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1 Vitamin D Receptor Deficit Induces Activation of Renin Angiotensin System

2 Via SIRT1 Modulation in Podocytes

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27 Running Head: VDR negatively regulates RAS

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29

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Abstract

Vitamin D receptor (VDR) deficient status has been shown to be associated with the activation of renin angiotensin system (RAS). We hypothesized that lack of VDR would enhance p53 expression in podocytes through down regulation of SIRT1; the former would enhance the transcription of angiotensinogen (Agt) and angiotensinogen II type 1 receptor (AT1R) leading to the activation of RAS. Renal tissues of VDR mutant (M) mice displayed increased expression of p53, Agt, renin, and AT1R. *In vitro* studies, VDR knockout podocytes not only displayed up regulation p53 but also displayed enhanced expression of Agt, renin and AT1R. VDR deficient podocytes also displayed an increase in mRNA expression for p53, Agt, renin, and AT1R. Interestingly, renal tissues of VDR-M as well as VDR heterozygous (h) mice displayed attenuated expression of deacetylase SIRT1. Renal tissues of VDR-M mice showed acetylation of p53 at lysine (K) 382 residues inferring that enhanced p53 expression in renal tissues could be the result of ongoing acetylation, a consequence of SIRT1 deficient state. Notably, podocytes lacking SIRT1 not only showed acetylation of p53 at lysine (K) 382 residues but also displayed enhanced p53 expression. Since renal tissues of VDR-M mice also showed enhanced expression of PPAR-y, it is plausible that either the deficit of SIRT1 has de-repressed expression of PPAR-y or enhanced podocyte expression of PPAR-y (in the absence of VDR) has contributed to the down regulation of SIRT1. Based on our experimental data, we conclude that VDR deficit activates the RAS via SIRT1 modulation.

53

54 Vitamin D has been demonstrated to be a negative regulator of renin (20). Interestingly,
55 VDR knockout mice have also been demonstrated to activate Renin Angiotensin
56 System (RAS) without displaying a vitamin D deficient state (20). These mice developed
57 hypertension and cardiac hypertrophy as a consequence to the activation of the RAS
58 (31). However, levels of angiotensinogen (Agt) and AT1R mRNA were not altered in
59 cardiac myocytes. Contrary to these findings, renal tissues of VDR knockout diabetic
60 mice displayed enhanced renin and Agt expression (34). We hypothesized that lack of
61 VDR would modulate the transcription of the Agt and AT1R in kidney cells of VDR
62 knockout mice. Further, these effects of VDR deficit status could be mediated through
63 attenuated Human Silent Information Regulator Type (SIRT) 1 resulting into enhanced
64 p53 expression by kidney cells.

65 SIRT1 is a NAD^+-dependent deacetylase that regulates cell phenotype including
66 cell death/survival, senescence, and metabolism (3). It is involved in transcriptional
67 silencing of genes by chromatin modification via histone deacetylation, DNA damage
68 response, and life span extension secondary to calorie restriction (5, 15, 30). SIRT1 is
69 also a repressor of nuclear receptors such as PPARγ by docking with co-repressors -
70 nuclear receptor corepressor (NCor1) and silencing mediator for retinoid or thyroid-
71 hormone receptors (SMRT, NCor2) (21). Interestingly, SIRT1 negatively regulates p53
72 expression by deacetylating p53 in response to DNA damage (2, 27, 29). Conversely,
73 elevation of cellular p53 expression enhances expression of SIRT1 as a negative
74 feedback.

75 Recently, liganded VDR has been shown to modulate expression of FOXO3a
76 target genes through deacetylation of FOXO3A via SIRT1 in SCC25 cells (4). In these
77 studies, lack of VDR as well as SIRT1 was associated with phosphorylation of FOXO3a.
78 In the present study, we have evaluated the effect of VDR down regulation on podocyte
79 SIRT1 expression and associated up regulation of p53 expression.

80 We and other investigators previously reported the role of p53 in the transcription
81 of angiotensinogen and AT1 receptors in cardiac myocytes in high glucose milieu (18).
82 These effects of p53 were associated with the activation of renin angiotensin system
83 and cardiac myocyte hypertrophy (18). However, in these studies the role of VDR and
84 SIRT1 was not explored. We have recently reported that high glucose down regulated
85 podocyte VDR expression both *in vitro* and *in vivo* studies (24). In these studies, high
86 glucose-induced down regulation of VDR was associated with the activation of the RAS.
87 However, we did not explore the role of SIRT1 and p53 in the induction of activation of
88 the RAS in podocytes.

89 In the present study, we evaluated the effect of lack of VDR from the genome of
90 kidney cells on SIRT1 expression both *in vivo* and *in vitro* studies. We delineated the
91 involved molecular mechanisms of the activation of renin angiotensin system in
92 podocytes lacking VDR.

93

94

95 **Material and Methods**

96 **VDR Mutant mice**

97 VDR $^{tm1MBD+/-}$ were purchased from Jackson Laboratories (Bar Harbor, Maine), and bred
98 to develop homozygous VDR mutant (VDR-M, VDR $^{tm1MBD+/+)\ mice}$) on FVB/N
99 background.

100 **Human podocytes**

101 Human podocytes (HPs) were obtained from Dr. Moin A. Saleem (Children's
102 renal unit and academic renal unit, University of Bristol, Southmead Hospital, Bristol,
103 UK). Human podocytes were conditionally immortalized by introducing temperature-
104 sensitive SV40- T antigen by transfection. The cells have additionally been transfected
105 with a human telomerase construct. These cells proliferate at permissive temperature
106 (33˚C, conditionally immortalized human podocytes) and enter growth arrest
107 (conditionally immortalized differentiated human podocytes) after transfer to the non-
108 permissive temperature (37˚C). The growth medium contains RPMI 1640 supplemented
109 with 10% fetal bovine serum (FBS), 1x Pen-Strep, 1 mM L-glutamine and 1x ITS
110 (Invitrogen) to promote expression of T antigen. Since incubation media being deficient
111 (contained only 10% serum) in vitamin D, majority of podocyte VDR was unliganded.

112 **Silencing for VDR and SIRT1**

113 HPs were transfected with 25 nM VDR siRNA, control-siRNA (Santa Cruz
114 Biotechnology, Santa Cruz, CA), or SIRT1 siRNA (Cell Signaling) with Siport Neofax

115 transfection reagent and left in optiMEM media for 48 hrs. Control and transfected cells
116 were used under control and experimental conditions.

117 **Western blotting studies**

118 Protein blots of control and experimental cells/renal tissues were processed as
119 described previously (9). Nitrocellulose membranes were then processed further for
120 immunostaining with primary antibodies against SIRT1 (anti-rabbit, Santa Cruz
121 Biotechnology, Santa Cruz, CA), p53 (antimouse, Abcam, Cambridge, San Francisco,
122 CA), Ac^{k382}-p53 (anti-rabbit, Abcam), angiotensinogen (anti-rabbit, Abcam), AT1R (anti-
123 rabbit, Santa Cruz), renin (Santa Cruz), or PPAR-y (anti-rabbit, Cell Signaling, Danvers,
124 MA) and subsequently with horseradish peroxidase (HRP) labeled appropriate
125 secondary antibodies. The blots were developed using a chemiluminescence detection
126 kit (PIERCE, Rockford, IL) and exposed to X-ray film (Eastman Kodak Co., Rochester,
127 NY). Equal protein loading and the protein transfer were confirmed by immunoblotting
128 for determination of actin protein using a polyclonal α-Actin antibody (I-19, Santa Cruz,
129 CA) on the same (stripped) Western blots.

130 **Reverse Transcription PCR Analysis**

131 Control and experimental podocytes were used to quantify mRNA expression of VDR,
132 p53, renin, Agt, AT1R, and SIRT1 as described previously (15).Quantitative PCR was
133 carried out in an ABI Prism 7900HT sequence detection system using the primer
134 sequences as shown below:

135 **VDR (Human)**

136 5 : GACTTTGACCGGAACGTGCCC -3

137 5: CATCATGCCGATGTCCACACA -3

138 **Agt (Human)**

139 F: 5-CTGCAAGGATCTTATGACCTGC-3

140 R: 5-TACACAGCAAACAGGAATGGGC-3

141 **Renin (Human)**

142 F: 5-AAATGAAGGGGGTGTCTGTGG-3

143 R: 5-AAGCCAATGCGGTTGTTAACGC-3

144 **SIRT1 (Human)**

145 F:5'-CAGGTTGCGGGAATCCAAAG-3'

146 R:5'-GCTGGGCACCTAGGACATCG-3'

147 SYBR green was used as the detector and ROX as a stabilizing dye. Results (means ±
148 S.D.) represent number of samples as described in the legend. The data was analyzed
149 using the Comparative C_T method ($\Delta\Delta^{CT}$ method). Differences in C_T are used to quantify
150 relative amount of PCR target contained within each well. The data was expressed as
151 relative mRNA expression with reference to control, normalized to quantity RNA input
152 by performing measurements on an endogenous reference gene, GAPDH.

153 **Statistical analysis**

154 For comparison of mean values between two groups, the unpaired t test was
155 used. To compare values between multiple groups, analysis of variance (ANOVA) was
156 applied and a Bonferroni multiple range test was used to calculate a P-value. Statistical
157 significance was defined as P<0.05. All values are displayed as mean ± SD.

158

159 **Results**

160 **Renal tissues lacking VDR display enhanced expression of p53 and activation of**

161 **renin angiotensin system**

162 Since p53 is known to enhance cardiac myocyte expression of angiotensinogen and
163 AT1R expression (18) we asked whether renal tissues of VDR-M would display
164 enhanced expression of p53 and associated downstream signaling. Protein blots of
165 renal tissues of control and VDR mutant mice were probed for p53, angiotensinogen
166 (Agt), renin, and AT1R and reprobed for actin (n=4). Representative gels in duplicate
167 are displayed in Fig 1A. Cumulative densitometric data (n=4) are shown as bar graphs.
168 VDR lacking renal tissues displayed 8 fold increases in p53 expression when compared
169 to control mice (Fig. 1B). Renal tissues from VDR mutant mice displayed 3 fold increase
170 in Agt expression (Fig. 1C), 2 fold increase in renin expression (Fig. 1D), and 5-fold
171 increase in AT1R expression (Fig. 1E). These findings indicate that lack of VDR in these
172 mice is associated with an upregulation of renal tissue p53 expression leading to the
173 activation of renin angiotensin system.

174 **VDR deficient podocytes display enhanced expression of p53 and activation of**

175 **renin angiotensin system**

176 To confirm the effect of the lack of VDR on podocyte p53 expression and associated
177 activation of the RAS, podocytes were partially silenced for VDR. Podocytes were
178 either silenced for VDR by transfecting them with siRNA-VDR or control (scrambled)-
179 siRNA. Protein blots of SiCon/HPs or SiVDR/HPs (n=4) were probed for p53 and then
180 reprobed for Agt, renin, and actin. Representative gels (in duplicates) are shown in Fig.

181 2A. Cumulative densitometric data are shown as bar graphs. siRNA/HPs displayed
182 down regulation of VDR expression by 50% when compared to siCon/HPs (Fig. 2B).
183 VDR deficient podocytes showed 1.8 fold increase in their p53 expression (Fig, 2C) and
184 similar increase in Agt (Fig. 2D) and renin (Fig 2E) expressions.

185 To determine the effect of podocyte VDR silencing on the transcription of VDR, p53,
186 and molecules involved in the RAS, podocytes were transfected with either scrambled
187 SiRNA (SiCon) or SiRNA-VDR (SiVDR). cDNAs from siCon and SiVDR were amplified
188 with specific primers for VDR, p53, Agt and renin. Transfection of HPs with SiVDR
189 decreased VDR expression in podocytes by 50% (Fig. 3A). This decrease in podocyte
190 VDR transcription was associated with increased p53 transcription only by 25% (data
191 not shown). Nonetheless there was a significant increase in p53 protein expression (Fig.
192 2C) in podocyte lacking VDR. Increased functionality of p53 in podocytes silenced for
193 VDR was further confirmed by display of enhanced transrcription of both Agt (Fig. 3B)
194 and renin (Fig. 3C) by approximately two fold.

195 **Lack of VDR is associated with down regulation of SIRT1 in VDR-M mice**

196 SIRT1 is a deacetylase and modulates p53 expression through deacetylation (3, 5). On
197 that account, lack of SIRT1 has been reported to enhance p53 expression (29). We
198 asked whether lack of VDR is associated with down regulation of SIRT1. Protein blots of
199 renal tissues (n=4) of control and VDR-M mice were probed for SIRT1 and the same
200 blots were reprobed for GAPDH. Representative gels are displayed in Fig. 4A. Renal
201 tissues of VDR-M mice displayed attenuated expression of SIRT1. Since renal tissues
202 of VDR-M mice barely displayed any expression of SIRT1, we also evaluated renal

203 tissue SIRT1 expression in VDR-heterozygous mice (h). Protein blots of renal tissues of
204 control (n=4) and VDR-H (n=5) were probed for SIRT1 and reprobed for GAPDH. Gels
205 are displayed in Fig. 4B. Densitoemetric data are shown as bar graphs in Fig. 4C. Renal
206 tissues of VDR-h mice displayed attenuated expression of SIRT1.

207 **p53 acetylation at lysine (K) 382 residues is prevailed in renal tissues lacking**
208 **VDR**

209 Since acetylation of p53 at k382 residues has been shown to be associated with
210 enhanced p53 expression, we expected lack of VDR in renal tissues would also be
211 associated with acetylation p53 at K382 residues. Renal tissues of control (n=3) and
212 VDR-M (n=3) were probed for SIRT1. The same blots were stripped and reprobed for
213 p53, Ac^{k382}-p53, Agt, AT1R, PPAR-y, and GAPDH. Gels are displayed in Fig. 5. Renal
214 tissues of VDR-M mice displayed attenuated expression of SIRT1 but enhanced
215 expression of p53 and acetylation at the 382 residue site. These findings indicate that
216 down regulation of SIRT1 in mice lacking VDR may be enhancing p53 expression
217 through acetylation at K382 residues. Renal tissues of VDR-M mice also displayed
218 enhanced expression of Agt and AT1R. Interestingly, renal tissues of VDR-M mice
219 displayed enhanced expression of PPAR-y. Since SIRT1 is a component of PARP-y
220 repressor complex, lack of SIRT1 is likely to disrupt repressor complex (21).

221 **Podocytes partially lacking SIRT1 from their genome display enhanced**
222 **acetylation of p53 at K382 residues in podocytes**

223 Since SIRT1 is a deacetylase, we asked whether partial deficit of SIRT1 from the
224 genome of podocytes would enhance acetylation of p53 at K382 residues. Human

225 podocytes were transfected either control (C)- siRNA or SIRT1-siRNA. Control cells, C-
226 siRNA- and SIRT1-siRNA transfected cells were incubated in media for 24 hours and
227 extracted for protein and RNA.

228 cDNAs were prepared and amplified with a probe specific for SIRT1. Results are
229 displayed in Fig. 6A. Podocytes transfected with SIRT1-siRNA displayed 50% down
230 regulation of SIRT1 mRNA expression.

231 Protein blots were probed for SIRT1, p53, ac-k382p53, and GAPDH. Gels are
232 displayed in Fig. 6B. Podocytes- transfected with SIRT1-siRNA displayed modest down
233 regulation (30% only) of SIRT1 protein expression. Nonetheless, podocytes transfected
234 with SIRT1-siRNA displayed both robust acetylation of p53 at K382 residues and an
235 increased p53 protein expression. Thus, it appears that despite 50% down regulation of
236 SIRT1 gene expression in the genome of podocytes, protein expression of SIRT1 in
237 SIRT1-siRNA transfected podocytes was not reduced to the same magnitude. We
238 would like to clarify this discrepancy in mRNA and protein expression of SIRT1 in these
239 studies. Downstream signaling in the form of robust increase in p53 acetylation at K382
240 residues as well as abundant p53 protein expression confirmed the deficit in
241 functionality of SIRT1 in these podocytes. We speculate that sustained increase in
242 podocyte expression of p53 stimulated SIRT1 expression in podocytes partially lacking
243 SIRT1 from their genome as a negative feedback. Moreover, these findings are
244 consistent with the observations of other investigators (5, 15).

245 **High Glucose milieu down regulates SIRT1, enhances p53 acetylation and RAS**
246 **activation**

247 We and other investigators have previously reported down regulation of podocyte
248 VDR expression in adverse milieus both *in vitro* and *in vivo* studies (8, 9, 25, 26, 34).
249 We asked whether high glucose milieu would also down regulate SIRT1 and enhance
250 podocyte p53 expression and associated downstream signals. Human podocytes were
251 incubated in media containing either normal glucose (5 mM, control) or high glucose (35
252 mM) for 24 and 48 hours. Protein blots were probed for SIRT1. The same blots were
253 reprobed for p53, Agt, and GAPDH. Gels are displayed in Fig. 7A. High glucose
254 attenuated podocyte SIRT1 expression but enhanced expression of both p53 and Agt at
255 24 hours. However, after 48 hours SIRT1 expression is also up regulated when
256 compared to 24 hours treatment. However, these findings are consistent with the other
257 investigators indicating that in due course of time elevated levels of p53 would also
258 enhance SIRT1 expression as a negative feedback (as mentioned above, Fig. 6B; 5, 6).
259 Therefore, acetylation of p53 and its expression is the determinant of functionality of the
260 SIRT1.

261 To determine the time course effect of high glucose on podocyte SIRT1 and p53
262 expression, human podocytes (HPs) were incubated in media containing either normal
263 glucose (5 mM) or high glucose (35 mM) for variable time periods (4, 12, 24, and 48
264 hours). Protein blots were probed for SIRT1. The same blots were reprobed for p53 and
265 GAPDH. Gels are displayed in Fig. 7B. High glucose down regulated SIRT1 expression
266 up to 24 hours. However, it is normalized to some extent as a negative feedback at 48
267 hours. Conversely, p53 expression was upregulated up to 24 hours and then declined at
268 48 hours. These findings further support the involved dynamics in SIRT1 and p53
269 expression, which are consistent both under physiological and pathological states.

270

271 **Enhanced podocyte VDR expression is associated with increased SIRT1**
272 **expression**

273 Since down regulation of VDR modulated podocyte SIRT1 expression, we expected
274 enhanced podocyte VDR expression would be associated with enhanced SIRT1
275 expression. To determine the effect of enhanced podocyte VDR expression on SIRT1
276 expression, podocytes were transfected with control plasmid (pcDNA), SIRT1, or VDR.
277 In parallel sets of experiments podocytes were incubated in media containing VDR
278 agonist (VDA, EB1089, 1 nM) for 24 hours. Protein blots were probed for SIRT1 and
279 reprobed for GAPDH. Gels are displayed in Fig. 8A. Podocytes transfected with VDR
280 or treated with VDA displayed enhanced expression of SIRT1.

281 To determine the time course effect of VDA on podocyte SIRT1 expression, podocytes
282 were incubated in media containing VDA (EB1089, 1 nM) for variable time periods (0,
283 2,4, 8, 12, 24 hours). Protein blots were probed for SIRT1 and reprobed for GAPDH.
284 VDA enhanced SIRT1 expression as early as 2 hours (Fig. 8B).

285 Proposed scheme depicting the role of VDR in the activation of the RAS is shown in Fig.
286 9. Lack of VDR in podocytes induces down regulation of SIRT1, which enhances
287 podocyte p53 expression through allowing acetylation of p53. Enhanced p53 expression
288 induces transcription of Agt and AT1R leading to the activation of the RAS.

289 **Discussion**

290 VDR knockout mice have been demonstrated to activate RAS as well as its
291 downstream effects in the form of cardiac hypertrophy (31). It was suggested that the
292 activation of the RAS was a consequence of disruption of vitamin D and VDR nexus
293 (20). In the present study, we determined that lack of VDR was associated with
294 enhanced expression of angiotensinogen and AT1 by renal tissues as well as by
295 podoctyes. This effect of VDR deficit seems to be mediated by enhanced expression of
296 p53 both in renal tissues and podocytes. Occurrence of acetylation of p53 at K382
297 residues contributed to enhanced expression of p53 in renal tissues lacking SIRT1.

298 Recently, high Ang II states including high glucose and HIV milieus have been
299 reported to cause down regulation of VDR in podocytes (8, 9, 25, 26, 34).High Ang II
300 states down regulate podocyte VDR by multiple mechanisms including enhanced VDR
301 degradation via proteasomal pathway and through transcription of CYP24A via de-
302 repression of co-repressor complexes at CYP24A1 promoter (26). Since CYP24A1
303 metabolizes vitamin D, enhanced podocyte CYP24A1 state would be associated with
304 low vitamin D which would lead to the accelerated degradation of VDR. Additionally,
305 Ang II as well as conditions associated with high Ang II states such as high glucose and
306 HIV milieus are associated with enhanced podocyte expression of SNAIL (19, 32); the
307 latter is a repressor of transcription of VDR (17, 32). Knocking of VDR in kidney cells
308 has been demonstrated to enhance the activation of renin angiotensin system (RAS)
309 (8,9,25, 26, 34). Thus, it appears that Ang II perpetuates its production through down
310 regulation of VDR in kidney cells.

311

312 We have previously reported that high glucose enhanced kidney cell p53 and
313 angiotensinogen expression in podocytes (28). We and other investigators also
314 demonstrated similar phenomenon in cardiac myocytes (18). In the present study, we
315 asked whether high glucose induced activation of the RAS is mediated through down
316 regulation of SIRT1 and upregulation of p53. SIRT1 is a negative regulator of p53
317 because of its deacetylase activity (3); conversely, p53 enhances SIRT1 expression as
318 a negative feedback (5, 15). Because of this complex inter-relationship between these
319 molecules, downstream functionality in the form of p53 acetylation or p53 expression
320 seems to be a better marker of SIRT1 activity rather than to mere SIRT1 protein
321 expression. In the present study too, podocytes partially silenced for SIRT1 displayed
322 robust acetylation of p53 and abundant expression p53 and thus confirming a deficit in
323 functionality of SIRT1. Since p53 was in abundance it is likely to increase expression of
324 SIRT1 in these cells, as negative feedback. As expected, despite lack of SIRT1 in the
325 genome of these podocytes, these cells displayed moderate SIRT1 expression. This
326 aspect was better exemplified in time course effect of high glucose on podocyte SIRT1
327 expression. High glucose attenuated podocyte SIRT1 expression but enhanced p53
328 expression during early time period (up to 24 hours); however, at 48 hours, podocyte
329 SIRT1 expression was up regulated and p53 expression diminished partially. These
330 findings highlight ongoing dynamic relationship between these molecules during
331 sustained exposure of adverse milieus.

332 SIRT1 protein directly interacts with PPARγ to form a complex and may thus
333 control acetylation and deacetylation status of PPARγ (13, 23). PPARγ also binds to the
334 promoter of *SIRT1* gene and control the expression *SIRT1* gene (23). Thus, both

335 PPARγ and SIRT1 inversely regulate each other's expression. The involved mechanism
336 of SIRT1 down regulation in cells lacking VDR is very well understood to date. Both
337 VDR and PPARγ compete for binding with RXR for their functionality (1). Therefore,
338 PPAR-γ binding with RXR will go unchallenged in cells lacking VDR. Since PPARγ
339 negatively regulates SIRT1 expression, it would down regulate SIR1 expression in
340 conditioned cells lacking VDR. Additionally, SIRT1 is also a part of PPAR-y-RXR
341 repressor complex docking on DNA binding site of PPRE (21); therefore, lack of SIRT1
342 would disrupt the repressor complex and de-repress the target gene expression such as
343 PPAR-y. In the present study, renal tissues lacking VDR displayed enhanced
344 expression of PPARy and attenuated expression of SIRT1. We speculate that either
345 PPARy would be contributing to down regulation of SIRT1 expression in podocytes
346 lacking VDR or lack of SIRT1 de-repressing the expression of PPARy. It would be worth
347 while exploring this aspect of the investigation in our future studies.

348 p53 is normally maintained at a low concentration in quiescent cells by
349 continuous ubiquitination and proteasome-mediated degradation (6). However, p53
350 expression gets up regulated in cells under stress because of suppression of p53
351 ubiquitination and enhancement of p53 acetylation. Under stress, SIRT1, a deacetylator
352 of p53 is destabilized and degraded via proteasomal pathway (7). Reduction of
353 deacetylation of p53 leads to the acetylation of p53 (33). On the other hand, up
354 regulation of SIRT1 inactivates p53 (3, 5). In the present study, renal tissues of VDR
355 mutant mice as well as VDR-silenced podocytes displayed enhanced expression of
356 podocyte p53. Since lack of VDR in renal tissues was not only associated with down
357 regulation of SIRT1 but was also associated with acetylation of p53 at 382 residues of

358 renal tissues, it is likely upregulation of p53 expression was contributed by lack of
359 SIRT1 to some extent.

360 Deficiency of vitamin D has been incriminated a variety of chronic diseases
361 including tuberculosis (16), HIV (10), hypertension (12), colon cancer (11) and chronic
362 kidney diseases (22). However, outcome of clinical trials of vitamin D therapy in clinical
363 trials of disease has not shown very promising data (14). Since vitamin D worked
364 through VDR, functionality of vitamin D could be better be monitored by VDR status. It
365 has been shown in patients of colon cancer that vitamin D receptor could not be
366 optimally raised with vitamin D therapy in several instances and these patients did not
367 respond to vitamin D therapy (17). We have recently reported that HIV down regulated
368 podocyte VDR expression thorough CpG methylation at VDR promoter (8). In these
369 studies, Vitamin D could not up regulate podocyte VDR expression optimally; however,
370 use of a demethylating agent in combination with vitamin D could optimally increase
371 podocyte VDR expression in HIV milieu (8). We propose that discrepancy in vitamin D
372 clinical trials may be related to the inability of vitamin D in upregulating VDR optimally
373 because of the down regulation as a consequence of epigenetics. It will be worth
374 investing this aspect of investigation in future studies.

375 Vitamin D has been reported to be a negative endocrine regulator of renin
376 transcription (20). This effect of vitamin D has been attributed to liganded-VDR which
377 blocks the binding of CRP binding protein (CBP) at renin promoter and thus preventing
378 the transcription of renin. On that account, vitamin D is being used to down regulate
379 renin angiotensin system in patients of chronic kidney diseases (22). In the present
380 study, we observed that lack of unliganded- VDR enhances transcription of several

381 molecules involved in the activation of the RAS. During vitamin D deficient state, there
382 is a reduction of total VDR because of decreased transcription of VDR and enhanced
383 degradation of unliganded VDR (See Scheme in Fig. 10). Thus, vitamin D deficient
384 status is associated with a decrease in both unliganded (UL) and liganded (L) VDR. In
385 this scenario, lack of liganded VDR would sustain the transcription of renin, whereas,
386 lack of unliganded VDR would enhance transcription of angiotensinogen and AT1R via
387 modulation of p53 expression. Theoretically, unliganded VDR-induced AT1R activation
388 should down regulate renin gene expression; however, this negative feedback effect of
389 AT1R activation is likely be nullified or neutralized because of deficient liganded VDR
390 mediated-enhanced renin transcription. Thus, it appears that lack of VDR or VDR
391 deficient state has multiple ways to activate the RAS.

392 In conclusion, we report for the first time that lack of VDR in the genome kidney
393 cells enhances expression of p53 via down regulation of SIRT1 resulting into enhanced
394 transcription of Agt and AT1R that further leads to the activation of the RAS.

395

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Figure legend

Fig. 1 Renal tissues lacking VDR display enhanced expression of p53 and activation or renin angiotensin system

A. Protein blots of renal tissues of control (FVB/N) and VDR mutant (M) mice were probed for p53, angiotensinogen (Agt), renin, and AT1R and reprobed for actin (n=4). Representative gels in duplicate are displayed.

B. Cumulative densitometric data (n=4) of protein blots probed for p53 and actin in the form of bar graphs.

C. Mean Agt/Actin ratio of renal tissue protein blots from control and VDR-M mice (n=4).

D. Mean Renin/Actin ration of renal tissue protein blots from control VDR mutant mice (n=4)

E. Mean AT1R/Actin ratio of renal tissues from control and VDR-M mice (n=4).

*P<0.05 compared to respective FVB/N

Fig. 2. VDR deficient podocytes display enhanced expression of p53 and activation of renin angiotensin system

Podocytes were either silenced for VDR by transfecting them with siRNA-VDR (Si/VDR) or control (scrambled)-siRNA (SiCon). Protein blots of Si-Con or SiVDR (n=4) were probed for p53 and then reprobed for Agt, renin, and actin.

A. Representative gels (in duplicates) are shown.
B. Cumulative densitometric data (VDR/Actin ratios) are shown as bar graphs (n=4).

535 C. Mean p53/actin ratios from SiVDR and SiCon protein blots are shown as bar
536 graphs (n=4).
537 D. Mean Agt/Actin ratios from SiVDR and SiCon protein blots are shown as bar
538 graphs (n=4).
539 E. Mean Renin/Actin ratios SiVDR and SiCon protein blots are shown as bar graphs
540 (n=4).
541 *P<0.05 compared with respective SiCon.

542 **Fig. 3. Effect of VDR silencing on podocyte Agt and renin mRNA expression**

543 Podocytes were transfected with either scrambled siRNA (SiCon) or siRNA-VDR
544 (SiVDR) (n=4). cDNAs from SiCon and SiVDR were amplified with specific
545 primers for VDR, Agt, and renin.
546 A. VDR mRNA expression by SiCon and SiVDR is displayed as bar graphs.
547 B. Agt mRNA expression by SiCon and SiVDR is displayed as bar graphs.
548 C. Renin mRNA expression by SiCon and SiVDR. is displayed as bar graphs.
549 *P<0.05 vs. SiCon

550 **Fig. 4. Lack of VDR is associated with down regulation of SIRT1 in VDR-M mice**

551 A. Protein blots of renal tissues (n=4) of control and VDR-M mice were probed for
552 SIRT1 and the same blots were reprobed for GAPDH. Representative gels in duplicate
553 are shown.

554 B. Protein blots of renal tissues of control (n=4) and VDR-heterozygous (h) (n=5) were
555 probed for SIRT1 and reprobed for GAPDH. Gels are displayed.

556 C. Densitoemetric data (SIRT1/GAPDH) of protein blots of Fig. B are shown as bar
557 graph.

558 *P<0.05 compared with FVB/N

559 **Fig. 5. Lack of SIRT1 is associated with p53 acetylation at K382 residues in renal**
560 **tissues of VDR-M mice**

561 A. Renal tissues of control (n=3) and VDR-M (n=3) were probed for SIRT1. The same
562 blots were stripped and reprobed for p53, Ac^{k382}-p53, Agt, AT1R, PPAR-y, and GAPDH.
563 Gels are displayed.

564 B. Cumulative densitometric data (variable/GAPDH ratio) are shown.

565 *P<0.05 compared with respective FVB/N

566 **Fig. 6. Podocytes partially lacking SIRT1 from their genome display enhanced**
567 **acetylation of p53 at K382 residues in podocytes**

568 A. Human podocytes were transfected either control (C)- siRNA or SIRT1-siRNA.
569 Control cells, C-siRNA- and SIRT1-siRNA transfected cells were incubated in
570 media for 24 hours. Total RNA as well as proteins was harvested. cDNAs were
571 prepared and amplified with a probe specific for SIRT1. Results are displayed as
572 bar graph.

573 B. Protein blots of control (C)- siRNA or SIRT1-siRNA. Control cells, C-siRNA- and
574 SIRT1-siRNA transfected cells were probed for SIRT1, p53, ac-K382p53, and
575 GAPDH. Gels are displayed.

576 **Fig. 7. High glucose down regulates SIRT1 in a time dependent manner**

A. Human podocytes were incubated in media containing either normal glucose (5 mM, control) or high glucose (35 mM) for 24 and 48 hours. Protein blots were probed for SIRT1. The same blots were reprobed for p53, Agt, and GAPDH. Gels are displayed.

B. Human podocytes were incubated in media containing either normal glucose (5 mM) or high glucose (35 mM) for variable time periods (4, 12, 24, and 48 hours). Protein blots were probed for SIRT1. The same blots were reprobed for p53 and GAPDH. Gels are displayed.

Fig. 8. Enhanced podocyte VDR expression is associated with increased SIRT1 expression

A. Podocytes were transfected with either control plasmid (pcDNA), SIRT1, or VDR. In parallel sets of experiments podocytes were incubated in media containing VDR agonist (VDA, EB1089, 1 nM) for 24 hours. Protein blots were probed for SIRT and reprobed for GAPDH. Gels are displayed.

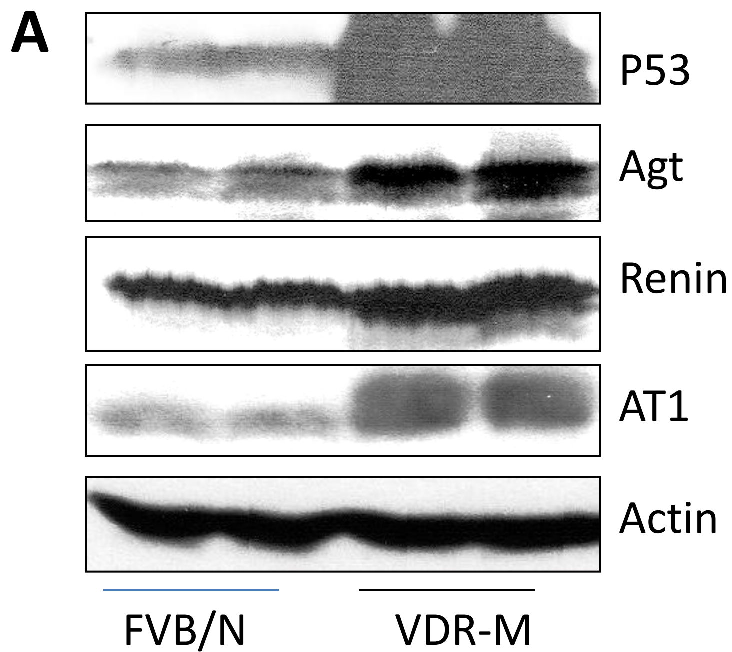
B. Podocytes were incubated in media containing VDA (EB1089, 1 nM) for variable time periods (0, 2, 4, 8, 12, 24 hours). Protein blots were probed for SIRT1 and reprobed for GAPDH. Gels are displayed.

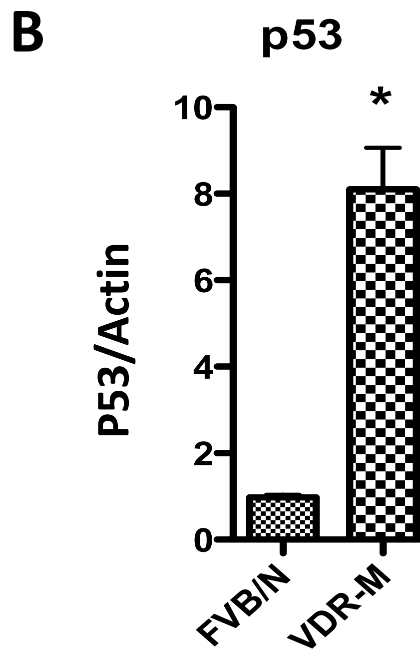
Fig. 9. Proposed scheme

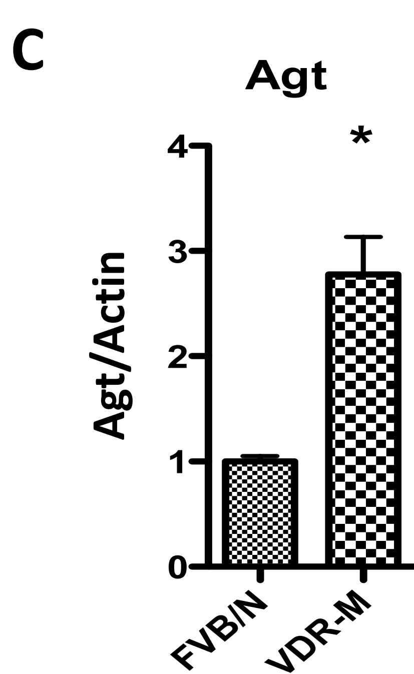
Lack of VDR in podocytes induces down regulation of SIRT1, which enhances podocyte p53 expression through allowing acetylation of p53. Enhanced p53 expression induces transcription of Agt and AT1R leading to the activation of the RAS.

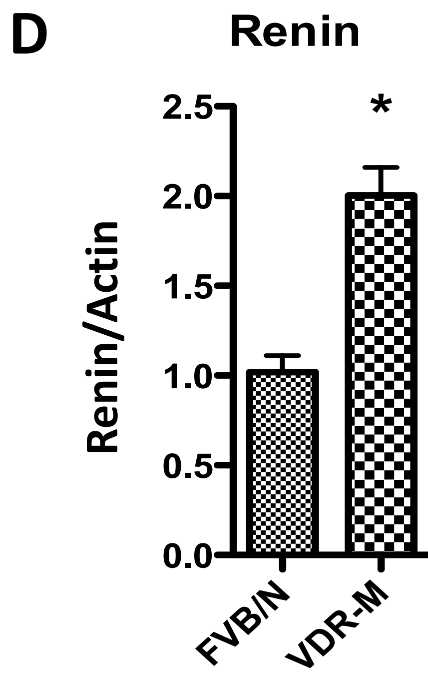
598 **Fig. 10. Unliganded VDR complements liganded VDR-mediated renin transcription**
599 **in vitamin D deficient state**

600 Vitamin D deficient state lacks both liganded and unliganded VDR. Lack of liganded
601 VDR would enhance the transcription of renin, whereas, lack of unliganded VDR would
602 stimulate the transcription of angiotensinogen and AT1R. Unliganded VDR-induced
603 AT1R activation should down regulate renin gene expression; however, this negative
604 feedback effect of AT1R activation would be neutralized because of deficient liganded
605 VDR-stimulated renin transcription.









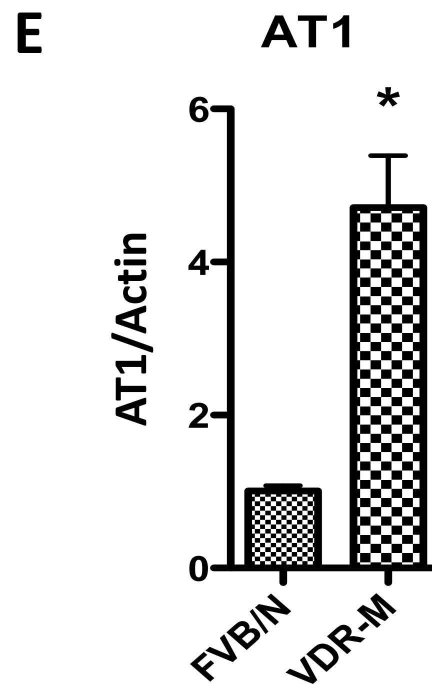


Fig. 1

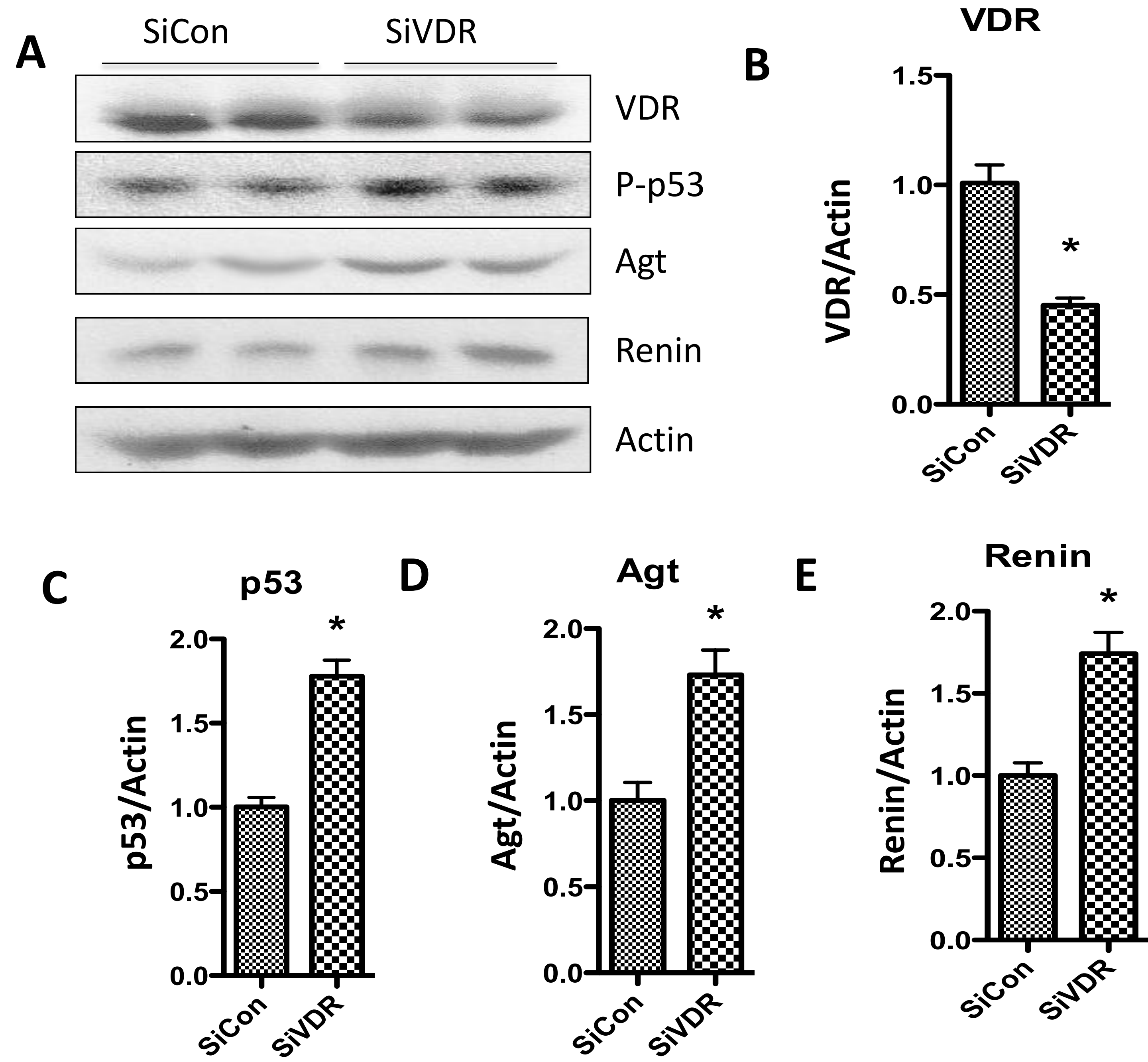
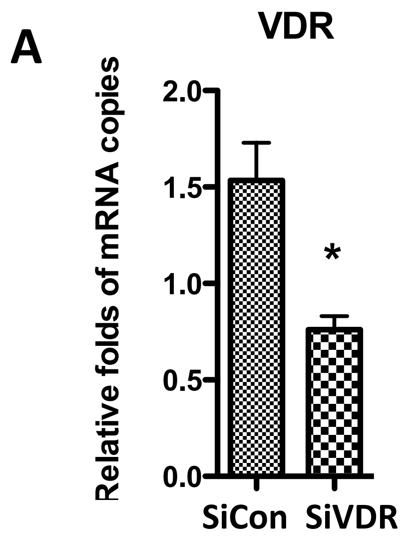
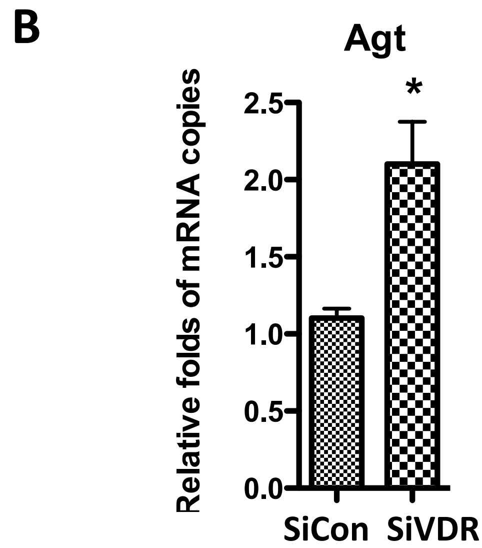


Fig. 2





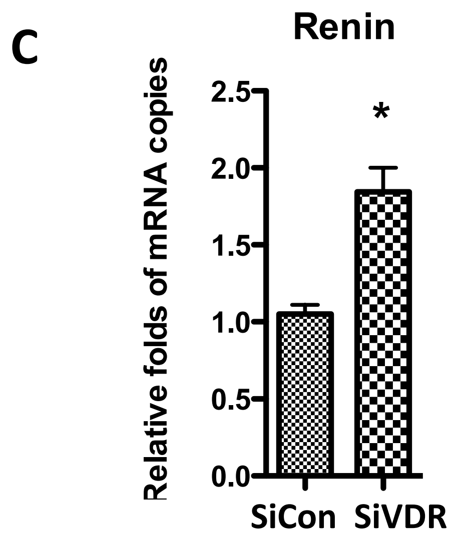
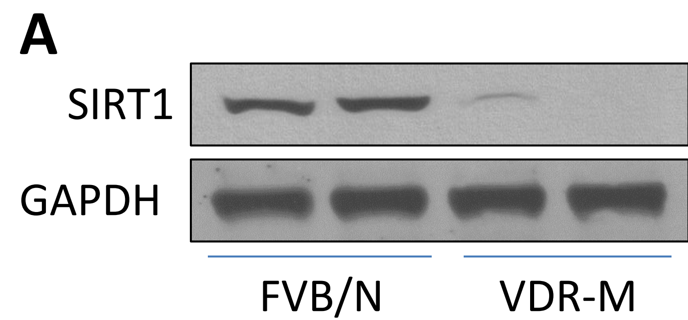
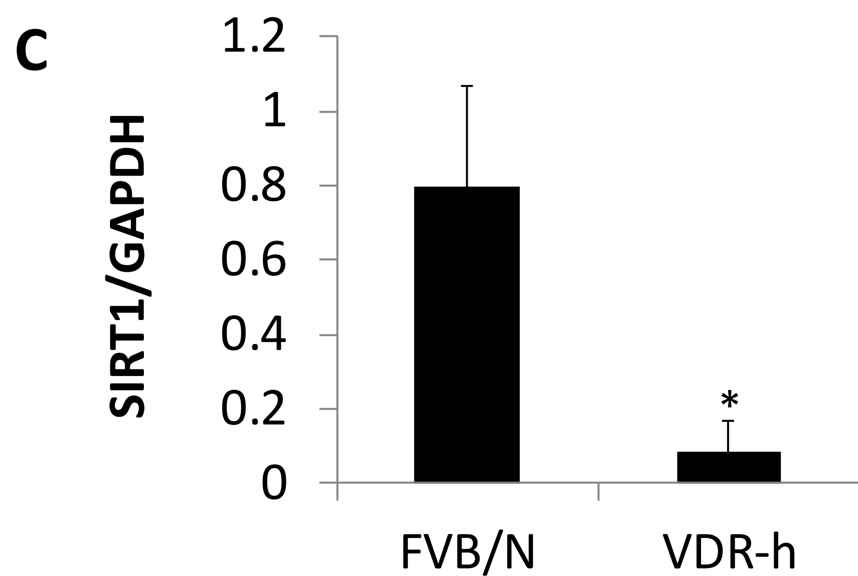


Fig. 3





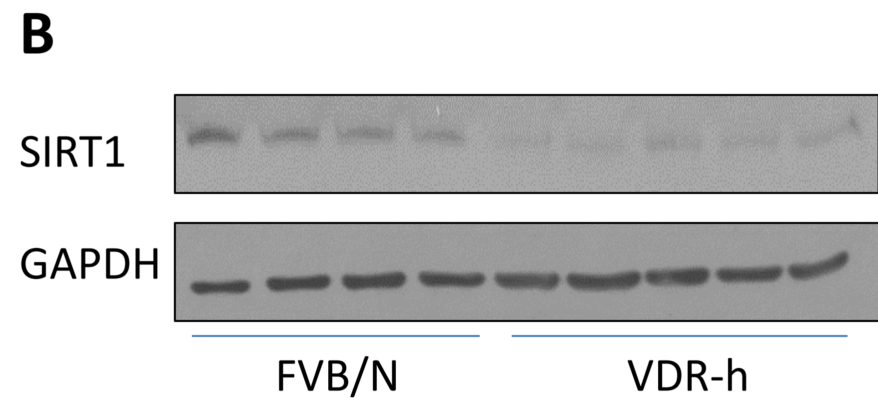
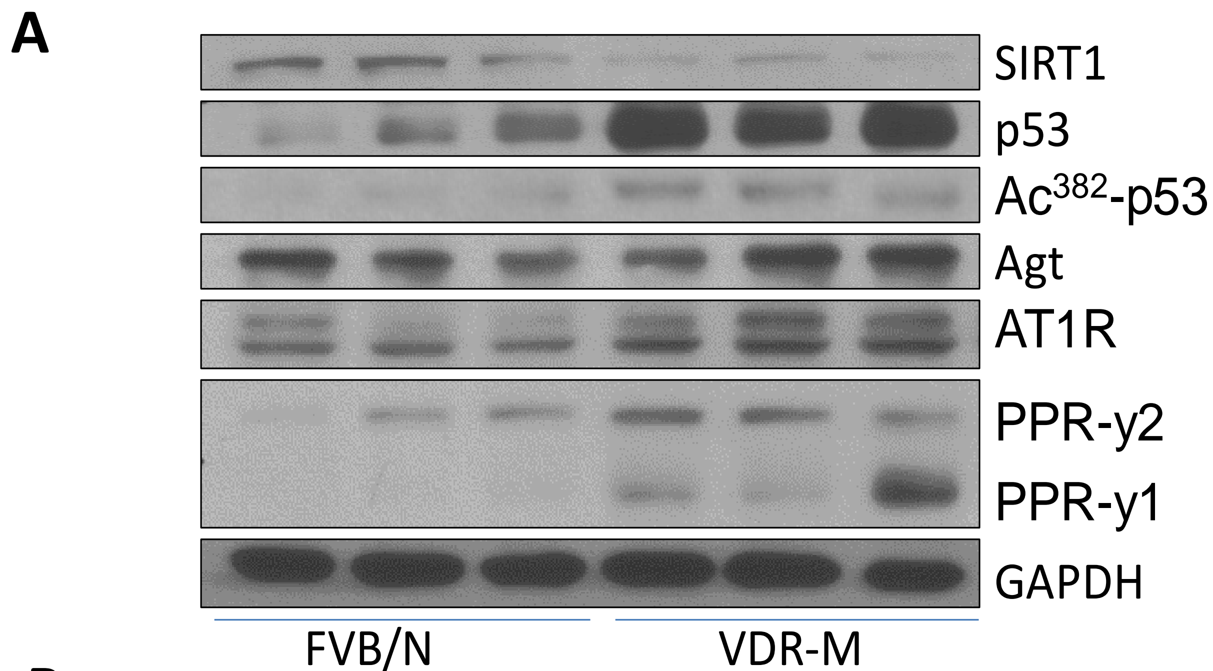


Fig. 4



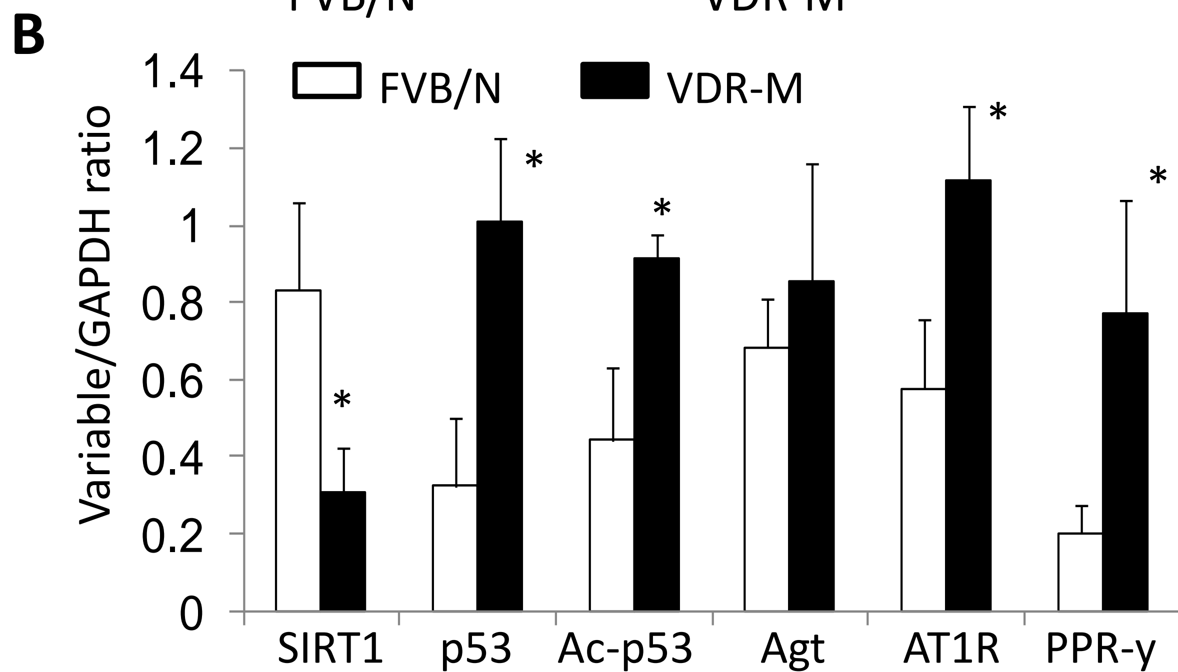
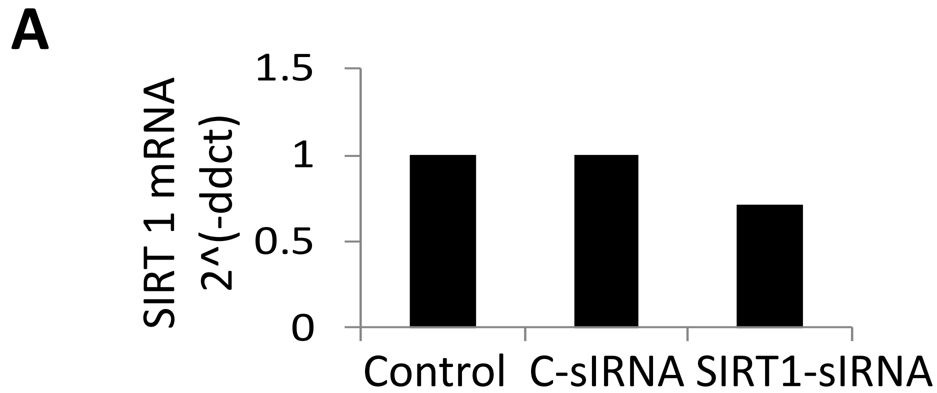


Fig. 5

A



B

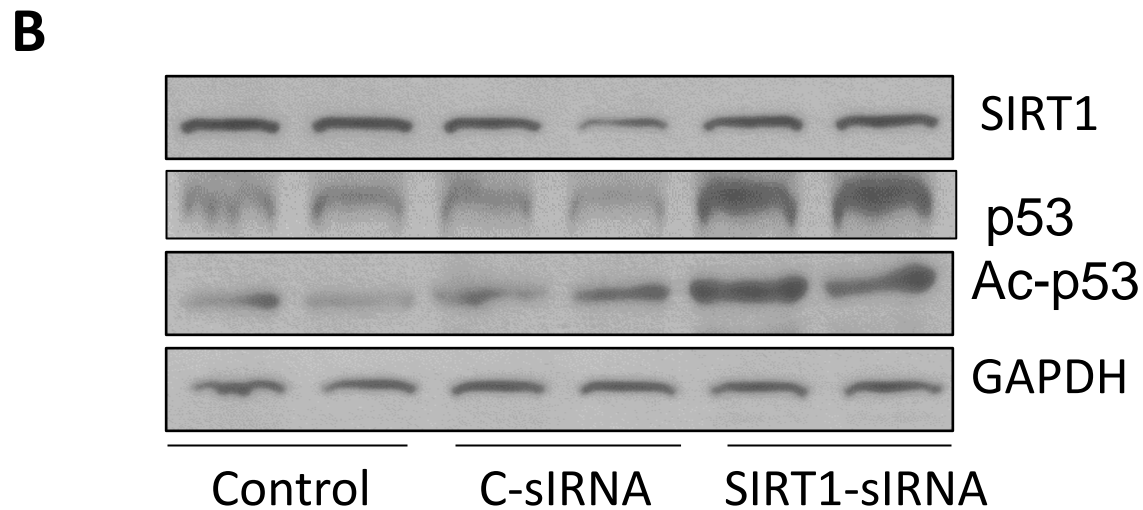
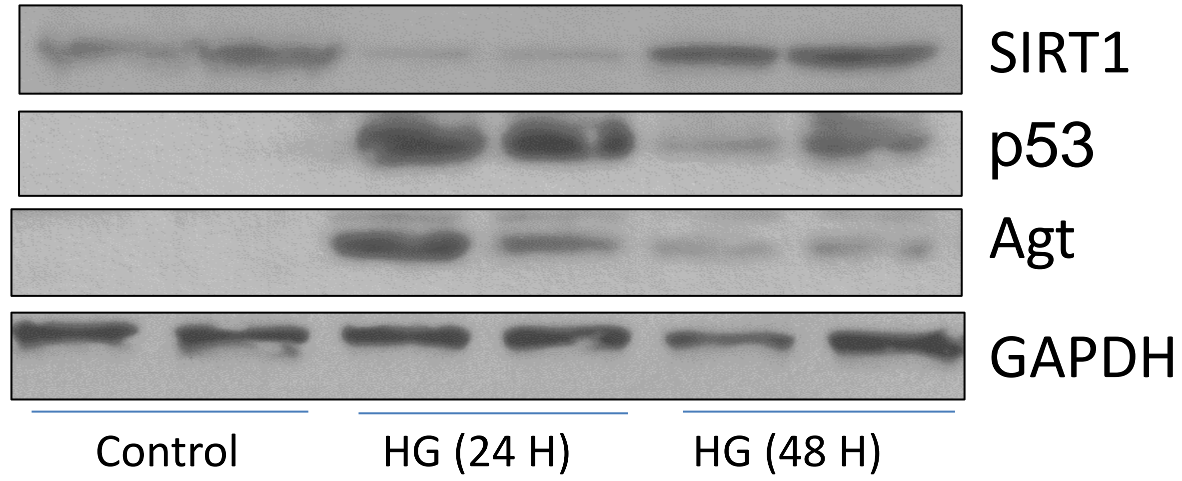


Fig. 6



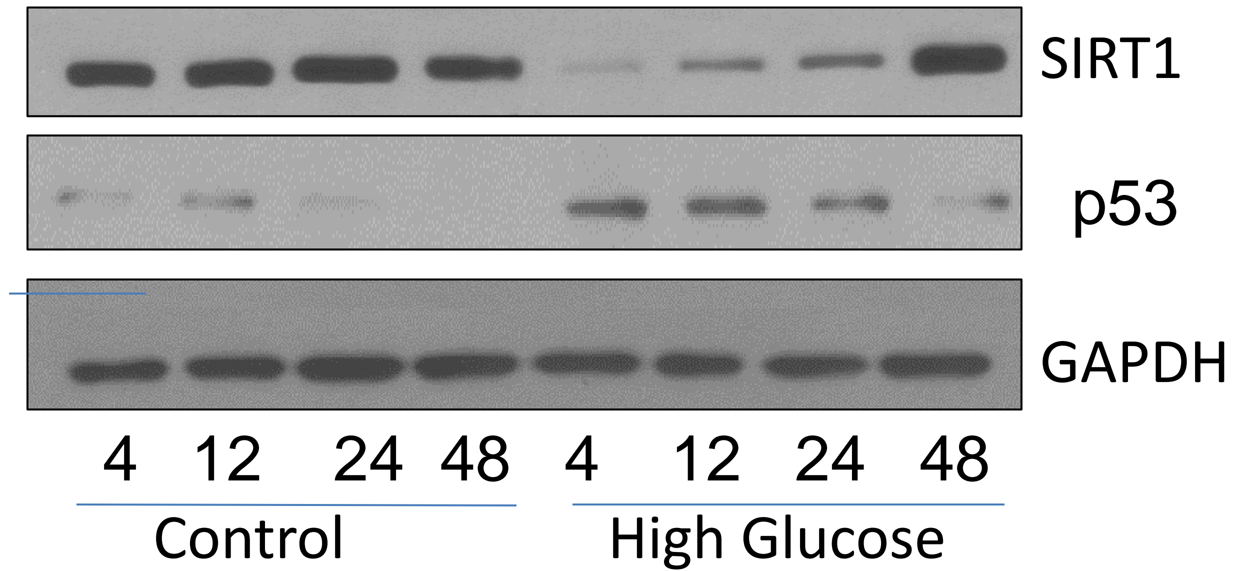
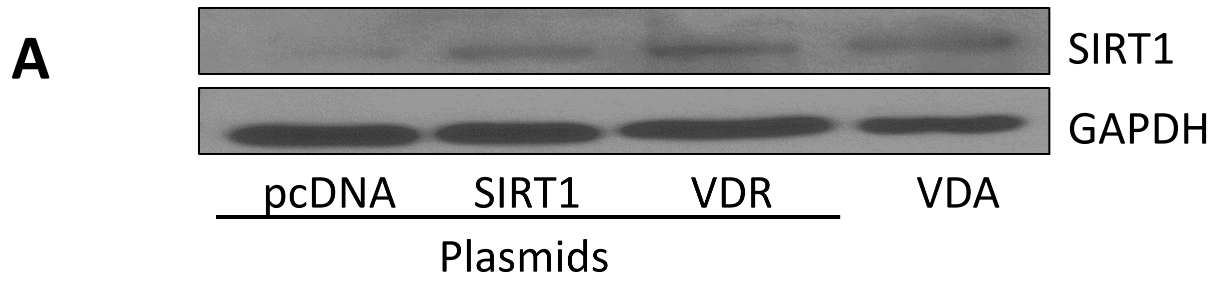


Fig.7



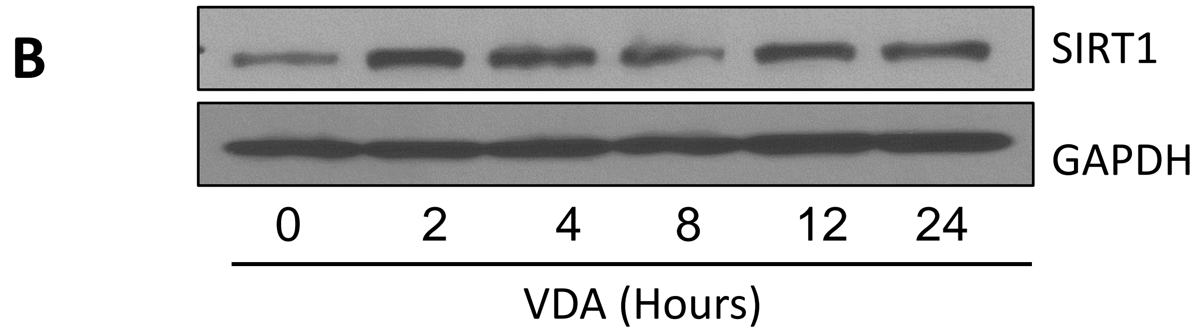


Fig. 8

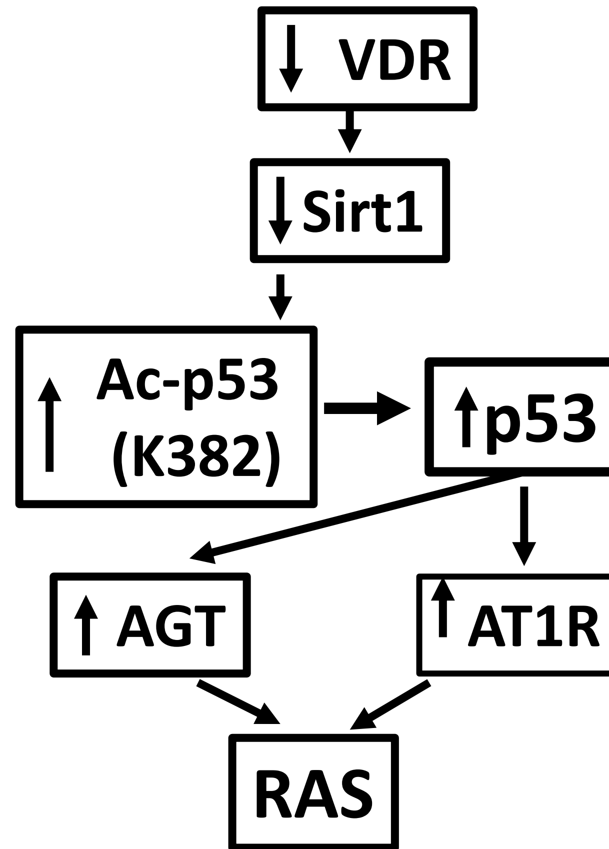


Fig. 9

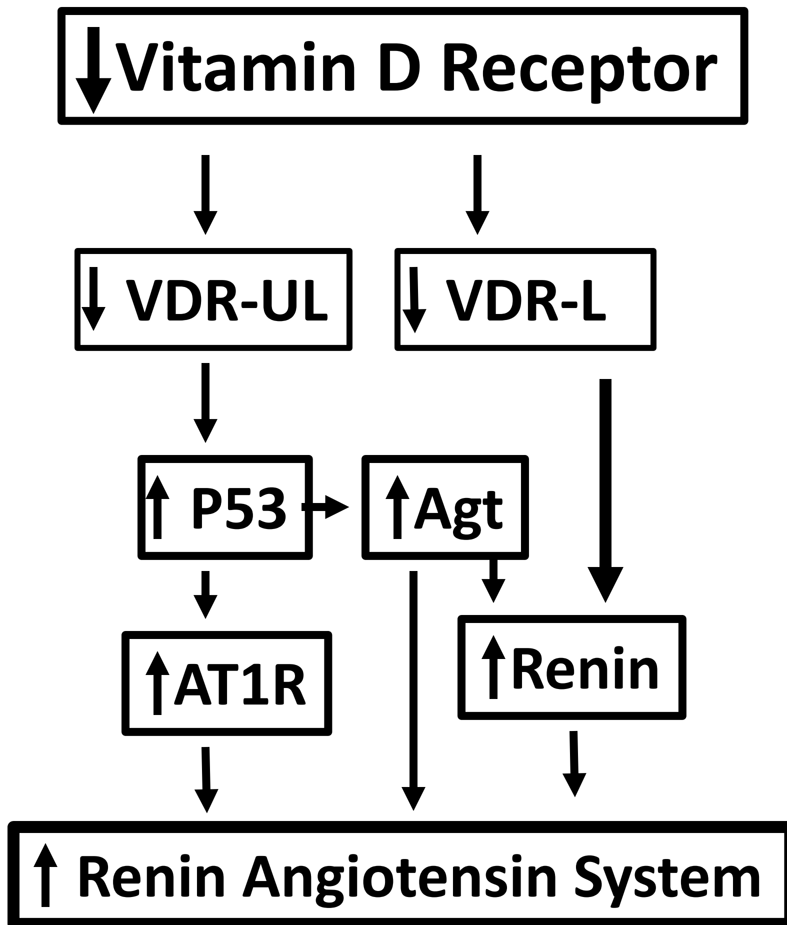


Fig. 10