Diabetic Microangiopathy in Ischemic Limb Is a Disease of Disturbance of the Platelet-Derived Growth Factor-BB/Protein Kinase C Axis but Not of Impaired Expression of Angiogenic Factors

Mitsugu Tanii, Yoshikazu Yonemitsu, Takaaki Fujii, Yasunori Shikada, Ri-ichiro Kohno, Mitsuho Onimaru, Shinji Okano, Makoto Inoue, Mamoru Hasegawa, Toshihiro Onohara, Yoshihiko Maehara, Katsuo Sueishi

Abstract—Diabetic foot is caused by microangiopathy and is suggested to be a result of impaired angiogenesis. Using a severe hindlimb ischemia model of streptozotocin-induced diabetic mice (STZ-DM), we show that diabetic foot is a disease solely of the disturbance of platelet-derived growth factor B-chain homodimer (PDGF-BB) expression but not responses of angiogenic factors. STZ-DM mice frequently lost their hindlimbs after induced ischemia, whereas non-DM mice did not. Screening of angiogenesis-related factors revealed that only the expression of PDGF-BB was impaired in the STZ-DM mice on baseline, as well as over a time course after limb ischemia. Supplementation of the PDGF-B gene resulted in the prevention of autoamputation, and, furthermore, a protein kinase C (PKC) inhibitor restored the PDGF-BB expression and also resulted in complete rescue of the limbs of the STZ-DM mice. Inhibition of overproduction of advanced-glycation end product resulted in dephosphorylation of PKC-α and restored expression of PDGF-BB irrespective of blood sugar and HbA1c, indicating that advanced-glycation end product is an essential regulator for PKC/PDGF-BB in diabetic state. These findings are clear evidence indicating that diabetic vascular complications are caused by impairment of the PKC/PDGF-B axis, but not by the impaired expression of angiogenic factors, and possibly imply the molecular target of diabetic foot. (Circ Res. 2006;98:55-62.)

Key Words: diabetic microangiopathy □ PDGF-BB □ PKC □ advanced-glycation end product □ pericyte

Critical limb ischemia is often caused by severe stenosis of feeder arteries or occlusion of a remaining below-knee artery. Bypass surgery and transluminal angioplasty are the efficient treatments of critical limb ischemia, and the focus of these interventions in patients with critical limb ischemia is to either restore arterial blood flow to at least 1 tibial vessel or to amputate the limb when tissue loss or pain becomes intractable.

Diabetes mellitus (DM), affecting 135 million people worldwide, is characterized by a chronic state of hyperglycemia. DM accompanies macro- and microangiopathy in multiple organs and is 1 of the major causes of morbidity and mortality of patients with the disease. Diabetic foot is an intractable disease categorized by DM-related vascular complications, and patients with it have a much higher risk of gangrene and amputation of the lower extremities. Collateral vessel development is insufficient to support the loss of blood flow through occluded arteries in patients with peripheral vascular disease, and the problem is exacerbated in DM patients. Furthermore, surgical and catheter interventions are usually difficult to treat limb ischemia of DM patients because vascular diseases are located at small vessels.

Therapeutic angiogenesis has been expected as a novel approach to treat patients with limb ischemia, including diabetic foot, without indication for bypass surgery or angioplasty. Current clinical results of therapeutic angiogenesis, however, have shown relatively limited outcome; therefore, further studies of the basic mechanisms of therapeutic angiogenesis should be performed.

With this point of view, we have performed some important studies assessing the levels and genes of angiogenic factors to be delivered to show the optimized therapeutic effect for murine and rabbit critical limb ischemia models using highly efficient gene transfer vector, namely recombinant Sendai virus. These studies revealed the following: (1) more than 2-fold overexpression of vascular endothelial growth factor (VEGF) compared with its baseline level accelerated limb loss, whereas, in contrast, basic fibroblast
growth factor (bFGF/FGF-2) gene transfer constantly showed therapeutic effect inducing morphologically matured capillary vessels; and (2) therapeutic effect of FGF-2 gene transfer depended on the harmonized induction of multiple endogenous angiogenic factors, including VEGF, as well as hepatocyte growth factor (HGF). In pericytes and stromal cells via PDGF-AA/PDGF-α-receptor (PDGFRα)/p70S6 kinase pathway. These results indicate FGF-2 gene transfer as a therapy via multiple angiogenic factors in vivo; however, no information is now available indicating whether or not these cascade-like system for therapeutic angiogenesis function under chronic diabetic state.

DM-related vascular complications have been widely explained by disorganized expression of angiogenic factors including VEGF. For instance, it has been demonstrated that advanced-glycation end products (AGEs) induced by chronic hyperglycemia regulates the expression of VEGF in retinal epithelial cells, suggesting the possible link between chronic DM state and hypervascularization of diabetic retinopathy. As reviewed in a recent article, however, DM is a paradoxical disease associated with hypervascularization in the retina and, inversely, with impaired collateral development in the ischemic limbs and hearts.

As such, because less information is available on the molecular mechanisms of the diabetic foot, particularly under limb ischemia, we investigated the essential factors related to angiogenic responses under chronic hyperglycemia using streptozotocin-induced diabetic mice (STZ-DM).

Materials and Methods

Reagents and Antibodies

The following intracellular signal inhibitors were dissolved at each concentration, which was previously demonstrated to sufficiently work in vivo: protein kinase C (PKC) inhibitor bisindolylmaleimide (bis-I) and its inactive control bis-V (100 mmol/L, Sigma-Aldrich, Tokyo, Japan); p70S6K inhibitor rapamycin (1 mmol/L, Sigma); and mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor U0126 (10 mmol/L; Promega, Madison, Wis). One hundred microliters of these inhibitors were intraperitoneally administered daily from day 0 to 28.

Enzyme-Linked Immunosorbent Assay

The protein contents in the limb muscles and culture medium were determined using Quantikine Immunoassay systems for human FGF-2 (available for both humans and mice; R&D Systems Inc, Minneapolis, Minn), murine VEGF-A (recognizes both 164 and 120 amino acid residue forms; R&D Systems), human PDGF-BB (specific for humans; R&D Systems), rat HGF (available as murine HGF; Institute of Immunology Inc, Tokyo, Japan), and murine PDGF-BB (BioSource International Inc, Camarillo, Calif) according to the instructions of the manufacturer, as previously described.

Real-Time PCR

The procedure was described previously. The total RNA was extracted from the ischemic limb muscles followed by treatment with RNase-free DNase I. The RNA was then reverse-transcribed and amplified with the TaqMan EZ RT-PCR kit and a Sequence Detection System, model 7000 (PE Biosystems, Foster City, Calif). The nucleotide sequences of the PCR primers and TaqMan probes are listed in the supplementary Table in the online data supplement available at http://circres.ahajournals.org. The murine GAPDH was used as the internal standard. The target quantity was determined from the relative standard curves constructed with serial dilutions of the control total RNA (PE Biosystems), according to the instructions of the manufacturer.

Transmission Electron Microscopy

Each harvested thigh muscle was fixed with 3% glutaraldehyde. After post fixation with 2% osmium tetroxide, the tissues were dehydrated in a graded series of ethanol and embedded in Epon 812. The ultra-thin sections were cut, stained with uranyl acetate, and dehydrated in a graded series of ethanol and embedded in Epon 812. The ultra-thin sections were cut, stained with uranyl acetate, and examined under a JEOL 1200 EX transmission electron microscope (Nippon Denshi Ltd, Tokyo, Japan) at 80 kV.

Western Blotting

Each harvested thigh muscle was homogenized, the supernatant was separated on a 10% SDS-PAGE, and the proteins were transblotted. After blocking using 3.0% nonfat dried milk, the membrane was reacted with anti–phospho-PKCs antibody (rabbit polyclonal, no. 9371, Sigma), which recognizes PKC-α, -β1, -βII; and anti–nonphospho-PKC-α antibody (rabbit polyclonal, P4434, Sigma).
Statistical Analysis

All data except for limb survival were expressed as mean±SEM and were analyzed by 1-way ANOVA with Fisher’s adjustment. For the survival analysis, the survival rate expressed by the limb salvage score was analyzed using the Kaplan–Meyer method.9–11 The statistical significance of the survival experiments was determined using the log-rank test. $P<0.05$ was considered to be statistically significant in all analyses.

Results

Tolerance Against Induced Hindlimb Ischemia Is Impaired in STZ-DM Mice

C57BL/6 mice were induced with DM by receiving an intraperitoneal injection of STZ (1.5 mg/body) for 5 days. In the following experiments, all animals were used after confirming significant upregulation of both the free blood sugar (pre-STZ: 161.4±17.2 mg/dL; 4 weeks later: 585.0±55.9 mg/dL) and HbA1c (pre-STZ: 1.78±0.9%; 4 weeks later: 5.20±1.0%) at 4 weeks.

We first discovered that the STZ-DM mice frequently lost their hindlimbs at various levels after surgically induced severe limb ischemia, whereas the non-DM mice did not. Quantitative analysis of the degree of autoamputation using the limb salvage score9–11 demonstrated impaired limb survival in the STZ-DM mice (Figure 1a).

FGF-2–Mediated Angiogenic Responses Are Not Impaired in STZ-DM Mice

Next, to investigate whether impairment of limb survival might be related to the expression of angiogenic factors, we examined the expression of typical angiogenic factors, namely FGF-2, VEGF, and HGF, following the administration of recombinant SeV-mediated murine FGF-2 gene transfer.9–11 Similar to our previous findings,9–11 a boost in FGF-2 resulted in the upregulation of endogenous VEGF and HGF, irrespective of the diabetic state (Figure 1b). Furthermore, the FGF-2 gene transfer prevented limb amputation in the STZ-DM mice by significantly restoring the blood flow (data not shown), suggesting that angiogenic responses were not seriously impaired in the STZ-DM mice.

PDGF-BB Is the Essential Factor That Determines the Tolerance Against Hindlimb Ischemia of STZ-DM Mice

To explain the causes of disturbed tolerance of limb ischemia in STZ-DM mice, we conducted an extensive assessment of the baseline gene expression of angiogenesis-related factors and their receptors. Quantitative analysis by real-time RT-PCR revealed that the PDGF-B gene expression was solely downregulated in the limb muscles of STZ-DM mice among the genes tested (VEGF-A and -C, HGF, FGF-2, PDGF-A and -B, angiopoietin-1 and -2) (Figure 2a), as well as their receptors (tie-2, flk-1, FGFR1, flt-4, PDGFRα, and -β) (data not shown). The disturbed expression of the PDGF-B gene in the STZ-DM mice was sustained after induced limb ischemia (Figure 2b), a finding that was not observed in the other genes tested (data not shown). These results possibly suggested that impaired expression of PDGF-BB might be a contributor for the diabetic vascular dysfunction, at least, in the case of STZ-DM mice, a relevant model for type 1 diabetes.

To confirm that the disturbed expression of PDGF-BB might be common in diabetic state, baseline expression mPDGF-BB protein in thigh muscles of a well-accepted model of type 2 diabetes, namely ob/ob mice, which are leptin-deficient C57BL6.17 Downregulated expression of PDGF-BB was evident in both DM mice, STZ-DM and ob/ob (Figure 2c, right graph), whereas the elevation of serum blood sugar and HbA1c in ob/ob mice was rather milder than those seen in STZ-DM mice (Figure 2c, left). These results suggest that the disturbed expression of PDGF-BB may be common in both types of diabetic states, at least, of C57BL6 strain.

Next we examined the impact of impaired expression of PDGF-BB on the ultrastructure of vessels in thigh muscles of STZ-DM mice. Electron microscopic examination occasionally demonstrated the apparent dissociation of pericytes from the capillary tube (Figure 2d, middle; 1 to 3 vessels/each tissue section containing 32 to 128 capillaries in 5 animals) in nonischemic limb muscles (left adductor muscles) of STZ-DM mice, a finding that was not seen at all in the
non-DM mice (5 animals; Figure 2d, left) as well as STZ-DM mice intramuscularly treated with plasmid DNA expressing human PDGF-B gene (pCEP4-hPDGFB, 5 animals; Figure 2d, right), suggesting that the impaired expression of PDGF-BB could be 1 cause for the morphological abnormality of the capillaries, namely, the pericyte loss seen in the PDGF-B–deficient mice. Inversely, these results suggest that the supplementation of PDGF-BB is sufficient to rescue the DM-based abnormality of ultrastructure of capillaries. Whereas irregular dilatation and microaneurysm formation can be observed in the capillaries of the brain of PDGF-B–deficient mice and the quadriceps muscles of diabetic individuals, it was not observed in the adductor muscles of the STZ-DM mice without any treatment (middle) or with intramuscular injection of plasmid DNA expressing human PDGF-B gene (right, pCEP4-hPDGFB). The dissociation of pericytes from the capillary channels were occasionally seen in the adductor muscles of the STZ-DM mice (middle, arrowheads) but not in those of the non-DM mice (left) as well as STZ-DM mice supplemented with human PDGF-B gene (right). PC indicates pericytes; EC, endothelial cells; RBC, red blood cells.

To investigate the functional role of the disturbed expression of PDGF-BB in STZ-diabetic mice, we performed a supplementation study by the plasmid-based intramuscular gene transfer of human PDGF-B (pCEP4-hPDGFB). The PDGF-B gene transfer at day −2 resulted not only in the sustained expression of human PDGF-B gene for at least 2 weeks (Figure 3a) but also in the complete prevention of autamputation in the STZ-DM mice (Figure 3b). Next, we performed another set of experiment assessing the effect of hPDGF-B gene transfer to the recovery of blood flow evaluated by LDPI. As shown in Figure 3c, preinjection of pCEP4-hPDGFB significantly improved the disturbed blood flow of the STZ-DM mice.
perfusion in STZ-DM mice, indicating that the supplementation of PDGF-BB was sufficient to restore the tolerance against hindlimb ischemia in STZ-DM mice.

**Spontaneous Phosphorylation of PKC Is the Determinant of Impaired Expression of PDGF-BB in STZ-DM Mice**

We further investigated the essential intracellular signals that are related to endogenous PDGF-B expression as well as limb salvage in diabetic mice. Among some of the inhibitors tested (MAPK: U0126; p70S6K: rapamycin; phosphatidylinositol 3-kinase: wortmannin; PKC: bis-I), only bis-I restored the PDGF-BB protein level in the ischemic muscles of diabetic mice, finding not seen in the use of a control compound (bis-V) (Figure 4a). The spontaneous phosphorylation of PKC-α+β (α>>β; assessed by RT-PCR, data not shown) and its inhibition by bis-I treatment at that concentration administered in vivo were confirmed by Western blotting (Figure 4b). Furthermore, the daily intraperitoneal administration of bis-I prevented the ischemic limb amputation of STZ-DM mice (Figure 4c), indicating that the PKC signal-transduction pathway is essential for PDGF-BB expression as well as for the tolerance against limb ischemia in the diabetic foot.

**AGE Is Important to Spontaneous Phosphorylation of PKC-α As Well As Impaired Expression of PDGF-BB in STZ-DM Mice**

A recent important study indicated that AGE directly activated PKC, particularly PKC-α, among various PKC isoforms in diabetic kidney, which could be inhibited by an AGE cross-breaker ALT-711 and an inhibitor of AGE formation, AMG.22 As the final assessment, we tested whether AGE/PKC-α pathway might be a major stream for PKC/PDGF-BB axis in STZ-DM mice.

One month after induced DM by STZ, plasma AGE level was significantly increased (non-DM versus DM: 1.8±0.1 versus 2.3±0.5 U/mL, n=10 and 9, respectively; \( P<0.05 \))
without significant alteration in free blood sugar and HbA1c (data not shown). The increase of AGE was significantly inhibited by AMG (1 g/L in drinking water during day 0 to 28) (Figure 5a). As expected, both the spontaneous phosphorylation of PKC-α and impaired expression of PDGF-BB

Figure 4. a, Effects of typical inhibitors for signal transduction (PKC: bis-I; phosphatidylinositol 3-kinase: wortmannin; p70S6K: rapamycin; and MAPK/MEK: U0126) on PDGF-BB expression in the thigh muscles of STZ-DM mice. The day before tissue sampling, 100 µL of inhibitor solution was intraperitoneally injected. The thigh muscles were subjected to the ELISA. A control compound for bis-I, bis-V, was also used. b, Effect of broad PKC inhibitor (bis-I) or its control compound (bis-V) on the PDGF-BB mRNA expression and confirmation of spontaneous PKC phosphorylation in thigh muscles in STZ-DM mice. The PKC inhibitor was intraperitoneally injected on the day before tissue sampling, and the thigh muscles were subjected to quantitative real-time RT-PCR (n=3, each group) or Western blot analysis, which was done in triplicate, and showed similar results. c, Limb prognosis curve according to the limb salvage score after intraperitoneal injection of bis-I, bis-V, or buffer (PBS) in STZ-DM C57/BL6 mice. These curves were obtained using the Kaplan–Meyer method, and the data were analyzed using the log rank test.

Figure 5. a, Bar graph indicating that daily and oral administration of aminoguanidine (AMG, 1 g/L, day 0 to 28) diminishes the increase of plasma AGE level in STZ-DM mice. #P<0.05, b, Effect of AMG on activation of PKC-α. The panels indicate the results of Western blot analysis for phosphorylated (pPKC-α) (top) and nonphosphorylated (bottom) PKC-α in thigh muscles of non DM or STZ-DM mice. The panels present the results of 3 animals in each group. The bar graph indicates the relative levels of pPKC-α/PKC-α in thigh muscles of STZ-DM mice by densitometric analysis standardized by that in non DM mice. AMG treatment significantly reduced the relative level of pPKC-α/PKC-α in STZ-DM mice. *P<0.01. c, Bar graph indicating that daily and oral administration of AMG attenuated the expression level of PDGF-BB protein in thigh muscles of STZ-DM mice. #P<0.05, *P<0.01.
protein were significantly restored by AMG treatment (Figure 5b and 5c), suggesting that impairment of PDGF-BB in diabetic state might be largely attributable to AGE/PKC-α-related pathway.

Discussion

The key observations made in this study are summarized as follows. (1) Although the STZ-DM mice showed disturbed tolerance against hindlimb ischemia, the baseline expression of angiogenesis-related genes as well as the FGF-2–mediated responses of the induction of VEGF and HGF were not impaired, except for the PDGF-B expression, suggesting that the angiogenic responses were preserved, even under the diabetic state. (2) The disturbed expression of PDGF-BB might relate to the morphological abnormality of the capillaries, namely, the drop off of pericytes from the endothelial tubes. (3) Supplementation of the PDGF-B gene expression was sufficient to prevent autoamputation of the ischemic limb of diabetic mice. (4) The PKC inhibitor was also effective in preventing autoamputation and restored the expression of endogenous PDGF-BB. (5) AGE is probably an upstream regulator of PKC/PDGF-BB axis. These findings are clear evidence that the PKC/PDGF-BB axis is essential to the formation of hyperglycemia-related vascular complications and imply that the PKC/PDGF-BB signal-transduction pathway could be a promising molecular target for treating DM microangiopathy.

In our current study, it was surprising for us that an approximately 40% to 50% reduction of PDGF-B expression (Figures 2a and 2c and 4a) was critical to inducing functional and morphological vascular changes, namely, the dissociation of pericytes from the capillaries in muscles, in STZ-DM mice. This seems to be paradoxical, because brain capillaries in PDGF-B−/− mice showed no significant reduction in the number of pericytes, unlike in the homozygous PDGF-B−/− mice.18,19 In a more recent study, however, both the PDGF-B−/+ and STZ-DM mice revealed not so frequent, but significant, reduction of the pericyte lining in the retinal capillaries, a finding that had been synergistically enhanced by the coexistence of low PDGF-B and DM,21 supporting our current findings. The absence of microaneurysm formation and the relatively small number of pericyte-dissociated capillaries in the current study, a subacute model (∼4 weeks), might be explained by the more recent study referred to above, in which a chronic model (∼6 months) was used.21

Although PKCs have been shown to be activated spontaneously under hyperglycemic conditions, and the favorable effects of PKC inhibitors against diabetic complications have been reported,23–26 very little knowledge is available regarding the target molecules that are downstream from PKCs in diabetic disease. An important advance of our current study, therefore, was in identifying PDGF-BB not only as an essential regulator for the function of capillary vessels but also as a target of spontaneously activated PKC in ischemic limbs under hyperglycemia. Our current study thus scientifically supports the results of a multicenter clinical trial indicating that treatment using gel that contained human recombinant PDGF-BB was highly effective (95% healing rate in 9 weeks) for treating diabetic ulcers in type 2 DM patients.27

Limitations of the current study include the fact that, even though AGE/PKC-α pathway was suggested to mediate the impairment of PDGF-BB expression in STZ-DM mice, a typical type 1 DM model, other PKCs may still be involved in diabetic vascular complications. Furthermore, we have not assessed yet whether disturbance of AGE/PKC/PDGF-BB is the common pathway in vascular disorder in any types of diabetes. We here demonstrated that a C57BL6-based well-accepted type 2 DM model, namely leptin-deficient ob/ob mice, exhibited disturbed expression of PDGF-BB as well (Figure 2c). A previous report, however, demonstrated a conflicting result to our current findings; baseline and ischemia-induced expression of VEGF was considerably disturbed in nonobese diabetic mice.28 In addition, our preliminary study using db/db mice, an alternative and well-used mouse model of type 2 DM, demonstrated very low level of PDGF-BB expression at baseline that was equal level to that seen in control db/+ mice (Y.Y. and T.F., unpublished data, 2005). These paradoxical results suggest that other factors, including genetic status, may also affect the angiogenic responses under DM state. Further extensive studies, therefore, should be carried out to determine the exact role of AGE/PKC/PDGF-BB in each type of diabetic state.

In addition to PKC/PDGF-BB axis, it has been revealed that AGE also impaired the angiogenic process via the other pathway. A recent important study by Tamarat et al using STZ-DM mouse model showed that AGE-related disturbance of ischemia-induced angiogenesis via inhibition of activity of matrix metalloproteinases (MMPs).29 Although the direct interaction system between PKC/PDGF-BB and MMPs has not been well understood, MMPs are possibly downstream players of PKC30 and PDGF-BB via membrane type 1–MMP, which activates MMPs.31 This notion may be reasonable, because data obtained in the current study showed the clear limb salvaging effect of PKC inhibitor as well as hPDGF-B gene transfer that could activate MMPs.

Over the last several years, we demonstrated that boost of FGF-2 constantly showed highest limb salvaging effect to non-DM mouse model of severe hindlimb ischemia9 and that such efficacy of FGF-2 was guaranteed by the downstream expression of VEGF and HGF, indicating that FGF-2 gene transfer is a multiple angiogenic therapy.9–11 Whether is FGF-2 sole therapy effective to diabetic foot too? It is still premature to draw the conclusion, but this may be possible because we obtained the data indicating the sustained upregulation of PDGF-BB by FGF-2 gene transfer in ischemic muscles of STZ-DM mice (data not shown). In turn, it has been demonstrated that the synergistic effect on vascular stability by a combination of PDGF-BB and FGF-2,32 suggesting that this combination may show far superior effect to diabetic foot to that by each sole therapy. Again, further study should be done to determine such synergism under diabetic state.

In conclusion, we demonstrated that disturbed tolerance against severe limb ischemia under hyperglycemia was solely attributable to the disturbance of the PKC/PDGF-BB axis, not of the angiogenic responses, and that the supplementation of
the PDGF-B gene expression was sufficient to prevent autoamputation caused by limb ischemia in STZ-DM mice. Therefore, PKC/PDGF-BB could be an attractive molecular target for treating intractable diabetic foot disease in patients with diabetic vascular complications.

Acknowledgments
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References
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**Supplementary Table.**

Sequences of primers and probes used for real-time RT-PCR

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### Supplementary Table

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**murine VEGFR3 amplicon size**: 109 bp
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- **probe**: 5’-FAM-TCCACCATCTCCGACAGATCC-TAMRA-3’

**murine Tie2 amplicon size**: 93bp
- **forward**: 5’-CCAACACGTAGTGCTGGCTCTATG-3’
- **reverse**: 5’-AGAGCTCGCGCAAGCGTC-3’
- **probe**: 5’-FAM-CTTAGGAGGCACCCCCCTACTGCAGGC-TAMRA-3’

**GAPDH amplicon size**: 117 bp
- **forward**: 5’-CCTGGAGAAAACCTGCCAAGAT-3’
- **reverse**: 5’-TTGAGAAGTCGAGAGACACCT-3’
- **probe**: 5’-FAM-TGCGCTTCCACCCCCTTCTGATG-TAMRA-3’