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Original Article

Antioxidation and wound healing activity of a recombinant protein from *Penaeus monodon*

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Abstract

Pm-fortilin is a recombinant protein of *Penaeus monodon* expressed in *Escherichia coli*. Activities regarding anti-oxidation and wound healing of the purified *Pm*-fortilin were investigated *in vitro* using normal fibroblast cell line L929. Relative viabilities of the cells were determined after exposure to either hydrogen peroxide (0.5 mmol/l for 4 h) or UV radiation (366 nm for 20 min). Significantly decreased viability was observed for the UV exposed cells compared with those incubated with the peroxide. Interestingly, proliferation of the stress-induced cells was improved by *Pm*-fortilin treatment. The protein's curing effect was dose dependent. The scratch method was used in the wound healing test on L929 cells. The injured cells distinctively migrated if pre-incubation with *Pm*-fortilin was carried out. However, such migration ability was compromised if the injured cells were continually in contact with *Pm*-fortilin. The transcriptions of *sydecan-2*, *dkk1*, *β-catenin*, *lrp6*, and *bmi1* genes in MG63 and HOS cancerous cells were down-regulated following incubation with *Pm*-fortilin for 5 days compared to those transcribed by L929 cells. The results suggested that *Pm*-fortilin might be non-oncogenic and is premised for use as a protective or repair agent in topical pharmaceutical products.

Keywords: *Penaeus monodon*, *Pm*-fortilin, cell migration, wound healing, antioxidant

1. Introduction

Endogenous overproduction or incorporation of free radicals from the environment into the body results in cellular oxidative stress and are causative agents to various chronic and degenerative diseases, including skin aging (Uttara, Singh, Zamboni, & Mahajan, 2009). Antioxidants from natural sources are of interest recently since they may elicit lower risks of side effects (Balekar, Katkam, Nakpheng, Jehtae, & Srichana, 2012). Sericin from silk worms (Kitisin, Maneekan, & Luplertlop, 2013) and thymosin beta-4 ($T\beta_4$) (Kumar & Gupta, 2011) are examples of natural antioxidant proteins reported to date. Sericin is revealed to function as a strong free radical scavenger and an inhibitor of lipid peroxidation or reactions catalyzed by tyrosinase enzyme

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(Lucconi, 2012). Tβ4 can protect cells exposed to UVB (Ho, Chen, & Lee, 2010) and H_2O_2 (Kumar & Gupta, 2011) by stimulating superoxide dismutase and catalase activities and inactivating caspase-9 (Wei, Kim, & Gupta, 2012). The protein also regulates the expression of matrix metalloproteinases that help increase wound healing (Philp *et al.*, 2006).

A translationally controlled tumor protein (TCTP) from *Penaeus monodon* was initially isolated in our laboratory after the organism was infected by white spot syndrome virus (WSSV) (Bangrak, Graidist, Chotigeat, & Phongdara, 2004). The protein was later called *Pm*-fortilin. Evidence indicated that *Pm*-fortilin mediates stress response and was responsible for the host immune response by increased expression after the viral infection (Nupan, Phongdara, Saengsakda, Leud, & Loc, 2011; Panrat *et al.*, 2012; Tonganunt *et al.*, 2008). In *Fenneropenaeus indicus*, increased reactive oxygen species (ROS) levels in the mitochondria by a WSSV infection were eliminated by a TCTP of the organism which resulted in decreased apoptosis (Rajesh, Kamalakannan, & Narayanan, 2014). In *Eriocheir sinensis* (a Chinese mitten crab), A TCTP was involved in eradication of toxic Cu^{2+} ions coming in from the environment (Wang, Fang, Li, Wang, & Jiang, 2011). In a Chinese hamster ovary cell line, a TCTP increased by several folds following oxidative induced cellular stress (Nagano-Ito, Banba, & Ichikawa 2009). In Chinese herbal medicine, a topical formulation containing *Angelica sinensis* extract was applied in patients for treatment of a skin injury which resulted in increased expression of a TCTP in the patient (Hsiao, Hung, Tsai, & Chak, 2012). Based on these findings, this study aimed to evaluate the ability of *Pm*-fortilin as a protein-born antioxidant and wound healing agent using cell culture models.

2. Materials and Methods

2.1 Purification of *Pm*-fortilin from *Escherichia coli*

The gene of *Pm*-fortilin fused to 6x histidine tag (His-Tag) was expressed in Escherichia coli (*E. coli*) M15 according to our previous publication (Tonganunt *et al.*, 2008). The recombinant protein was purified using a sequential procedure. Cells of recombinant *E. coli* were harvested by centrifugation at 500g for 10 min. The cell pellet was lyzed by a few milliliters of lysis buffer (50 mmol/l NaH_2PO_4 pH 8.0, 300 mmol/l NaCl, 10 mmol/l imidazole, and 1 mmol/l phenylmethanesulfonyl fluoride [PMSF]). Then, the lysate was overlaid on a Ni–NTA chromatographic column (QIAGEN). After washing several times with washing buffer (50 mmol/l NaH_2PO_4, pH 8.0, 300 mmol/l NaCl and 20 mmol/l imidazole), the column was eluted with elution buffer (50 mmol/l NaH_2PO_4 pH 8.0, 300 mmol/l NaCl, 250 mmol/l imidazole, and 1 mmol/l PMSF) to obtain the recombinant protein. The presence of *Pm*-fortilin in the flow-through was ascertained by Western blotting using 12.5% sodium dodecyl sulfate polyacrylamide gel coupled with specific antibody against His-Tag. Concentrations of *Pm*-fortilin were quantitatively determined using the Bradford protein assay kit (Bio-Rad) in which bovine serum albumin was used to prepare the standard calibration curve.

2.2 Cells and culture conditions

L929 is a cell line of fibroblastic origin from ATCC. It was obtained as a gift from Dr. Teerapol Srichana, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10% antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) in a 5% CO_2 incubator at 37 °C. The cells were sub-cultured every 3-4 days.

Human cancerous cell lines, namely MG63 and HOS, were purchased from ATCC. The cells were routinely grown in α-minimum essential media, supplemented with 10% FBS and 10% antibiotics under an atmosphere of 5% CO_2 at 37 °C, and sub-cultured every 2-3 days. All media and chemicals used in the cell culture technique were obtained from Gibco.

2.3 Preparation of stress-induced cells and measurement of cell viability

A cell suspension of L929 at a density of $3x10^4$ cells/ml in complete DMEM medium was prepared. Each of 200 µl was separately seeded in a well of 96-well plates and grown overnight before induction or treatment. There were two types of stress-inducing factors: i) hydrogen peroxide (H_2O_2) at concentrations ranging between 0.1 and 1.0 mmol/l for 4 h of incubation and ii) UV radiation at 366 nm for 20, 40, and 60 min of exposure. The cells in the plates were divided into two subgroups, namely the pre-induction group and the pre-treatment group. In the former group, the cells were induced with each of the inducers, followed by treatment with *Pm*-fortilin at a concentration of 50 or 150 ng/ml for 24 h. In the later group, however, the cells were treated with *Pm*-fortilin at a defined concentration for 24 h and then induced with each of the inducers. Cell viability was then assessed using the methyl tetrazolium (MTT) method (Dash, Acharya, Bindu, & Kundu, 2008). The cells that were thoroughly incubated in complete DMEM medium were used as the controls. Results were calculated by comparing with the control and expressed as relative viability. Each experiment was performed in quadruplicate.

2.4 Wound healing ability

The monolayer cells of 90% confluence in a well of 24-well plates were pre-incubated with serum-free DMEM containing 150 ng/ml *Pm*-fortilin for 24 h. After gentle washing with phosphate buffered saline (PBS) solution, a linear wound was made on the pre-treated monolayer cells using a sterile pipette tip according to the scratch assay (Liang, Park, & Guan, 2007). Any cellular debris was removed by gentle washing with PBS. The scratched cells were post-cultured in either serum-free DMEM or serum-free DMEM containing 150 ng/ml *Pm*-fortilin for an additional 3 days. Cell migration around the wound was monitored on each of 3 consecutive days using a 4x-magnification light micro-scope (Olympus CK2). The cells cultured in serum-free DMEM for the whole experimental period were the controls.

Percent migration was calculated using the equation below (Balekar, Katkam, Nakpheng, Jehtae, & Srichana, 2012). The results are expressed as relative migration after normalization by the control.

$$\% \text{ Migration} = \frac{\text{Average distance of the scratch on day 0} - \text{Average distance of the scratch on day 1, 2 or 3}}{\text{Average distance of the scratch on day 0}}$$

2.5 Evaluation of oncogenic property of *Pm*-fortilin

It is important to verify the oncogenic properties of any test compound which elicits a growth promoting effect. To serve this objective, two cancerous cell lines (MG63 and HOS) and a normal cell line (L929) were employed and treated with *Pm*-fortilin. Real-time polymerase chain reaction (RT-PCR) technique was used. The transcriptions of genes associated with the cancerous phenotype of the Wnt signaling pathway, such as *β-catenin*, *lrp6*, and *bmi-1*, were investigated and compared to those of antioncogenic genes such as *dkk1* and *syndecan-2*.

2.5.1 Isolation of total RNA

The cells of MG63, HOS or L929 previously incubated with *Pm*-fortilin at a concentration of 150 ng/ml for 5 days were subjected to total RNA extraction using Trizol reagent (Invitrogen). The extraction procedures were in accord with the manufacturer's instructions. Purity and quantity of the resulting RNA were ascertained measuring the optical density at wavelengths of 260 and 280 nm.

2.5.2 Quantitative RT-PCR (qPCR)

The first cDNA strands were synthesized from 1 µg total RNA using oligo (dT) and reverse transcriptase Superscript III kit (Invitrogen), according to the manufacturer's instructions. qPCR was performed on a Light Cycler Nano Machine (Roche) using Brilliant II SYBR Green QPCR Master Mix (Stratagene). Sequences of the primers used are shown in Table 1. PCR was performed in a thermal cycle program of 40 cycles which consisted of denaturation at 95 °C for 15 sec, annealing at a defined temperature (Table 1) for 15 sec, and extension at 72 °C for 15 sec. The threshold cycle for any test gene was recorded and compared to that of the *gapdh* gene. Results were expressed as relative gene transcription in folds increment.

2.6 Statistical analysis

Data are expressed as means±standard deviation (SD). Student's t-test was used to compare two small data sets in which $P<0.05$ was considered statistically significant. SPSS software v. 16 was used for the statistical analysis.

3. Results and Discussion

3.1 Effects of *Pm*-fortilin on oxidative stress

Pm-fortilin is indicated as a multifunctional and highly conserved protein (Bommer & Thiele, 2004). Its over-expression is necessary to decrease oxidative stress attributed by infected microorganisms and to fight any invaders (Munirathinam & Ramaswamy, 2007; Nagano-Ito, Banba, & Ichikawa, 2009). To date, it is certain that increased cellular oxidative stresses are causes of various degenerative disorders in humans. This is the rationale to study antioxidant activities of natural compounds for prevention and treatment of diseases induced by oxidative stresses.

Table 1. Primer sequences used for RT-PCR technique.

Primer's Name	Primer sequence (5′→3′)	Tm (°C)	PCR product size (bp)
β-catenin	F: CACAAGCAGAGTGCTGAAGGTGC R: AAGGAGGCCTTCCATCCCTTC	55	189
lrp6	F: CAGGGTGGAATGAATGTGC R: GTGGATGGGAAGGATGATG	59	224
dkk1	F: CCGCGCCGGGAATCCTGTAC R: GCAGCGTTTTCGGCGCTTCC	59	179
syndecan-2	F: GCATGAGAAAGAAGGATGAAGG R: CTGCTATTCACAGAACACTGCA	59	311
bmi1	F: TGGAGAAGGAATGGTCCACTTC R: GTGAGGAAACTGTGGATGAGGA	55	222

In this research, *Pm*-fortilin was expressed as a recombinant protein fused to His-Tag in *E. coli*, and its potential as a natural antioxidant or an agent with wound healing ability was investigated.

The relative viabilities of L929 cells in the pre-induction group exposed to H_2O_2 and UV_{366} are shown in Figures 1a and 2a**.** The results indicated that H_2O_2 at a concentration of 1 mmol/l markedly induced cell death. Growth of the remaining viable cells was barely rescued and there was little recovery by the following incubation of 150 ng/ml *Pm*-fortilin. In contrast to those induced by using 0.1 and 0.5 mmol/l H_2O_2, their growth improved significantly after treatment by the protein. With the low toxic effect of 0.1 mmol/l H_2O_2, the curing effect of *Pm*-fortilin was found to be dose dependent**.** In addition, after exposure to UV_{366} radiation, the damaged cells actively proliferated following *Pm*-fortilin treatment. However, growth promoting activity of this protein was not consistent with the doses used. In fact, the extent of growth recovery was dependent on the duration of UV_{366} exposure and significantly increased by the 60-min contact time. The effect of UV_{366} on growth inhibition was apparent to be lower than by 1 mmol/l H_2O_2 for 4 h of incubation. Furthermore, the relative viabilities of L929 cells in the *Pm*-fortilin pre-treatment group that were subsequently challenged with H_2O_2 and UV_{366} are shown in Figure 1b and 2b. By *Pm*-fortilin pre-treatment, the numbers of viable cells were maintained after exposure to higher concentrations of H_2O_2. Growth of the cells in continual contact with 1 mmol/l H_2O_2 improved. The protein, at a concentration as low as 50 ng/ml, showed a cyto-protective effect on H_2O_2 induced toxicity. In addition, when *Pm*-fortilin was used at a low concentration of 50 ng/ml in pre-treatment, the cells were protected from the growth interfering effect of the UV_{366} radiation. As indicated by the constant viability, the cells still survived after exposure to the radiation for 60 min. Indeed, the growth inhibitory effect of UV_{366} could be avoided by *Pm*-fortilin pre-treatment. Results were evident that *Pm*-fortilin exhibited a growth promoting effect on L929 cells previously injured by H_2O_2

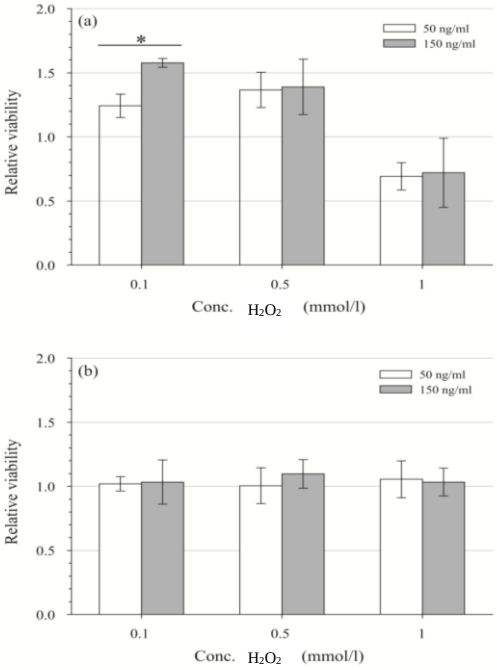

Figure 1. Effect of H_2O_2 on *Pm*-fortilin-treated cells. (a) Effect of *Pm*-fortilin on H_2O_2-induced cells: L929 cells grown in 96-well plate were induced with H_2O_2 of 0.1, 0.5 or 1.0 mmol/l for 4 h and subsequently treated with *Pm*-fortilin of 50 or 150 ng/ml for 24 h. (b) Effect of H_2O_2 on *Pm*-fortilin-treated cells: The cells in a 96-well plate were treated with *Pm*-fortilin of 50 or 150 ng/ml for 24 h and next induced with H_2O_2 of 0.1, 0.5, or 1.0 mmol/l for 4 h. For both conditions, cell viability was then assessed by MTT assay. Each experiment was performed in triplicate. The relative viabilities are mean±SD expressed by comparing with the results acquired for the controls. * indicates significantly different ($P<0.05$).

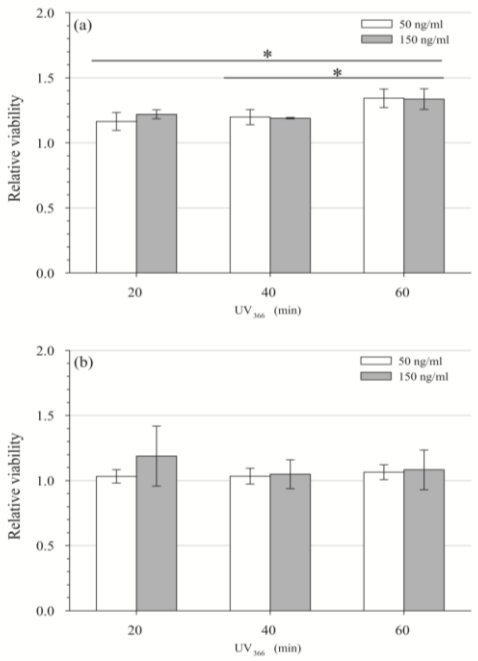

Figure 2. Effect of *Pm*-fortilin on UV_{366}-treated cells. (a) Effect of *Pm*-fortilin on UV_{366}-induced cells: L929 cells grown in a 96-well plate were induced with UV_{366} for 20, 40 or 60 min and subsequently treated with *Pm*-fortilin of 50 or 150 ng/ml for 24 h. (b) Effect of UV_{366} on *Pm*-fortilin-treated cells: The cells in a 96-well plate were treated with *Pm*-fortilin of 50 or 150 ng/ml for 24 h and next induced with UV366 for 20, 40 or 60 min. For both conditions, cell viability was assessed by MTT assay. Each experiment was performed in triplicate. The relative viabilities are mean± SD expressed by comparing with the results acquired for the controls. * indicates significantly different ($P<0.05$).

and UV_{366}. In addition, the cells were protected from these harmful factors if pre-treated by the protein. Clear demonstrations of the protective and therapeutic potential of *Pm*-fortilin were demonstrated. These results are consistent with those reported previously, which suggested that a recombinant TCTP from banana prawn (*Penaeus merguiensis*) elicited the capability to reduce apoptosis of HEMA-treated dental pulp cells (Kongsaengkaeo, Chotigeat, & Kedjarune-Leggat, 2013; Lucibello *et al.*, 2011). UV radiation is an external important factor, which can damage DNA and cause numerous cell deaths (Svobodova, Walterova, & Vostalova, 2006). Exposure to UV light may result in extremely increased ROS in cells, especially in keratinocytes of the skin (Lee, Wu, Hong, Yu, & Wei, 2013). Protection of UV-induced DNA damage by over-expressed TCTP was achieved by mechanisms associating p53 activation and inhibition of cytochrome c leakage (Zhang *et al.*, 2012). In fact, cytoplasmic TCTP needs to be trans-ferred to the cell nuclei for active functioning. To support this idea, parts of any TCTP have been identified to contain small ubiquitin-like modifier (SUMO) motifs, which act as responsive sequencing signals (Munirathinam & Ramaswamy, 2012). In this research, the putative SUMO motif of *Pm*-fortilin was also characterized which is located between amino acid residues of 82-85 (unpublished data). Therefore, *Pm*-fortilin may exert antioxidant activity by using a mechanism consistent with that previously described.

3.2 Effect of *Pm*-fortilin on cell migration

Due to the protective effect of *Pm*-fortilin on cellular oxidative stress, this prompted us to further investigate its benefit on wound healing. The skin contains the three layers of epidermis, dermis, and hypodermis. Most of the cells in the epidermis are keratinocytes. They originate from cells in the deepest layer of the epidermis called the basal layer. New keratinocytes slowly migrate up toward, and once they reach the surface of the skin they are gradually shed and replaced by newer cells pushed up from below. Fibroblasts are the main cells in the dermis, essentially located in the dermal papillae close to the epidermis. Below the dermis lies a layer called hypodermis or fat layer. Fat contained in fat cells helps insulate the body from heat and cold, provides a protective padding, and serves as an energy storage area. When the skin is broken, cascading biochemical events are instantly set in

motion to repair the damage. Although, fibroblasts begin to enter the wound site about two or three days after the wound occurs, their active proliferation is ultimately required for proper healing (Martin, 1997). In this study, the fibroblast cell line L929 was used to determine the wound healing ability of *Pm*-fortilin. The monolayer cells of L929 were incubated with 150 ng/ml *Pm*-fortilin for 1 day, and then damaged by scratching. The results of cell migration in regard to the presence/absence of *Pm*-fortilin in the culture medium are illustrated in Figures 3a and 3b. By continually growing the injured cells in *Pm*-fortilin-supplemented medium, their migration was significantly less active than the cells grown in *Pm*-fortilin-free medium, but was comparable to those thoroughly grown in FBS-free medium. On the other hand, the migration was inhibited if the protein was concurrently present in the cell culture. It was possible that inflammatory mediators released by the damaged cells might have impeded the wound healing ability of the protein (Balekar, Katkam, Nakpheng, Jehtae, & Srichana, 2012). More informative data are warranted by a future *in vivo* study of immune response. Indeed, our observation was consistent with previous studies that suggested up-regulation of TCTP to present a growth stimulatory effect during wound healing (Bae, Kim, Lee, & Lee, 2014; Hsiao, Hung, Tsai, & Chak, 2012).

3.3 The oncogenic property of *Pm*-fortilin

Lastly, an important question that must be answered when using a compound for wound healing activity is whether it is oncogenic. Increased transcription of genes associated with the Wnt/β-catenin signalling pathway is malignant to cells (Polakis, 2012). In the present work, the genes of interest for this pathway included *β-catenin*, *lrp6*, and *bmi1*. This is in contrast to the *syndecan-2* and *dkk1* genes, which when actively transcribed, are anti-oncogenic (Barbouri *et al.*, 2014). The normal cell line L929 and the two cancerous cell lines MG63 and HOS were employed in verification for the safety of *Pm*-fortilin. These cells were challenged with the protein for a few days before the transcriptions of the genes of interest were determined using the RT-PCR technique. *Syndecan-2* and *dkk1* are representatives of anti-oncogenes, whereas *β-catenin*, *lrp6*, and *bmi1* are the genes with oncogenic properties. The transcriptions of these genes in the L929 cells were approximately greater than those transcribed by the MG63 and HOS cells following incubation with *Pm*-fortilin for 5 days (Figure 4). In other words, the gene transcriptions were down-regulated in the MG63 and HOS cells. Furthermore, the gene transcriptions in the MG63 cells were less active compared to those of the HOS cells. The results indicated a lower tendency for *Pm*-fortilin to cause cancers.

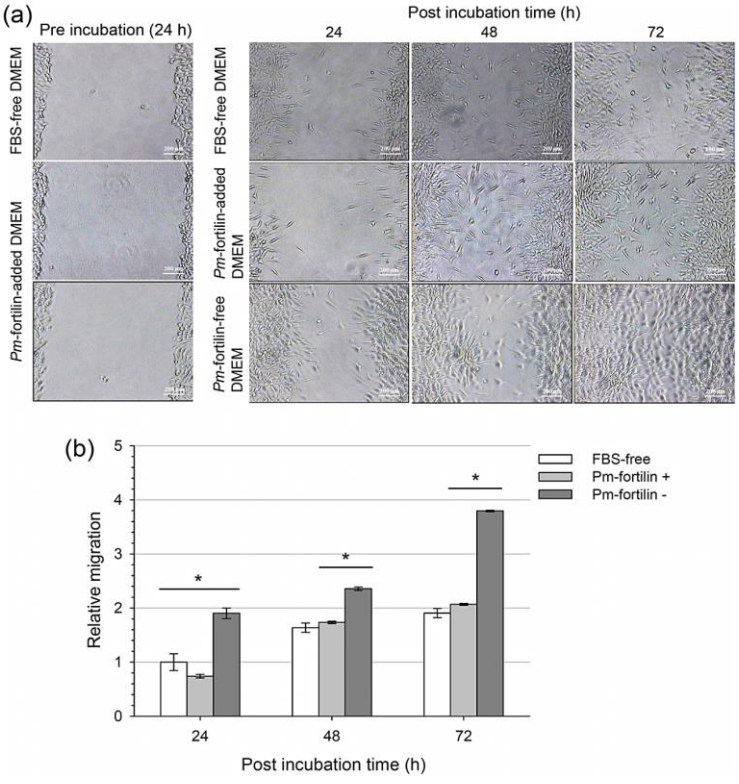

Figure 3. Effect of *Pm*-fortilin on cell migration. The monolayer cells of L929 at 90% confluence were grown in a 24-well plate and were pre-incubated with FBS-free DMEM containing 150 ng/ml of *Pm*-fortilin for 24 h. Then, a linear wound was made on the pre-treated well using a sterile pipette tip according to the Scratch method. The damaged cells were post-incubated in either FBS-free DMEM or FBS-free DMEM containing 150 ng/mL *Pm*-fortilin for another 72 h. In the control groups, the cells were absolutely grown in FBS-free DMEM for 4 days. (a) Cell migration was monitored at 24-, 48-, and 72-h post-incubation using a 4x-magnification light microscope, (b) The percent of cell migration was calculated (n=3). The relative migration was mean±SD expressed after normalization with the control. * indicates significantly different ($P<0.05$).

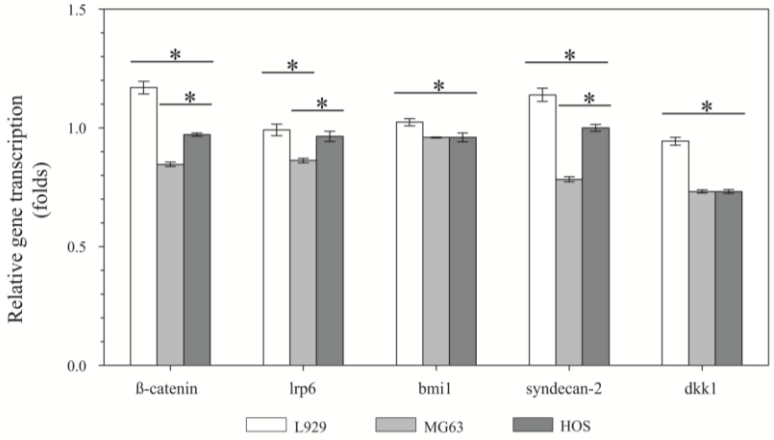

Figure 4. Relative transcriptions of genes, including *β-catenin, lrp6, bmi-1, syndecan-2,* and *dkk1* measured by the RT-PCR technique. The cells of L929, MG63, and HOS were incubated with 150 ng/ml *Pm*-fortilin for 5 days before extraction of total RNAs. The first cDNA strands were reversely transcribed from 1 µg total RNA using oligo(dT) primer. qPCR was performed on a LightCycler Nano Machine (Roche) using SYBR Green qPCR Master Mix (Stratagene). The threshold cycle for the gene of interest was recorded (n=3). Relative gene transcription (folds) are mean±SD expressed by comparison with the *gapdh* gene. * indicates significantly different ($P<0.05$).

4. Conclusions

It was evident in this study that *Pm*-fortilin elicited antioxidant and wound healing properties in response to oxidative stresses as a result of externally induced factors and cell injury. This protein shows promise for incorporation into pharmaceutical products for prevention or treatment or both of skin aging.

Acknowledgements

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