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14. ABSTRACT

We conducted renalgraft experiments to study the role of YY1 in prostate cancer. We used the mouse prostate epithelial cells (MPECs) with either depleted endogenous YY1 or overexpressed exogenous YY1. The grafts of YY1-depleted MPECs did not show significant growth. We reasoned this to the culturing of urogenital mesenchyme (UGM) and this study experiment will be repeated. In YY1 increase study, we successfully generate renalgrafts, but did not observe any significant change in the histology and weight in response to YY1 increase. One possible reason is that YY1 does not play an etiological role in prostate tumorigenesis. Another possible reason is that the CMV promoter used to drive YY1 expression was silenced in vivo. We are currently testing chicken beta-actin promoter and an inducible system to overexpress YY1 and repeat the experiments. We have determined the effects of different YY1 levels on the proliferation of MPECs in a 3-D culture system. This will provide insight on choosing an appropriate YY1 expression level in animal studies. We further defined the binding region of Mdm2 on YY1 from previously determined 95 residues to currently 26 residues. This will help us in the structural/functional studies to be carried out in next step.

15. SUBJECT TERMS
YY1, Prostate Cancer, Lentivirus, siRNA, knockdown, overexpression, renalgraft, mouse model.

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A. Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer and the second leading cause of cancer-related deaths among men in the United States. Genetic changes, such as mutations of tumor suppressor genes, and epigenetic alterations, defined as heritable changes in gene expression without changes in DNA sequence, contribute to prostate malignancy [1]. Yin Yang 1 (YY1) [2] is overexpressed in several types of cancers, including prostate cancer (PCa) [3-6]. YY1 is a multifunctional protein that acts as a transcription factor and plays a crucial role in epigenetic regulation, including mediating histone acetylation, deacetylation and methylation [7-9]. Data from our lab and others indicated that YY1 is overexpressed in both human PCa [3] and the PCa of a transgenic mouse model. The goal of this project is to determine the functional role of overexpressed YY1 in PCa. Our overall hypothesis is that overexpressed YY1 is essential to the development and progression of PCa. We will perform *in vivo* studies to test this hypothesis. In addition, we will also investigate how the interplay of YY1-p53-Pten and YY1-Ezh2 contributes to the tumor development of PCa. Our study will improve the understanding of the mechanism that leads to the aberrant epigenetic regulation in PCa. This will provide fundamental support to the development of therapeutic approaches to conquer PCa by reversing its epigenetic abnormality.

B. Body:

Task 2. To study the effects of YY1 expression to the PCa formation *in vivo* (**Months 9-17**):

  a. Start the PCa tumor formation experiments in mice by renal grafting (1) Pten$^{-/-}$ MPECs expressing the scrambled siRNA and *yy1* siRNA; (2) Pten$^{+/+}$ and Pten$^{+/-}$ MPECs containing the YY1 expression cassette and the empty vector (**Months 9-12**).
  b. Data analysis of the *in vivo* study, including dissection of the graft, histopathological study, immunohistochemistry and the assessment of the results (**Months 13-15**).
  c. Determine whether YY1 overexpression in PCa is regulated at the transcriptional or posttranslational level (**Months 16-17**).

B1. To study the effects of YY1 knockdown and overexpression on PCa formation in a renalgraft mouse model.

B1.1. To study the effect of YY1 knockdown on the tumor formation ability of Pten$^{-/-}$ cells.

As described in our previous report, we individually subcloned the expression cassettes for U6/scrambled siRNA and *yy1* siRNA into two different lentiviral vectors (Figures 1A and 1B) that express red fluorescent protein (RFP) and puromycin resistant gene (Puro), respectively. We produced lentiviruses carrying these siRNA expression cassettes and used them to infect mouse prostate epithelial cells (MPECs). Western blot analyses indicated that we achieved efficient knockdown of YY1 protein in the cells transduced by U6/*yy1*-containing lentivirus, when compared with the
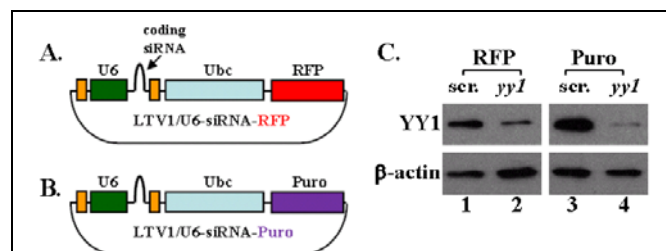
Figure 1. Lentiviral vectors carrying (A) RFP and (B) puromycin resistant gene used to introduce siRNA expression cassettes and the Western blot studies of YY1 knockdown in Pten$^{-/-}$ MPECs.
scr.: scrambled siRNA.

control cells infected by U6/scrambled siRNA-containing lentivirus (compare lanes 2 & 4, with 1 & 3, respectively, Figure 1C).

The Puro-containing lentivirus allowed us to selectively culture the infected MPECs by adding puromycin in the medium and the cell exhibited more pronounced YY1 depletion, compared to the lentivirus carrying the DsRed2 as a marker (Figure 1C). Therefore, we used the cells expressing "U6/siRNA+Puro" in the mouse renalgraft studies, following the procedure described in Figure 2.

Figure 2. Schematic procedure of renal grafting study.
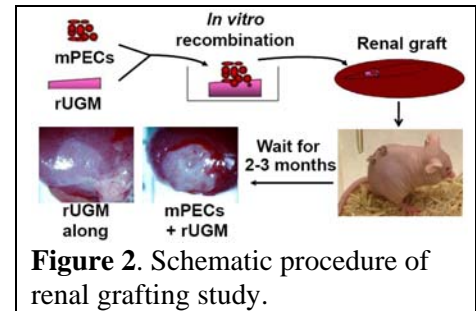
Results and discussion:

We transplanted the following cells into mouse renal capsule: (graft numbers are indicated in the brackets)

(1) MPECs only ($\times$2);
(2) UGM (urogenital mesenchyme) alone ($\times$2);
(3) U6/scrambled siRNA transduced Pten$^{-/-}$ MPECs + UGM ($\times$6);
(4) U6/scrambled siRNA transduced Pten$^{-/-}$ MPECs ($\times$6).

Figure 3 shows the representative results of this renalgraft study. The control groups (1) and (2) did not show any significant growth of the implanted cells (columns 1 and 2, Figure 3). However, the recombination of the siRNA-transduced MPECs barely formed any renalgraft (representative images shown in the columns 3 and 4, Figure 3). This observation is unexpected and we predict the following possible factors may have led to this technically negative result:

(1) The isolated UGM might have lost their function in supporting the MPECs forming prostate-like tissues. UGM isolation is technically very challenging. In this particular experiment, the 18-day

Figure 3. Renalgrafts of the control groups (MPECs alone and UGM alone, columns 1 and 2) and the MPECs treated with either a scrambled siRNA or *yy1* siRNA (columns 3 and 4).

pregnant rat produced much fewer (5) embryos than what we can normally get (average 10-12 embryos). As a result, we did not harvest enough UGM for their immediate *in vitro* recombination with MPECs.
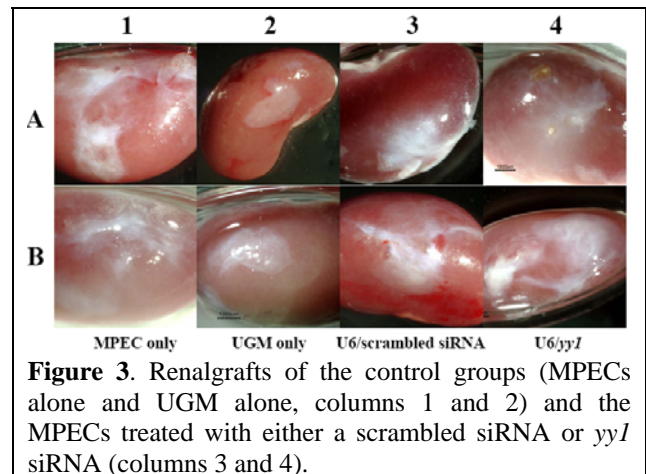
To circumvent this problem, we used an alternative approach, which is based on a previous report from Dr. Witte group [10]. This study indicated that the isolated UGM can be cultured for up to two passages before being used in the *in vitro* recombination with MPECs for renalgraft studies. We therefore cultured these UGM cells following this protocol and also consulting the authors [10], and hoped to get sufficient amounts of UGM for this experiment. The UGM cells were cultured for 10 days and then used for the in vitro recombination with MPECs and renal implantation experiment. The failure in obtaining significant renalgraft growth indicated that the culturing process of the UGM might have led to the loss of original functions of these cells. Indeed, we observed that these cultured UGM cells exhibited much larger cell dimension than these freshly isolated.

(2) The Pten$^{-/-}$ MPECs were directly isolated from the tumors formed in Pten$^{-/-}$ mouse prostate. After multiple passages, the cells may have become transformed or terminally differentiated, and therefore lost the properties of progenitor cells.

Ongoing and future studies:
(1) We are repeating the renalgraft study using these Pten$^{-/-}$ MPECs with freshly isolated UGM.
(2) We have isolated the Pten$^{flox/flox}$ MPECs from normal mouse prostate. We will delete the Pten *in vitro* using lentivirus carrying Cre recombinase. The generated Pten$^{-/-}$ cells will be used in mouse renalgraft experiments to study how YY1 knockdown will affect the PCa formation.

B1.2. To study whether increased YY1 will endow the tumor formation ability to Pten^{wt} cells

To determine the effects of YY1 increase on PCa formation, we used two different lentiviral vectors carrying either ZsGreen or puromycin resistant cDNA (Puro) (Figures 4A and 4B) to deliver YY1 expression cassette into MPECs with wild type (wt) Pten (Pten^{wt}). Cells infected by pSL2-YY1-ZsGreen lentivirus showed increased expression of YY1 (compare lane 2 with lane 1 in Figure 4C). However, cells infected by pSL2-YY1-Puro lentivirus (Figure 4B), cultured in medium containing 2 µg/ml puromycin, exhibited robust expression of YY1 (compare lane 4 with other lanes in Figure 4C).

Figure 4. Lentiviral vectors carrying (A) ZsGreen and (B) puromycin resistant gene used to introduce YY1 expression cassettes and the Western blot studies of YY1 overexpression in Pten^{wt} MPECs. V.: empty vector.

Prior to the *in vivo* renalgraft experiments, we first carried out *in vitro* studies to determine the effects of the YY1 increase after the lentiviral infection on cell proliferation. We used the collagen three-dimensional (3-D) culture system developed by our collaborator, Dr. Cramer [11]. In this 3-D culture system, we observed that the cells transduced by the pSL2-YY1-ZsGreen showed increased proliferation (compare the images of the center and the left, Figure 5B). However the MPECs infected by the pSL2-YY1-Puro (expressing robustly increased YY1, Figure 5A) exhibited retarded growth (Figure 5B, compare the image at the right with the ones in the center and left). These results indicated that a modest increase of YY1 in MPECs (lane 2, Figure 5A) could significantly enhance cell growth in the collagen 3-D culture system, while a robust YY1 elevation in these cells (lane 3, Figure 5A) adversely affected the cell proliferation. Therefore, we chose the cells infected by pSL2-YY1-ZsGreen in our renalgraft experiment.

Figure 5. The correlation between YY1 increase and mPEC growth in a 3-D culture system. A. Western blot to detect YY1 and β-actin expression in MPECs transduced by an empty lentiviral vector, pSL2-YY1-ZsGreen and pSL-YY1-Puro. **B.** Branching structures formed by MPECs (4×10^4 cells/well in a 24-well plate) transduced by the lentiviruses (top). The whole collagen culture matrix in each well was the individually digested by collagenase. All of the pelleted cells were re-plated to 6-cm dishes and stained by crystal violet after 2-day culture (bottom). **C.** YY1 antibody and hematoxylin co-staining of the dissected ductal branches formed by the MPECs. No meaningful structure could be captured from the collagen inoculated with pSL2-YY1-Puro-transduced MPECs. **D.** Pixels in the dishes of Figure 5B to quantify the cell proliferation.

The MPECs transduced by the pSL2 empty vector and pSL2-YY1-ZsGreen were incubated with *freshly isolated* UGM for *in vitro* recombination and then implanted into mouse renal capsules, following the procedure described in Figure 2.
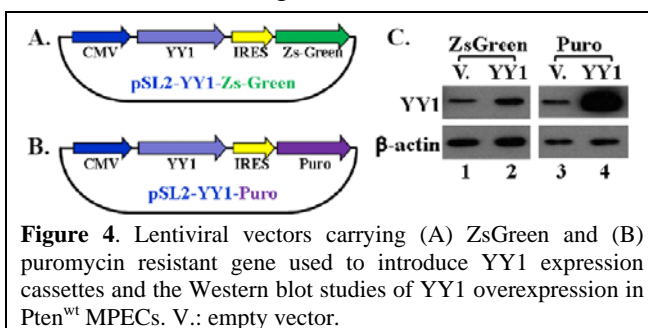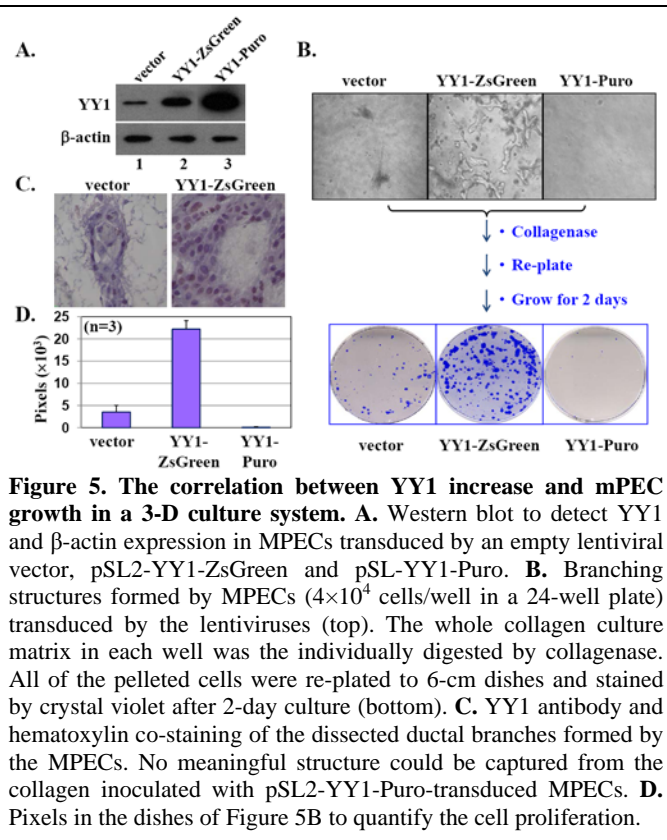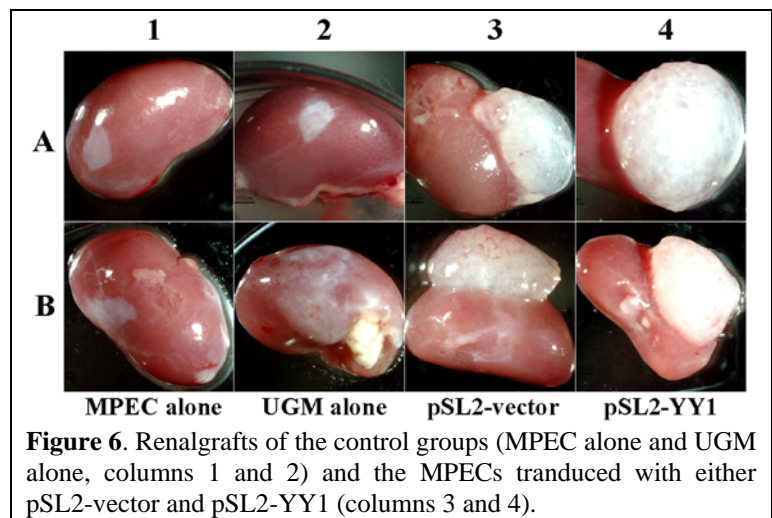
Result and discussion:
We transplanted the following cells into mouse renal capsules: (graft numbers are indicated in the brackets)
(1) MPECs only (×2);
(2) UGM alone (×4);
(3) pSL2-vector transduced Pten^{wt} MPECs + UGM (×6);
(4) pSL2-YY1 transduced Pten^{wt} MPECs + UGM (×8).

At the termination of the renal grafting experiment (10 weeks after the renal implantation), the animals were euthanized. The kidneys with the grafts were removed intact and individually placed in culture dishes with sterile HBS buffer. Excess tissue was removed with the aid of a dissecting microscope. As shown in Figure 6, the two control groups (MPECs alone and UGM alone) did not show significant growth at the implanted sites (columns 1 and 2). The pSL2-vector and the pSL2-YY1 transduced cells generated large renalgrafts (see representative images in columns 3 and 4). The morphology of the formed renalgrafts indicated a successful experimental procedure using the freshly isolated UGM for the *in vitro* recombination. These grafts were individually excised from the

Figure 6. Renalgrafts of the control groups (MPEC alone and UGM alone, columns 1 and 2) and the MPECs tranduced with either pSL2-vector and pSL2-YY1 (columns 3 and 4).

kidneys and weighed. The weights of the renalgrafts are shown in Figure 7. The grafts formed by MPECs alone and UGM alone are small and neglectable when compared with the other two groups (A and B, Figure 7). The weights of the grafts generated by the pSL2-YY1 transduced MPECs and UGM are more scattered than the ones with pSL2-vector (compare D and C in Figure 7). Although the mean weight of pSL2-YY1 grafts is 50.9 mg more than that of the pSL2-vector grafts, this difference is not statistically significant ($p = 0.34$).

A portion of each graft was excised from the rest part, placed in 10% formalin, embedded and sectioned for histological analysis (hematoxylin/eosin staining) and immunohistochemical (IHC) analysis using an YY1 antibody (H-414, Santa Cruz). The representative H&E staining images of the sectioned renalgrafts generated from the pSL2-vector and pSL2-YY1 transduced MPECs in four individual mice are shown in Figure 8. The renalgrafts
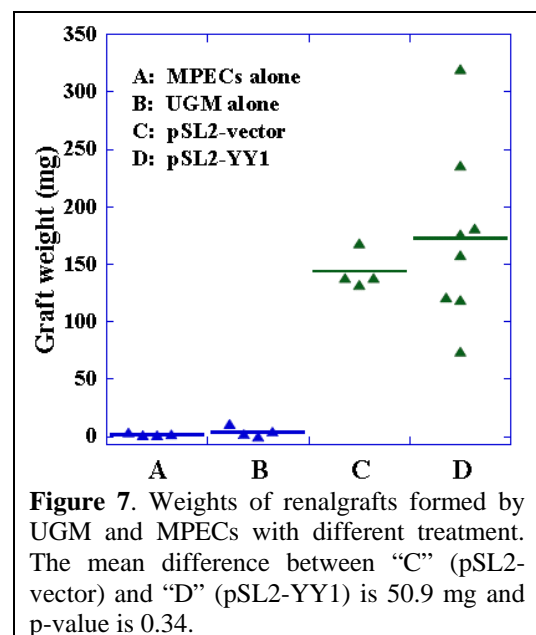
Figure 7. Weights of renalgrafts formed by UGM and MPECs with different treatment. The mean difference between "C" (pSL2-vector) and "D" (pSL2-YY1) is 50.9 mg and p-value is 0.34.

formed at both conditions exhibited structures that resemble to the normal prostate-like glands, as

previously presented by Dr. Cramer and his colleagues [12]. No obvious prostate hyperplasic or tumorigenic lesion was observed. In the IHC studies, YY1 expression is overall at medium levels and no significant YY1 increase was observed (Figure 9).

We predict that the following possible factors could lead to this observation:

(1) It is possible that YY1 increase will not endow normal MPECs with proliferative or tumorigenic properties, although we have observed it could enhance the cell growth in 3-D collagen culture system (Figure 5D). This will suggest that YY1 increase may not play an etiological role in prostate cell tumorigenesis.

(2) The CMV promoter used to drive YY1 expression in pSL2 lentiviral vector could have been attenuated or even silenced after the MPECs were implanted into the mouse kidneys. When searching the literature, we found a previous study indicating that CMV promoter was attenuated by epigenetic regulation [13]. To circumvent this problem, we planned to repeat this experiment using the chicken β-actin promoter (generously provided by Dr. Miyazaki, [14]) to drive YY1 cDNA. Recently, we observed that this promoter could stably sustain the expression of YY1 cDNA for more than 2 months (data not shown).

Ongoing and future studies:
We have generated pSL5-YY1 that employs the chicken β-actin promoter [14] to drive YY1 expression. Although the initial ectopic YY1 expression after lentiviral infection is not as high as the MPECs transduced by pSL2-YY1 (with CMV promoter), we observed a long-lasting increase of YY1 expression. We are currently screening for individual MPECs clones that can exhibit 4-6-fold YY1 increase compared to the endogenous YY1. These clones will be used for new renalgraft experiments.

H&E staining:

pSL2-vector   pSL2-YY1
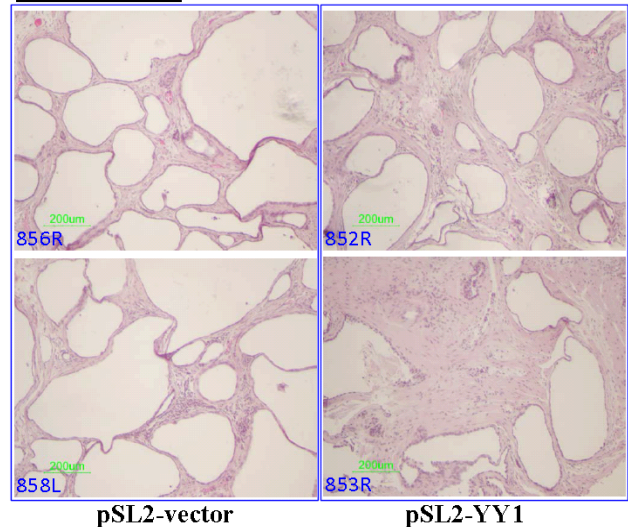
Figure 8. Representative H&E (hematoxylin/eosin staining) staining images (10×) of sectioned renalgrafts generated from the MPECs transduced by pSL2 vector (left) and pSL2-YY1 (right) lentiviruses. The number at the left-bottom corner of each image indicates the animal number and the side of the kidney.

YY1 antibody (H-414):

pSL2-vector   pSL2-YY1
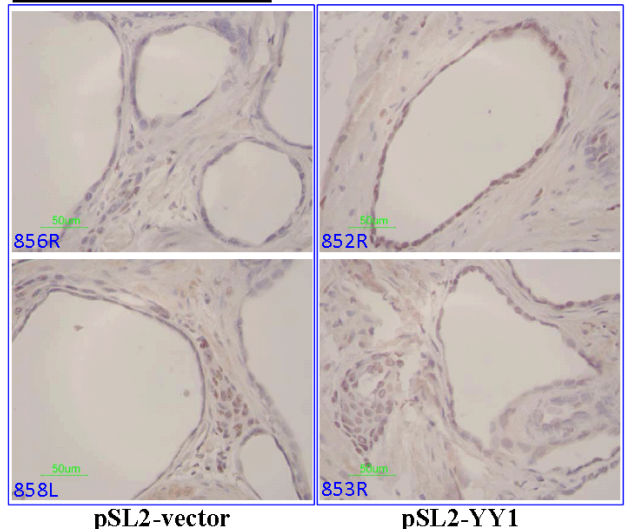
Figure 9. Representative IHC staining images (40×) using YY1-H414 antibody (Santa Cruz) of sectioned renalgrafts generated from the MPECs transduced by pSL2 vector (left) and pSL2-YY1 (right) lentiviruses. The number at the left-bottom corner of each image indicates the animal number and the side of the kidney.

Task 3. To study the tumor formation with disrupted YY1-p53 interaction (**Months 18-26**):

     a.   Produce lentivirus containing the vector or the expression cassette for the YY1 mutant deficient in mediating p53 degradation. Use the lentivirus to generate sable Pten$^{-/-}$

MPECs carrying the vector or expressing this YY1 mutant (**Months 18-19**).
  b. Start the *in vivo* tumor formation experiment by renal grafting these MPECs (**Months 20-22**).
  c. Data analysis of the *in vivo* study, including the dissection of the renal graft, histopathological study, immunohistochemistry and the assessment of the results (**Months 23-24**).
 d. Determine if the interactions of YY1-p53 and YY1-Pten are exclusive (**Months 25-26**).

B2. To study whether YY1-p53 interaction is essential to PCa formation.
B2.1. To generate an YY1 mutant with a further defined region that is responsible for mediating p53 degradation.

Previous studies including ours demonstrated that YY1 is essential to Mdm2-mediated p53 ubiquitination and degradation [15-17]. Therefore, we hypothesize that the disruption of this regulatory function of YY1 will let p53 avoid the Mdm2-mediated ubiquitination. We have reported that p53 interacts with two different sites of YY1 protein [15]: one is located at the residues 142-224 and the other one is at the C-terminal zinc finger region (residues 331-414). Therefore, we predict that an YY1 mutant with disrupted p53 binding sites will need mutating these two regions, which will most likely have a large impact on YY1's other essential functions, especially its DNA activity. In our previous study, we also demonstrated that YY1 mutant with deleted spacer domain (residues 201-295) lost its function of enhancing Mdm2-p53 interaction, although its affinity to p53 did not change compared to wt YY1 [15].

Thus, we proposed to use an YY1 mutant with disrupted interaction with Mdm2 to study whether YY1-regulated p53 degradation is essential to prostate cancer formation.

In our previous report, the Mdm2 interacting site was mapped to a relatively large region of YY1 with 95 residues [15]. Disruption of this region may still exert a big impact to the function and/or structure of YY1 protein. Thus, we decided to further define this region of YY1 to narrow down the residues that are responsible

Figure 10. Further defining the Mdm2-interacting domain on YY1. A. The domain structure of wt YY1 and the three mutants. p53 binding sites [15] and further defined Mdm2 binding site (*see* **Figure 10B**) are indicated on the top. The domain mapping results of **Figure 10B** are summarized in the right panel. "**+/-**" means very weak interaction. **B.** *In vitro* protein binding of wt YY1 and its mutants with GST-Mdm2 and GST-p53. The bands marked by red pentagrams indicate the significantly decreased Mdm2 interaction.

for interacting with Mdm2. As shown in Figure 10A, in addition to the previously reported YY1 (Δ201-295) mutant [15] (designated as Mut 1), we generated two new mutants, Mut 2: YY1 (Δ201-226) and Mut 3: YY1 (Δ227-295). We carried out an *in vitro* protein binding experiment to assess the interaction of these His-tagged YY1 mutants with GST-Mdm2 and GST-p53. The interaction of these YY1 mutants with Mdm2 and p53 are shown in Figure 10B and summarized in Figure 10A (right
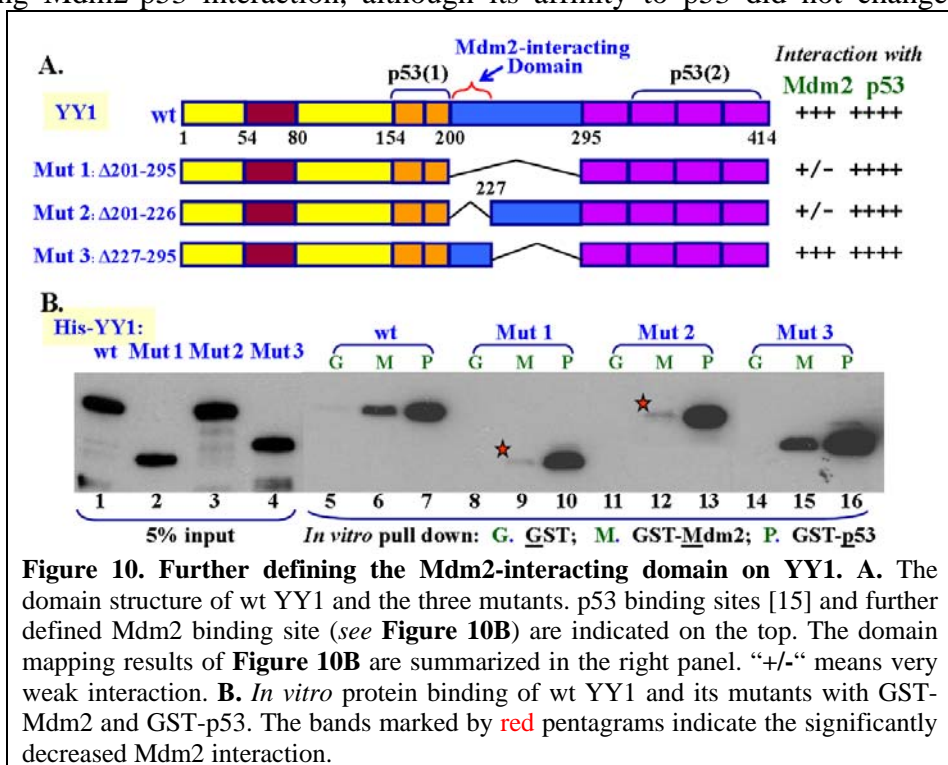
panel). The three YY1 mutants retain strong affinity to p53, which is comparable to the wt YY1 (compare lanes 7, 10, 13 and 16 in Figure 10B). However, Mut 2 exhibits a significantly compromised ability to interact with Mdm2, essentially the same as Mut 1 (compare lane 12 with lane 9 for the bands marked by ★ in Figure 10B). Mut 3 possesses similar Mdm2 binding affinity to wt YY1 (compare lane 15 with lane 6 in Figure 10B). In summary, the results indicate that a small region of YY1 (residues 201-226) is involved in interacting with Mdm2.

B2.2. To study whether the substitution of endogenous YY1 by an YY1 mutant deficient in promoting Mdm2-mediated p53 degradation will compromise the tumor formation ability of Pten$^{-/-}$ MPECs.

We will simultaneously knockdown the endogenous YY1 and ectopically express the YY1(Δ201-226) to determine whether the YY1/Mdm2-mediated p53 degradation is essential to prostate cancer formation.

As described above, we predict that CMV promoter may be silenced when used in *in vivo* studies and we are testing whether chicken β-actin promoter can be used in renalgraft study. Once this can be determined, we will use this promoter to drive the expression of YY1(Δ201-226) mutant in the renalgraft studies. Meanwhile, we are also testing a *Tet-on* inducible system and want to use this system to inducibly express YY1 after the implantation of MPECs. We have generated an "all-in-one" vector, as depicted in Figure 11A. We have used this system to express YY1 cDNA in cultured MPECs. YY1 induction can reach nearly 6-fold of the endogenous YY1 (see the quantification of the YY1 bands in Figure 11B). We will test whether this inducible YY1 expression can be maintained for a long term, before using these MPECs in the renalgrafting experiment.
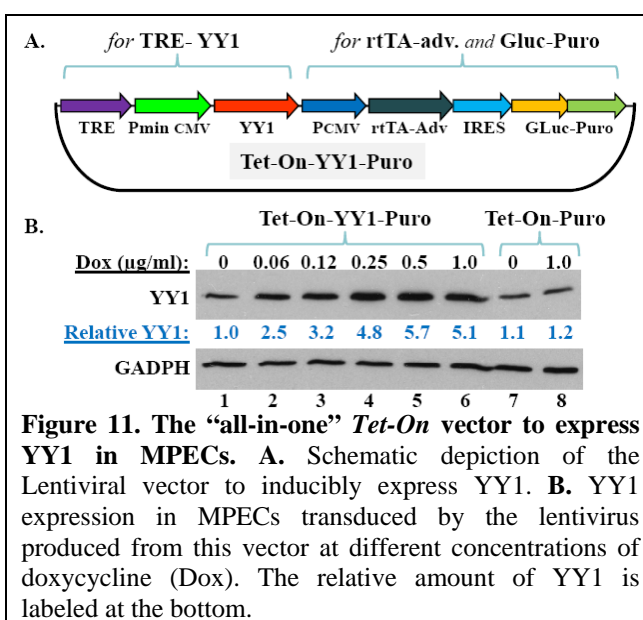
Figure 11. The "all-in-one" *Tet-On* vector to express YY1 in MPECs. A. Schematic depiction of the Lentiviral vector to inducibly express YY1. **B.** YY1 expression in MPECs transduced by the lentivirus produced from this vector at different concentrations of doxycycline (Dox). The relative amount of YY1 is labeled at the bottom.

C. Key Research accomplishment:
1. We have further defined the Mdm2 binding site on YY1 to a much smaller region compared to our previously reported data.

2. We determine that medium levels of YY1 increase exert proliferative effects on MPECs, but a robust expression of YY1 will have adverse effect on the proliferation of MPECs.

3. We can successfully carry out renalgraft experiment using an YY1-overexpression vector transduced MPECs, although we currently did not observe any effect of YY1 on prostate cancer formation.

4. We have generated two new lentiviral vectors. The first one uses the chicken β-actin promoter to drive the expression of YY1. The second one is an "all-in-one" *Tet-on* inducible lentiviral vector. Upon addition of doxycycline, this vector can inducibly express YY1 *in vitro* and potentially *in vivo*.

D. Reportable Outcomes:
1. We compared the effects of different levels of YY1 increase on the proliferation of MPECs using 3-D collagen culture. We observed that medium level of YY1 increase facilitates the growth of MPECs in the 3-D culture system. However, a robust increase of YY1 adversely affects the growth of MPECs. The results of this study will help us to determine which levels of YY1 increase should be used in the *in vivo* renalgraft studies.

2. We carried out the renalgraft studies using MPECs with ectopically overexpressed YY1. The results from the last animal study did not show any malignant change of the renalgraft tissue generated by YY1-overexpressed MPECs. We reasoned this to the attenuated CMV promoter that was used to drive YY1 expression.

3. We have further defined the Mdm2 binding site on YY1 from previous 96 residues to current 26 residues. This result helped us generate a more defined YY1 mutant that will be used to study the role of YY1-mediated p53 degradation in prostate cancer formation.

E. Conclusion:
We encountered some technical difficulties in one renalgraft study, when investigating the effect of YY1 decrease on prostate cancer formation. We will repeat this YY1 knockdown study. We are also seeking for alternative approaches, such as using a *Tet-On* inducible siRNA system to knockdown endogenous YY1.

In the YY1 overexpression study, we did not observe any hyperplasic or tumorigenic lesion in the renalgrafts formed by YY1-overexpressed MPECs. One of the possible reasons is that the CMV promoter used to drive YY1 expression may have been silenced in the *in vivo* study. We have started to use two alternative expression systems. The first one uses chicken β-actin promoter to drive YY1 expression. Our *in vitro* experiment indicated that YY1 overexpression driven by the chicken β-actin promoter could last two months, suggesting that it is a long term expression system. Secondly, we also generated an "all-in-one" *Tet-on* expression vector that will be used to overexpress YY1 in the implanted MPECs in an inducible manner.

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