

## Original Article

# Modulated expression levels of tyrosine kinases in spontaneously developed melanoma by single irradiation of non-thermal atmospheric pressure plasmas

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**Abstract:** Development of therapy for melanomas without BRAF<sup>V600E</sup> mutation, which account for about half of all melanomas, is an urgent issue because effective therapy is currently being developed for patients who have melanomas with BRAF<sup>V600E</sup> mutation. *RET*-transgenic mice (*RET*-mice) carrying the *RFP-RET* oncogene under the control of *metallothionein-I* promoter spontaneously developed skin melanomas without BraF<sup>V600E</sup> mutation from benign melanocytic tumors. We previously showed decreased expression levels of cell cycle regulators and matrix metalloproteinases in melanoma from *RET*-transgenic mice by single irradiation of non-equilibrium atmospheric pressure plasmas (NEAPPs). In this study, we focused on *RFP-RET*, *c-Ret*, Epidermal growth factor receptor (*Egfr*), Vascular endothelial growth factor receptor 2 (*Vegfr2*) and *c-Src* kinases, which are correlated with melanoma. We first confirmed significantly increased transcript expression levels of the 5 kinases in melanomas compared to those in benign tumors in *RET*-mice. We then found that transcript expression levels of *c-Ret* and *Egfr*, but not those of *RFP-RET*, *Vegfr2* and *c-Src*, were significantly decreased by single irradiation of NEAPP. Since EGFR-mediated promotion of melanoma has been reported, we further focused on the mechanism of NEAPP-mediated decrease in the level of *Egfr*. Transcript expression level of *Y box protein 1 (Ybx1)*, but not those of *p53*, *Early growth factor 1 (Egr1)*, *GC-rich sequence DNA binding factor 2 (Gcf2)* and *Kluppel-like factor 10 (Klf10)*, was significantly decreased by single irradiation of NEAPP. These results suggest that NEAPP decreased *Egfr* expression level through decrease of *Ybx1* expression. Our results indicate that NEAPP irradiation to melanoma without BRAF<sup>V600E</sup> mutation is a possible novel therapy.

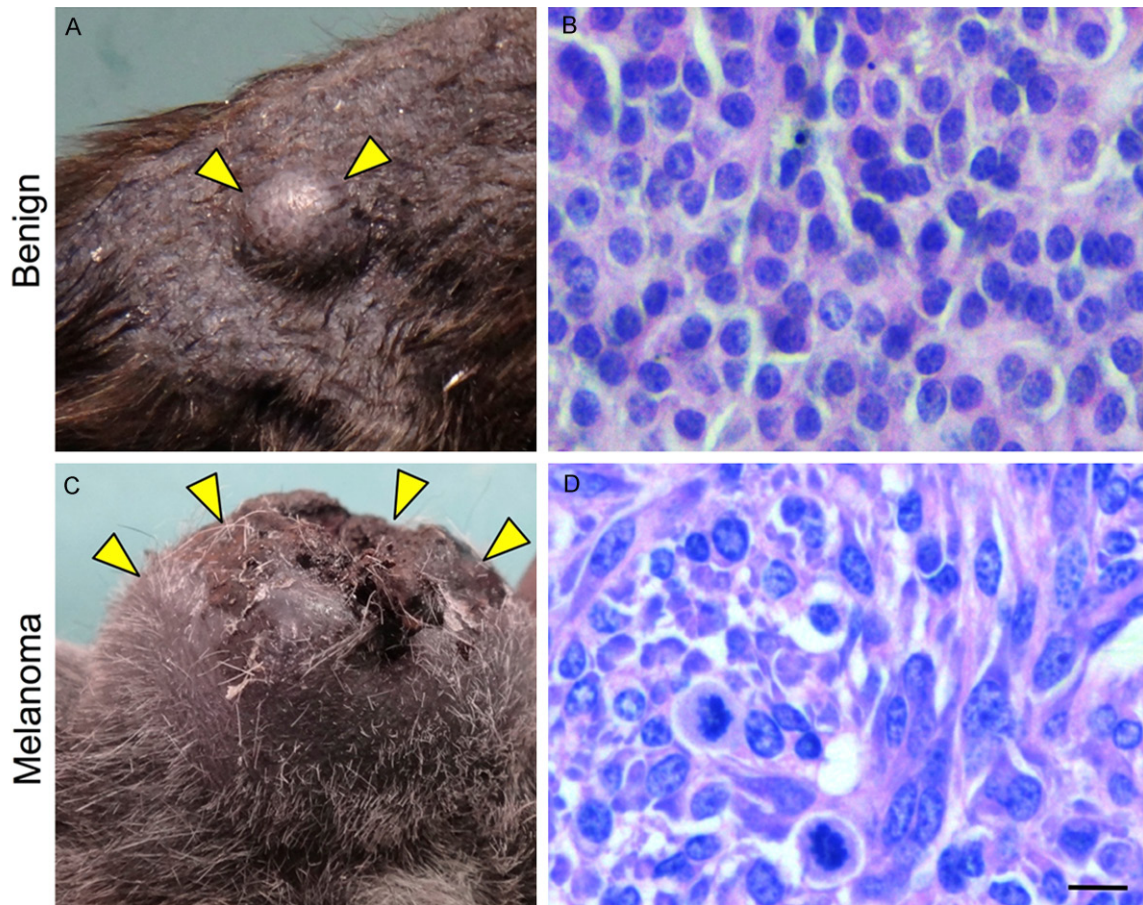
**Keywords:** Non-thermal atmospheric pressure plasmas (NEAPPs), melanoma, tyrosine kinase, Epidermal growth factor receptor (*Egfr*), *Y box protein 1 (Ybx1)*

## Introduction

The worldwide incidence of melanoma, the most aggressive cutaneous cancer, has recently become higher than that of any other cancer. Recent molecular-targeted therapy for melanomas with BRAF<sup>V600E</sup> mutation is developing in humans despite recurrence of the melanomas [1]. In fact, the prognosis of patients who have melanoma with BRAF<sup>V600E</sup> mutation is better than that of patients who have melanoma with-

out BRAF<sup>V600E</sup> mutation [2]. Therefore, the development of a novel therapy for melanomas without BRAF<sup>V600E</sup> mutation, which account for about half of all melanomas, is an urgent issue.

Previous studies showed that protein tyrosine kinases (PTKs) play a crucial role in various cancers including melanoma. *c-Ret/RET* protein is a receptor-type PTK and is activated by dimer formation through its ligands such as a glial cell line-derived neurotrophic factor (GDNF) [3, 4].

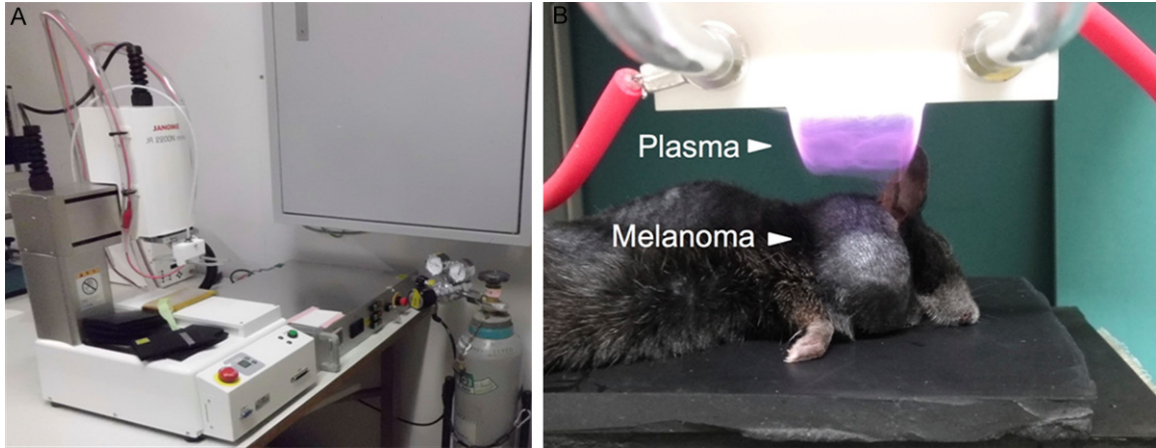


**Figure 1.** Macroscopic and microscopic appearances of tumors in RET-mice. (A-D) Representative macroscopic (A, C) and microscopic (B, D) appearances of a cutaneous benign melanocytic tumor (A, B) and melanoma (C, D) in RET-mice at 4 months and 12 months of age, respectively, are presented. Crusts covered the ulcer on the surface in melanoma (C) but not in the benign melanocytic tumor (A) in RET-mice. Arrowheads indicate a benign melanocytic tumor (A) and melanoma (C). Hematoxylin and eosin staining was used for histopathological analysis (C, D). Scale bar: 10  $\mu$ m (D).

We have shown that anchorage-dependent growth of human melanoma cells was increased by interaction of c-RET and GDNF after confirming c-RET expression levels in plural human melanoma cell lines [5]. c-SRC protein, which is expressed in melanoma, is directly associated with c-RET protein and regulates the function of c-RET kinase [6]. *RFP-RET* of the hybrid gene from c-RET and *RFP* has an oncogenic activity and is constitutively activated without stimulation of ligands [3, 7]. *RFP-RET*-transgenic mice (RET-mice) carrying *RFP-RET* under the control of *metallothionein-I* promoter spontaneously develop benign melanocytic tumors and melanomas without *Braf*<sup>FV600E</sup> mutation in a stepwise manner [7, 8]. Increased expression levels of epidermal growth factor receptor (Egfr) and vascular endothelial growth

factor receptor 2 (Vegfr2) in human melanoma have been reported [9, 10]. These results suggest that *RFP-RET*, c-RET, Egfr, Vegfr2 and c-Src molecules are involved in the pathogenesis of melanoma.

Attention has recently been paid to medical applications of non-thermal atmospheric pressure plasmas (NEAPPs) consisting of an ionized gas. Previous studies showed anti-cancer effects of NEAPP irradiation *in vitro* [11-13]. We have also reported suppressed growth of benign melanocytic tumors in RET-mice by repeated NEAPP irradiation via decreased expression levels of cell cycle regulators and matrix metalloproteinases (MMPs) [14-16]. In our previous study, we showed an anti-cancer effect of single NEAPP irradiation to melano-



**Figure 2.** Non-thermal atmospheric pressure plasma (NEAPP) irradiation. (A, B) Device used for NEAPP irradiation (A) and a bright field of NEAPP irradiation to a melanoma in a RET-mouse (B) are shown. While Ar gas was provided through a tube, plasma in the main discharge region between two electrodes was excited by applying 10 kV from a 60 Hz commercial power supply. The distance between the two electrodes was 20 mm. Single irradiation for 30 sec was performed on malignant tumors from RET-mice. The RET-mouse was placed on a rubber plate to keep the distance from the plasma source at 13 mm.

mas in RET-mice via decreased levels of cell cycle regulators and MMPs [8]. In this study, we examined the effect of NEAPP irradiation on expression levels of RFP-RET, c-Ret, Egfr, Vegfr2 and c-Src in melanomas without BRAF<sup>V600E</sup> mutation from RET-mice.

### Materials and methods

#### *RET-mice spontaneously developed tumors in the skin*

Benign melanocytic tumors (**Figure 1A, 1B**) and melanomas (**Figure 1C, 1D**) developed spontaneously in our original RET-mice with an intact immune system [7, 8]. Therefore, RET-mice could be a strong tool to develop various therapies for melanomas [17, 18]. Experiments using recombinant DNA were approved by the Recombination DNA Advisory Committee of Nagoya University (no. 12-39, 13-59, 13-76). The animal experiments were approved by the Animal Care and Use Committee of Nagoya University (approval no. 27241).

#### *NEAPP irradiation to tumors in RET-mice*

Benign melanocytic tumors and melanomas with or without NEAPP irradiation in RET-mice were used. The NEAPP device used in the present study is shown in **Figure 2A**. Melanomas were collected and analyzed 6 hours after single NEAPP irradiation for 30 sec for melanoma

in RET-mice (**Figure 2B**) following the method previously described [8].

#### *Quantitative PCR (Q-PCR)*

After extracting total RNA from tumors of RET-mice, transcript expression levels were measured by the method described previously [14, 16]. Sequences of primers used are shown in [Supplementary Table 1](#).

### Results

#### *Diagnosis of tumors in RET-mice*

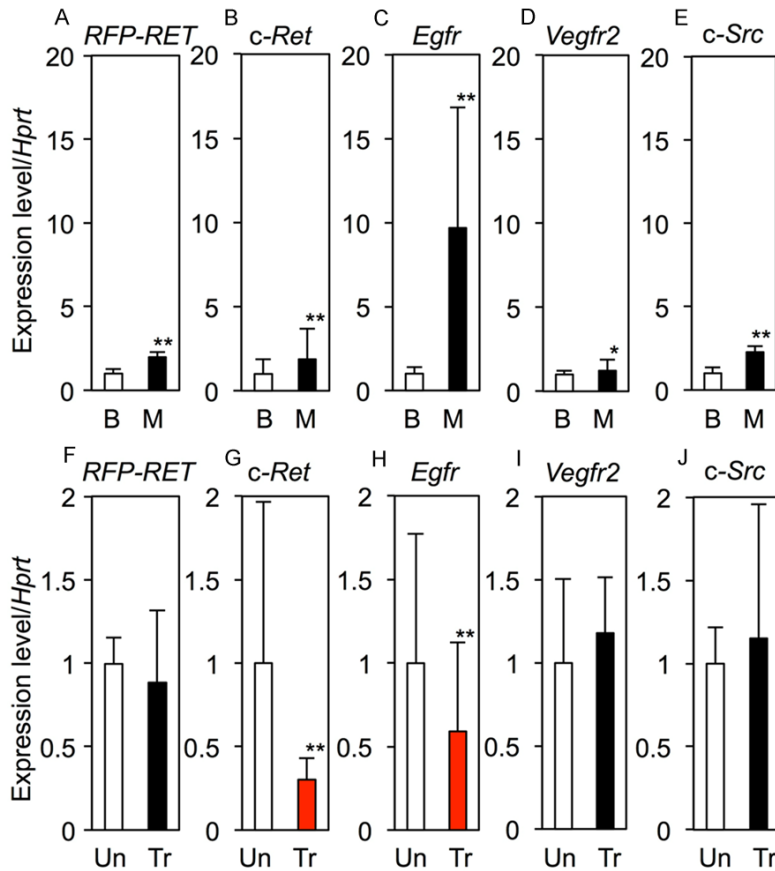
Benign melanocytic tumors and melanomas in RET-mice were preliminary selected by macroscopic observation (**Figure 1A, 1C**). After collection of tumors with or without NEAPP irradiation, all of the tumors were histopathologically diagnosed by a trained pathologist (**Figure 1B, 1D**). A benign melanocytic tumor consisted of uniform cells having round nuclei without mitosis (**Figure 1B**). Melanoma consisted of atypical cells having various sizes and shapes of nuclei with high mitotic activity (**Figure 1D**).

#### *Effects of single NEAPP irradiation on expression of 5 tyrosine kinases*

Transcript expression levels of RFP-RET, c-Ret, Egfr, Vegfr2 and c-Src in melanomas were 2.0-fold, 1.9-fold, 9.7-fold, 1.2-fold and 2.3-fold higher than those in benign melanocytic



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**Figure 3.** Transcript expression levels of *RFP-RET*, *c-Ret*, *Egfr* and *Vegfr2* in tumors from RET-mice. (A-E) Relative transcript expression levels (means  $\pm$  SD) of *RFP-RET* (A, *RFP-RET*), *c-Ret* (B), *Epidermal growth factor receptor* (C, *Egfr*), *Vascular endothelial growth factor receptor 2* (D, *Vegfr2*) and *c-Src* (E) in benign melanocytic tumors (B, n=6) and melanomas (M, n=5) from RET-mice are shown. (F-J) Relative transcript expression levels of *RFP-RET* (F), *c-Ret* (G), *Egfr* (H), *Vegfr2* (I) and *c-Src* (J) in untreated (Un, n=5) and NEAPP-treated (Tr, n=6) melanomas from RET-mice are presented. The transcript expression levels were determined by quantitative PCR and normalized with the transcript expression level of *hypoxanthine ribosyltransferase* (A-J, *Hprt*). Statistical significance was evaluated by Student's *t*-test. \*\* $P < 0.01$ , \* $P < 0.05$ .

tumors, respectively, in RET-mice (**Figure 3A-E**). Single NEAPP irradiation decreased expression levels of *c-Ret* and *Egfr* transcripts by 70% and 41%, respectively (**Figure 3G, 3H**). However, the expression levels of *RFP-RET*, *Vegfr2* and *c-Src* transcripts were comparable in single NEAPP irradiated- and unirradiated-melanomas in RET-mice (**Figure 3F, 3I, 3J**).

### Effects of NEAPP irradiation on expression of transcription factors for *Egfr*

Effects of single NEAPP irradiation on transcript expression levels of *Y box protein 1* (*Ybx1*), *p53*, *Early growth factor 1* (*Egr1*), *GC-rich sequence DNA binding factor 2* (*Gcf2*) and

*Kluppel-like factor 10* (*Klf10*) in melanomas from RET-mice were further examined. Single NEAPP irradiation decreased the expression level of *Ybx1* transcript by 27% (**Figure 4A**). However, there were comparable expression levels of *p53*, *Egr1*, *Gcf2* and *Klf10* transcripts in melanomas from RET-mice despite single NEAPP irradiation (**Figure 4B-E**).

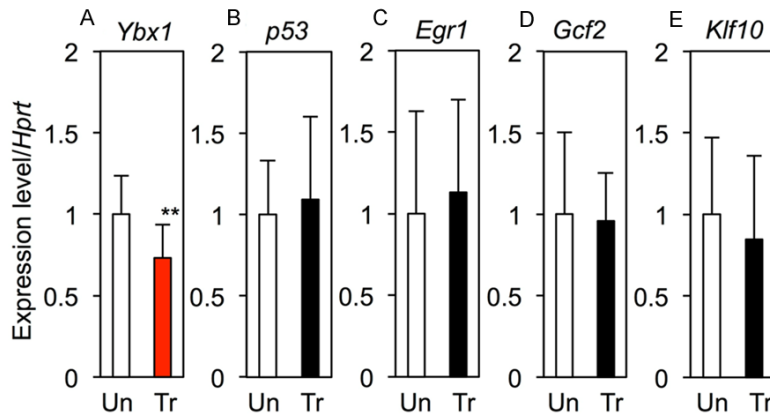
### Discussion

In this study, we first confirmed higher expression levels of *RFP-RET*, *c-Ret*, *Egfr*, *Vegfr2* and *c-Src*, all of which were previously reported to be associated with melanoma [5, 6, 9, 10], suggesting that the kinases are also associated with melanomas in RET-mice. Then we demonstrated for the first time that expression levels of *c-Ret* and *Egfr* transcripts, but not those of *RFP-RET*, *Vegfr2* and *c-Src* transcripts, in melanomas from RET-mice were decreased by single NEAPP irradiation. Our results showed that the difference in *Egfr* expression level between benign melanocytic tumors and

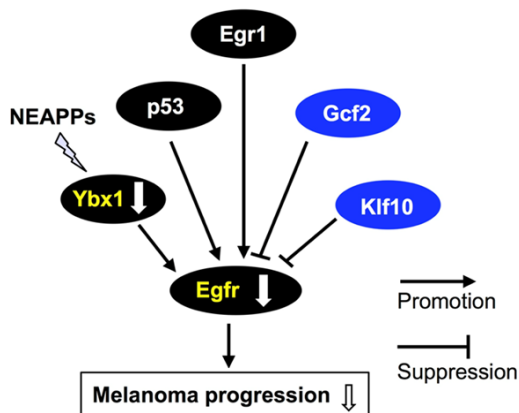
melanoma is the largest among the 5 kinases. Moreover, the effects of endogenous *c-Ret* may be replaced by the effects of the introduced *RFP-RET* in RET-mice. In fact, kinase activity of *c-Ret* was found to be lower than that of *RFP-RET* in RET-mice in our previous studies [3, 4, 19]. Together with a previous report showing that EGFR regulates progression and metastasis of human melanoma [20], we focused on *Egfr* as a representative molecule that was decreased by single NEAPP irradiation.

*YBX1*, *p53* and *EGR1* bind to the promoter of the *EGFR* gene and positively control its transcription in human osteosarcoma, breast cancer and colon cancer cells [21-23]. *GCF2* and

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**Figure 4.** Transcript expression levels of *Ybx1*, *p53*, *Egr1*, *Gcf2* and *Klf10* in irradiated melanomas from RET-mice. (A-E) Relative transcript expression levels (means  $\pm$  SD) of *Y box protein 1* (A, *Ybx1*), *p53* (B), *Early growth factor 1* (C, *Egr1*), *GC-rich sequence DNA binding factor 2* (D, *Gcf2*) and *Kluppel-like factor 10* (E, *Klf10*) in untreated (Un; n=5) and NEAPP-treated (Tr, n=6) melanomas from RET-mice are presented. Statistical significance was evaluated by Student's t-test. \*\*P<0.01.



**Figure 5.** A schematic pathway for regulation of *Egfr* expression level. *Y box protein 1* (*Ybx1*), *p53* and *Early growth response 1* (*Egr1*) progressively regulate *Egfr* expression level. *GC-rich sequence DNA binding factor 2* (*Gcf2*) and *Kluppel-like factor 10* (*Klf10*) negatively regulate *Egfr* expression level. Our results suggest that single irradiation of NEAPP decreases *Egfr* transcript expression level via suppression of *Ybx1* transcript expression. Since *EGFR*, a regulator for melanoma progression, has been suggested to be a clinical target for melanoma therapy [20], NEAPP may be useful for melanoma therapy.

*KLF10* have been reported to be transcriptional repressors of the *EGFR* gene in human melanoma cells and breast cancer cells, respectively [24, 25]. Our results further showed that single irradiation decreased the transcript expression level of only *Ybx1* among the 5 transcription factors for *Egfr*. These results suggest

that single NEAPP irradiation decreases *Egfr* expression level via decreased level of *Ybx1*, a transcription factor promoting expression of *Egfr*, in melanomas without *Braf*<sup>V600E</sup> mutation from RET-mice (Figure 5). Since *EGFR* could be a clinically potential therapeutic target for melanoma [20], our results suggest that NEAPP irradiation is a novel option for therapy of melanoma without *BRAF*<sup>V600E</sup> mutation (Figure 5).

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### Disclosure of conflict of interest

None.

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**Supplementary Table 1.** Sequences of primers used in quantitative PCR

Gene	Strand	Sequence
<i>RFP-RET</i>	Forward	5' TCACAGGGGATGCAGTATCT 3'
<i>RFP-RET</i>	Reverse	5' CCTGGCTCCTCTTCACGTAG 3'
<i>c-Ret</i>	Forward	5' TGACATCAGCAAGGATCTGG 3'
<i>c-Ret</i>	Reverse	5' CCCATCGTCATACAGCAGTG 3'
<i>Egfr</i>	Forward	5' CAAGTGGATGGCTTTGGAAT 3'
<i>Egfr</i>	Reverse	5' TCCATCATAAGGCTTGACC 3'
<i>Vegfr2</i>	Forward	5' TAAAATCTTTGGTGGGAAGCC 3'
<i>Vegfr2</i>	Reverse	5' GGCCTTCCATTCTGTACCA 3'
<i>c-Src</i>	Forward	5' TCGTGAGGGAGAGTGAGAC 3'
<i>c-Src</i>	Reverse	5' GCGGGAGGTGATGTAGAAAC 3'
<i>Ybx1</i>	Forward	5' GGTGCAGGAGAGCAAGGTAG 3'
<i>Ybx1</i>	Reverse	5' TGCCATCCTCTCTAGGCTGT 3'
<i>p53</i>	Forward	5' TGTCACGCTTCTCCGAAGAC 3'
<i>p53</i>	Reverse	5' ATCGTCCATGCAGTGAGGTG 3'
<i>Egr1</i>	Forward	5' GAGCGAACAACCCTATGAGC 3'
<i>Egr1</i>	Reverse	5' TTTGGCTGGGATAAECTCGTC 3'
<i>Gcf2</i>	Forward	5' AACTTCCACAAGTGCCAAGG 3'
<i>Gcf2</i>	Reverse	5' GGAAACTTTTCTCGCCACTG 3'
<i>Klf10</i>	Forward	5' AGCAAGGGTCACTCCTCAGA 3'
<i>Klf10</i>	Reverse	5' TGAAAGGTTTTTCCCCTGTG 3'
<i>Hprt</i>	Forward	5' TATGTCCCCGTTGACTGAT 3'
<i>Hprt</i>	Reverse	5' CTTTGCTGACCTGCTGGATT 3'