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Transcriptional Regulation of MMP-9 and MMP-14 by p53 and its Mutant R280K

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Abstract of the Thesis

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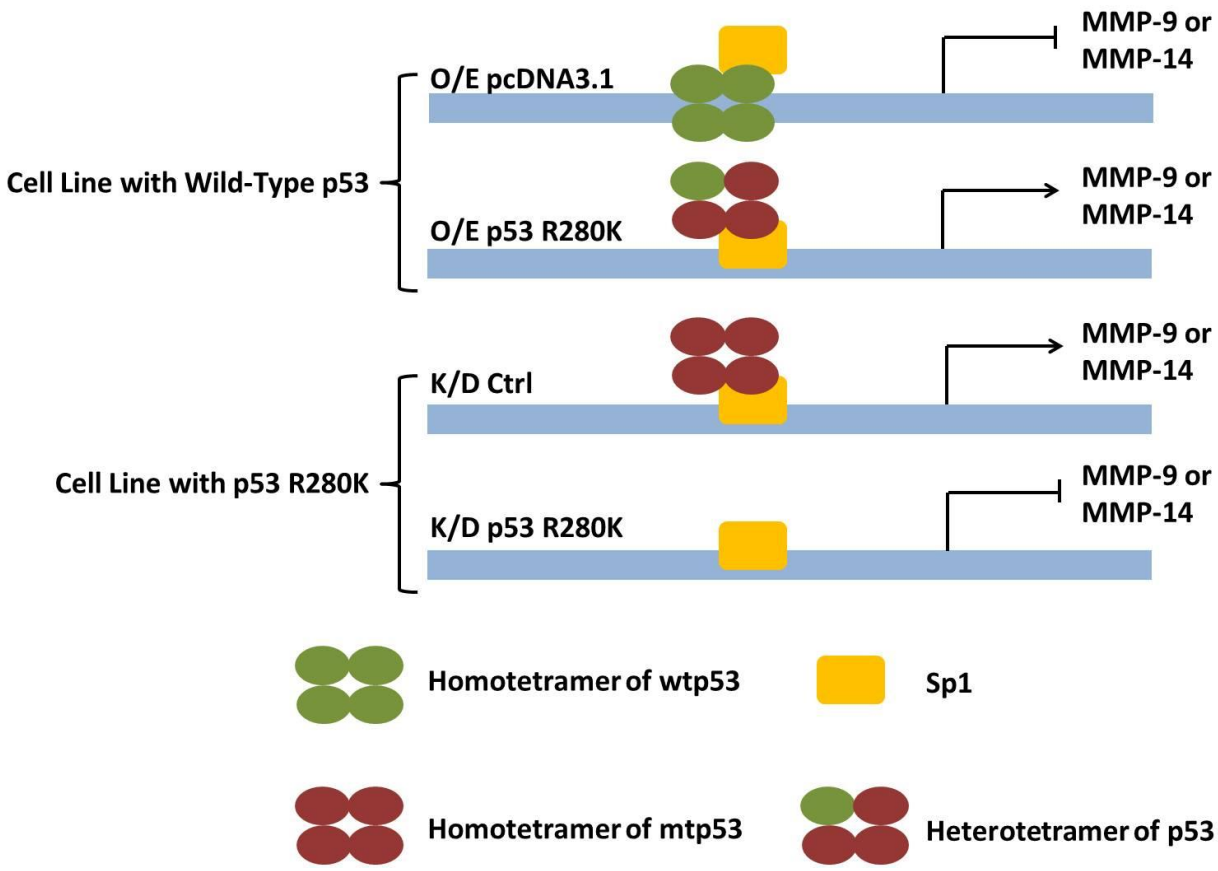
Breast cancer is the most common cancer in women and metastasis is the primary cause for mortality. Matrix metalloproteinases (MMPs) play important roles in cancer cell migration and invasion. Tumor suppressor gene *TP53* is the most frequently mutated gene in cancer, and p53 mutation occurs in invasive breast cancer with higher frequency than in non-invasive breast cancer. However, the nature of relationship between p53 and MMPs remains inconclusive. Here we show that wild type p53 could repress transcriptional activity of MMP-9 and MMP-14, while the DNA-contact mutant p53 R280K could upregulate the transcription of MMP-9 and MMP-14. This regulation might be Sp1-dependent as there is a p53/Sp1 overlapping binding site on the promoters of MMP-9 and MMP-14. Although additional study is needed to further confirm this mechanism, our finding would provide a potential target for anti-metastatic therapy.

Frontispiece



In cell lines with p53 R280K, p53 forms homotetramers and cannot bind to DNA sequences, which renders the opportunity for Sp1 to exert its transactivation function. Even so, this contact mutant do not lose the interaction with Sp1. Upon p53 knockdown, there is no endogenous p53 and the binding site is occupied by Sp1 alone. Since transcription was repressed, it is suggested that Sp1 functions in a p53-dependent pattern. In cell lines with wild type p53, p53 forms homotetramers and occupies that specific binding sites and exert its transrepression function. After overexpression of p53 R280K, heterotetramers are formed with compromised DNA-binding ability. As a consequence, Sp1 stimulates transcription of MMP-9 and MMP-14.

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List of Abbreviations

ER	Estrogen Receptor
PgR	Progesterone Receptor
HER2	Human Epidermal Growth Factor receptor 2
TNBC	Triple-Negative Breast Cancer
EMT	Epithelial-to-Mesenchymal Transition
ECM	Extracellular Matrix
MMP	Matrix Metalloproteinase
TIMP	Tissue Inhibitor of Matrix Metalloproteinase
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
PEA3	Polyomavirus Enhancer A-binding Protein-3
AP-1	Activator Protein-1
Sp-1	Specificity Protein 1
SAF-1	Serum Amyloid A-Activating Factor
DMEM	Eagle's Minimal Essential Medium
SFM	Serum-Free Media
PEI	Polyetherimide
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
SDS	Sodium Dodecyl Sulfate
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
DBD	DNA-Binding Domain
GOF	Gain of Function
wtp53	Wild Type p53
mtp53	Mutant p53
ConA	Concanavalin A
EGFP	Enhanced Green Fluorescent Protein
ChIP	Chromatin Immunoprecipitation

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1 Introduction

1.1 Invasive Breast Cancer and Cancer Metastasis

Breast cancer is the most common cancer in women[1]. Breast cancer can be classified into several subtypes based on the expression of three makers: estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2), all of which are molecular targets of therapeutic agents. Hormone receptor (ER or PgR) -positive breast cancer and HER2-positive breast cancer account for 75-80% and 15-20% of breast cancer cases, respectively, and about half of HER2-positive cases coexpress hormone receptors [2,3]. The remaining 10–15% is triple-negative breast cancer (TNBC), as characterized by lack of the expression of these three proteins. Therefore, this aggressive disease is resistant to existing targeted treatments, such as trastuzumab and hormonal treatments[4]. However, chemotherapy is still the primary established treatment option for both early-stage and advanced-stage of TNBC.

Since a majority of basal-like cancers are also TNBCs and approximately 80% of TNBCs are also basal-like breast cancers[5], the basal-like cancers are often referred to as TNBCs. Triple-negative and basal-like tumors are usually invasive ductal carcinomas with high histologic grade. They are difficult to be detected due to their rapid growth and frequent occurrence in young women[6]. Furthermore, a higher incidence of visceral and cerebral metastases and a higher rate of local relapse have been reported in patients with TNBCs, compared with patients with other breast-cancer subtypes[7,8]. Metastasis remains the cause of 90% of deaths from solid tumors, and is the leading cause for mortality in breast cancer. Metastasis is the multi-step process, beginning with epithelial-to-mesenchymal transition (EMT), which is a significant step in the invasive cascade, local migration and invasion of cancer cells from the primary tumor to the surrounding host tissue, intravasation into blood or lymphatic vessels, dissemination through the blood or lymphatic stream, extravasation to distant organ, survival in dormancy and finally proliferation and angiogenesis within the organ[9-11].

Metastasis fundamentally involves the process of migration and invasion. Cancer cell migration is regulated by matrix-degrading proteinases, integrins and other cell adhesions molecules[12]. A molecular depiction of cell migration involves 5 steps: dynamic cytoskeletal changes and protrusion of the leading edge, cell-matrix interactions and formation of focal contacts, recruitment of surface proteases to extracellular matrix (ECM) contacts and focalized proteolysis, actin-myosin contractions, and detachment of the trailing edge[13]. Particularly, in step 3, surface proteases become concentrated near substrate binding sites[14]. Close to the cell surface, proteases cleave ECM components, such as collagen, fibronectin and laminins, as well as pro-MMPs, creating active soluble matrix metalloproteinases (MMPs) as a result, such as MMP-2[15,16]. MMP-14 (also called MT1-MMP), MMP-1 and other collagenases cleave native collagens and other ECM macromolecules into smaller fragments, which are followed by subsequent degradation by gelatinases (MMP-2 and MMP-9)[15,17,18].

1.2 MMPs and Their Involvement in Cancer Metastasis

ECM-degrading enzymes, such as MMPs, are frequently upregulated in tumor cells, and promote migration *in vitro*, as well as dissemination and metastasis *in vivo*[16,17,19-23]. Increased expression of MMP-2 and MMP-9 has been observed in different types of cancers, including breast, lung, colon, skin, ovary and prostate cancer[24]. Along with enhanced gelatinase expression, cancers often exhibit increased invasiveness and metastasis and decreased overall survival. In

several epithelial cancer models, inhibition of MMPs impairs tumor-cell migration *in vitro*[25-27] and metastasis after orthotopic implantation[22,28,29].

Protease activity of MMPs is not only required for the degradation of matrix, but also indispensable in disruption of cell-matrix adhesion and cell-cell interactions, exposure of cryptic migration promoting sites, and cleavage of matrix-associated latent growth factors[30]. Since proteases produced by cancer cells have been shown to play important roles in invasive processes, pharmacological protease inhibitors have been developed as cancer therapeutics[13]. However, these compounds have been shown ineffective in slowing late-stage tumor progression and metastasis.

MMPs are a family of zinc-dependent endopeptidases responsible for both physiological and pathophysiological tissue remodeling[31]. MMP-2 and MMP-9, also known as the gelatinase A and B respectively, have been long recognized as major contributors to the proteolytic degradation of ECM during tumor invasion. They contain an N-terminal predomain required for the correct secretion, a prodomain forming an essential contact with the catalytic zinc ion and maintaining the latency of the MMPs, a catalytic domain containing the characteristic signatures for zinc-dependent metalloenzymes, a collagen binding domain, and a hemopexin -like domain[30].

The MMPs are secreted as zymogens and require activation via proteolysis for full catalytic activity. The activation pathway of MMP-2 on the cell surface is by the formation of a molecular complex containing MMP-2, MMP-14 and tissue inhibitor of matrix metalloproteinase (TIMP)-2[32,33]. MMP-14 is synthesized as a proenzyme, activated within the Golgi network by the proprotein convertase furin and then transported on the cell membrane where it cleaves the latent 72 kDa proMMP-2 to an intermediate 68 kDa form, which autocatalytically converts into a 62 kDa active form[33] (see Figure 1.1 and 1.2). MMP-2 activation and high levels of MMP-14 are well correlated with cellular invasion and tumor spread[34]. As for MMP-9, several activation mechanisms have been reported, and the consequence is 82 kDa active MMP-9 yielded from 92kDa proMMP-9.

A primary difference between MMP-2 and MMP-9 is the differential regulation of expression due to their promoter elements[35,36]. MMP-9 expression is highly inducible[37]. MMP-9 is positively regulated by multiple factors, including E-26 transcription factors , nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), polyomavirus enhancer A-binding protein-3 (PEA3), activator protein-1 (AP-1), specificity protein 1 (Sp-1), and serum amyloid A-activating factor (SAF)-1[38]. However, MMP-2 is constitutively expressed with only modest upregulation or downregulation under various conditions[39], because MMP-2 has fewer inducible promoter elements such as binding sites for transcription factors[40]. Similar to MMP-2, MMP-14 is also expressed in a constitutive fashion because of lacking other obvious promoter features besides GC boxes.

1.3 p53 and Transcriptional Regulation of its Target Genes

p53 is a critical tumor suppressor that prevents cells with damaged DNA from replicating by inducing either DNA repair or apoptosis. It is mutated in approximately 50% of reported human tumor cases, making it a target for anticancer therapy[41]. p53 is a transcription factor acting as a homotetramer (see Figure 1.3), and each monomer consist of an N-terminal transactivation domain, a proline-rich domain, a DNA-binding domain, a tetramerization domain and a C-terminal regulatory domain[42] (see Figure 1.3). It has been reported that over 95% of the malignant mutations occur in the DNA-binding domain (DBD)[43], and about 40% of them occur

at only six hot-spots (R175, G245, R248, R249, R273 and R282) [44].The mutants that affect DBD folding are usually referred to as structural mutants, such as R110P, R175H, R248Q, R249S and R282W; the mutants that affect DNA binding without altering the overall conformation of the p53 molecule are referred to as contact mutants, such as R248W and R273H[42]. Contact mutants compromise the ability of p53 to bind to specific DNA sequences with high affinity. Among the residues that interact with the DNA, R280 makes the highest number (six) of contacts within 4 Å of base atoms in the major groove[45], suggesting that R280K might be a contact mutant.

During the process of carcinogenesis, TP53 mutations mostly arise sporadically in one allele, leading to cells expressing both wild-type p53 (wtp53) and mutant p53(mutp53), and the latter might suppress the tumor suppressor activities of the former by dominant-negative effect through the tetramerization domain[46] (see Figure 1.3). During tumor progression, the remaining wtp53 allele is often mutated further enhancing tumorigenesis, and this phenomenon is called loss of heterogeneity. Several mechanisms of p53-mediated transcriptional repression of target genes have been studied. p53 binds to its target promoters and competes and forms a complex with other transactivators for binding to DNA, leading to the sequestration or changes in their activity or affinity for DNA. These transactivators include TATA binding protein, Sp1, and NF-κB. Increasing evidence has demonstrated that mtp53 and wtp53 often exert opposite effect on the same biochemical pathway or biological process. Mtp53 protein might acquire novel activities, which can contribute to various aspects of tumor progression, described as gain of function (GOF)[47]. Mtp53 exerts its GOF partially because of aberrant interaction with other transcription factors. Mtp53 has been shown to interact with several sequence-specific transcription factors, which possess binding sites on genes that are responsive to mtp53. For example, the interaction of mutp53 with Sp1 was shown to elicit cooperative effects and amplify the activating effects of Sp1 on transcription.

More specifically, p53 can interact with Sp1 protein, rendering Sp1 inactive for Sp1-mediated transcription, and granulocyte/macrophage colony-stimulating factor-dependent proliferation of human erythroleukemia cell line is accompanied by the formation of DNA binding heterocomplexes between Sp1 and a mtp53[48]. The presence of Sp1 increases p53 binding to its recognition sequence in the HIV-1 long terminal repeat[49]. p53 is able to repress Sp1-stimulated promoter activity of the DNA polymerase δ catalytic subunit gene and this repression is significantly due to the loss of the sequence-specific interaction between Sp1 protein and the Sp1-binding site, which overlaps the p53-binding site; mutations in the p53 DNA-binding domain completely abolished the p53 transrepression activity[50]. p53 was also reported to sequester Sp1 to prevent its binding to the cis elements in the promoter of O^6-Methylguanine-DNA methyltransferase gene and inhibit expression[51]. In addition, p53 is able to repress the Sp1-stimulated promoter activity of Ki-67, which is strictly associated with cell proliferation[52].

As for transcriptional regulation of MMPs by p53, several studies have also shown similar regulation pattern. It has been reported that wtp53 could inhibit NF-κB-induced MMP-9 promoter activation and thus inhibit growth and metastasis of human soft tissue sarcoma[53]; p53 could downregulate MMP-1 by disrupting the communications between the transactivator AP-1 and the basal transcriptional complex[54]; also, MMP-13 was downregulated by wtp53 and this repression could be reversed by overexpression of mtp53[55]. Unlike most MMPs, however, MMP-2 transcription was shown to be positively regulated by wtp53[56].

Basal-like tumors showed a highest frequency of TP53 mutations (80%) compared with other subgroups[57] and higher capacity of migration and invasion. Nevertheless, transcriptional

regulation of MMP-9 and MMP-14 by p53 has not been investigated in breast cancer, about which this paper mainly studied and discussed.

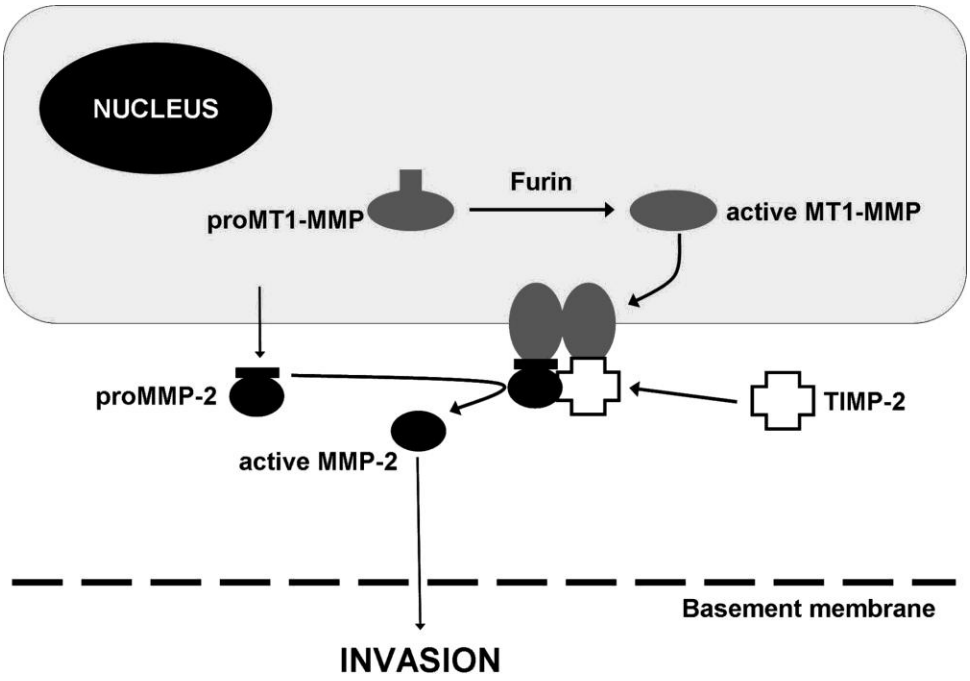

Figure 1.1 MMP-2 activation pathway[58]

MMP-14 is activated intracellularly by the proprotein convertase furin. Regulated positioning of activated MMP-14 to lamellipodia and invadopodia enables focal degradation of ECM during cell migration. MMP-14 also plays a role in MMP-2 activation. ProMMP-2 is activated on the cell surface in a ternary complex involving active MMP-14 and TIMP-2. Generation of active MMP-2 subsequently promotes cellular invasion.

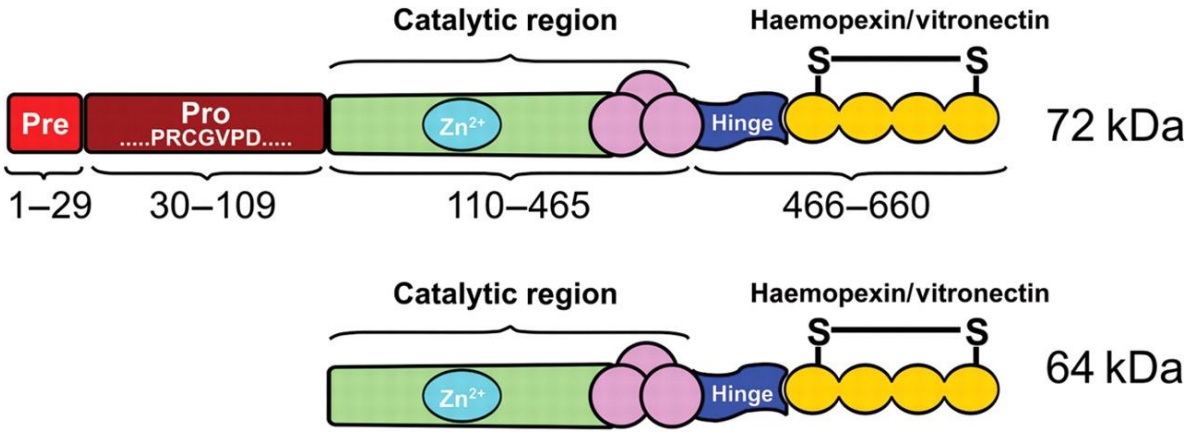

Figure 1.2 Schematic structure of 72 and 64 kDa MMP-2 isoforms[59]

The N-terminal signal sequence (indicated as 'Pre') is followed by the propeptide ('Pro') domain. The catalytic site contains the essential zinc ion-binding site. The catalytic domain of MMP-2 (and MMP-9) contains three fibronectin repeats (pink circles), with the ability to bind to denatured collagen. The flexible proline-rich hinge region and a C-terminal haemopexin domain which functions in substrate recognition mediate interaction with enzyme substrates.

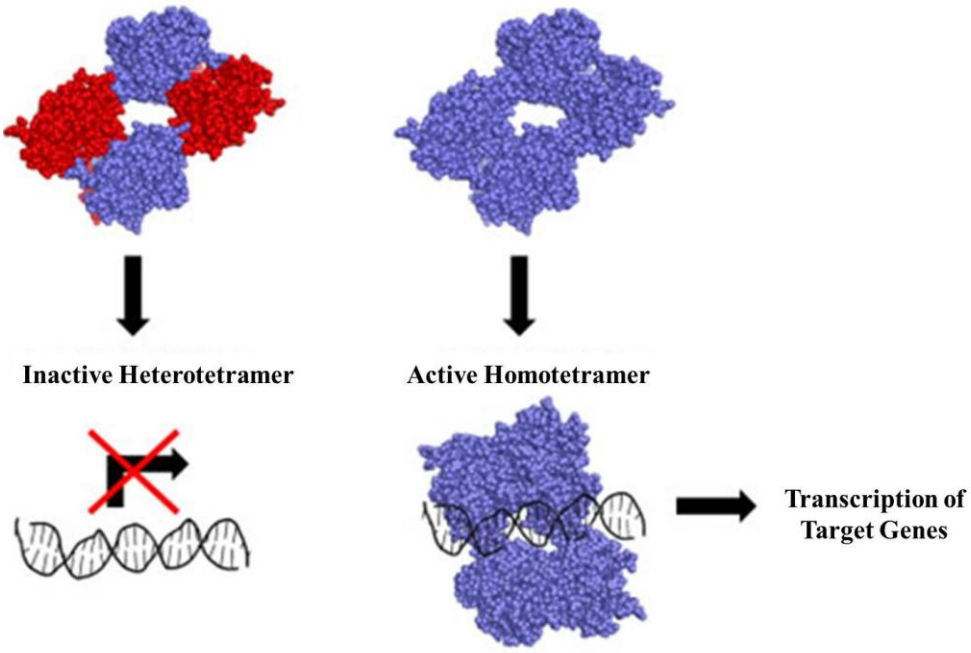

Figure 1.3 Tetramerization of p53[60]

Homotetramers with wtp53 activity can bind DNA to transactivate p53-target genes. Within cancer cells, mutant p53 can interact with active wtp53 forming inactive heterotetramers. The domain negative effect can sequester the anti-cancer function of wtp53.

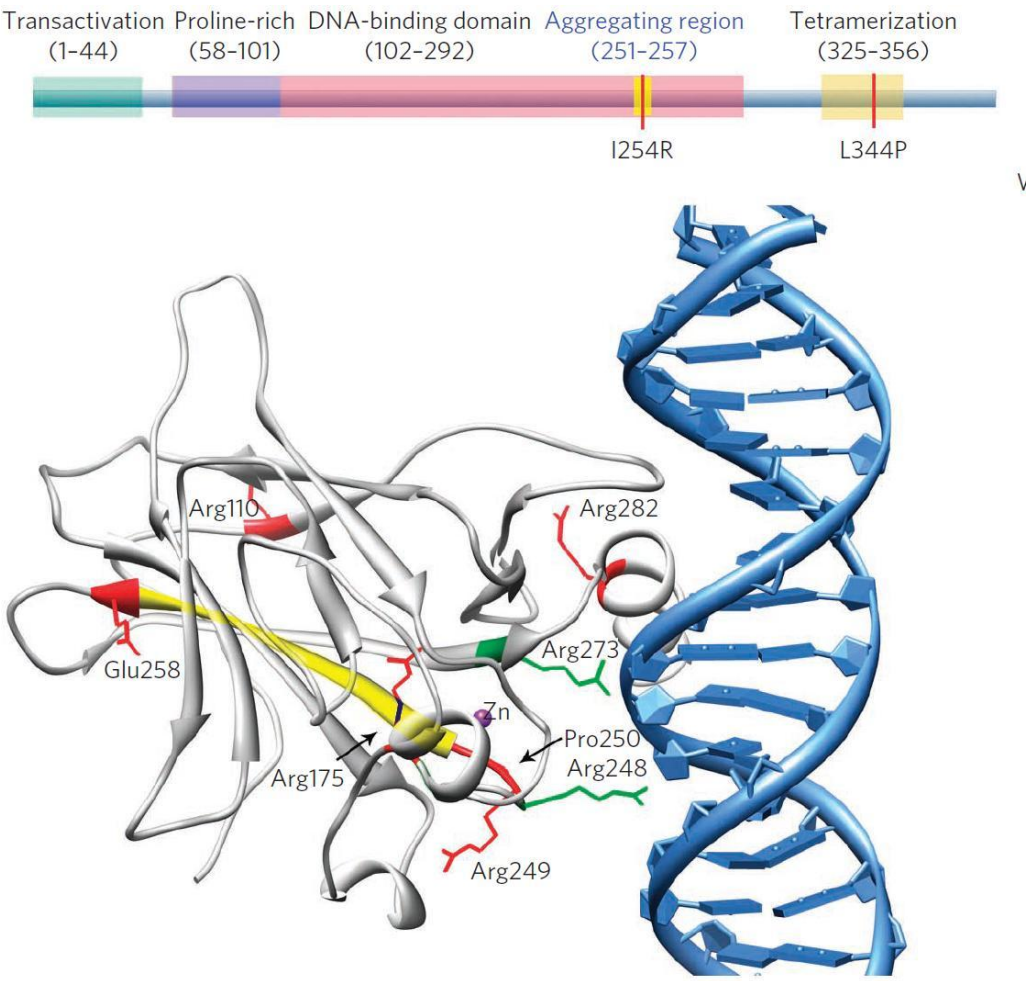

Figure 1.4 Structure of p53 protein[42]

The schematic domain structure of p53 is shown in the upper panel. The structure of p53 DNA-binding domain is shown in the lower panel. The structural mutations and the contact mutations are labeled in red and green, respectively. The aggregation-prone sequence is shown in yellow. (PDB ID: 1TUP)

2 Materials and Methods

2.1 Cell Culture

Cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cell lines used include human breast epithelial cancer MDA-MB-231 and monkey kidney fibroblast COS-1, both of which were maintained in the DMEM (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were cultured in tissue culture dishes and maintained in a humidified environment at 37℃ and 5% CO2 for all experiments.

2.2 Constructs

0.6 kb fragment of MMP-9 promoter and 0.5 kb fragment of MMP-14 promoter were amplified by PCR and the resultant PCR fragments were cloned into pGL3 vector containing the firefly luciferase reporter gene. Wild type p53 or p53 R280K was cloned into pcDNA3.1 vector. p53 stable knockdown cells were generated by lentivirus-based infection of shRNA.

2.3 Transfection

MDA-MB-231 and COS-1 cells were plated on 3 cm dished and were transfected when cells were 70% confluent. MDA-MB-231 cells were transfected using 3 μl of Lipofectamin 2000 transfection reagent with 1 μg of plasmid in serum-free media (SFM); COS-1 cells were transfected using 1 μl of PEI transfection reagent with 1 μg of plasmid in complete medium. Cells were harvested 24-48 hours following transfection.

2.4 Gelatin Zymography

Gelatin zymography was performed as described (Zucker et al., 1995)[61]. After electrophoresis, the gels were incubated in 2.5% Triton X-100 to replace SDS followed by incubation in a Tris-based buffer overnight at 37℃. Staining was accomplished using Coomassie Brilliant Blue, and cleared areas were indicative of gelatinolytic activity.

2.5 Real-Time Quantitative PCR

RNA from cells was isolated using Qiagen RN easy Kit (Germantown, MD) according to the manufacturer's instructions. RNA was reverse transcribed to generate cDNA using Reverse Transcriptase (BioRad iScript cDNA Synthesis Kit). Quantitative real-time PCR was performed using Invitrogen Superscript VILP MasterMix on a BioRad iQ5 Real Time PCR machine. Relative expression was calculated using the ΔΔCt method. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as internal controls.

2.6 Western Blot

The transfected cells were lysed by 2 × SDS gel-loading buffer. The samples were resolved by 10% polyacrylamide gel electrophoresis, and proteins were transferred to nitrocellulose membranes and probed with antibodies. Molecular weight was determined using prestained protein standards.

2.7 Promoter Analysis

Promoter sequences of MMP-9 and MMP-14 were obtained from NCBI Nucleotide Database. The promoters were analyzed for identification of putative transcription factor binding

sites by the online tool PROMO using version 8.3 of TRANSFAC.
http://alggen.lsi.upc.es/cgibin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3

2.8 Dual Luciferase Assay

To examine the promoter activity, COS-1 cells were transiently transfected with the promoter constructs along with Renilla luciferase reporter gene. After 24 hours of transfection, cells were treated with GLO Lysis Buffer. Firefly and Renilla luciferase activities were measured using the Promega Dual-Glo Luciferase Assay System.

2.9 Two-Dimensional Dot Migration Assay

MDA-MB-231 cells were mixed with 3 mg/ml neutralized type I collagen. The mixture was dotted into each well of a 96-well plate and allowed to solidify at 37℃ (about 5 minutes). After collagen solidification, cell-matrix dots were overlaid with complete medium. Cells were allowed to migrate up to 8 hours. Cells were then stained in Hoechst/PBS (1:2000), and images were captured using the Nikon Eclipse TE2000-S equipped with a Sutter Instruments SmartShutter System and a QiClick QImaging camera. Migration was then quantified by counting nuclei using the Nikon Elements Basic Research Software analysis tools.

2.10 Immunofluorescent Staining

Immunofluorescent staining was performed by fixing treated cells in 4% paraformaldehyde (PFA) in PBS at room temperature for 20 minutes, followed by permeabilization in 0.2% Triton X-100 at room temperature for 10 minutes. Blocking solution was composed of 1% bovine serum albumin and 5% normal goat serum in PBS. After 1-hour blocking at room temperature, cells were exposed to, visualized using the complementary secondary fluorescent antibody (anti-rabbit Alexa Fluor 568), counterstained with Hoechst, and imaged on the Nikon microscope.

3 Results

3.1 Expression of MMP-9 and MMP-14 in MDA-MB-231 Cells

As triple-negative breast cancer cell line MDA-MB-231 harbors homozygous mutation of *TP53* (R280K), we want to ask whether the mutant p53 has an impact on MMPs' expression. We designed short hairpin RNA against *TP53* (shp53) to knockdown the endogenous mutated p53 protein, and thus we obtained stable transfected MDA-MB-231 cell lines with shCtrl or shp53. p53 expression after silencing was checked by both Real-Time Quantitative PCR (RT-qPCR) and Western Blot (see Figure 3.2 and Figure 3.3). By comparing the levels of MMPs in these two cell lines, we could determine the function of p53 R280K on MMPs. Therefore, proteolytic activity, transcription, and expression level of MMPs were measured in shCtrl and shp53 cells.

As zymography is a simple assay to measure the amounts of MMP-2 and MMP-9, we firstly performed zymography assay to test the influence of p53 on MMP-2 and MMP-9. Besides, the status of MMP-2 activation would reflect the level of MMP-14. Conditioned medium from COS-1 cells that overexpress MMP-9 or MMP-2 were used as positive controls for pro/active-MMP-9 or pro/active-MMP-2 respectively. Before collecting conditioned medium, MDA-MB-231 cells were treated with SFM. Upon p53 knockdown, the amount of pro-MMP-9 was reduced significantly. Since active-MMP-2 could not be observed, MDA-MB-231 cells were treated with SFM plus 20μg/ml concanavalin A (ConA), which could induce cell surface activation of pro-MMP-2. However, it did not make any difference. Due to the low level of endogenous MMP-2, MDA-MB-231 cells were treated with SFM plus 20ug/ml ConA and 25ul/ml MMP-2 conditioned medium (from COS-1overexpressing cells). Compared with control group, more pro-MMP2 and less active-MMP-2 were observed in p53 knockdown group. In addition, active-MMP-9 was slightly increased upon p53 knockdown. (See Figure 3.1)

Since MMP-14 plays a critical role in MMP-2 activation, less active-MMP-2 implied lower level of MMP-14. Although the total amount of MMP-2 did not show dramatic change, its activation status lead us to ask whether this change was due to differential expression level of MMP-14.On the other hand, although p53 knockdown did not lead to significant MMP-9 activation, difference in MMP-9 total amount forced us to further test the influence of p53 R280K on expression level of MMP-9.

In order to test the protein levels of MMP-9 and MMP-14, Western blot was performed. Decreased protein levels of MMP-9 and MMP-2 were confirmed in p53 knockdown group (see Figure 3.2(a)). Besides, the efficiency of p53 knockdown was also verified. MDA-MB-231 cell were also transiently transfected with enhanced green fluorescent protein (EGFP) or wtp53 with GFP tag. Even though MDA-MB-231 cell line is not readily transiently transfected, decreased MMP-9 and MMP-14 were observed due to wtp53 (see Figure 3.2(b)).

Then we asked whether p53 R280K could promote transcription of MMP-9 and MMP-14. RT-qPCR was performed to test this surmise. As expected, transcription level of MMP-9 was reduce about 50 folds and transcription level of MMP-14 was reduce about one fold upon p53 knockdown (see Figure 3.3).

Taken together, these results suggested that the p53 mutant R280K promoted transcription of MMP-9 and MMP-14,while wild type p53 decreased them.

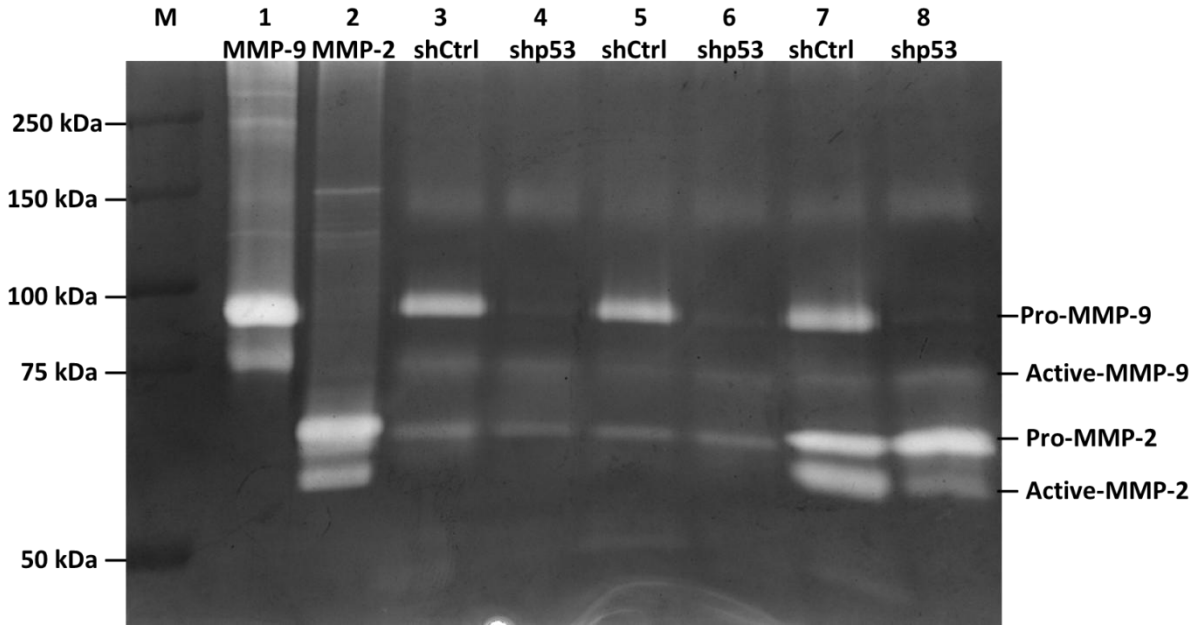

Figure 3.1 Gelatin zymography performed to measure proteolytic activities of MMP-2 and MMP-9

Lane 1,2: MMP-9 or MMP-2 condition media collected from stable transfected COS-1 cell line, which overexpressed MMP-9 or MMP-2 respectively. Lane 3-8: Conditioned medium collected from stable transfected MDA-MB-231 cell line with shCtrl or shp53 as labelled; lane 3,4: treated with SFM overnight; lane 5,6: added 20ug/ml ConA in SFM and treated overnight; lane 7,8: added 20ug/ml ConA and 25ul/ml MMP-2 conditioned media in SFM and treated overnight. Molecular weights are shown on the left.

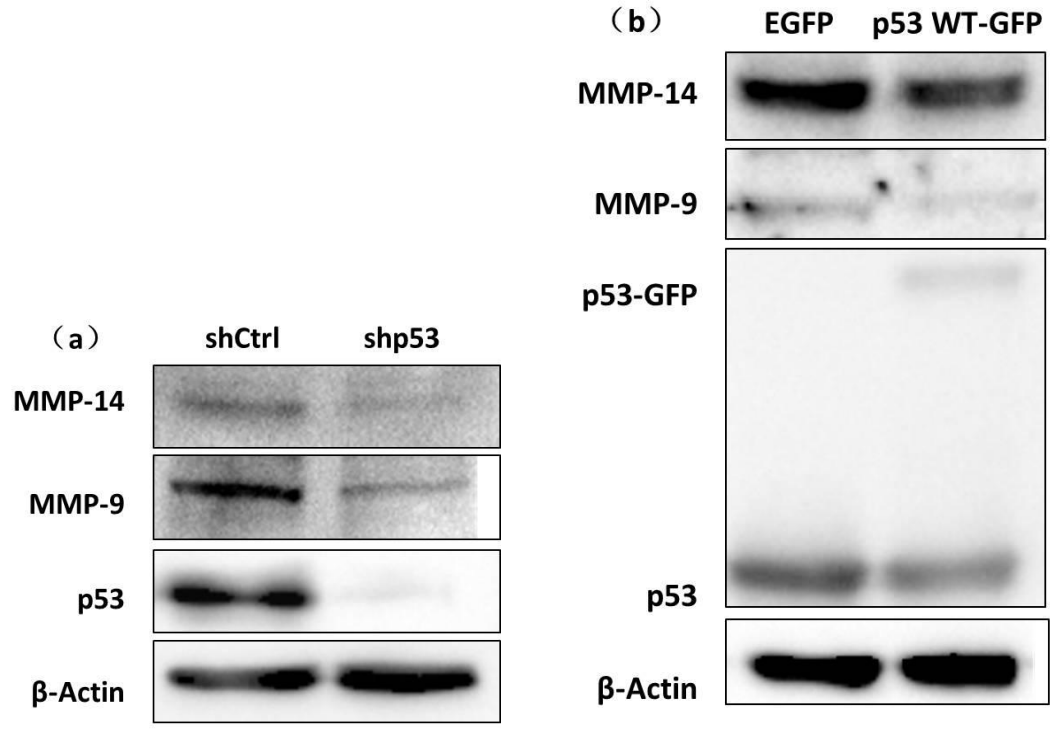

Figure 3.2 Western blot performed to test protein levels of MMP-9 and MMP-14

(a) Stable transfected MDA-MB-231 cell lines with shCtrl or shp53. (b): Transient transfected MDA-231 cell line with EGFP or wild type p53-GFP.

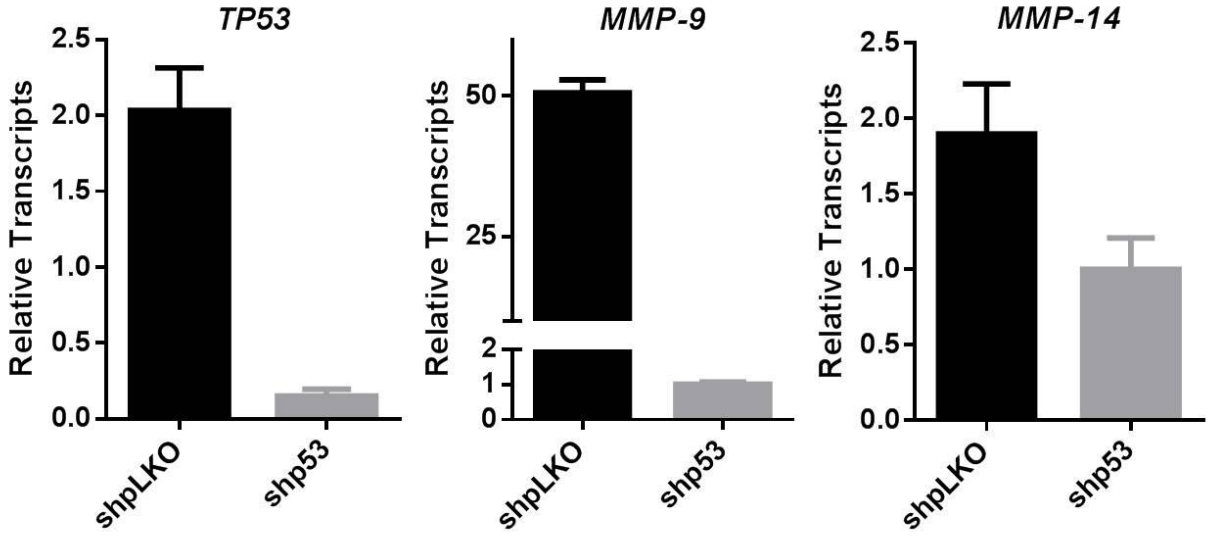

Figure 3.3 RT-qPCR performed to measure the transcripts of p53, MMP-9 and MMP-14

Stable transfected MDA-MB-231 cell lines with shCtrl or shp53 were used to measure the mRNA levels of p53, MMP-9 and MMP-14. The transcripts were normalized using HPRT as an inertial control.

3.2 Analysis of the Putative Transcription Factor-Binding Sites within the MMP-9 and MMP-14 Promoters

As previous studies have reported that multiple MMPs could be regulated by p53, we asked whether MMP-9 and MMP-14 can be directly regulated by p53 and its mutant R280K. Therefore, the promoters of MMP-9 and MMP-14 were analyzed by PROMO. A p53 and Sp1 overlapping binding site was predicted on each promoter (see Figure 3.4).

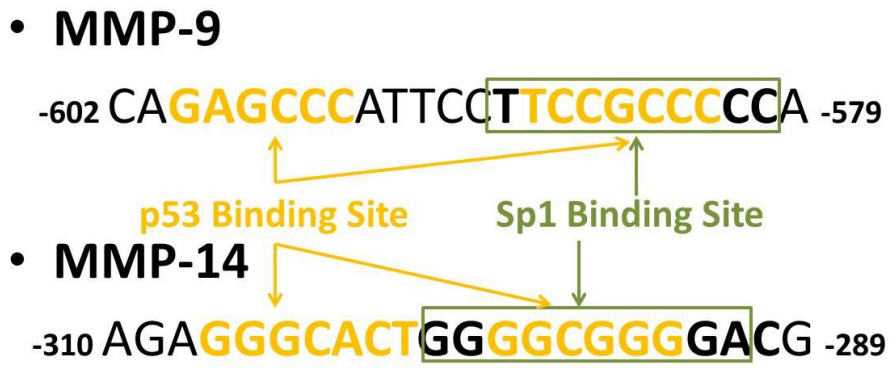

Figure 3.4 Potential binding sites for p53 and Sp1 MMP-9 and MMP-14 promoter

p53 is able to recognize a specific consensus sequence consisting two copies of a 10-bp motif, 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3', separated by 0-13 bp. Sp1 is able to bind the consensus sequence 5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3'. The location of nucleotide is relative to translation start site.

3.3 Involvement of p53 in Transcriptional Activity of the MMP-9 and MMP-14 Promoters

To further verify that wild type p53 and the p53 mutant R280K regulate transcriptional activity of MMP-9 or MMP-14 differentially, luciferase reporter gene assay was performed. The MMP-9 and MMP-14 promoter regions that contained predicted binding sites were fused to firefly luciferase gene respectively. COS-1 cells were transiently transfected with plasmids of promoter, p53 and Renilla. The results showed that wtp53 could repress the transcriptional activity of both MMP-9 and MMP-14, while p53 R280K did in the opposite way (see Figure3.5).

It has been reported that structurally destabilized p53 has the propensity to aggregate in cytoplasmic, while wtp53 and contact mutants predominantly stay in the nucleus[42]. To prove that p53 R280K did not form aggregation in cytoplasm, immunofluorescence assay was performed using MDA-MB-231 cells. It showed that p53 was perfectly co-localized with nucleus (see Figure 3.6), suggesting that p53 R280K is a contact mutant but not a structurally destabilized mutant.

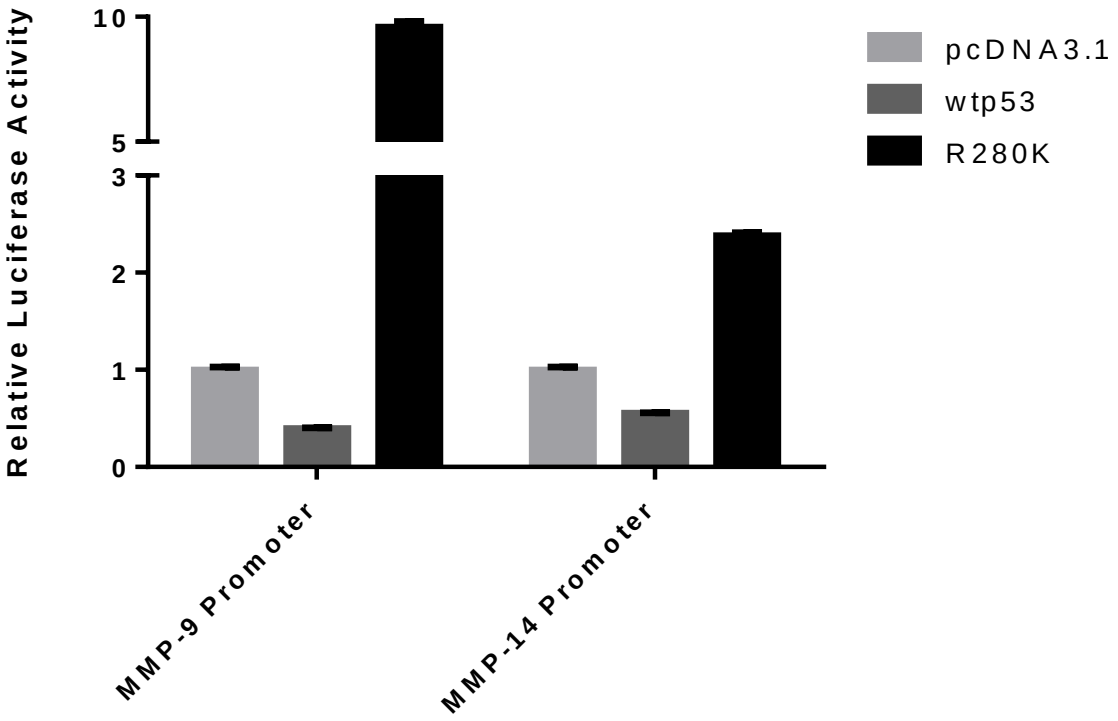

Figure 3.5 Luciferase reporter gene assay performed in COS-1 cell line

COS-1 cells were co-transfected with MMP-14 or MMP-9 promoter, Renilla gene, and pcDNA3.1, wild type p53 or p53 R280K. Firefly luciferase activity was normalized to Renilla luciferase activity.

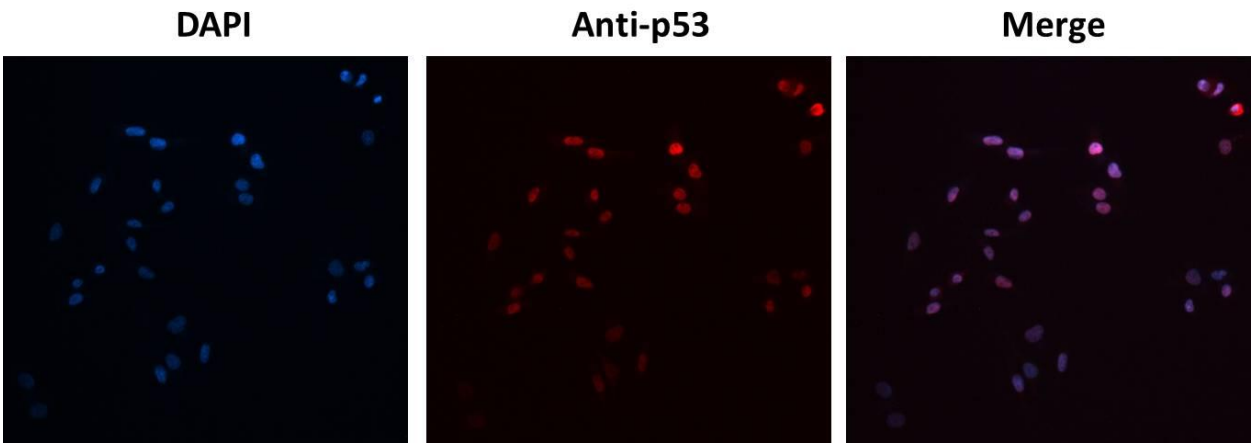

Figure 3.6 Immunofluorescence in MDA-MB-231 cells

3.4 p53 R280K Promotes Cell Migration

In functional study, migration ability of MDA-MB-231 cells were measure through 2-dementinal dot assay. Representative images were shown in Figure 3.7. It is obvious that knockdown of mutant p53 impaired the migration capacity of MDA-MB-231 cells. It might be partially due to the decreased levels of MMP-9 and MMP-14, which are involved in tumor cells migration and invasion.

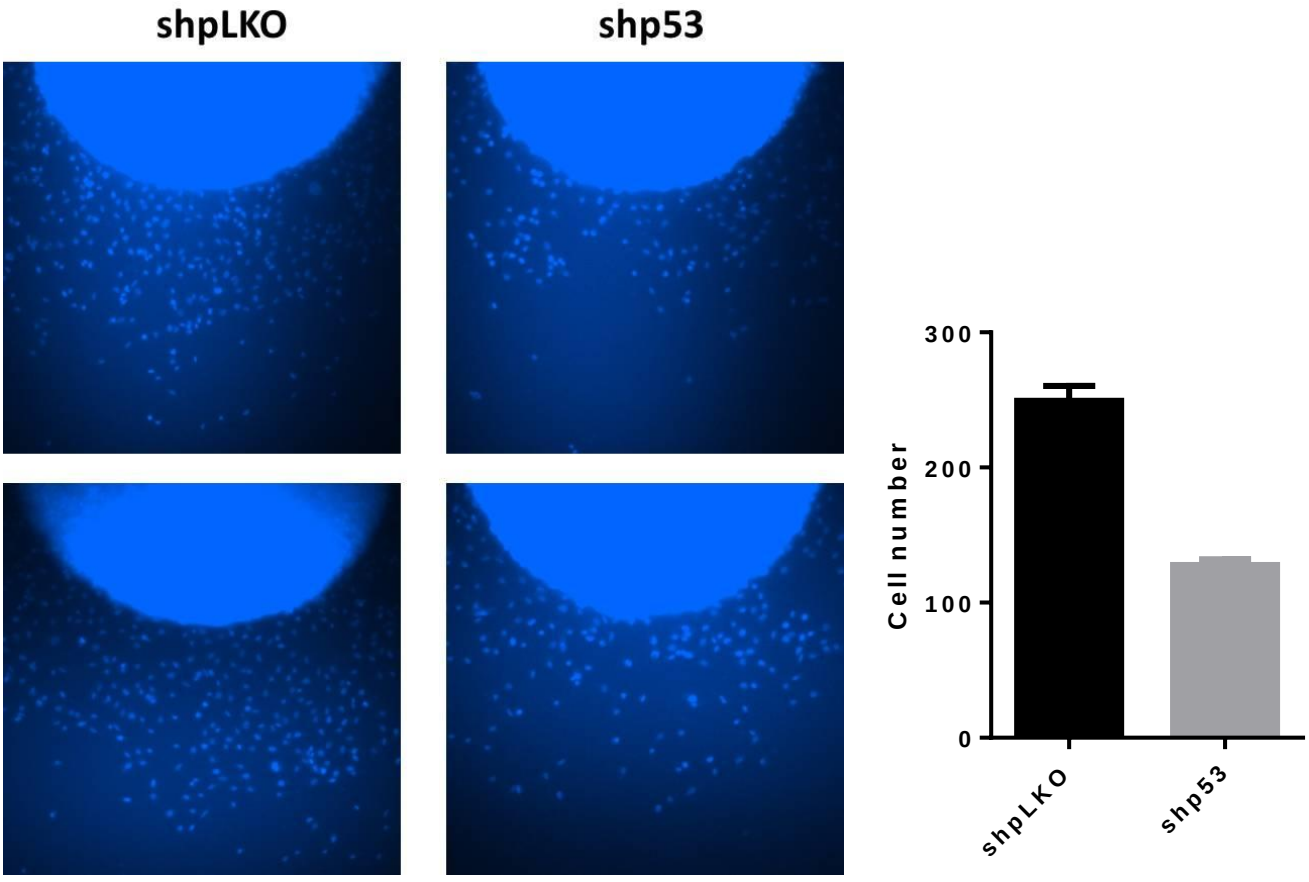

Figure 3.7 2-D dot migration performed in stable MDA-MB-231 cells with shCtrl or shp53

4 Discussion

It has been reported that p53 R280 makes the most number of contacts within 4 Å of base atoms in the major groove[45], and the R280K mutant was also observed in nucleus rather than forming aggregation in the cytoplasm. These facts confirm that p53 R280K is a DNA-contact mutant. If p53 functions alone on the promoters of MMP-9 and MMP-14, there should not be different consequences between control group and knockdown p53 in MDA-MB-231 cells, because the in both cases no p53 could bind to the promoters and p53 could not exert its transrepression function.

However, transfection of shp53 repressed the transcription of MMP-9 and MMP-14 in MDA-MB-231 cells, which suggested that p53 did not act alone on the promoters. Indeed, Sp1 binding site was predicted overlap with one of the p53 binding sites, implying that MMP-9 and MMP-14 might be co-regulated by p53 and Sp1. As previous studies have shown that p53 was able sequester Sp1 to prevent its binding and further repress the Sp1-stimulated promoter activity, it is reasonable to surmise that p53 and Sp1 function in the same pattern on the promoters of MMP-9 and MMP-14. If it is true, all the results can be explained.

Firstly, in MDA-MB-231 control group, p53 R280K forms homotetramers and cannot bind to DNA sequences, which renders the opportunity for Sp1 to exert its transactivation function. Even so, this contact mutant does not lose the interaction with Sp1 (see Figure 4.1(c)). Upon p53 knockdown, there is no endogenous p53 and the binding site is occupied by Sp1 alone. Since transcription was repressed, it is suggested that Sp1 functions in a p53-dependent pattern (see Figure 4.1(d)). Later, in COS-1control group, endogenous wild type p53 forms homotetramers and occupies that specific binding sites and exert its transrepression function (see Figure 4.1(a)). After overexpression of p53 R280K, heterotetramers are formed with compromised DNA-binding ability. As a consequence, Sp1 stimulates transcription of MMP-9 and MMP14 (see Figure 4.1(b)).

Although this model seems to be reasonable, some questions remain to be answered. For example, whether interaction between Sp1 and p53-DNA complex really exists, needs to be demonstrated by chromatin immunoprecipitation (ChIP) assay. Besides, whether the predicted transcription factor binding sites really play a critical role should be confirmed by truncated mutants or point mutants. Moreover, whether this regulation is Sp1-dependent, needs to be addressed by Sp1 knockdown.

Overall, our data implies a co-regulation mechanism of p53 and Sp1 on the transcriptional activity of MMP-9 and MMP-14. In addition, the p53 mutant R280K, which is common in triple-negative breast cancer, is confirmed to upregulate MMP-9 and MMP-14 and thus promotes tumor cells to migrate and invade. Additional studies will be needed to further verify this mechanism. And further understanding of this mechanism could provide a novel therapeutic strategy for preventing cancer metastasis.

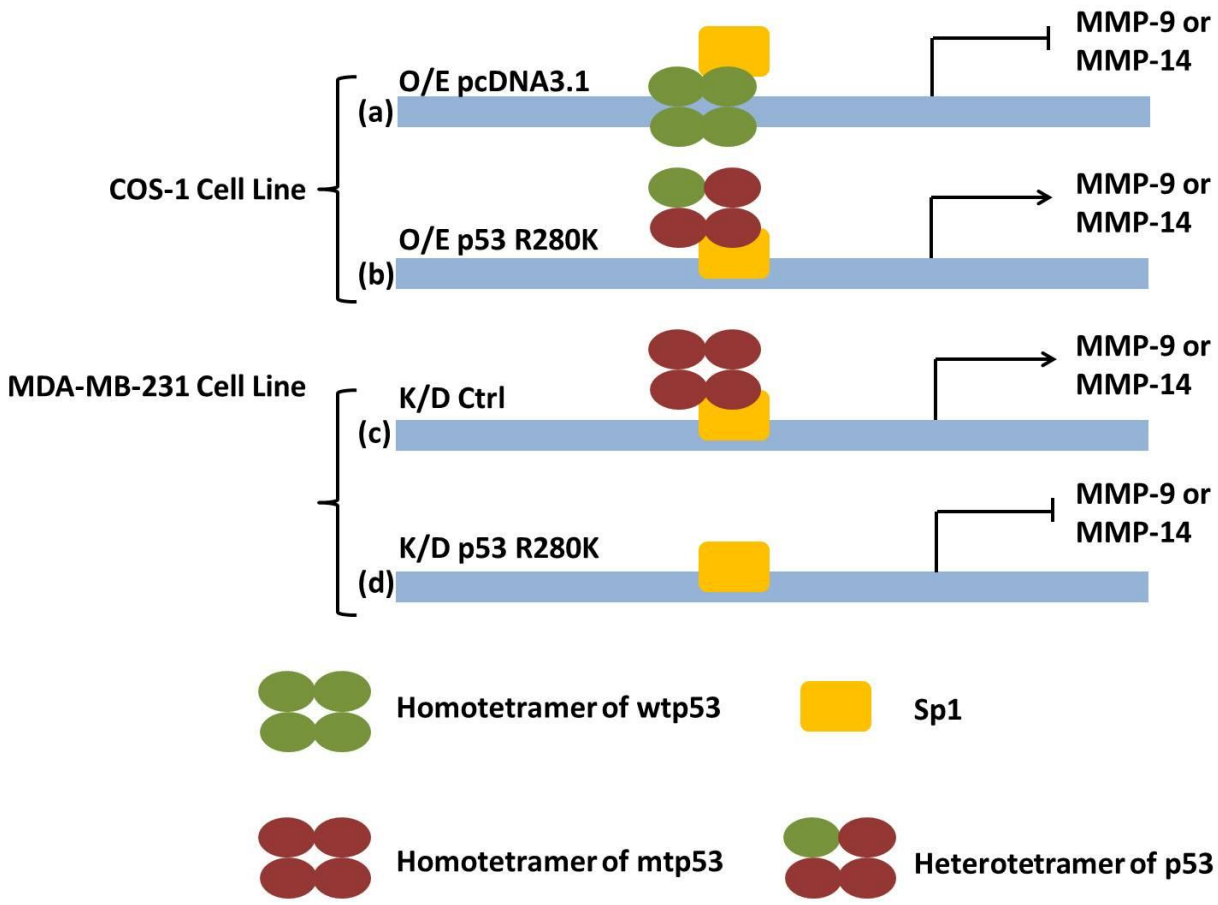

Figure 4.1 Working model of p53/Sp1 co-regulation of MMP-9 and MMP-14 transcription

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