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1 **Vitamin D limits inflammation linked microRNA expression in adipocytes in vitro and in**  
2 **vivo : A new mechanism of the regulation of inflammation by vitamin D**

3

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6

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10

11 Abbreviated title : Vitamin D blunts inflammatory miRs

12 Keyterms : vitamin D, adipose tissue, adipocyte, anti-inflammatory strategy, miR, inflammation.

13

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18

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20

21 **Abstract**

22

23 Inflammation of adipose tissue is believed to be a contributing factor to many chronic diseases  
24 associated with obesity. Vitamin D (VD) is now known to limit this metabolic inflammation by  
25 decreasing inflammatory marker expression and leukocyte infiltration in adipose tissue. In this  
26 study, we investigated the impact of VD on microRNA (miR) expression in inflammatory  
27 conditions in human and mouse adipocytes, using high throughput methodology (miRNA PCR  
28 arrays). Firstly, we identified three miRs (miR-146a, miR-150 and miR-155) positively regulated  
29 by TNF $\alpha$  in human adipocytes. Interestingly, the expression of these miRs was strongly prevented  
30 by 1,25(OH) $_2$ D preincubation. These results were partly confirmed in 3T3-L1 adipocytes (for miR-  
31 146a and miR-150). The ability of VD to control the expression of these miRs was confirmed in  
32 diet-induced obese mice: the levels of the three miRs were increased following High Fat (HF) diet  
33 in epididymal white adipose tissue and reduced in HF diet fed mice supplemented with VD. The  
34 involvement of NF- $\kappa$ B signaling in the induction of these miRs was confirmed in vitro and in vivo  
35 using aP2-p65 transgenic mice. Finally, the ability of VD to deactivate NF- $\kappa$ B signaling, via p65  
36 and I $\kappa$ B phosphorylation inhibition in murine adipocyte was observed, and could constitute a  
37 driving molecular mechanism.

38 This study demonstrated for the first time that VD modulates the expression of miRs in adipocytes  
39 in vitro and in adipose tissue in vivo through its impact on NF- $\kappa$ B signaling pathway, which could  
40 represent a new mechanism of regulation of inflammation by VD.

41

42 **Keywords:** Inflammation; vitamin D; adipocyte; miRs; aP2-p65 transgenic mice, NF- $\kappa$ B

43

## 44 **Introduction**

45

46 Adipose tissue was originally considered as a passive reservoir for energy storage, mechanical and  
47 heat insulation, and participating to the regulation of thermogenesis <sup>1</sup>. Now, this tissue is also  
48 known to synthesize and secrete a large variety of pro-inflammatory and anti-inflammatory  
49 adipokines, cytokines and chemokines <sup>2</sup>, that act at both autocrine/paracrine and endocrine level <sup>3</sup>.  
50 Other markers such as microRNAs (miRs) have been associated with inflammatory response in  
51 adipose tissue during obesity <sup>4-6</sup>. miRs are small non-coding RNAs that can modulate  
52 transcriptional networks, influencing most of biological processes. miRs regulate gene expression  
53 by degrading complementary mRNA targets or by inhibition of traduction, depending of the  
54 complementarity of sequence with the target mRNA <sup>7</sup>. Several miRs are involved in adipocyte  
55 differentiation <sup>8</sup>, fat cell behavior <sup>9</sup> and oxidative stress <sup>4</sup>. The relevance of miRs in adipose tissue  
56 biology is increasingly recognized, being intrinsically linked to different pathways, including  
57 obesity-related inflammation. This is notably the case for miR-155 which was reported to be  
58 increased in inflamed adipocytes <sup>10</sup>, but also in subcutaneous adipose tissue biopsies of obese  
59 subjects <sup>11</sup>, and which displays a strong impact on the regulation of inflammatory tone and  
60 adipocyte differentiation <sup>11</sup>

61 Similarly to the increased pro-inflammatory markers, a low vitamin D (VD) status is common and  
62 well documented in obesity <sup>12</sup>. Whether low VD status is the consequence of obesity, or whether  
63 this factor accelerates the development of obesity in humans is presently unknown. However, the  
64 VD insufficiency could influence the development of obesity-related diseases, including  
65 inflammation and insulin resistance <sup>12-16</sup>. Indeed, it was shown that 1,25(OH)<sub>2</sub>D significantly  
66 decreased the release of cytokines and chemokines by human preadipocytes <sup>17</sup> and by human  
67 adipocytes <sup>18</sup>. We also demonstrated the anti-inflammatory properties of 1,25(OH)<sub>2</sub>D in murine  
68 and human adipocytes, in both basal and TNF $\alpha$ -stimulated conditions <sup>19</sup>. Similarly, 1,25(OH)<sub>2</sub>D

69 reduced the expression of cytokines in human adipose tissue biopsies submitted to IL-1 $\beta$   
70 stimulation in vitro <sup>20</sup>. The molecular mechanisms have been investigated, and VDR and NF- $\kappa$ B  
71 signaling pathways and p38 MAP kinases were shown to be involved in 3T3-L1 adipocytes <sup>19</sup> and  
72 in human adipocytes <sup>21</sup>. Interestingly, Zoico et al. recently demonstrated that VD, similarly to  
73 1,25(OH)<sub>2</sub>D, was able to blunt the LPS-mediated pro-inflammatory effect in human adipocytes <sup>22</sup>.  
74 Using a microarray approach, we recently demonstrated that 1,25(OH)<sub>2</sub>D was able to downregulate  
75 a large set of chemokines in human and murine adipocytes, and consequently reduced macrophage  
76 migration mediated by adipocyte-conditioned medium <sup>23</sup>. Finally, we demonstrated for the first  
77 time this effect in vivo, in acute and chronic models of adipose inflammation <sup>23</sup>.

78

79 In the present study, we aimed to explore the impact of VD on adipose inflammation associated  
80 miRs. To this end, we evaluated the impact of 1,25(OH)<sub>2</sub>D on miR expression in inflammatory  
81 conditions in human and mouse adipocytes, using a highthroughput methodology (miRNA PCR  
82 arrays). We also confirmed in vivo the anti-inflammatory effects of VD in a chronic mice model of  
83 adipose tissue inflammation and identified molecular mechanisms involved in miR regulation by  
84 VD.

85

## 86 **Results**

87

### 88 **1,25(OH)<sub>2</sub>D modulates microRNA expression in human and murine adipocytes**

89 To examine, in detail, the impact of 1,25(OH)<sub>2</sub>D on the modulation of miRs in human adipocytes  
90 submitted to inflammatory stress, human adipocytes primocultures were preincubated with or  
91 without 1,25(OH)<sub>2</sub>D for 24 h followed by incubation with TNF $\alpha$ . Using a miRNA PCR array  
92 approach, we quantified 84 miRs and 6 housekeeping genes. Results analysis showed that 76 miRs  
93 were expressed in adipocytes in basal condition. Among these miRs, only 3 were positively  
94 regulated by TNF $\alpha$  in our conditions. As shown in Figure 1A, the inflammatory response mediated  
95 by TNF $\alpha$  significantly increased the expression levels of miR-146a, miR-150 and miR-155 (4.3,  
96 1.9 and 2.1-fold, respectively, Figure 1). Interestingly, their expression decreased in adipocytes  
97 preincubated with 1,25(OH)<sub>2</sub>D in inflammatory conditions by 2.4, 2.1 and 1.6-fold, respectively  
98 compared with TNF $\alpha$  condition (Figure 1).

99 Our results were partly confirmed in 3T3-L1 adipocytes, where the levels of miR-146a and miR-  
100 155 increased in TNF $\alpha$ -treated cells (by 6.7 and 8-fold, respectively) and decreased in the  
101 adipocytes pretreated with 1,25(OH)<sub>2</sub>D and incubated with TNF $\alpha$  (by 3.1 and 1.3-fold,  
102 respectively, Figure 2). No effect on miR-150 was observed in this cell model.

103

### 104 **Vitamin D<sub>3</sub> supplementation limits microRNA expression in epididymal white adipose tissue**

105 To further examine the effect of VD on miR expression in vivo, a model of chronic inflammation  
106 induced by a HF diet was used. In this preclinical model, the induction of the miR-146a, miR-150  
107 and miR-155 levels was observed after 10 weeks of HF diet (by 2.4, 1.4 and 1.5-fold, respectively)  
108 compared with control mice (Figure 3). Interestingly, VD supplementation for 10 weeks strongly  
109 limited the expression of these three miRs in epididymal adipose tissue (2.1, 1.3 and 1.3-fold,  
110 respectively, Figure 3).

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**1,25(OH)<sub>2</sub>D limits microRNA expression through NF-κB signaling in 3T3-L1 adipocytes.**

To identify signaling pathways involved in miR-146a and miR-155 regulation by TNFα, 3T3-L1 cells were incubated with specific inhibitors of MAP kinases (JNK, p38) and NF-κB signaling, and then stimulated or not with TNFα. NF-κB signaling inhibition resulted in a significant decrease in miR-146a and miR-155 expression (by 2.7- and 1.6-fold compared with TNFα condition) (Figure 4A) whereas no effect of JNK and p38 inhibitors were observed,. To confirm the role of NF-κB in miR-146a and miR-155 regulation in vivo, we used a transgenic model overexpressing NF-κB p65 subunit in adipose tissue (aP2-p65 mice<sup>24</sup>). Results showed that miR-146a and miR-155 were significantly increased in epididymal adipose tissue of aP2-p65 mice compared with controls (by 2.2- and 10-fold, respectively; Figure 4B). Altogether these data strongly support the specific role of NF-κB on the regulation of these two miRs.

Thus, we examined the impact of 1,25(OH)<sub>2</sub>D preincubation (10 nM and 100 nM) for 24 hours followed by a 24-hour incubation with TNFα on NF-κB signaling. To this end, the phosphorylation levels of p65 and IκB were quantified by ELISA. Expectedly, the phosphorylation levels of p65 and IκB were significantly increased into 3T3-L1 adipocytes incubated with TNFα (by 4.1- and 3.6-fold, respectively), whereas incubation with 1,25(OH)<sub>2</sub>D strongly limited the phosphorylation of p65 and IκB, suggesting that 1,25(OH)<sub>2</sub>D reduced NF-κB activation in 3T3-L1 adipocytes (Figure 4C).

131 **Discussion**

132

133 Our results indicate that obesity-related inflammation increased the production of miR-146a, miR-  
134 150 and miR-155 in adipocytes. We also reported a beneficial role of VD or its active form  
135 (1,25(OH)<sub>2</sub>D) on these miR expression in human and murine adipocytes, but also in inflamed  
136 adipose tissue of mice. Molecular mechanisms for these effects were investigated and the NF-κB  
137 signaling pathway is suggested to be involved in miR regulation and to be targeted by 1,25(OH)<sub>2</sub>D.  
138 If the anti-inflammatory effect of VD has largely been reported based on cytokines and chemokines  
139 expression in adipocytes as described in the introduction, its impact regarding its ability to  
140 modulate inflammatory-linked miRs has never been reported.

141

142 In a preliminary study, we identified miR-146a, miR-150 and miR-155 as adipocyte inflammatory  
143 stimulus modulated miR. These miRs have been largely suspected to be involved in inflammatory  
144 tone regulation. Indeed, miR-155, which is described as a typical multifunctional microRNA <sup>25</sup>,  
145 has been associated with the regulation of different immune-related processes, such as  
146 haematopoiesis <sup>26</sup>, innate immunity <sup>27</sup>, cancer <sup>28</sup> and B-cell and T-cell differentiation <sup>29</sup>. In the  
147 context of obesity onset and/or obesity comorbidities, we have recently shown that miR-155 was  
148 induced in adipocytes submitted to inflammatory stress and in subcutaneous adipose tissue  
149 biopsies of obese subjects <sup>11</sup>. We also showed that this miR induced inflammatory response,  
150 chemokine expression, and macrophage migration in 3T3-L1 adipocytes <sup>11</sup>. A recent study also  
151 demonstrated that the deletion of miR-155 in female mice prevented diet-induced obesity,  
152 improved insulin sensitivity and energy uncoupling machinery and abrogated HF diet induced  
153 adipocyte hypertrophy and white adipose tissue inflammation <sup>30</sup>. Together these data confirm the  
154 importance of miR-155 in adipose tissue function, notably in the control of inflammation.

155 To the best of our knowledge, no specific effect of miR-146a in adipocyte or adipose tissue biology  
156 has been reported. However, in inflammatory context, it has been reported that miR-146a is  
157 involved in the control of innate immunity. Indeed, miR-146a was found to be inducible upon  
158 stimulation with LPS in a NF- $\kappa$ B-dependent manner in human monocytes<sup>31</sup>. Such induction of  
159 miR-146a could contribute to the establishment of endotoxin tolerance in monocytes and to the  
160 regulation of TNF $\alpha$  production<sup>32</sup>. In addition, exposure of human lung alveolar epithelial cells  
161 resulted in a pronounced increase of miR-146a levels, that in turn induced a decrease of IL-8 and  
162 RANTES chemokines<sup>33</sup>. Finally, miR-146a deficiency during diabetes led to increased expression  
163 of M1 activation markers and pro-inflammatory cytokines and suppression of M2 markers in  
164 macrophages<sup>34</sup>. Together these data strongly suggest that miR-146a displays an anti-inflammatory  
165 effect. However, its role in adipose tissue will require further investigations.

166 Regarding miR-150, inflammatory or anti-inflammatory properties are less clear. A recent study  
167 demonstrated that miR-150 could physiologically modulate metabolic activities and inflammatory  
168 response both in cells and animals by regulating lipid metabolism and inflammatory response.  
169 Indeed, overexpression of miR-150 in macrophages resulted in an increase in lipid accumulation,  
170 associated with an elevated expression of several pro-inflammatory cytokines<sup>35</sup>. Conversely, when  
171 miR-150 knockout mice were challenged with a HF diet, these mice presented reduced whole body  
172 weight with less fat accumulation, improved systemic glucose tolerance and insulin sensitivity<sup>35</sup>.  
173 In contrast, another study reported that miR-150 knockout mice showed exacerbated obesity-  
174 associated tissue inflammation and systemic insulin resistance<sup>36</sup>. Such versatility has already been  
175 suggested in human cancer, where the role of miR-150 is context-dependent, i.e. this miR can have  
176 either oncogenic or tumor suppressor activity in cells that originate from different tissues<sup>37</sup>. Thus  
177 its specific role in adipose tissue and notably in the control of inflammatory tone will require  
178 further investigations.

179 To identify molecular mechanisms involved in miR regulation several approaches were combined.  
180 First, we demonstrated that miR-146a and miR-155 regulations were primarily NF- $\kappa$ B dependent  
181 in 3T3-L1 adipocytes, through the use of specific NF- $\kappa$ B inhibitor that blunted TNF $\alpha$ -mediated  
182 induction of these two miRs. The involvement of this signaling pathway was also confirmed in  
183 vivo, through the use of aP2-p65 transgenic mice which display a specific adipose tissue  
184 overexpression of p65<sup>24</sup> and that presented a strong induction of these two miRs. Based on this  
185 putative role of NF- $\kappa$ B in the induction of miR-146a and miR-155, the effect of the VD on the  
186 phosphorylation of two intermediates of the NF- $\kappa$ B signaling pathway (p65 and I $\kappa$ B) has been  
187 investigated. As previously demonstrated<sup>19,23</sup>, we reported that 1,25(OH)<sub>2</sub>D has a strong limiting  
188 effect on NF- $\kappa$ B signaling in 3T3-L1 adipocytes through the reduction of the phosphorylation  
189 levels of p65 and I $\kappa$ B. This effect could be related to the ability of 1,25(OH)<sub>2</sub>D to bind VDR and  
190 to interact with IKK<sup>38</sup> or could result from the induction of phosphatases involved in the  
191 dephosphorylation of p65 and I $\kappa$ B. Altogether these data strongly suggested that the inhibition of  
192 miRs by VD is largely mediated by its ability to reduce NF- $\kappa$ B signaling.

193 Interestingly, we observed both in vitro in human and murine adipocytes and in vivo in mice, a  
194 global upregulation of the miRs in pro-inflammatory conditions, suggesting that these three miRs,  
195 independently of their inflammatory effect (pro- or anti-), actively participated to inflammatory  
196 process in adipocytes and in adipose tissue. We also observed that VD, through the down-  
197 regulation of these three miRs, displayed an overall blocking effect of the inflammatory pathway  
198 in adipocytes. If the modulation of miRs by VD is reported here for the first time in the context of  
199 obesity associated comorbidities, similar ability has been widely described in cancer context.  
200 Indeed, in prostate cancer cells<sup>39</sup>, in bladder cancer cells<sup>40</sup> or in lung cancer<sup>41</sup>, it has been shown  
201 that 1,25(OH)<sub>2</sub>D differentially regulated miR expression profiles.

202 These original observations not only reinforce the role of VD as an anti-inflammatory agent in  
203 adipose tissue and adipocytes, but it also demonstrates the relevance of the miR regulation as new

204 anti-inflammatory mechanism in adipose tissue / adipocytes, that could represent an interesting  
205 new target to limit metabolic inflammation.

206

207 **Material and Methods**

208

209 **Reagents** - Dulbecco's modified Eagle's medium (DMEM) was purchased from Life  
210 Technologies (Cergy Pontoise, France), and fetal bovine serum (FBS) was obtained from PAA  
211 Laboratories (Les Mureaux, France). Isobutylmethylxanthine, dexamethasone and insulin were  
212 purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). TRIzol reagent was obtained  
213 from Life Technologies (Saint Aubin, France). QuantiTect SYBR Green PCR Master Mix,  
214 miScript Universal Primer and miScript Primer assays were purchased from Qiagen (Courtabœuf,  
215 France).

216

217 **Cell culture** - Adipocyte cells were grown at 37 C in a 5% CO<sub>2</sub> humidified atmosphere. The human  
218 preadipocytes (3 independent cultures) were obtained from Promocell and cultured according to  
219 the manufacturer's instructions. The mature adipocytes (day 12) were incubated with 1,25(OH)<sub>2</sub>D  
220 (100 nM, 24 h) followed by a 24-hour incubation with TNF $\alpha$  (15 ng/mL). Experiments were  
221 performed in triplicate, on 3 independent cultures.

222 The 3T3-L1 preadipocytes (ATCC, Manassas, VA) were seeded in 3.5 cm-diameter dishes at a  
223 density of  $15 \times 10^4$  cells/well, and grown in DMEM supplemented with 10% FBS, at 37 C, as  
224 previously reported<sup>42</sup>. To induce differentiation, two-day postconfluent 3T3-L1 preadipocytes  
225 (day 0) were stimulated for 72 h with 0.5 mM isobutylmethylxanthine, 0.25  $\mu$ mol/L  
226 dexamethasone and 1  $\mu$ g/mL insulin in DMEM supplemented with 10% FBS. The cultures were  
227 then treated with DMEM supplemented with 10% FBS and 1  $\mu$ g/mL insulin. The mature  
228 adipocytes (d 8) were incubated with 1,25(OH)<sub>2</sub>D (100 nM, 24 h) followed by a 24-hour incubation  
229 with TNF $\alpha$  (15 ng/mL). Experiments were performed in triplicate, on 3 independent cultures.

230

231 **3T3-L1 adipocytes incubations** - To identify signaling pathways involved in microRNA  
232 regulation, 3T3-L1 cells were treated with specific inhibitors of MAP (Mitogen Activated Protein)  
233 kinases (JNK, p38) and NF- $\kappa$ B signaling (JNK inhibitor II (10  $\mu$ M), SB 202190 (20  $\mu$ M) and BAY  
234 117082 (10  $\mu$ M), respectively) for 1 h (all obtained from Calbiochem, Merck Millipore, Darmstadt,  
235 Germany) and then stimulated or not with TNF $\alpha$  (15 ng/mL) for 24 h. All the treatments were  
236 performed on day 8.

237

238 **NF- $\kappa$ B activation** - The levels of p65 (Ser536) and I $\kappa$ B $\alpha$  (Ser32/36) phosphorylation were  
239 quantified using the ELISA Instant One kit according to the manufacturer's instructions  
240 (eBiosciences SAS, Paris, France).

241

242 **miRNA PCR arrays** - The miRNA PCR arrays (Qiagen, Courtabœuf, France) were used to  
243 quantify miRs extracted from human culture adipocytes, according to the manufacturer's  
244 instructions. Reactions were performed in a 12.5  $\mu$ L volume containing 6.25  $\mu$ L of 2X QuantiTect  
245 SYBR Green PCR Master Mix (Qiagen, Courtabœuf, France), 1.25  $\mu$ L of 10X miScript Universal  
246 Primer (Qiagen, Courtabœuf, France). After an initial incubation step of 15 min at 95°C,  
247 amplification reaction was performed in 40 cycles comprising 3 steps (94°C, 15 s; 55°C, 30 s and  
248 70°C, 30 s). For each condition, the expression was quantified in duplicate and the SNORD68 was  
249 used as the endogenous control in the comparative cycle threshold ( $C_T$ ) method.

250

251 **RNA isolation and qPCR** - Total cellular RNA was extracted using TRIzol reagent according to  
252 the manufacturer's instructions. To quantify miR-146a, miR-150 and miR-155, cDNAs were first  
253 synthesized from 1  $\mu$ g of total RNA in 20  $\mu$ L using 5X miScript Hiflex Buffer, 10X nucleic mix  
254 and miScript reverse transcriptase according to the manufacturer's instructions (Qiagen). Real-  
255 time quantitative RT-PCR analyses were performed using the Mx3005P Real-Time PCR System

256 (Stratagene, La Jolla, CA) as previously described <sup>43</sup>. Reactions were performed in a 12.5  $\mu$ L  
257 volume containing 6.25  $\mu$ L of 2X QuantiTect SYBR Green PCR Master Mix (Qiagen), 1.25  $\mu$ L of  
258 10X miScript Universal Primer (Qiagen), 1.25  $\mu$ L of 10X miScript Primer Assay (Mm\_miR146\_1  
259 miScript Primer Assay, Mm\_miR-150\_1 miScript Primer Assay, Mm\_miR-155\_1 miScript Primer  
260 Assay and Hs\_SNORD68 miScript Primer Assay (Qiagen)) and 2.5  $\mu$ L of RNase-free water. For  
261 each condition, the expression was quantified in duplicate, and the SNORD68 was used as the  
262 endogenous control in the comparative cycle threshold ( $C_T$ ) method.

263

264 **Animal experiments** – The protocol was approved through the local ethics committee. Six-week-  
265 old male C57BL/6J mice were obtained from Janvier (Le Genest Saint Isle, France). The mice  
266 were fed ad libitum (chow diet A04, Safe, Augy, France), with full access to drinking water. The  
267 animals were maintained at 22°C under a 12 h light: 12 h dark cycle with a 50% humidity level.  
268 To assess the impact of vitamin D (cholecalciferol) on miRs expression, three experimental groups  
269 of mice were fed ad libitum with a low (10% of total energy as fat; TD06416; Harlan, Indianapolis,  
270 Indiana, USA) or HF diet, providing 45% of total energy as fat (TD06415, Harlan, Indianapolis,  
271 Indiana, USA), supplemented (3000 IU/kg of body weight, i.e. around 90 UI/j/mouse, Sigma-  
272 Aldrich, Saint Quentin Fallavier, France) or not (300 IU/kg of body weight, i.e. around 9  
273 UI/j/mouse) with cholecalciferol. The mice (10 per group) were assigned into one of the three  
274 experimental groups depending on the diet, i.e., control, HF and HF plus vitamin D (HFVD), for  
275 10 weeks as previously described <sup>44</sup>. Epididymal adipose tissue was collected and stored at -80°C.  
276 aP2-p65 mice were generated on the C57BL/6J background as described elsewhere <sup>24, 45</sup>. All of  
277 the mice were housed in the animal facility at the Pennington Biomedical Research Center with a  
278 12:12-h light-dark cycle and constant temperature (22–24°C). The male mice were fed with a chow  
279 diet (MF 5001, 11% calorie in fat) and the epididymal fat tissue was collected at 20 weeks. The  
280 mice were housed at four per cage with free access to water and diet.

281

282 **Statistical analysis** - The data are expressed as the means  $\pm$  SEM. Significant differences between  
283 the control and treated group were determined using ANOVA, followed by the PLSD Fischer post  
284 hoc test using Statview software, and  $p < 0.05$  was considered statistically significant. Significant  
285 differences between two groups were determined using *t* test.

286

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290

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418

419

420 **Legends for figures**

421

422 **Figure 1. 1,25(OH)<sub>2</sub>D limits microRNA expression levels in human adipocytes.** Cells were  
423 preincubated with 1,25(OH)<sub>2</sub>D (100 nM) for 24 h and incubated with TNF $\alpha$  (15 ng/mL) for 24 h.  
424 The miR expression levels were quantified through miRNA PCR arrays approach. SNORD68 was  
425 used as the endogenous control. The values are presented as the means  $\pm$  SEM. Bars not sharing  
426 the same letters are significantly different,  $p < 0.05$ . Experiments were reproduced in triplicate, at  
427 least 3 independent times.

428

429 **Figure 2. 1,25(OH)<sub>2</sub>D limits microRNA expression levels in murine adipocytes.** 3T3-L1  
430 adipocytes were preincubated with 1,25(OH)<sub>2</sub>D (100 nM) for 24 h and incubated with TNF $\alpha$  (15  
431 ng/mL) for 24 h. The expression levels of miR-146a and miR-155 were quantified through qPCR  
432 in 3T3-L1 adipocytes. SNORD68 was used as the endogenous control. The values are presented  
433 as the means  $\pm$  SEM. Bars not sharing the same letters are significantly different,  $p < 0.05$ .  
434 Experiments were reproduced in triplicate, at least 3 independent times.

435

436 **Figure 3. Vitamin D limits miR-146a, miR-150 and miR-155 expression in mice epididymal**  
437 **adipose tissue.** The expression levels of miR-146a, miR-150 and miR-155 were quantified through  
438 qPCR in epididymal adipose tissue of mice fed standard, HF or VD-supplemented HF diets for 10  
439 weeks (n=10) and expressed relative to SNORD68 in the white adipose tissue. The data are  
440 expressed as relative expression ratios. The values are presented as the means  $\pm$  SEM. Bars not  
441 sharing the same letters are significantly different,  $p < 0.05$ .

442

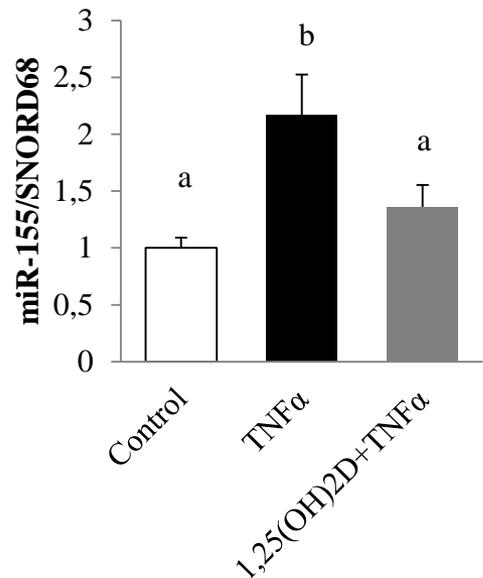
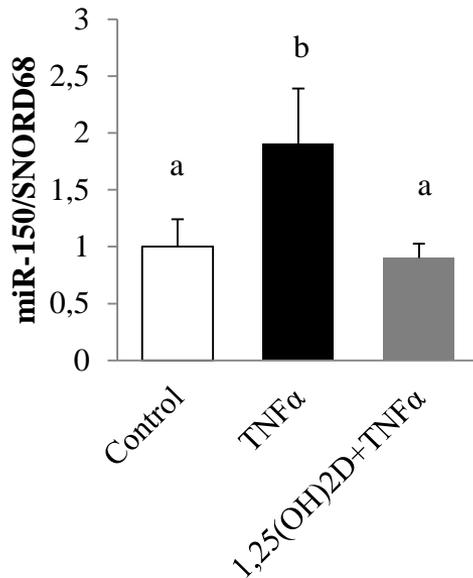
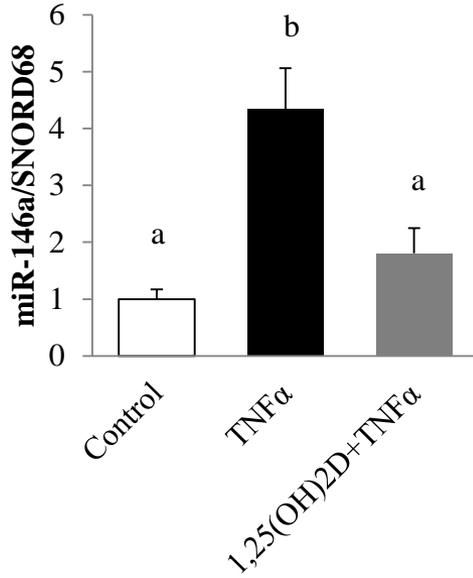
443 **Figure 4. 1,25(OH)<sub>2</sub>D limits NF- $\kappa$ B activation. A.** 3T3-L1 cells were treated with specific  
444 inhibitors of MAP (Mitogen Activated Protein) kinases (JNK, p38) or NF- $\kappa$ B signaling (JNK

445 inhibitor II (10  $\mu$ M), SB 202190 (20  $\mu$ M) and BAY 117082 (10  $\mu$ M), respectively) for 1 h and  
446 then stimulated with TNF $\alpha$  (15 ng/mL) for 24 h, and miR-146a and miR-155 expression were  
447 measured by qPCR. SNORD68 was used as the endogenous control. The data are expressed as  
448 relative expression ratios. The values are presented as means  $\pm$  SEM. Bars not sharing the same  
449 letters are significantly different,  $p < 0.05$ . Experiments were reproduced in triplicate, at least 3  
450 independent times. **B.** The expression of miR-146a and miR-155 were quantified by qPCR in  
451 epididymal adipose tissue of aP2-p65 transgenic mice. SNORD68 was used as the endogenous  
452 control. The data are expressed as relative expression ratios. The values are presented as means  $\pm$   
453 SEM. Bars not sharing the same letters are significantly different,  $p < 0.05$ . **C.** The cells were  
454 preincubated with 1,25(OH) $_2$ D (10 and 100 nM) for 24 h in dose-dependent manner and incubated  
455 with TNF $\alpha$  (15 ng/mL) for 5 min. The phosphorylation levels of the NF- $\kappa$ B subunits (p65 and I $\kappa$ B)  
456 were evaluated using ELISA. The data are expressed as relative expression ratios. The values are  
457 presented as the means  $\pm$  SEM. Values not sharing the same letters are significantly different,  $p <$   
458  $0.05$ . Experiments were reproduced in triplicate, at least 3 independent times.

459



Figure 1



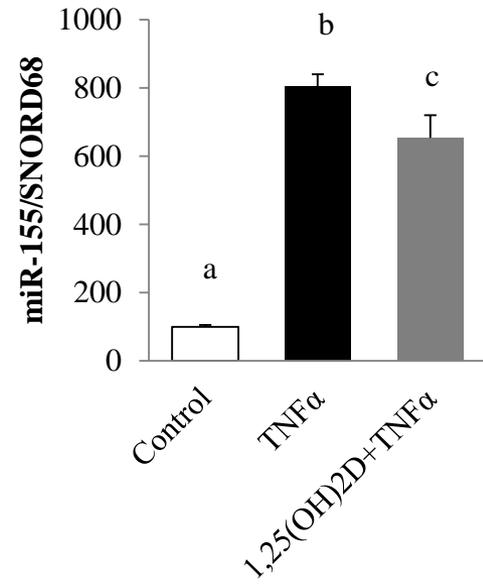
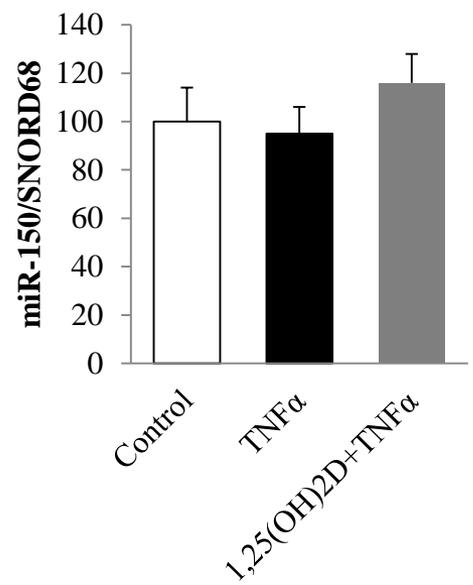
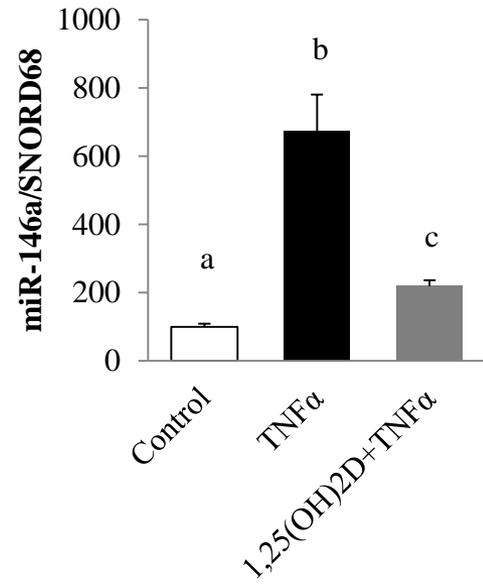
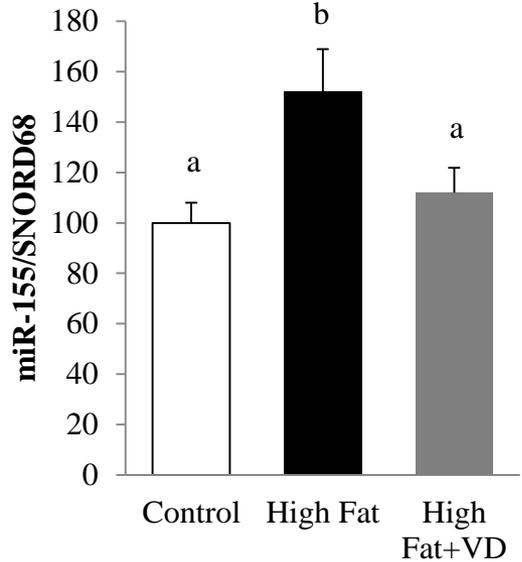
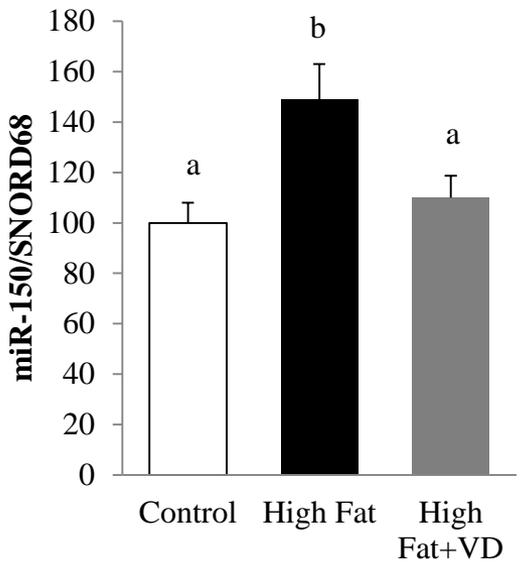
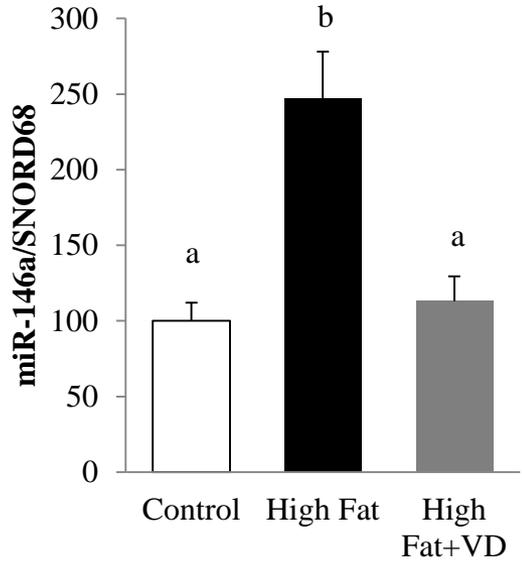
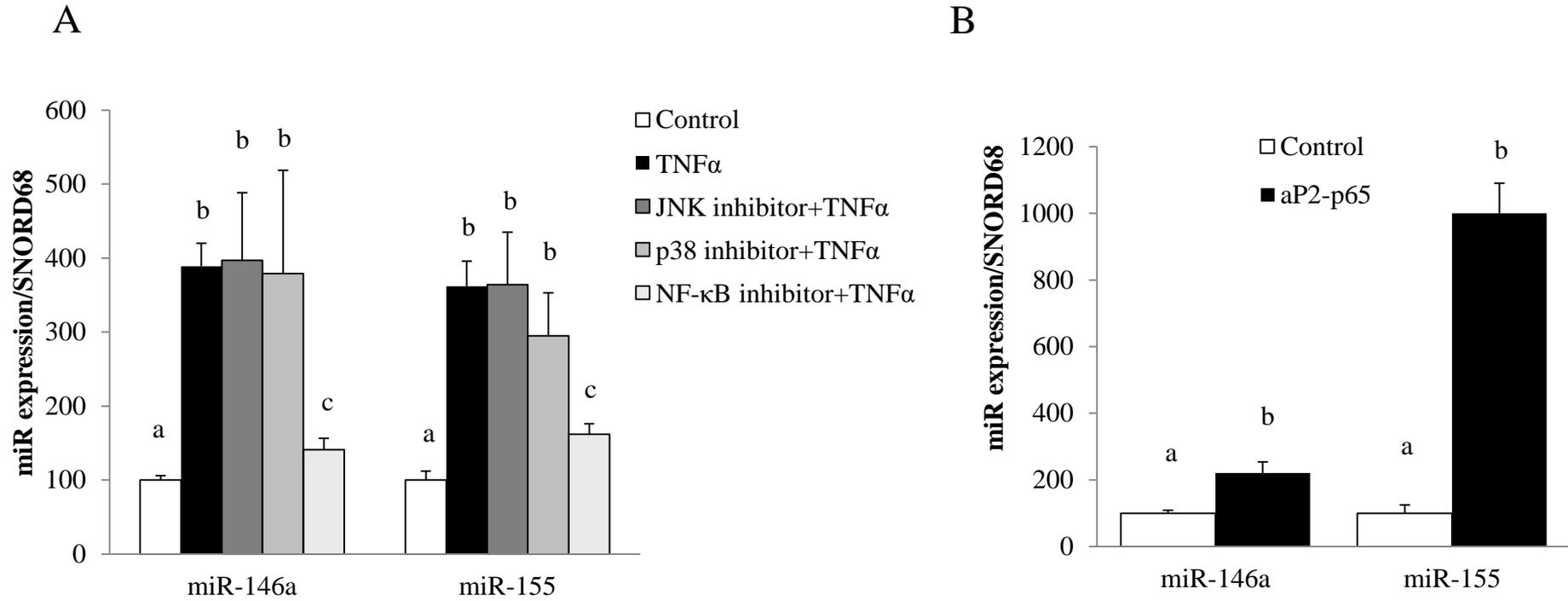


Figure 3





**C**

	Control	1,25(OH) <sub>2</sub> D (10 nM)	1,25(OH) <sub>2</sub> D (100 nM)	TNF $\alpha$	1,25(OH) <sub>2</sub> D (10 nM) +TNF $\alpha$	1,25(OH) <sub>2</sub> D (100 nM) +TNF $\alpha$
p65 phosphorylation	0.61±0.01 <sup>a</sup>	0.87±0.014 <sup>a</sup>	0.73±0.01 <sup>a</sup>	2.53±0.013 <sup>b</sup>	1.14±0.008 <sup>a</sup>	1.27±0.025 <sup>a</sup>
I $\kappa$ B phosphorylation	0.61±0.007 <sup>a</sup>	0.44±0.005 <sup>a</sup>	0.62 ±0.013 <sup>a</sup>	2.2±0.021 <sup>b</sup>	0.99±0.01 <sup>a</sup>	0.85±0.01 <sup>a</sup>