture. White arrows indicate cell clumps that the microscope was not able to count them.

Because of the technical issues we faced with microscope nuclei cell count, we did total cell count using chamber slide technique. Also, in this manual total cell count assay, the results were also not reliable because the aggressive cells harboring *HRAS* mutation formed clumps (Figure.3.3) that didn't adhere very well to cell culture plates and were washed out during cell count procedure. Figure.3.4 shows a representative result that we obtained using total cell count technique. To try to overcome the technical problems we faced with previous techniques, we tried to learn about cell proliferation behavior by measuring cell metabolic activity by using XTT assay. But also, here we could not get reliable results for the same reasons mentioned above. As shown in Figure.3.5, the highest proliferative rate was observed with MCF10A cells with *TP53* Wtres and *HRAS G12V*, which is not in concordance with our eye observations.

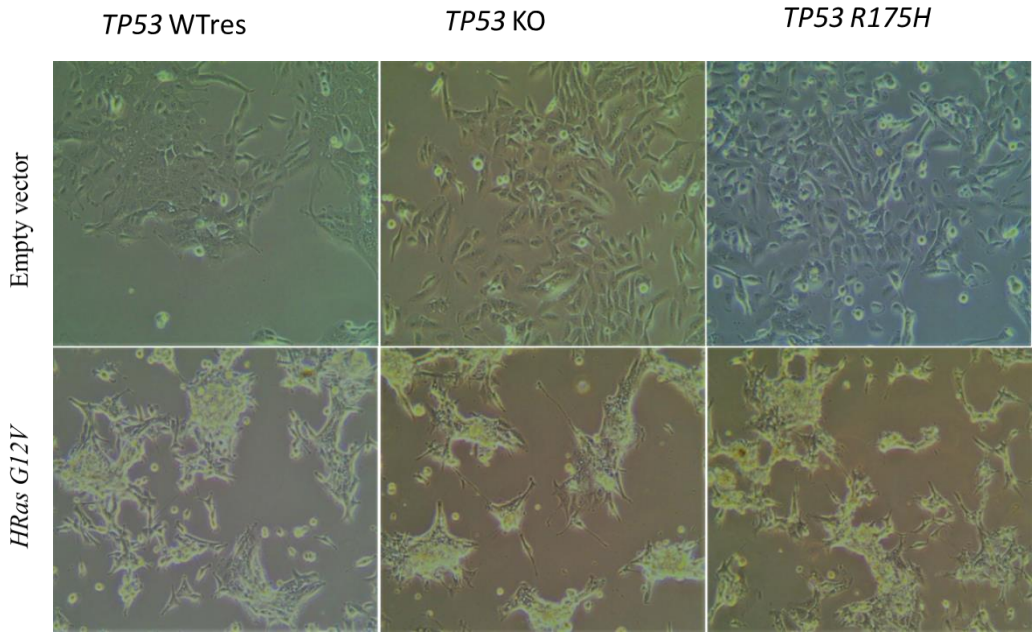


Figure 3.3: Transduced MCF10A cell morphology. Representative micrographs showing differences in cell morphology that accompany the different transduction combinations. The upper photos represent the cells with empty vector and the lower photos represent the cells with *HRAS G12V*.

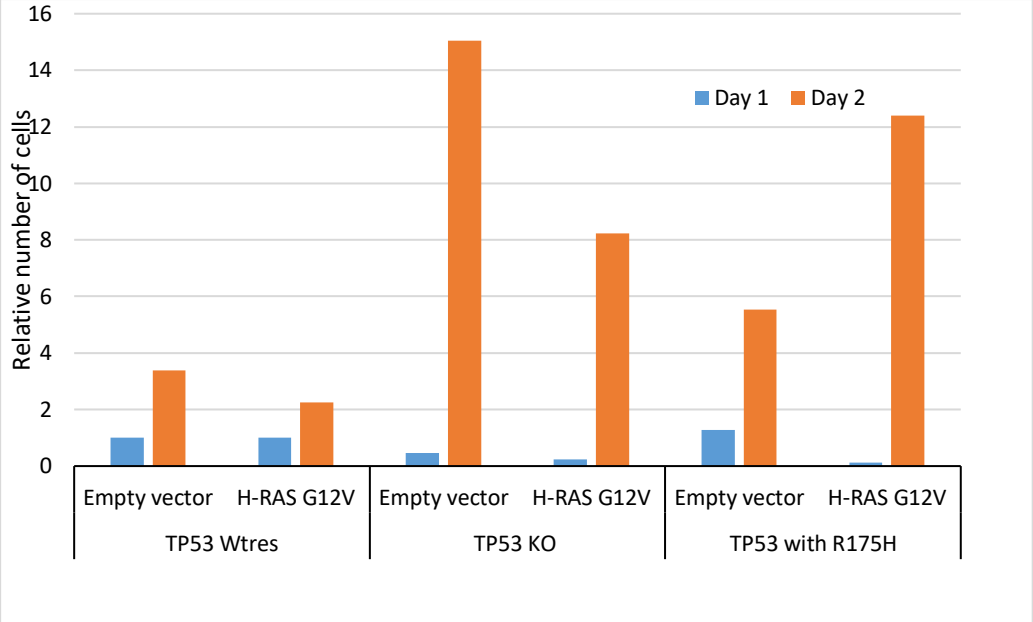


Figure 3.4: Relative proliferation rate for the transduced MCF10A clones using total count assay. The bars show the effects of *HRAS* overexpression on cell proliferation measured by total count assay for two days. Cells were counted in triplicates. Y axis represents the relative proliferation rate index correlated to *TP53* Wtres MCF10A cell lines at day 1.

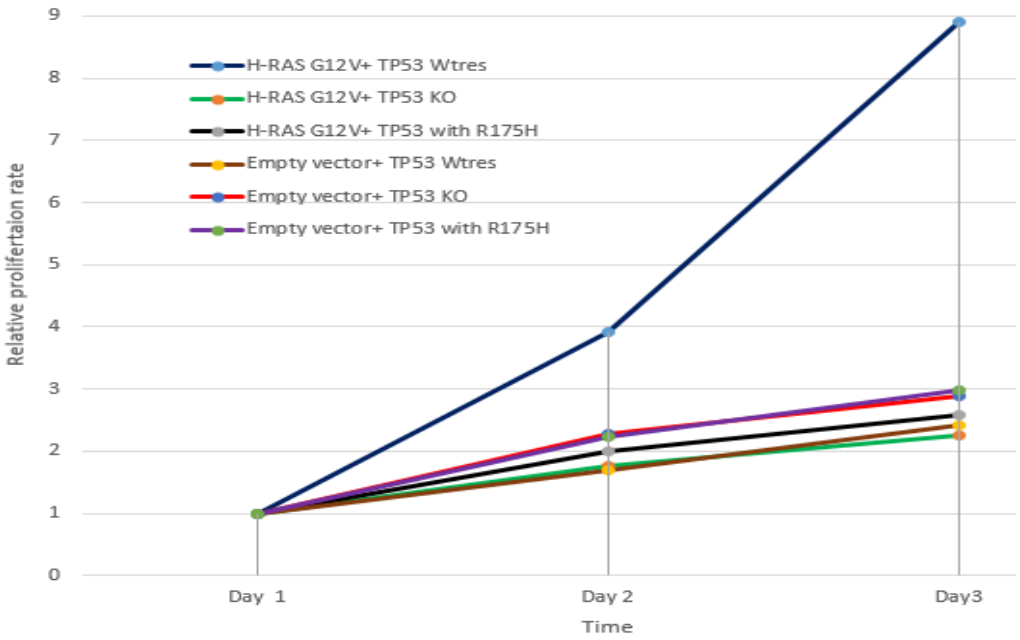


Figure 3.5: Relative proliferation rate for the MCF10A transduced clones using XTT assay. The graph shows the effects of HRAS overexpression on cell proliferation monitored by XTT assay for three days. Cells were tested in triplicates per each point of time. Y axis represents the relative proliferation rate index correlated to day zero.

3.2.1.2. *HRAS* overexpression and *TP53* variants effects on MCF10A cell migration

A further key cancer cells hallmark is the acquisition of cell migratory phenotype. *HRAS* overexpression is associated with enhancement of cell mobility and metastasis of tumor cells (Solomon et al., 2010). However, in normal cells as a defense mechanism to prevent cancer transformation, p53 is involved in senescence, cell-cycle arrest, or cell death (Lin et al., 1998) as well as in inhibiting migration and metastasis. When *TP53* is lost or has gain of function mutation, that increases the metastatic potential of breast cancer (Powell, Piwnica-Worms, & Piwnica-Worms, 2014). In fact, as migration is important for metastasis of the tumor cells, the combination between *HRAS G12V* and *TP53* KO or *TP53* with *R175H* mutation was hypothesized to increases MCF10A cells migration. To test this hypothesis, wound healing assay was performed. As shown in Figure 3.6, *HRAS* increased cell migration capacity in all cell lines compared to cells infected with EV. The most drastic effect on cell migration was observed in the combination of *HRAS G12V* with *TP53* KO in MCF10A cells. In *TP53 R175H* and *HRAS G12V* combination, the cells failed to form a monolayer because they tend to grow in clumps, which made it difficult to compare the migration capacity of these cells to others.

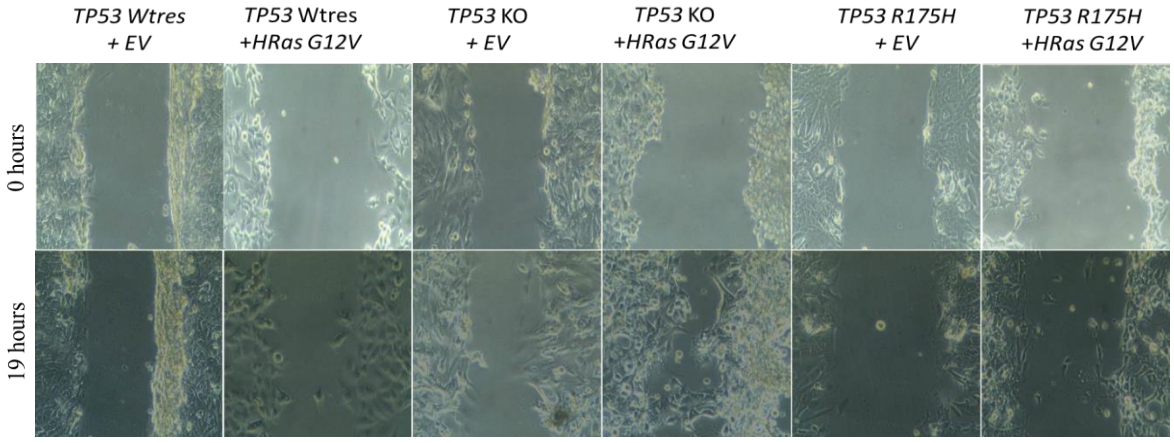


Figure 3.6: Effect of *HRAS* overexpression on migration of different *TP53* manipulated MCF10A clones. Representative micrographs taken for the indicated cells at 0 h as well as after 19 h after wounding cell monolayer.

3.2.2. Effect of *HRAS* overexpression on cell survival of different MCF10A *TP53* clones

One of cancer cell tumorigenesis features is independence on exogenous stimulus for growth and survival (Hanahan & Weinberg, 2000). *HRAS* overexpression correlates with increased cancer cell resistance to death (Solomon et al., 2010). In order to test the effect of *HRAS G12V* overexpression on different MCF10A *TP53* clone survival, we did colony formation assay.

Unexpectedly, *HRAS* showed an effect only on cells expressing Wt *TP53* (Fig.3.7). In contrary *HRAS* seemed to reduce cell survival in *TP53* KO and *TP53 R175H* cells (Fig. 3.7). Also, these results didn't not match out eye observation. Moreover, in these two combinations, cells grew in clumps and detached from the plates when we tried to stain and enumerate them by the end of the assay.

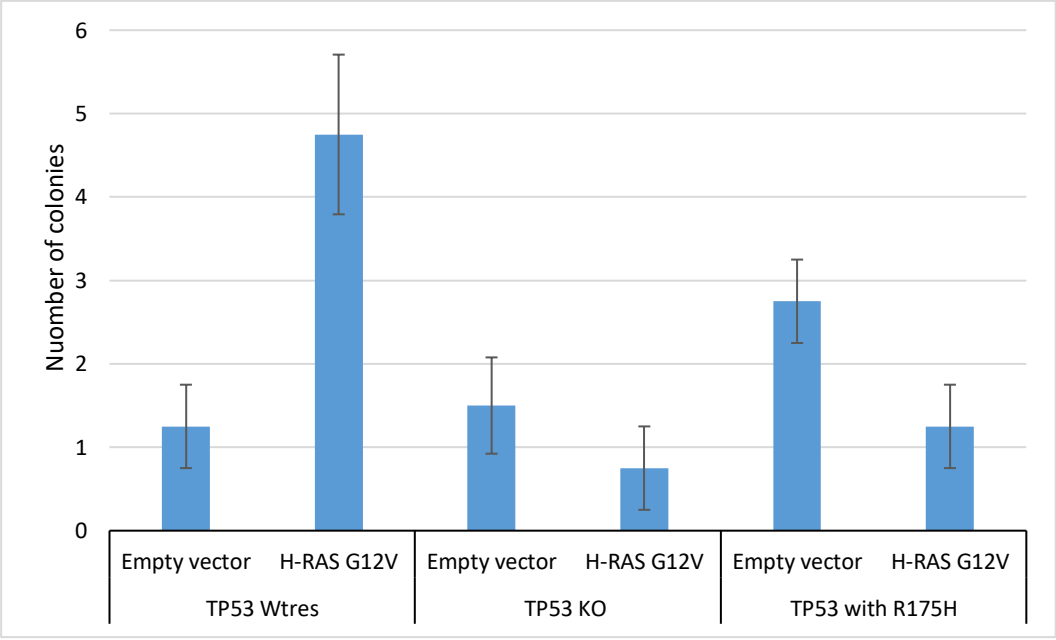


Figure 3.7: *HRAS* overexpression effect on survival of different MCF10A TP53 clones. Representative statistical analysis of the survival rate of *HRAS* transduced MCF10A cells monitored using colony formation assay. Cells were tested in triplicates for 12 days and the bars represent the standard error of the mean.

3.2.3. *HRAS* overexpression and *TP53* variants effect on MCF10A anchorage-independent growth

Anchorage-independent growth is one of the frequent events that could occur during cell transformation in solid tumors including breast cancer and it reflects the metastatic potential of transformed cells (Wise, Duhachek-Muggy, Qi, Zolkiewski, & Zolkiewska, 2016). The cell ability to grow in suspension, mamosphere formation, and anoikis (a form of apoptosis)-resistance are important for cancer cell to resist cell death in suspension and thus metastasis to distant organs (Dontu et al., 2003). Normal breast epithelium cell line MCF10A exhibits absence of tumorigenic feature and lack of the capability of anchorage-independent growth (Debnath, Muthuswamy, & Brugge, 2003). In this framework, *HRAS* overexpression with *TP53*KO or *TP53* with *R175H* mutation were hypothesized to stimulate anchorage-independent growth of MCF10A cells. Hence, anchorage-independent growth assay, by using coated and uncoated plates was used in order to prove this hypothesis. Cells were counted by using Trypan blue stain, which stains dead cells only. As we mentioned above, the cells form clumps, which hard to count and that give unreliable data as shown in Figure 3.8.

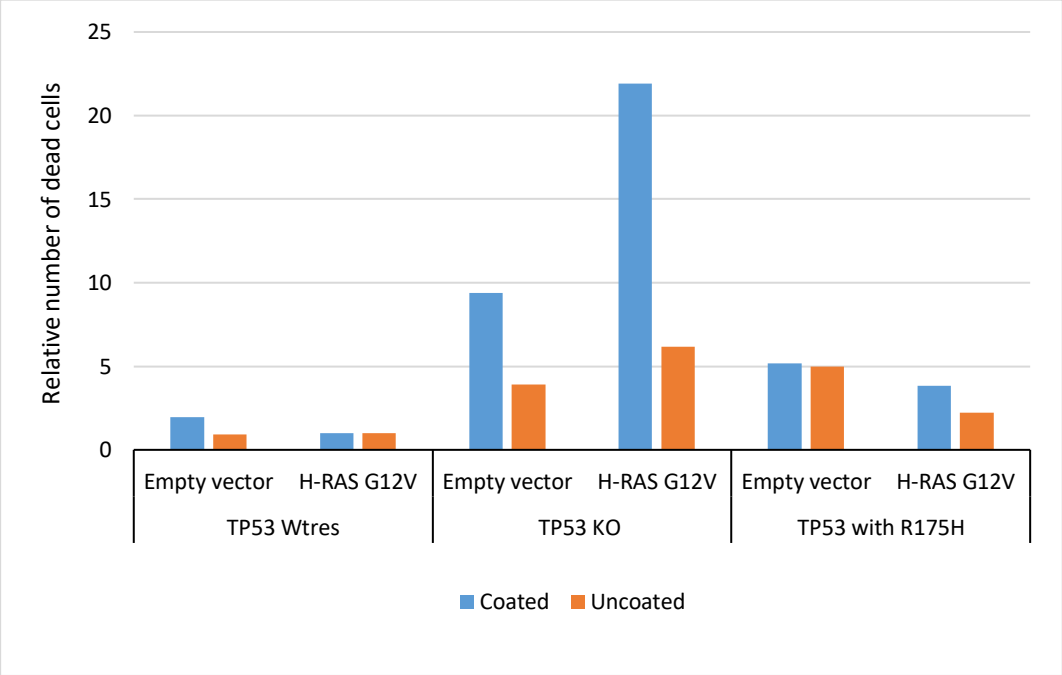


Figure 3.8: *HRAS* overexpression effect on MCF10A anchorage-independent growth. The figure shows the relative number of dead cells after 14 days of culture on coated and uncoated plates.

3.2.4. Effect of *HRAS* overexpression in transduced MCF10A with different *TP53* variants on encourage cell tumorigenicity

MCF10A cells are immortalize normal mammary epithelial cells (Soule et al., 1990). They grow as mammosphers, plorized, and glandular structure *in vitro*. Oncogenes overexpression and/or tumor suppresser inactivation leads to the disruption of this normal growth pattern. To demonstrate the effect of our manipulation on MCF10A normal growth pattern and to better characterize the acquired over growth phenotype that we observed but could not very well show in different assays because the cells form clumps, we did 3D Matrigel culture assay. As shown in Figure 3.9, the different combinations between different *TP53* variants had different effects on cell proliferation rate as shown by the size of the clumps form and on the invasive pattern of the cells as seen by more cells trying to migrate away from these clumps. Compared to the MCF10A parental cells that formed normal organized mammoshpers with small size, MCF10A cells without *HRAS* overexpression and *TP53*, *TP53* KO or *TP53 R175H* formed bigger mammoshpers, the biggest ones were the ones formed by *TP53 R175H* cells. Of note, *R175H* mutation was also enough to a little bit disrupt the normal mammaospher formation towards a more invasive one. Upon *HRAS* overexpression all clones formed very big cellular clumps indicating that *HRAS* increases cell proliferation in all our cell types. In comparison to each other, *TP53 R175H* cells clumps were the biggest followed by *TP53* KO cells and then *TP53* Wtres cells. In addition, to variable cell clump size, the invasive appearance of the clumps was also different with the most invasive growth was observed in *TP53 R175H* cells followed by *TP53* KO cells and then *TP53* Wtres cells. Altogether, these results indicate that *HRAS* overexpression differentially affects the growth rate and invasiveness of cells expressing different *TP53* variations.

Cell ability to grow independent of extracellular matrix and solid surface is one of cancer hallmarks (Hanahan & Weinberg, 2011). Soft agar assay is a widespread used technique for *in vitro* tumorigenic capacity evaluation (Borowicz et al., 2014). To study the effect of *HRAS* overexpression on the tumorigenicty of the different *TP53* cells and to verify our eye observation about the growth pattern of these cells, that was prevented by technical difficulties related to clump formation in 2D culture setup, we did soft agar assay. As shown in Figure 3.10, in all non *HRAS* transformed cell, cells failed to form any colonies with the exception of *TP53 R175H* cells. In *HRAS* transformed cells, all clones formed colonies of different sizes with the biggest formed by *TP53 R175H* cells followed by *TP53* KO and finally *TP53* Wtres cells. Our results here prove that transformation with *HRAS G12V* affects cell proliferation and tumorigenicty in a different manner and that combination between *TP53 R175H* and *HRAS* overexpression has the strongest effect on these phenotypes.

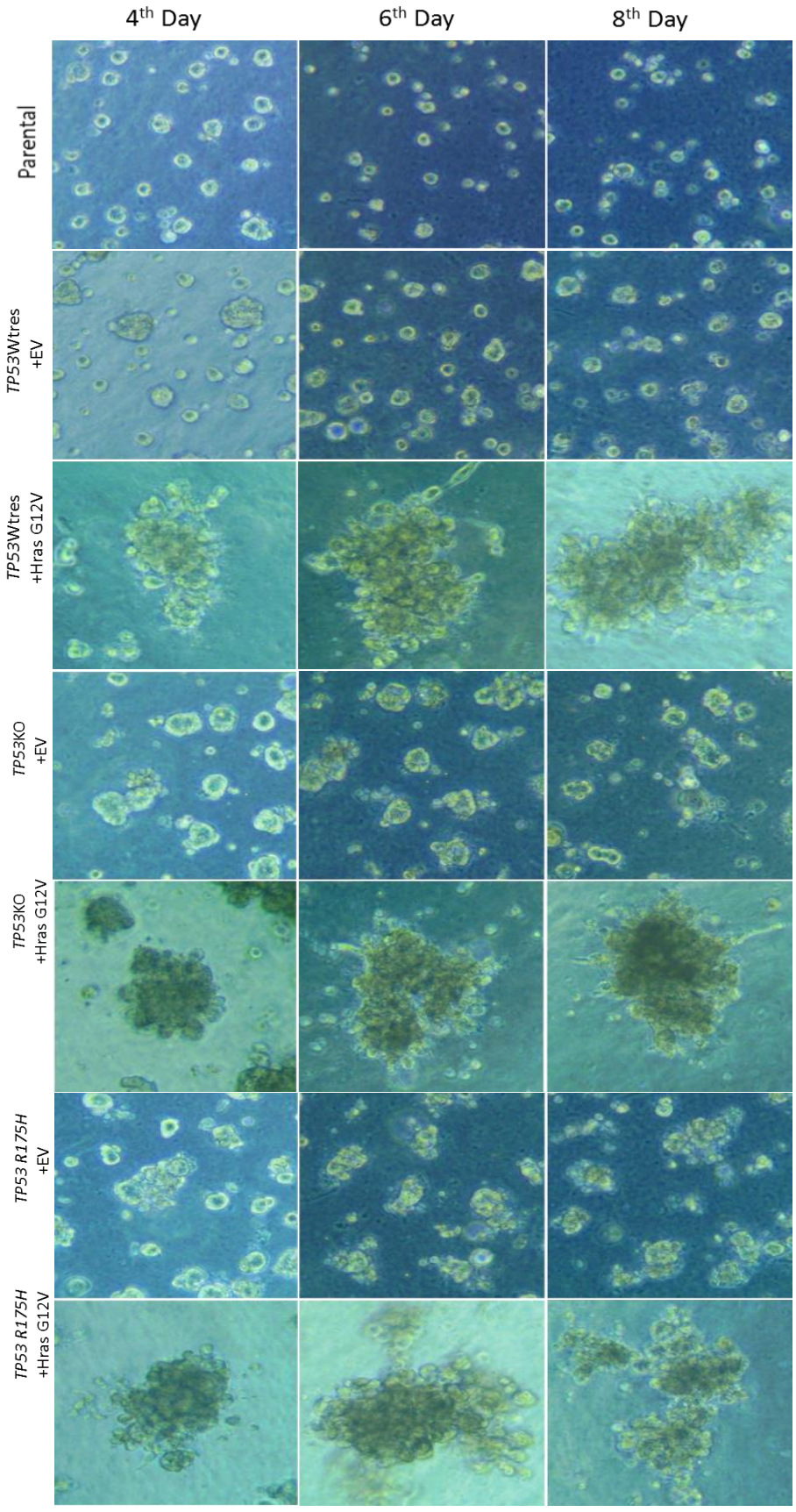


Figure 3.9: Matrigel 3D assay. Representative images at 10X shown the colonies organization of the MCF10A transduced clones with and without *HRAS* overexpression. The colonies formation was monitored for 6 days.

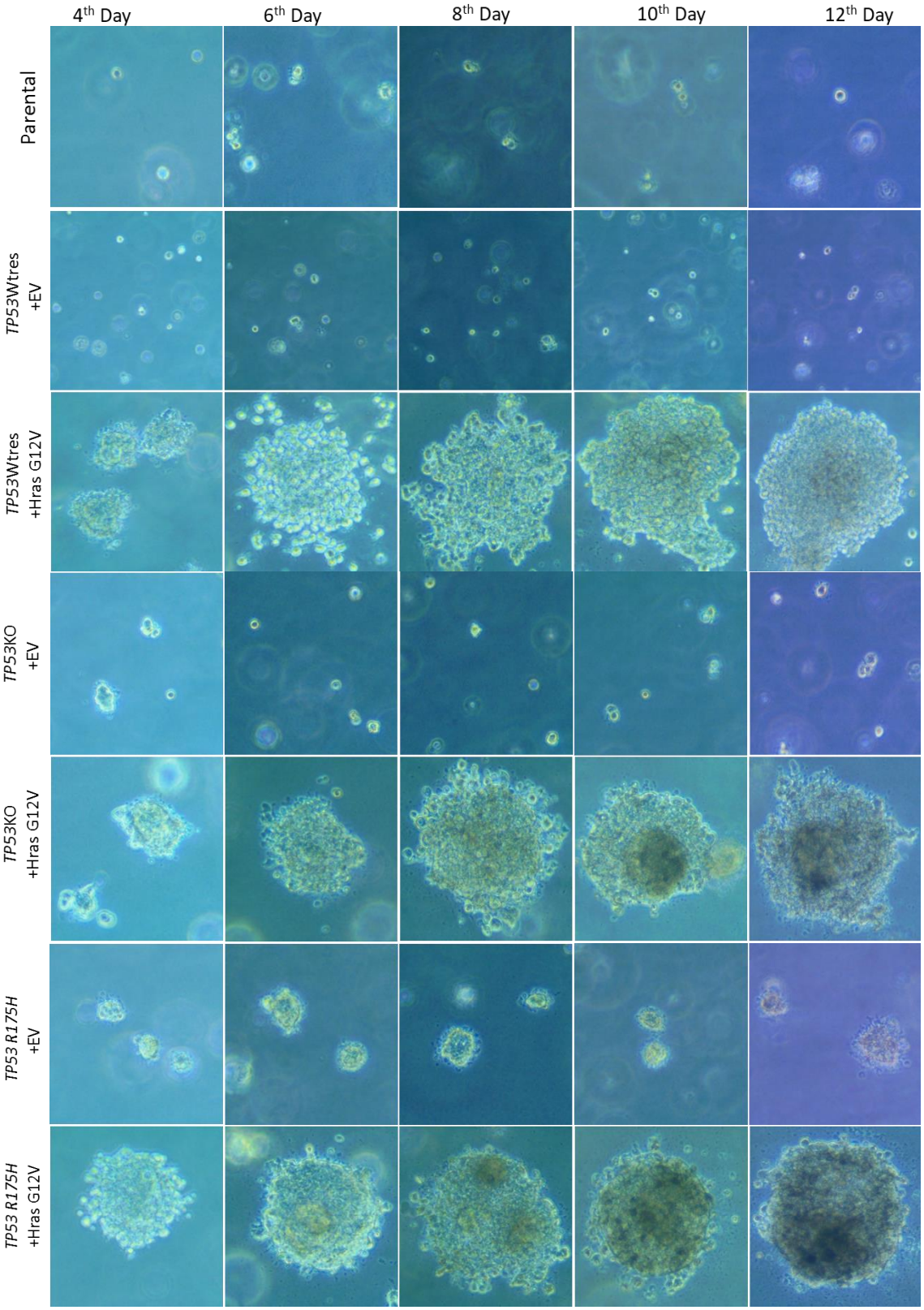
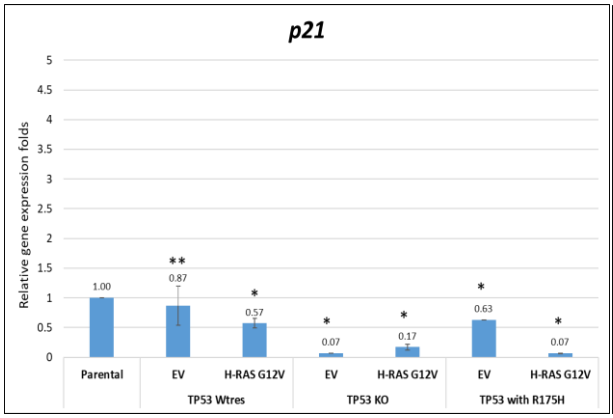


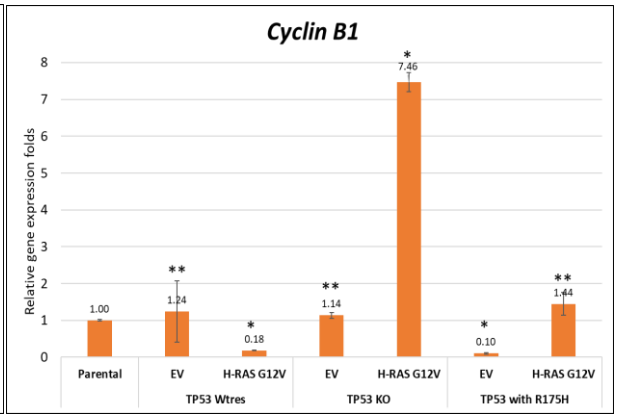
Figure 3.10: Soft agar assay. Representative images at 10X shown the colonies shape, size and migration of the MCF10A transduced clones with and without *HRAS* overexpression. The colonies formation was monitored for 16 days.

3.2.5. Effect of *HRAS* overexpression on the expression of genes related to different cancer phenotypes in cells harboring different *TP53* variants

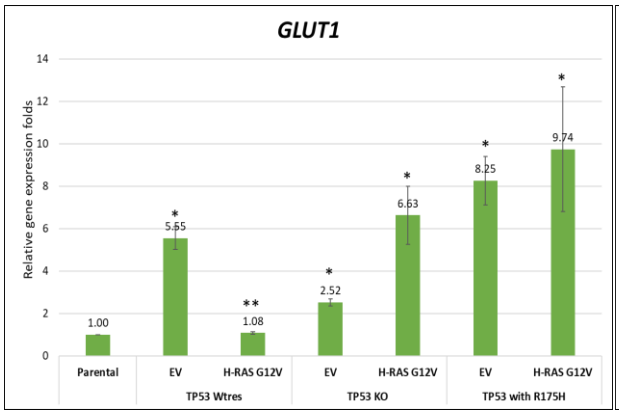
To test wither the phenotype we observed in the cells are related to the common gene expression pattern induced by *HRAS*, we did real time PCR to test the expression of genes related to different cancer cell phenotypes including cell cycle and cell proliferation (*p21*, *CyclinB1*), metabolism (*IDH1*, *GLUT1*), EMT (*Snail1*, *Slug*, *Twist1*, *Cyr61*, *Wnt5A*) and cell survival (*Bcl2*, *Bax*). As shown in Figure 3.11, *HRAS* overexpression affected different genes in different ways in different cells. For example, in cell proliferation-related genes, *p21* was affected more in *TP53 R175H* cells compared to other cell lines, while *CyclinB1* was affected more in *TP53* KO cells (Figure 3.11 A). In genes related to glucose uptake and metabolism, *HRAS* overexpression induced *GLUT1* expression in *TP53* KO cells compare to other cell lines. Of note, *TP53 R175H* was enough to induce *GLUT1* expression with no difference with *HRAS* overexpression (Figure 3.11 B). Regarding *IDH1*, while *HRAS* overexpression led to downregulation of *IDH1* in *TP53* KO cells, it significantly induced it in *TP53 R175H* cells (Figure 3.11 B). For EMT related marker genes, *Snail1* was the most affected. It was induced by *HRAS* in all cell lines but the highest expression was observed in *TP53 R175H* cells. For *Slug* and *Twist1*, which are also like *Snail1* target the epithelial marker e-cadherin and lower it, are not significantly affected in all cell lines. Interestingly, in *TP53* KO cells the EMT markers *Wnt5A* and *Cyr61* were reduced (Figure 3.11 C 1 and 2). For cell death and survival genes *Bcl2* was induced in all cells but most in *TP53 R175H* on the other hand *Bax* was affected mainly in *TP53* KO cells and to a lesser extent in *TP53* Wtres cells, while almost not affected in *TP53 R15H* cells (Figure 3.11 D). In conclusion, *HRAS* overexpression affected the expression of genes related to different oncogenic pathways in a differential manner.

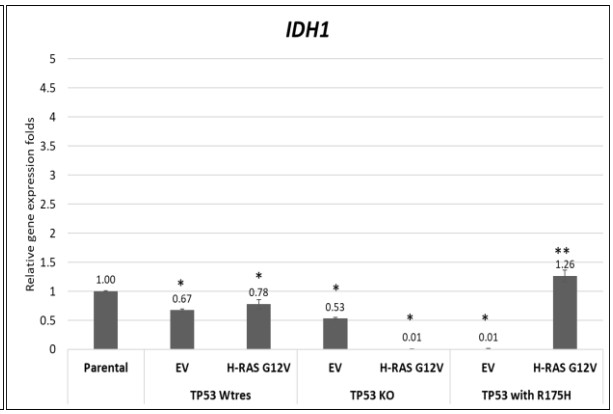
A. Cell proliferation related genes



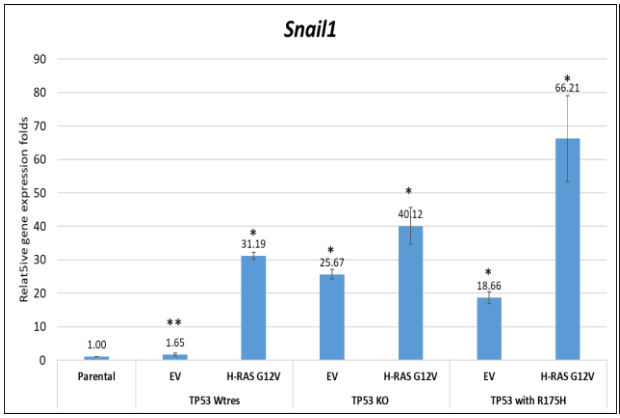


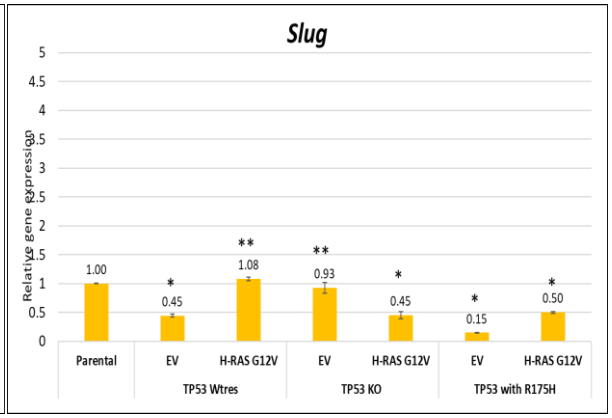
B. Glucose uptake and metabolism related genes



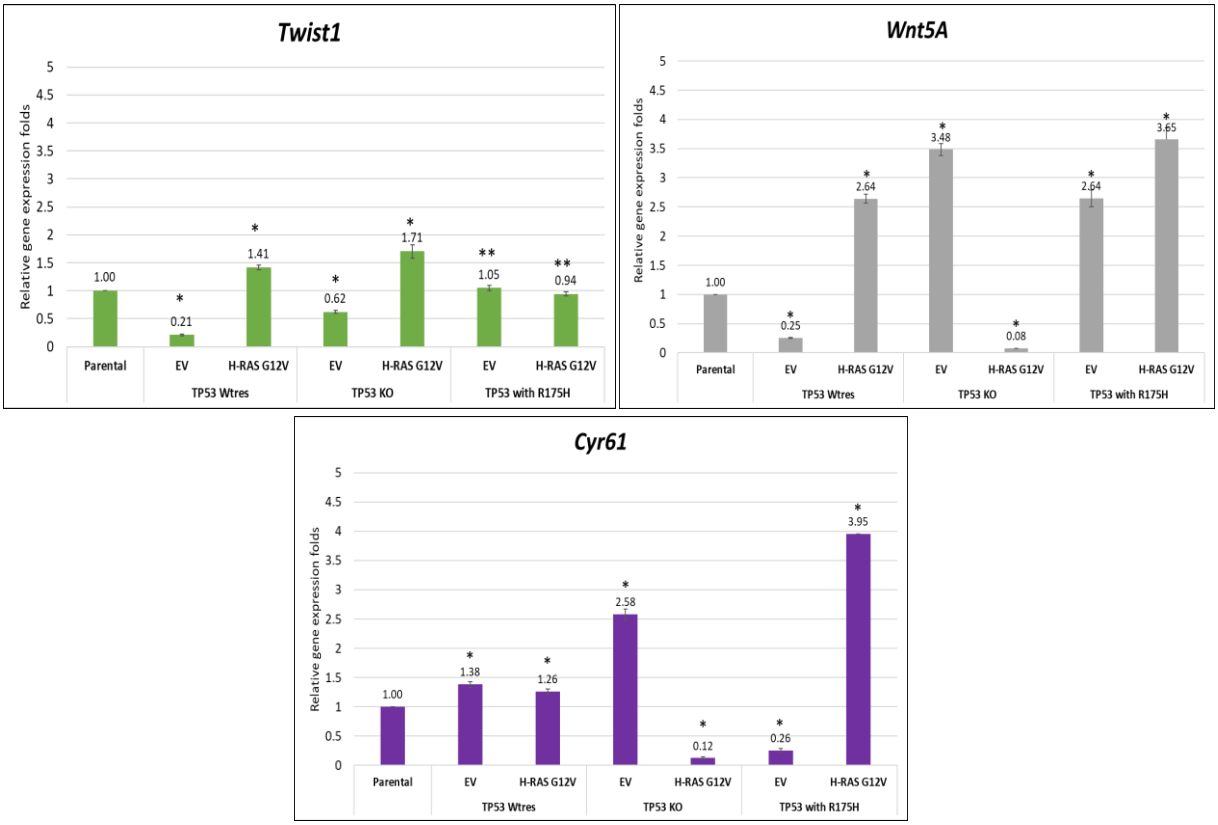


C.1 EMT related marker genes





C.2 EMT related marker genes



D. Cell death and survival related genes

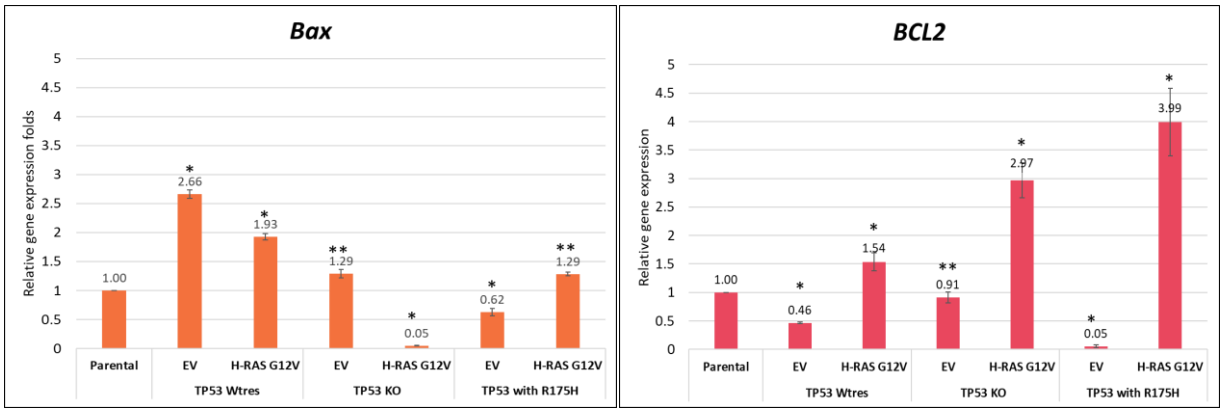


Figure 3.11: Genes expression related to different cancer phenotypes levels in transduced MCF10A cells. Real time PCR results showing the relative expression level of: (A) *p21*, *CyclinB1*, (B) *IDH1*, *GLUT1*, (C.1) *Slug*, *Snail1*, (C.2) *Twist1*, *Cyr61*, *Wnt5A*, and (D) *Bcl2*, *Bax*, relative to the house keeping gene *UBC*. All gene expression folds were calculated relative to the expression level in parental cells. Bars indicate the standard deviation of the mean of three different biological replicates. The statistical significance of the results was determined by measuring the p value. (** means that p-value>0.05 and * means that p-value <0.05).

Chapter 4

4. Discussion

Identification and understating of cancer transformation is a key stone in cancer biology. In the heart of cancer transformation is the interaction between tumor suppressor genes and oncogenes (Hanahan & Weinberg, 2011; L. D. Wood et al., 2007). Different studies described that the combination between RAS oncogenic activation and p53 inactivation occurs recurrently in many cancer types (Solomon et al., 2010; Zhang et al., 2013). RAS is in the center of upstream and downstream different signaling pathways that control various cellular functions in normal and cancer cells (Maertens & Cichowski, 2014; Myers et al., 2016). In breast cancer, different RAS family members , including *HRAS*, are involved in breast epithelia cells signaling pathways like P13K- AKT and RAF-MEK-ERK (Myers et al., 2016; Saini et al., 2013). *HRAS* plays a critical role in RAS-RAF-ERK signaling pathway, which is induced in different tumor types such as head, neck, skin and breast (Hobbs et al., 2016; McCubrey et al., 2007). p53 is a transcription factor that has different tumor suppressive functions in different types of cancer including breast cancer. p53 function is lost in most types of cancer, either by being deleted on the genomic level or by inactivating mutations (Walerych et al., 2012). Moreover, different p53 mutations not only inactivate p53 but can also lead to gain of oncogenic functions (Oren & Rotter, 2010). Oncogenic constitutively active *HRAS* (*HRAS G12V*) was shown to negatively regulate p53 tumor suppressive function (Ries et al., 2000).

As our preliminary data (not shown) suggests that different *TP53* variants have led to differential gene expression. We aimed to test the *HRAS G12V* transformation effect on breast cancer related phenotypes on MCF10A cells harboring different *TP53* variants *in vitro*. We stably transduced different variants of *TP53* into the *TP53* KO background to ectopically express wild-type and mutant versions of p53. We specifically used the p53 null background to overexpress mutant forms of the protein to circumvent dominant-negative effects that might occur in a Wt p53 background(Freed-Pastor & Prives, 2012). Using the MCF10A parental cells as additional controls we are thus able to distinguish dominant-negative from true gain-of-function activities of the different variants.

First, we validated the successful generation of our model. We found that all our cell lines expressed *HRAS* to the same extent except for cells expressing *TP53 R175H*, which showed a higher *HRAS* expression compared to other cells. This is an expected result since it was reported that p53 *R175H* increases *HRAS* expression through reduction of *HRAS* inhibitor BTG2 (Solomon et al., 2012). In fact, in order to show the expression of exogenous HRAS we should have used primers specific for G12V RAS. After measuring *HRAS* expression, we measured p53 expression using Western blot analysis. As expected the *TP53* KO cells showed no p53 expression at all. The *TP53* Wtres cells showed a low p53 expression compared to *TP53 R175H* cells. This variable expression pattern can be explained by the fact that Wt p53 is continually degraded by its E3 ubiquitin ligase MDM2. On the other hand, it is well documented that some p53 mutant variants are stabilized by escaping this post-translational regulation mechanism and accumulate in the cell(D. P. Liu et al., 2010; Shetzer, Molchadsky, & Rotter, 2016).

To study the effect of p53-*HRAS* combination on cancer cell hallmarks, we did different cancer transformation related functional assays. In concordance with our findings, different studies proved that *HRAS* overexpression enhances cell proliferation. For example, fibroblast cells with *HRAS G12V* in combination with *p27*KO exhibited higher proliferation and grew at high rate in an anchorage independent manner(Pellizzari et al., 2016). These fibroblasts produced tumors when were injected subcutaneously in nude mice (Pellizzari et al., 2016). In a further study, MCF10A cells with *HRAS* overexpression in combinations with wild-type p53 showed increased cell proliferation (Geyer et al., 2018). In our study, we did different cell proliferation assays on our different clones. Both direct and indirect cell proliferation assays did not give us reliable results and did not match with our eye observations. This was due to the fact that *HRAS* transformed cells form clumps that did not strongly adhere to cell culture surfaces and thus a big portion of the cells was washed away during the different proliferation assays. In future studies, we will try to overcome this technical obstacle by culturing the cells on either polylysine or gelatin coated cell culture plates. In agreement with our eye observations, it was clear from Matrigrl and soft agar assays that *HRAS* clones has higher proliferative index compared to non-transformed cells. Moreover, from the size of soft agar colonies and mammosphers generated by the cells in Matrigel assay, it was obvious that *TP53 R175H* cells have the highest proliferation rate. This is similar to what a previous study has described. It showed that breast cancer cells with *TP53 R175H* mutation gain oncogenic phenotypes including higher proliferation rates (Rieber & Strasberg Rieber, 2009). On the

molecular level, we tested some proliferation related genes and we found that *HRAS* overexpression lowered *p21* more in *TP53 R175H* cells compared to other cell lines. Indeed, this result is similar to previous results that showed that *TP53 R175H* lowers the expression of *p21* expression level in breast cancer (Rieber & Strasberg Rieber, 2009). In *cyclinB1* gene case, *CyclinB1* was increased more in *TP53* KO cells, which expected because p53 is an important regulator of cell cycle and prevents the transition between G2 and M phases by inhibition of *cyclin B1* expression (Hoffmann et al., 2011).

Another phenotype that changes with cell transformation is cell metabolism (Hanahan & Weinberg, 2011). Related to this phenotype, we tested the expression of two cell metabolism related-genes *GLUT1* and *IDH1*. *GLUTI* is the most widely expressed gene related to glucose uptake (I. S. Wood & Trayhurn, 2003). In breast cancer it was shown that GLUT1 knockdown correlates with low cancer invasiveness phenotypes including lower glucose uptake(Oh, Kim, Nam, & Shin, 2017). Regarding *IDH1*, *IDH1*gene encodes isocitrate dehydrogenase 1 enzyme, which is a metabolic enzyme in the Krebs cycle that convert isocitrate to α-ketoglutarate (α-KG) by using NAD(P)+. *IDH1* was found to be mutated in different cancer types including myeloid leukemia (AML), colon cancer , prostate cancer , lung cancer (B. Jiang et al., 2018) and breast cancer (Fathi et al., 2014; W. S. Liu et al., 2018). The mutated IDH1 converts α-KG to 2-hydroxyglutarate (2-HG) and that take place of isocitrate decarboxylation normal process. This results in 2-HG accumulation, which leads to cancer transformation through affecting histone methylation, DNA methylation and other cell events (Fathi et al., 2014). We noticed that *HRAS* overexpression raises *GLUTI* in both *TP53* KO and *TP53 R175H* cells. Whereas in Wtres cells this gene was downregulated when *HRAS* overexpressed. Which is expected because cancer transformation leads to more glycolytic dependent cell growth which ensures a higher glucose demand which is supplemented in cancer cells in part by increasing GLUT1 expression (Oh et al., 2017; I. S. Wood & Trayhurn, 2003).In support of our findings, a previous study described that *HRAS* overexpression leads to *GLUT1* upregulation by induction of H1F-1(Chen, Pore, Behrooz, Ismail-Beigi, & Maity, 2001). Also, another study showed that wild-type p53 downregulates GLUT1 expression level , while mutated p53 led to increase of *GLUT1* (Schwartzenberg-Bar-Yoseph, Armoni, & Karnieli, 2004). In comparison to GLUT1, we found that IDH1 expression is was drastically lowered in *TP53* KO cells which goes along with a more glycolytic growth phenotype. Interestingly and unexpectedly, *HRAS G12V* increased the expression of IDH1 in *TP53*

R175H cells, which ensures the essence of future investigations to try to understand how *R175H* mutation affects *IDH1* expression in breast cancer.

It is well established that cancer cell migration ability and EMT are associated with their metastatic potential. EMT and cell migration enable cancer cells to invade blood capillaries , circulate and survive in blood , integrate within secondary tissue and form colonies (Hanahan & Weinberg, 2011). In this study, we also tested the migratory capacity of our cells using wound healing assay. One more time, this assay did not show reliable results too, for the same difficulties we faced in cell proliferation assays as described above. However, luckily, in Matrigel assay, we were able to confirm our eye observations that indicated that *HRAS* transformation leads to a more migratory and invasive phenotype and that the strongest phenotype was clear in *R175H* cells. In addition, it was shown that *TP53 R175H* mutation induces migration and invasion when co-expressed with constitutively active *HRAS in vivo* and *in vitro* (D. Jiang et al., 2015). These findings meet with previous study results that showed that induction of *HRS G12V* increases MCF10A cell migration and invasion (Koh et al., 2016; Vuoriluoto et al., 2011), and that *R175H* mutation correlates with higher invasive phenotype (Kogan-Sakin et al., 2011).

p53 contributes to controlling different gene activity that are related to EMT, invasion and migration like *Slug*, *Snail* and *Twist* (Muller, Vousden, & Norman, 2011). Also, previous research demonstrated that oncogenic *RAS* activates EMT, induces migration and invasion by altering specific genes like, *Twist* (He et al., 2015) *Snail (*Horiguchi et al., 2009) *Slug* (Lamouille, Xu, & Derynck, 2014). In order to explain the invasiveness phenotypes on the molecular level, we tested different genes were shown to be affected by p53 and HRAS and related to cell migration, invasion and EMT. For this purpose, we tested the expression of *Slug*, *Snail*, *Twist1*, *Wnt5A* and *Cyr61*, which were shown to play very important roles in breast cancer progression (Fernandez-Cobo, Zammarchi, Mandeli, Holland, & Pogo, 2007; Huang, Lan, Lorusso, Duffey, & Ruegg, 2017; Vuoriluoto et al., 2011; Wang et al., 2016). Our results demonstrate that *TP53* variants showed differential expression of these markers and this can explain the differential migratory and invasiveness phenotype that were shown in Matrigel assay. *Snail1* was the most affected and the highest expression was observed in *TP53 R175H* cells. While, *Slug* and *Twist1* were not significantly affected in all cell lines, while remarkably, *Wnt5A* and *Cyr61* were reduced in *TP53* KO cells. Altogether, these results indicate that different p53 variants can induce the same invasive phenotypes but still by utilizing different gene repertoire.

Normal epithelial cells require adhesion to the extracellular matrix (ECM) and interaction with integrins to receive growth signals, proliferate and survive. When normal epithelial cells detached from ECM, they become unconnected to their environment and ECM depletion-dependent programmed cell death (anoikis) is induced (Kang & Krauss, 1996). Anchorage independent growth is an important hallmark of cancer transformation (Hanahan & Weinberg, 2011). Therefore, it reflects the tumorigenic potential and the aggressiveness of the cancer cells (Mori et al., 2009; Wise et al., 2016). In this study, soft agar assay showed that *HRAS* overexpression induces colony formation in comparison to MCF10A without *HRAS* overexpression. Moreover, we noticed that *R175H* mutation alone was sufficient to form small colonies. Previous studies demonstrated that that mutated p53 increases breast cancer cells survival and resistance to anoikis (Lim, Vidnovic, Ellisen, & Leong, 2009; Tan et al., 2015), while another study described that wild-type p53 increases normal mammary epithelial cells sensitivity to anoikis (Lim et al., 2009; Tan et al., 2015). Different studies mentioned that *HRAS* overexpression increases the potential for EMT, survival and cancer transformation (Datta et al., 2007; Geyer et al., 2018; Solomon et al., 2010). *TP53* KO and *TP53 R175H* in combination with *HRAS G12V* induced cancer related gene signature that is related to cell survival and formed tumors when injected to mice (Buganim et al., 2010). Because survival is controlled by the balance between apoptotic and anti-apoptotic genes, we tested the expression of the pro-apoptotic gene *Bax* and the anti-apoptotic gene *BCL2* (Chipuk et al., 2004). It was shown the accumulation of p53 in the cell lead to induce *Bax* (Chipuk et al., 2004) and that *BCL2* is downregulated in the presence of wild-type p53 (Nakazawa, Dashzeveg, & Yoshida, 2014; Ryan et al., 1994). Also, *HRAS* overexpression was shown to alter the levels of *BCL2* (Kinoshita, Yokota, Arai, & Miyajima, 1995) and *Bax (Figueiredo et al., 2012)*. Our analysis showed that *Bax* expression went down in all cells with *HRAS* overexpression, except in *TP53 R175H* cells where *Bax* levels were enhanced by *HRAS*. On the other hand, *BCL2* increased in all cells with *HRAS* overexpression and this in concordance with soft agar assay results.

5. Conclusion and future outlook

In this project, we were able to generate a proof of concept model that can be copied and extended to different other mutational combination models. These models can be employed to study common as well as model-specific mutations, find shared biomarkers and therapeutic targets for the diagnosis and treatment of breast cancer. The potential of this model is witnessed by our findings that clearly showed that although cancer cells share common phenotypes, these phenotypes are mediated by different gene expression pattern. Of course further analysis is needed in order to fully understand some of cell behaviour and the gene expression pattern related to them including *in vivo* studies.

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دراسة التحول السرطاني الناتج من تأثير ادخال جينات معدلة الى خلايا ثديية طبيعية

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الملخص

قمنا في هذا البحث بانشاء نماذج خلوية للتحول السرطاني التي من الممكن ان تحدث في سرطان الثدي ، من خلال ادخال جين*HRAS* (overexpressed)الى خلايا ثديية طبيعية تحتوي على نسخ جينية مختلفة من جين *TP53* .

بعد ذلك ، اختبرنا الاختلافات الناتجة من التحول السرطاني في النماذج التي قمنا بانشائها على مستوى الصفات الشكلية والتعبير الجيني لبعض الجينات المتعلقة بتلك الصفات. وتميزت تلك النماذج الخلوية بتغير واضح في صفاتها الشكلية وزيادة قابليتها للانتشار وقدرتها على التنقل ، ومقاومتها للموت الخلوي المبرمج . وتوضح هذه الدراسة دورًا مهمًا لاجتماع اكثر من طفرة جينية في التحريض على التحول السرطاني. وإجمالا ، نجحنا في توليد نماذج لدراسة التحول السرطاني لسرطان الثدي والذي بدوره قد يساعد في فهم سلوك خلايا سرطان الثدي وكيفية حدوث التحول السرطاني لها ، وتوظيفها في تحسين طرق الكشف والتشخيص والعلاج.