MiR-124 is Related to Podocytic Adhesive Capacity Damage in STZ-Induced Uninephrectomized Diabetic Rats

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Key Words
miRNA-124 • Integrin • Podocyte • Diabetic nephropathy

Abstract
Background: Diabetic nephropathy (DN) is the leading cause of end-stage renal disease. Podocyte plays a key role in the pathogenesis of DN. Adhesive capacity damage of podocytes is characteristic in DN. Emerging evidence suggests that microRNAs (miRNAs) play crucial roles in controlling many cell adhesion molecules thus contribute to normal cell adhesion. The roles of miRNA in podocytic adhesive capacity damage in diabetic conditions remain largely unknown. Methods: Diabetes was induced by tail vein injection of streptozotocin (STZ) into uninephrectomized male Wistar rats. Comparative miRNA expression array and real-time PCR analyses were conducted in sham group at week 0 (W0, n = 3) and STZ-induced uninephrectomized diabetic rats at week 1 (W1, n = 3) and week 2 (W2, n = 3) to demonstrate the greatest increased miRNA in renal cortex. At week 2, STZ-induced uninephrectomized diabetic rats were treated with vehicle (Group U, n = 9), chemically modified antisense RNA oligonucleotide (ASO) complementary to the mature miR-124 (Group O, n = 8), miR-124 mismatch control sequence (Group M, n = 8). Urine specimens were obtained for measurement of urine albumin concentration and urinary podocyte specific protein (nephrin and podocin) quantitation. Expression of integrin α3 were detected by immunohistochemistry and western blotting. Results: MiRNAs are differentially regulated in renal cortex of STZ-induced uninephrectomized diabetic rats relative to sham rats. Among the up-regulated miRNAs, miR-124 expression demonstrated the greatest increase. Administration of miR-124 ASO for two weeks significantly reduced urinary podocytic nephrin, podocin and albumin excretion and up-regulate integrin α3 expression. Conclusion: MiR-124 is related to podocytic adhesive capacity damage and may be implicated in the pathogenesis of DN.

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Introduction

Diabetic nephropathy (DN) is a major health epidemic and a serious complication that affects 25-40% of type 1 and type 2 diabetic patients [1, 2]. Although the mechanisms underlying the development of DN are incompletely understood, there has been a growing interest in the possible role of glomerular podocytic damage in the pathogenesis of DN [3, 4]. Podocytes are a type of terminally differentiated epithelial cells with a unique structure and function which are crucial in maintaining glomerular permselectivity, regulating the synthesis of glomerular basement membrane (GBM) extracellular matrix (ECM) proteins [5]. The loss of glomerular podocytes is a key event in the progression of DN resulting in proteinuria and declining function. Viable podocytes were found in the urine, suggesting that reduced adhesive capacity may be one of the mechanisms of podocyte damage [6]. Integrins are a major family of cell-surface proteins that mediate cell binding of DN [7]. Integrin α3β1 is the principal adhesion complex that attaches podocytes to the GBM and is abundantly expressed in the podocyte membrane [8]. In previous vitro study, integrin α3β1 expression was decreased in rat and human podocytes cultured in high glucose condition [9]. New experimental confirmation approaches about podocytic adhesive capacity damage in diabetes are desperately needed.

MicroRNAs (miRNAs) are a group of endogenous, noncoding small RNAs that have recently been implicated in the regulation of gene expression by translation repression or transcript degradation of specific target genes in multiple biologic processes [10]. Many studies have suggested that miRNAs may be involved in kidney disease especially in DN which provided us critical new information to advance the knowledge of DN [11]. Emerging evidence suggests that miRNAs play crucial roles in controlling many cell adhesion molecules thus contribute to normal cell adhesion [12]. MiR-29 [13] and miR-183 [14] have been found to regulate the expression levels of integrin β1. But up to now, the roles of miRNA in podocytic adhesive capacity damage in diabetic conditions remain largely unknown.

Our previous study showed that integrin α3 was down-regulated accompanied with increased podocyte leakage from urine in STZ-induced diabetic rats [15]. Based on this result, we hypothesize that miRNA expression patterns might serve as a signature of podocytic adhesion damage under diabetic condition. To prove this hypothesis in the present study, we tried to profile whether differences exist in kidney miRNA expression of sham rats and STZ-induced uninephrectomized diabetic rats. We found altered miRNA expression in kidneys of STZ-induced uninephrectomized diabetic rat, among which miR-124 demonstrated the greatest increase. The purpose of this study is to investigate whether the possible role of miR-124 is related to podocytic adhesive capacity damage in STZ-induced uninephrectomized diabetic rats and expected to produce novel insights into mechanisms of DN.

Materials and Methods

Animal studies

All experiments were performed with 6-week-old male Wistar rats weighing 180-220 g that were purchased from Center of Experimental Animal of Institute of Radiation Medicine of Peking Union Medical College. Animals were housed under controlled temperature and humidity with a 12:12-hour light-dark cycle, had free access to a standard rat diet and to tap water. 35 rats were anaesthetized by intraperitoneal injection with sodium pentobarbital 50 mg/kg body weight, and the left kidney was removed through a flank incision. We also prepared sham-operated rats, which underwent a similar flank incision followed by kidney exteriorization only (Group S, n = 10). Following the operation, uninephrectomized rats were injected with streptozotocin (STZ, 65 mg/kg, Sigma, St. Louis, MO, USA) dissolved in 0.1 mol/L sodium citrate buffer (pH 4.0) via a tail vein to induce diabetes. Uninephrectomized STZ-injected rats with blood glucose level of ≥16.7 mmol/L (300 mg/dl) were considered diabetic 72 hours later after the injection, thus 4 rats with blood glucose levels <16.7 mmol/L were excluded from the study. Three rats of Group S were sacrificed and right kidneys were harvested for miRNA microarray analysis at week 0 (W0, n = 3). At the
indicated time point of week 1 and week 2, diabetic rats were sacrificed and right kidneys were harvested for miRNA microarray analysis (W1, n = 3; W2, n = 3). At week 2, diabetic rats were randomly divided into three groups. One group was injected with chemically modified antisense RNA oligonucleotide (ASO) complementary to the mature miR-124 sequence via a tail vein (Group O, n = 8). One group was given the miR-124 mismatch control sequence (Group M, n = 8). The other group was given with an equal volume of citrate buffer similarly (Group U, n = 9).

Body weight was recorded weekly throughout the experimental period. On week 4, individual rats were placed in metabolic cages for 24-hour urine specimens collection. Urine specimens were tested for urinary albumin, creatinine concentration and urinary podocyte specific protein (nephrin and podocin). Urinary albumin concentration was measured by immunoturbidimetry (Sun Bioengineering, Shanghai, China). Urinary creatinine concentration was measured by basic picric acid method (Biosino Bio-technology and Science Corp., Beijing, China). Nephrin and podocin were detected by western blotting.

After urine collection, rats were sacrificed under anesthesia by intraperitoneal injection of sodium pentobarbital 50 mg/kg body weight. Blood was collected for concentration of plasma creatinine, plasma glucose, and plasma albumin by using a commercially available kit (Auto Biochemistry analyzer, HITACHI 7170A, Japan). The right kidney was removed, weighted, macroscopically divided into renal cortex and medulla. Some cortical tissue was frozen in liquid nitrogen, and kept at -80 °C for western blotting. Some cortical tissue was perfused and fixed with 10% neutral-buffered formalin, for immunohistochemistry. The other cortical tissue of right kidney was dissected into 1-2 mm sections, fixed in 2.5% glutaraldehyde for transmission electron microscopy. All procedures were carried out in accordance with the approval of the ethics committee of Tianjin Medical University.

MiRNA microarray analysis
Total RNAs were isolated from rat renal cortical tissue by using mirVana™ miRNA Isolation Kit (Ambion, Austin, Texas, USA) according to the manufacturer’s instructions. 5-μg good quality RNAs were used for miRNA expression analysis performed by TaqMan MiRNA Array v2.0 (Applied Biosystems, Foster City, CA, USA).

Real-time PCR
Real-time PCR using miRNA-specific stem-loop primers for reverse transcription and TaqMan probes for mature murine miRNA was performed in accordance with manufacturers’ protocols using an ABI 7900HT real-time PCR system (Applied Biosystems, Foster City, CA, USA). First-strand cDNAs were generated using SuperScript III (Invitrogen, Carlsbad, CA, USA). TaqMan probes for miR-128b, miR-124, miR-190, miR-223, miR-320, miR-343-3p and U87 were developed by Applied Biosystems. Data analysis was performed using the Applied Biosystems SDS Software package, version 2.2.

Urinary podocyte specific protein expression
Urine samples were centrifuged at 3000 g for 30 min and at 13 000 g for 5 min at 4 °C shortly after collection. Supernatant was then discarded and specimens were stored in -80 °C for nephrin and podocin detection.

Reagents
Chemically modified antisense RNA oligonucleotide complementary to the mature miR-124 and miR-124 mismatch control oligonucleotides for in vivo applications were purified by Sigma-Genosys (Cambridge, United Kingdom). The miR-124 ASO sequence was: GCACAAGUGUCGCCUGGAACUA; the miR-124 mismatch control sequence was: GCUCACGAGUGGCCUGCAACAA. All oligonucleotides were 2’-OMe modified and free of endotoxin for in vivo delivery. MiR-124 ASO or miR-124 mismatch control oligonucleotides were injected at 80 mg/kg body weight via a tail vein two weeks after uninephrectomized STZ-injected rats were induced.

Immunohistochemical analysis
Cortical tissue of right kidney was removed from rats and fixed in 10% phosphate buffered formalin. The fixed tissue was then embedded in paraffin at 60 °C, and cut transversely into 3 μm thick sections. Then the slides were preincubated with blocking solution containing bovine serum albumin (Zhongshan Goldenbridge, Beijing, China) for 10 minutes. Staining was performed by avidin biotin-peroxidase complex
technique in accordance to manufacturer’s protocol. Sections were incubated with primary antibodies, rabbit anti-rat integrin α3 monoclonal antibody (1:100, Bosider, Wuhan, China). Exposure was overnight at room temperature, followed by a 40-minute incubation with the secondary antibodies, PV-6001 goat anti-rabbit IgG-HRP multimer (Zhongshan Goldenbridge, Beijing, China). Negative control sections were incubated with PBS instead of primary antibody.

**Western blot analysis**

Renal cortices were lysed in hypotonic lysis buffer, and equal amounts of protein were loaded onto an 8% SDS-polyacrylamide gel. Separated proteins were transferred to a nitrocellulose membrane and blocked with 8% nonfat milk at room temperature for 1 hour. Membranes with proteins were incubated with rabbit anti-rat podocin polyclonal antibody (1:100, Santa-Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-rat nephrin polyclonal antibody (1:200, Santa-Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-rat integrin α3 monoclonal antibody (1:100, Bosider, Wuhan, China) overnight followed by incubation with secondary horseradish peroxidase antibodies according to the manufacturer’s instructions. All blots were developed using western blotting detection system of enhanced chemiluminescence.

**Electron microscopy**

For transmission electron microscopy, cortical tissue of kidney were dissected into 1-2 mm sections, double-fixed in PBS-buffered glutaraldehyde (2.5%) and osmium tetroxide (0.5%), dehydrated and embedded into Spurr’s epoxy resin (in Epon by standard procedures). Ultrathin sections (90 nm) were made and double-stained with uranyl acetate and lead citrate, and examined using a transmission electron microscope (JEM 100 CX-II, JEOL, Tokyo, Japan). Podocyte lesions including foot process effacement, basement membrane thickening were analysed.

**Statistical analysis**

All analyses in the current study were performed using SPSS 15.0 software. Differences were considered significant at \( P<0.05 \). Data were presented as Mean ± SEM and were compared by Student t test or ANOVA as appropriate.

**Results**

**Changes of specific miRNAs in kidneys of STZ-induced uninephrectomized diabetic rats**

To determine whether miRNAs play a role in the initiation and progression of DN, we performed a miRNA expression profiling in kidneys of STZ-induced uninephrectomized diabetic rats and sham rats. 39 miRNAs were found to be altered at week 1 and week 2 after animal model was made successfully. Most notable among the up-regulated miRNAs were miR-128b, miR-124, miR-190 and miR-223, while miR-320 and miR-343-3p were significantly down-regulated. Given that miRNAs are involved in the pathogenesis of DN, it is suggested that specific miRNAs may participate in the podocyte damage in STZ-induced uninephrectomized diabetic rats, see Figure 1.

For determining the findings with the miRNA array analysis, we tested the selected miRNAs (miR-128b, miR-124, miR-190, miR-223, miR-320 and miR-343-3p) that demonstrated the greatest alterations in the diabetic kidneys by real-time PCR assays. Consistent with the miRNA array analysis, the expression of miR-128b, miR-124, miR-190 and miR-223 was significantly increased, whereas the expression of miR-320 and miR-343-3p was significantly decreased relative to sham group. Both in the miRNA array and the real-time PCR analyses, miR-124 expression demonstrated the greatest increase in kidneys of STZ-induced uninephrectomized diabetic rats relative to sham samples, see Figure 2.

**Biochemical parameters of each group**

As shown in Table 1, Group U and M displayed higher blood glucose levels (27.19 ± 1.33 mmol/L, \( P<0.05 \) and 27.27 ± 2.03 mmol/L, \( P<0.05 \)) than Group S (6.12 ± 0.32 mmol/L) at
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Fig. 1. Analysis of miRNA microarray data. Hierarchical clustering heat map of miRNA expression profile was produced using fold change in miRNA expression. Analysis categorized 3 clusters corresponding to sham group at week 0 (W0), STZ-induced uninephrectomized diabetic rats at week 1 (W1), STZ-induced uninephrectomized diabetic rats at week 2 (W2). Method of complete linkage was used as a clustering method (n = 3).

Week 4. Administration of miR-124 ASO did not affect these changes in blood glucose (P>0.05). A lower concentration of plasma albumin was detected in Group U and Group M when compared with Group S (P<0.05). Treatment with miR124 ASO significantly increased plasma albumin (P<0.05), Group U and M presented higher levels of 24-hour urinary albumin (P<0.05), ratio of kidney/body weight (P<0.05), creatinine clearance (Ccr) (P<0.05), urine volume (P<0.05), which were corrected by miR-124 ASO administration (P<0.05).

**Expression changes of urinary nephrin and podocin**

Urinary expression of nephrin and podocin significantly increased at week 4 in Group U and M relative to Group S (P<0.05), treatment with miR-124 ASO significantly decreased podocyte excretion (P<0.05), see Figure 3.

Table 1. Biochemical parameters of each group on week 4

| Group | P-Glu (mmol/L) | P-Alb (g/L) | U-Alb (mg/d) | Rt-KW/BW (×10^3) | Ccr (ml/mi n/kg) | U-V (ml/d) |
|-------|---------------|-------------|--------------|------------------|-----------------|-----------|
| S (n=7) | 6.12±0.32    | 36.28±1.69  | 1.89±0.11    | 2.78±0.09        | 0.73±0.11       | 21.03±1.6 |
| U (n=9) | 27.19±1.33a   | 25.22±1.25a  | 7.33±0.95a   | 10.12±1.08a      | 2.77±0.32a      | 118.36±9.6a |
| M (n=8) | 27.27±2.03a   | 26.88±2.71a  | 8.10±1.16a   | 9.55±2.12a       | 2.89±0.18a      | 123.75±10.0a |
| O (n=8) | 26.98±1.57    | 32.75±1.62bc | 4.16±0.28bc  | 6.53±1.80bc      | 1.42±0.09bc     | 84.85±11.8bc |

P-Glu indicates plasma glucose; P-Alb, plasma albumin; U-Alb, 24-hour urinary albumin; Rt-KW/BW, ratio of right kidney weight to body weight; Ccr, creatinine clearance; U-V, urine volume. ^P<0.05 vs Group S, bP<0.05 vs Group U, cP<0.05 vs Group M. Values are mean ± SD.
Fig. 2. Expression of specific miRNAs was altered in sham group at week 0 and STZ-induced uninephrectomized diabetic group at week 1 and week 2 determined by real-time PCR assays. A: up-regulated miRNAs; B: down-regulated miRNAs. W0: sham group at week 0; W1: STZ-induced uninephrectomized diabetic rats at week 1; W2: STZ-induced uninephrectomized diabetic rats at week 2. *P<0.05 vs Group S, †P<0.01 vs Group S (n = 3).

Expression changes of miR-124 in the kidney cortices
As shown in Figure 4, real-time PCR analysis indicated that miR-124 expression in the kidney cortices was significantly up-regulated in Group U and M compared with Group S (P<0.05) at week 4 and was knocked down significantly after administration of miR-124 ASO (P<0.05).

Fig. 3. Effect of miR-124 ASO on nephrin and podocin excretion assessed by western blot. A: expression changes of nephrin, B: expression changes of podocin, C, D: The bar graph showed the densitometric quantification of nephrin and podocin under the indicated conditions. β-actin served as an internal control. Expression of nephrin and podocin were high in the Group U and Group M and significantly decreased in Group O. *P<0.05 vs Group S, †P<0.05 vs Group U, ‡P<0.05 vs Group M (n = 7-9).
Expression changes of integrin α3 in the kidney cortices

To assess the effect of miR-124 ASO on integrin α3 expression, we sought to carry out immunohistochemistry and western blot for integrin α3 detection. In the Group U and M, protein α3 were significantly lower compared to the sham group (P<0.05), whereas rats treated with miR-124 ASO had significantly greater expression of integrin α3 (P<0.05). Consistent with the above findings, immunohistochemical analysis also showed weak α3 staining in the Group U and Group M, but the signal was markedly stronger in the miR-124 ASO group, see Figure 4.

Electron microscopy observation

On week 4, transmission electron microscopy has shown that no pathological injury in glomeruli in the sham group, while foot process effacement, basement membrane thickening were detected in the Group U and Group M. In marked contrast, in rats given miR-124 ASO, the pathological alteration above improved, see Figure 5.
Discussion

Our data show that 1) there exists podocytic adhesive capacity damage in STZ-induced uninephrectomized diabetic rats presented as increased urinary podocyte excretion and decreased integrin α3 expression in renal cortical tissue. 2) miRNAs are differentially regulated in renal cortex of STZ-induced uninephrectomized diabetic rats relative to sham rats. 3) among the up-regulated miRNAs, miR-124 expression demonstrated the greatest increase and podocytic adhesive capacity is ameliorated via knocking down miR-124 in STZ-induced uninephrectomized diabetic rats. These results suggest that miR-124 is related to podocytic adhesive capacity damage and may be implicated in the pathogenesis of DN.

Many observations have described urinary podocyte excretion observed in proteinuric renal diseases including DN as consisting of a mixed population of viable and apoptotic podocytes [6, 16]. Meanwhile, the development of glomerular sclerosis in conditions such as diabetes, is associated with a decrease in the number or density of podocytes in the glomerular capillary tuft [4, 17]. There are several causes of podocyturia and podocytopenia (defined as a decrease in the podocyte number in the glomeruli), including apoptosis, detachment from the GBM, and the inability or lack of podocytes to proliferate [18]. Integrins are heterodimeric, transmembrane proteins which consist of a characteristic combination of α- and β-subunits that are receptors for cell adhesion to ECM [19]. Integrin α3β1 is the specific integrin anchored podocytes to the GBM [20, 21], down-regulation of which is causally related to the podocytic adhesion damage, leading to podocyturia and podocytopenia. The α3 integrin knockout mouse exhibits an immature GBM with podocyte foot process effacement and a reduction in the podocyte number [22]. In the present study, our data revealed that urinary specific podocytic protein (nephrin and podocin) excretion, urinary albumin excretion of STZ-induced uninephrectomized diabetic rats were significantly higher accompanied with down-regulation of integrin α3 at week four. These findings then led to us in-depth analysis and thinking: what participate in the podocytic adhesion damage by down-regulating integrin α3 in STZ-induced uninephrectomized diabetic rats?

MiRNAs are a class of endogenous, short (21–24 nucleotides), noncoding RNAs which have recently been implicated in the regulation of multiple biologic processes by inhibiting target gene expression [23]. For exploration of the functional roles of miRNA in podocyte adhesive capacity damage under diabetic conditions, we profiled that differences existed in miRNA expression in STZ-induced uninephrectomized diabetic rats and sham rats. MiRNA microarray analysis revealed that most notable among the up-regulated miRNAs were miR-128b, miR-124, miR-190 and miR-223, among which the increasing amplitude of miR-124 demonstrated the greatest at week 1 and week 2 after animal model replication. Real-time PCR validation of miRNA expression of kidney tissue also showed that miR-124 expression was persistently increased. Krützfeldt et al [24] demonstrated that miRNA ASO could effectively induce target miRNA silencing when administered systemically in mice. So we focused on miR-124 for further study by injection of miR-124 ASO via a tail vein in STZ-induced uninephrectomized diabetic rats. Administration of miR-124 ASO for two weeks significantly reduced nephrin and podocin excretion and up-regulate integrin α3 expression, which meant miR-124 might be related to the protective effects of podocyte adhesion. As far as we know, this is the first report that proves the administration of miRNA ASO is likely to contribute to decrease podocyte leakage and to prevent podocyte damage secondary to diabetes. Remarkably, the enhanced miR-124 expression may be located in different cell populations that undergo high glucose overload. The activity of miR-124 blockade via a tail vein to uninephrectomized diabetic rats could be partially offset by a potential miR-124 increase in other tissues, which reflects a complicated consequence after miR-124 ASO injection. It should also be noted that the efficiency of miR-124 blockade in treating uninephrectomized diabetic rats may not be as robust compared with those trasfected to podocytes in vitro. These may explain why miR-124 expression in Group O remained increased relative to Group S after a two-week miR-124 ASO injection.
Hemodynamic changes, especially glomerular capillary hypertension contribute greatly to the development of DN [25]. One of the consequences of glomerular capillary hypertension is the increasing mechanical stretch on resident glomerular epithelial cells which may favor a decrease in podocyte adhesion to GBM [26, 27]. In our present study, four weeks after animal model replication, renal integrin α3 expression was significantly down-regulated accompanied with increased kidney/body ratio and Ccr, of which the former indicates podocytic adhesive capacity damage, and the latter indicates hemodynamic changes and increasing mechanical stress of podocyte. A possible mechanism for the precise regulation of integrin α3 in podocytes might be through its regulation by miRNAs. Vitro experiment showed that podocyte miR-124, miR-190, miR-217 and miR-188 were significantly up-regulated under mechanical stress accompanied with down-regulation of integrin α3 in our latest study [28]. Bioinformatic assessment also showed that miR-124 was the predicted miRNA target site in Itga3 3′UTRs according to Targetscan (Release 6.2) and picTar (http://pictar.mdc-berlin.de/cgi-bin/new_PicTar_vertebrate.cgi?species=vertebrate), suggesting its possible role in regulating gene Itga3. Thus the influence of miR-124 on podocytic adhesive capacity damage is likely to be accomplished by down-regulation of integrin α3.

Besides hemodynamic changes, cytokines also play a very important role in the development and progression of DN which is partly due to complex interplay between different cytokines. In diabetes, glucose induces augmented expression of vasoactive factors such as endothelin-1 (ET-1) which promotes glomerular hyperfiltration with its consequent potential for renal injury [29, 30]. Latest research showed that miR-320 can negatively regulate expression of ET-1 in Human Umbilical Vein Endothelial Cells (HUVECs) treated with high glucose and STZ-induced diabetic rats [31]. In our present study, the expression of miR-320 was significantly decreased in STZ-induced uninephrectomized diabetic rats relative to sham group. In parallel with these changes, 24-hour urinary albumin, ratio of kidney/body weight, Ccr, urine volume and podocyte excretion were significantly increased which might be an evidence of the possible involvement of miR-320 in podocytic adhesive capacity damage and perfectly explain the reason why sole silencing of miR-124 in diabetic rats did not re-establish the normal physiologic parameters and podocyte phenotype.

**Conclusion**

Our study demonstrates the potential relationship between miR-124 and podocytic adhesive capacity damage in STZ-induced uninephrectomized diabetic rats which made us better understand the possible mechanisms of podocyte detachment from GBM in DN and other glomerular diseases. In order to further validate miR-124 binding sites, co-transfections of Itga3 3′UTR luciferase reporter and expression vectors for miR-124 remain to be performed. Such studies should be significantly valuable, as they may suggest much more precise ways in which the podocyte-GBM interaction can be modulated for therapeutic benefit.

**Conflict of Interests**

The authors of this study state that they have nothing to disclose.

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