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Molecular Studies on Regulation of Human Cancer : Effect of Environmental Stresses and Their Interventions

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Ling LI

Abstract

Cancer, also known as malignant neoplasm is a group of diseases involving uncontrolled abnormal cell growth and is extremely complex to understand and treat. According to World Health Organization (WHO), cancer caused 8.8 million deaths in 2015 and 30-50% of them could be prevented either by early diagnosis or improvement in therapeutic strategies. Current therapeutic solutions comprise surgical removal of detectable tumors and chemotherapy that is complicated by high toxicity, undesired secondary effects on normal body functions and drug resistance. Novel diagnostic markers and therapeutic drugs to improve the treatment are hence required.

MicroRNAs (miRNAs), single-stranded RNA of 18-24 nucleotides, first discovered in the early 1990s, have been recognized as noncoding regulators of gene functions including control of proliferation, differentiation, inflammation, stress response, apoptosis, carcinogenesis and metastasis. Several miRNAs have been shown not only to act as tumor suppressors or oncogenes, but also serve as diagnostic markers important for the pathophysiology of tumors. Many miRNAs have been shown to be downregulated in tumors and hence have been called tumor suppressors. On the other hand, several others miRNAs have been found to be upregulated in tumors and are ascribed to upregulate oncogenic functions. Downregulation of miR-451 has been reported in a variety of tumors including glioma, breast carcinoma, gastrointestinal carcinoma, non-small cell lung carcinoma (NSCLC), hepatoma, nasopharyngeal, esophageal, bladder, osteosarcoma, epithelial ovarian, renal and thyroid carcinomas. In a loss-of-function screening by recruiting retrovirus mediated arbitrary manipulation of genome coupled with escape of cells from 5-Aza-2'-deoxycytidine (5Aza-dC)-induced senescence, analysis of miRNA pool from these cells have identified miR-451 as one of the upregulated miRs and characterized its functional relevance to drug resistance, cell growth, tumor suppressor proteins p53 and pRb, and stress response. This study reports that miR-451 caused growth arrest in cells leading to their resistance to 5Aza-dC-induced senescence. miR-451-induced growth arrest in miR-451 transfected cells, was found to be essentially mediated by increase in p21WAF1 (independent of p53 status) and consequent decrease in Cyclin D1, CDK4, pRB and E2F5. At molecular level, investigation using mRNA and 3'UTR reporter assays revealed that Collaborator of ARF (CARF) protein is a new target of miR-451 that intermediates its function in tumor suppressor and stress signaling. Enrollment of environmental factors including fuel, edible products,

plastic, aromatic, disposal, chemical, and emotional stresses demonstrated tight correlation of miR-451 and CARF expression during stress exposure and recovery. This study reports that miR-451 plays role in regulation of CARF in response to various endogenous and exogenous/environmental stresses causing growth arrest.

Consistent to the adopted loss-of-function screening strategy, we next adopted interventional approach to investigate effect of environmental and metabolic stresses on the melanogenesis process in the aging skin using skin cancer/melanoma cell model system. Using shRNA-mediated loss-of-function screening in conjunction with induction of melanogenesis by OAG (diacylglycerol 1 -oleoyl-2-acetyl-sn-glycerol) in human melanoma G361 cells, we intent to identify the molecular factors involved in human melanogenesis and role of environmental factors and their possible interventions. Gene targets of the shRNAs that led to the loss of OAG-induced melanogenesis were considered as candidate cellular factors crucial for melanogenesis. At the end of 4^{th} screenings, we identified 40 gene targets. Bioinformatics and pathway analyses revealed that these gene targets are involved in the regulation of cell proliferation, apoptosis, stress responses and mitochondrial functions. Based on these we investigated the role of mitochondrial stress chaperone, mortalin in melanogenesis and found that (i) it is an important regulator of melanogenesis (ii) it showed upregulation in clinical samples of keloids and (iii) it may serve as a molecular target for manipulation of melanogenesis suggesting its value for cosmetics and therapeutic manipulation of skin color and other characteristics regulating stress, tolerance and pathologies. Altogether, using loss-of-function screening with 5Aza-dC or shRNA approaches, in the present study we identified (i) tumor suppressor activity of miR-451 and its new target CARF to induce growth-arrest/senescence as validated with endo-and exo-genous/environmental stresses; (ii) mortalin as a molecular target in melanogenesis process. Conclusively, present report using loss-of-function strategies in human cancer model exposed with environment stresses revealed molecular regulation of growth arrest or senescence/aging and melanogenesis processes and identified miR-451, CARF and Mortalin as key molecular targets to develop interventional approaches.

Keywords: Cancer; Environment stress; microRNA; Growth arrest; Melanogenesis.

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Abbreviations

ARF	Alternate reading frame
BCL-2	B-cell lymphoma 2
CARF	Collaborator of ARF
CDKs	Cyclin dependent kinases
DMEM	Dulbecc's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
GFP	Green fluorescent protein
HSPs	Heat shock protein families
HDM2	Human double minute 2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PARP	Peroxisome proliferator activated receptor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PVDF	Ploy vinylidene fluoride
RB	Retinnoblastoma
RNA	Ribonucleic acid
ROS	Reactive oxygen sulfate
SDS	Sodium dodecyl sulfate

Chapter 1 Introduction

1.1 Cancer

1.1.1 Cancer is multifactorial disease

Cancer, also known as neoplasm is a group of diseases involving out of control abnormal cell growth, and is extremely complex to understand and manage. The most common types of cancer in males are lung cancer, colorectal cancer, skin cancer and bone cancers. In females, the most common types are breast cancer, colorectal cancer and cervical cancer [1]. The risk of cancer increases significantly with age and many cancers occur more commonly in developing nations because of environmental factors. Although cancer survival tends to be poorer in developing countries, its most likely cause is combination of a late stage at diagnosis and limited access to timely and standard treatment [2]. A substantial proportion of the worldwide burden of cancer could prevent through the application of existing information about cancer by controlling environmental factors and timely diagnostics / therapeutics.

World Health Organization database showed that in China the total number of new bone cancer cases were 46723 in 2012, predicted that approximately 60000 newer cases will be diagnosed by 2020. Osteosarcoma is the one of the commonly diagnosed primary bone tumors produced by osteoid malignant cells [3, 4], representing approximately 56% of malignancies in younger patients [5]. The previous study showed that approximately 53% osteosarcoma patients were diagnosed at age 0-24 years, 28% at age 25-59 years and about 19% at age over 60 years. The relative 5-year survival rate for younger year onset cases was 61.6% [6]. Cytogenetic studies have shown various complex haphazard genetic [7]. Current therapeutic solution comprises of chemotherapy, surgical removal of detectable tumors including metastases, and radiation [8]. However, these are associated with severe adverse consequences such toxicity and drug resistance due to chemotherapy, recurrence of disease after surgical removal and radiation poisoning to the normal cells [9]. Many studies have suggested the possible molecular mechanisms in osteosarcoma, but because of its highly complex machinery to cause carcinogenesis its progression still remains obscure.

Skin is the largest organ of the body. It is the most important tissue that interfaces and interacts with environment and defends the body from variety of stresses caused by chemicals, radiations and micro-organisms [10, 11]. Although the skin color is genetically determined, it is also appreciably influenced by environmental and endocrine factors that cause its modulation

either temporarily or permanently. The color of skin is determined by the amount and distribution of melanin pigment, which is a complex polymeric pigment synthesized by melanosomes [12]. Melanosomes are subcellular organelles in specialized cells in skin called melanocytes [10, 13]. Melanoma, also known as malignant melanoma is a type of skin cancer that develops from the mutations in melanocytes. It is one of the most dangerous types of skin cancer. The primary known contributing factor of melanoma is ultraviolet light (UV) exposure induced DNA damage in those with low levels of skin pigment. Melanin pigment provides protection against UV-induced DNA damage [14, 15]. The earliest stage of melanoma starts when melanocytes begin to proliferate out of control [11]. According to the World Health Organization (WHO), about 25% melanoma cases have an active history of moles. In 2015 from a total of about 3.1 million active diseases, 59,800 eventually died. Therefore, it is necessary to investigate novel markers for melanoma cancer, which hopefully can upgrade therapeutic strategies and improve disease prognosis.

1.1.2 Cancer and tumor suppressor mechanisms

Tumor suppressors refer to a large group of molecules that are capable of restricting cell division, promoting apoptosis and suppressing disease metastasis. A loss of function of a tumor suppressor may lead to cancer due to uncontrolled cell division. Cancer cells are no more than the transformed normal cells. This transformation usually requires genetic mutations [16]. These mutations in proto-oncogenes or tumor suppressors can cause loss or reduction in tumor suppressors function and activation of oncogenes [17, 18]. Loss of tumor suppressors have previously shown to be more vigorous than oncogene activation in carcinogenesis [17, 18]. Once a tumor suppressor becomes inactive, cell division may happen uncontrollably leading to cancer. The identification of oncogenes such as H-RAS and tumor suppressor genes such as that encoding retinoblastoma protein (RB) involves a combination of functional cloning, linkage analyses, positional cloning or mutational analyses of genetically predisposed individuals [19-21]. Many tumor suppressors such as p53 are inactive in normal cells and activate by potential carcinogens which could cause sustained DNA damage [22, 23]. p53 is one of the most important tumor suppressor genes; loss of functional of p53 is associated with about 50% of known human cancer cases [24]. Normal cells have kept low-level p53 by MDM2, a p53 partner protein. Cellular stress induces posttranslational modification of p53 and MDM2, allowing p53 [25, 26] binding to DNA in a sequence-specific manner to induce cell cycle checkpoint activation [25]. This can trigger DNA repair processes and induce the transcription of other tumor suppressors such as $p21^{WAF1}$ and p16, contributing apoptosis, cellular senescence or autophagy [22, 25, 27-29]. RB is also

an important tumor suppressor gene, which assists the cell cycle from G_1 to S phase by E2F transcription factors that stimulate the expression of genes required for cell cycle progression [30]. Cyclin D1/CDK4 complex phosphorylates RB, driving its dissociation from RB/E2F complex. The freed E2F activates S-phase gene transcription. Deregulation of RB pathway occurs commonly cancer, and is mediated either by loss of function mutation or expression of negative players like RB- and CDK-inhibitors ($p21^{WAF1}$) [30-32]. To date, four major mechanisms have been revealed for tumor suppressors: suppression of cell division, induction of apoptosis, DNA damage repair and inhibition of metastasis.

1.1.3 Cancer and stress chaperones

Stress or heat shock proteins (HSPs) were first discovered by Ferruccion Ritossa in 1962 [33]. Most HSPs function as molecular chaperones. They constitute up to 5-10% of the total protein content in a healthy growing cell under normal conditions and play an impotent role in cell survival. HSPs are generally classified based on their approximate molecular size as HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs (sHSPs) with molecular sizes ranging from 15 to 30 kDa [34]. HSPs are often overexpressed in cancer cells and this constitutive expression is necessary for cancer cell survival. HSPs may have oncogene-like functions. Cancer cells must extensively rewire their metabolic and signal transduction pathways, thereby becoming dependent on proteins, like stress-inducible HSPs [35]. Therefore, the cytoprotective functions of HSPs mentioned above are necessary to maintain cancer cells survival. HSPs depletion or inhibition frequently reduces the size of the tumors and can even cause their complete involution. Clinical studies have often shown a close association of high HSPs expression with poor clinical outcome [36].

Mortalin is one of the HSP70 family proteins. It was first cloned from the cytoplasmic fraction of normal mouse fibroblasts. Stress chaperone mortalin is an essential protein often enriched in cancer cells, and promotes their pro-proliferative characteristics by multiple pathways [37-39]. It has been shown to interact with tumor suppressor protein-p53 (both wild and mutant types) and inhibits its pro-apoptotic function in cancer cells. Mortalin binds to p53 and prevents its nuclear localization. Targeting mortalin-p53 interaction with either mortalin small hairpin RNA or a chemical or peptide inhibitor could induce p53-mediated tumor cell-specific apoptosis in cancer cells [38]. As a chaperone protein, mortalin confers cellular resistance on various stress conditions. At the same time, it functions in many constitutive cellular processes, including maintenance of mitochondrial function. Many studies involving mortalin have shown that it is essential for the translocation of cytosolic precursor proteins across two mitochondrial membranes [40-42].

Heat shock protein 60 (HSP60) is a mitochondrial chaperonin that is typically responsible for the transportation and refolding of proteins from the cytoplasm into the mitochondrial matrix [43, 44]. HSP60 functions as a chaperonin to assist folding linear amino acid chains into their respective three-dimensional structure. HSP60 has been deemed essential in the synthesis and transportation of essential mitochondrial proteins from the cytoplasm into the mitochondrial matrix [45]. The upregulation of HSP60 production contributes to the maintenance of other intracellular processes, especially during stress. HSP60 has been shown to influence apoptosis in tumor cells [46], and is upregulated in many kinds of tumors [46, 47]. Studies have also showed that both HSP60 and HSP70 (mortalin) are expressed in the mitochondria [45, 48]. Furthermore, some studies have shown co-regulation and co-modification of HSP60 and mortalin during cellular stresses, including cytotoxicity, injury, mitochondrial hyperactivity and tumorigenesis [48, 49]. Overall, the two mitochondrial chaperones are closely related and interact in close proximity for their functional involvement proliferation and cellular senescence [50].

1.1.4 Cancer and environmental stress

The global burden of cancer continues to increase largely because of aging and expanding world population, and remarkable exposure to carcinogens particularly smoking and environmental factors, especially within the developing countries. Cancer is by large a phenotypic representation of abnormal and uncontrolled genetic mutation. The genes inherited from the parents to their progenies have only limited impact on life outcome. They actively determine the associated risk factors for a disease, but not the cause or the final. Biology is the science involving complex chemical and physical interactions, filled with exceptions, varying with environmental factors and the genetic coding [51]. Cancer is caused by both internal factors such as inherited mutations, hormones, and immune conditions and environmental factors. Enormous environmental factors specifically those we exposure to in our routine life such as polluted substances of fossil fuel, edible products, plastics, chemical, aromatic and consumable compounds and chronic stress produced from physiological causes, are categorized as potential environment carcinogenic factors. Environmental factor play a very important role in carcinogenesis [52]. Exposures of above environment factors over the time period contributed to the recent rise in cancer incidence, and thus marked them as emerging carcinogenic factors [53]. Involuntary exposure to the carcinogens in the environment, including air (2-Nitro-9-fluorenone, 1-Hydroxypyrene), chemical (Formaldehyde), consumables (Vanadium (V) oxide, Cadmium nitrate tetrahydrate) and plastic (Chloroformate) account for the recently growing incidences of cancer. Some major concerns adding up to it are (1) outdoor air pollution associated with

polycyclic aromatic hydrocarbons [54, 55]; (2) indoor air pollution by environmental smoke, formaldehyde and volatile aromatic compounds [56], and (3) carcinogenic food additive (Vanadium(V) oxide) particularly affecting children [54]. Although the exact proportion of risk attributable to environmental factors is still unknown, list of carcinogenic and especially mutagenic factors support to our base hypothesis according to which numerous cancers may in fact be prevented by slight modification in our environment [51, 57] (Fig. 1-1).

1.2 microRNAs

1.2.1 Biogenesis and regulation of microRNAs

MicroRNAs (miRNAs) were first discovered in the early 1990s, the researchers discovered a gene named lin-4 that could control the timing of *C. elegans* larval development. It did not code for a protein but instead produced a pair of small RNAs [58]. To date, over 2,500 potential human miRNAs have been recorded in miRBase, a biological database that acts as an archive of miRNA sequences and annotations [59]. miRNA coding gene is transcribed by RNA polymerase II, generating long primary transcripts (pri-miRNAs). Subsequently, the RNase III–type enzyme (Drosha) processes the long primary transcripts into ~70-nucleotide hairpin precursors (pre-miRNA). The pre-miRNAs are then exported into the cytoplasm and undergo additional processing in which a double- stranded RNA of ~22 nucleotides complementarily couples in structure. Next, the less stable of the two strands in the double-helix is incorporated into the miRNA-induced silencing complex (miRISC). The mature miRNA strand is preferentially retained in the functional miRISC complex and negatively regulates its target genes [60-64]. A mature miRNA is a single-stranded RNA approximately 18-24 nucleotide (nt) in length [65], and it regulates basic cellular functions including proliferation, differentiation and death. It does so via gene-regulation through pairing to the mRNAs of protein-coding genes and directing their posttranscriptional repression [65, 66]. It is clear that miRNAs regulate gene expression through binding to the 3'UTR (3' non-translational region) of target mRNAs causing degradation or inhibition of mRNA translation [67]. About one-third of human mRNAs are potentially controlled by miRNAs, because of the short microRNA–mRNA binding site which is commonly 6–8 base pairs. Each microRNA has the potential to target multiple mRNAs, while a single mRNA may also have target sites for multiple microRNAs (Fig. 1-2) [68].

The general role of miRNAs is designed according to the target genes. For the majority of miRNAs, the phenotypic consequences of disrupted or altered miRNAs regulation are not known. However, computational approaches are being developed to find the regulatory targets of the

miRNAs, providing clues to miRNA function based on the known roles of these targets [69]. The experiments supporting the identity of these targets typically fall into two classes. miRNA is thought to specify mRNA cleavage, the cleavage products can be reverse-transcribed, cloned, and sequenced. The preponderance of sequences that end precisely at the predicted site of cleavage provides experimental validation that this mRNA is a cleavage target of the complementary miRNA [70, 71].

1.2.2 Cancer and microRNAs

The molecular features make miRNA can act as tumor suppressors and oncogenes that mediate processes in tumorigenesis, such as inflammation, cell cycle regulation, stress response, differentiation, apoptosis and invasion [72]. miRNA targeting is mostly achieved through specific base-pairing interaction between the 5'end of the miRNA and sites within coding and untranslated regions (UTRs) of mRNAs. Target sites in the 3'UTR lead to more effective mRNA destabilization. Since miRNAs frequently target hundreds of mRNAs, miRNA regulatory pathways are complex [72]. miRNA pathway components could either be mis-expressed in tumors or mutated [73, 74]. miRNA dysregulation could be used as a diagnostic tool, even if the particular miRNAs do not serve any regulatory function. Many studies provides evidence that miRNA expression patterns may be specific biological marks of tumors and biological activities important for the pathobiology of malignant tumors and can serve as both diagnostic markers and therapeutic targets for many different tumor types [75]. For example, some studies have shown overexpression of tumor suppressor miRNAs such as let-7g can reduce tumor burden in K-RAS murine lung cancer model. On the other hand, overexpression of the oncogenic miR-17-92 in MYC-driven B cell lymphomas dramatically increased its tumorigenicity [76, 77]. miRNA regulate a large number of genes, few estimate miRNA regulation up to 60% in the human genome, making it challenging to attribute a phenotype after mis-expression of a particular miRNA through its action on only a subset of targets. Examples of miRNA regulated cancer pathways include differentiation, apoptosis, proliferation and stem cell maintenance, a process important for disease relapse and or metastasis. miRNAs demonstrate their potential as early diagnostic tumor markers, when their profile correlate with the tumor embryonic origin, thus distinguishing tumors of unknown origin by histology and clinical information [67, 78, 79].

1.2.3 Stress and microRNAs

Cellular stress induced by environment factors can be defined as changes that disturb cellular homeostasis and cause damage to macromolecules such as DNA, RNA and proteins. Cells respond to stress via survival mechanisms, which either lead to the restoration of cellular

homeostasis or adaptation to environmental conditions through growth arrest, repair or clearance of damaged macromolecules and changes in the gene expression programs. There are studies confirming the critical role of miRNAs in cellular stress responses [80-83]. miRNAs are post-transcriptional regulators of gene expression during stress, which target multiple transcripts at the same time. Numerous studies have shown that DNA damage stress changes the global profile of miRNA expression [84]. An example of transcriptional regulation of miRNA expression upon stress is DNA damage response, can activate transcription factors, including p53, NF-κB, c-Myc and c-Jun, which upregulate or downregulate the expression of specific miRNAs [85]. Study on miRNAs provides us an insight into the underlying mechanisms of oncogenesis, and offers a promising opportunity for cancer detection, diagnosis, and prognostic assessment.

1.3 Cancer and melanogenesis and their manipulation

1.3.1 Melanogenesis are stress responses

Skin is the largest organ of the body and is the most important tissue that interfaces and interacts with environment and regulates signaling pathways evoking adaptation or defense to a variety of stresses involving chemicals, heat, radiations and microbes. Skin color ranges from the darkest brown to the lightest hues. An individual's skin color is typically a result of hereditary genotyping, inherited from both the biological parents' gene pool. Many parameters affect the skin color thereafter via regulation of synthesis of the pigment melanin [86]. Melanogenesis is directed by a variety of signal transduction pathways. Melanin is produced by the oxidation of the amino acid tyrosine, in a specialized group of cells known as melanocytes. Melanin synthesized in melanocytes is transferred to keratinocytes and determines skin color by virtue of its characteristics including type and proportion of small molecule constituents. There are three basic types of melanin eumelanin (EM), pheomelanin (PM) and neuromealanin (NM). The most common type is eumelanin. Eumelanin gives black and brown coloration, and is the most abundant type in humans. Pheomelanin imparts pink and red color and is concentrated in the lips, nipples and glands [15, 87]. In the human skin, melanogenesis is triggered by the exposure to UV radiation, causing the skin to tan. Melanin is an effective absorbent of light. The pigment is able to dissipate over 99.9% of absorbed UV radiation [10, 15]. Melanin pigment protects against UV-induced DNA damage. Besides its role in skin coloration, melanin has been assigned functions including stress protection and its susceptibility to disorders ranging from minor itching to metastatic cancers. For example, neuromealanin (NM) is a dark pigment produced in specific catecholaminergic neurons in human brain, and has been shown to play crucial role in protection

against metal toxicity and regulation of apoptosis in brain cells [88, 89]. The transcription factor, MITF has been implicated to play a key role not only in the process of melanocyte development and melanogenesis but also in proliferation and survival of melanocytes [90]. Molecular mechanism of regulation of melanin synthesis and its photo protective action, in context to its melanogenic, antioxidant and cell survival have not been fully elucidated yet.

1.3.2 Skin pigmentation, melanogenesis and cancer

In the skin melanin acts as an optical and chemical photo protective filter, which reduces the penetration of all wavelengths of light into subepidermal tissues. Skin cancer is the appearance and proliferation of abnormal cells from the skin, which have the ability metastasize. There are mainly three types of skin cancer: basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and melanoma (Fig. 1-3). The BCC and SCC along with a number of less common skin cancers collectively are known as non-melanoma skin cancer [91]. Melanomas are the most aggressive type of skin cancer. Symptoms such as a mole that has changed in size, shape, color, has irregular edges, has more than one color, is itchy or bleeds [92]. Epidemiologic evidence suggests that exposure to ultraviolet (UV) radiation and the sensitivity of an individual's skin to UV radiation are risk factors for skin cancer, though the type of exposure and pattern of exposure may differ among the three main skin cancer types. All three types of skin cancer are more likely to occur in individuals of lighter complexion substantially exposed to sun, therefore, these cancers are more common towards the equatorial region. In addition, the immune system may play a role in pathogenesis of skin cancers[13, 93]. In the human skin melanogenesis by melanocytes is initiated to protect skin cells from UV radiation. Malignant melanoma is a type of cancer that develops from the pigment-containing cells known as melanocytes [14]. Melanomas typically occur in the skin, but may rarely occur in the mouth, intestines or eye. Sometimes they develop from a mole [14].

1.4 Novel targets and structure of the thesis

This experimental study provided novel evidences for upregulation of miR-451 mediated growth arrest of cancer cells and melanogenesis regulation by stress chaperone mortalin. It indicated a new target for miR-451 and identified mortalin that is related to proliferation, stress response and apoptotic signaling also regulated melanogenesis in human melanoma cells.

Chapter 1 Introduction

In this chapter, the background of this research was introduced, including basic concepts of cancer, relationship between cancer and environmental stress, micro RNAs, and melanogenesis. Motivation and the structure of this study were also addressed.

Chapter 2 Tumor suppressor function of miRNA451

In this chapter, miR-451 was screened. Sets of experiments were applied for exploring the new target for miR-451.

Chapter 3 Cell based screening for genes involved in melanogenesis and validation of results-involvement of mortalin

In this chapter, stress chaperone mortalin was also screened. Its functional characterization and effect on melanogenesis regulation was discussed as well.

Chapter 4 Conclusions and future research

In this chapter, the main results are summarized. In particular, the structures of this research (Fig. 1-4) and the future research points were also directed.

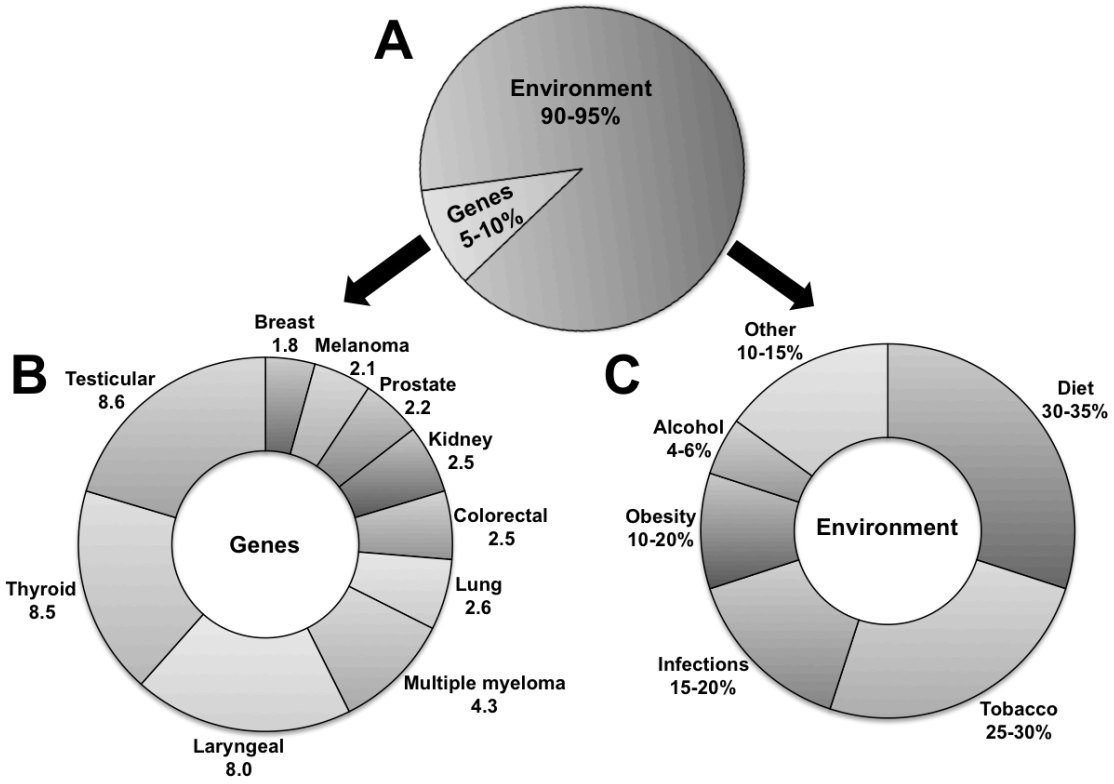


Fig. 1-1 The role of genes and environment in the development of cancer. (A) The percentage contribution of genetic and environmental factors for cancer. The contribution of genetic factors and environmental factors towards cancer risk is 5–10% and 90–95% respectively. (B) Family risk ratios for selected cancers. The numbers represent familial risk ratios, defined as the risk to a given type of relative of an affected individual divided by the population prevalence. The data shown here is taken from a study conducted in Utah to determine the frequency of cancer in the first-degree relatives (parents + siblings + offspring). The familial risk ratios were assessed as the ratio of the observed number of cancer cases among the first degree relatives divided by the expected number derived from the control relatives, based on the years of birth (cohort) of the case relatives. In essence, this provides an age-adjusted risk ratio to first-degree relatives of cases compared with the general population. (C) Percentage contribution of each environmental factor. The percentages represented here indicate the attributable-fraction of cancer deaths due to the specified environmental risk factor [51, 56].

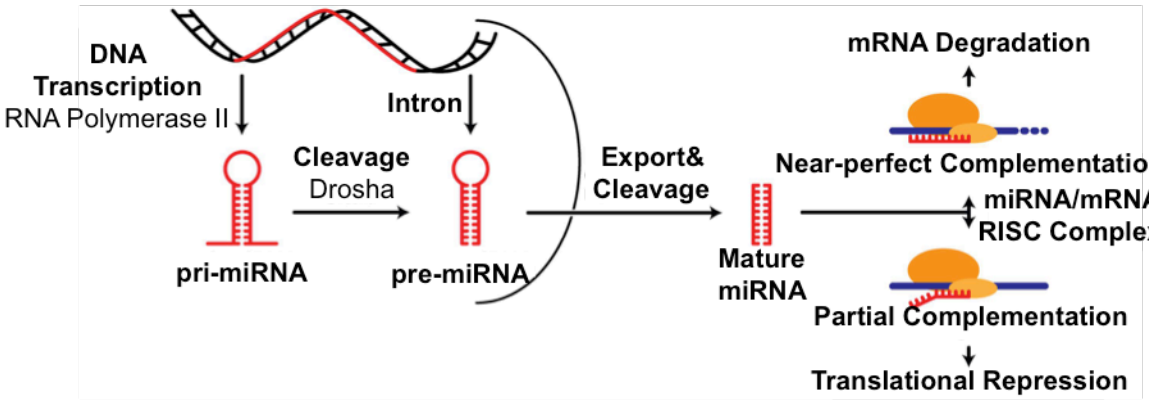


Fig. 1-2 Model of microRNAs biogenesis [68]

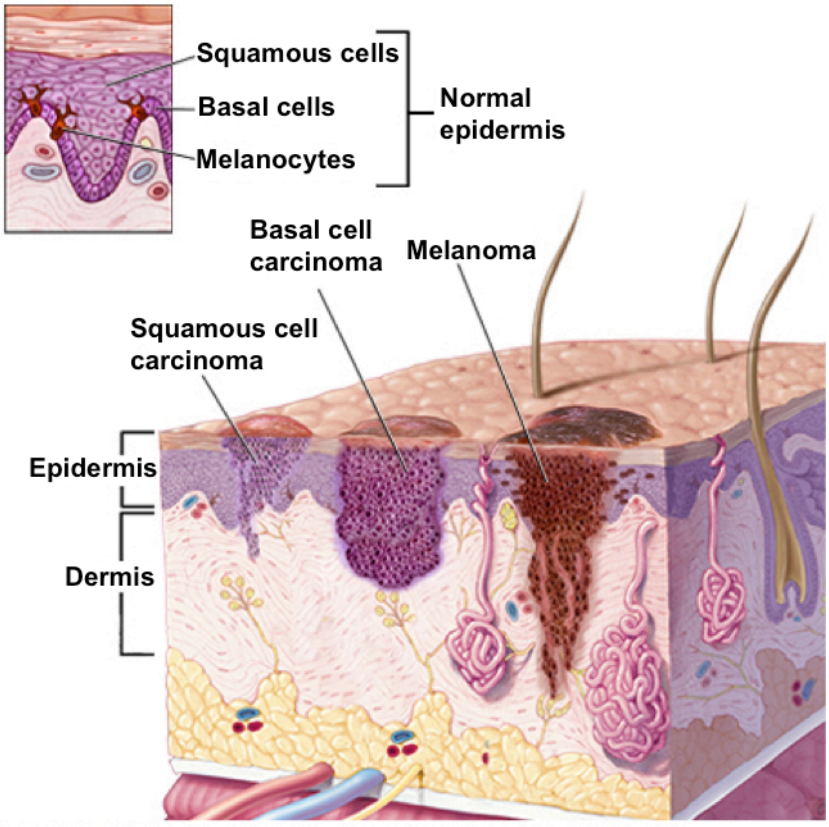


Fig. 1-3 Model of melanogenesis and skin cancer develops *http://www.mayoclinic.org/diseases-conditions/skin-cancer/multimedia/where-skin-cancer-develops/img-20007623*

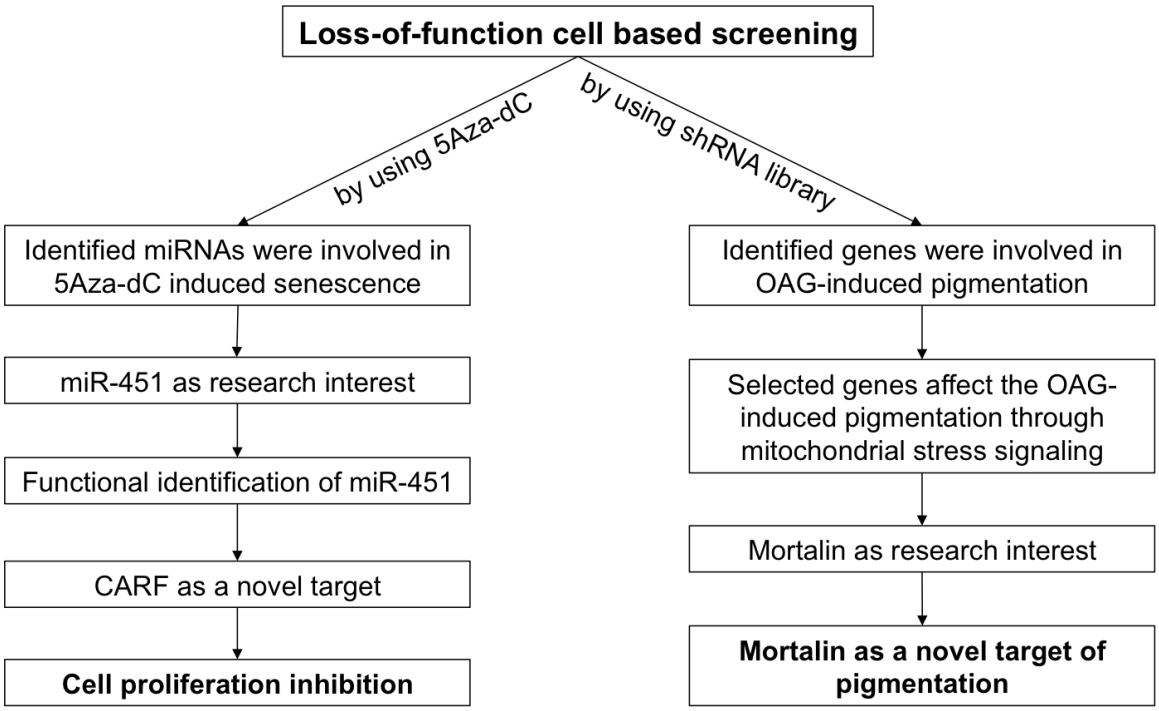


Fig. 1-4 Outline of this research

Chapter 2 Tumor suppressor function of miRNA451

2.1 Introduction

Cancer is a complex disease. It is regulated by multifaceted network of signaling pathways driven by loss of activities of tumor suppressor proteins, gain of function of oncogenes and several epigenetic mechanisms [94-96]. MicroRNAs (miRs) are a class of highly conserved small non-coding molecules (about 21-25 nucleotides long) that act as gene repressors by either causing mRNA degradation or translational block. They are transcribed as pre-miRNAs and are subsequently processed into short hairpin structured molecules by Drosha, the double-stranded RNA specific ribonuclease. Their involvement in diverse biological processes ranging from normal development to a variety of pathogenesis has been implicated. Hence, miR profiling has been considered to yield valuable outcomes not only in the understanding of regulation of basic biological phenomena, but also in disease, diagnosis, therapy and prognosis [97-99]. Several miRNAs have been shown not only to act as tumor suppressors or oncogenes, but also serve as diagnostic markers important for the pathobiology of tumors [65, 100]. Many miRNAs have been shown to be downregulated in tumors, hence called tumor suppressors [65, 72, 100]. On the other hand, several others have been found to be upregulated in tumors, and ascribed to upregulate oncogenic functions [65, 72, 101, 102]. Studies have provided evidence that miRNA expression patterns may be specific to tissue biology and pathophysiological activities. Hence, miRNAs may serve as both diagnostic markers and therapeutic targets for tumor subtypes [75]. Many miRNAs including miR-214, miR-124, miRNA-181a have been shown to be downregulated in tumors and are considered as tumor suppressor candidates [79, 103, 104]. Similarly, several others including miR-21, miR-196a, miR195b have been found to be upregulated in tumors and are shown to possess oncogenic functions [101, 102].

Downregulation of miR-451 has been reported in a variety of tumors including glioma [105-108], breast carcinoma [109], gastrointestinal carcinoma [110], non-small cell lung carcinoma (NSCLC) [111-114], Hepatoma [115-118], nasopharyngeal [119], esophageal [120, 121], bladder [122], osteosarcoma [123, 124], epithelial ovarian [125], renal [126] and thyroid [127] carcinomas. In several studies, it was shown to be correlated with clinical stages of the tumor including metastasis and poor response to neoadjuvant chemotherapy and recurrence [123, 124]. Authors previously examined the miR-451 expression in 115 ovarian cancer and 34 normal ovarian tissues, and found correlation with clinicopathological factors and prognosis. Low level of miR-451 in ovarian cancer as compared with normal tissue was associated with advanced FIGO stage, higher serum CA125 expression

level, and lymph node metastasis. Furthermore, transfection of miR-451 mimics in ovarian cancer cells reduced their cell proliferation, promoted cell apoptosis, and inhibited cell invasion suggesting that miR-451 is a potential candidate for therapy [125]. Study showed that reduced miR-451 was significantly correlated with advanced clinical stage, metastasis and worse disease-free or overall survival in HCC tissues. Reconstitution of miR-451 caused growth arrest of HCC, increased their chemo- or radio-sensitivity and reversed epithelial to mesenchymal transition (EMT) [117, 122]. Furthermore, decrease in Bcl-2, AKT and p-AKT expression of resulting in increase in apoptosis in esophageal carcinoma was also reported in miR-451 overexpressing cells [120]. miR-451 has been shown to target PSMB8 in renal cell carcinoma and lung cancer [113, 126], MIF, LKB1/AMPK, AMPK/mTOR and Fascin1 in thyroid, glioma, nasopharyngeal and gastric carcinomas [106, 107, 110, 119, 127], IKK-β in HCC [116], CXCL16 in osteosarcoma [123], CDKN2D and MAP3K1 in esophageal carcinoma [121], liver receptor homolog-1 (LRH-1) that plays crucial role in the onset and progression of many cancer types [128], c-Myc/Erk1-2 and ATF2 in hepatocarcinoma [117, 118] and PI3K/Akt/mTOR in multiple myeloma [129]. Reconstitution of miRNA-451 inhibited cell cycle progression, cellular migration and the invasive ability of NSCLC cells. It was shown to target Ras-related protein 14 (RAB14) [112] and serves as a novel therapeutic drug to treat NSCLC patients. Study showed that ectopic overexpression of miR-451 inhibited growth and induced apoptosis in A549 cells and sensitized them to cisplatin by inactivation of Akt signaling pathway [114]. The other study also reported that miR-451 regulates the expression of multidrug resistance 1 gene. Transfection of the MCF-7/DOX-resistant cells with miR-451 sensitized them to DOX suggesting its implication for treatment of drug resistant cells [109].

Collaborator of ARF, also known as CARF was first reported at 2002, where two CARF-interacting proteins were identified [130]. In the previously study shown CARF was key factor of cell growth arrest, cellular senescence and apoptosis by regulated p53-p21^{WAF1}-HDM2 pathway [131, 132]. Later, it was found evidence to prove that CARF regulated human cells proliferation via dose-dependent manner regulated DNA damage signaling, the level of CARF was crucial for checkpoint response of cells trough AMT/CHK1/CHK2, p53 and ERK pathways [133, 134]. Therefore, in human cancer cells the level of CARF determine the proliferative fate and malignant phenotypes, suppression of CARF could inducing apoptosis by activation of the mitochondrial stress and caspase-dependent pathways via induction of DNA damage and interference cell cycle [135-137]. Recently study demonstrated tumor suppresser miR-335 was directly targeting CARF, in turn regulates cell cycle [138].

In the present study, a bicistronic vector containing GFP reporter was used to arbitrarily induce miRs in human osteosarcoma [138]. Cells expressing such random library of miRs were subjected to 5-Aza-dC induced senescence for 3-5 days. miR pool of cells that escaped senescence was subjected to miR-array analysis with respect to the control cells. Out of several upregulated miRs, the function of miR-451 was characterized in the present study. This study report miR-451 caused growth arrest of cells that showed increase in $p21^{WAF1}$, and decrease in CyclinD1, CDK4, phospho-pRB and E2F5 proteins. Expression and reporter assays demonstrated that these effects are mediated by targeting of CARF by miR-451.

2.2 Materials and methods

2.2.1 Cell culture, transfections and drug treatments

All human cancer and normal cells were purchased from Japanese Collection of Research Bioresources (JCRB, Japan). Human osteosarcoma (U2OS, MG-63 and Saos-2), colorectal adenocarcinoma (DLD-1 and SW620) breast adenocarcinoma (MCF7), lung carcinoma (A549), immortalized lung fibroblasts (MRC5/hTERT)and normal human fibroblast (TIG-3 and MRC-5) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL), and 1% (v/v) penicillin/streptomycin in the presence of 5% CO_2 at 37℃. Transfection was performed using X-tremeGENE 9 DNA transfection reagent (Roche Applied Sciences, Basel, Switzerland). Transfected cells were selected using blasticidin (10 μg/ml). Cells were treated with 20 μM of 5-Aza-2'deoxycytidine (5-Aza-dC) (Sigma-Aldrich, St. Louis, MO, USA) for 48-96 h.

2.2.2 Induction of arbitrary miR library and screening for miRNAs involved in escape from 5Aza-dc induced senescence

To construct a retroviral vector pMXGbu, a short linker with 5'-gaattAGCGGAGGACAGTACTCCGATCGGAGGACAGTACTCCGTtcgac -3' was inserted between EcoRI and SalI sites of pMXCRGb [139] by removing CMV-RFP cassette. Upon transduction, the upstream LTR of the vector drives GFP-Bsd fusion gene and the downstream LTR initiates random transcription. The transduced cells were selected in blasticidin (10 μg/ml) supplemented medium and were then subjected to 5-Aza-dC (20 μM) for 3-5 days. The cells were subsequently harvested to prepare total RNA (RNeasy Plus Mini Kit (QIAGEN).

2.2.3 Coning of miR-451, expression plasmid and transfection

pCXGb-miR-451 encoding GFP and miR-451 driven by promoter was generated by amplification of miR-451 from human genomic DNA by PCR using following primers: Sense 5'AAAGTCGACAAGCTCTCTGCTCAGCCTGTC3' and antisense 5'AAAATATCTC GAGCCCCCACCCCTGCCTTGT3'. The PCR product was digested with Sal I and EcoRV and introduced into pCXGb plasmid as described earlier [138]. Cells were transfected with miR-451 plasmid using X-remeGENE HPDNA transfection reagent (Roche Applied Science, Indianapolis, USA). Typically, 1 μg and 5 μg of miR-451 plasmid were used for 6-well dish and 10-cm dish of cells at around 70% confluency, respectively. The transfection efficiency was determined by GFP fluorescence. Vector containing GFP encoding, but no miR, sequence was used as empty control.

2.2.4 Senescence-associated β-galactosidase assay (SA-β-gal)

SA-β-gal detection kit (Senescence Cells Histochemical Staining Kit, Sigma-Aldrich) was used following manufacturer's instructions. Cells showing blue staining were considered positive and counted under the microscope. Quantitation from three independent experiments was performed.

2.2.5 Cell viability, proliferation and colony forming assays

For short term cell viability, 5000 control or the miR-451 transfected cells were plated in 96-well plates, incubated at 37℃ for 48h followed by addition of MTT (100 μl, 5mg/ml in PBS) (Sigma-Aldrich, Missouri, USA) to each well and incubation at 37℃ for 4h. The supernatant was replaced with 100 μl of dimethyl sulfoxide (DMSO) and the chromophore was quantitated at 570nm using microplate reader (Infinite M200 PRO, TRCAN).

For cell proliferation assay, equal number of control and transfected cells were plated in 12-well plates. Cultured cells were harvested at indicated time points. The viable cells were counted by trypan blue exclusion assay using TC20^{TM} Automated Cell Counter (Bio-Rad, Hercules, CA, USA). Growth curves were generated for each cell line from three independent experiments.

Effect of miR-451 on long-term proliferation of cells was determined by colony forming assay. Control or transfected cells (500/ well) were plated in 6-well plates. The cells were cultured for next two weeks with regular (every third day) change of culture medium until colonies appeared. Colonies were fixed with methanol/acetone (1/1, v/v) for 10 min at 4℃, stained with 0.1% Crystal violet for overnight, destained with water. Plates were dried and photographed using

scanner (EOSON). Colonies were counted and statistical analysis was performed as described below.

2.2.6 RNA extraction, reverse transcription and real-time reverse transcription-polymerase chain reaction

Total RNA was isolated and quantitated from cells using the Relia Prep RNA Cell Miniprep System (Promega, Madison, WI, USA) and NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA), respectively. Pure RNA with A260/A280 ratio > 1.9 was used for reverse transcription using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The cDNA thus obtained was used for real-time qRT-PCR (50°C for 2 min; 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min; and 72°C for 30 s) using SYBR®Select Master Mix (Applied Biosystems, CA, USA) in triplicate on the EcoTM real time system (lllumina, San Diego, CA, USA). The results of real-time qRT-PCR were normalized to the geometric mean of the 18s internal control gene for variability in expression levels, then calculated as $2^{-[(Ct,\ Target\ gene\ -\ Ct,\ 18s)\ Time\ x\ -(Ct,\ Target\ gene\ -\ Ct,\ 18s)\ Time\ 0]}$ following the manufacturer's instructions, where Ct represents the threshold cycle for each transcript. The primer sets included: p21WAF1, 5'- GAGGCCGGGATGAGTTGGGAGGAG -3' (forward) and 5'- CAGCCGGCGTTTGGAGT GGTAGAA -3' (reverse); Cyclin D1, 5'- TCCTCTCCAAAATGCCAGAG -3' (forward) and 5'- GGCGGATTGGAAATGAACTT -3' (reverse); CDK4, 5'- TCGAAAGCCTCTCTTCTG TG -3' (forward) and 5'- TACATCTCGAGGCCAGTCAT -3' (reverse); pRb, 5'- GGAAGC AACCCTCCTAAACC -3' (forward) and 5'- TTTCTGCTTTTGCATTCGTG -3' (reverse); p53, 5'- TAACAGTTCCTGCATGGGCGGC -3' (forward) and 5'- AGGACAGGCACAAA CACGCACC -3' (reverse); HDM2 5'- TAGTATTTCCCTTTCCTTTGATGA -3' (forward) and 5'- CACTCTCCCCTGCCTGATAC -3' (reverse); CARF, 5'- TCAAAGTGACAGATG CTCCA -3' (forward) and 5'- CGTTGAACTGTTTTCCTGCT -3' (reverse); 18s, 5'- CAGG GTTCGATTCCGTAGAG -3' (forward) and 5'- CCTCCAGTGGATCCTCGTTA -3' (reverse).

In order to detect miR-451 expression level in cells, the corresponding miR-451 (Assay ID: 464419 mat) and RNU6B (Assay ID: 001093) primers and TaqMan® MicroRNA assay were performed with manufacturer's instructions (Applied Biosystems, CA, USA). The stem loop sequence included: RNU6B, GCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATT TTT; miR-451, UGGGUAUAGCAAGAGAACCAUUACCAUUACUAAACUCAGUGG GUAUAGCAAGAGAACCAUUACCAUUACUAAACUCAG. miR-451Real-time qRT-PCR was performed at 95℃ for 10min, 40 cycles of 95℃ for 15 s and 65℃ for 1 min. The miR-451

expression data were normalized to the endogenous control based on the threshold cycle (C_t) calculated as $2^{-[(Ct, miR\text{-}451 - Ct, RNU6B) Time\ x - (Ct, miR\text{-}451 - Ct, RNU6B) Time\ 0]}$.

2.2.7 Flow cytometry analysis

Equal number of control and transfected cells were seeded in 10 cm dishes. After 24 h of seeding, cells were cultured in serum free medium for 24 h followed by harvesting using 0.25% trypsinization. Cell pellets were washed with cold PBS and then added, drop by drop into the pre-cooled 70% ethanol to fix the cells. The fixed cells were stored at -20℃ for 24 h to until further use. The cells were centrifuged at 500 xg for 5 min, washed with cold PBS twice, re-suspended in 1 ml cold PBS and were treated with RNase A (100 µg/ml) at 37℃ for 1 h to avoid false DNA-PI staining. RNase A-treated cells were centrifuged to discard the supernatant. The pellet was re-suspended in 200 µl of Cell Cycle Guava reagent (Millipore, Billerica, MA, USA), mix gently and incubated for 30 min in dark. The stained cells were subjected to cell cycle analysis using Guava PCA flow cytometer (Millipore), and FlowJo Software (version 7.6, Flow Jo, LLC, USA).

2.2.8 Apoptosis assay

2×10^5 of U2OS cells and miR-451 or miR-335 [138] stabilized transfection cells were seeded in 6-well plates, 12 h later stabilized transfection cells were transfected with different plasmid (miR-335 or miR-451) and cultured in medium for 48 h. For collect all cell samples, cells were harvested by using trypsin (0.25%) along with culture medium centrifuging at 500xg for 10 min at 4℃, then re-suspended in adequate medium to make the cells number between 2×10^5 to 5×10^6 per ml. 100 µl of cells were incubated with 100 µl of Guava Nexin Reagents (Millipore, Billerica, MA, USA) for 20 min in dark, and analyzed by Guava PCA flow cytometer (Millipore). The data were further analyzed by using FlowJo Software (version 7.6, Flow Jo, LLC, USA).

2.2.9 Luciferase reporter assay

2×10^5 of U2OS cells and miR-451 or miR-335 [138] stabilized transfection cells were seeded in 6-well plates, 12h later stabilized transfection cells were transfected with different plasmid (miR-335 or miR-451) and cultured in medium for 48h. For collect all cell samples, cells were harvested by using trypsin (0.25%) along with culture medium centrifuging at 500 xg for 10 min at 4℃, then re-suspended in adequate medium to make the cells number between 2×10^5 to 5×10^6 per ml. 100 µl of cells were incubated with 100 µl of Guava Nexin Reagents (Millipore,

Billerica, MA, USA) for 20min in dark, and analyzed by Guava PCA flow cytometer (Millipore). The data were further analyzed by using FlowJo Software (version 7.6, Flow Jo, LLC, USA).

2.2.10 Western blotting

Cells were lysed in RIPA buffer (Thermo Scientific, Waltham, MA, USA) supplemented with a protease inhibitor cocktail (Roche). The protein concentrations were determined by using the Pierce BCA Protein Assay Kit (Thermo Scientific). 20 μg of protein for each sample were resolved in SDS-polyacrylamide gel, and electroblotted onto PVDF membranes (Millipore). Membranes were blocked in 3% of BSA in TBS-T. Then membranes were incubated with following primary antibodies: anti-p53 (DO-1), anti-MDM2 (HDM2-232), anti-cyclin D1 (72-13G), anti-Cdk4 (C-22), anti-E2F-5 (MH5), anti-NFκB p65 (sc-109), anti-GFP (B-2) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-p21 Waf1/Cip1 (12D1), anti-Phospho-Rb (Ser780), anti-Rb (4H1) from Cell signaling (Danvers, MA, USA) and anti-CARF (FL-10) [140]. Anti-β-actin antibody (AC-15) (Abcam, Cambridge, MA, USA) was used to determine the level of actin expression (as an internal loading control). All experiments were performed in triplicates. Quantitation of immunoblots was performed using the ImageJ software (National Institute of Health, Bethesda, MD).

2.2.11 Immunostaining

Control and transfected cells (5×10^4 cells/well) were seeded on 18-mm glass coverslips placed in 12-well culture dish. Cells were washed with cold PBS, fixed by using pre-cold methanol/acetone (1:1) mixture for 10 min at 4℃ and permeabilized using 0.5% Triton X-100 in PBS (PBS-T) for 10 min. The fixed cells were blocked with 0.2% BSA in PBS and were then incubated with specific primary antibodies as described above, for 1 h at room temperature or overnight at 4℃. After washing cells with PBS-T for three times, they were incubated with Alexa-594-conjugated goat anti-mouse or anti-rabbit (Molecular Probes, Invitrogen, USA) secondary antibodies. Nuclear staining was performed with Hoechst 33342 (Sigma, USA) for 10 min in dark after washings (thrice) with PBS-T. Following three further washings with PBS-T, the cells were examined under Carl Zeiss microscope (Axiovert 200 M, Tokyo, Japan). Images were quantified by ImageJ software (National Institute of Health, Bethesda, MD).

2.2.12 *In vivo* tumor formation assay

Four-weeks old female BALB/c nude mice were used for subcutaneous xenograft experiments. Mice (five per group) were subcutaneously injected with 5×10^6 human lung cancer cells A549 (control and miR-451 transfected cells) in 0.2 ml of PBS. Cells formed tumors

in 10 days. Mice body weight; general activity (movements and eating behavior) and tumor volume were monitored every 2 days. Volume of the subcutaneous tumors was calculated as V = L × W2/2, where L was length and W was the width of the tumor, respectively. All animal experiment was performed following the protocols for animal experiments recognized by the Animal Care and Use Committee, Institute of Laboratory Animal Science of Peking Union Medical College (ILAS-PG-2014-018).

2.2.13 miRNA array

Control (non-transduced and untreated) and virus transduced and 5-Aza-dC (20 μM) treated cells (pooled colonies) that escaped senescence were harvested. For miRNA microarray, small RNAs (less than 200 nt including precursor and mature miRNAs) were extracted using mirVana miRNA isolation kit (Ambion, Austin, TX, USA) following the manufacturer's protocol. Purified RNA was labeled with Cy3 or Cy5 using the mirVana miRNA labeling kit (Ambion). Labeled RNA was hybridized with oligonucleotides against human miRs arrayed on slides (Hokkaido-System Science, Japan), and detected by a scanner (Agilent Technologies, Santa Clara, CA, USA).

2.2.14 miR-451 target prediction

Sequence-based prediction of miR-451 recognition site in CARF transcript was analyzed using TargetSpy, a knowledge based online portal comprising broad-range compositional, physical, and combinatorial base-pairing algorithms. While, analysis of the conserved miR-451 sequence was performed using TargetScan that predicts biological targets of miRNAs via searching 8-mer, 7-mer, 6-mer sites in the transcript seed region. Prediction results comprised efficacy of targeting, their probability of conserved targeting (PCT) as conferred by similarities to 3' UTRs and their orthologs, also detected within ORFs.

2.2.15 Statistical analysis

All experiments were carried out at least three times, and data were expressed as mean ± standard deviation (SD). As shown in figures the data were respected to control that were set either at 100 or 1. Two-tailed Student's t-test or nonparametric Manne Whitney U-test, whichever was applicable, was used to determine the degree of significance between the control and experimental sample. Statistical significance was defined as significant (*p-value < 0.05), very significant (**p-value < 0.01) and very very significant (***p-value < 0.001).

2.3 Results and discussion

2.3.1 Identification of miR-451 in loss-of-function screening of miRs involved in escape of 5-Aza-dC induced sentence

A retroviral vector constituting two long terminal repeat (LTR) promoters on the 5' and 3' ends of the gene for GFP was generated in a way that the random integration of this vector in the genome would result into expression of GFP; detected by green fluorescence and its integration-dependent arbitrary manipulation of the host cell genome. The latter may yield loss of function phenotype due to altered expression of either proteins or their noncoding regulators miRs. This system was coupled with induction of senescence in human cancer cells (U2OS) by demethylating drug (5-Aza-dC). The cells that escaped senescence were selected by their propagation and were subjected to microRNA array with respect to the untreated control cells (Fig. 2-1). I found that several miRNAs (miR-101, miR-145, miR-335, miR-451, and miR-558) were upregulated in vector-transduced cells that showed resistance to 5-Aza-dC induced senescence (Fig. 2-1). Of note, two of these miRs (miR-145 and miR-335) have been reported to undergo hypermethylation-mediated silencing in a large variety of cancers [141, 142] and hence their upregulation by 5-Aza-dC induced demethylation was justified. Furthermore, our data suggested that these miRs are involved in regulation of induction of cellular senescence, proliferation and drug response of cells. I aimed to characterize such functions and targets of miR-451 in this study.

An expression plasmid encoding primary miR-451 was transfected in to U20S cells. Treatment of control or miR-451 overexpression cells with 5-Aza-dC and detection of senescent cells by senescence associated β-gal staining showing that miR-451 sensitized to 5-Aza-dC induced senescence (Fig. 2-2 A). However, this result was contrary to our expectation. Growth curve of control and miR-451 overexpressing cells showed growth arrest in response to 5-Aza-dC treatment (Fig. 2-2 B), suggesting that miR-451 induced growth arrest may prevent incorporation of 5AZA-dC in the cell genome and contribute to fast recovery of cells during subsequent culture in 5AZA-dC free medium.

2.3.2 miR-451 possesses a tumor suppressor activity

Downregulation of miR-451 has been reported in several kinds of cancers in many recent studies [113, 117, 124, 143]. In light of this data, Real-time PCR assay was performed in a variety of cancer and normal cells. Consistent with the other reports, that all cancer cells possessed low level (~2-6 fold) of expression of miR-451 as compared to the normal cells (Fig. 2-3). Of note, *in*

vitro immortalized lung fibroblasts (MRC5/hTERT) and lung tumor derived cells (A549) showed lower level of expression as compared to the normal lung fibroblasts (MRC5) (Fig. 2-3). Furthermore, amongst several cancer cells, U2OS showed the lowest level of miR-451 expression (Fig. 2-3). I next overexpressed miR-451 in U2OS cells, and examined their proliferation rate with respect to the control cells. As shown in Fig. 2-4, miR-451 overexpressing derivatives showed decrease in viability (Fig. 2-4 A), cell growth (Fig. 2-4 B), long-term survival and colony forming capacity (Fig. 2-4 C). Cell cycle analysis of control and miR-derivatives revealed G_0/G_1 arrest of in the latter (Fig. 2-5 A). These data suggested that miR-451 caused growth arrest of cells that may have contributed at least in part, escape of cells to 5-Aza-dC induced senescence as hypothesized above. In order to confirm the growth inhibitory activity of miR-451, I performed *in vivo* tumor progression assays using subcutaneous xenograft model. As shown in Figs. 2-5 B and C, significant growth retardation of miR-451 overexpressing A549 derivatives as compared to the control demonstrated that miR-451 is a tumor suppressor miR.

2.3.3 Tumor suppressor activity of miR-451 is mediated by upregulation of $p21^{WAF1}$ but not p53

In order to investigate the mRNA targets and clarify the mechanism of miR-451-mediated growth suppression, I performed expression analyses of major tumor suppressor proteins. As shown in Fig. 2-6 A and B, miR-451 overexpressing cells (as identified by GFP fluorescence), showed decrease in pRB, its phosphorylated form and E2F-5 (Fig. 2-7 B) signifying cell cycle arrest. Consistent with these, upstream regulators of pRB-phosphorylation, CDK4 (Fig. 2-6 A and B) and Cyclin D1 (Fig. 2-7 A), showed decrease, in line with an increase in expression level of $p21^{WAF1}$ (an established mediator of growth arrest and inhibitor of cyclin/CDK complexes [144]) (Fig. 2-6 A and B). These data were supported by immunofluorescence assay with specific antibodies. I performed real time RT-PCR for these candidate target genes, and found that in agreement with the protein expression data, mRNA for pRB, Cyclin D1 and CDK-4 was decreased in miR-451 derivatives (Fig. 2-8 A). $p21^{WAF1}$ on the other hand, showed increase that was also supported by $p21^{WAF1}$ promoter-luciferase assays (Fig. 2-8 B). Contrary to the increase in $p21^{WAF1}$, p53 specific promoter-luciferase assays did not real revealed significant change in the miR-451 derivatives suggesting that $p21^{WAF1}$ increase might be p53-independent (Fig. 2-8 B). I examined the expression of p53 (its major transcriptional activator) and indeed found its decrease, both at the protein (Fig. 2-9 A and B) as well as mRNA levels (Fig. 2-9 C), in miR-451 derivatives. In line with this, HDM2 (antagonist for p53) protein and mRNA showed increase (Figs. 2-10 A, B and C) that may account for decrease in p53.

2.3.4 miR-451 targets CARF and results in an upregulation of $p21^{WAF1}$

Collaborator of ARF (CARF) was first reported as a binding partner and collaborator of ARF ($p14^{Alternate\ Reading\ Frame}$ protein) by yeast two-hybrid screening [130]. It has been shown to regulate proliferative fate of cells; cellular senescence, apoptosis and malignant transformation by dose dependent two-way regulatory pathways [131, 133-135, 145]. It has been established that CARF is essential for cell survival; its knockdown causes apoptosis. Furthermore, whereas overexpression of CARF was shown to be associated with senescence related growth arrest of cells, its super high level of expression was correlated with malignant transformation of cancer cells [134]. Consistent with these findings, CARF has been shown to be upregulated in a variety of cancer cells [135, 146] and involved in cancer metastasis [117]. Recently study observed that the tumor suppresser miR-335 directly targets CARF and regulates cell cycle [138].

The mechanism of miR-451 induced growth arrest that was marked by increase in $p21^{WAF1}$ and hypo-phosphorylation of pRB were explored (Fig. 2-6 and Fig. 2-8). There have earlier reported that CARF (Collaborator of ARF) acts as a transcriptional repressor of HDM2 and $p21^{WAF1}$. Based on the above results on upregulation of both $p21^{WAF1}$ and HDM2 in miR-451 derivatives, I predicted that CARF could be one of the targets of miR-451. Analyses of protein as well as mRNA expression of CARF in control, vector and miR-451 derivatives revealed its remarkable decrease in the latter (Fig. 2-11). Furthermore, increase in $p21^{WAF1}$, HDM2 and decrease in CARF was observed in p53 deficient Saos-2-miR 451 derivative cells (Fig. 2-12 A) suggesting that increase in $p21^{WAF1}$ was independent to that of p53, and CARF is a candidate new target for miR-451. For conclusive validation, reporter assays was performed by using CARF, p53 and p21 3'UTR-luciferase constructs. As shown in Fig. 2-12 B, miR-451 caused reduction in CARF, but not p53 or $p21^{WAF1}$ mRNA. These data confirmed that miR-451 targets CARF, but not p53 or $p21^{WAF1}$. In order to confirm that the effect was specific to miR-451, two other miRs (miR-101 and 558) with identical vector and cell line were used for comparing. miR-101, miR-558 and miR-451 were overexpressed in U2OS cells, and examined their proliferation rate with respect to the control cells. As shown in Fig. 2-13 A, miR-451 overexpressing derivatives showed decrease in viability but miR-101 or miR-558 overexpressing promoted cell proliferation (Fig. 2-4 A), cell growth (Fig. 2-13 B), long-term survival and colony forming capacity (Fig. 2-13 C). In order to further examine miR-451 driven regulation of CARF, sequence based analyses was performed by using TargetSpy, a knowledge based online portal comprising broad-range compositional, physical, and combinatorial base-pairing algorithms that

predicted miR-451 target sequence in CARF/CDKN2AIP 3'UTR transcript (NM 017632) located at 448-465 base position (Fig. 2-13 D).

2.3.5 CARF targeting by miR-451 and miR335 induce apoptosis

CARF has been shown to pose two-way control on cell proliferation [131, 133-135, 145]. Its knockdown was lethal for cells *in vitro* and *in vivo* suggesting that it is an essential protein for cell survival [135]. A variety of cellular senescence models endorsed that the overexpression of CARF caused growth arrest of cancer cells and plays a definitive role in replicative as well as stress-induced senescence [133, 135, 137, 145]. Super-high level of CARF expression has been associated with malignant transformation of cancer cells. Molecular mechanism(s) on the role of CARF in such dual regulation of cell proliferation has not been resolved as yet. In the present study recruited retroviral vector constituting two long terminal repeat (LTR) promoters on the 5' and 3' ends of the gene. Upon random integration of the vector in the genome, it resulted into expression of GFP; detected by green fluorescence. At the same time, it caused integration-dependent arbitrary manipulation of the host cell genome leading to altered expression of either proteins or miRs, and thereby loss-of-function phenotype, escape from induction of senescence in cancer (U2OS) cells by 5-Aza-dC (demethylating drug). The cells that evaded senescence were selected and subjected to microRNA array [138]. There were several miRNAs (miR-101, miR-335, miR-145, miR-451, and miR-558) were upregulated in vector-transduced cells that showed resistance to 5-Aza-dC-induced senescence [138]. In the earlier study CARF has be identified as target for miR-335 [138]. It was shown that miR-335 caused growth arrest in cancer cells and hence inhibited incorporation of 5AZA-dC in genome[138]. Similar to miR-335, miR-451 caused growth suppression *in vitro* and *in vivo*. In view of this, the cells were co-transfected with miR-451 and miR-335 and examined the cell phenotype. As shown in Fig. 2-14 A, cells co-transfected with miR-335 and miR-451 showed stronger knockdown of CARF resulting in apoptosis as confirmed by apoptotic markers including cleavage of pro-caspase-3 and decrease in anti-apoptotic protein, Bcl-2. FACS analysis of control and double (miR-335 and 451)-transfected cells also confirmed higher rate of apoptosis in the latter (Fig. 2-14 B).

In order to further prove the regulation of CARF by miR-451, seven chemical stress models that represented a variety of environmental stresses were used in this study (Fig. 2-15). Normal human fibroblasts (TIG-3) were treated with stress inducing agents that caused apoptosis, endorsed by decrease in CARF (Fig. 2-16 A). Of note, consistent with the decrease in CARF, miR-451 showed increase (Fig. 2-16 B). Furthermore, this study showed that the cells treated with a herbal extract were protected from apoptosis. They showed recovery in CARF expression

(Fig 2-16 A). Of note, increase in CARF was accompanied by decrease in miR-451 in stress-recovered cells (Fig. 2-16 B). These data supported that miR-451 regulates CARF as well as stress response of cells.

2.4 Summary

This study demonstrated that CARF is a new target of miR-451 that mediates its tumor suppressor function. Targeting of CARF by both miR-451 and miR-335 was confirmed by double transfections of cells that caused stronger knockdown of CARF. This study showed that miR-451-induced growth arrest was mediated by increase in $p21^{WAF1}$ and consequent decrease in Cyclin D1, CDK4, $pRB^{Phospho}$ and E2F5 (Fig 2-17). These changes were independent of p53 status of the cells and mediated by targeting CARF, as endorsed by protein, mRNA and 3'UTR reporter assays. It was found that miR-451 plays role in such regulation of CARF. It showed increase in response to a variety of stresses that caused decrease in CARF. Of note, when cells were protected against stress by treatment with a herbal extract, both miR-451 and CARF showed some recovery to the normal unstressed levels suggesting a tight correlation of miR-451 with CARF.

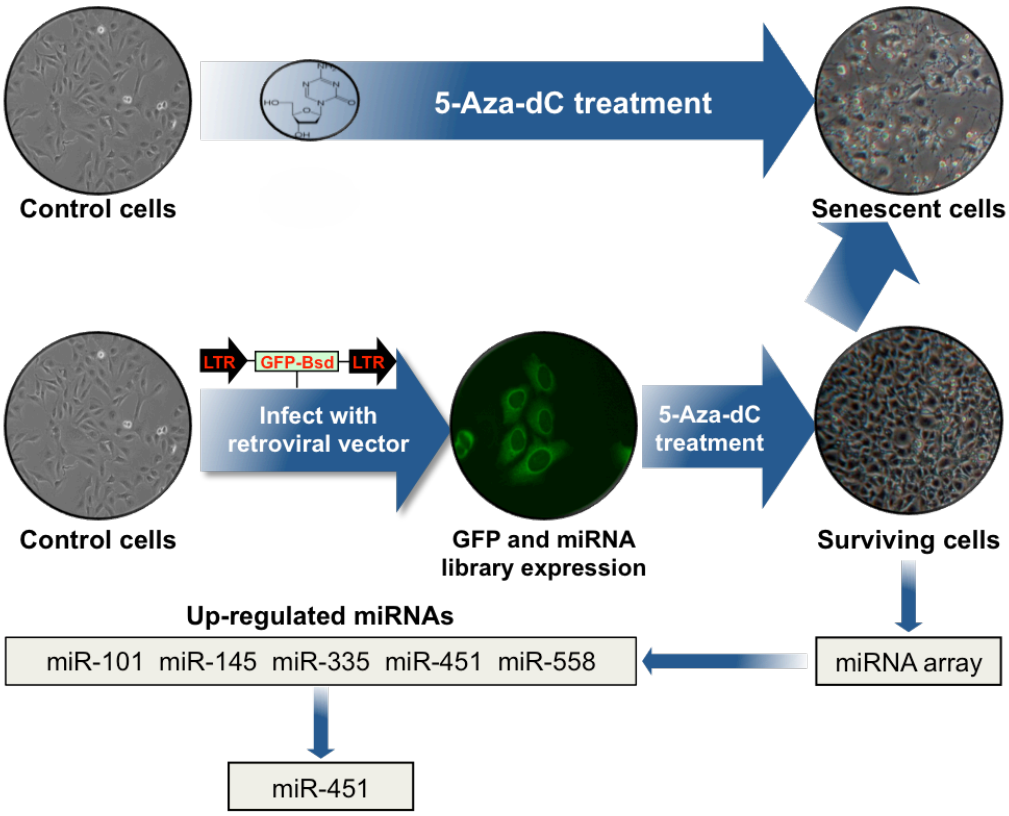


Fig. 2-1 Loss-of-function screening of miR library in human cancer cells for identification of miR-451. The cells infected with bicistronic vector constituting of two promoters and GFP were treated with 5-Aza-dC. Whereas control uninfected cells showed induction of senescence, colonies emerged in the virus harboring cells. These colonies were expanded and subjected to miR-array analysis with respect to the uninfected control cells. miR array analysis resulted in identification of miR-451 as one of the upregulated miR in cells that escaped 5-Aza-dC induced senescence.

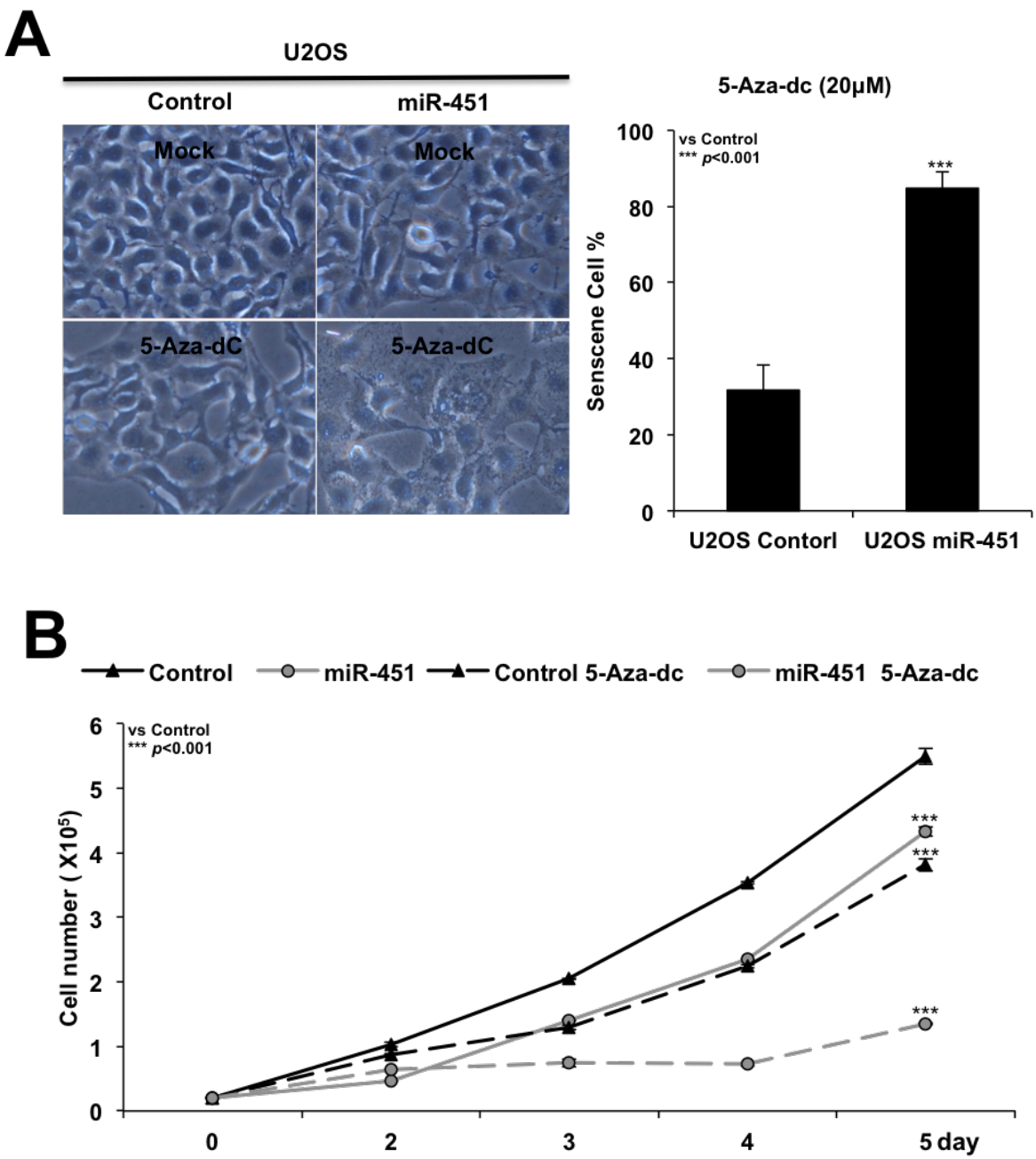


Fig. 2-2 Cell overexpressing miR-451 showed sensitized to 5-Aza-dC induced senescence as examined by senescence associated β-gal staining (A). Growth curve of control and miR-451 overexpressing cells showed growth arrest in response to 5-Aza-dC treatment (B).

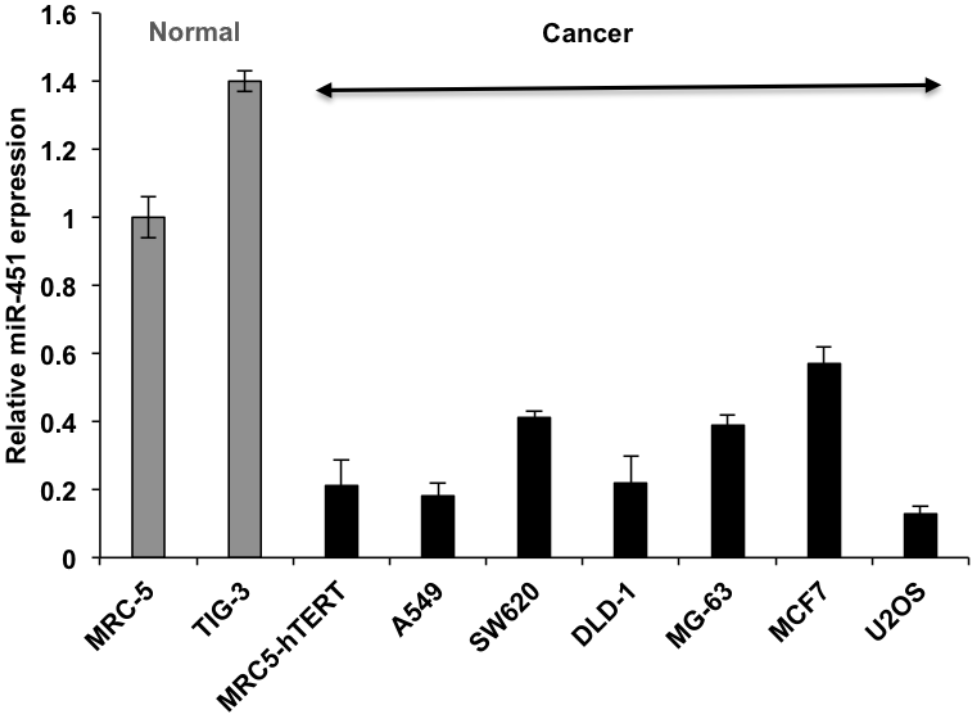


Fig. 2-3 miR-451 is downregulated in cancer cells. Real-time PCR analysis of miR-451 in human normal and cancer cells showed its higher level of expression in normal cells. Cancer cells showed 2 to 1- fold less expression.

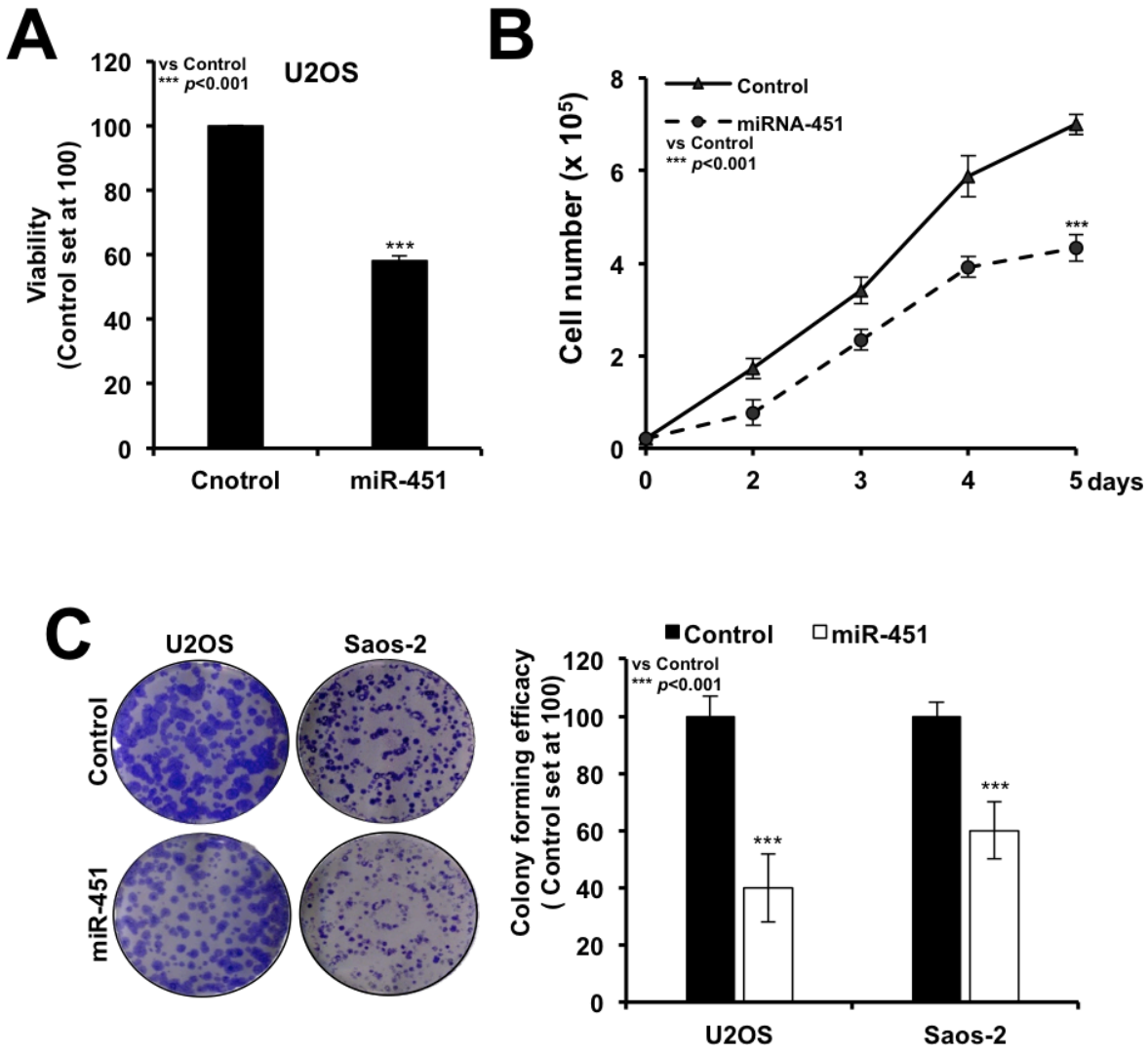


Fig. 2-4 Viability of control and miR-451 overexpression cells showed less number of viable cells in the latter (A). Growth curve analysis revealed that miR-451 overexpression suppresses U2OS cell growth (B). Long-term survival by colony forming assay showed reduction (60% and 40% in U2OS and Saos-2, respectively) of cells colonies in miR-451 overexpressing derivatives (C).

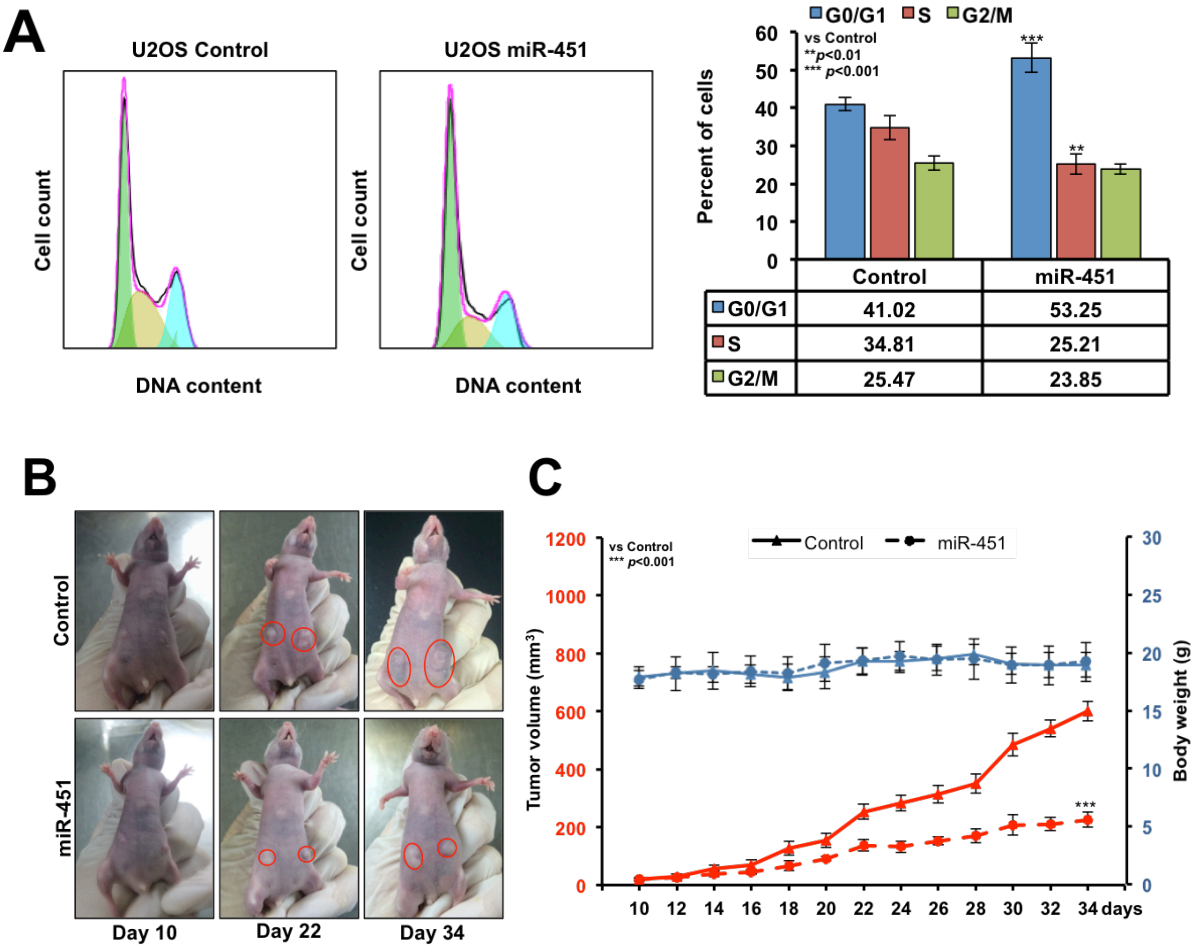


Fig. 2-5 Cell cycle analysis revealed increase in number of cells at G_0/G_1 phase and decrease in number of cells at G_2/M and S phases (A). Tumor growth assays in subcutaneous xenograft of control and miR-451 overexpressing cells showed reduced tumor forming capacity of the miR-451 derivatives as compared to the control cells (B, C). Body weight of the mice during the course of experiment showed no difference (C).

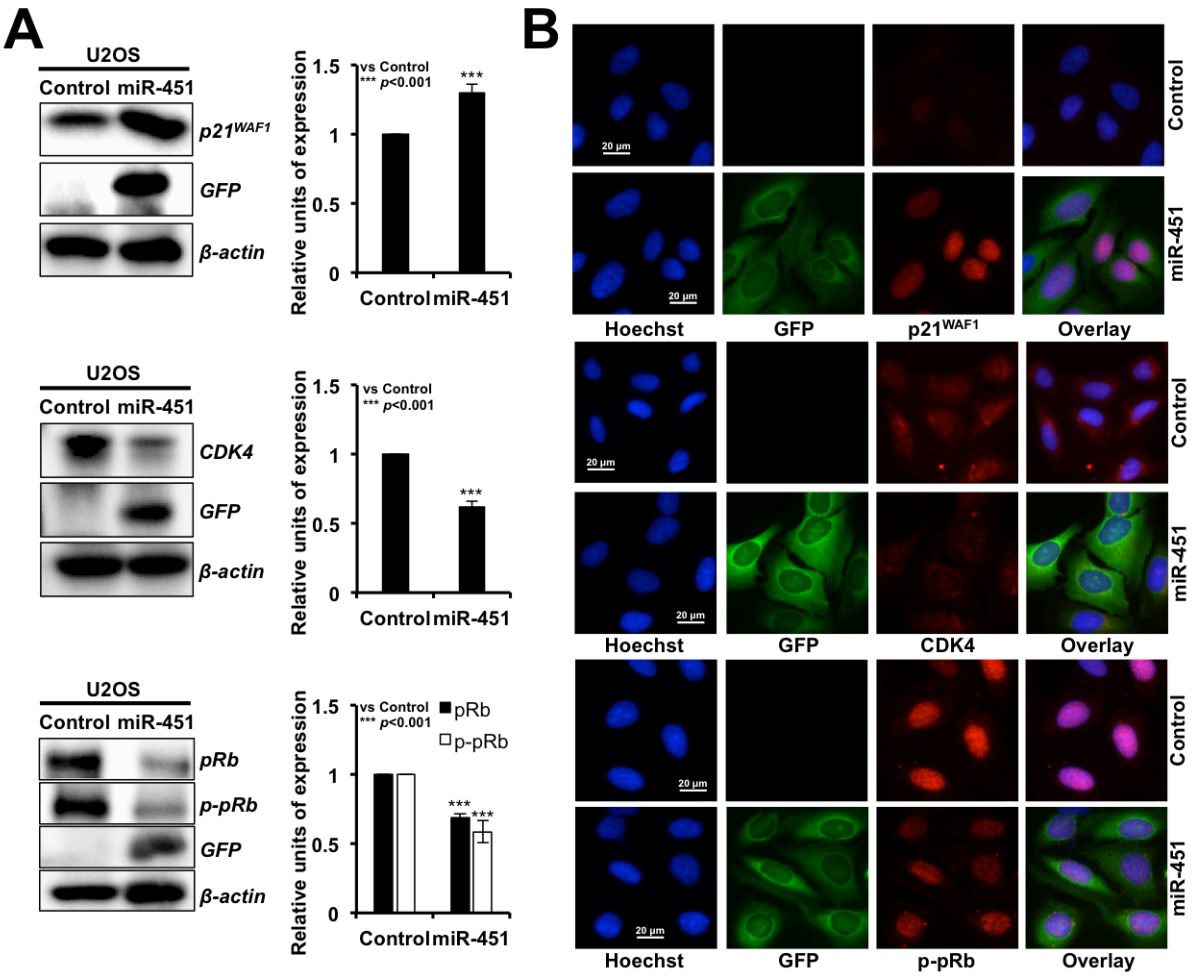


Fig. 2-6 Western blotting (A) and immunostaining (B) showed miR-451 overexpressing cells possessed higher level of expression of $p21^{WAF1}$, and lower of expression pRB, $pRB^{Phospho}$, CDK4.

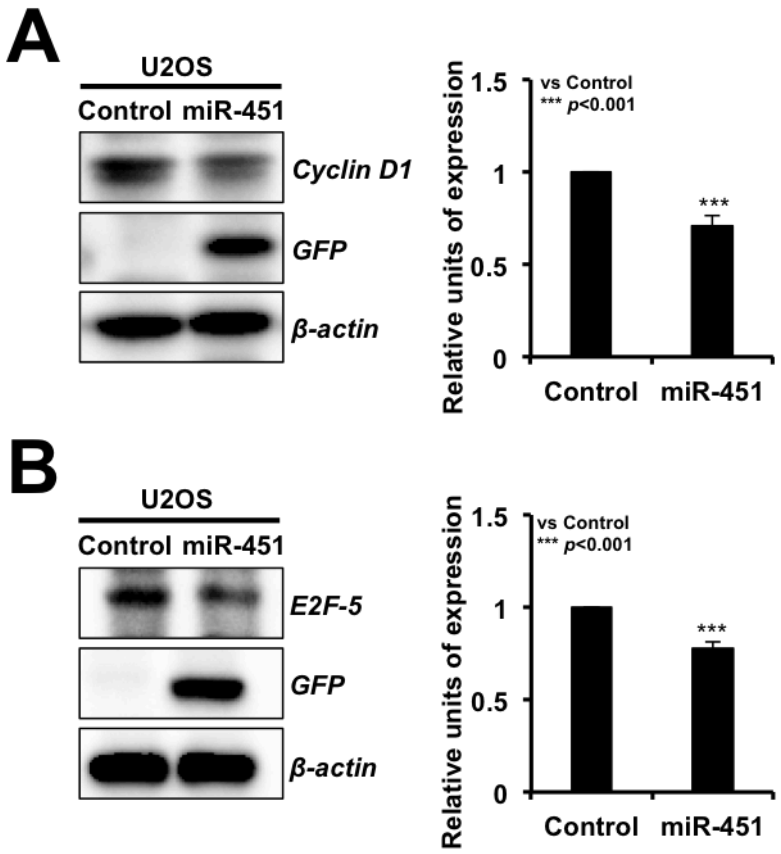


Fig. 2-7 miR-451 overexpressing derivatives that showed growth arrest possessed lower level of expression Cyclin D1 and E2F-5 by western blotting.

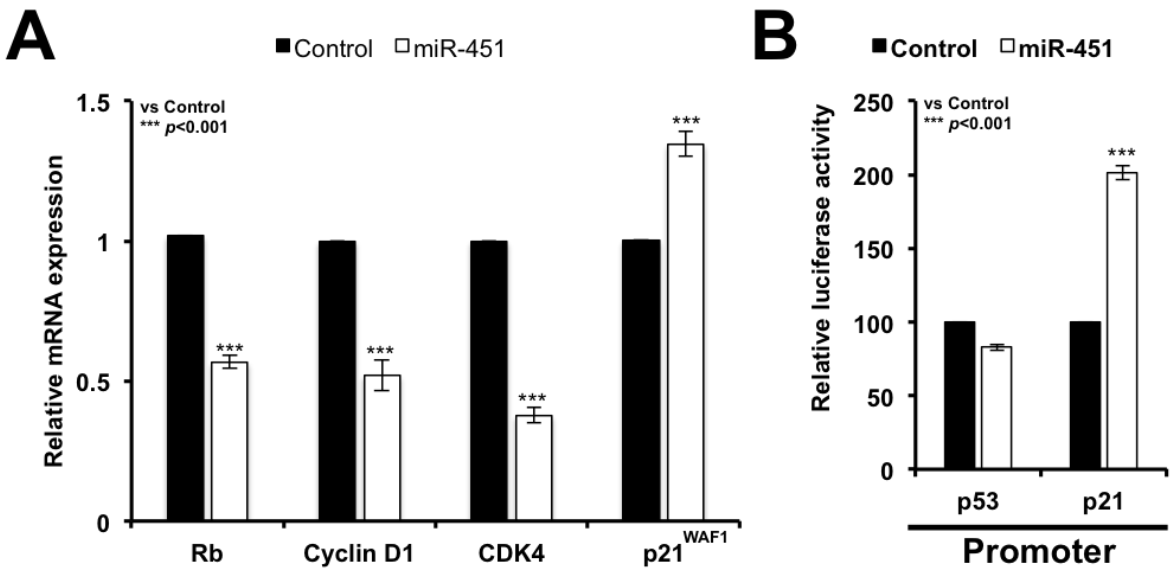


Fig. 2-8 mRNA expression revealed, decreased level of pRB, Cyclin D1, CDK4 and increased level of expression of $p21^{WAF1}$ in miR-451 overexpressing cells (A). p53 dependent promoter driven-reporter assays in control and miR-335 transfected cells showed upregulation of $p21^{WAF1}$ and no change in p53 (B).

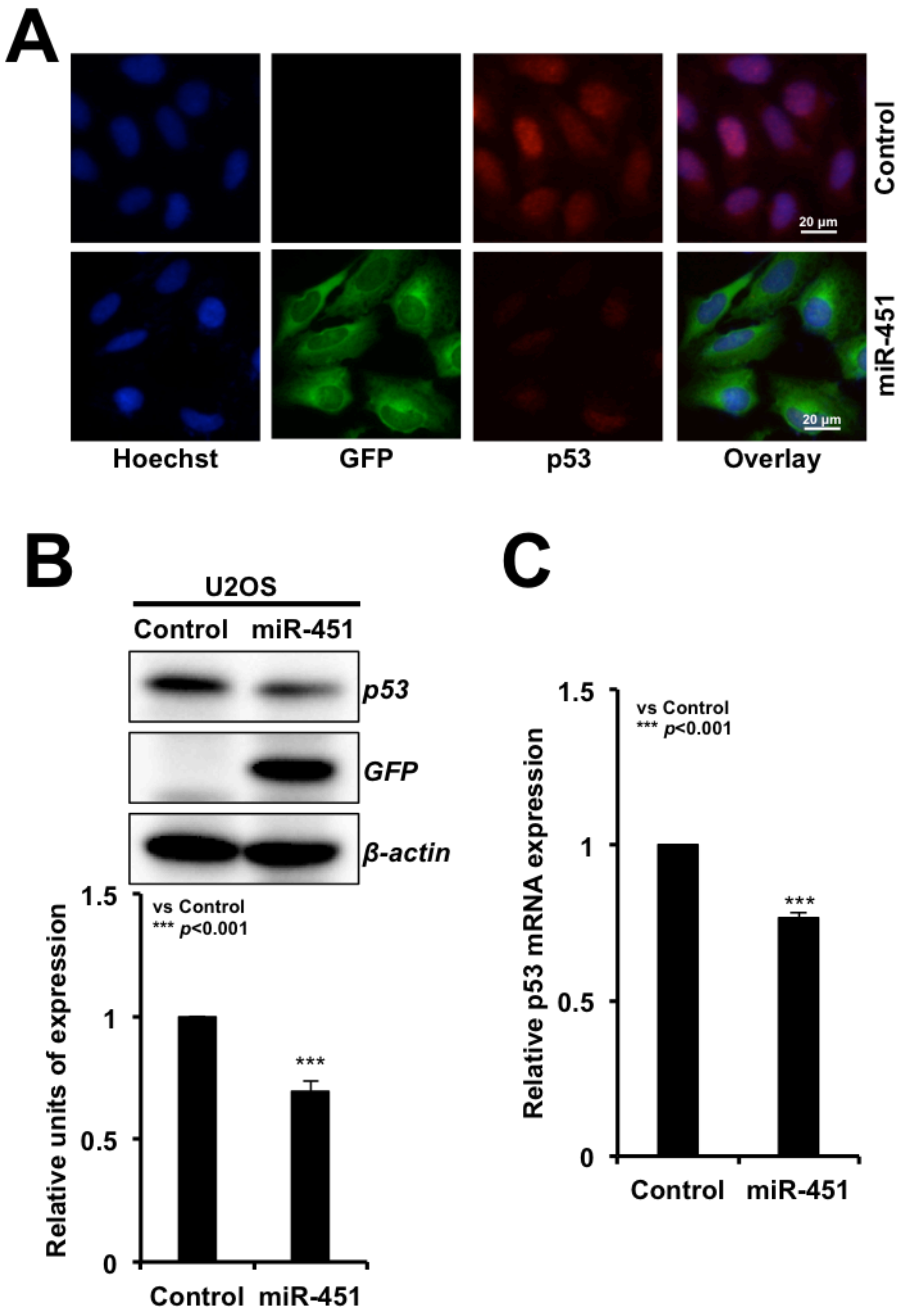


Fig. 2-9 miR-451 overexpressing cells showed downregulation of p53. miR-451 overexpressing derivatives possessed lower level of expression p53 at protein and both mRNA protein levels (A-C).

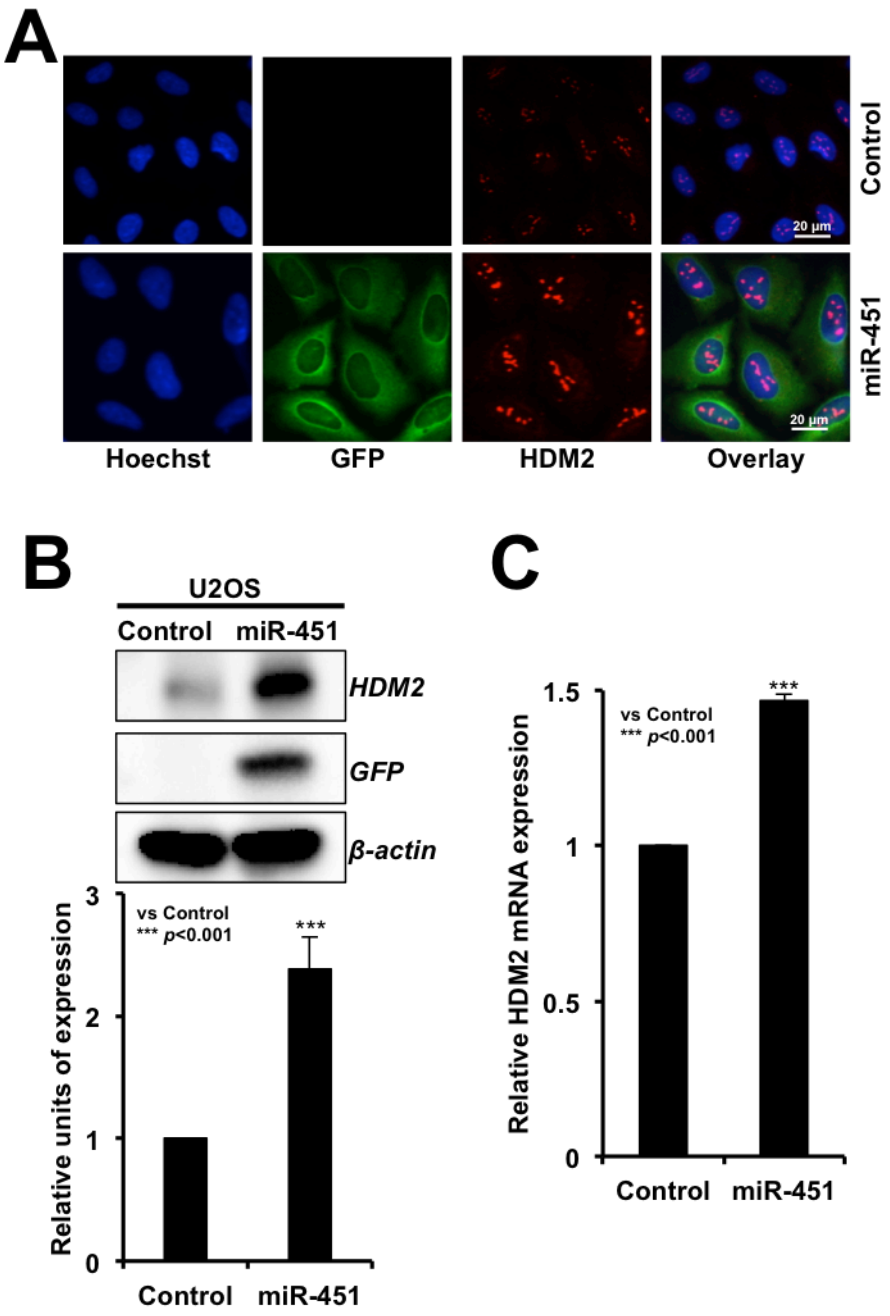


Fig. 2-10 miR-451 overexpressing cells showed upregulation of HDM2. miR-451 overexpressing derivatives possessed higher level of expression HDM2 at protein and both mRNA protein levels (A-C).

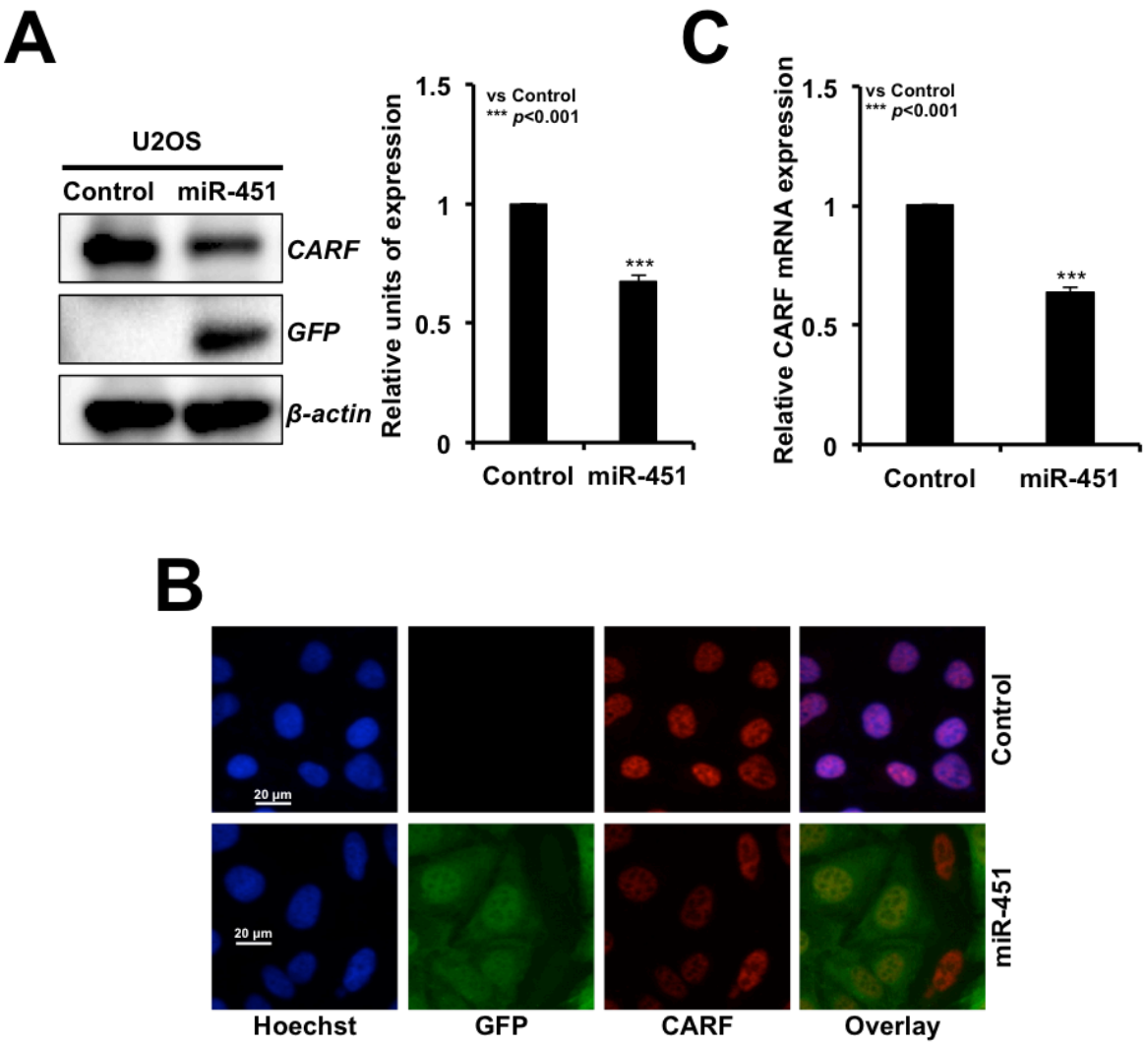


Fig. 2-11 Western blotting (A) immunostaining (B) and qRT-PCR (C) analysis for CARF showed decreased of expression in miR-451 overexpression cells.

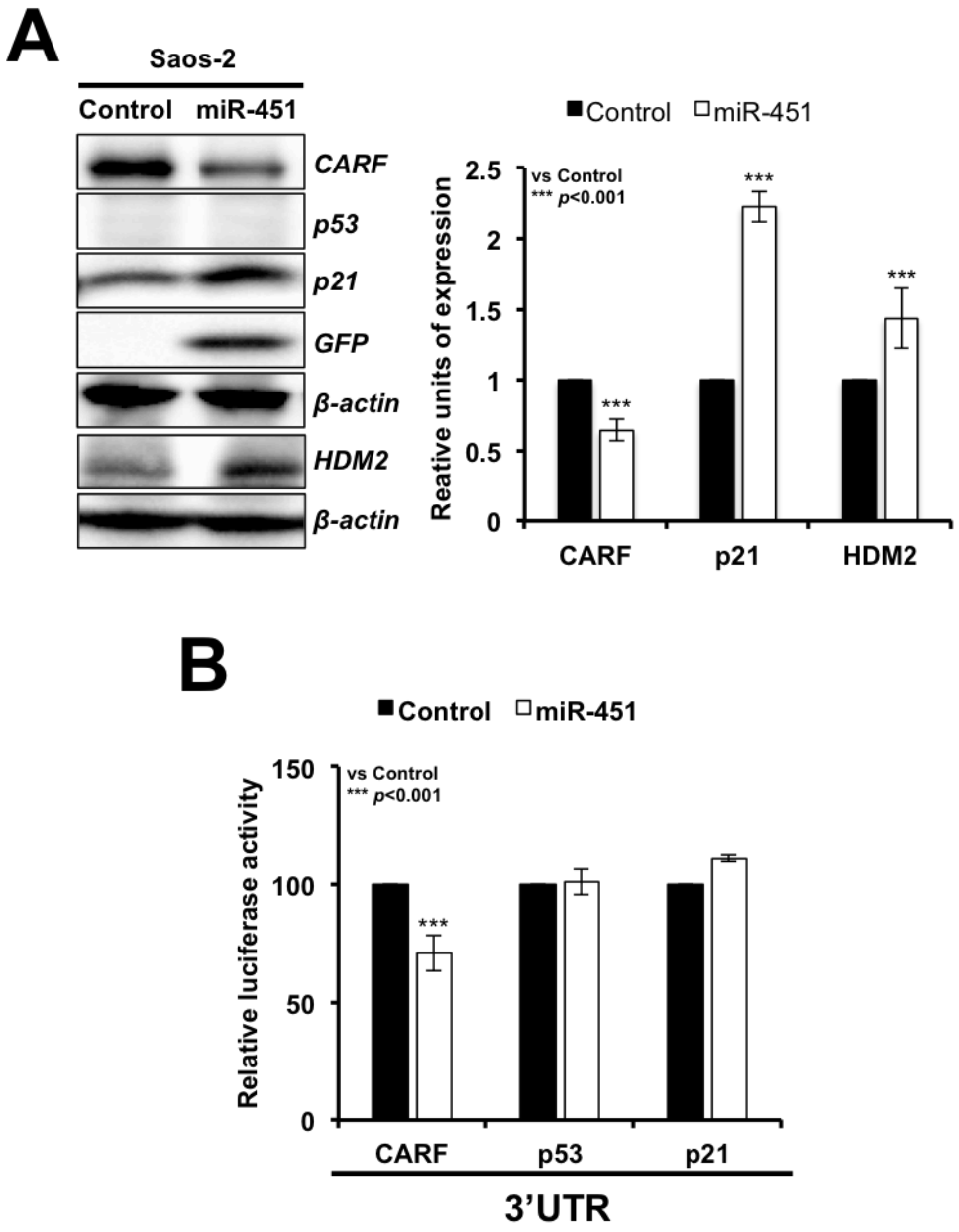
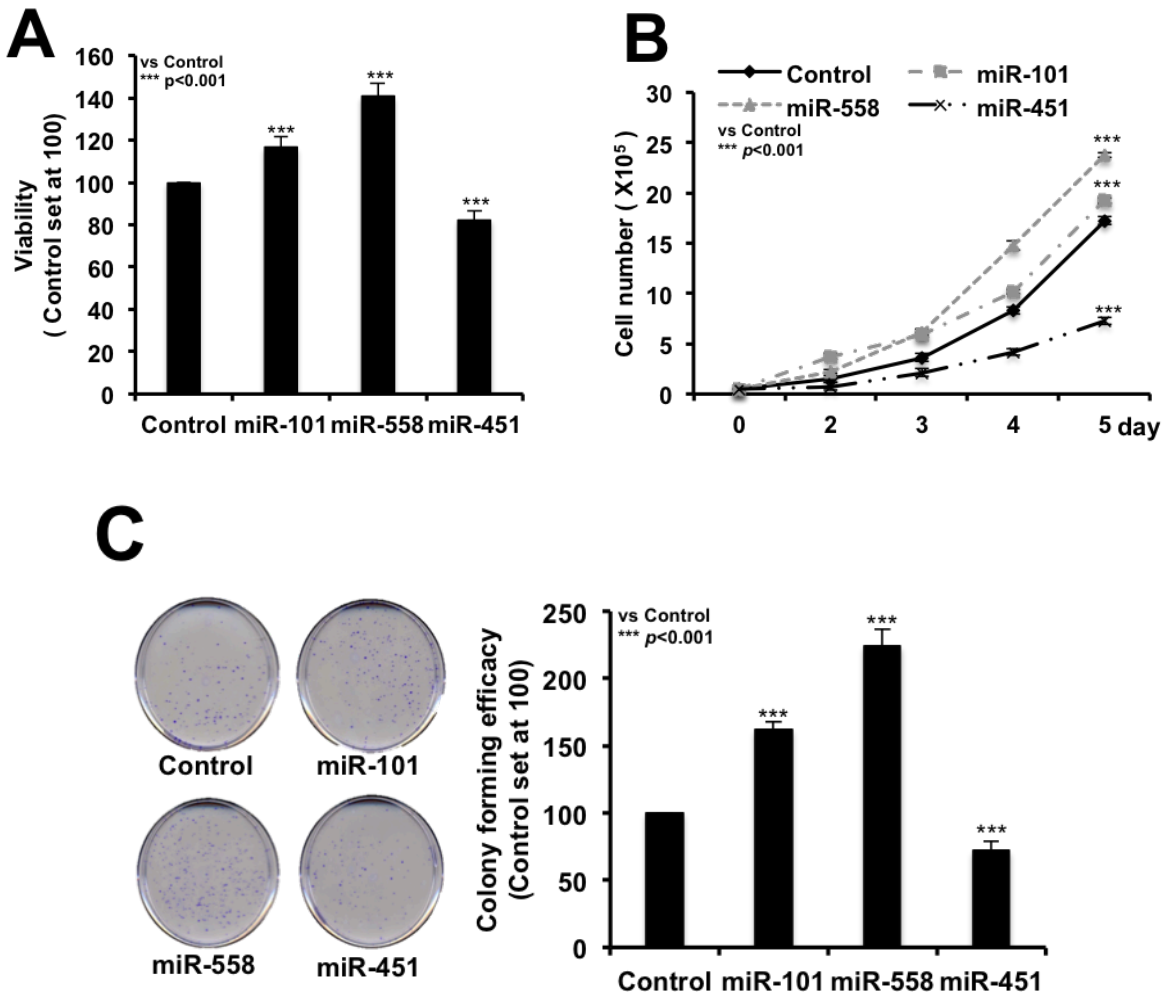


Fig. 2-12 miR-451 directly targets CARF, but not p53. Western blotting (A) showed decreased in CARF, increase in $p21^{WAF1}$ and HDM2 occurred in p53-/- (Saos-2) cells. 3'UTR reporter assay for CARF, $p21^{WAF1}$, p53 in control and miR-451 overexpressing cells showed that miR-451 directly targets CARF (B).



D

MicroRNA	Gene	Position in 3'UTR	Structure	Energy	Score
miR-451	NM 017632	448-465	TTGCCCGTAATGTTGAACGTGTCT |||||| |||| || | UUGAGUCAUUACCA-UUGC-CAAA	-8.8	0.99

Fig. 2-13 Specificity of miR-451 in cell growth-arrest assays and targeting of CARF. Viability of control, miR-101, miR-558 and miR-451 transfected cells showing decreased number of viable cells in the latter (A). Growth curve analysis revealed that miR-451, but not the miR-101 and miR-558, suppressed cell growth (B). Long-term survival by colony forming assay showed reduction of colonies in miR-451, but not miR-101 and miR-558, overexpressing derivatives (C). Target site of miR-451 in 3' UTR of CARF/CDKN2AIP is shown (D).

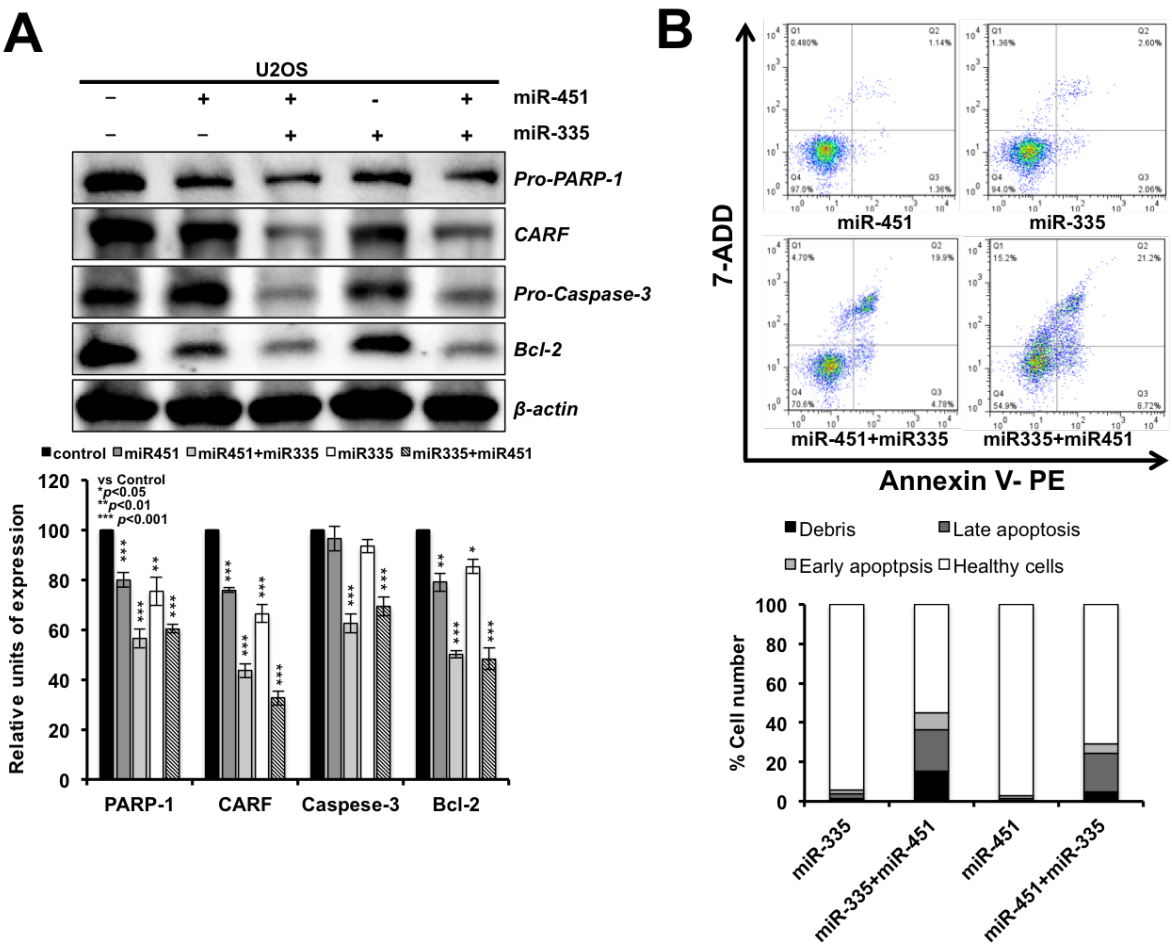
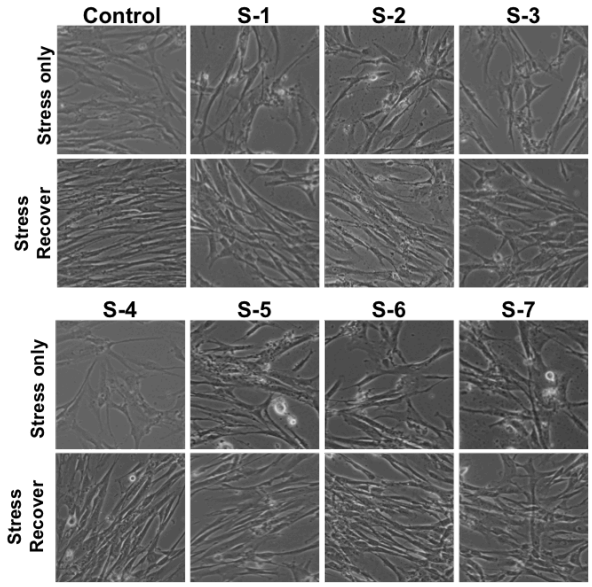


Fig. 2-14 miR-335 and miR-451 co-transfection caused stronger knockdown of CARF resulting in apoptosis of cells. miR-335 and miR-451 double derivatives showed stronger knockdown of CARF resulting in decrease in pro-caspase-3, pro-PARP-1 and BCL-2 (A) and apoptosis (B).



	Stress types/Source	Chemical model
S-1	Environmental/Diesel fuel	2-Nitro-9-fluorenone
S-2	Environmental/Cans	Vanadium(V) oxide
S-3	Environmental/Plastic	Chloroformate
S-4	Environmental/Aromatic	1-Hydroxypyrene
S-5	Environmental/Painting	Formaldehyde
S-6	Environmental/Battery	Cadmium nitrate tetrahydrate
S-7	Physiological environments/ Anger	Epinephrine

Fig. 2-15 miR-451 regulates stress response of cells through CARF signaling. Phenotype of human fibroblasts under normal and a variety of stressed conditions (as shown in the table).

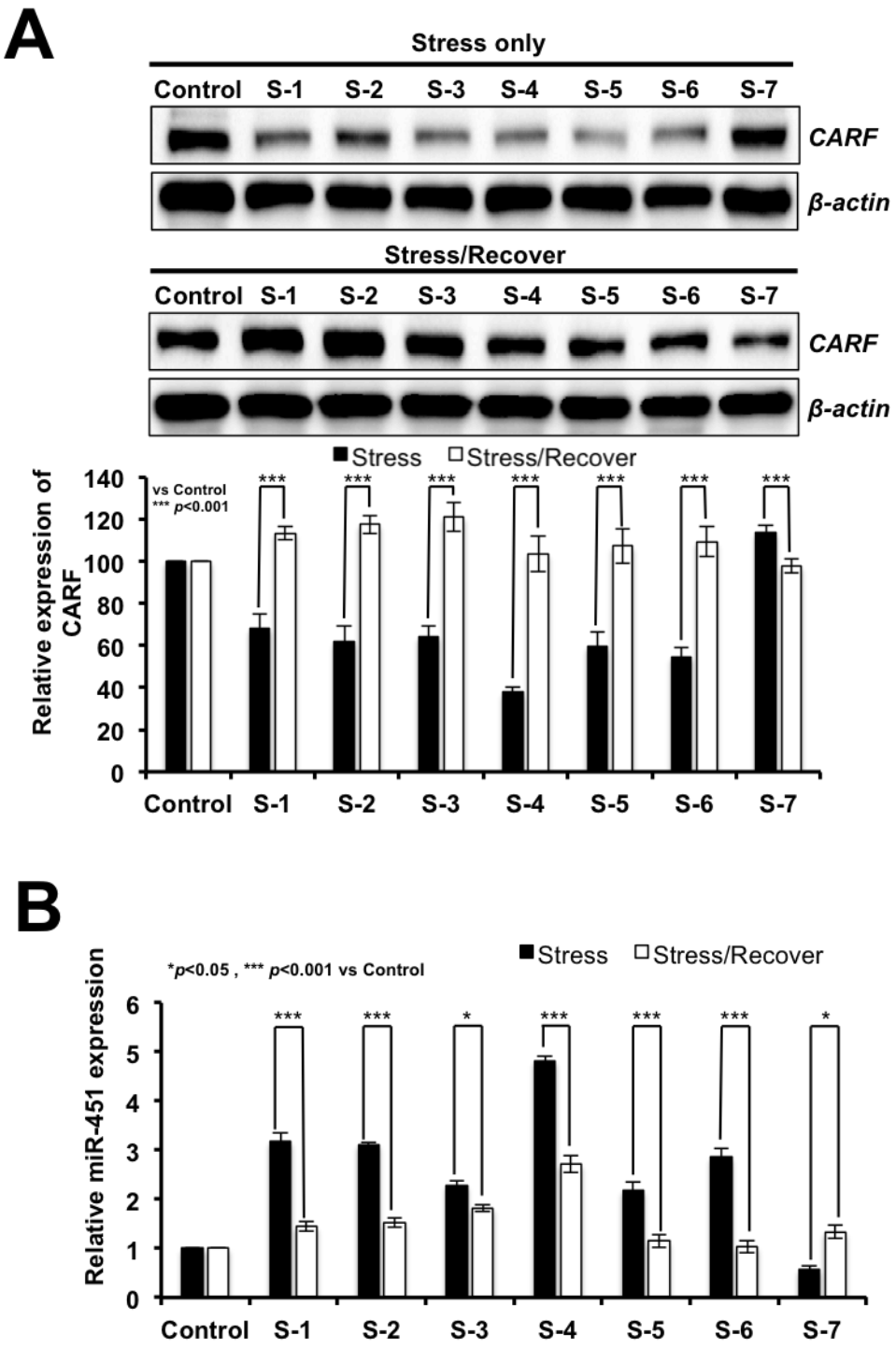


Fig. 2-16 Western blotting showing decrease in CARF associated with apoptosis of stressed cells and its recovery in cells treated with medium (A). miR-451 expression was upregulated in stressed cells and showed downregulation in recovered cells (B).

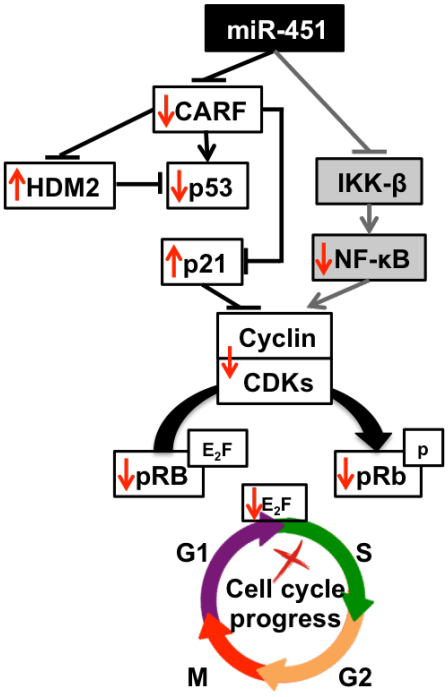


Fig. 2-17 Schematic presentation of miR-451 targets as resolved in this study.

Chapter 3 Cell based screening for genes involved in melanogenesis and validation of results- involvement of mortalin

3.1 Introduction

Skin, the largest organ of the body, is the most important tissue that interfaces and interacts with environment and regulates signaling pathways evoking adaptation/ defense to a variety of stresses involving chemicals, heat, radiations, and microbes [10]. Skin color, although a heritable trait, is highly influenced by environmental and endocrine factors that cause its modulation either temporarily or permanently [13, 15, 147]. It is determined by the amount, type, and distribution of melanin pigment, synthesized by a complex process of melanogenesis in subcellular organelles (melanosomes), in specialized cells (melanocytes) and involves melanogenic enzyme, tyrosinase [10]. Melanin synthesized in melanocytes is transferred to keratinocytes and determines skin color by virtue of its characteristics including type and proportion of small molecule constituents. Eumelanin (EM) that gives black and brown coloration is the most abundant melanin in humans. Pheomelanin (PM) imparts pink and red color and is concentrated in the lips, nipples, and glands [15, 87, 148]. Besides its role in skin coloration, melanin has been assigned functions including stress protection and its susceptibility to disorders ranging from minor itching to metastatic cancers.

Induction of photo-damage and oxidative stress are established as the initiating steps of skin carcinogenesis including melanomas, the most aggressive form of skin cancer [149, 150]. Melanin pigment provides protection against UV-induced DNA damage. Several population studies have shown that the regular use of sunscreens causes reduction in the lifetime incidence of ultraviolet-induced skin cancers [150]. Light complexion has been linked to high incidence of dysplastic nevi (DN, atypical moles) [151]. X-ray microanalysis of melanosomes from DN and melanomas revealed high sulfur (an indicator of pheomelanin), iron, and calcium (involved in oxidative stress) as compared to the normal skin melanocytes from the same individual suggesting that the pheomelanogenesis is associated with oxidative imbalance [151]. DN cells also showed significantly high reactive oxygen species (ROS) and DNA fragmentation than the normal melanocytes from the same donor [152]. In oxidative-stressed melanoma cells, tyrosinase and microphthalmia transcription factor (MITF), the two main regulators of melanogenesis, were downregulated suggesting that the oxidative stress might lead to hypopigmentation [153]. However, it has not been clinically associated with either increased photo-damage/skin aging or cancer due to high levels of wild-type functional dermal p53 protein, implicated in control of DNA damage [154].

Melanogenesis is regulated by a variety of signal transduction pathways. In mammals, more than 100 genes have been shown to be involved in the process of melanogenesis. Several of these have also been implicated in oxidative stress response. The expression level of catalase, a main enzyme responsible for degrading H_2O_2 in melanocytes, was correlated with melanin content [155]. Darkly pigmented melanocytes therefore possess two protective mechanisms (high melanin and catalase activity) that may act synergistically for protection against UV. Induction of p53 expression, a hallmark for genotoxic stress, was shown to cause induction of pigmentation in human melanocytes [156]. Several synthetic and natural antioxidants have been shown to modulate skin color and its characteristics to protect against UV damage and cancer [157]. Molecular mechanisms of regulation of melanin synthesis and its photoprotective action, in context to its melanogenic, antioxidant, and cell survival activities, have not been fully elucidated. Understanding these mechanisms is of high significance in cosmetic (skin whitening from beauty perspectives and skin tanning products for reducing the potential risk from skin cancer) as well as pharmaceutical (therapy for pigmentary diseases) applications.

In the present study, loss-of-function screening was using human shRNA library in conjunction with OAG-induced (1-oleoyl-2- acetyl-glycerol, an established inducer of pigmentation) melanogenesis in human melanoma cells [158, 159]. Pathway and molecular analyses of the identified gene targets revealed that the proliferation, stress, and apoptotic signaling mediated by heat shock 70 family chaperone mtHsp70/ mortalin are critically involved in the process of melanogenesis [160].

3.2 Materials and methods

3.2.1 Cell culture, transfections, and drug treatments

Human skin melanoma (G361) cells obtained from the Japanese Collection of Research Bioresources (JCRB, Japan) were cultured in McCoy's 5A medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. Primary human melanocyte cells were obtained from Kurabo Industries Ltd (Osaka) and cultured in DermaLife Basal Medium (Life Line Cell Technology, Carlsbad, CA). OAG (Sigma, Japan), dissolved in dimethylsulfoxide (DMSO), was added to the subconfluent (60–70% confluence) cells at a concentration of 15 μg/ml for primary human melanocytes and 30 μg/ml for G361 melanoma. Repeated freezing and thawing of OAG was avoided by making small aliquots of the stock solution and storage at −20°C. Transfections of shRNA were performed using X-tremeGENE 9 (Roche Applied Science, Indianapolis, USA). Conditions for the best transfection efficiency were determined by using

GFP expression plasmid. Typically, 100 ng and 2 μg of plasmid DNA were used per 96-well and 6-cm dish of cells, respectively, at 60–70% confluency. Cells were selected in puromycin (2 μg/ml)-supplemented medium and then used for assays as described below. Epolactaene (ETB, an heat shock protein 60 (HSP60) inhibitor) (3 μM), creatine (inducer of mitochondrial fragmentation) (10 μM), and carbonilcyanide *p*-triflouromethoxyphenylhydrazone (FCCP, an uncoupler of mitochondrial oxidative phosphory- lation) (10 μM) were used to inhibit mitochondrial functions. MKT-077 at a subtoxic dose (100 nM) was used as an inhibitor of mortalin.

3.2.2 Preparation of shRNAs and screening of shRNA library

shRNAs were cloned in a U6-driven expression vector as described earlier[161]. Cells were plated in 96-well plates and transfected at about 70 % confluency with 100 ng of the plasmid DNA. Twenty-four hours post-transfection, cells were selected in puromycin (2 μg/ml)-supplemented medium for 48–72 h, expanded to 70% confluency, and then treated with OAG. shRNAs that resulted in abrogation of OAG-induced increase in melanin content and tyrosinase activity were selected. Cells were transfected with these selected shRNAs, and OAG-induced melanogenesis was determined by quantitative assays for melanin and tyrosinase once again. Similar cycle of selection was repeated four times.

3.2.3 Melanin content

To estimate the melanin concentration, cells (5 × 103/well for primary human melanocytes and 2 × 103/well for G361 melanoma) plated in 96-well dish were treated with shRNA and/or OAG for 24 h as described. The cells were incubated with 0.85 N KOH (100 μl) overnight with slow shaking at room temperature (RT). Melanin content was estimated by reading absorbance at 405 nm using a spectrophotometer (Tecan, Switzerland). Relative amount of melanin was calculated by using synthetic melanin (Sigma) as a standard in similar assays and normalized against protein content.

3.2.4 Tyrosinase ELISA

Cells were plated in 96-well plates (NUNC-IMMUNO, Maxisorp) and cultured until (24 or 48 h) they attached well to the surface. The cells were treated with OAG and shRNA, as indicated, for 24 h followed by two washing with cold PBS (shaking for 5 min each time). Cells were lysed with RIPA buffer (Thermo Fisher Scientific Inc., IL) and stored in −80°C until further processing. Protein concentration was estimated using Pierce BCA Protein Assay Kit (Thermo Scientific, IL, USA). Equal amounts of the protein from control and treated cells were diluted in coating buffer (0.1 M sodium bicarbonate pH 9.6 with 0.02% sodium azide) and incubated in

plates for either 3 h at RT or overnight at 4°C. The uncoated proteins were removed by aspirating the protein lysate softly. Plates were washed with washing buffer (PBS–0.5% Tween, pH 7.4) by shaking for 10 min (twice). Cells were blocked in blocking buffer (1 % bovine serum albumin and 0.02% NaN_3, pH 7.4) by incubation at either RT (for 3 h) or 4°C (overnight). Blocking buffer was then discarded, and plates were washed with washing buffer (twice, 5 min shaking each time). Cells were then incubated with anti-tyrosinase polyclonal antibody (M-19)-R (1:5000 dilution in blocking buffer) for 1 h at RT. Cells were washed thrice with washing buffer and then incubated with secondary antibody (alkaline phosphatase-goat anti-rabbit IgG) (1:1000 dilution in blocking buffer) followed by washing in washing buffer (with shaking for 5 min × 5 times). Cells were then incubated with AP substrate *p*-nitrophenyl phosphate (pNPP; 1 mg/ml) (PIERCE) in substrate buffer (50 mM $NaHCO_3$ and 10 mM $MgCl_2 \cdot 6H_2O$, pH 9.8) at RT for 30 min, followed by measurement of absorbance at 405 nm. In order to avoid evaporation, plates were kept sealed during the process.

3.2.5 Pathway analysis

Gene network and pathway analyses of the 40 selected candidate genes were performed using STRING database v9.1 and Ingenuity Pathway Analysis software[162, 163]. Outcome of these analyses was also compared with the analysis performed using STRING database v9.1 and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. The top ten path- ways with high probability scores were selected and investigated for their interaction with the melanogenesis regulatory genes.

3.2.6 Western blotting

Cells were harvested upon completion of treatment (as indicated). They were washed with PBS and lysed in RIPA buffer containing complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Whole cell lysate (20 μg) was resolved using SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane using semidry transfer apparatus (ATTO Corporation, Japan). The membrane was blocked with 3 % BSA/TBS, and the expression level of indicated proteins was determined by incubating the membrane with specific primary antibodies tyrosinase (M-19)-R, mortalin, p53 (DO-1) (Santa Cruz Biotechnology, Santa Cruz, CA) Hsp60 (N-20) followed by incubation with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and detection by ECL PLUS (GE Healthcare, UK). The membranes were probed by anti β-actin antibody (Abcam, Cambridge, UK) as an internal loading control.

3.2.7 Immunostaining

Cells were plated on coverslips placed in a 12-well culture plate. After the indicated treatments, cells were fixed with pre-chilled methanol/acetone (1:1) for 5–10 min. After washing with PBS and PBS with 0.2% Triton X-100 (PBS-T) for 10 min each, cells were incubated with primary antibodies, anti-p53 (DO-1; Santa Cruz Biotechnology), anti-myc tag (Cell Signaling), anti-melanosome (HMB45- recognizes 10-kD segment of a sialylated glycoconjugate) (Dako), and anti- mortalin at 4°C overnight. Cells were washed extensively with PBS-T (four times, 10 min each) followed by incubation with fluorochrome-conjugated secondary antibodies (Alexa-488-conjugated goat anti-rabbit or anti-mouse or Alexa-594-conjugated goat anti-rabbit or anti-mouse) (Molecular Probes). Cells were processed for imaging after extensive washing with PBS-T. Mitochondrial membrane potential was determined in control and treated cells by using JC-1 Assay Kit (Cell Technology Inc., USA) that uses a unique cationic dye (5, 5, 6, 6′-tetrachloro-1, 1′, 3, 3′- tetraethylbenzimidazoly- lcarbocyanine iodide) to signal the loss of mitochondrial membrane potential. While healthy mitochondria are stained bright red, the collapse of mitochondrial membrane potential is seen as green fluorescence. ROS were detected by fluorescent staining using the Image-IT^{TM} LIVE Green ROS Detection Kit (Molecular Probes, Eugene, OR). Images, in all cases, were captured on a Zeiss Axiovert 200M microscope and analyzed by AxioVision 4.6 software (Carl Zeiss Microimaging, Thornwood, NY).

3.2.8 Immunohistochemistry

Keloid tissues were obtained from the Institute for Human Tissue Restoration, Department of Plastic & Reconstructive Surgery, College of Medicine, Yonsei University. Keloid and normal abdominal skin tissues were obtained for fibroblast culture, histology, and immunohistochemical analyses with excision. Keloid fibroblasts were obtained from both the central dermal layer of keloids. All the experiments involving humans were performed in adherence to the Helsinki Guidelines. Keloid and normal abdominal skin tissue sections were incubated at 4 °C overnight with an in-house anti-mortalin monoclonal antibody (Clone C1-3), followed by incubation with secondary antibody (Dako EnvisionTM Kit, Dako, Glostrup, Denmark) at RT for 20 min. Diaminobenzidine/hydrogen peroxidase (DAKO, Carpinteria, CA) was used as the chromogen substrate. All the slides were counterstained with Mayer's hematoxylin. The expression level of mortalin was semi- quantitatively analyzed using MetaMorph® image analysis software (Universal Image Corp., Buckinghamshire, UK). Results were expressed as the mean optical density for six different digital images.

3.2.9 Statistical analysis

All the experiments were performed, at least, three times. Quantitation of data was performed using ImageJ software (NIH, MA). Significance values were $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

3.3 Results and discussion

3.3.1 OAG-induced pigmentation in melanoma cells and human primary melanocytes

In order to identify the cellular factors involved in human melanogenesis, human melanoma (G361) and primary melanocytes from Caucasian skin (PM-C) were used in this study. The cells were subjected to OAG at a dose predetermined to cause induction of melanogenesis but not apoptosis, in independent experiments including cell morphology and protein assays. From these experiments, results determined that 15 and 30 μg/ml OAG were non-toxic to G361 and PM-C cells, respectively. As shown in Fig. 3-1 A and B, OAG-treated G361 cells and PM-C cells showed an increase in melanosome staining. Quantitative assay on melanin content revealed nearly twofold increase, normalized by treatment with vitamin C (a positive control for depigmentation) (Fig. 3-1 C). In consistent with the melanin assays, tyrosinase expression was detected by immunostaining, Western blotting, and ELISA. The results showed increased expression level of tyrosinase in OAG-treated cells (Fig. 3-2 A and B) and decreased in the presence of vitamin C (Fig. 3-2 C). Suggesting that OAG-induced melanogenesis is a reliable and sensitive assay to screen cellular factors involved in this process.

3.3.2 Screening of shRNA library for identification of genes involved in melanogenesis

shRNA-induced loss-of-function screening was performed by using G361 cells and OAG-induced melanogenesis. As shown in Fig. 3-3, cell transfection conditions were determined by microscopic observations of GFP protein in pEGFPC1-transfected G361 cells. In order to further rule out the differences in the transfection efficacy of the shRNA plasmids, the transfected cells were selected in puromycin- supplemented medium for 24–48 h and then treated with OAG. Increase in melanin was parallely examined by melanosome staining, melanin content, and tyrosinase activity assays. The shRNAs that caused abrogation of OAG-induced increase in melanosome, melanin content, and tyrosinase activity were selected, and the process was repeated for next round of screening. The effect of 2044 shRNAs was investigated by conducting four rounds of screenings, and 40 shRNAs that caused reduction in all the three parameters were finally selected (Fig. 3-4 A). Next for investigated the effect of these genes on tyrosinase expression during OAG-induced melanogenesis. The western blotting data showing knockdown

of several selected genes but not all, abrogated OAG-induced increase in tyrosinase (Fig. 3-4 B) suggesting the possibility of identification of new gene/cellular factors that may regulate melanogenesis, independent to that of tyrosinase signaling. Involvement of selected gene targets in melanogenesis was assessed by gene interaction and pathway analyses using bio-informatic tools (Fig. 3-6). The analysis revealed the involvement of tyrosinase-mediated melanin pathway suggesting the reliability of the screening assay. Seven genes (p53, p21, p14, Hsp60, Bcl-2, Bcl-xL, and mortalin) involved in control of cell proliferation, stress signaling, and mitochondrial functions for further analyses were selected for this.

3.3.3 Investigate the role of selected genes in melanogenesis

In order to investigate the role of selected genes in melanogenesis, first examined the expression in control and OAG-treated G361 cells. As shown in Fig. 3-6, induction of melanogenesis was associated with an increase in the expression of p53, p21, p14, Hsp60, Bcl2, Bcl-xL, and mortalin. Of note, cell cycle regulatory proteins (p53, p21, p14) showed higher increase as compared to the stress regulatory and anti-apoptotic proteins (Hsp60, mortalin, Bcl-2, and Bcl-xL). Melanin content assays in cells compromised for the target gene expression by specific shRNA caused a decrease in OAG-induced increase in melanin (Fig. 3-7 A). Tyrosinase assay in control, OAG-treated, and shRNA-transfected cells also exhibited decrease, although to a variable level, in shRNA-treated cells suggesting that these genes are involved in melanogenesis (Fig. 3-7 B). In order to further validate their role in melanogenesis, we generated G361 cells with overexpression of these proteins. Melanin assay in these cells revealed small increase in OAG-induced melanin (Fig. 3-7 A). However, it was not associated with an increase in tyrosinase activity (Fig. 3-7 B) suggesting that these genes may regulate melanogenesis by tyrosinase-independent pathways. Together with the data shown in Fig. 3-7 A and B, it was suggestive that these genes are involved in melanogenesis; their knockdown compromised OAG-induced increase in melanin in a tyrosinase-dependent manner. However, an increase in expression of these genes that resulted in small increase in OAG-induced melanin occurred through tyrosinase-independent pathway. Terrain, a fungal metabolite derived from *Aspergillus terreus*, has been shown to have a variety of biological activities including anti-inflammatory, antioxidant, anticancer, and inhibition of melanogenesis. It was shown to affect melanin synthesis via downregulation mitochondria integrity leading to increase in p53, p21, ERK, and Bax expression levels suggesting a link between proliferation control and melanogenesis signaling [164, 165].

3.3.4 Selected genes affect the OAG-induced pigmentation through mitochondrial stress signaling

All the data described above on the selection of stress and mitochondrial proteins involved in melanogenesis, hypothesis was that the melanogenesis might represent a stress-survival response mediated through mitochondria (Fig. 3-8 A). Therefore, investigated the role of mitochondria and mitochondrial stress proteins, Hsp60, and mtHsp70/mortalin in OAG-induced melanogenesis. First using mitochondrial inhibitors and found that the OAG-induced increase in melanin was compromised in cells treated with mitochondrial inhibitors (creatine induces mitochondrial fragmentation and FCCP, an uncoupler of oxidative phosphorylation) (Fig. 3-8 B). Under the similar conditions, mitochondrial inhibitors did not affect OAG-induced increase in tryrosinase activity (Fig. 3-8 C). These data suggested that the functional mitochondria is involved in the process of melanogenesis and may not have direct effect on tyrosinase. I next investigated the functional significance of mitochondrial stress proteins, Hsp60, and mtHsp70/mortalin in melanogenesis. Both Hsp60 and mortalin are essential proteins that localize in multiple subcellular sites and shown to be involved in protein assembly and folding, oxidative, and anti-apoptotic stress signaling [166]. Both Hsp60 and mortalin have been shown to complex with Bax in the cytoplasm and deregulate apoptosis in cancer cells [47, 167]. In light of these reports and all the data above, I investigated the role of Hsp60 in OAG-induced melanogenesis. G361 cells treated with ETB, an inhibitor of Hsp60, showed a de- crease in OAG-induced increase in melanin (Fig. 3-8 D). Furthermore, co-treatment with ETB and mitochondrial inhibitors showed stronger effect (Fig. 3-8 D). These data supported the role of Hsp60 in regulation of OAG-induced pigmentation in human cells.

3.3.5 Investigate the functional significance of mitochondrial stress protein mortalin in melanogenesis

Mortalin, a stress chaperone, predominantly localizes in the mitochondria. Its functions have been classified into two major classes based upon subcellular location: (i) functions in mitochondria including import of nuclear-encoded proteins into the mitochondrion, nascent protein folding, protein degradation, and interaction with sub-mitochondrial constituents to maintain its integrity and function and (ii) extra- mitochondrial function including its interaction and functional regulation of several cytoplasmic, endoplasmic reticulum and nuclear proteins, centrosomes, growth factors, immune system constituents, and metabolic constituents [168, 169] . Mortalin is similar to Hsp60, influenced OAG-induced melanogenesis. So far there is no report on the involvement of mortalin in the process of melanogenesis. Therefore, extended this finding

to further analyses. melanin content was examined in G361 cells and their mortalin-overexpressing derivatives and found that the latter possess higher level of melanin (Fig. 3-9 A and B) that was also associated with increase in tyrosinase expression (Fig. 3-9 C). Anti-mortalin molecule, MKT-077 (100 nM), caused reduction in OAG-induced melanin, similar to the one caused by anti-mortalin shRNA (Fig. 3-9 D), not only in G361 cells but also in normal primary melanocytes, as examined by immunostaining assays (Fig. 3-10). Furthermore, the inhibitor treated cells showed a decrease in the expression of tyrosinase suggesting that mortalin is an important factor for melanogenesis (Fig. 3-11).

3.3.6 Mortalin was upregulated in oxidative stress-induced melanogenesis

In view of the established relationship of oxidative stress and melanogenesis [170], next H_2O_2 and UV-induced oxidative stress models were recruited and examined if mortalin was upregulated in oxidative stress-induced melanogenesis. As shown in Fig. 3-13 A and B, both UV and H2O2-induced upregulation of melanin was associated with increase in mortalin. In order to investigate further the physiological relevance of role of mortalin in melanogenesis, its expression in the clinical samples of keloids was examined that possess hyperpigmentation. As shown in Fig. 3-14, the hyperpigmentation of skin in keloids was tightly associated with the higher expression of mortalin. Take all the data together provide evidence that (i) the hyperpigmented keloid skin possesses significantly higher level of mortalin expression, (ii) mortalin is increased during oxidative stress and drug-induced melanogenesis, and (iii) overexpression of mortalin causes an increase in pigmentation and its compromise leads to reduction in melanin content.

3.4 Summary

In this study previously established shRNA-mediated loss-of-function screening in conjunction with induction of melanogenesis by OAG (diacylglycerol 1 -oleoyl-2-acetyl-sn-glycerol) in human melanoma G361 cells. Cells were transfected with shRNA library and assayed for induction of melanogenesis by multidimensional approach, involving quantitative biochemical and visual determination of the melanin content and tyrosinase activity. Gene targets of the shRNAs that led to the loss of OAG-induced melanogenesis were considered as candidate cellular factors crucial for melanogenesis. 40 gene targets were identified. Bioinformatics and pathway analyses revealed that these gene targets are involved in the regulation of cell proliferation, apoptosis, stress response and mitochondrial functions. Based on these data, the role of mitochondrial stress chaperone, mortalin in melanogenesis was discovered.

This study demonstrate (i) its use as a molecular target for manipulation of melanogenesis and (ii) whitening effect of some natural and synthetic compounds in OAG-induced melanogenesis in cell culture models.

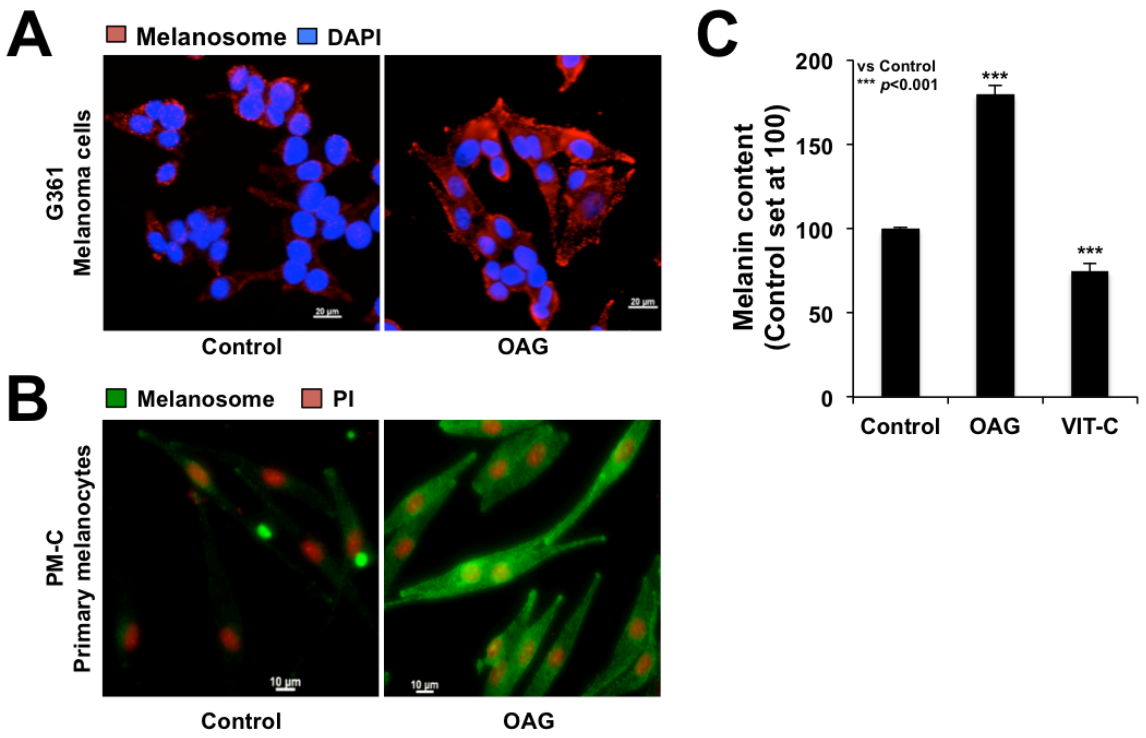


Fig. 3-1 G361 cells were treated with OAG (30 μg/ml) for 24-48 h. Upregulation of melanin was examined by immunostaining (A). Primary melanocytes (PM-C) were treated with OAG (15 μg/ml for 24 h) showed upregulation of melanin by immunostaining (B). Melanin content assay showed increase in melanin content in G361 cells treated with OAG and its inhibition by Vitamin C.

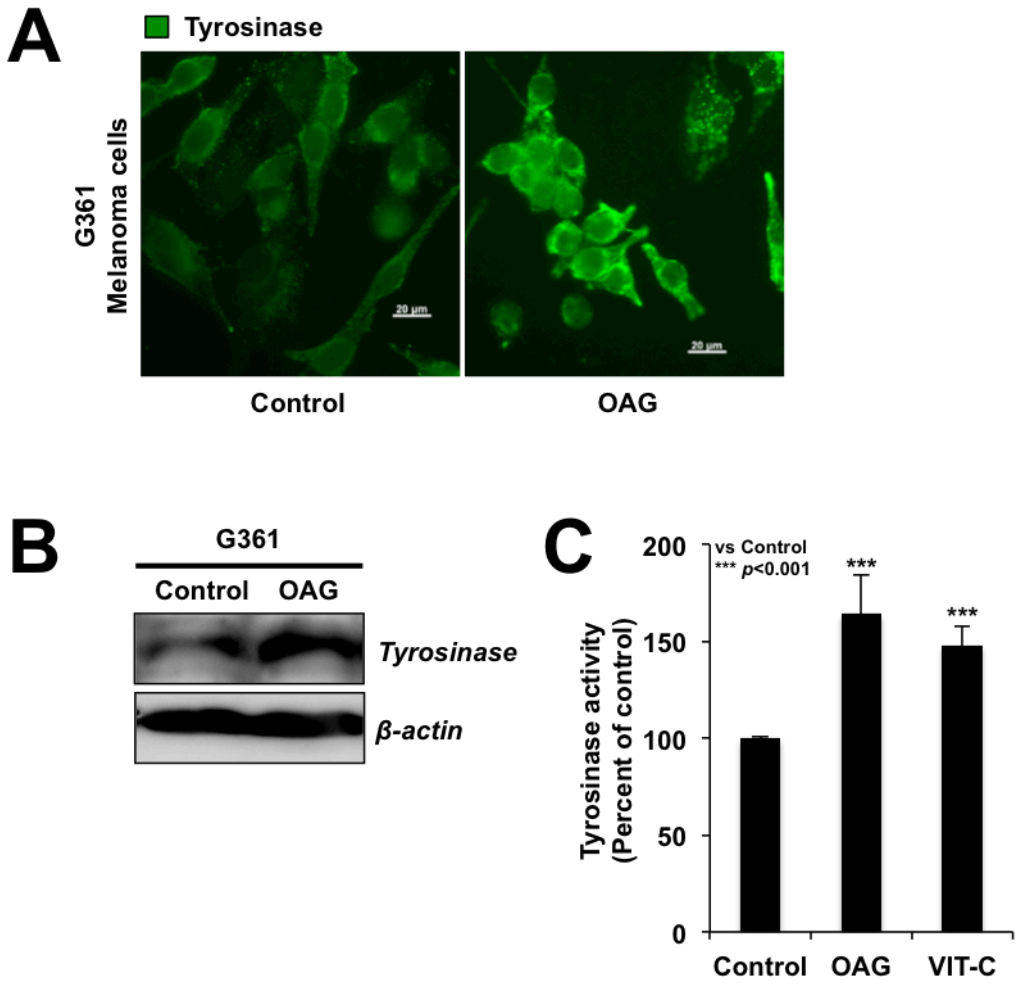


Fig. 3-2 Tyrosinase was detected by immunostaining (A), western blotting (B) and tyrosinase ELISA (C). All the assays revealed an increase in melanin as well as tyrosinase in OAG treated cells. Vitamin C (VIT-C), used as a positive for depigmentation, caused downregulation of melanin as shown in (C).

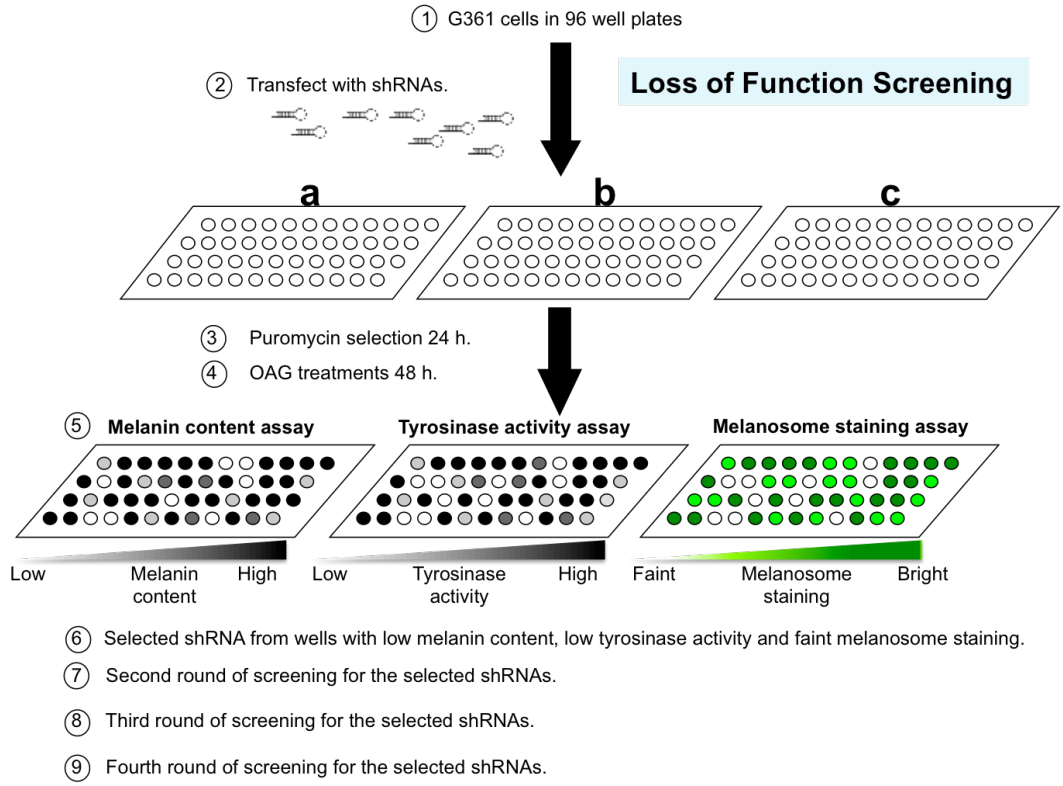


Fig. 3-3 Screening of shRNAs library for identification of genes involved in melanogenesis. G361 cells were transfected with shRNAs library. In order to rule out the differences in the transfection efficacy of the shRNA plasmids, the transfected cells were selected in puromycin-supplemented medium for 24-48 h and then treated with OAG. The shRNAs that caused abrogation of OAG-induced increase in melanosome, melanin content, and tyrosinase activity were selected, and the process was repeated for next round of screening.

A

	Gene Name		Gene Name
1	Cyclin-dependent kinase 5 (CDK5)	21	TNFR-associated factor 6 (TRAF)
2	Fibroblast growth factor (FGFG3)	22	G kinase anchoring protein 1 (FKSG2)
3	Retinoic acid receptor, alpha (RARA)	23	DNA binding Ku antigen protein (G22P1)
4	Mitogen-activated protein kinase 7 (MAP2K7)	24	Synovial apoptosis inhibitor 1, synoviolin (HRD1)
5	Interleukin-1 receptor-associated kinase 3 (IRAK3)	25	Mitogen-activated protein kinase 8 interacting protein 1 (MAPK8IP1)
6	Mitogen-activated protein kinase 7 (MAPK7)	26	Phosphatase 11 (RNA/RNP complex 1-interacting) (DUSP11)
7	p21(CDKN1A)-activated kinase 7 (PAK7)	27	Protein phosphatase 1 regulatory (inhibitor) subunit 3A (PPP1R3A)
8	p16 cyclin dependent kinase inhibitor (p16INK4A)	28	Serine/threonine kinase 38 like (STK38L)
9	Heat shock protein 60 (HSP60)	29	Adenylate kinase 5 (AK5)
10	Mitochondrial heat shock protein 70 (HSP9A)	30	Protein kinase C and casein kinase substrate in neurons 3 (PACSIN3)
11	Pyruvate dehydrogenase kinase, isoenzyme 2 (PDK2)	31	Liver-specific bHLH-Zip transcription factor (LISCH7)
12	Polymerase-gamma 22 (POLG-22)	32	T-cell leukemia, homeobox 2 (TLX2)
13	Transcription factor 1 (ATF1)	33	Engrailed homolog 2 (EN2), mRNA.
14	Tyrosine phosphatase receptor type C (PTPRC)	34	CCAAT/enhancer binding protein alpha (CEBPA)
15	Tyrosine phosphatase non-receptor type 3 (PTPN3)	35	Activating transcription factor 7 (ATF7)
16	Phosphatase 2 regulatory subunit A (PR 65)	36	ETS oncogene family member (ELK1)
17	Aurora kinase C (AURKC)	37	TGFB-induced factor (TALE family homeobox) (TGIF)
18	Tyrosine kinase (TXK)	38	Nijmegen breakage syndrome 1 (nibrin) (NBS1)
19	Mixed-lineage leukemia translocated to 6 (MLLT6)	39	Germ cell specific Y-box binding protein (YBX2)
20	Lymphotoxin alpha TNF superfamily member 1 (LTA)	40	Bagpipe homeobox homolog 1 (Drosophila) (BAPX1)

B

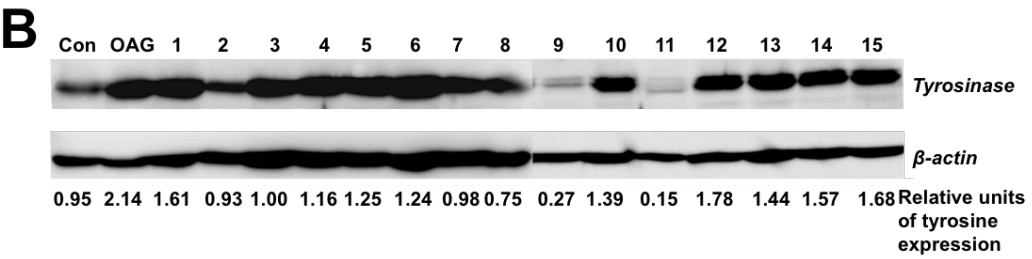


Fig. 3-4 Identification and validation of genes involved in melanogenesis pathway. Gene targets of the 40 selected shRNAs that caused loss of OAG-induced upregulation of melanogenesis, from four rounds of screening, are listed. Representative western blot of tyrosinase in shRNA-transfected (corresponding to the gene targets shown in table) cells showing a decrease in its expression with some but not all (B).

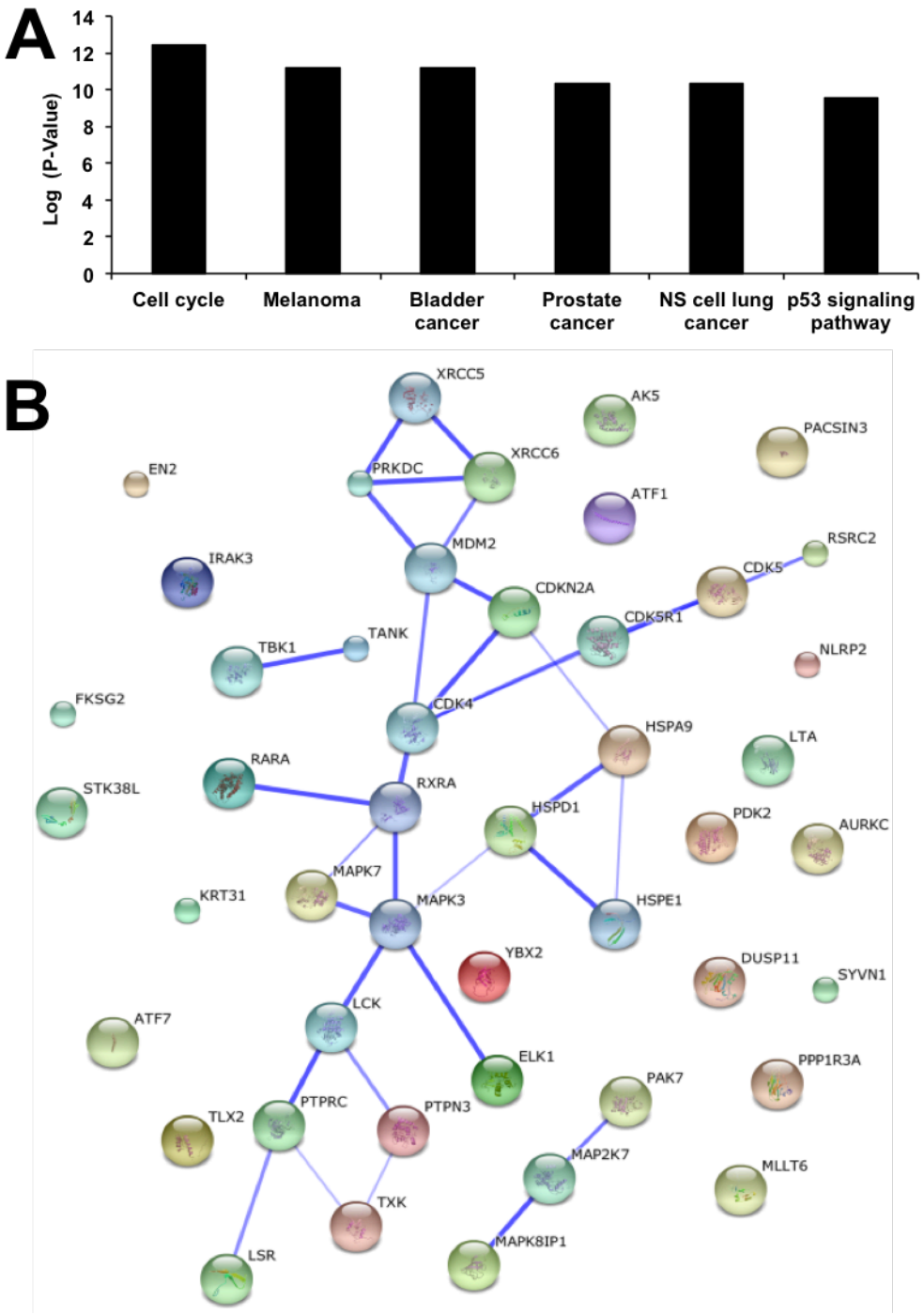


Fig. 3-5 The target genes showed their connection with cellular mechanisms involving cell cycle, cancer, and p53 signaling (A and B).

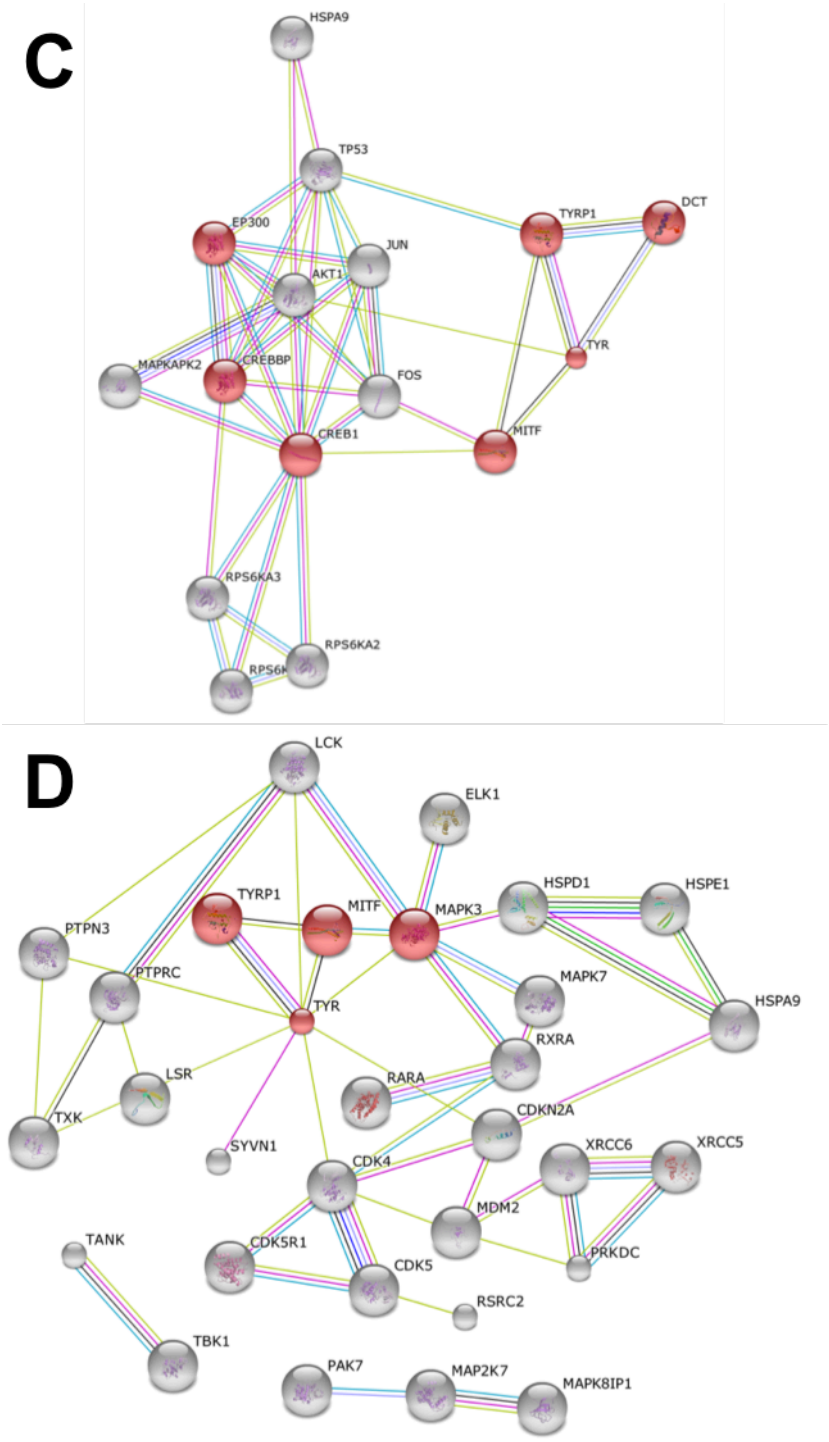


Fig.3-5 Melanogenesis genes, i.e., MIFT, TYR, TYRP1, DCT, and CREB (red circle), showed connections with HspA9/mortalin, p53 (C) and HspD1, HspE1, CDKN2A, and CDK4 (D). Analyses were performed using STRING and Ingenuity Pathway/Kyoto Encyclopedia of Genes and Genomes (KEGG)

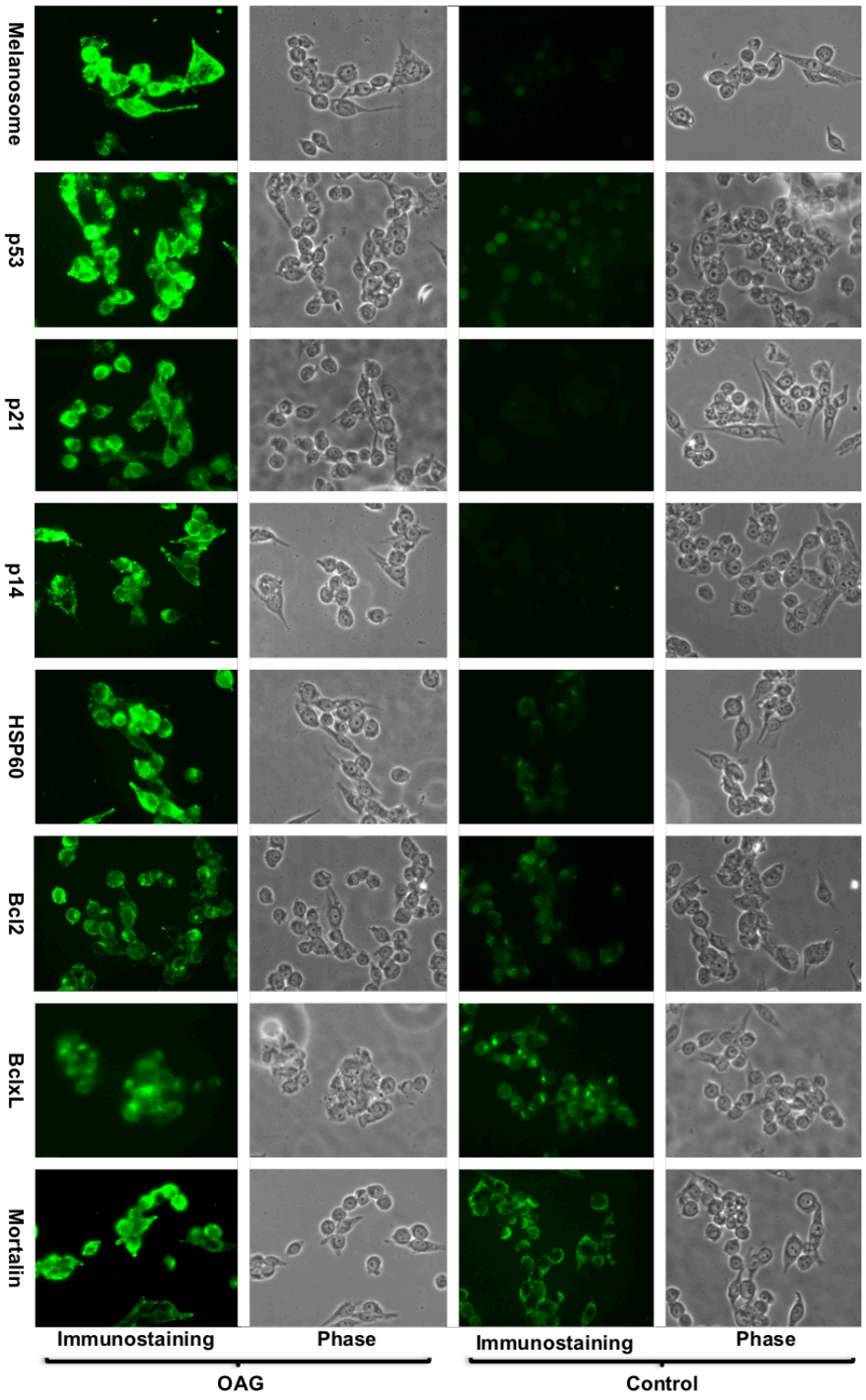


Fig. 3-6 Expression analyses of the selected genes in control and OAG (10 μM)-treated cells. Immunostaining of control and OAG-treated cells for indicated proteins showed OAG-induced upregulation of their expression. Images were scanned and quantitated with ImageJ software. Expression level in control and OAG-treated cells and fold increase in the latter are indicated on the images (white) and bottom of the panel, respectively.

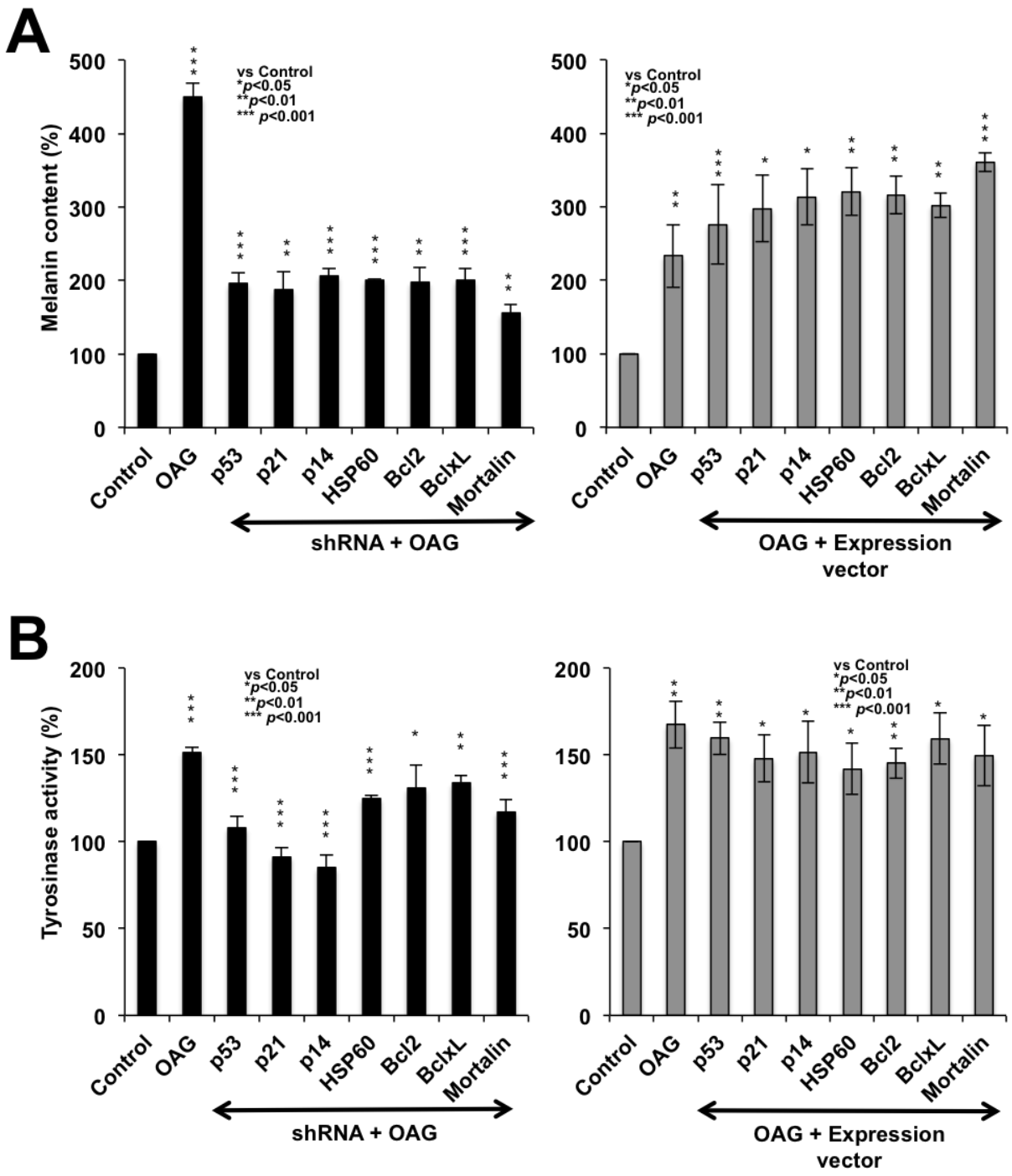


Fig. 3-7 Quantitative estimation of melanin and tyrosinase activity in cells transfected with either shRNAs (left) or expression plasmids (right) in the presence of OAG. Cells compromised for the indicated genes showed significant attenuation of OAG-induced increase in melanin content (A, left). Decrease in tyrosinase activity in all cases, although to a variable extent, was also observed (B, left). Overexpression of proteins caused small enhancement of OAG-induced increase in melanin content (A, right) and was not associated with increase in tyrosinase activity (B, right)

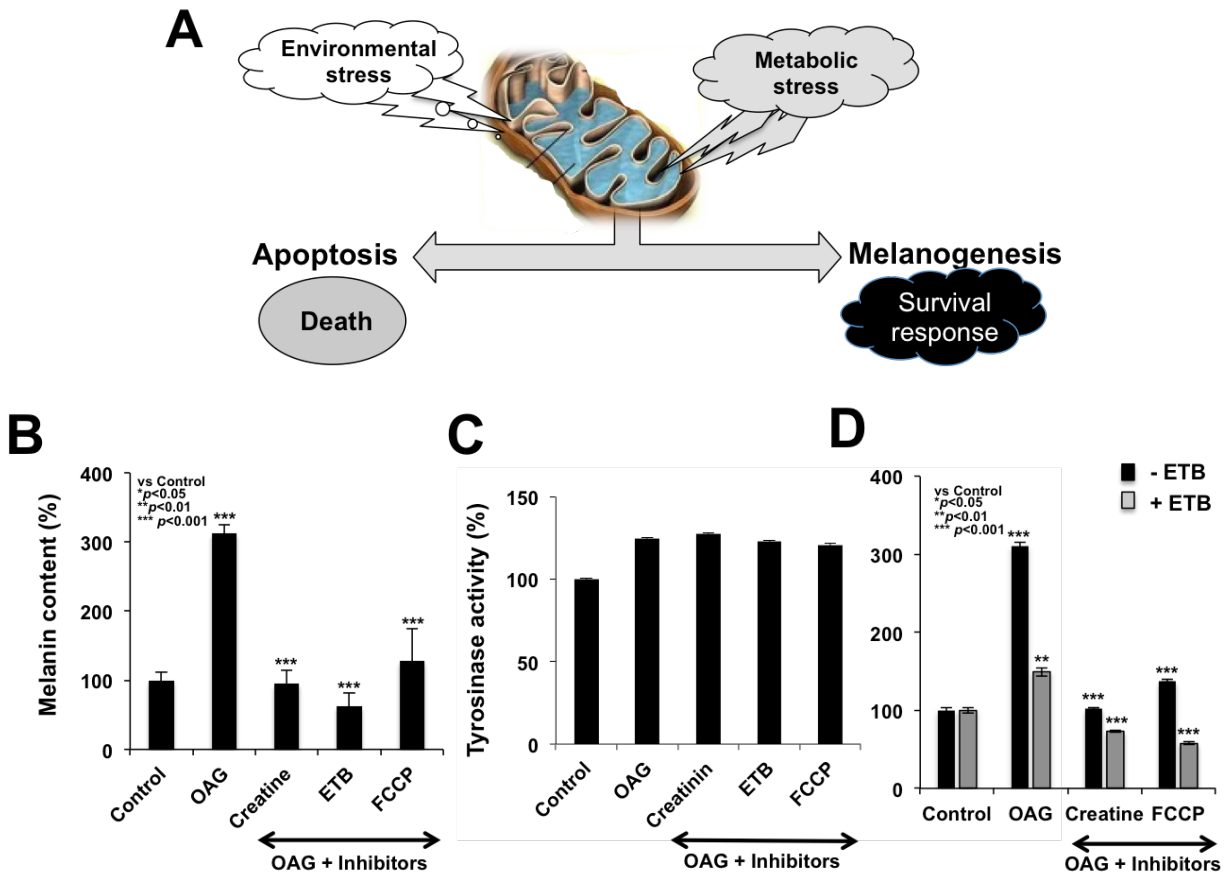


Fig. 3-8 Effect of mitochondrial inhibitors on melanogenesis. A drawing showing melanogenesis as a survival response triggered in mitochondria in response to internal/external stresses (A). G361 cells were treated with mitochondrial inhibitors (Creatine, 10 μM; FCCP, 10 μM) and Hsp60 inhibitor (ETB, 3 μM) for 24–48 h with/without OAG (20 μg/ml). As shown, the inhibitors caused abrogation of OAG-induced upregulation of melanin content in G361 (B). Under the similar conditions, OAG-induced increase in tyrosinase activity was not affected by mitochondrial inhibitor creatine (C). ETB potentiated the inhibition of OAG-induced melanogenesis by mitochondrial inhibitors (Creatine and FCCP) (D)

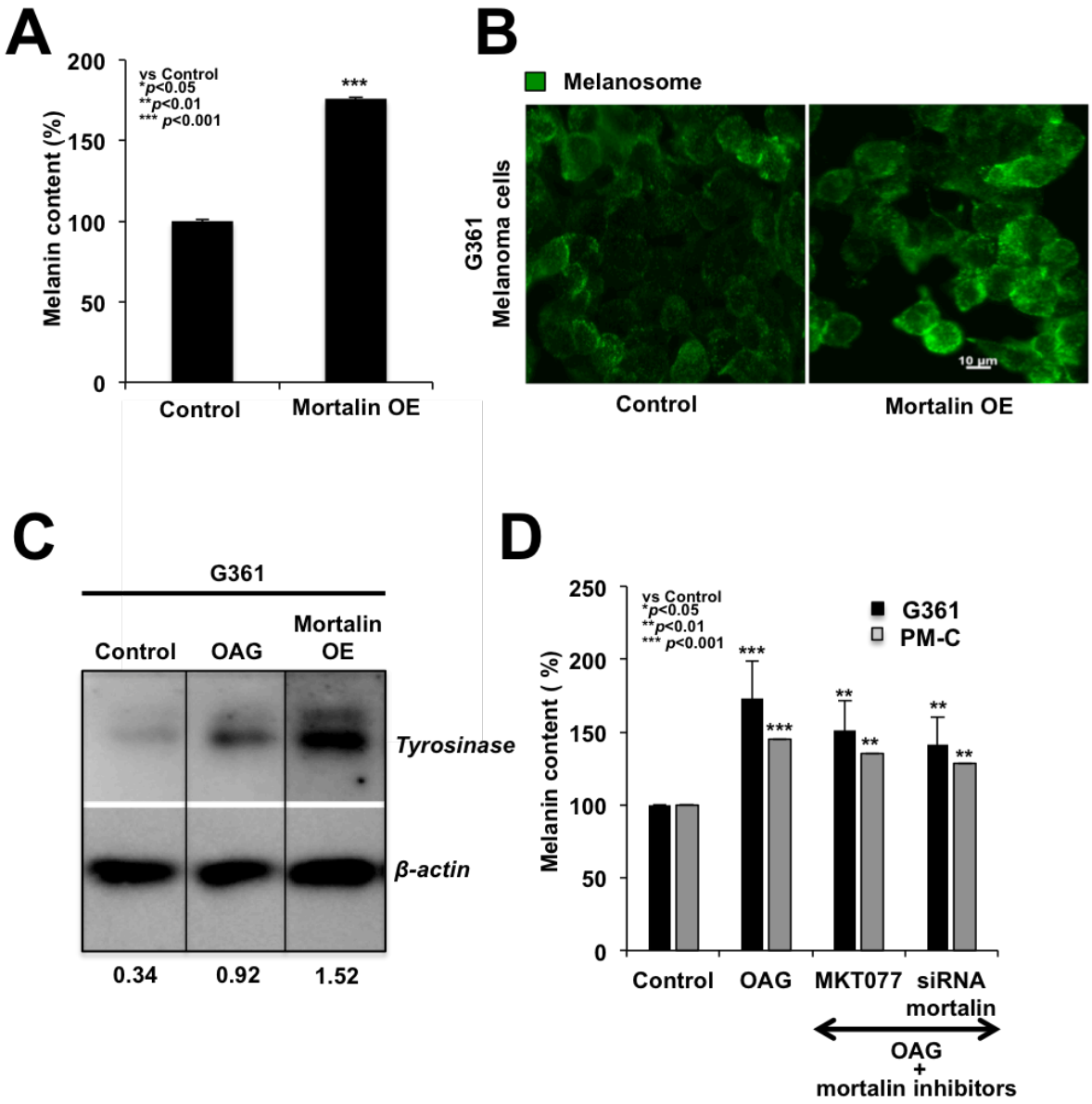


Fig. 3-9 Upregulation of melanogenesis in mortalin-overexpressing cells. Mortalin overexpressing G361 derivatives showed increase in melanin content (A and B) and tyrosinase expression (C). Treatment of cells with mortalin inhibitor, MKT-077 (50–100 nM for 24 h), caused a decrease in OAG-induced melanin (D) similar to the effect caused by anti-mortalin shRNA.

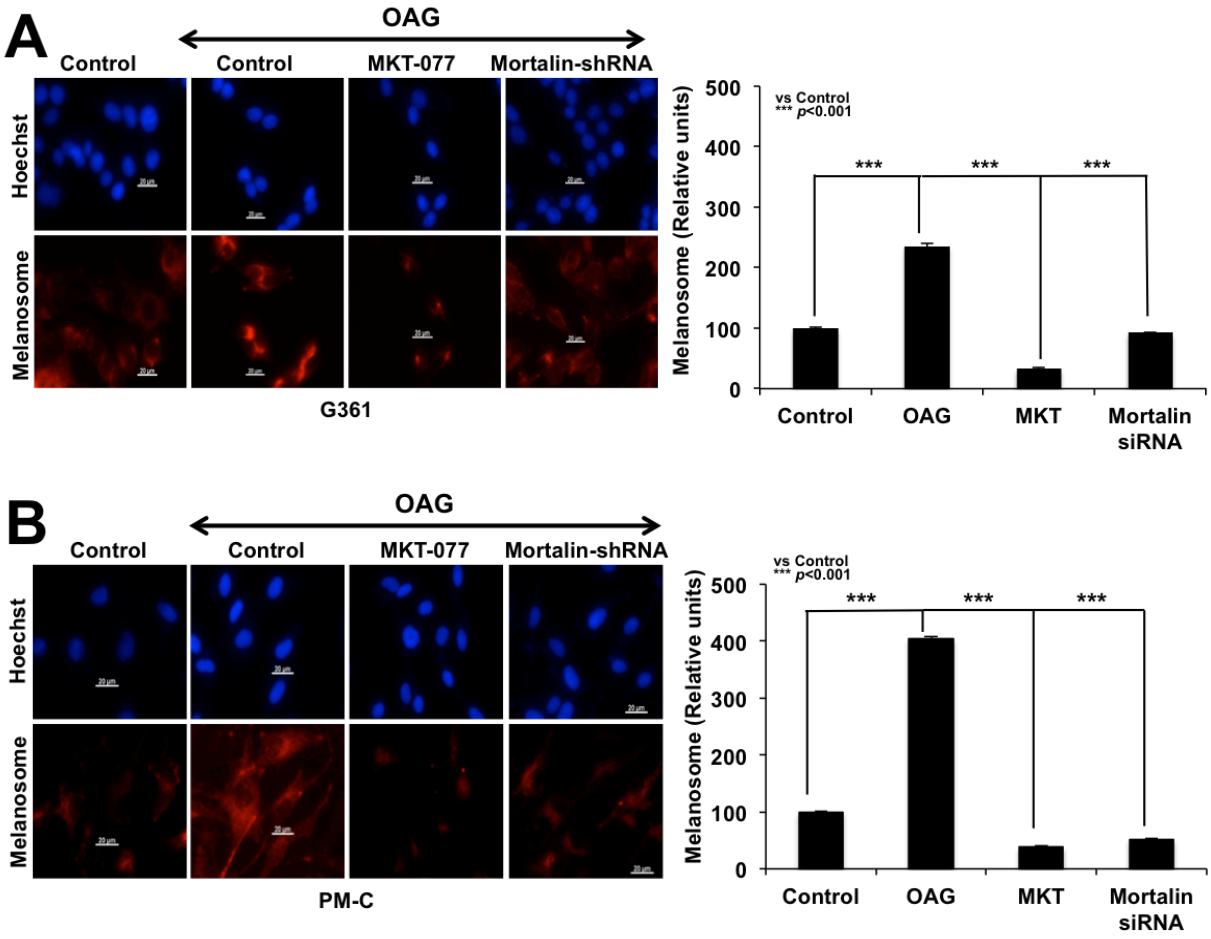


Fig. 3-10 Immunostaining of melanosome showed decrease level of express in cells treated with mortalin inhibitor (MKT-077) and anti-mortalin shRNA. Densitometric quantitation of representative staining was from at least three independent experiments**.**

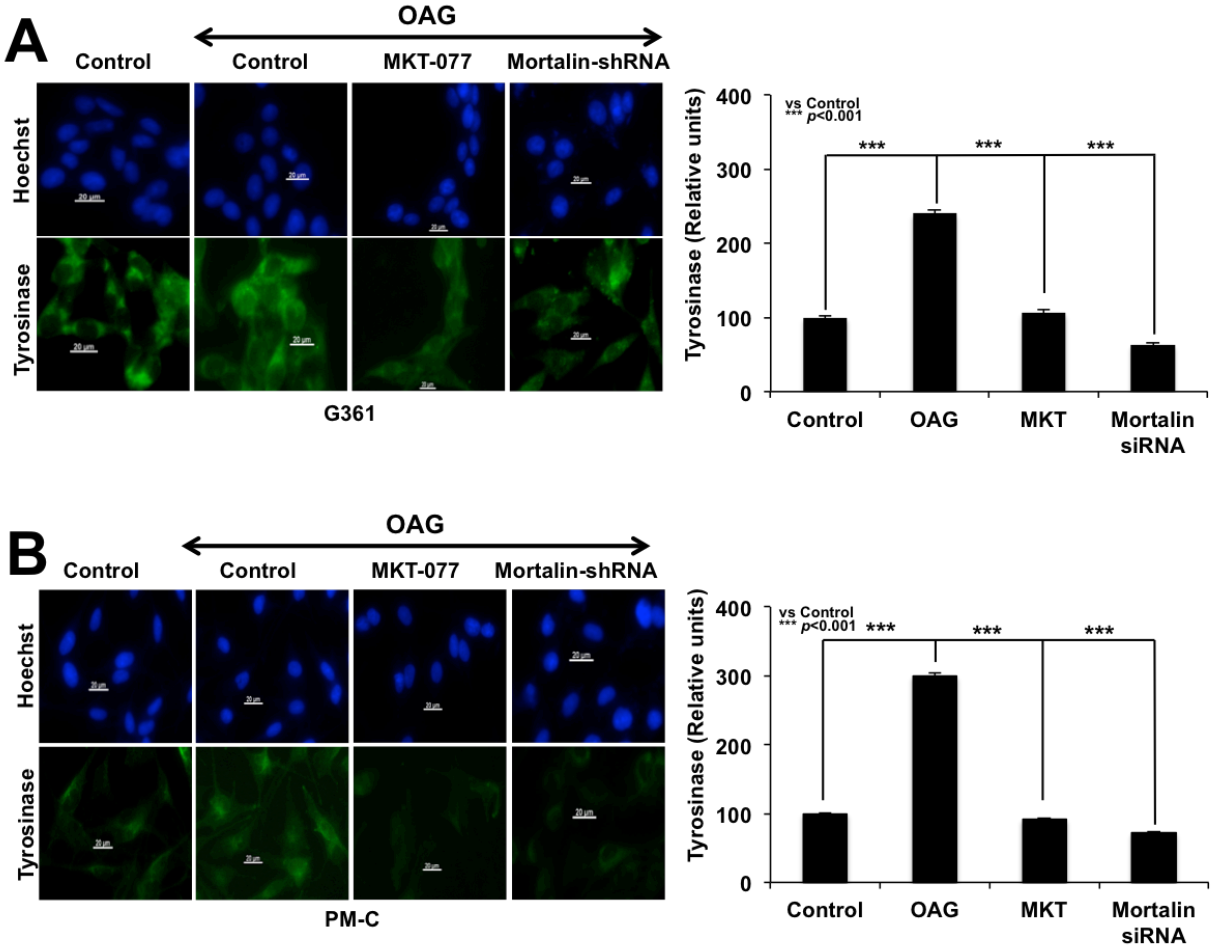


Fig. 3-11 Immunostaining of Tytosinase showed decrease level of express in cells treated with mortalin inhibitor (MKT-077) and anti-mortalin shRNA. Densitometric quantitation of representative staining was from at least three independent experiments.

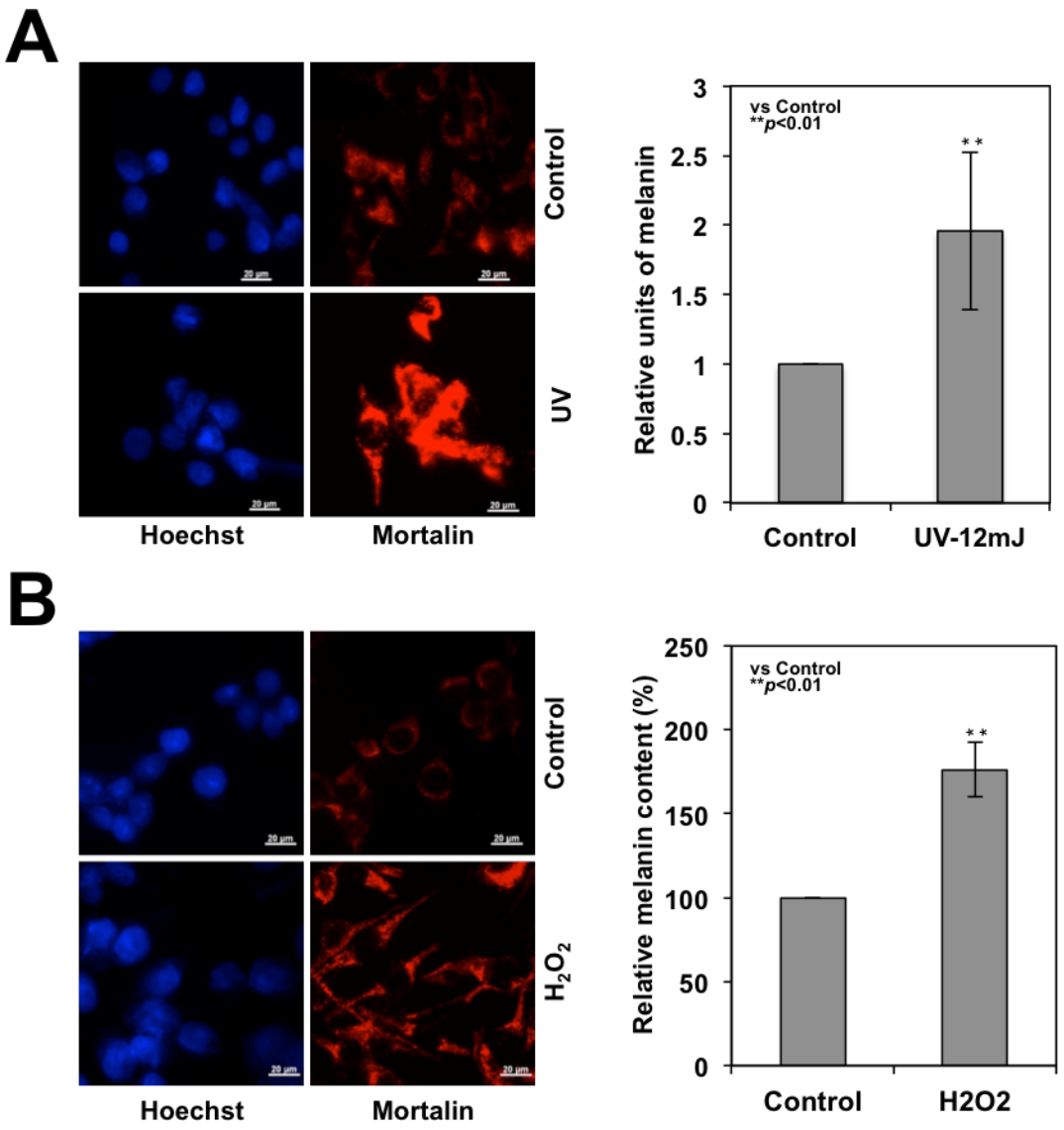


Fig. 3-12 Upregulation of mortalin in oxidatively stressed cells. G361 cells with oxidative stress by exposure to either UV (12 mJ/ cm^2) or H_2O_2 (40 μM) for 24–48 h showed an increase in melanin (A, quantitation from three independent experiments is shown) as well as mortalin (B, images; scanned and quantitated by ImageJ software) revealed two- to three-fold increase in mortalin.

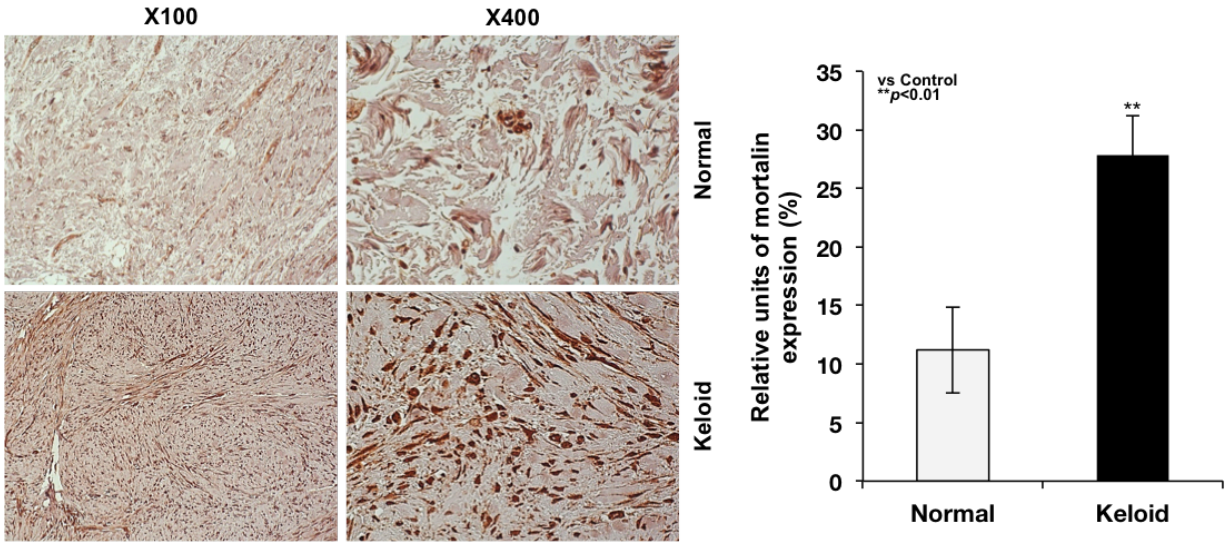


Fig. 3-13 Upregulation of mortalin in hyperpigmented keloid skin. Immunohistochemical staining of mortalin in keloid skin sections. Quantitation was performed using MetaMorph® image analysis software and the statistical significance of the data were calculated by Statistical Package for the Social Sciences (SPSS) software.

Chapter 4 Conclusions and future research

Despite characterization of key molecular signaling in the cancer cell, intricate regulation of carcinogenesis and impact of various environmental factors on the process have been largely elusive. With cumulating burden of various environmental factors on human health, producing mutagenesis and other health anomalies, precise assessment of their molecular regulatory effects and development of an interventional approach has become indispensible. Present report using loss-of-function approaches in human cancer model (subjected to various environment stress exposures), identified miR-451, CARF and Mortalin as key molecular targets growth arrest or senescence/aging and melanogenesis processes respectively. The loss-of-function strategies using 5Aza-dC and shRNA-based approaches, distinguished molecular regulators involved in above two pathways in human cancer cell model system. Present report provided the molecular evidences that how miR-451 instigates growth arrest in cells leading to their resistance to 5Aza-dC-induced senescence. miR-451-induced growth arrest at molecular level was found to essentially be mediated by increase in $p21^{WAF1}$. With enrollment of environmental stress factors, mRNA and 3'UTR reporter assays, Collaborator of ARF (CARF) protein was found to be a new target of miR-451. Consistent to the loss-of-function screening (shRNA-based approach), study on the effect of environmental and metabolic stresses on the melanogenesis process in the aging skin (using skin cancer/melanoma cell model), revealed mitochondrial stress chaperone, mortalin as a key regulator of melanogenesis. The silent findings of the present study are enlisted below.

In this thesis, cell-based model of loss-of-function screening using miRNA or shRNA library, revealed that-

1) miR-451 is one of the upregulated miRs in the cells that escape 5-Aza-dC-induced senescence.
2) miR-451 is one of the tumor suppresser that is downregulated in many cancers.
3) miR-451-induced growth arrest is essentially mediated by increase in $p21^{WAF1}$ (independent of p53 status) and consequent decrease in Cyclin D1, CDK4, pRB and E2F5.
4) miR-451 targets CARF as analyzed by mRNA and 3'UTR reporter assays, and cause growth arrest of cancer cells by complex interactions with its downstream targets.
5) miR-451 regulates stress response through CARF-related cellular signaling, and functions in drug resistance, cell growth, tumor suppressor proteins p53 and pRb, and stress response.
6) shRNA mediated loss-of-function screening revealed potential genes involved in pigmentation process in skin cancer cells.

7) Melanogenesis process evolves mortalin, p53, p21, p14, Hsp60, Bcl2, and Bcl-xL proteins.
8) Melanogenesis is an intricate process and involves multiple genes including the ones involved in cell proliferation control and stress signaling.
9) Melanogenesis process involves mitochondria-based signaling and mitochondrial proteins, mortalin and Hsp60.

Convincingly, present study using 5-Aza-dC or shRNA-based loss-of-function strategies in human cancer model, revealed molecular regulation of growth arrest or senescence/aging and melanogenesis processes and identified miR-451, CARF and Mortalin as key molecular targets to develop interventional approaches.

Present study would strengthen understanding of systematic regulation of intricate carcinogenesis and melanogenesis process in cancer cell, along with accessing the impact of diverse environmental stresses. With enrolled loss-of-function approaches, identification of miR-451, CARF and Mortalin as key molecular targets of growth arrest or senescence/aging and melanogenesis processes, would essentially further pave the path to develop novel interventional strategies to regulate and manipulate above processes. Although, identification of the function of miR-451 to instigate growth arrest/senescence mediated by $p21^{WAF1}$ through CARF suppression has provided the initial molecular lead, initiative to develop miR-451 based translational approach to control cancer cell proliferation yet remained crucial. Similarly, identification of mitochondrial stress chaperone- mortalin as an important regulator of melanogenesis, provided as a key for manipulation of melanogenesis process in the skin cell. Largely, based on these findings documented in the present report, the key future perspectives warrant further studies on (i) validate upregulation of miR-451 for cancer therapies, (ii) elucidate the molecular mechanism of the role of tyrosinase in mortalin-mediated melanogenesis and (iii) validate mortalin as a target and drug discovery tool for manipulation of skin pigmentation for therapeutic and cosmetic purposes.

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