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Early molecular events in the development of the diabetic cardiomyopathy*

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Summary. Oxidative damage to DNA has been well documented in cardiac cells isolated from diabetic patients and rats with streptozotocin-induced diabetes mellitus (DM). This study evaluates possible molecular mechanisms for early events in the development of DM-induced cardiomyopathy.

Methods: To analyze the mechanism of overexpression of $p21^{WAF1/CIP1}$ and inhibition of cyclin D_1 expression in cardiomyocytes of diabetic rats we examined the methylation status of these genes by MS-PCR and assessed the possibility of epigenetic control of their expression.

Results: We found that the steady-state expression of both genes is influenced by their methylation status, as an epigenetic event, of their 5′-flanking regions upon development of diabetes.

Conclusions: Oxidative damage contributes to the development of cardiomyopathy via p53-dependent activation of cardiac cell death. This pathway includes *de novo* methylation of the P53-inducible $p21^{WAF1/CIP1}$-gene encoding a protein which binds to and inhibits a broad range of cyclin-cyclin-dependent kinase complexes.

Keywords: Diabetes mellitus – Cardiomyopathy – $p21^{WAF1/CIP1}$ – Cyclin D_1 – CpG Methylation – Gene regulation

Introduction

Heart rate variability is reduced in diabetics, suggesting the presence of abnormalities in neural regulatory mechanisms. More than 50% of patients with type 2 diabetes have coronary heart disease, related to silent ischemia and autonomic denervation of the heart. Oxidative damage to DNA has been well documented in cardiac cells isolated from diabetic patients and rats with streptozotocin-induced diabetes mellitus (DM) (Frustaci et al., 2000; Murata et al., 1999). This well-characterized animal DM-model shows structural changes in vascular tissue typical for the development of atherosclerosis already seven days after onset of disease. Metabolic pathways regulating these pathologic cardiovascular events by DM are still unclear.

Streptozotocin (STZ) is known to induce insulin-dependent DM possibly via DNA damage in experimental animals (Murata et al., 1999). DNA damage of mammalian cells is controlled at "checkpoints" of the cell cycle in a p53-dependent manner. p53 acts as a transcriptional activator and induces the transcription of genes with p53 response elements. The known cdk inhibitor $p21^{WAF1/CIP1}$ is one of these genes (Gartel et al., 1996; Mantel et al., 1999). Interaction of this protein with the cdk2/cyclin E complex is thought to inhibit progression of cells into S phase (Chen et al., 1996; Ogryzko et al., 1997). There is an excellent correlation between p53 expression status, capability for G_1 arrest, and $p21^{WAF1/CIP1}$ induction after treatment with DNA-damaging agents (Coffman and Studzinski, 1999; Stein et al., 1999). The balance of growth-stimulatory and growth-inhibitory signals regulates the transition between proliferation and quiescence (Pardee, 1992). In mammalian cells, passage from G_0 to G_1 is controlled by cyclin-dependent kinases (cdks) regulated by D-type cyclins. D-cyclins act as growth factor sensors, are induced as part of the delayed early response to growth factor stimulation, and assembled with cdk4 and cdk6 in a growth factor-dependent way. Among several cyclin-CDK complexes, cyclin D-dependent kinases play critical roles in regulating G_1 progression and entry into S phase.

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Whereas oxidative stress is provoked under diabetic conditions the implication of $p21^{WAF1/CIP1}$ in the glucose toxicity was examined and its induction by suppressing cell proliferation and insulin biosynthesis upon development of diabetes was shown (Kuan et al., 1998; Al-Douahji et al., 1999; Kaneto et al., 1999). The molecular mechanism of $p21^{WAF1/CIP1}$ induction upon development of diabetes is not completely undersood. For both $p21^{WAF1/CIP1}$ and cyclin D_1 methylation status of their CpG-islands, located in the promoter regions of these genes is known to have an important regulatory effect on the gene expression (Allan et al., 2000; Kitazawa et al., 1999). The mechanisms of gene silencing by methylated cytosin are varied among promoters. The most generally reported mechanism is the repression of transcription by methyl-CpG-binding proteins (MeCP1 and MeCP2) that bind DNA in a sequence independent manner: binding of methyl-CpG-binding proteins results in alternating the chromatin structure and preventing the transcriptional factors like Sp1 from DNA binding (Lewis et al., 1992; Tate et al., 1996; Simmen et al., 1999).

This study evaluates possible molecular mechanisms for early events in the development of DM-induced cardiomyopathy in view of the possible epigenetic control of the $p21^{WAF1/CIP1}$ and cyclin D_1 gene expression.

Materials and methods

Experimental Diabetes mellitus

All animals received care in accordance with the national guidelines for animal protection and were approved by the Animal Care and Use Committees of the General Hospital of Gent, Belgium. After an overnight fast, 6 pathogen-free female Wistar rats (Iffa Credo, Brussels, Belgium) weighting 200 to 250 g received a single intravenous injection of streptozotocin (Pfanstiel, Davenham, UK, 40 mg/kg) dissolved in citrate buffer (0.05 mol/L, pH 4.5). Six control nondiabetic animals received citrate buffer only. Animals with blood glucose levels greater than 20 mmol/L at 48 hours after streptozotocin injection were deemed diabetic. Before each experiment and sacrifice, the diabetic state was reconfirmed. The animals selected for study had blood glucose levels 20–25 mmol/L at all time points tested. Blood glucose was measured with an automated test strip employing the glucose oxidase (Gluco Touch, Lifescan, Beerse, Belgium) method. The rats were fed standard laboratory chow and were alowed free access to food and water in an air-conditioned room with a 12-hour light/12-hour dark cycle. All animals were sacrificed 6 weeks after streptozotocin injection. Myocardial tissue was snap frozen in liquid nitrogen.

Laser microdissection (LMD)

The frozen heart samples from both DM- and control-rats were used for LMD of cardiomyocytes with the "Microbeam-Moment"-technique as described earlier (Böhm et al., 1997). 5-μm-thick serial sections were cut, placed on numbered Superfrost/Plus slides (Fisher), and put on dry ice immediately. The numbered sections were air dried, fixed in 70% ethanol and washed with sterile water. A Laser-Pressure-Catapulting-device was used to collect specimen focusing onto the collection substrate using 40× magnifying objective. The laser cut tissue spots with cardiomyocytes were collected in sterile tubes, and used for chromosomal DNA isolation.

Isolation of chromosomal DNA was carried out from cardiomyocyte samples pooled from LCM using DNA isolation kit (Machery-Nagel, Düren, Germany, Cat. No. 740 952.50) according to the manufacturer's instructions. The amount of chromosomal DNA and its quality was detected using UV-spectrophotometer measurements.

Bisulfite DNA modification

DNA (1 μg) in a volume of 50 μl was denatured by NaOH (final concentration, 0.2 M) for 10 min at 37°C. Thirty microliters of 10 mM hydroquinone (Sigma, Deisenhofen, Germany) and 520 μl of 3 M sodium bisulfite (Sigma, Deisenhofen, Germany) at pH 5, both freshly prepared, were added and mixed; samples were incubated under mineral oil at 50°C for 16 hr. Modified DNA was purified using the silica-DNA-purification kit (AGS, Heidelberg) and eluted into 50 μl of sterile DNAse free water. Modification was completed by NaOH (final concentration, 0.3 M) treatment for 5 min at room temperature, followed by ethanol precipitation. DNA was resuspended in water and stored at −20°C before use for methylation-specific PCR (MS-PCR).

Primer design for MS-PCR

Primers for $p21^{WAF1/CIP1}$ (Table 1) were designed from its 120 bp 5′UTR sequence (Fig. 1) containing 15 CpG-islands (Allan et al., 2000).

Primers for cyclin D_1 (Table 1) were designed from the 525 bp 5′-flanking sequence (Fig. 4) rich in CpG-islands with putative Sp1-, CRE- and E2F-binding domains upstream of four transcription initiation start sites (Kitazawa et al., 1999).

Methylation-specific PCR (MS-PCR)

MS-PCR were perfomed as described previously (Herman 1996). Primer pairs (Table 1) were synthesized by MWG-Biotech

```
          10        20        30        40        50
5´-CGGTGTCCGGTCCCCGCTCGGCGGCGCGCCCTCGGGGACAGGGAGTCTGGG
   CGGTGTCCGGTCCCCGC
   sense primer (5´→3´)

          60        70        80        90        100
   CGGCCCTGTCCACCCTGCCAGCCCGGGAAGCGCAGAGGATCCCACCCACC
     ↑                   ↑              ↑
    HaeIII              SmaI           BamHI

        110       120
   GCGGCCCCACCACGACCCGG-3´
       GGGTGGTGCTGGGCC
       antisense primer (3´→5´)
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Fig. 1. CpG rich promoter sequence of the rat $p21^{WAF1/CIP1}$ gene. The first 25 nucleotides show the sense primer (5′ → 3′) and the final 29 nucleotides show the antisense primer (3′ → 5′) both representing methylated wild type chromosomal DNA. Restriction endonuclease recognition sites are shown in bold letters and arrows

Table 1. PCR Primers used for MS-PCR

Primer	Sense primer (5′ → 3′)	Antisense primer (3′ → 5′)	Size of PCR product (bp)	T_m (°C)	Pol. (sec)
$p21^{WAF1/CIP1}$-W	CGGTGTCCGGTCCCCGC	CCGGGTCGTGGTGGGCG	120	61	15
$p21^{WAF1/CIP1}$-M1	CGGTGTTCGGTTTTCGT	CCGAATCGTAATAAACG	120	46	15
$p21^{WAF1/CIP1}$-M2	TGGTGTTTGGTTTTTGT	CCAAATCATAATAAACA	120	41	15
cyclin D_1-W	GTGTTGATGAAATTGAAAGAAGTCG	ACTTTGCAACTTCAACAAAACTCCCCTGT	525	61	15
cyclin D_1-M	GTGTTGATGAAATTGAAAGAAGTTG	ACTTTACAACTTCAACAAAACTCCCCTAT	525	61	15

Tm, melting temperature; *Pol.*, polymerization time; *W*, wild type; *M*, modification (s. Material and methods); *M1*, mild modification; *M2*, complete modification

(Ebersberg). The PCR mixture contained 1 × PCR buffer (16.6 mM ammonium sulfate/67 mM Tris, pH 8.8/6.7 mM $MgCl_2$/10 mM 2-mercaptoethanol), dNTPs (each at 1.25 mM), primer pairs (100 pM each per reaction), and intact (unmodified DNA (50 ng) or bisulfite-modified DNA (50 ng) in a final volume of 50 μl. PCR specific for unmodified DNA also included 5% dimethyl sulfoxide. Reactions were hot-started at 95°C for 5 min before the addition of 1.5 units of *Taq* polymerase (Red-hot, ABgene, Hamburg, Cat. No. AB-0406/B) at the annealing temperature (Table 1) followed by the polymerization at 72°C for 1 min. Amplification was carried out in a Perkin Elmer temperature cycler for 35 cycles (denaturation for 45 sec at 95°C, annealing for 45 sec at the annealing temperature listed in Table 1, and polymerization at 72°C for the time listed in Table 1), followed by a final 4 min extension at 72°C. Negative controls without DNA as well as with unmodified DNA used in the reaction with primers designed for modified DNA were performed for each set of PCR experiments. PCR products (50 μl) were directly loaded onto 3% agarose gels ("Wide Range"-Agarose for analysis of DNA fragments longer than 50 bp, Sigma), stained with ethidium bromide after electrophoresis, and directly visualized under UV illumination.

Restriction analysis

PCR products underwent a phenol/chlorophorm extraction and ethanol precipitation before digestion. They were digested in a final volume of 50 μl with 20 units of each restriction endonuclease for 4 hr, according to conditions specified by the manufacturer (La Roche, Switzerland). For digestion of the cyclin D_1 PCR product endonucleases *Hpa*II, *Alu*I and *Hae*III were used. For the digestion of the $p21^{WAF1/CIP1}$ PCR product endonucleases *Bam*HI, *Sma*I, *Hae*III were used. Digested DNA fragments were directly loaded onto 3% agarose gels ("Wide Range"-Agarose for analysis of DNA fragments longer than 50 bp, Sigma), stained with ethidium bromide after electrophoresis, and directly visualized under UV illumination.

Results

Substantial changes in the methylation status of the $p21^{WAF1/CIP1}$-gene and the cyclin D_1-gene have been detected in DM-rats (Fig. 2 and 5).

MS-PCR performed with the primer pairs designed to the completely methylated and mild methylated sequences of the $p21^{WAF1/CIP1}$-gene (Table 1) show no products after amplification of the bisulfite modified chromosomal DNA from DM-rats, whereas the MS-PCR with the primer pair designed to the completely demethylated sequence results in a single product of 120 bp (Fig. 2A). The parallel MS-PCR with control samples shows a single product of 120 bp with the primer pair designed to the completely methylated sequence (Fig. 2B). Both products of 120 bp of the amplification with DM- and control-samples underwent restriction analysis (Fig. 3) confirming the amplification of the target DNA fragment with the sequence shown in Fig. 1.

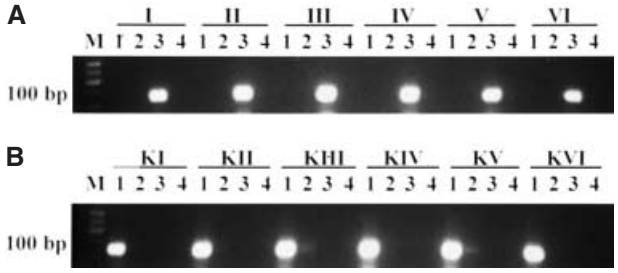

Fig. 2. MS-PCR of the CpG rich promoter region of the rat p21$^{WAFI/CIPI}$ gene. Primers used for MS-PCR are shown in Table 1. The PCR product is analysed electrophoretically in 3% agarose gels; **A** the 120 bp p21$^{WAFI/CIPI}$ PCR product from chromosomal DNA of 6 diabetic rats (*I–VI*); M = 50 bp DNA ladder (GeneRuler, MBI Fermentas), lane 1 = the methylated 120 bp PCR product, lanes 2 + 3 = the unmethylated 120 bp PCR product, and lane 4 negative control of the amplification (unmodified chromosomal DNA with primer set designed to modified unmethylated DNA); **B** the 120 bp p21$^{WAFI/CIPI}$ PCR product from chromosomal DNA of 6 control rats (*KI–KVI*); M = 50 bp DNA ladder (GeneRuler, MBI Fermentas), lane 1 = the methylated PCR product, lanes 2 + 3 = the unmethylated PCR product, and lane 4 = negative control of the amplification (unmodified chromosomal DNA with primer set designed to modified unmethylated DNA)

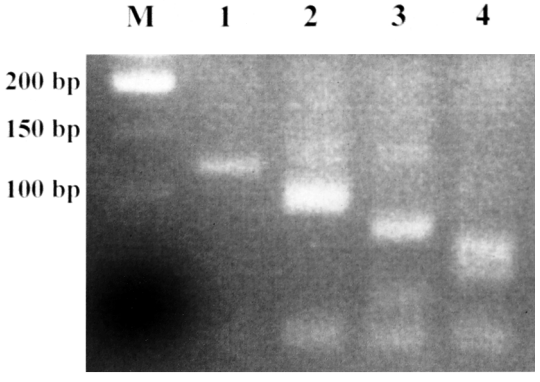

Fig. 3. Restriction analysis of the 120 bp rat p21$^{WAFI/CIPI}$ product of MS-PCR. The digested and non-digested PCR product is analysed electrophoretically in a 3% agarose gel. Lane M = 50 bp DNA ladder (GeneRuler, MBI Fermentas), lane 1 = the non-digested MS-PCR product (120 bp), lanes 2 − 4 = the PCR product digested with *Bam*HI (87 bp, 33 bp), *Sma*I (74 bp, 46 bp), and *Hae*III (67 bp, 53 bp) respectively

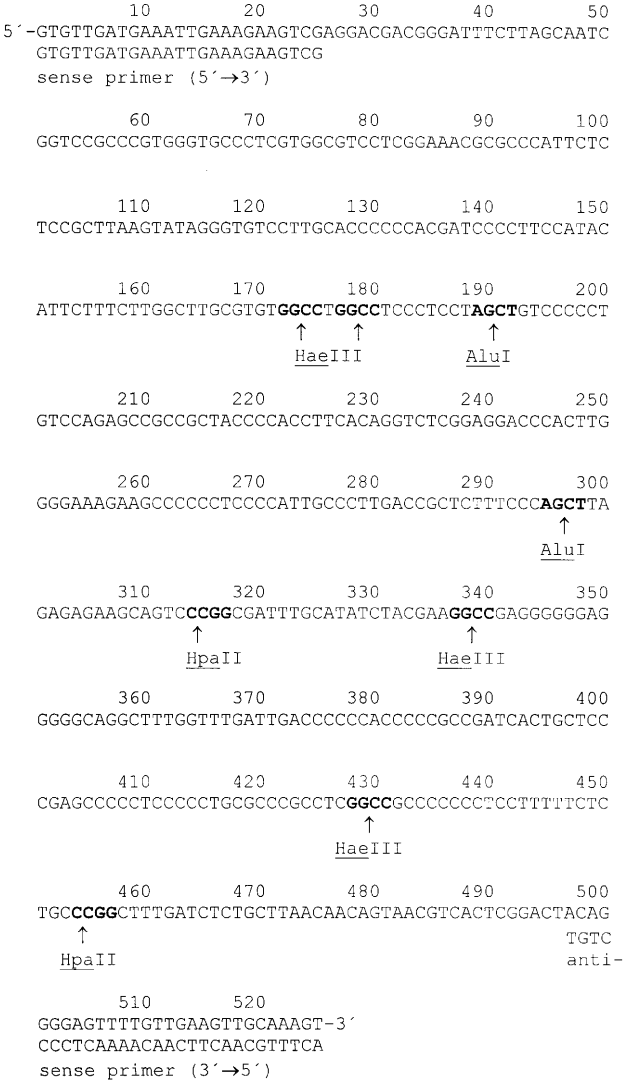

Fig. 4. CpG rich promoter sequence of the rat cyclin D_1 gene. The first 17 nucleotides show the sense primer (5′ → 3′) and the final 15 nucleotides show the antisense primer (3′ → 5′) both representing methylated wild type chromosomal DNA. Restriction endonuclease recognition sites are shown in bold letters and arrows

MS-PCR performed with the primer set designed to the demethylated sequences of the cyclin D_1-gene (Table 1) show no products after amplification of the bisulfite modified chromosomal DNA from DM-rats whereas the MS-PCR with the primer set designed to the completely methylated sequence result in a single product of 525 bp (Fig. 5A). The parallel MS-PCR with control samples show a single product of 525 bp with the primer pair designed to the demethylated sequence (Fig. 5B). Both products of 525 bp underwent restriction analysis (Fig. 6) confirming the amplification of the target DNA fragment with the sequence shown in Fig. 4.

Discussion

The renin-angiotensin system is upregulated in diabetes mellitus, and may contribute to the development of cardiomyopathy. Angiotensin II together with the high synthesis of NO may lead locally to oxidative damage, activating cardiac cell death via a p53-dependent pathway (Frustaci et al., 2000).

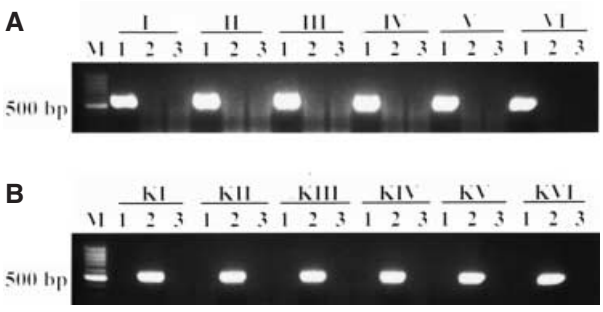

Fig. 5. MS-PCR of the CpG rich promoter region of the rat cyclin D_1 gene. Primers used for MS-PCR are shown in Table 1. The PCR product is analysed electrophoretically in 3% agarose gels; **A** the 525 bp cyclin D_1 PCR product from chromsomal DNA of 6 diabetic rats (I–VI); M = 50 bp DNA ladder (GeneRuler, MBI Fermentas), lane 1 = the methylated 525 bp PCR product, lane 2 = the unmethylated 525 bp PCR product, and lane 3 = negative control (unmodified chromosomal DNA with primer set designed to modified unmethylated DNA); **B** the 525 bp cyclin D_1 PCR product from chromosomal DNA of 6 control rats (KI–KVI); M = 50 bp DNA ladder (GeneRuler, MBI Fermentas), lane 1 = the methylated PCR product, lane 2 = the unmethylated PCR product, and lane 3 = negative control of the amplification (unmodified chromosomal DNA with primer set designed to modified unmethylated DNA)

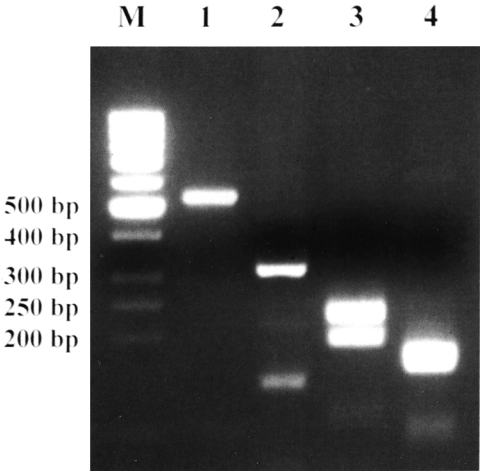

Fig. 6. Restriction analysis of the 525 bp cyclin D_1 product of MS-PCR. The digested and non-digested PCR product is analysed electrophoretically in a 3% agarose gel. Lane M : 50 bp DNA ladder (GeneRuler, MBI Fermentas), lane 1 = the non-digested MS-PCR product (525 bp), lane 2 − 4 = the PCR product digested with *Hpa*II (314 bp, 140 bp, 71 bp), *Alu*I (229 bp, 190 bp, 106 bp), and *Hae*III (173 bp, 160 bp, 96 bp, 91 bp, 5 bp) respectively

The mechanism of DNA damage induction by STZ was investigated *in vitro*, using human cell lines and ^{32}P-labeled DNA fragments isolated from human genes (Murata et al., 1999). STZ induced cellular DNA damage and apoptosis. DNA modification via a methylation activity of STZ to guanines has been suggested for the mechanism of DNA damage in experimental animals (Murata et al., 1999). Methylation of chromosomal DNA has generally important regulatory effects on gene expression, especially when involving CpG-islands located in the promoter regions of many genes (Bird, 1992). Considerable changes in the methylation status of CpG-islands have been detected by patients with transient neonatal diabetes (TND) within the TND critical region situated on chromosome 6 (Temple et al., 2000). TND is a rare type of diabetes that presents soon after birth, resolves by 18 months, and predisposes to diabetes later in life. Moreover, CpG motifs have been used in plasmid constructs for DNA vaccination of nonobese diabetic (NOD) mice spontaneously developing insulin-dependent diabetes mellitus, and have been shown to have an inhibitory effect on the development of diabetes (Quintana et al., 2000). The mechanism of the protection against diabetes by CpG oligonucleotides remains to be clarified. Nevertheless an important role of CpG methylation in the pathogenesis of NOD has been supposed.

Our results show that the pathomechanisms of STZ-induced diabetes mellitus include *de novo* methylation of genes. Complete methylation of the cyclin D_1-gene (inactivation of the gene) and complete demethylation of the P53-inducible $p21^{WAF1/CIP1}$-gene (activation of the gene) detected precisely using MS-PCR indicates rather the individual regulation of the methylation status of these genes by p53-dependent G_1 arrest due to DNA damage then an aberrant methylation due to the DNA modifying effect of STZ. The basic promoter structure of 5′-flanking region with a cAMP response element and two continuous Sp1-binding sites is crucial for the steady-state expression of cyclin D_1, a G_1/S cell cycle-regulating oncogene (Kitazawa et al., 1999). The methylation status especially close to Sp1-sites was found to be epigenetically important for the regulatory mechanism determinig the steady-state expression level in rat leukemic cells. Expression of D type cyclins is the earliest event in G_1 phase that leads to cell division (Sherr, 1993). Treatment with insulin of MCF-7 cells increased cyclin D_1, cyclin E and p21 gene expression, induced the formation of active Cdk4 complexes but resulted in only minor increases in cyclin E-Cdk2 activity, likely due to recruitment of the CDK inhibitor $p21^{WAF1/CIP1}$ into these complexes (Lai et al., 2001). $p21^{WAF1/CIP1}$ encoding a protein which binds to

and inhibits a broad range of cyclin-cyclin-dependent kinase complexes. Thereby, $p21^{WAF1/CIP1}$ is supposed to inhibit DNA synthesis preventing the replication of damaged DNA. Activated $p21^{WAF1/CIP1}$ inhibits DNA replication by its ability to bind proliferating cell nuclear antigen (PCNA), which is required for both replicative DNA synthesis and DNA repair (Waga et al., 1994; Li et al., 1994). However, $p21^{WAF1/CIP1}$ has no inhibitory effect on the DNA repair function of PCNA and may play a central role in preventing the replication of mutations incurred after exposure of cells to DNA damage (Allan et al., 2000).

This study shows that epigenetic control of the gene expression of both $p21^{WAF1/CIP1}$ and cyclin D_1 plays an important role for early events in the development of DM-induced cardiomyopathy.

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