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Creating cellular model for studying novel mechanisms of transcriptional reprogramming in breast carcinogenesis

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Dedication

To my God who guides me to success, then to those who encouraged and helped me to fulfill my aims. The passion I have to the most precious parents mother and father who made the cloud rain all the love. True love and truth that does not change, to my brothers, sisters my precious family and my dearest friends especially Mohammad Shana'a and Ali Jahajha, to my supervisor Dr Zaidoun Salah and our lab team, to my beloved town " Dahiet Al Bareed", to my teachers in collage of Science & Technology especially Mr Raid Rezeq. To my dearest colleagues, to all those who helped me accomplishing the following thesis satisfyingly, to my beloved University; Al-Quds University, at last but not least to my beloved homeland, Palestine.

Yosef Sameer Ishaq Torman

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.....

Yousef Sameer Ishaq Torman

Date: 12 / 5 /2018

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I am glad to thank my father, mother and family who supported and encouraged me during this period.

List of abbreviations

°C	Celsius
Ml	Microliter
Min	Minutes
H	Hours
Nm	Nanometer
Ng	Nano gram
DMSO	Dimethyl sulfoxide
EGF	Epidermal growth factor
PBS	Phosphate buffer saline
RPM	Round per minute
OD	Optic density
SFM	Serum free media
Ml	Milliliter
MG	Matrigel
cDNA	Complementary DNA
RT-PCR	Real time- polymerase chain reaction
LB	Lysogeny broth
TF	Transcription factor
CpG	Cytosine phosphate guanine
EMT	Epithelial mesenchymal transition
CGS	Cancer related Gene Signature
Bp	Base pair
Wt	Wild type
RT-buffer	Reverse transcriptase buffer
EMC	Extracellular matrix

Abstract

Breast cancer is one of the most common and heterogonous cancer types, and the first cause of death related to cancer in women worldwide. Breast cancer is triggered by endogenous and/or exogenous factors. These factors lead to critical mutations and/or eipmutations in important genes including oncogenes and tumor suppresser genes. These genetic and epigenetic changes lead to cancer initiation and cancer progression. During these processes, cells gain alteration and dysregulation in gene expression at different levels. The most important and critical level of gene expression alteration in cancer is the transcriptional level.

Our current project is part of a larger project, in which we hypothesized that breast cancer transformation might have common transcriptional reprograming events, that are associated with misregulation in gene expression, which reflects on cellular activity and homeostasis. In this part of the project, the aim was to test the ability to generate a proof of concept breast cancer transformation model that can be used to study transcriptional reprogramming in breast cancer and identify specific TFs that can be used as biomarkers for diagnosis, prognosis or even treatment of breast cancer. An *in vitro* breast cancer transformation model using HRAS overexpression in immortalized non transformed normal epithelial mammary gland cells (MCF10A) was generated. After HRAS overexpression, different cell phenotypes were tested, known to be induced by HRAS overexpression, that in order to ensure successful transformation. the transformed cells were then tested (not by us in this part of the project), for transcriptional reprogramming. Using different techniques, the model, indeed, showed massive genome wide transcriptional re-programming. Among the different transcription site activities that were lost are transcription sites of p53 and p63. In order to evaluate the role of these transcription factors in this transformation model, the functions of these two important TFs (p53 by using Nutlin-3a, and p63 by its overexpression) were reactivated. Our results, showed that the induction of these TF functions was enough to revert to certain extent some the transformation process-related phenotypes.

In conclusion, our transformation model can be used as an efficient tool to learn about transcriptional re-programing during cellular transformation, to identify and study the role of specific TFs in transformation. This may contribute to identifying some target genes involved in breast carcinogenesis and employ them in prognosis, diagnosis and treatment.

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Chapter 1

1. Introduction

1.1. Cancer

Cancer is a multifactorial disease. It can be initiated by multiple exogenous and endogenous factors. Exogenous factors including physical factors, like ionizing or UV irradiations, chemical factors, like cigarette smoking and industrial waste products, and biological factors, like different types of viruses and bacteria. Endogenous factors include metabolic and hereditary risk factors. regardless the nature of the carcinogenic factor, it must cause critical mutations in oncogenes such as RAS and PIK3CA or tumor suppressor genes such as p53 and pRb or both (Ritchie et al., 2001). These mutations eventually induce tumorigenesis progression through diverse genetic and epigenetic alteration that drive the transformation of normal cells into malignant derivatives. This diversity results in different carcinogenic phenotypes and cancer subtypes (Johnson, Warmoes, Shen, & Locasale, 2015).

The morphogenesis diversity of cancer and the multistep transformation of normal cells to a neoplastic state are accompanied by eleven main hallmarks including, sustained proliferative state by reducing their dependence on exogenously derived signals in the normal tissue microenvironment, evading growth suppressors, resisting cell death, enabling replicative immortality inducing angiogenesis, activating invasion and metastasis, genome instability and mutation, inflammation, metabolic alterations evading immune destruction, tumors exhibit another dimension of complexity and deregulating cellular energetics (Hanahan & Weinberg, 2000, 2011).

Genetic alterations are mutations that occur in the gene sequence. These alterations might come in the form of deletions, insertions, or substitutions. these alterations might also affect chromosomes, resulting in either numerical or structural changes that include insertions,

deletions, inversion or translocation. While epigenetic alterations, are any change in gene expression that occur without altering the primary DNA sequence. like DNA methylation or histone modifications including; methylation, ubiquitination, phosphorylation, acetylation and sumolyation. All these cause adjusting gene expression profiles by chromatin –modifying enzymes that affect the binding pattern of transcription factors that will result in altering the expression pattern of other cancer cell genes (Jones & Baylin, 2007; Polak et al., 2015).

1.2. Breast cancer

Breast cancer is the most common cancer in women and a leading cause of cancer-related female deaths worldwide (Yue et al., 2017), Breast cancer, like other types of cancer is, a very heterogeneous cancer, with several molecular subtypes having variable profiles, which leads to difficulty in diagnosis and treatment (Banerji et al., 2012). Gene expression profile represents the most fundamental biological change that gives the apparent phenotype of cancer. Different gene expression regulatory levels that were studied in breast cancer and other cancer types (Curtis et al., 2012). However, the most critical level in the regulation of gene expression is the intermediate level, which include transcription (Yeh, Toniolo, & Frank, 2013).

1.3. Transcription

The most important process involved in gene expression regulation (T. I. Lee & Young, 2000) Transcriptional regulation in eukaryotic cells is very organized and complex. It involves a network of transcriptional factors, chromatin organization and chromatin-modifying enzymes, which reflects the genomic interaction with environment (Cox & Goding, 1991). During cancer initiation and progression, many genes become silent or active, which alter the expression of several proteins including transcription factors such as MYC and p53, co-factors, as well as histones and DNA modifying enzymes (Dawson, Kouzarides, & Huntly, 2012).

1.4. Transcription regulation

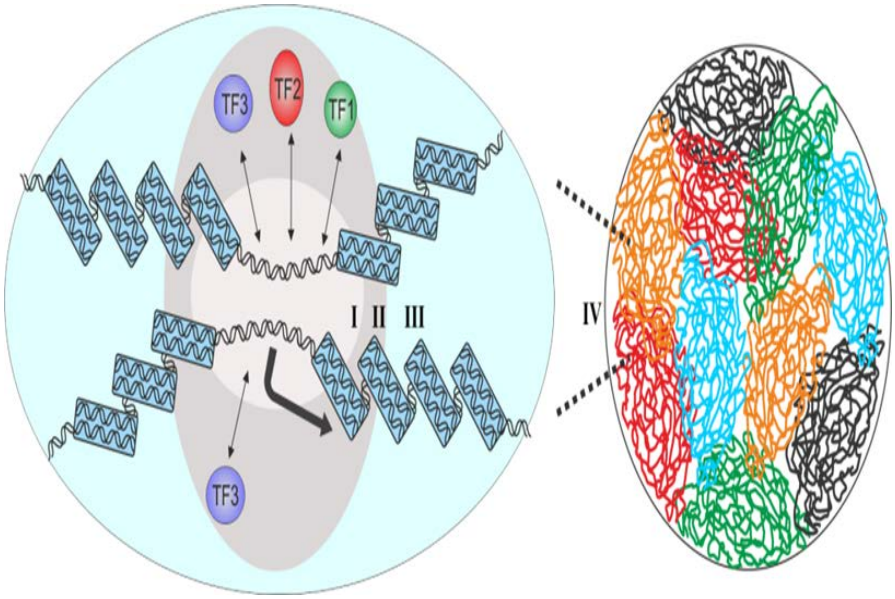

Figure 1.1 Graphical representation of the four transcription regulatory:
(I) Accessibility, (II) Binding, (III) Enhancer-promoter association, (IV) Genome high order organization (Salah et al, unpublished data)

1.4.1. Chromatin accessibility

Specific DNA sequence provides initial accessibility of DNA elements within active chromatin that permits TF binding. Moreover, accessibility to chromatin is also determined by modifications that takes place on histones or DNA itself. Histones are proteins on which DNA is wrapped to form the first level of chromatin organization; the nucleosome. In chromatin, there are different types of histones (H2A, H2B, H3 and H4). Different chemical modifications that take place on different amino acids results in differential chromatin accessibility (Karlic, Chung, Lasserre, Vlahovicek, & Vingron, 2010; Schneider & Grosschedl, 2007). For example, histone acetylation causes more accessible chromatin (Karlic et al., 2010). On the DNA level, methylation that takes place on CpG islands, makes the chromatin less accessible to TF, while methylation makes chromatin more accessible to these factors (Vinarskaja, Schulz, Ingenwerth, Hader, & Arsov, 2013).

1.4.2. Binding of transcription complex

Transcription initiation starts by binding of specific proteins, named general transcription factors, to the promoter or enhancer sequences of which target genes within the accessible chromatin, which leads to the assembly of pre-initiation complex with RNA polymerase to activate transcription.

The regulation of TF function tack placed on different levels including TF synthesis, modification and localization. which, TF levels can differ under different cellular physiological conditions. On the TF localization level, it is well documented that the function of different transcription factors is dependent on post translational modifications including phosphorylation, ubiquitination, sumoylation, etc. where, phosphorylation of some TFs leads to their cytoplasmic sequestration, or nuclear translocation (Guertin & Lis, 2013).

1.4.3. Enhancer-Promoter association

Enhancers are a specific segments of DNA, which are located few hundreds up to mega bases upstream from the promoter region of target gene. Enhancers can regulate transcription by creating a loop with the promoter after binding to specific TFs called the activators that can be closed by an insulator. Enhancers activity is regulated by histone modifications that affect the binding of TFs (Bulger & Groudine, 2011). For example, in the embryonic stem cells, master transcription factors, including Oct4 and Sox2 bind to an enhancer with a mediator to activate specific embryonic stem cell gene expression (Whyte et al., 2013).

1.4.4. Genome high order organization

The genome is organized into high-order complex structures by hierarchical DNA folding, which raises chromatin fibers, chromosome domains, and eventually chromosomes. The high-order organization of the genome significantly affects gene regulation and the control of gene expression program. The chromosomal regions can be dramatically repositioned. localizing

activated genes on the chromosome to be concentrated along the peripheral regions. while inactivated genes are located in the interior of the chromosomal territories (Schneider & Grosschedl, 2007). For example, it has been shown that in quiescent cells HOXB gene cluster is inactive because it is hidden in the interior regions of chromosome 17. Upon differentiation, the chromosomal territory that harbors this gene cluster is relocalized, an event that results in the active expression of this cluster (Chambeyron & Bickmore, 2004).

HRAS

Harvey Rat Sarcoma (HRAS) is a member of the RAS family that also contains NRAS and KRAS. It is classified as a small G protein. It has intrinsic GTPases activity, and plays important roles in cell growth, differentiation, and survival through the activation of different downstream signaling cascades (X. Chen et al., 2009; Fernandez-Medarde & Santos, 2011).

RAS activation is initiated by receptor Tyrosine kinases. These receptors have two subunits that have the ability to phosphorylate each other. When ligand binds to the extracellular domain of a tyrosine kinase receptor, the two subunits of receptor dimerise and get activated after phosphorylation. This activation is responsible for the binding of growth factor receptor-bound protein 2 (GRB2) protein to the cytosolic domain of the receptor tyrosine kinase. Afterwards, son of sevenless (SOS) protein binds to the GRB2. Followed by RAS binding to the complex, and its GDP is displaced by GTP in the presence of Guanine nucleotide exchange factor (GEF). This reaction transforms RAS to “on” state. When the initial signal disappears, the GTPase activity will hydrolyze the GTP to GDP and convert RAS to the “off” state as described in Fig 1.2 (Margolis & Skolnik, 1994).

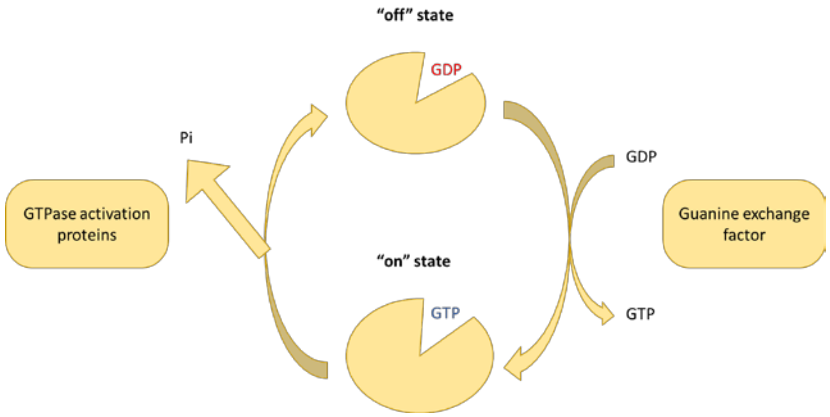

Figure 1.2 regulation of HRAS (a GTPase) activity.

Activation of RAS proteins subsequently stimulate different effector proteins that activate several signaling cascades within the cell. The three important downstream RAS pathway signaling cascades are described in Fig 1.3

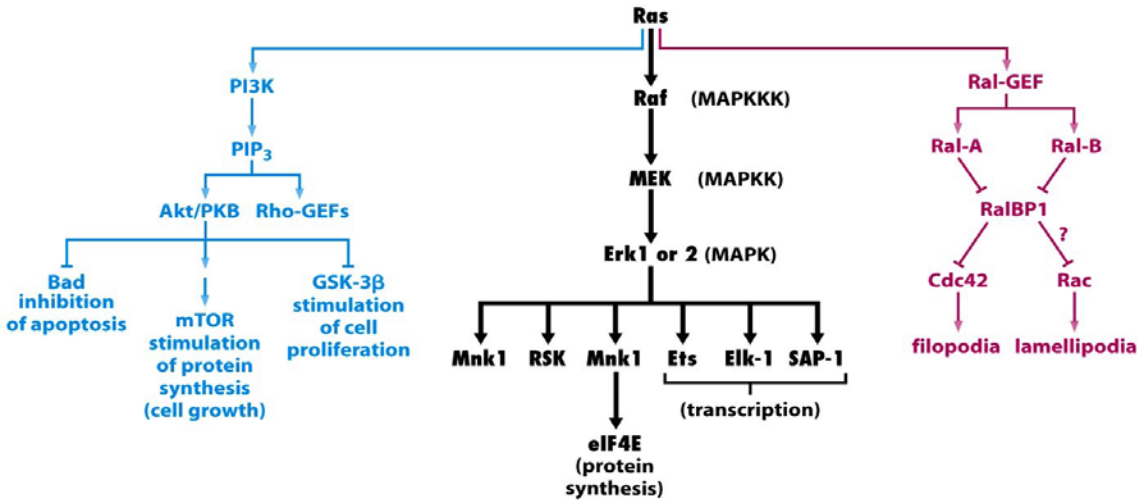

Figure 1.3 HRAS downstream cascade pathways (Weinberg, 2007).

Some mutations in the *RAS* gene prevent the GTPase to hydrolize GTP to GDP and therefore stabilizes RAS in the "on" state, converting HRAS to a constitutively active form. This event is one of the most common mutations associated with many types of human cancers. It is estimated that 30% of human tumors harbor a somatic mutation in RAS subfamily (Prior, Lewis, & Mattos, 2012). The most common mutations that lead to "on" state of RAS are substitution mutations in codons 12,13,61 and 146 of the *RAS* gene (Fernandez-Medarde &

Santos, 2011). Many studies have shown the capability of mutant RAS to transform normal cells to cancer cells both *in vitro* and *in vivo* systems. In one study, it was shown that the co-expression of HRAS G12V with LBX1 in MCF10A leads to various changes in these normal cell physiology. In this breast tumorigenesis model, the morphology of LBX1 + G12V HRAS infected cells were transformed to prominent elongated spindle-shaped cells. Analysis of mesenchymal and epithelial cell markers in these transformed cells showed a cooperative effect of LBX1 and G12V HRAS in inducing characteristic features of EMT. Furthermore, the ability of these two proteins to cooperate in generating tumors following subcutaneous inoculation into nude mice was tested and the results demonstrated that while MCF-10A cells expressing LBX1 or G12V HRAS alone developed tumors after 3 months, cells expressing LBX1 + G12V HRAS developed tumors within 1 month. In another study, it was shown that, when HRAS was co-expressed with the anti-apoptotic gene *Bim-1* (B cell-specific Moloney murine leukemia virus integration site 1) and cells were injected into mammary fat pad of female mice, cells showed accelerated tumor development (have big tumor in 1 month), compared to cells over expressing either HRAS or Bmi-1 alone (Datta et al., 2007).

P53

The p53 is a tumor suppressor protein that belongs to a larger family which also includes *p63* and *p73.* the *p53* gene is located on the short arm of chromosome 17 in position 13.1. and the p53 protein consists of 393 amino acids with four functional domains. The first domain is the transactivation domain (TAD). Subdivided into two subdomains, AD1 (residues 1-42) and AD2 (residues 43-63). The N-terminal domain can interact with many regulatory proteins such as the negative regulatory protein MDM2 that controls p53 levels, acetyltransferases p300 and CBP which act as co-activators to p53-mediated transcription, and stabilization of the p53-DNA C-terminus complex (C. W. Lee, Martinez-Yamout, Dyson, & Wright, 2010). The other domains, the core DNA-binding domains (DBD) (residues 94-292), which consists of two decameric half sites as a dimer, and contains the sequence 5′ – Pu Pu Pu C (A/T) | (T/A) G Py Py Py – 3′ (where Pu indicates a purine and Py indicates a pyrimidine) separated by 0 to 13 base pairs (el-Deiry, Kern, Pietenpol, Kinzler, & Vogelstein, 1992; Funk, Pak, Karas, Wright, & Shay, 1992). The two half sites form a tetramer in the oligomerization domain (residues

317- 353) (Ho, Fitzgerald, & Marmorstein, 2006), which confers flexible link with DNA-binding domain to give p53 the accessibility to bind with different DNA sequences resulting in transcriptional activation or repression for wide range of genes (Y. Chen, Dey, & Chen, 2010; Malecka, Ho, & Marmorstein, 2009). Finally, the C-terminus domain (CTD) (residues 353-393), acts as a negative or positive regulator for sequence-specific DNA binding domain. For example, p53 C-terminus domain binds with chromatin to access p21 promoter (Espinosa & Emerson, 2001; Fischbach et al., 2017).

Tp53 plays a critical role in modulating the expression of genes involved in cell cycle arrest and apoptosis. In stress conditions caused by DNA damage like UV, chemicals or viruses, or hypoxia, Tp53 stabilization leads to transcriptional modulating for different Tp53-response genes, which produce proteins that regulate a large number of signal transduction pathways and a number of auto regulatory feedback loops in the presence of p53 effect. One of the p53 target genes is *p21* which is a potent cyclin-dependent kinase inhibitor that causes cell cycle arrest. It is an important regulator of cell cycle transition from G1 to S phase (Tang et al., 1998), Other p53 target genes include BCL2 associated X (*BAX*) that acts as an accelerator for apoptosis (Miyashita & Reed, 1995), and Mouse double minute 2 (MDM2). Transcription of *MDM2* can be induced by binding of p53 or RAF to its promoters region (Ries et al., 2000). MDM2 is a major negative regulatory protein of p53, it binds to p53 and transfers it to the cytoplasm for degradation (Iwakuma & Lozano, 2003). A new chemical that was discovered recently, Nutlin-3a is used to increase the stability of p53 through binding to MDM2 (B. Wang, Fang, Zhao, Xiang, & Wang, 2012). p53 dysfunction or inactivation due to DNA mutations, is an important driver for carcinogenesis. Studies showed that p53 is mutated in more than 50% of human cancers (Espinosa & Emerson, 2001). Other studies showed that, the activation of the oncogene HRAS G12V and inactivation of the tumor suppressor p53 gene are strongly associated with cancer transformation (Solomon, Brosh, Buganim, & Rotter, 2010). In an *In vivo* study on HRAS activation and p53 inhibition, this combination activates EMT and increases stemness in urothelial progenitor cells (He, Melamed, Tang, Huang, & Wu, 2015). In another study, it was suggested that there is a cross talk between p53 and activated HRAS G12V. In this study, it was demonstrated that the B-cell translocation gene 2 (BTG2) binds to HRAS G12V and represses its activity by reducing its GTP loading state, which in

turn causes a reduction in cancer-related gene signature (CGS) expression. In addition, activating transcription factor 3 (ATF3) binds directly to the CGS promoters following p53 stabilization and represses their expression.(Buganim et al., 2010).

p63

This is a member of the p53 family located on the long arm of chromosome 3 at position 28. The protein is encoded by alternative promoters to yield two main isoforms. These isoforms either contain an N-terminal p53-homologous transactivation domain (TAp63) or lack this domain (ΔNp63)(Yang et al., 1998). Both TA and ΔN transcripts are alternatively spliced at the 3′ end to generate different C-terminal isoforms, called α, β and γ (Di Como et al., 2002; Ghioni et al., 2002), as explained in Fig 1.4..

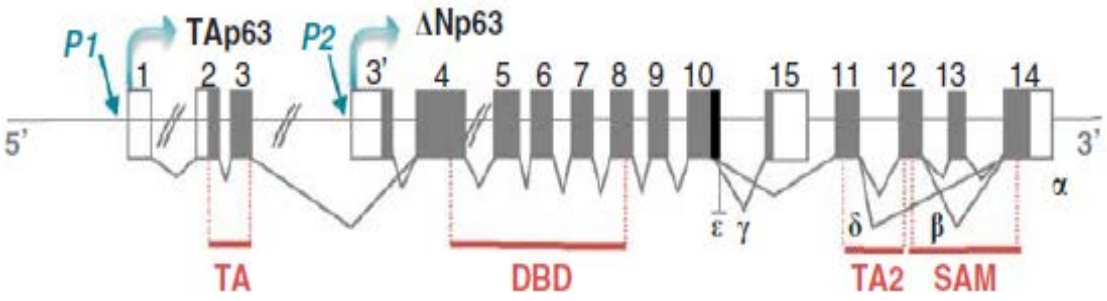

Figure 1.4 Human p63 isoforms. Exon schema and corresponding domains of the human Tp63 gene. Alternative promoter usage produces TA (transactivation) and N-terminally truncated (ΔN) isoforms, and alternative splicing produces C-terminal variants **(Melino, 2011)**

p63 has transactivation domain, DNA binding domain, and an oligomerization domain similar to that of p53, but are different in the C-terminal domain. The similarity in the domains produces redundancy in p53 and TAp63, which function in cell cycle arrest and cell death induction. The ΔNp63, has a critical role in cellular differentiation and epithelial development. Both isoforms act as inhibitors of cancer progression (Bergholz & Xiao, 2012; Jung, Qian, Yan, & Chen, 2013).

In our work, lentiviral particles a cellular transformation model by creating stable MCF10A clones that overexpress the oncogene HRAS was generated using lentiviral vectors. To confirm transformation, we tested different cancer cell hallmarks. Using this transformation model, we were able to identify important transcriptional reprogramming that involved many genes including *p53* and *p63*. In order to evaluate the role of these transcription factors in cell transformation, we reactivated the functions of these genes in our cell model and tested for the same cancer hallmarks that we tested before. Our results, showed that the reactivation of p53 and p63 was enough to revert to certain extent some the transformation process-related phenotypes.

1.5. Problem Statement and motivation of study

Cancer is a genetic disease. Mutations and epigenetic alterations result in altered specific gene expression programs as well as misregulation of cellular signaling and communication (Hanahan & Weinberg, 2011). While pathogenic genetic changes frequently affect the activities of oncogenes and desregulate tumor suppressor genes, epimutations mostly affect TF binding and as such gene regulatory modules. In conjunction, these events abrogate cellular homeostasis, resulting in transformation and ultimately tumor formation and metastasis (Northcott et al., 2014). Current efforts to characterize the pathways contributing to breast cancer development mostly focus on either the identification of upstream mutations (Cancer Genome Atlas, 2012) or downstream RNA and protein expression patterns of cancer cells (Curtis et al., 2012). However, demarcating the intermediate molecular events that trigger gene expression programs and holds great promise for generating novel and improved diagnostic tools and identifying relevant targets for therapeutic interventions (Yeh et al., 2013). This is especially invaluable for aggressive subtypes such as triple-negative/basal-like breast cancer which poses a major challenge in the clinic due to the current lack of effective targeted therapies (Mayer, Abramson, Lehmann, & Pietenpol, 2014).

This work is part of a comprehensive project that aims to find common transcriptional reprograming events that results from cancerous transformation. Here, we aimed to test our ability to generate an in vitro transformation model and use it in our research. The model can potentially be used to validate our findings from transcription regulation studies (Fig. 1.5).

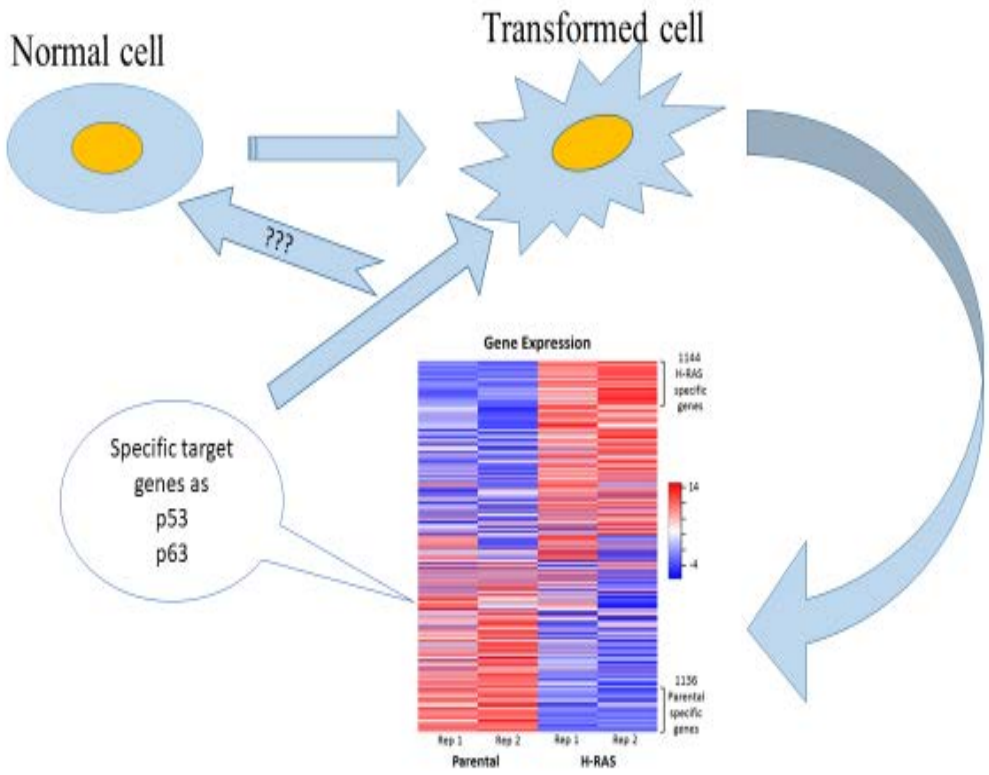

Figure 1.5 Our working model. Generation of a cellular model, its validation and evaluation of the role of specific TF in the transformation process. (Salah et al, unpublished data)

1.6. Hypothesis

Our current project is based on our hypothesis that cancer cell transformation that results from various molecular mechanisms must involve common transcriptional reprogramming events that can be used as biomarkers for early detection or as targets for therapy. Our preliminary data suggest that both p53 and p63 functions are lost in transformed cells. Thus our hypothesis

the loss of function of these genes is important for cell transformation and that the re-expression would at least partially reverses this transformed phenotype.

1.7. Objective of study

1.7.1. To generate an *in vitro* transformation model for breast cancer using a known HRAS mutation.

1.7.2. Validation of the system utility to reverse the transformation phenotype using TFs known to be involved in the transformation process.

Chapter 2

2. Methodology

2.1. Materials

Table 2.1-A: list of material used in methodology

No	Material	Manufacture
1	MDEM/F12 media	Biological industries
2	RPMI (1640) media	Gibco Thermofisher
3	Horse serum	Biological industry
4	Fetal bovine serum	Gibco Thermofisher
5	Hydrocortisone	Sigma
6	Insulin	Sigma
7	Epidermal growth factor (EGF)	Sigma
8	Cholera toxin	Sigma
9	Glutamine	Biological industries
10	Penicillin/streptomycin	Biological industries
11	Dimethyl sulfoxide	Sigma
12	Sterile phosphate buffer saline PBS	Biological industries
13	PWZL-hygro HRAS	Addgene
14	PWZL-hygro EV	Addgene
15	NOPLX305-TP63-HA-LRES-PURO-K4	Gift from Prof.Stefan Wiemann
16	GAG-pol plasmid	Addgene
17	VSV-G plasmid	Addgene
18	Mirus TransLTi	Mirus Bio
19	Ampicillin	Sigma
20	Puromycin	Sigma
21	Maxi prep kit	Qaigen
22	Mini prep kit	Qaigen
23	Q5® Site-Directed Mutagenesis Kit	BioLabs
24	XTT kite	Biological industries
25	Trypane blue	Biological industries

Table 2.1-B: list of material used in methodology

No	Material	Manufacture
26	Isopropanol biological gradient	Sigma
27	Ethanol biological gradient	Sigma
28	Chloroform biological gradient	Sigma
29	qScript™cDNA synthesis kit	Quanta Biosciences
30	SYBR® Green	Applied Biosystems
31	TRIZOL	Sigma
32	Nutlin-3a	Sigma
33	Polyvinylpyrrolidone-free polycarbonate filters	Costar Scientific
34	Diff-Quick System	Dade Behring
35	2-Hydroxyethyl Agarose	Sigma
36	MCF10A KO p53	Gift from Prof.Stefan Wiemann

2.2.Equipment and tools

Table 2.2:list of Equipment and tools used in methodology

No	Equipment or tool	Company	Industrial country
1	Inverted microscope	Olympus ck40-SLP	Japan
2	Biological hood (HERA guard)	Heraeus	Germany
3	Biofuge Stratos Reconditioned	Heraeus 75005289R	Germany
4	Biofuge Fresco	Heraeus 75005521	Germany
5	Hera cell 150 CO2 Incubator	Heraeus	Germany
6	Labofuge 200 centrifuge	Heraeus	Germany
7	Autovortex SA6	Stuart Scientific	U.K
8	Water Bath Orbital Shaking	Grant OLS200	U.K
9	Water Bath	Grant LTD6G	U.K
10	Ultracentrifuge	BECKMAN COULTER optima LE80H	U.S.A
11	SPIN-micropipette site	Nano Spinreact	china
12	Digital dry bath	Labnet	U.S.A
13	Elisa reader	BioTek EL-X800	U.S.A
14	Analytical Balance	METLER TOLEDO AB104	Switzerland
15	Autoclave	HIRAYAMA HV-110	U.S.A
16	RT-PCR	(Applied Bio-systems 7500 FAST Real Time PCR	Singafora
17	PCR machine 96 well	Applied Biosystem #9902	Singapore

2.3. Methods

2.3.1. Cell culture

2.3.1.1. Media preparation

MCF10A cells were grown in DMEM/F12 supplemented with 5% horse serum, 20 ng/mL EGF, 10 mg/mL insulin, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, 1% glutamine and 1% Penicillin/Streptomycin.

HEK293T cells were grown in RPMI (1640) supplemented with 10% fetal bovine serum, 1% glutamine and 1% Penicillin/Streptomycin.

Freezing media was prepared to contain 70% growth media, 20% fetal bovine serum and 10% DMSO.

2.3.1.2. Cell passage

To pass cells, old culture media was aspirated and cells were washed with 0.5 ml PBS X1. Afterwards, PBS was removed, and 1.0 ml trypsin was added. Then, part of trypsin was removed and incubated in CO_2 incubator at 37 ^{0}C until the cells detached from the plate. Finally, cells were mixed with the media and certain amount of cells was passed, depending on the experiment needs, and incubated in CO2 incubator at 37 ^{0}C.

2.3.1.3. Freezing cells

Cells were washed and trypsinized as mentioned above. When ready, cells were collected in freezing media that contain 70% growth media, 20% fetal bovine

serum and 10% DMSO. and transferred to cyrotubes, gradually frozen and finally stored in liquid nitrogen.

2.3.1.4. Cell thawing

To restore cells from liquid nitrogen storage, cryotubes containing the cells were immediately transferred from the liquid nitrogen tank to a 37 ^{0}C water path. After thawing, cells were transferred to a new conical tube containing 5 ml fresh medium. Afterwards, cells were resuspended in fresh media and plated.

2.3.2. Preparation of competent bacteria (DH5α)

To prepare chemically competent bacterial cells, one DH5α colony was grown in 250 ml LB media until the OD for a 1/5 dilution at 600 nm is between 0.04- 0.08. Cells were centrifuged at 4000 RPM for 10 min and the cell pellet was re-suspended in 50 ml of 0.1 M $CaCl_2$ and incubated on ice for 15 min. After that, cells were centrifuged for 10 min at 4000 RPM at 4 ^{0}C. Finally, the pellet was resuspended in 10 ml of 0.1 M $CaCl_2$ containing 350 µl DMSO, split into Cryotubes, and immediately frozen in liquid nitrogen.

2.3.3. Bacterial transformation using heat shock

Competent bacteria (DH5α) tube was taken from liquid nitrogen and directly incubated on ice for 15-30 min, then 50 µl of DH5α was put in cold Eppendorf tube. Then 2-10 ng of plasmid was added to the tube. After that the tube was incubated on ice for 30 min. After incubation the tube was quickly transferred and incubated at 42 ^{0}C in water bath for 1min. Then the tube was returned to ice for 5 min. Finally, the bacteria (DH5α) was grown in LB agar plate with selection antibiotic for the plasmid and was cultured in incubator at 37 ^{0}C for 24 h.

2.3.4. Lentivirus preparation

Lentivirus particles were prepared by a three-plasmid expression system, in which 293T cells were cotransfected with the following three vectors: packaging (CMVΔR8.91), envelope (CMV-VSV-G), and transfer vector. One day before transfection, 293T cells were plated to 60% confluency. The next day, cells were fed with fresh medium and transfected with the three plasmids using transfection reagent (Mirus TransLTi). Medium was changed 24 h after transfection. On days 2 and 3 after transfection, medium was collected to recover excess viral particles. The collected medium was filtered and kept at -80 °C.

2.3.5. Infection and selection

To prepare cell clones harboring the gene of interest, lentivirus particles were added to cells and incubated with them for 48 h in CO_2 incubator at 37 ^{0}C. After two days, the infection media was changed with fresh media containing the appropriate selection antibiotic. The selection process was continued until complete death of control cells that were not infected. Control cells for HRAS and p63 clones were cells infected with empty vector (EV) of the viral vector backbone (pwzl-hygro for HRAS and NOPLX305 -HA-LRES-PURO-K4 for p63).

2.3.6. Primer design

Primer 3 software was used (http://primer3.ut.ee/) for Primers design and used in our research as listed in table 2.3.

Table 2. 1: list of our primers.

No	gene	AN	Primer	AT
1	*HRAS*	NM_001130442.2	F.P 5'- tgccatcaacaacaccaagt-3' R.P 5'- agccaggtcacacttgttcc-3'	53 ^{0}C
2	*p21*	NM_001291549.1	F.P 5'- cgtcaaatcctccccttcct-3' R.P 5'- atgggttctgacggacatcc -3'	50 ^{0}C
3	*BAX*	NM_001291428.1	F.P 5'- ggttgtcgccctttctact-3' R.P 5'-aagtccaatgtccagcccat -3'	54 ^{0}C
4	*p63*	NM_003722.4	F.P 5'- acaggaagacagagtgtgct-3' F.P 5'- catccctccaacacaactgc-3'	53 ^{0}C
5	*HUBC*	NM_021009.6	F.P 5'- gtcgcagttcttgtttgtgg-3' R.P 5'-gatggtgtcactgggctcaa-3'	53 ^{0}C

2.3.7. Site direct mutagenesis

To generate G12V mutant plasmid, we did site directed mutagenesis of the wt HRAS in the PWZL-hygro HRAS plasmid using QuikChange XL Site-Directed Mutagenesis Kit from Agilent, according to manufacturer instructions. Afterwards, the reaction product was used to transform DH5α cells. Then this transformation reaction was cultured on LB plates containing ampicillin as a selection marker for successful transformation. To search for successful mutant palsmids, 10% of growing colonies were grown in 5ml liquid LB media containing ampicillin. From these cultures plasmid was prepared using NucleoSpin kit, according to manufacturer instructions. These plasmids were then sent for Sanger sequencing to ensure mutagenesis of the specific site.

2.3.8. Plasmids extraction by Maxiprep

After transforming the bacteria with the needed plasmid, one colony of transformed bacteria was grown in 250 ml liquid LB containing the selection antibiotic and grown at 37 ^{0}C for 16-18 h. later, bacteria collected and plasmid was prepared using Maxiprep Kit from Invitrogen according to manufacturer instruction.

2.3.9. Cell count

$3x10^{4}$ cells were seeded in 6 well plate in triplicates, after 1 day of incubation (D0) the culture media and the adherent cells were collected in 15 ml tubes. The growth media was collected to count floating cells, while adhering cells were collected to assess for differences in cell count. After centrifugation at 1200 RPM for 10 min, the supernatant was removed and cells were resuspended in a specific volume of media. After complete resuspension of cells, we put 10 µl of the homogenous supernatant in champers slide and counted the cells.

2.3.10. XTT test

2 $X10^{3}$ cells were seeded triplicate in 96 well plate. for 24, 48, and 72 h later, cell proliferation was measured for 3 days using the XTT test, according to the manufacture's instructions.

2.3.11. Wound healing assay

$25x10^{4}$ cells/well were seeded in 12 well plate wells to form a 100% confluent layer. One day after the layer was wounded using the 10µl pipet tip and monitored for wound healing at 0 h and 24h in minimal growth factor medium.

2.3.12. Survival assay

200 and 400 cells were seeded in triplicates in 6 well plate. The media was changed every 3-4 days until colonies were visible to the naked eye. Then the media was removed and wells were washed with PBS X1, aspirated and wells were left to dry. After drying, cells were fixed using absolute methanol for about 15 min. Afterwards, wells were left to dry and then stained using Coomassie blue. Finally, the stain was removed and wells were washed using tap water.

2.3.13. Matrigel invasion assay

Blind well chemotaxis chambers with 13-mm-diameter filters were used for this assay. Polyvinylpyrrolidone-free polycarbonate filters, 8-mm pore size, were coated with basement membrane Matrigel (25 µg/filter). Briefly, the Matrigel was diluted to the desired final concentration with cold distilled water, applied to the filters, and dried under a hood. Cells (2×10^5 to 3×10^5), were suspended in DMEM containing 0.1% bovine serum albumin (BSA), were added to the upper chamber. Conditioned medium of 3T3 fibroblasts was applied as a chemoattractant and placed in the lower compartment of the Boyden chamber with the addition of EGF (20ng/ml). Assays were carried out at 37 °C in 5% CO2. More than 90% of the cells attached to the filter after incubation overnight. At the end of the incubation, the cells on the upper surface of the filter were removed by wiping with a cotton swab. The filters were fixed and stained with Diff-Quick System. Cells from various areas of the lower surface were counted and each assay was done in triplicate.

2.3.14. Soft agar assay

The assay media is composed of an upper and a lower layer. lower layer (2 ml) is composed of MCF10A media containing 0.6% 2-Hydroxyethyl Agarose. After pouring the lower media in 6 well plates, the plates were incubated at 4 ^{0}C for 1 h. Then $2x10^4$ cells were seeded at the upper layers (1.2 ml) for each well. The upper media contained

MCF10A media with 0.3% 2-Hydroxyethyl Agarose. After adding the upper layer, plates were incubated in CO_2 incubator until the point where colonies were able to be seen by naked eye. The top layer was changed every 3-4 days.

2.3.15. 3D culture assay

Matrigel was incubated on ice overnight, then 3000 cells were seeded on a solidified layer of growth factor reduced Matrigel measuring approximately 1–2 mm in thickness. The cells were grown in an assay medium containing 5 ng/ml EGF and 2% Matrigel then incubated in CO2 incubator at 37 ^{0}C.

2.3.16. RNA Extraction

$90x10^4$ cells were seeded in 10 cm plate. After 24 h of incubation the old media was removed and the plate was incubated on ice for 1min. Then 1 ml Trizol reagent was put to each plate and incubated on ice with shacked for 5 min. After that the plate was washed well with Trizol reagent and collected in RNase free tube with incubation on ice. Then 200 µl chloroform was added to each tube with shacked well and incubated on ice for 15 min. After incubation the tube was centrifuged at 12000 RPM for 15 min on 4 ^{0}C. After centrifugation the first supernatant layer was transferred to RNase free tube and 500 µl cold isopropanol was added with mixed well then incubated in ice for 15 min. After incubation we centrifuged on 4 ^{0}C at 12000 RPM for 15 min. After that we decanted the supernatant and put 500 µl 70% Ethyl alcohol. Then we centrifuged on 4 ^{0}C at 12000 RPM for 10 min. After centrifugation we decanted the supernatant and dry the pellet for 1 min. Then resuspended the pellet in ultra-pure water and incubated in dray bath at 60 ^{0}C for 5 min. Finally, we checked the RNA by measured the concentration and run on gel electrophoresis.

2.3.17. cDNA synthesis

1 µg of RNA samples were used to synthesis cDNA, mixed with 4 µl RT buffer, and 1µl enzyme in a PCR tube and completed the volume to 20 µl by added ultra-pure water. Then was transferred the tube to PCR machine to synthesis cDNA at specific program according to manufactures instructions.

2.3.18. RT-PCR

1 µl of primer (10 µM), 3 µl of cDNA 1 µg/µl (1:10) was diluted, 6 µl ultra-pure water and 10 µl SYBR® Green were put in each well of 96 well RT-PCR plate. Then the seal was put on the RT-PCR plate and the plate was centrifuged for 5 min. Finally, the plate was put in RT-PCR machine, and was worked in the program of table

Table 2.4: RT-PCR program for SYBR® Green mix

Pre-conditioning	activation	Denaturation	Anneal/Extend
50 ^{0}C/2 min	95 ^{0}C/10 min	95 ^{0}C/15 sec	60 ^{0}C/1 min
		40 cycles	

2.3.19. polyhema Coating and Anoikis assay

Polyhema was prepared by dissolving 12 g in 1 L of 95% ethanol, and incubated at 60 ^{0}C overnight, finally the mixture was filtered by 0.2 µm filter. Then, enough amount was put in the plate to coat the plate surface, and was left overnight in the hood until completely evaporated, then the plate was stored at 4 ^{0}C until use. To assess for cell death through Anoikis, we plated 50X10^3 cells in 12 well plate wells coated with Polyhema. 4 days later, cells were collected from the wells and counted manually using Trypan blue exclusion assay to differentiate between dead and living cells.

Chapter 3

3. Results and discussion

3.1. Generation of HRAS G12V

To generate hot spot mutation from wt HRAS, we used site directed mutagenesis to covert codon 12 of the HRAS gene from glycine to valine in pwzl-hygro HRAS plasmid. Sequencing results showed that we had successfully converted wt to V12 as shown in Fig 3.1.

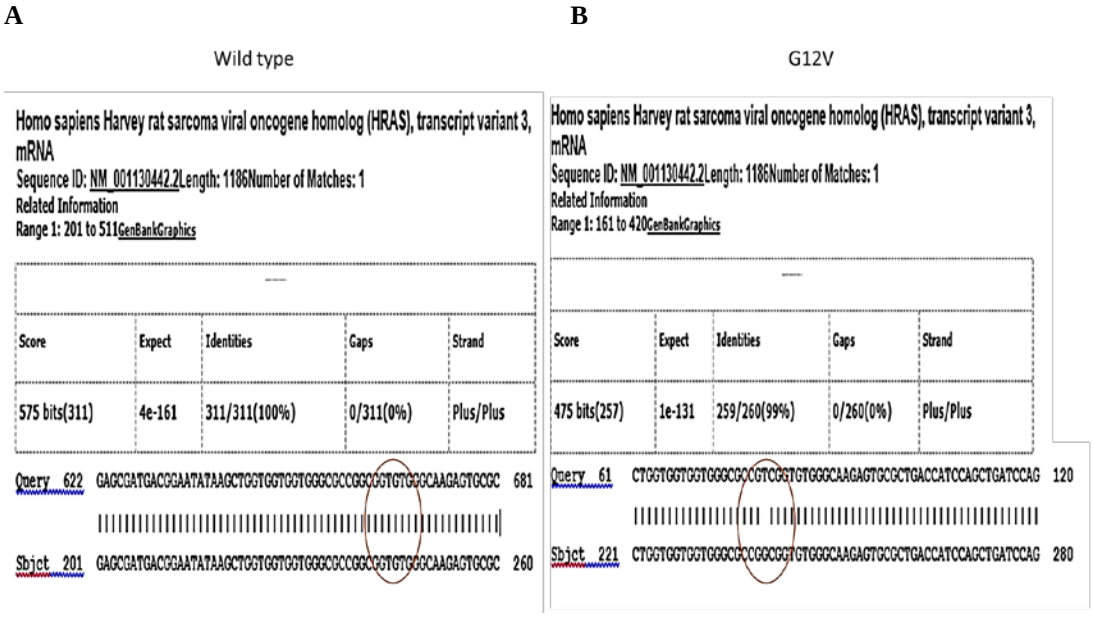

Figure 3. 1 Alignment between pwzl-hygro HRAS plasmid and *HRAS* gene sequence by BLAST tool. Sequencing results where (A) HRAS Wt and (B) HRAS G12V.

3.2. Validation of HRAS overexpression in HRAS clones.

Our first aim in this project was to create a transformed cellular model harboring known HRAS mutation (G12V). To generate HRAS MCF10A clone of cells overexpressing HRAS, we infected MCF10A cells with either lentiviral vectors either harboring HRAS or just the empty viral vector (EV, to be used as control). 48 h post infection, we did clone selection

using the selection marker (antibiotic resistant gene) present in the viral vector backbone (pwzl-hygro plasmid). Selection was completed after 3-4 days using100 µg/ ml hygromycin.

To validate HRAS overexpression in our HRAS clones, we did RT-PCR using HRAS specific primer (table 2.3). As shown in Figure 3.2 our HRAS clone expressed about 7 folds HRAS mRNA relative to control (EV) cells.

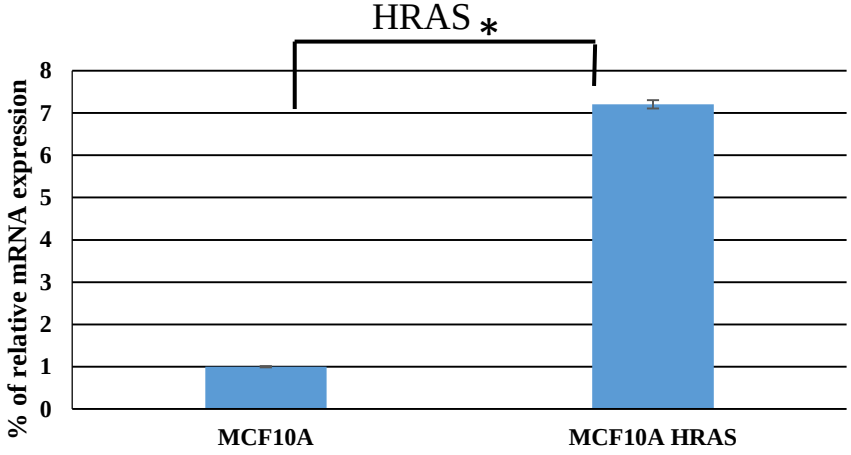

Figure 3.2 Expression level of HRAS in MCF10A clones. Real time PCR results on mRNA extracted from the cells indicated in the graph. Error bars represent the standard error of the mean of three experiments done in triplicates. to defined the significances different between MCF10A and MCF10A HRAS cells by T-test to calculate *p-value.* (** means that *p-value>0.05 and * means that p-value <0.05),* to a significant results the *p-value* <0.05.

3.3. Phenotypic changes in MCF10A cells with HRAS induction.

To make sure that the transformation process was succeeded, different tumor hallmarks related to HRAS function were tested.

3.3.1. Effect of HRAS overexpression on cell proliferation

G12V is a hot spot mutation in *HRAS* gene that results in cancer cells in uncontrolled cell proliferation. To test the effect of HRAS on MCF10A cell proliferation, we used XTT test. As shown in Fig 3.3 HRAS clones showed higher proliferative index compared to control cells.

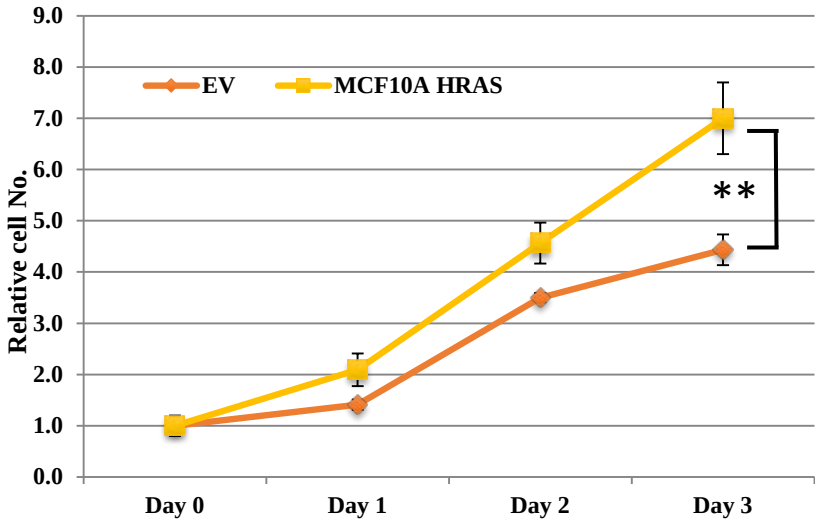

Figure 3. 3 HRAS increases cell proliferation of MCF10A cells. Representative graph showing the relative proliferation rate of HRAS compared to control cells using XTT assay. Cell growth was monitored over three days. Cell were assessed in triplicates per each time point. Y axis represent the relative cell proliferation index related to day zero. Bars represent standard error of the mean of three readings per point. to defined the significances different between MCF10A and MCF10A HRAS cells by T-test to calculate *p-value.* (** means that *p-value>0.05 and * means that p-value <0.05),* to a significant results the *p-value* <0.05.

Previous *in vitro* and *in vivo* studies showed that over expression HRAS harboring G12V increases proliferation in transformed cells, exactly similar to what we got in our experiments. One of these studies showed that fibroblast cells (IMR90, MEF, and REF52) that were infected with HRAS harboring G12V, the proliferation rate was accelerated compared to control cells (Serrano, Lin, McCurrach, Beach, & Lowe, 1997). In another *in vivo* study, it was shown that HRAS transformed MCF10A can create tumor within three months after injection into mice (Datta et al., 2007). These studies indicate that HRAS has a role in the transformation of normal cells to cancer, whereas prevents the GTPase activity and allow the proliferation downstream signaling pathways to be on all the time.

3.3.2. HRAS induces higher cell survival in MCF10A cells

Throughout tumorigenesis, cancer cells acquire the capacity of being autonomous, ie. independent of external signals to grow and survive. In order to check for this capacity in the HRAS clones, cell survival assay was used. Here, cultured cells with a very low number of cells in cell culture plates to prevent cell-cell communication. As shown in Fig 3.4, mutant HRAS clones have a higher survival index in comparison to control cells. Of note HRAS clone colonies were larger in diameter that of control cells indicating also a higher proliferative rate in these cells.

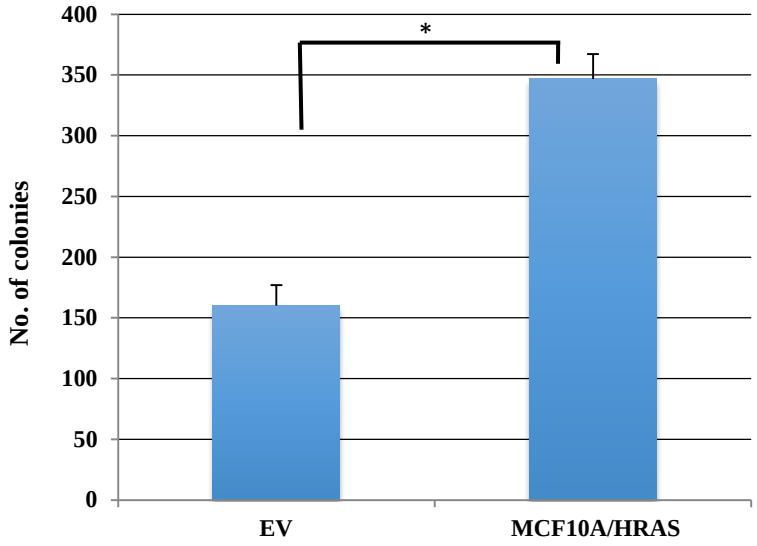

Figure 3. 4 HRAS transformed MCF10A clones have more survival index. Representative statistical analysis of the survival rate of HRAS transformed cells measured using cell survival assay. Experiments were done in triplicates and the bars represent the standard error of the mean. to defined the significances different between MCF10A and MCF10A HRAS cells by T-test to calculate *p-value.* (** means that *p-value>0.05 and * means that p-value <0.05),* to a significant results the *p-value* <0.05.

This result is consistent with HRAS function to induce and stimulate downstream signaling pathways without need to extracellular signaling pathways activation. These results show that each cell has the ability to grow and create colony without the need for cell-cell interaction, a phenomenon that was connected to HRAS transformation (Datta et al., 2007; Yu et al., 2009). On the molecular level, different studies have

shown that HRAS induces the cell survival by activating the PI3K or the RAF-MEK-ERK pathways, which play a critical role in promoting cell survival and proliferation (McCain, 2013; Rajalingam, Schreck, Rapp, & Albert, 2007).

3.3.3. HRAS overexpression in MCF10A induces cell tumorigenicity *in vitro*

One of the phenotypes that cancer cells acquire during transformation process is their ability to grow and survive independent of the extracellular matrix (in suspension). To that in the MCF10A transformed cells, we did soft agar assay (one of the most popular techniques used to test for transformation). Was done as expected, HRAS overexpression in MCF10A lead to the formation of many colonies in soft agar compared to non-transformed control cells (Fig 3.5).

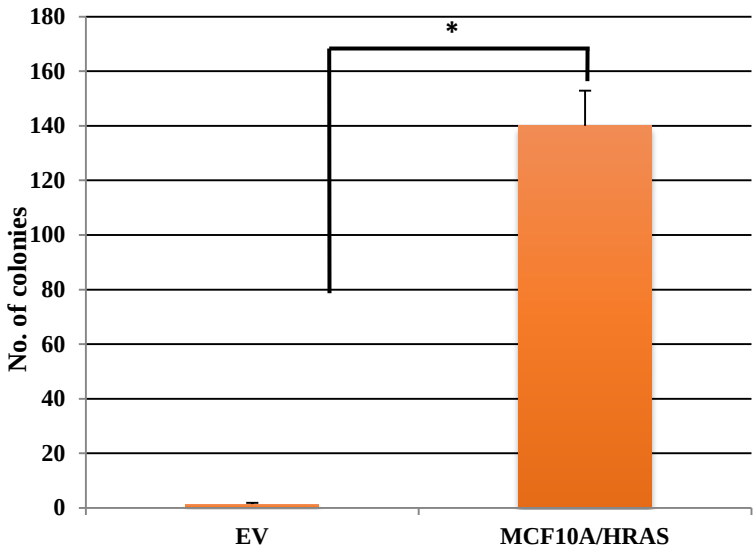

Figure 3. 5 HRAS transforming in MCF10A clones have more ability to create colonies. the figure show the number of colony created in agar layer. Experiments were done in triplicates and the bars represent the standard error of the mean to defined the significances different between MCF10A and MCF10A HRAS cells by T-test to calculate *p-value*. (** means that *p-value>0.05 and * means that p-value <0.05),* to a significant results the *p-value* <0.05.

Similar to our results, different previous studies have shown that overexpression of HRAS lead to tumorigenesis *in vitro* (Datta et al., 2007). In one study, it was shown

that primary human mammary epithelial cell transformation with HRAS and co-expression of SV40 large-T antigen and *hTERT* genes increased tumorigenesis as measured by soft agar assay (Elenbaas et al., 2001).

3.3.4. HRAS transformation of MCF10A cells lead to Anoikis resistance

Anoikis is a form of programmed cell death (apoptosis) that results from growing adherent cells in suspension. In cancer, resistance to this type of cell death is extremely important for cancer cells to survive in the blood stream during invasion and metastasis, which is a cancer hallmark (Hanahan & Weinberg, 2011). To elucidate whether HRAS overexpression leads to gaining this important cancer hallmark, cells were depleted from their interaction with the extracellular matrix (ECM) by culturing them on plates coated with polyhema. To differentiate between dead and living cells, we used Trypan blue exclusion assay. Upon calculating the percentage of dead cells, we noticed that around 80% of control cells died after growing for 48 in suspension, while only around 30% of HRAS cells died over the same period of time (Fig 3.5).

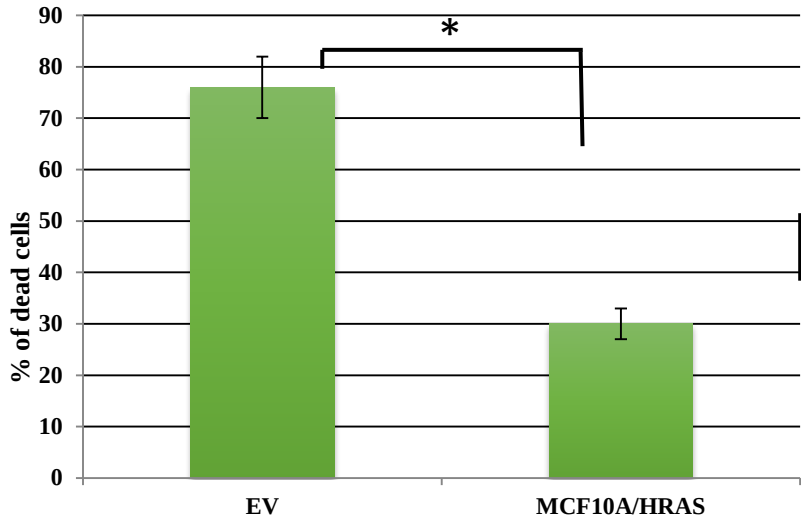

Figure 3. 6 Anchorage independent cell growth of HRAS MCF10A clones. The figure shows the percentage of dead cells cultured on polyhema coated plates. different between MCF10A and MCF10A HRAS cells by T-test to calculate *p-value.* (** means that *p-value>0.05 and * means that p-value <0.05),* to a significant results the *p-value* <0.05.

In order to resist Anoikis, epithelial cells must loose their epithelial identity and become more mesenchymal. Indeed and in concordance with our findings, previous studies proved that HRAS is capable of transforming epithelial cells into mesenchymal cells that are able to resist Anoikis (Makrodouli et al., 2011; Yu et al., 2009). This HRAS induced phenotype was shown to be related to the activation of different signaling pathways including RAS–RAF–MEK–ERK and PI3K pathways. (Rajalingam et al., 2007).

3.3.5. HRAS induces invasiveness in MCF10A cells

The transformation of epithelial cells by oncogenes like HRAS leads to the acquisition of mesenchymal phenotype that usually has the ability to migrate and invade ECM. To test whether our clones are also more invasive, we measured their invasive potential using Boyden chamber Matrigel invasion assay. In comparison to non-transformed

control cells, HRAS clone cells invaded the Matrigel coated filter as observed in Fig 3.7.

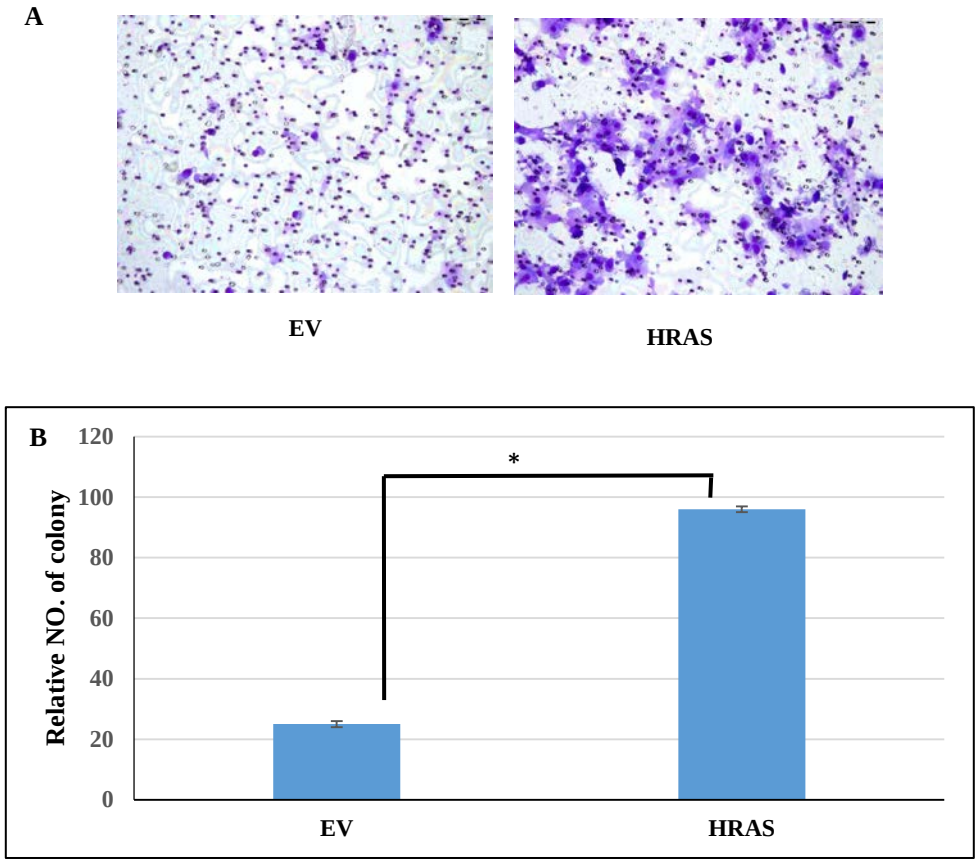

Figure 3. 7 Invasive phenotypes of HRAS clones. A: Representative micrograph showing cells that were capable of invading Matrigel coated filters in Boyden chamber Matrigel invasion assay. Invading cells were fixed and stained with Diff-Quick System Kit. Experiments were performed in triplicates. B: quantitative representation of the data in A. Bar Bars represent standard error. different between MCF10A and MCF10A HRAS cells by T-test to calculate *p-value.* (** means that *p-value>0.05 and * means that p-value <0.05),* to a significant results the *p-value* <0.05.

This result shows that HRAS overexpression in MCF10A leads to cell transformation from epithelial to a more mesenchymal phenotype (capable of forming colonies in soft agar, and more resistant to Anoikis). (Thus it was expected that these observation clones will be more migratory and more invasive). This, similar to many previous studies that have shown HRAS increases cell invasiveness *in vitro* (Datta et al., 2007) by inducing EMT (Makrodouli et al., 2011), Moreover, it was shown that HRAS G12V

induces EMT and the invasive phenotype of MCF10A by activating PI3K pathway and its downstream effector protein Rac1 (Shin, Kim, Song, Kim, & Moon, 2005).

3.4. p53 stabilization in MCF10A HRAS clones using Nutlin-3a was capable of reversing the tumorigenic phenotype of HRAS clones.

As mentioned above, one major goal of this research project is to assess how transformation results in transcriptional reprogramming. Upon testing the pattern of motif binding on the whole genome level, our results revealed that the binding motif of p53 is the most prominent in the regulatory loci that were lost and associated with down-regulated genes upon HRAS expression (Salah *et al*, not shown, unpublished data). To test whether induction of p53 function would be sufficient to rescue cell phenotypes induced by HRAS transformation, small-molecule antagonist of MDM2 Nutlin-3a, was used to restore p53 protein function. Of note Nutlin-3a is known and wildly used in research to restore p53 function (Drakos et al., 2011; Drakos et al., 2007).

3.4.1. Nutlin-3a inhibits cell proliferation and induces cell death in MCF10A-HRAS cells.

Uncontrolled cell proliferation and resistance to cell death are major phenotypes acquired throughout cancer transformation. As shown above, HRAS induces cell proliferation and decreases cell sensitivity to death. To test whether p53 function induction reverses these phenotypes in HRAS transformed cells, direct cell count and XTT assays after inducing p53 by using Nutlin-3a were evaluated. As shown in Fig 3.8 and 3.9, respectively, induction of p53 decreased cell proliferation and increased cell death. To further prove the effect of p53 reactivation on cell proliferation, and to show that the effect of Nutlin-3a is mediated through p53, we used XTT proliferation assay on both WT and KO p53 MCF10A cells. As shown in Fig 3.10, the activation of p53 resulted in reduced cell proliferation rate compared to untreated cells. As expected this effect was not observed upon using p53 KO cells, showing that Nutlin-3a effect is

through p53. Altogether, these results prove that HRAS mediates its effect on cell proliferation and cell death by inactivating p53 function.

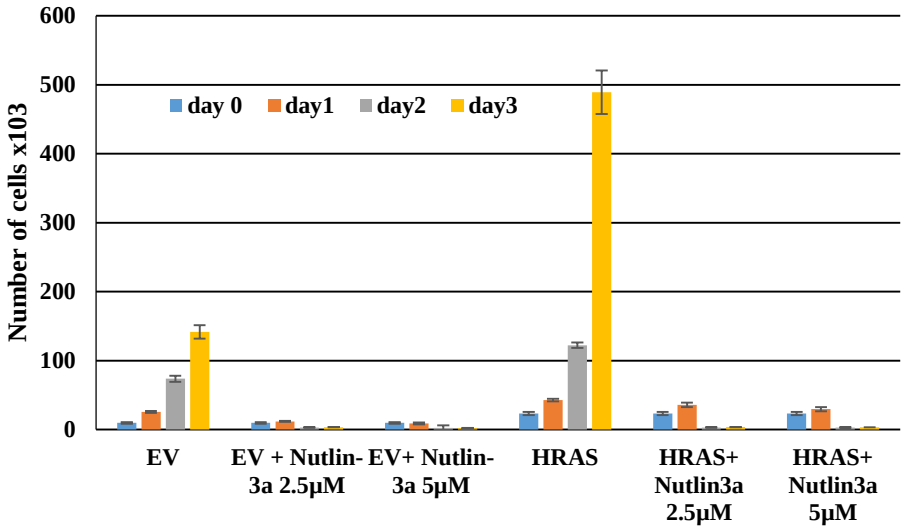

Figure 3. 8 Nutlin-3a reverses HRAS effect on cell proliferation. Representative graph showing the relative proliferation rate of HRAS with and without Nutlin-3a treatment using cell count assay. Cell growth was monitored over three days. Cell were assessed in triplicates per each time point per each Nutlin-3a concentration. Bars represent standard error of the mean of three readings per point.

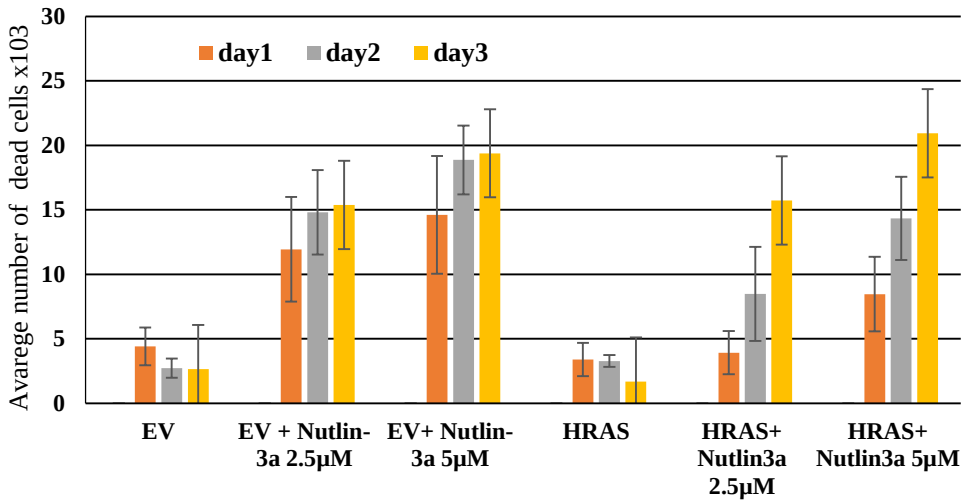

Figure 3. 9 Nutlin-3a reverses HRAS effect on cell death. Representative graph showing the relative proliferation rate of HRAS with and without Nutlin-3a treatment using cell count assay. Cell growth was monitored over three days. Cell were assessed in triplicates per each time point per each Nutlin-3a concentration. Bars represent standard error of the mean of three readings per point.

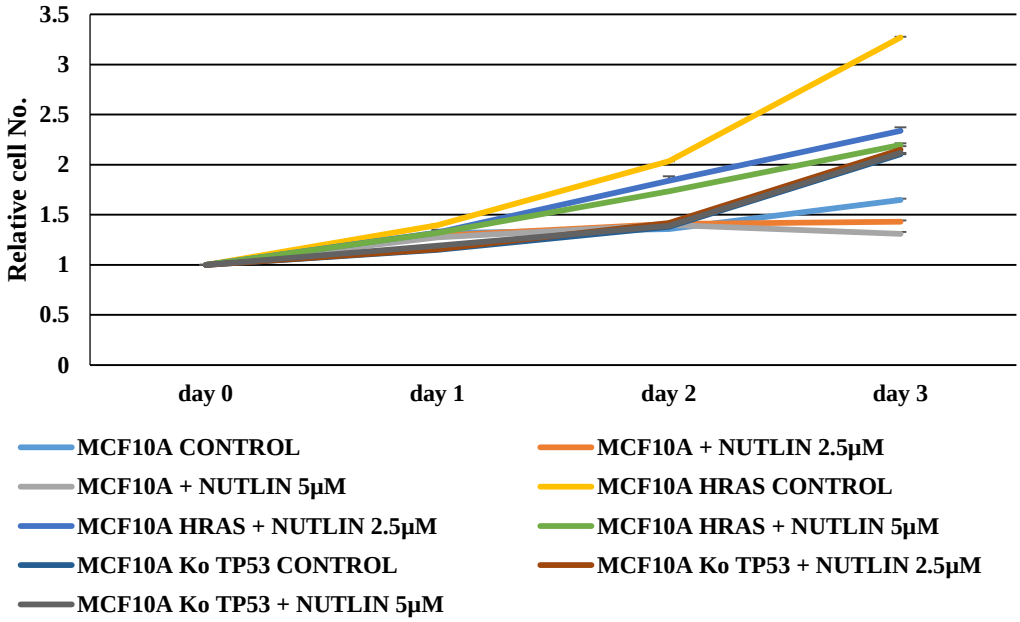

Figure 3. 10 Nutlin-3a reverses HRAS effect on cell proliferation through p53. Representative graph showing the relative proliferation rate of HRAS with and without Nutlin-3a treatment on WT and KO p53 cells, using cell count assay. Cell growth was monitored over three days. Cell were assessed in triplicates per each time point per each Nutlin-3a concentration. Bars represent standard error of the mean of three readings per point.

P53 is a tumor suppressor gene which is inactivated in many types of cancer, including breast cancer (Espinosa & Emerson, 2001). Thus, in addition to our not shown unpublished data that demonstrates that p53 regulatory sites are one of the mostly lost upon HRAS transformation, an expected, the reactivation of p53 in HRAS clones would rescue some of the tumorigenic phenotypes gained upon HRAS transformation. And indeed, our data here shows that p53 activation was enough to rescue some of HRAS oncogenic phenotypes. Moreover, it was published that Nutlin-3a was able to inhibit cell growth and induce apoptosis by increasing BAX and PUMA, and also leads to cell cycle arrest by increasing p21 expression in diffuse large B-cell lymphoma, of course as a result of p53 stabilization in these cells (Drakos et al., 2011).

3.4.2. Nutlin-3a inhibits cell migration of both MCF10A and MCF10A-HRAS cells.

One of the phenotypes that accompany cancer transformation is gaining of cell migratory phenotype. Above, we have shown that HRAS increase cell migration and invasion (Fig 3.7). Here we wanted to test the effectiveness of Nutlin-3a on HRAS induced cell migration. To do so, we did cell migration assay on cells with, or without Nutlin-3a treatment. Of note, in this assay, Nutlin-3a was added for a short period of time to ensure that the effect on cell migration is not as a result of cell death because of Nutlin-3a treatment. As shown in Fig 3.11. While in HRAS transformed cells were able to close the wound, Nutlin-3a-treated cells didn't. These results show that HRAS induces this phenotype by inactivating p53 and that reactivation of p53 is enough to reverse this phenotype.

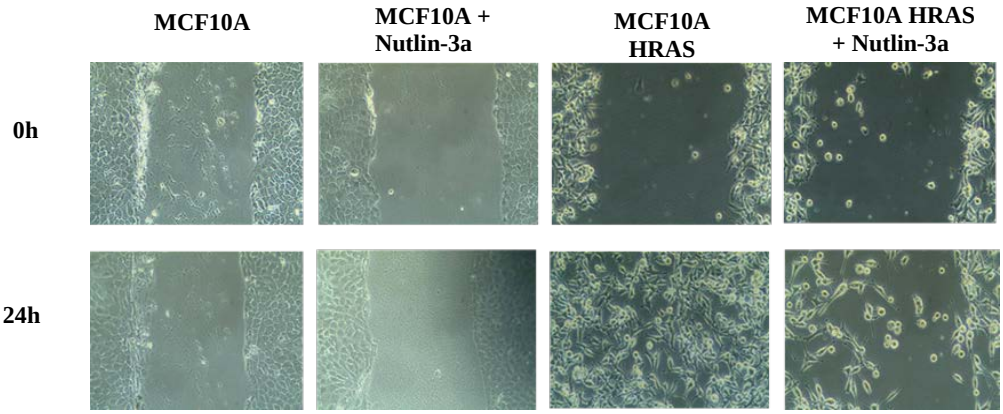

Figure 3. 2 Nutlin-3a reverses HRAS effect on cell migration through p53. Representative images showing the migration capability of HRAS with and without Nutlin-3a treatment on cells, using wound heling assay. Cell growth was monitored for one day.

Our results like previous studies showed the capability of HRAS to confer migration for transformed cells (Fig 3.10). On one previous study showed that Nutlin-3a not only induced cell cycle arrest and apoptosis, but also decreased migration capability by inhibiting the *RhoA* and *Rac1* expression in p53 wt human cancer cells, these two gene induce the lamellipodia formation, this formation proved the capability of cells to migration (Moran & Maki, 2010).

3.4.3. Nutlin-3a inhibits cell survival of both MCF10A and MCF10A-HRAS cells

More cancer cells resistance to variant conditions that leads to death were seen in HRAS transformed cells Fig 3.4. To test the effect of p53 reactivation on this phenotype we conducted cell survival assay on Nutlin-3a treated and untreated cells. In this assay, we were able to show that p53 activation is capable of reversing cell survival phenotype induced by HRAS transformation (Fig 3.12). In conclusion, this result shows that, while HRAS enhances cell survival, Nutlin-3a was able to reverse this acquired cancer hallmark. This finding, here is supported by previous studies that showed that Nutlin-3a decreased colony formation in DoHH2, MCA, OCI-LY3 and EJ cells (Drakos et al., 2011).

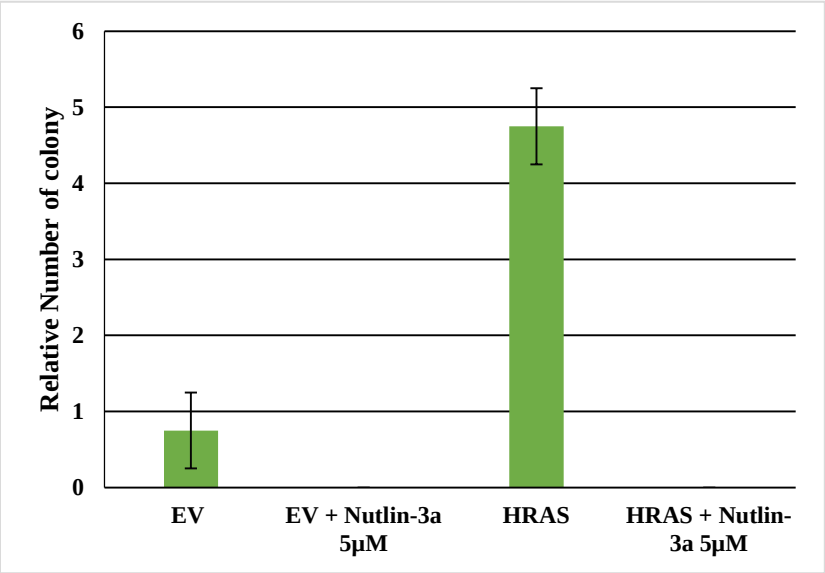

Figure 3. 3 p53 activation reverses cell survival phenotype induced by HRAS transformation. Representative statistical analysis of the survival rate of HRAS transformed cells with or without Nutlin-3a treatment as measured using colony formation assay. Experiments were done in triplicates and the bars represent the standard error of the mean.

3.4.4. Nutlin-3a reverses HRAS effect on mammosphere formation in 3D culture

Disruption of cellular morphogenesis and organization is a hallmark of transformation. As we discussed above, HRAS transformation of MCF10A cells changed normal cell growth phenotypes. One of the normal cell growth phenotypes of MCF10A cells is their ability to form mammospheres in 3D cell culture setup. To test whether Nutlin-3a is capable of reversing the effect of HRAS overexpression on mammosphere formation, we cultured transformed and non-transformed MCF10A cells in Matrigel 3D cell culture with and without Nutlin-3a treatment. Because Nutlin-3a leads to cell death, we used very low Nutlin-3a concentration for limited time to avoid cell death. This assay too, we were able to show that p53 can reverse the effect of HRAS transformation on mammosphere formation *in vitro*. As shown in Fig 3.13, MCF10A cells formed, as expected, very well organized mammospheres that resembles mammary gland cell growth *in vivo* (top most panel). Also, even though, smaller in size, MCF10A cells formed mammospheres after Nutlin-3a treatment (second panel from the top). On the other hand, upon transformation with HRAS, MCF10A cells

failed to form these regular mammospheres. On the contrary, these HRAS clones formed very big irregular masses of cells that lacked any organization (third panel from the top). However, upon Nutlin-3a treatment, HRAS clones were able to successfully grow in a very well organized mammospheres that recapitulated the normal growth of MCF10A before transformation (lower most panel).

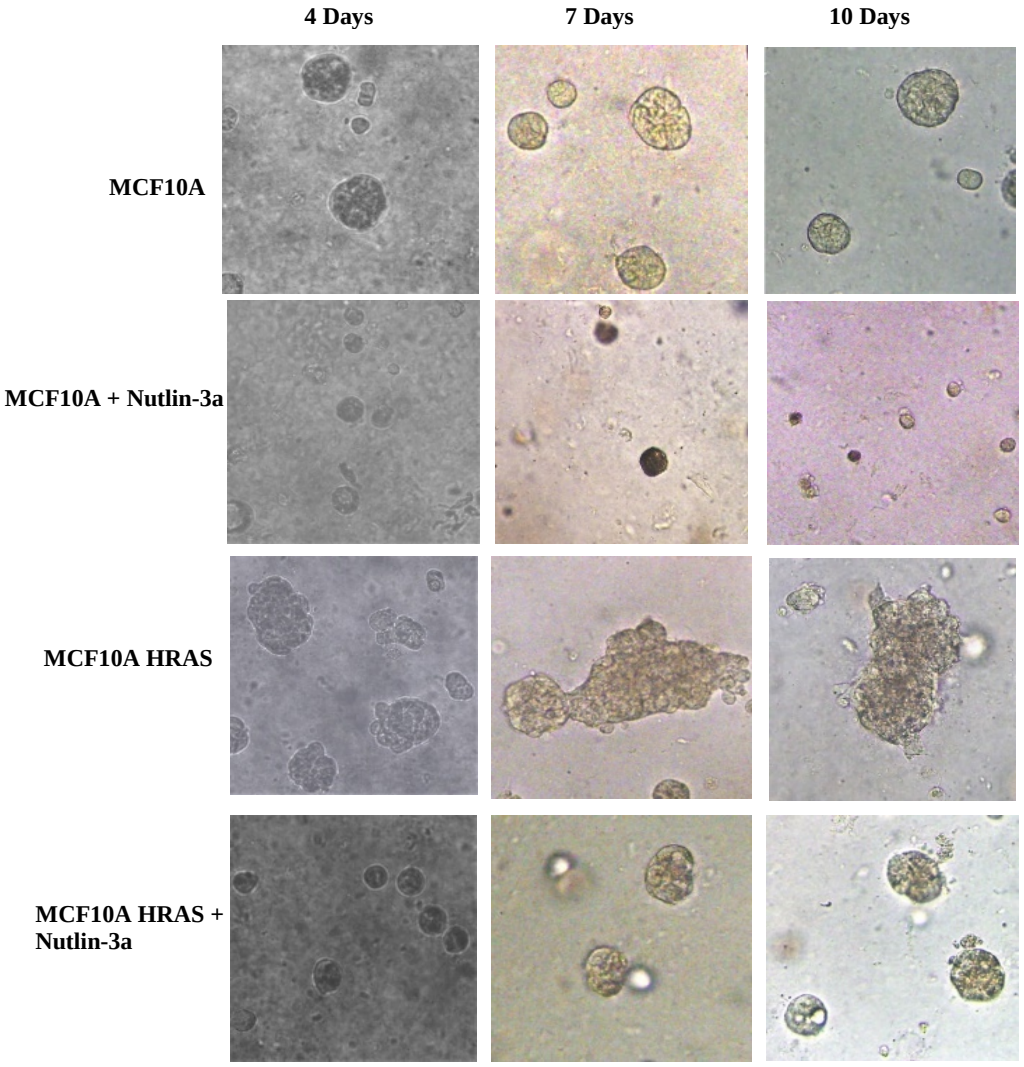

Figure 3. 4 p53 activation effect on mammosphere formation induced by HRAS transformation. Representative images at 20X shown the colony organization in 3D of HRAS with and without Nutlin-3a treatment on cells, using 3D culture assay. The mammosphere formation was monitored for 10 days.

These results show that HRAS leads to the formation of disorganized clusters of MCF10A cells without central lumen formation and loss of polarity. This is in

concordance with a previous study which showed that HRAS transformation blocks normal luminal differentiation and dysregulated morphogenesis (Yu et al., 2009). Moreover, our results show that Nutlin-3a, by stabilizing p53, reverses the mammosphere organization to normal. This indicates that p53, by activating downstream pathway(s), is capable of reversing the imbalance of cellular hemostasis in the context of normal tissue development.

3.4.5. Nutlin-3a effect on gene expression of p53 target genes

To show that the genomic activity of p53 is rescued by Nutlin-3a, we measured the expression level of p53 direct target genes, *p21* and *BAX* by RT-PCR with specific primers that shown. This regard, our results show that while HRAS transformation was able to reduce *p21*, and as expected (Y. Wang et al., 2013) increase BAX level. Nutlin-3a was able to induce their *p21* expression about 4 folds and server induce *BAX* level (Fig. 3.14 A&B). Overall, our gene expression results here, might explain some of the phenotypes induced by HRAS overexpression, like resistance to cell cycle arrest. BAX is a pro-apoptotic protein that when activated leads to cell death (Chipuk et al., 2004), while p21 is a cyclin dependent kinase inhibitor that leads to cell cycle arrest (Kachnic et al., 1999). Moreover, our data here prove that cell physiology reprogramming is dependent in part on transcriptional reprogramming that results from attenuating the function of important transcription factors like p53,

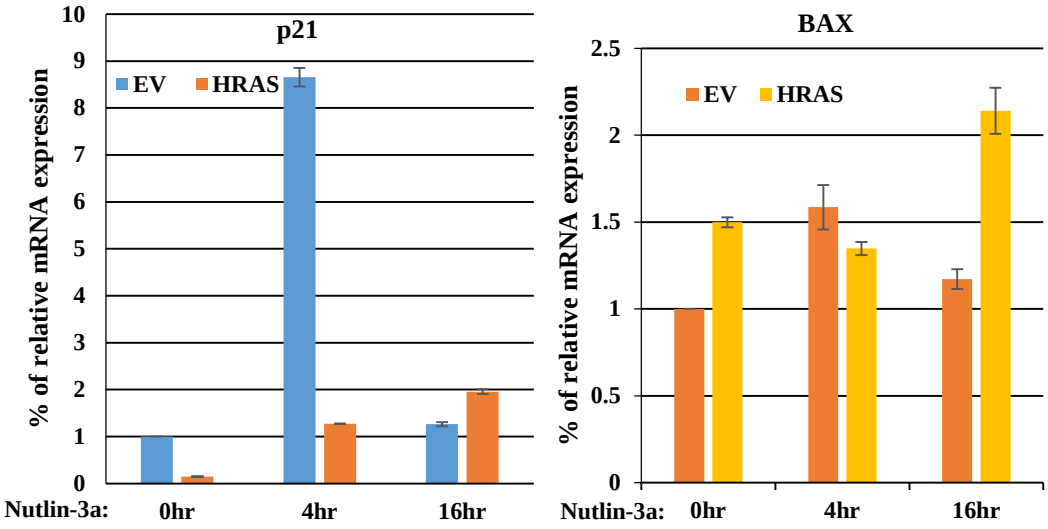

Figure 3. 5 Nutlin-3a rescues genomic p53 function. Real time PCR showing the effect of Nutlin-3a on the expression of p53 target genes; *p21* and *BAX* in HRAS transformed cells. In all figures, error bars represent the standard deviation of at least three different biological experiments done in triplicates.

3.5. Infected MCF10A HRAS with p63

Throughout searching for the differential expression of different transcription factors between control and HRAS transformed cells, p63, a p53 homologue, was among the highly affected transcription factors (Salah et al un published data) and Fig3.15. Also , it was reported before that HRAS overexpression in MCF10A cells represses p63 transcription (Yoh et al., 2016).

In order to evaluate the role of p63 in HRAS transformation, we generated MCF10A HRAS clones that overexpress p63 using a lentiviral vector using NOPLX305-TP63-HA-LRES-PURO-K4 as transfer plasmid then selected by 1µg/µl Puromycin for 4-5 days. In order to test for the successful generation of these clones, we did RT- PCR using p63 specific primer (table 2.3) and *hUBC* one of the internal housing keep gene control. As our RT- PCR results show in Fig.3.15, indeed we were able to generate p63 clones that express ≈ 4 folds more *p63* mRNA relative to control cells.

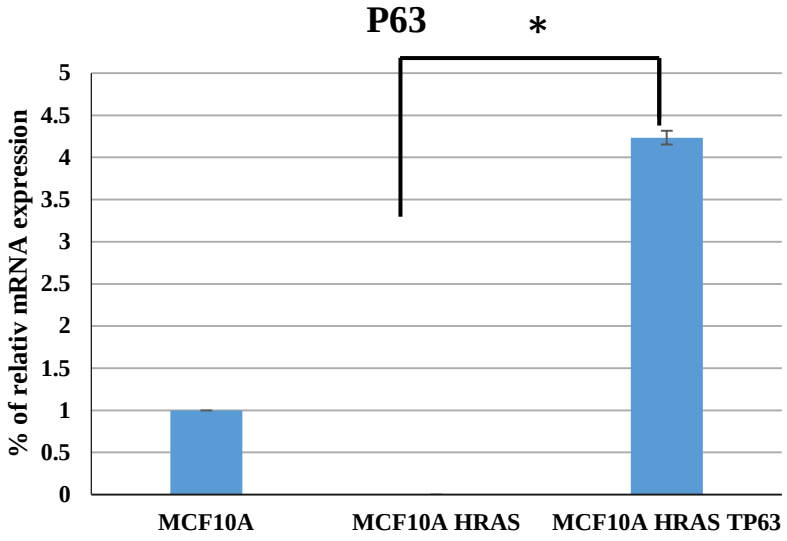

Figure 3. 6 Expression level of *p63* in HRAS MCF10A p63 clones. Real time PCR results on mRNA extracted from the cells indicated in the graph. Error bars represent the standard error of the mean of three experiments done in triplicates. to defined the significances different between MCF10A HRAS and MCF10A HRAS TP63 cells by T-test to calculate *p-value.* (** means that p-value>0.05 and * means that p-value <0.05), to a significant results the p-value <0.05.

3.6. Characterization of p63 expression in HRAS transformed MCF10A cells

After generating the *p63* clones, we wanted to test the effect of p63 restoration on HRAS induced phenotypes. In order to do so, we tested some cell phenotypes known to be affected by oncogenic HRAS overexpression, including, cell proliferation, and cell migration, and genomic activity of p63. As shown in Fig. 3.16 A and B, using cell count and XTT cell proliferation assay, p63 cells have lower proliferative index compared to control cells. In HRAS clones, we noticed changes in cell morphology towards a more mesenchymal morphology that was accompanied with a more invasive potential (Fig 3.11). To evaluate the role of p63 in this acquired phenotype, we tested the migratory potential of p63 clones in wound healing assay. As shown in Fig.3.16 C, while HRAS were able to close the wound, p63 clones failed to do so, indicating that p63 clones are less migratory than HRAS clones. To assess p63 genomic function, the expression of *p21* as a target p63 gene was evaluated. As shown in Fig 3.16 D using RT-PCR, HRAS transformed cells reduced *p21* mRNA level

while p63 restored *p21* expression in these cells. Altogether, our data here show that p63 restoration in HRAS clones was able to reverse some cellular phenotypes related to HRAS transformation, which indicates that loss of p63 function plays a role in HRAS cell induced transformation. In comparison to p53 effects, p63 effects are mild. This is seen by not always having a very significant differences (*p-value*>0.05). These findings provide another evidence that changes in cellular phenotypes that accompany cellular transformation involves transcriptional reprogramming, that might be the heart of cell transformation into a cancer cell.

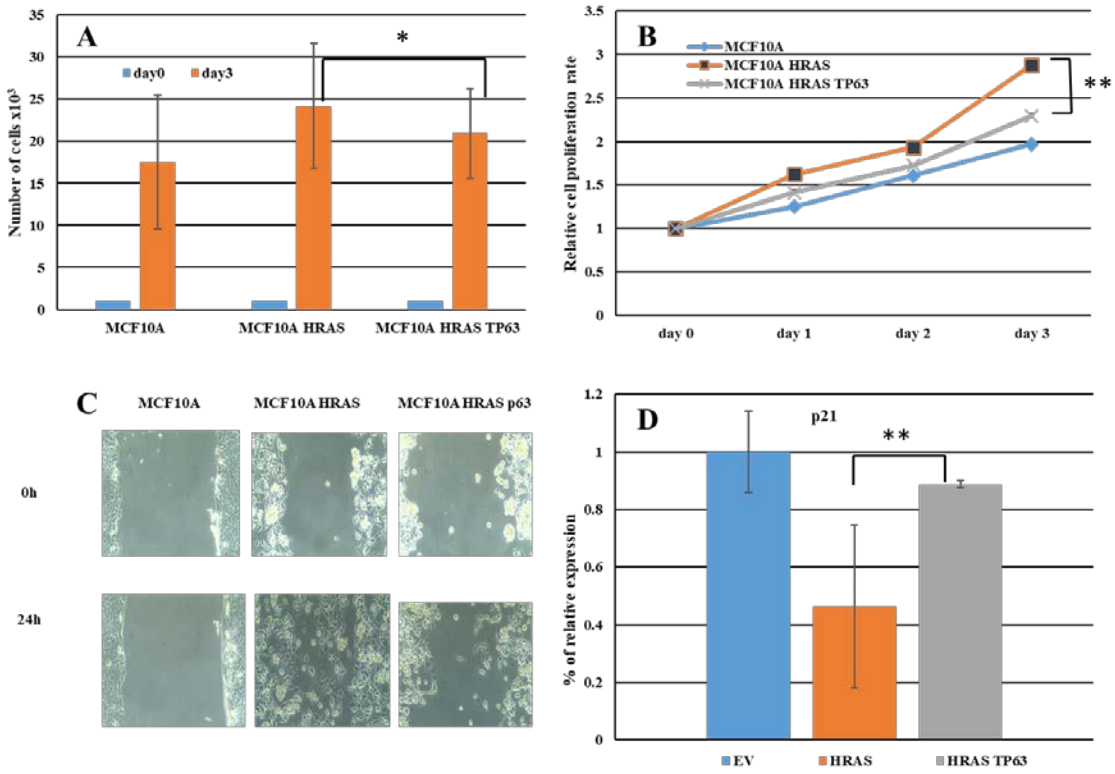

Figure 3. 7 Characterization of p63 clones.

(A). Cell count assay showing the proliferation rate of the different cell clones indicated in the figure **(B).** XTT cell proliferation assay results showing the effect of p63 on HRAS clones growth rate over 3days**. (B).** Colony formation assay showing MCF10 cell survival rates after transforming cells with HRAS as compared with non-transformed cells. **(C).** Representative images of wound healing assay of different cell clones and control cells**. (D).** Real time PCR showing the effect of p63 on the expression of p63 target p21 in HRAS transformed cells. to defined the significances different between MCF10A HRAS and MCF10A HRAS TP63 cells by T-test to calculate *p-value.* (** means that *p-value>0.05 and * means that p-value <0.05),* to a significant results the *p-value* <0.05.

Chapter 4

4. Conclusion

A model that can be used for transformation-associated transcriptional reprogramming studying. Indeed; the data showed the successful ability to develop an *in vitro* model for studying transcriptional reprogramming of cellular transcription machinery and were able to identify important transcription factors involved in this reprogramming event. More important, the manipulation of these transcription factors were able to reverse in part some transformation-related phenotypes indicating that such a model can be used to identify important factors that can be utilized in breast cancer therapy or at least be utilized to identify biomarkers for breast cancer diagnosis or prognosis.

References

1. Banerji, S., Cibulskis, K., Rangel-Escareno, C., Brown, K. K., Carter, S. L., Frederick, A. M., . . . Meyerson, M. (2012). Sequence analysis of mutations and translocations across breast cancer subtypes. *Nature, 486*(7403), 405-409. doi:10.1038/nature11154
2. Bergholz, J., & Xiao, Z. X. (2012). Role of p63 in Development, Tumorigenesis and Cancer Progression. *Cancer Microenviron, 5*(3), 311-322. doi:10.1007/s12307-012-0116-9
3. Buganim, Y., Solomon, H., Rais, Y., Kistner, D., Nachmany, I., Brait, M., . . . Rotter, V. (2010). p53 Regulates the Ras circuit to inhibit the expression of a cancer-related gene signature by various molecular pathways. *Cancer Res, 70*(6), 2274-2284. doi:10.1158/0008-5472.CAN-09-2661
4. Bulger, M., & Groudine, M. (2011). Functional and mechanistic diversity of distal transcription enhancers. *Cell, 144*(3), 327-339. doi:10.1016/j.cell.2011.01.024
5. Cancer Genome Atlas, N. (2012). Comprehensive molecular portraits of human breast tumours. *Nature, 490*(7418), 61-70. doi:10.1038/nature11412
6. Chambeyron, S., & Bickmore, W. A. (2004). Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription. *Genes Dev, 18*(10), 1119-1130. doi:10.1101/gad.292104
7. Chen, X., Mitsutake, N., LaPerle, K., Akeno, N., Zanzonico, P., Longo, V. A., . . . Fagin, J. A. (2009). Endogenous expression of Hras(G12V) induces developmental defects and neoplasms with copy number imbalances of the oncogene. *Proc Natl Acad Sci U S A, 106*(19), 7979-7984. doi:10.1073/pnas.0900343106
8. Chen, Y., Dey, R., & Chen, L. (2010). Crystal structure of the p53 core domain bound to a full consensus site as a self-assembled tetramer. *Structure, 18*(2), 246-256. doi:10.1016/j.str.2009.11.011
9. Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., & Green, D. R. (2004). Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science, 303*(5660), 1010-1014. doi:10.1126/science.1092734
10. Cox, P. M., & Goding, C. R. (1991). Transcription and cancer. *Br J Cancer, 63*(5), 651-662.
11. Curtis, C., Shah, S. P., Chin, S. F., Turashvili, G., Rueda, O. M., Dunning, M. J., . . . Aparicio, S. (2012). The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature, 486*(7403), 346-352. doi:10.1038/nature10983
12. Datta, S., Hoenerhoff, M. J., Bommi, P., Sainger, R., Guo, W. J., Dimri, M., . . . Dimri, G. P. (2007). Bmi-1 cooperates with H-Ras to transform human mammary epithelial cells via dysregulation of multiple growth-regulatory pathways. *Cancer Res, 67*(21), 10286-10295. doi:10.1158/0008-5472.CAN-07-1636
13. Dawson, M. A., Kouzarides, T., & Huntly, B. J. (2012). Targeting epigenetic readers in cancer. *N Engl J Med, 367*(7), 647-657. doi:10.1056/NEJMra1112635
14. Di Como, C. J., Urist, M. J., Babayan, I., Drobnjak, M., Hedvat, C. V., Teruya-Feldstein, J., . . . Cordon-Cardo, C. (2002). p63 expression profiles in human normal and tumor tissues. *Clin Cancer Res, 8*(2), 494-501.
15. Drakos, E., Singh, R. R., Rassidakis, G. Z., Schlette, E., Li, J., Claret, F. X., . . . Medeiros, L. J. (2011). Activation of the p53 pathway by the MDM2 inhibitor nutlin-3a overcomes BCL2 overexpression in a preclinical model of diffuse large B-cell lymphoma associated with t(14;18)(q32;q21). *Leukemia, 25*(5), 856-867. doi:10.1038/leu.2011.28

16. Drakos, E., Thomaides, A., Medeiros, L. J., Li, J., Leventaki, V., Konopleva, M., . . . Rassidakis, G. Z. (2007). Inhibition of p53-murine double minute 2 interaction by nutlin-3A stabilizes p53 and induces cell cycle arrest and apoptosis in Hodgkin lymphoma. *Clin Cancer Res, 13*(11), 3380-3387. doi:10.1158/1078-0432.CCR-06-2581
17. el-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., & Vogelstein, B. (1992). Definition of a consensus binding site for p53. *Nat Genet, 1*(1), 45-49. doi:10.1038/ng0492-45
18. Elenbaas, B., Spirio, L., Koerner, F., Fleming, M. D., Zimonjic, D. B., Donaher, J. L., . . . Weinberg, R. A. (2001). Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes Dev, 15*(1), 50-65.
19. Espinosa, J. M., & Emerson, B. M. (2001). Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. *Mol Cell, 8*(1), 57-69.
20. Fernandez-Medarde, A., & Santos, E. (2011). Ras in cancer and developmental diseases. *Genes Cancer, 2*(3), 344-358. doi:10.1177/1947601911411084
21. Fischbach, A., Kruger, A., Hampp, S., Assmann, G., Rank, L., Hufnagel, M., . . . Mangerich, A. (2017). The C-terminal domain of p53 orchestrates the interplay between non-covalent and covalent poly(ADP-ribosyl)ation of p53 by PARP1. *Nucleic Acids Res*. doi:10.1093/nar/gkx1205
22. Funk, W. D., Pak, D. T., Karas, R. H., Wright, W. E., & Shay, J. W. (1992). A transcriptionally active DNA-binding site for human p53 protein complexes. *Mol Cell Biol, 12*(6), 2866-2871.
23. Ghioni, P., Bolognese, F., Duijf, P. H., Van Bokhoven, H., Mantovani, R., & Guerrini, L. (2002). Complex transcriptional effects of p63 isoforms: identification of novel activation and repression domains. *Mol Cell Biol, 22*(24), 8659-8668.
24. Guertin, M. J., & Lis, J. T. (2013). Mechanisms by which transcription factors gain access to target sequence elements in chromatin. *Curr Opin Genet Dev, 23*(2), 116-123. doi:10.1016/j.gde.2012.11.008
25. Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70.
26. Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674. doi:10.1016/j.cell.2011.02.013
27. He, F., Melamed, J., Tang, M. S., Huang, C., & Wu, X. R. (2015). Oncogenic HRAS Activates Epithelial-to-Mesenchymal Transition and Confers Stemness to p53-Deficient Urothelial Cells to Drive Muscle Invasion of Basal Subtype Carcinomas. *Cancer Res, 75*(10), 2017-2028. doi:10.1158/0008-5472.CAN-14-3067
28. Ho, W. C., Fitzgerald, M. X., & Marmorstein, R. (2006). Structure of the p53 core domain dimer bound to DNA. *J Biol Chem, 281*(29), 20494-20502. doi:10.1074/jbc.M603634200
29. Iwakuma, T., & Lozano, G. (2003). MDM2, an introduction. *Mol Cancer Res, 1*(14), 993-1000.
30. Johnson, C., Warmoes, M. O., Shen, X., & Locasale, J. W. (2015). Epigenetics and cancer metabolism. *Cancer Lett, 356*(2 Pt A), 309-314. doi:10.1016/j.canlet.2013.09.043
31. Jones, P. A., & Baylin, S. B. (2007). The epigenomics of cancer. *Cell, 128*(4), 683-692. doi:10.1016/j.cell.2007.01.029
32. Jung, Y. S., Qian, Y., Yan, W., & Chen, X. (2013). Pirh2 E3 ubiquitin ligase modulates keratinocyte differentiation through p63. *J Invest Dermatol, 133*(5), 1178-1187. doi:10.1038/jid.2012.466
33. Kachnic, L. A., Wu, B., Wunsch, H., Mekeel, K. L., DeFrank, J. S., Tang, W., & Powell, S. N. (1999). The ability of p53 to activate downstream genes p21(WAF1/cip1) and MDM2, and

cell cycle arrest following DNA damage is delayed and attenuated in scid cells deficient in the DNA-dependent protein kinase. *J Biol Chem, 274*(19), 13111-13117.
34. Karlic, R., Chung, H. R., Lasserre, J., Vlahovicek, K., & Vingron, M. (2010). Histone modification levels are predictive for gene expression. *Proc Natl Acad Sci U S A, 107*(7), 2926-2931. doi:10.1073/pnas.0909344107
35. Lee, C. W., Martinez-Yamout, M. A., Dyson, H. J., & Wright, P. E. (2010). Structure of the p53 transactivation domain in complex with the nuclear receptor coactivator binding domain of CREB binding protein. *Biochemistry, 49*(46), 9964-9971. doi:10.1021/bi1012996
36. Lee, T. I., & Young, R. A. (2000). Transcription of eukaryotic protein-coding genes. *Annu Rev Genet, 34*, 77-137. doi:10.1146/annurev.genet.34.1.77
37. Makrodouli, E., Oikonomou, E., Koc, M., Andera, L., Sasazuki, T., Shirasawa, S., & Pintzas, A. (2011). BRAF and RAS oncogenes regulate Rho GTPase pathways to mediate migration and invasion properties in human colon cancer cells: a comparative study. *Mol Cancer, 10*, 118. doi:10.1186/1476-4598-10-118
38. Malecka, K. A., Ho, W. C., & Marmorstein, R. (2009). Crystal structure of a p53 core tetramer bound to DNA. *Oncogene, 28*(3), 325-333. doi:10.1038/onc.2008.400
39. Margolis, B., & Skolnik, E. Y. (1994). Activation of Ras by receptor tyrosine kinases. *J Am Soc Nephrol, 5*(6), 1288-1299.
40. Mayer, I. A., Abramson, V. G., Lehmann, B. D., & Pietenpol, J. A. (2014). New strategies for triple-negative breast cancer--deciphering the heterogeneity. *Clin Cancer Res, 20*(4), 782-790. doi:10.1158/1078-0432.CCR-13-0583
41. McCain, J. (2013). The MAPK (ERK) Pathway: Investigational Combinations for the Treatment Of BRAF-Mutated Metastatic Melanoma. *P T, 38*(2), 96-108.
42. Melino, G. (2011). p63 is a suppressor of tumorigenesis and metastasis interacting with mutant p53. *Cell Death Differ, 18*(9), 1487-1499. doi:10.1038/cdd.2011.81
43. Miyashita, T., & Reed, J. C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell, 80*(2), 293-299.
44. Moran, D. M., & Maki, C. G. (2010). Nutlin-3a induces cytoskeletal rearrangement and inhibits the migration and invasion capacity of p53 wild-type cancer cells. *Mol Cancer Ther, 9*(4), 895-905. doi:10.1158/1535-7163.MCT-09-1220
45. Northcott, P. A., Lee, C., Zichner, T., Stutz, A. M., Erkek, S., Kawauchi, D., . . . Pfister, S. M. (2014). Enhancer hijacking activates GFI1 family oncogenes in medulloblastoma. *Nature, 511*(7510), 428-434. doi:10.1038/nature13379
46. Polak, P., Karlic, R., Koren, A., Thurman, R., Sandstrom, R., Lawrence, M., . . . Sunyaev, S. R. (2015). Cell-of-origin chromatin organization shapes the mutational landscape of cancer. *Nature, 518*(7539), 360-364. doi:10.1038/nature14221
47. Prior, I. A., Lewis, P. D., & Mattos, C. (2012). A comprehensive survey of Ras mutations in cancer. *Cancer Res, 72*(10), 2457-2467. doi:10.1158/0008-5472.CAN-11-2612
48. Rajalingam, K., Schreck, R., Rapp, U. R., & Albert, S. (2007). Ras oncogenes and their downstream targets. *Biochim Biophys Acta, 1773*(8), 1177-1195. doi:10.1016/j.bbamcr.2007.01.012
49. Ries, S., Biederer, C., Woods, D., Shifman, O., Shirasawa, S., Sasazuki, T., . . . McCormick, F. (2000). Opposing effects of Ras on p53: transcriptional activation of mdm2 and induction of p19ARF. *Cell, 103*(2), 321-330.
50. Ritchie, M. D., Hahn, L. W., Roodi, N., Bailey, L. R., Dupont, W. D., Parl, F. F., & Moore, J. H. (2001). Multifactor-dimensionality reduction reveals high-order interactions among

estrogen-metabolism genes in sporadic breast cancer. *Am J Hum Genet, 69*(1), 138-147. doi:10.1086/321276
51. Schneider, R., & Grosschedl, R. (2007). Dynamics and interplay of nuclear architecture, genome organization, and gene expression. *Genes Dev, 21*(23), 3027-3043. doi:10.1101/gad.1604607
52. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., & Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell, 88*(5), 593-602.
53. Shin, I., Kim, S., Song, H., Kim, H. R., & Moon, A. (2005). H-Ras-specific activation of Rac-MKK3/6-p38 pathway: its critical role in invasion and migration of breast epithelial cells. *J Biol Chem, 280*(15), 14675-14683. doi:10.1074/jbc.M411625200
54. Solomon, H., Brosh, R., Buganim, Y., & Rotter, V. (2010). Inactivation of the p53 tumor suppressor gene and activation of the Ras oncogene: cooperative events in tumorigenesis. *Discov Med, 9*(48), 448-454.
55. Tang, H. Y., Zhao, K., Pizzolato, J. F., Fonarev, M., Langer, J. C., & Manfredi, J. J. (1998). Constitutive expression of the cyclin-dependent kinase inhibitor p21 is transcriptionally regulated by the tumor suppressor protein p53. *J Biol Chem, 273*(44), 29156-29163.
56. Vinarskaja, A., Schulz, W. A., Ingenwerth, M., Hader, C., & Arsov, C. (2013). Association of PITX2 mRNA down-regulation in prostate cancer with promoter hypermethylation and poor prognosis. *Urol Oncol, 31*(5), 622-627. doi:10.1016/j.urolonc.2011.04.010
57. Wang, B., Fang, L., Zhao, H., Xiang, T., & Wang, D. (2012). MDM2 inhibitor Nutlin-3a suppresses proliferation and promotes apoptosis in osteosarcoma cells. *Acta Biochim Biophys Sin (Shanghai), 44*(8), 685-691. doi:10.1093/abbs/gms053
58. Wang, Y., Godin-Heymann, N., Dan Wang, X., Bergamaschi, D., Llanos, S., & Lu, X. (2013). ASPP1 and ASPP2 bind active RAS, potentiate RAS signalling and enhance p53 activity in cancer cells. *Cell Death Differ, 20*(4), 525-534. doi:10.1038/cdd.2013.3
59. Weinberg, R. A. (2007). The Biology of Cancer © *Garland Science*.
60. Whyte, W. A., Orlando, D. A., Hnisz, D., Abraham, B. J., Lin, C. Y., Kagey, M. H., . . . Young, R. A. (2013). Master transcription factors and mediator establish super-enhancers at key cell identity genes. *Cell, 153*(2), 307-319. doi:10.1016/j.cell.2013.03.035
61. Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dotsch, V., . . . McKeon, F. (1998). p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell, 2*(3), 305-316.
62. Yeh, J. E., Toniolo, P. A., & Frank, D. A. (2013). Targeting transcription factors: promising new strategies for cancer therapy. *Curr Opin Oncol, 25*(6), 652-658. doi:10.1097/01.cco.0000432528.88101.1a
63. Yoh, K. E., Regunath, K., Guzman, A., Lee, S. M., Pfister, N. T., Akanni, O., . . . Prywes, R. (2016). Repression of p63 and induction of EMT by mutant Ras in mammary epithelial cells. *Proc Natl Acad Sci U S A, 113*(41), E6107-E6116. doi:10.1073/pnas.1613417113
64. Yu, M., Smolen, G. A., Zhang, J., Wittner, B., Schott, B. J., Brachtel, E., . . . Haber, D. A. (2009). A developmentally regulated inducer of EMT, LBX1, contributes to breast cancer progression. *Genes Dev, 23*(15), 1737-1742. doi:10.1101/gad.1809309
65. Yue, X., Zhao, Y., Xu, Y., Zheng, M., Feng, Z., & Hu, W. (2017). Mutant p53 in Cancer: Accumulation, Gain-of-Function, and Therapy. *J Mol Biol*. doi:10.1016/j.jmb.2017.03.030

تصميم نموذج خلوي من أجل دراسة آليات جديدة لإعادة برمجة النسخ الجيني في سرطان الثدي

إعداد: يوسف سمير اسحاق طرمان

اشراف: الدكتور زيدون صلاح

الملخص:

سرطان الثدي هو واحد من أكثر أنواع السرطان شيوعا وتنوعا ، ويعد المسبب الاول لوفاة النساء اللاتي أصبن بالسرطان في جميع أنحاء العالم. يتأثر سرطان الثدي بعوامل داخلية أو خارجية المنشأ، وهذه العوامل تؤدي الى طفرات جينيه (mutation) أو فوق جينيه (epimutation) أو الاثنتين معا في جينات مهمة وحساسة تحتوي الجينات المسرطنة(oncogenes) أو الجينات المثبطة للسرطان (tumor suppresser genes) أو الاثنتين معا. هذه التغيرات على المستوى الجيني و الفوق جيني تؤدي الى نشأة السرطان وبعد ذلك الى تقدم السرطان. و في أثناء هذه المراحل تكتسب الخلايا تغيرا وسوء تنظيم في التعبير الجيني(gene expression) على مستويات مختلفة. و يعد مستوى نسخ الجينات (transcription) أكثر المستويات أهمية وحساسية على تغيرات التعبير الجيني في السرطان.

مشروعنا الحالي هو جزء من مشروع كبير، افترضنا فيه أن التحول لسرطان الثدي قد يتتضمن أحداثا مشتركة في إعادة تنظيم النسخ الجيني. وهي مرتبطة مع الخلل التنظيمي في التعبير الجيني الذي ينعكس على النشاط والتوازن الخلوي. وفي هذا الجزء من المشروع الذي أقدمه هنا، هدفنا فيه كان اختبار مقدرتنا على إنشاء نموذج على التحول السرطاني لنسيج الثدي، واستخدام هذا النموذج لتحديد عوامل النسخ الجيني التي يمكن استخدامها كعلامات حيوية، وتوظيفها في التشخيص والوقاية أو على مستوى علاج سرطان الثدي. ومن أجل الوصول إلى هذه النهايه قمنا بإنشاء نموذج لتحول سرطان الثدي في المختبر بوساطة الإفراط في التعبير ل HRAS (overexpression) في خلايا متمايزة وغير متحولة، طبيعية طلائية من الغدة الثديية (MCF10A). كما قمنا بفحص عدة طرز شكلية للخلية، من المعروف بأنه يسببها الإفراط في التعبير ل HRAS؛ وذلك من أجل ضمان التحول الناجح. ثم تم اختبار هذه الخلايا المتحوله، في بحث متمم لهذا البحث (لم نقم نحن بهذا الجزء من المشروع)، لفحص إعادة برمجة النسخ الجيني (transcription reprograming). باستخدام تقنيات مختلفة. في الواقع أظهر نموذجنا الحجم

الهائل لإعادة برمجة في نسخ الجينات على مستوى المحتوى الجيني. بين مختلف مواقع النسخ الجيني التي فقدت كانت مواقع النسخ الجينية ل p53 و p63. من أجل تقيم دور عوامل النسخ الجيني هذه في نموذج التحول هذا. لقد قمنا بإعادة إظهار وظائف هذين العاملين المهمين للنسخ الجيني، p53 عن طريق استخدام Nutlin-3a وp63 عن طريق الإفراط في التعبير عنه. نتائجنا، هنا تبين أن تحفيز وظائف عوامل النسخ هذه كان كافيا إلى حد معين لاعادة بعض الطرز الشكلية المتعلقة بعملية التحول الى الطرز الشكلية للخلايا قبل عملية التحول.

مجتمعه، يمكن استخدام نموذج التحول الخاص بنا كأداة فعالة للتعلم عن إعادة البرمجة للنسخ الجيني خلال التحول الخلوي، وتحديد دراسة دور عوامل النسخ الجيني الخاصة في التحول، والتي ربما قد تساهم في تحديد بعض الجينات المستهدفة المشاركة في سرطان الثدي، وتوظيفها في الوقايه والتشخيص و العلاج.