A mutation in signal peptide of rat resistin gene inhibits differentiation of 3T3-L1 preadipocytes[1]

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KEY WORDS resistin; mutation; adipocyte differentiation; obesity

ABSTRACT

AIM: To detect the resistin expression of white adipose tissue in diet-induced obese (DIO) versus diet-resistant (DR) rats, and to investigate the relationship of mutated resistin and 3T3-L1 preadipocytes differentiation. **METHODS:** RT-PCR and Western Blot were used to detect gene /protein expression. 3T3-L1 cells were cultured, transfected, and induced to differentiation using 0.5 mmol/L 3-isobutyl-1-methyxanthine (MIX), 1 mg/L insulin, and 1 μmol/L dexamethasone. Oil red O staining was applied to detect the degree of preadipocytes differentiation. **RESULTS:** Expression of resistin mRNA was upregulated in DIO rats and downregulated in DR rats. However, the expression levels varied greatly within the groups. Sequencing of the resistin genes from DIO and DR rats revealed a Leu9Val (C25G) missense mutation within the signal peptide in one DR rat. The mutant resistin inhibited preadipocyte differentiation. Local experiments and Western blotting with tagged resistin fusion proteins identified both mutant and wild type proteins in the cytoplasm and secreted into the culture medium. Computer predictions using the Proscan and Subloc programs revealed four putative phosphorylation sites and a possible leucine zipper motif within the rat resistin protein. **CONCLUSION:** Resistin-increased differentiation may be inhibited by the mutation-containing precursor protein, or by the mutant non-secretory resistin isoform.

INTRODUCTION

Resistin, a newly-discovered hormone[1,2], is secreted mainly in adipose tissue and has been proposed to form a biochemical link between obesity and type 2 diabetes[3,4]. Resistin gene expression and plasma concentration are significantly higher in genetic or diet induced obese rodents as well as in obese patients[4,5], while treatment with rosiglitazone (TZD), a drug that enhances insulin action by activating nuclear peroxisome proliferator-activated receptor γ (PPARγ), can markedly down-regulated its mRNA expression in adipose tissue of obese mice[4]. Administration of resistin was reported to impair glucose tolerance and insulin action, whereas administration of an anti-resistin antibody significantly improved insulin action[4,6-8]. In addition, insulin-stimulated glucose uptake by adipocytes was enhanced by neutralization of resistin, and conversely reduced by addition of resistin[4]. However, Savage and coworkers[5] found that resistin mRNA levels

[1] Project supported by the National Natural Science Foundation of China (No 30371502), the Natural Science Foundation of Jiangsu Province (No BK2001120), the Innovation Foundation of Nanjing Medical University (No CX2001005), and a grant from Educational Committee of Jiangsu Province (No 01KJD320025).
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Received 2004-03-11 Accepted 2004-07-29

were very low in freshly-isolated human adipocytes, and were undetectable in adipocytes from a severely insulin-resistant subject with a dominant-negative mutation in the PPARγ gene. Similarly, resistin is expressed at very low levels in several genetically obese rodents, its mRNA expression has been found to increase following treatment with TZDs PPARγ agonists and insulin itself *in vivo*, and yet suppressed by the same treatment *in vitro*[9,10]. Thus, the reports to date seem to present a contradictory function for resistin, and there remains a debate as to whether resistin stimulates or inhibits obesity. As diet-induced obese (DIO) and diet-resistant (DR) rodent model can mimic many characteristics of human obesity, including polygenic inheritance and insulin resistance[11,12], here we sought to address this question by determining the levels of resistin expression in DIO and DR rats.

When the resistin genes from DIO and DR rats were sequenced, a Leu9Val (C25G) missense mutation within the resistin gene signal peptide in one DR rat was uncovered. As wild type resistin has been shown to be up-regulated during the process of preadipocyte differentiation, and was suggested to be an important feedback regulator of adipogenesis[10], we next questioned whether mutant resistin is able to promote preadipocyte differentiation at levels similar to those of wild type resistin[13]. So in our study, the differentiation of 3T3-L1 cells stably expressing mutant resistin was observed. To explore the subcellular location of mutant resistin and its secretion by adipocytes, local experiment and Western blot were also applied.

MATERIALS AND METHODS

Animals and diets Twenty-five male Sprague-Dawley (SD) rats (111-131 g; Animal Center of Jiangsu Province, Grade II, Certificate No SCXK Jiangsu 2002-0031, China) were housed at 21-23 ºC with a 7:00 AM-19:00 PM light:dark cycle. All rats were allowed free access to food and water for 6 d, after which they were switched to a high-energy (HE) diet of 8 % corn oil, 44 % sweetened condensed milk, and 48 % rat chow, fed *ad libitum*[11,12]. After 2 weeks on the HE diet, the 9 rats with the highest body weight gain were designated DIO and the 9 rats with the lowest body weight gain were designated DR[11,12]. The remaining 7 rats, which showed intermediate weight gains, were designated chow-fed controls. The DIO and DR animals received the HE diet for four more weeks; the controls were fed straight rat chow. All animal experiments were conducted in accordance with the highest standards of care.

RNA preparation, RT-PCR amplification, and sequencing Total RNA was isolated from epididymal adipose tissue of rats using the TRI_{ZOL} reagent (Invitrogen, USA). The concentration of RNA was determined by absorbance at 260 nm, and samples were separated by electrophoresis on 1 % agarose gels, stained with 0.1 mg/L ethidium bromide and visualized under UV light for confirmation of RNA integrity. The total RNA (1 μg) was then converted to cDNA with 200 U Moloney murine leukemia virus reverse transcriptase (RT; Promega, USA) in 20 μL of buffer containing 0.5 mmol/L dNTPs, 20 U RNase inhibitor, and 0.1 μg oligo $(dT)_{15}$ primer (Promega, USA). An RT-free negative control was amplified for each sample to rule out genomic DNA contamination. An aliquot (10 %) of the cDNA was amplified with truncated *Thermus aquatious* DNA polymerase (Promega, USA) using primers specific for resistin (396 bp product; sense, 5′-CTG AGC TCT CTG CCA CGT ACT-3′; antisense; 5′-GCT CAG TTC TCA ATC AAC CGT CC-3′) and the internal control β-actin (241 bp product; sense, 5′-TAA AGA CCT CTA TGC CAA CAC AGT-3′; antisense, 5′-CAC GAT GGA GGG GCC GGA CTC ATC-3′). The PCR conditions were as follows: 25 (β-actin) or 27 (resistin) cycles of 94 ºC for 30 s, 64 ºC (resistin) or 58 ºC (β-actin) for 30 s, and 72 ºC for 40 s. Following amplification, 5 μL of each PCR product was separated on an agarose gel, stained with ethidium bromide and visualized under UV light. PCR products were sequenced for confirming specific amplification, and a C25G mutation was identified from a DR rat.

For this semi-quantitative RT-PCR assay, the cycle numbers and reaction temperatures were optimized to provide a linear relationship between the amount of input template and the amount of PCR product generated over a wide concentration range of total RNA (0.5 to 10 μg).

Construction of resistin expression plasmids To examine the differences of subcellular location and function between mutated and wildtype resistin, we generated constructs capable of expressing two different rat resistin fusion proteins with same pairs of primers. The full-length coding cDNA of the rat resistin gene (360 bp) was RT-PCR amplified with specific primers (sense, 5′-CGC TCT TCG ATG AAG AAC CTT TCA TTT CTC CTC C-3′; antisense 5′-CGC TCT TCC AAG GGA ACC AAC CCG CAG GGT AC-3′), digested with

Eam 1104 I, and inserted into the pDual GC expression vector (Stratagene, Germany) for generation of a resistin fusion protein that included a thrombin cleavage site, three copies of the c-myc epitope tag, and a single copy of the 6×His tag. Similarly, the resistin cDNA (359 bp) was amplified with a second set of primers (sense, 5′-GAT CTC GAG ATG AAG AAC CTT TCA TTT CTC-3′; antisense, 5′-TAC GAA TTC GAA CCA ACC CGC AGG GTA CA-3′), digested with *EcoR* I and *Xho* I, and inserted into the pEGFP-N2 subcellular location expression vector (Clontech, USA) for expression of a resistin-EGFP fusion protein. The constructed plasmids were verified by sequencing. The predicted molecular weight of both resistin fusion protein was 22.38 kDa (not including the signal peptide).

Transfection and selection of stably expressing 3T3-L1 cells To generate cell lines stably expressing the mutated or wild-type rat resistin gene, the appropriate resistin expression vectors were transfected into 3T3-L1 cells using Fugene 6 (Roche, USA) according to the manufacturer's protocol. Transfected cells were selected for 2 weeks in medium containing 1 kg/L G418. The cell culture supernatants were then screened for resistin expression by Western blot, and stably expressing transfectants were further grown in culture medium supplemented with 300 mg/L G418.

Culture and differentiation of 3T3-L1 cells 3T3-L1 preadipocytes were cultured and induced to differentiate as previously described[14]. Briefly, cells were cultured in DMEM containing 10 % FBS in a 5 % CO_2 atmosphere. Two days after the cells reached confluence (d 0), differentiation was induced by replacement of the medium with DMEM containing 10 % FBS, 0.5 mmol/L 3-isobutyl-1-methyxanthine (MIX) (Sigma, USA), 1 mg/L insulin (Sigma, USA) and 1 μmol/L dexamethasone (Sigma, USA). On d 2, the medium was replaced with DMEM containing 10 % FBS plus 1 mg/L insulin. On d 4, the medium was replaced with DMEM containing only 10 % FBS, and changed with the same medium every two days thereafter.

Oil Red O staining To determine the degree of differentiation, 3T3-L1 cells were stained with Oil Red O and hematoxylin as described[15]. Briefly, 10 % formalin (pH 7.4)-fixed 3T3-L1 cells were stained for 5 min with Oil Red O (stock solution: 3 g/L dissolved in isopropanol; working solution: 60:40 Oil Red O stock: distilled water) and counterstained with hematoxylin for 1 min. The red lipid droplets were visualized by microscopy.

aP2 mRNA measurement To further determine the degree of differentiation of 3T3-L1 cells, the aP2 gene expression was also detected by RT-PCR. Total RNA was isolated from 3T3-L1 cells of varied days (d 0, d 2, d 4, d 6, and d 8). The protocol of RT-PCR was the same as that mentioned before. The primers specific for aP2 (232 bp product; sense, 5′-GAT GCC TTT GTG GGA ACC TG-3′; antisense; 5′-TCC TGT CGT CTG CGG TGA TT-3′) and the internal control β-actin (241 bp product; sense, 5′-TAA AGA CCT CTA TGC CAA CAC AGT-3′; antisense, 5'-CAC GAT GGA GGG GCC GGA CTC ATC-3′) were used. The PCR conditions were as follows: 25 (β-actin) or 28 (aP2) cycles of 94 ºC for 30 s, 57 ºC (aP2) or 58 ºC (β-actin) for 30 s, and 72 ºC for 40 s.

Western blot analysis Conditioned media were collected from 3T3-L1 cells expressing wild type and mutant resistin-6×His, and untransfected 3T3-L1 cells. Protein concentrations were determined by BCA assay (Pierce, USA), and 1 μg protein was subjected to 12 % SDS-polyacrylamide gel electrophoresis and electroblotted onto a PROTRAN nitrocellulose membrane (Schleicher & Schuell, Germany). The proteins were then immunodetected using mouse anti-6×His antibody (Clontech, USA) and goat anti-mouse Ig G-horseradish peroxidase conjugate (Amersham Biosciences, Sweden) and visualized with an ECL Western Blot analysis system. (Amersham Biosciences, Sweden).

Statistical analysis All results were expressed as mean±SD. Data were analyzed using one-way ANOVA with a correction for multiple comparisons of SPSS 10.0 statistic software package, as appropriate.

RESULTS

Body weight divergence during the grouping period Two weeks after the HE diet, the DIO rats showed higher body weights than the chow-fed rats. In contrast, the DR rats weighed less than chow-fed controls. These trends remained consistent throughout the study period (Tab 1).

Resistin expression in DIO, DR, and control rats In contrast to the previous reports of Steppan and Way[4,9], in the epididymal adipose tissue of our tested rats, resistin expression was up-regulated in most diet induced obese (DIO) rats, and down-regulated in most diet resistant (DR) rats, as compared with chow-fed controls. Within each group, the expression levels varied significantly, and each group contained individuals

Tab 1. Body weight increase in rats fed with HE diet and chow for 6 weeks. Mean±SD. [b]*P*<0.05, [c]*P*<0.01 *vs* chow-fed control group. [e]*P*<0.05, [f]*P*<0.01 *vs* DR group.

	DIO (n=9)	Control (n=7)	DR (n=9)	F value	P value
W0 (g)	159±80[bf]	153±8	153±11	1.20	>0.05
W1 (g)	210±11[bf]	191±15[e]	173±20	12.42	<0.001
W2 (g)	257±12[cf]	231±12[f]	196±22	31.36	<0.001
W3 (g)	301±20[cf]	259±9[f]	219±21	47.45	<0.001
W4 (g)	345±24[cf]	312±9[f]	254±24	42.79	<0.001
W5 (g)	375±26[cf]	334±12[f]	280±32	30.52	<0.001
W6 (g)	396±27[cf]	347±19[f]	295±38	25.77	<0.001

in which resistin expression was not detected (Fig 1).

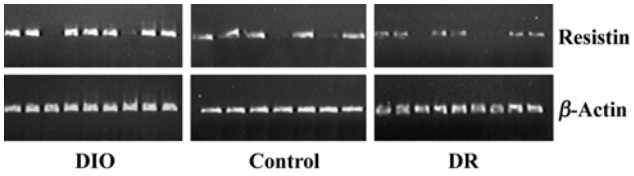

Fig 1. RT-PCR of resistin gene expression in the epididymal adipose tissue of DIO, DR, and control rats. Resistin gene expression of epididymal adipose tissue was upregulated in most DIO rats and suppressed in most DR rats. However, a great deal of variation was evident within each group, with undetectable expression noted in individuals of all three groups.

The Leu9Val missense mutation in signal peptide did not affect its secretory function RT-PCR amplicans were sequenced to confirm the specification of PCR amplification, and a C25G mutation site was identified. As the mutation site located in the signal peptide, we speculate that the mutated resistin may lose its secretory function. However, we detected trimer of resistin-6×His fusion protein in culture medium of mutated resistin stably expressed 3T3-L1 cells (Fig 2), which indicate that the secretory function of the mutated resistin was not affected.

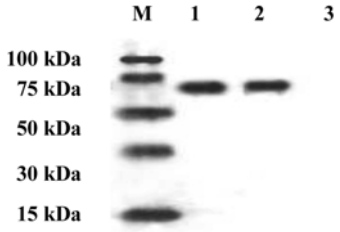

Fig 2. Mutant and wild type resistin are secreted. Wild type and mutant pDual GC-resistin expression vectors were stably transfected into 3T3-L1 cells. Culture media were separated on 12 % SDS-PAGE gels and detected by Western blotting with an anti-His antibody. M: marker; 1: wild type resistin-transfected cells; 2: mutant resistin-transfected cells; 3: pDual GC empty vector-transfected cells.

Mutated resistin inhibits preadipocyte differentiation Since the Leu9Val missense mutation in the resistin signal peptide should not affect its secretory function, we questioned whether the mutant resistin could promote preadipocyte differentiation as effectively as wild type resistin[13]. Surprisingly, compared with pDual GC empty vector-transfected 3T3-L1 cells, mutated resistin-transfected cells underwent less extensive adipoconversion, as judged by lipid staining using Oil Red O (Fig 3) and aP2 mRNA measurement (Fig 4).

Subcellular distribution of mutated and wild type resistin Our evidence suggested that the mutant resistin inhibited preadipocyte differentiation, prompt-

Tab 2. Consensus phosphorylation sites and putative leucine zipper in the rat resistin precursor.

Rat resistin 114A.A (precursor molecule)

1 MKNLSFLLLF LFFLVLGLLG PSMSLCPMDE AIS*KKINQDF SSLLPAAMKN TVLHCWSVS*S
61 RGRLAS*CPEG TTVTSCSCGS GCGSWDVRED TMCHCQCGSI DWTAARCCT*L RVGS

Protein kinase C phosphorylation (PKC) sites (33-35, 59-61, 109-111): [**ST**]-x-[RK]
Casein kinase II phosphorylation (CK2) sites (66-69): [**ST**]-x(2)- [DE]
Leucine zipper pattern (4-25): L-x(6)-L-X(6)-L-x(6)-L

* Phosphorylatable residue

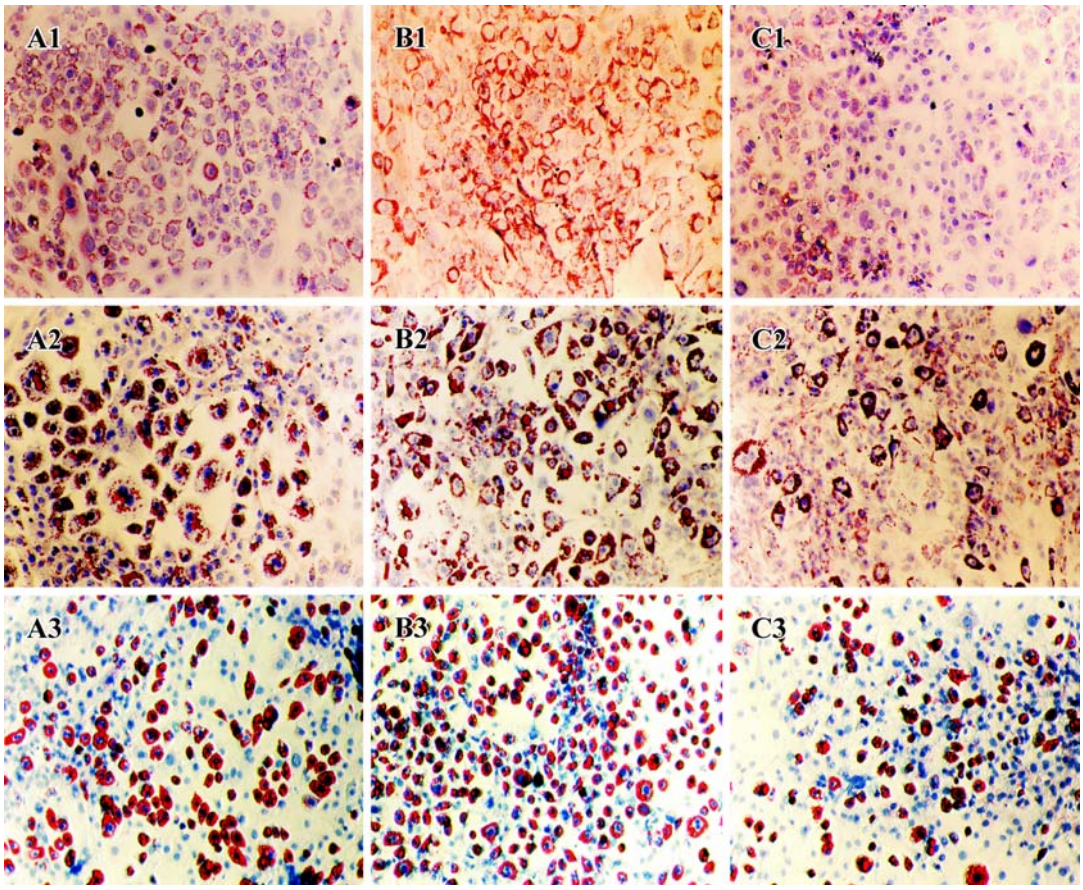

Fig 3. Wild type and mutant resistin proteins respectively stimulate and inhibit preadipocyte differentiation. 3T3-L1 preadipocytes were induced to differentiate using DMEM plus 15 % FBS containing 1mg/L insulin, 0.5 mmol/L MIX, and 1 μmol/L DEX. During the process (d 2, d 5, and d 8), cells were fixed and stained with Oil Red O for visualization of lipid droplets. A: normal 3T3-L1 cells; B: pDual GC-resistin transfected 3T3-L1 cells; C: pDual GC-mutant resistin-transfected 3T3-L1 cells. 1: d 2 of differentiation; 2: d 5 of differentiation; 3: d 8 of differentiation. cells; 3: pDual GC empty vector-transfected cells.

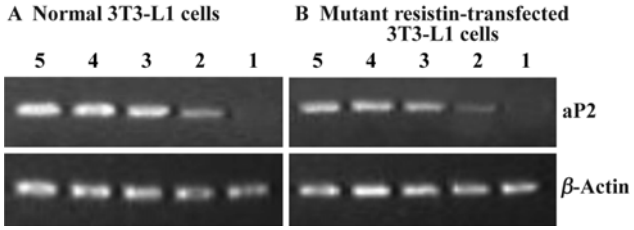

Fig 4. The mRNA expression of aP2 during preadipocyte differentiation. pDual GC-mutant resistin-transfected or non-transfected 3T3-L1 cells were induced to differentiate into adipocytes as above. The mRNA expression of aP2, a marker of adipocyte differentiation, was detected by RT-PCR. During the process of differentiation, the mRNA expression of aP2 in pDual GC-mutant resistin-transfected 3T3-L1 cells was downregulated when compared with that in non-transfected 3T3-L1 cells. 1: d 0 of differentiation; 2: d 2 of differentiation; 3: d 4 of differentiation; 4: d 6 of differentiation; 5: d 8 of differentiation. resistin-transfected cells; 2: mutant resistin-transfected cells; 3: pDual GC empty vector-transfected cells.

ing us to question whether resistin synthesized within preadipocytes may have multiple, functionally distinct subcellular locations. This was supported by the observation of a putative N-terminal leucine-zipper motif, which could suggest a nuclear transcription factor role. As shown in Fig 5, we observed only faint fluorescence from EGFP-tagged wild type and mutated resistin only in the cytoplasm. In addition, Western blotting revealed secreted resistin in the culture medium collected from both wild type and mutated resistin transfected 3T3-L1 cells (Fig 2). Together, these results seem to indicate that wild type and mutant resistin show similar localizations.

Primary structure analysis To examine possible functional differences between wild type and mutated resistin, we used the Proscan and NetPhos programs to analyze the primary structures of these resistin proteins. Our analysis identified a putative leucine zip-

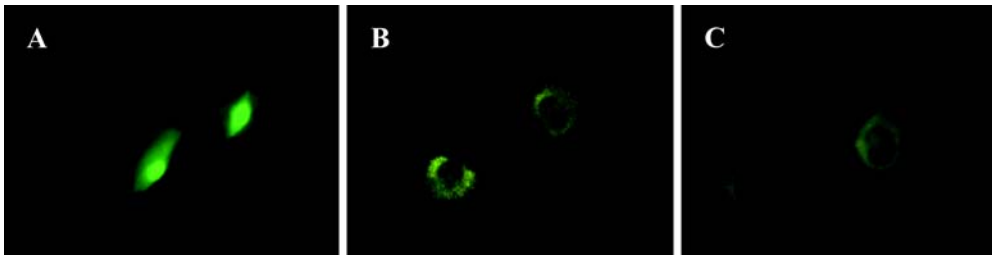

Fig 5. Subcellular localization of wild type and mutant resistin. The full-length coding sequences of the rat mutant and wild type resistin genes were inserted into pEGFP-N_2 and stably transfected into 3T3-L1 cells. Faint fluorescence could be observed only in the cytoplasm of 3T3-L1 cells. A: cells transfected with empty pEGFP-N_2 vector; B: wild type resistin-pEGFP-transfected 3T3-L1 cells; C: mutant resistin-pEGFP-transfected 3T3-L1 cells.

per and four potential phosphorylation sites (Tab 2).

DISCUSSION

Diet-induced obese (DIO) and diet-resistant (DR) rodent model was an ideal model for obesity research [11,12], because both environmental factors (ad libitum high-fat diets, limited physical activity in small cages) and genetic factors (different susceptibility to obesity, polygenic inheritance) were recruited in model-preparing. Based on this model, our study found that most (but not all) DIO rats showed up-regulation of resistin expression in the epididymal adipose tissue, whereas most (but not all) DR rats showed resistin down-regulation. Combined with the conclusions that more insulin resistant existed in DIO rats than in DR rats[16,17], these results are reasonable to support the notions that insulin resistance is related to increased resistin expression in adipose tissue and resistin may be the important link between obesity and type 2 diabetes. However, the inter-group expression levels were extremely diverse, with samples from each group showing no evidence of resistin expression. The mechanisms by which rats with similar degrees of obesity show greatly different expression levels of resistin are unknown. As resistin level-independent impaired glucose metabolism was previously observed in resistin-overexpressing transgenic rats[18] in a manner similar to our observations, it is possible that the functional status, not the absolute serum concentration, may be the key determinant of resistin function.

When RT-PCR amplicans of resistin gene were sequenced to confirm the specification of PCR amplification, a Leu9Val missense mutation within the resistin signal peptide was identified in one DR rat. As several studies have shown that resistin is a secretory protein[1,4], and resistin mRNA levels are markedly increased during 3T3-L1 and primary preadipocyte differentiation[4,10,19,20], we questioned whether mutant resistin is loss of its secretory function. Surprisingly, *In vitro* experiments showed that the mutant is appropriately secreted, but that it unexpectedly suppresses differentiation of 3T3-L1 preadipocytes. As our recent studies has reported that the wild type resistin promotes 3T3-L1 preadipocyte differentiation[13], and an N-terminal leucine zipper (a hallmark of transcription factors) within the resistin protein was predicted based on bioinformatic analysis, we next questioned whether resistin synthesized within the preadipocytes shows different subcellular distributions, perhaps associated with different functions. However, as shown in Fig 5, EGFP-tagged wild type and mutant resistin were both found in the cytoplasm, which suggests consistent localization. Together with that western blotting of cultured supernatants showed that resistin is secreted into the culture medium before and during the 3T3-L1 cell adipoconversion process, it was suggested that subcellular location was not the reason why the mutated and wildtype resistin represent contrary effects on 3T3-L1 cell differentiation.

Recently, an alternative splicing isoform of the rat resistin gene was reported to generate a novel non-secretory resistin protein, located mainly in the nucleus, while secretory resistin was located in endoplasmic reticulum and trans-Golgi network[21]. So, it is likely that when the start AUG codon is present, other downstream AUG codons are not used for translation initiation, which may explain why we were unable to detect resistin in the nucleus, where it might act as a transcription factor for inhibition of 3T3-L1 preadipocyte differentiation. It is also possible that the resistin-mediated increase of adipoconversion is inhibited by the pre-cleaved precursor protein. A similar interaction is seen in the case of TNFα, in which phosphorylation of the 26 KDa TNFα precursor has been reported in monocytes and is

suggested to play a role in regulating expression and proteolytic processing, modulating TNFα bioactivity, or take part in intracellular signaling by cell-surface TNFα[22,23]. Similar to TNFα, resistin is a serine/threonine-rich protein containing four putative phosphorylation sites (Tab 2). Thus, we speculated that Leu9Val mutation within the resistin signal peptide may alter the phosphorylation status of the resistin precursor, leading to inhibition of its adipoconversion-promoting activity. Additional studies will be needed to further explore the phosphorylation of wild type and mutated resistin proteins, and their possible implications in obesity and type 2 diabetes.

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