Master's thesis

A synthetic Isoflavone, DCMF, Promotes Human Keratinocyte Migration by Activating Src/FAK Signaling Pathway

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Department of Medicine

Graduate School

Jeju National University

February, 2016

새로운 플라보노이드 물질 (DCMF)의 Src/FAK 경로 활성화를 통한 각질세포의 이동성 증가 효과

지도교수 조 문 제

폴 소폴스

이 논문을 의학 석사학위 논문으로 제출함

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폴 소폴스의 의학 석사학위 논문을 인준함

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A synthetic Isoflavone, DCMF, Promotes Human Keratinocyte Migration by Activating Src/FAK Signaling Pathway

(Supervised by Professor **Moonjae Cho**)

A thesis submitted in partial fulfillment of the requirement for degree of Master of Science in Medicine

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This thesis has been examined and approved.

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LIST OF ABBREVIATIONS

DCMF	2',6Dichloro-7-methoxyisoflavone
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MMPs	Matrix metalloproteinases
ERK	Extracellular-signal-regulated kinases
P38	p38 mitogen-activvated protein kinases
Src	Proto-oncogene tyrosine-protein kinase Src
FAK	Focal adhesion kinase
AKT	Protein kinase B (PKB)
MAPKs	Mitogen-activated protein kinases
EMT	Epithelial-mesenchymal transition
ECM	Extracellular matrix
TMF	4′,6,7-trimethoxyisoflavone

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1. Abstract

Flavonoids are a class of secondary compounds produced by plants that contain various pharmacological properties. Our previous report showed that TMF, trimethoxyisoflavone, provided a good effect on wound healing by inducing keratinocyte migration. In this study, we screened derivatives of TMF for searching better pharmacological effects and found **2',6 Dichloro-7-methoxyisoflavone (DCMF)** is better candidate for wound healing agents. We investigate the effects and possible action mechanism **of 2',6 Dichloro-7-methoxyisoflavone (DCMF)** on wound healing using keratinocyte HaCaT cell line and in excisional wound animal model. We found DCMF markedly increased keratinocyte cell migrations but not proliferation. DCMF induced activation of ERK, AKT and p38 MAPK signaling pathways through Src kinase. We also found DCMF induced secretion of MMP-2 and MMP-9, and partial epithelial-mesenchymal transition (EMT). Finally, effects induced by DCMF were abolished when Src was inhibited. In *in vivo* excisional wound model, DCMF treated mice showed improvement of wound closure and re-epithelialization. In conclusion, DCMF induces cell migration through activation Src, ERK, AKT, p38 signaling pathway.

Key words: Flavonoid, Keratinocytes, Cell Migration, Wound healing

2. Introduction

The main function of the skin is a protective barrier against the any harm effects from environment. Loss of this largest organ caused from injury for illness may lead to disability or even death (Kamel, Ong, Eriksson, Junker, & Caterson, 2013; Singer & Clark, 1999). Wound healing is a dynamic and interactive process that requires both molecular and cellular event (Mendonça & Coutinho-Netto, 2009). Re-epithelialization is the restoration of an intact epidermal barrier through wound epithelialization that is an essential feature of a healed wound. The Migration, proliferation and differentiation of fibroblasts and Keratinocytes and interaction between these two cell types plays a crucial role in re-epithelialization and wound healing wound healing (Brun et al., 2014; Raja, Sivamani, Garcia, & Isseroff, 2007; Zhenxiang Wang, Wang, Farhangfar, Zimmer, & Zhang, 2012).

Src and Src family are known as proto-oncogenes protein kinase that plays a crucial role in regulation of cell morphology, motility, proliferation, and survival (Roskoski, 2004). The activation of Src is under the stimulation from plasma membrane receptors including receptor tyrosine kinase and integrins. The activation of Src results in activation of several biochemical cascades that thereby propagate signals generated extracellularly along intracellular interconnected transduction pathways (Guarino, 2010). The interaction of Src and tyrosine kinase FAK to form a Src/FAK Complex plays significant role in activating many signaling pathways to regulate cell adhesion and migration (Seong, Lu, & Wang, 2011). Moreover, Src/FAK complex is now identified as a crosstalk between integrin and cadherin-mediate adhesion of epithelial cells, particularly during the EMT process (Avizienyte & Frame, 2005). This complex also contributes to activating multiple downstream signaling pathways through phosphorylation of other proteins to regulate cellular function (Zhao & Guan, 2011). Three main MAPK cascades such as the extracellular signal-regulated kinases (ERK1/2), the c-Jun N-terminal kinases (JNK, also known as SAPK), and p38 are serine/threonine protein kinases that become phosphorylated both cytoplasmic and nuclear targets. (Chen et al., 2009; Rodriguez & Crespo, 2011). The activation of ERK and p38 was reported by *Hoq et al., 2011a* to be involved in catestatin mediated-keratinocyte migration. In addition, activation of ERK signaling

pathway is required for adiponectin mediated-keratinocyte migration and proliferation (S. Shibata et al., 2012). The activation of the p38 MAPK signaling pathway also involve in keratinocyte migration on collagen (W Li et al., 2001). Phosphatidylinositol-3-kinase (PI3K) is a lipid kinase and generates phosphatidylinositol-3,4,5-trisphosphate that is a second messenger essential for the translocation of Akt to the plasma membrane where it is phosphorylated and activated by phosphoinositide-dependent kinase (PDK) 1 and PDK2 to regulate central cellular functions including cell proliferation and survival by phosphorylating a variety of substrates (Osaki, Oshimura, & Ito, 2004). In chicken embryo fibroblast (CEF) cells, expression of active forms of PI3K, v-P3k or Myr-P3k, was reported to be sufficiently induced actin filament remodeling result in increasing cell migration, as well as the activation of Akt (Qian et al., 2004).

Flavonoids are plant pigments, phenolic substance, synthesized from phenylalanine. There are over 8000 individual compounds of flavonoids are known and isolated from wide range of vascular plants (Havsteen, 2002; Pietta, 2000). The Nobiletin, polymethoxy flavonoid, was shown to prevented UVB-induced photoinflammation and photoaging by inhibiting production of PGE, in which not only by suppressing the expression of COX-2 but also by decreasing the activity of cPLA2 in human keratinocytes (Tanaka, Sato, Akimoto, Yano, & Ito, 2004). A flavonoid, luteolin was reported by *Lodhi & Singhai, 2013* to induced wound healing in *in vivo* model. Moreover, our previous work demonstrated that isoflavone, TMF induced keratinocyte migration via induction of NOX2 pathway (Bui, Ho, Kim, Lim, & Cho, 2014). In this study we focus on the effect of an isoflavone derivative, 2',6 Dichloro-7-methoxyisoflavone (DCMF), that has a close chemical structure to TMF, on promotion of keratinocyte migration and wound healing. We demonstrated that DCMF promotes human keratinocyte migration via activating Src, ERK, AKT, p38 MAPK signaling cascade that might be implicated in in vivo wound healing.

3. Material and Method

3.1. Synthesized DCMF and Antibodies

The **2',6Dichloro-7-methoxyisoflavone (DCMF)** (Fig. 1B), was kindly provided by Professor Youngho Lim (Division of Bioscience and Biotechnology, Konkuk University, Seoul, Korea). The stock solution of DCMF was store at −20 °C, and 20μM of final concentration was diluted before use.

Phopho-FAK (Y97), FAK, Cyclin D1, Cyclin E, c-Src, Collage1A, and MMP-9 were obtained from Santa Cruz Biotechnology. Phopho-AKT (Y473), phopho-ERK1/2, phopho-Src (Y416), phopho-p38 MAPK, p38, Slug, Snail, Vimentin, MMP-2, and GAPDH were obtained from Cell Signaling and E-cadherin was obtained from BD Science,. The secondary antibodies used in the Western blotting were anti-mouse (PI-2000; Vector Laboratories) anti-rabbit (PI-1000; Vector Laboratories), and anti-goat (AP-107P; Millipore).

3.2. Culture of HaCaT cells

Briefly, the spontaneously immortalized keratinocyte cell line (HaCaT) was cultured in DMEM medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO) and 1% penicillin/streptomycin (PAA Laboratories GmBH, Strasse, Austria). Cells were maintained in a humidified 5% CO2 incubator at 37 °C.

3.3. Scratch Wound Healing Assay

HaCaT cells were seeded in a 48-well plate in $3.5.10^4$/well and allowed to growth until reach 80% of confluence. The wound area was made using a sterile 200 μL pipette across the bottom of the culture plate. Culture medium were then removed and replaced with fresh medium containing tested material. Photographs were captured at 4× magnification using an OLYMPUS IX70 microscope equipped with a digital camera at 0 and 24 hours. The width of the scratch was measured by the distance from the both edge of scratch by ImageJ software.

3.4. MTT

Cells were seeded on 96-well with 200 μl of density of 3×10^4 cells/ml for each wells. Cell viability was assessed by using the conversion of 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) into formazan via mitochondrial oxidation. MTT solution (10 μl of 5 mg/ml solution; Amresco) was added to each well and incubated for 37 °C for 4 h. The medium was gently removed and replaced with 150 μl of DMSO and then incubated for 30 min with gentling shaking. The Absorbance at 570 nm was recorded with a spectrophotometer.

3.5. Cell Counting

HaCaT cells were seed on 6-well plates in serum-containing medium for 24 hours. In the next day, cells were treated with different concentration of DCMF. After growing two days, cells were trypsinized and directly counted using hemocytometer under light microscope. The medium was changed every two days until six days.

3.6. Western Blot

Protein from cells was lysed using RIPA buffer for 30 min on ice. The protein concentration was determined using the bicinchoninic (BCA) assay (Thermo Scientific, Rockford, IL, USA). The protein in conditional medium (serum-free medium) was concentrated by Amicon (Milipore, Darmstadt, Germany) centrifugal following manufacture instruction. The protein in medium after centrifugation was determined the concentration using Bradford assay(Bradford, 1976). Equal amounts of protein (30 μg) were resolved by 8–10% SDS-PAGE and transferred to PVDF membranes. The membranes then were blocked with TBST solution (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween-20) containing 5% skim milk at room temperature for 2 hours, and then incubated with indicated primary antibodies at 4 °C for overnight. After 3 washes with TBST, membranes were room temperature-incubated with peroxidase-conjugated secondary antibodies for 1 hour and then washed 3 times. Signal was detected with an ECL-kit. The signal quantification was performed using ImageJ program.

3.7. Wounding Experiment

Briefly, 6-7 week old male ICR mice (n = 6 for each group) were chosen for the experiment. The hair was removed with an electronic hair clipper and removal cream. Dermal wound was made on the middle of a back using and 5 mm punch instrument. 200 µl of DCMF was applied to the wounds of the experimental group in concentrations of 200µM for 14 days. DMSO and Madecassol were used as negative and positive control respectively.

3.8. Histological Analyses

Wound tissues were isolated at day 7, 9 and 14 (two mice from each group) and then fixed in 10% buffered formalin saline embedded in paraffin wax. 4µM section of each tissue was stained with Hematoxylin and Eosin (H&E). Images were captured using an Olympus BX51 microscope (Olympus Corp., Tokyo, Japan) with the apochromatic objective lens and a 0.85 numeric aperture. The measurement of wound diameter and quantification of re-epithelialization. Was procedure as previously described by *Liu et al., 2014* and *Emmerson et al., 2012* respectively.

3.9. Statistical analysis.

All Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software). Data are expressed as mean value ± standard errors (S.E.). The significant of differences were analyzed using unpaired Student's *t* test. Value $P<0.05$ was considered statistically significance

4. Result

4.1. DCMF promotes HaCaT cell migration via activation of Src.

We previously reported that an isoflavone flavonoid, TMF, has a high effect on promotion of Keratinocyte cell migration by activation of NOX2 (Bui et al., 2014). To increase our knowledge on the effect of isoflavone derivative compound on cell migration, we performed scratch wound healing assay treated with 20μM with several derivatives of isoflavone that have similar chemical structure to TMF. We found that 2',6 Dichloro-7-methoxyisoflavone (DCMF) highly induced cell migration compared to untreated control and other tested compounds. Therefore, we chose DCMF as our best candidate for further study on its effects on cell migration and wound healing **(Fig. 1A and B)**.

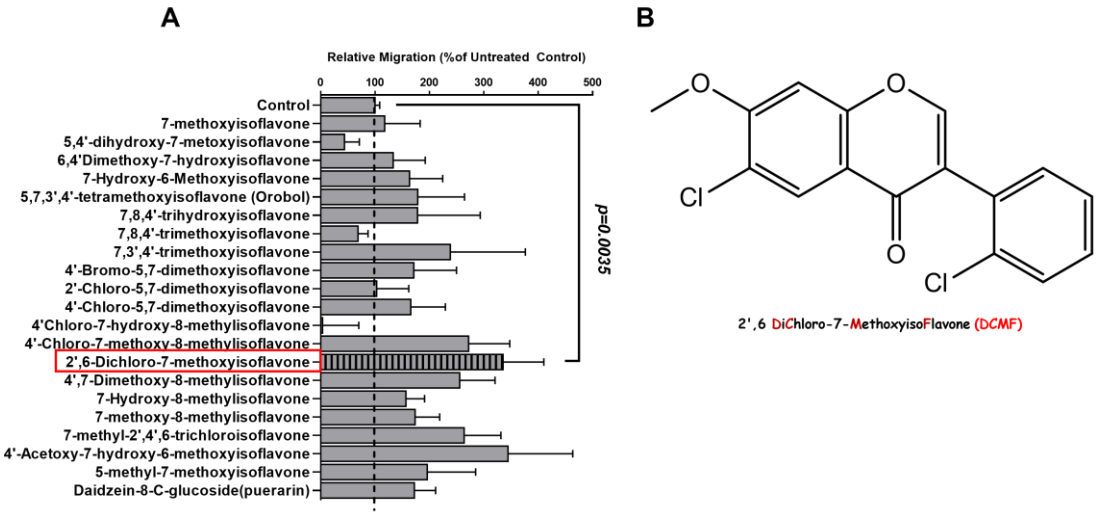

Figure 1: Effect of several compounds from flavonoid on cell migration.

(A) Relative migration from scratch assay is shown. Cells were incubated with of 20μM of DCMF for 24h. P-value, versus untreated control. (B) Chemical structure of DCMF.

To further investigate the effect of DCMF on cell migration, we performed a 24h scratch wound-healing assay treated with different concentrations of DCMF on HaCaT Cells. The results showed that DCMF significantly enhanced migration of HaCaT cells with a dose dependent manner **(Fig. 2).**

To verify whether increasing of cell migration induced by DCMF was not involved by cell proliferation, we performed MTT assay treated with DCMF (5–10μM) for 24 hours. MTT data revealed DCMF slightly promoted HaCaT cell proliferation without dose dependent. However, there is no significant difference compared to untreated control **(Fig. 3A).** To confirm this finding, we directly counted cells treated with DCMF as the same concentration and time as MMT assay. We found no significant different of cell growth curve compared to control **(Fig. 3B)**.

Cyclin E and Cyclin D play significant role in regulation of cell cycle. (Kozar & Sicinski, 2005; Möröy & Geisen, 2004). To test effect of DCMF on cyclin E&D expression, we performed western blotting of total cells lysate treated with different dose of DCMF for 24. There is no significant change on protein level of cyclin E and D **(Fig. 4)**. Collectively, these data indicate that 267D7M strongly induces migration but has no effect on proliferation of keratinocytes.

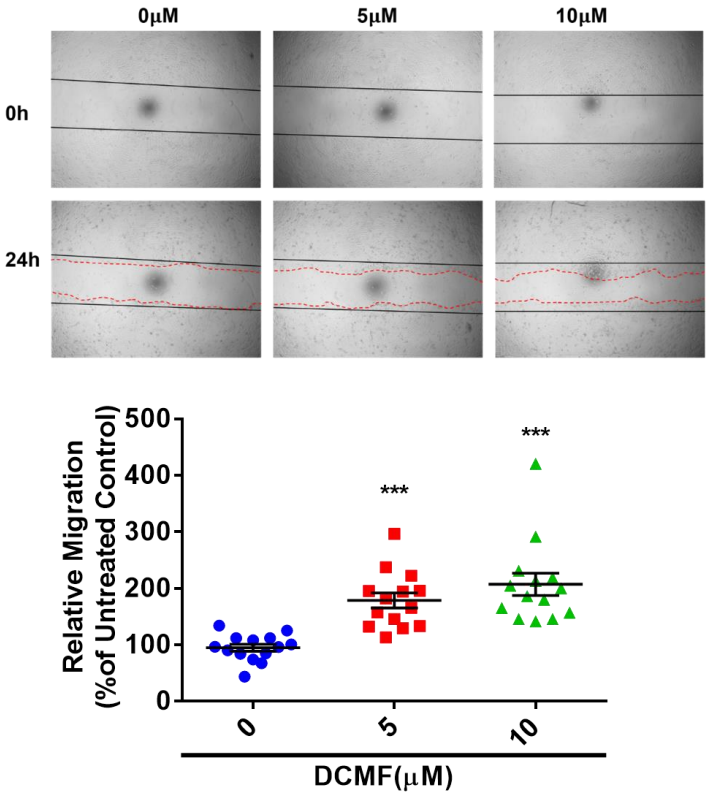

Figure 2: Effect of DCMF on HaCaT cell migration.

Cells were incubated with different doses of DCMF (5–10μM) for 24h. Scratch wound healing assay of HaCaT cells treated with DCMF. Relative migration from is shown as graph. All Data represent means±S.E. ***P<0.001 versus untreated control.

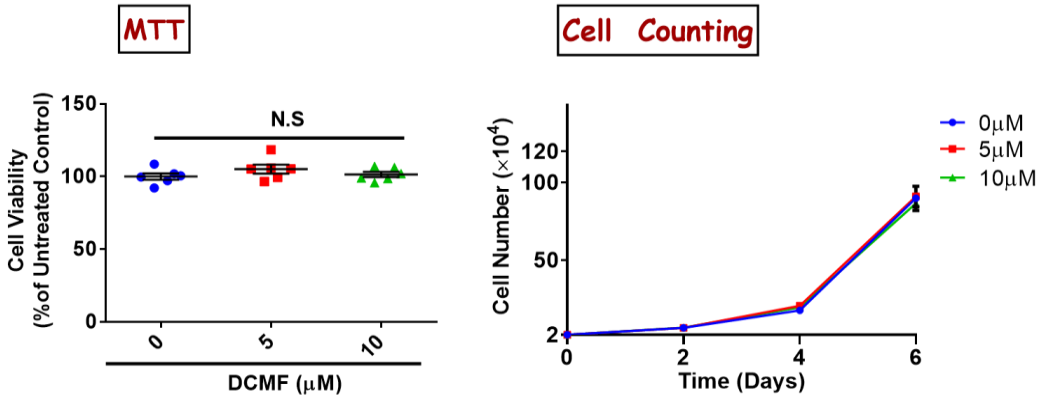

Figure 3. Effect of DCMF on HaCaT cell proliferation.

(A) Cells were incubated with different doses of DCMF (5–10μM) for 24h. The effect of DCMF was evaluated by MTT assay. (B) Cell were seed on 6-well plate for 24h and then replaced with fresh medium contain tested material. Cells were trypsinized and counted with indicated time points. All Data represent means±S.E. N.S: no significant versus untreated control.

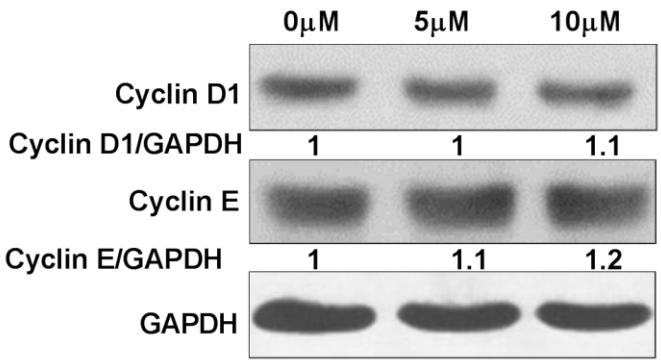

Figure 4: Effect of DCMF on Cyclin D and Cyclin E

Western blotting of cell cycle regulatory proteins from total cell lysate treated with different doses of DCMF (5–10μM) for 24hours. Ratio of relative intensity versus untreated control is shown.

To gain insight the molecular mechanism of keratinocyte migration induced by DCMF, we investigate the effect of DCMF on activation of FAK and Src. Focal adhesion kinase FAK is a cytoplasmic tyrosine kinase that plays important role in integrin-regulate signal transduction (Zhao & Guan, 2011). FAK-Src interaction is a dual complex. Activation of FAK-Src plays roles in the regulation of cell motility (Mitra & Schlaepfer, 2006). To elucidate the effect of DCMF on phosphorylation of Src and FAK, cells were treated with different doses and times of DCMF. The phosphorylation of Src and FAK was greatly elevated with a dose dependent manner in DCMF treated cells compared to un treated control **(Fig. 5A and B).** In addition, phosphorylation of FAK induced by DCMF peaked at 6 hours and Src did at 12 hours **(Fig. 5C -E)**. Saracatinib (AZD0530) is known as a potent c-Src/Abl kinase inhibitor that was previously reported by *Hennequin et al., 2006*. In HaCaT cells, we found that 1μM treatment of AZD0530 inhibited activation of Src induced by DCMF efficiently **(Fig. 6A**.) To verify whether activation of Src is required for DCMF-induced HaCaT cell migration, we performed wound healing assay of HaCaT cells pre-treated with 1μM of AZD530 30 min and then incubated with 10μM of DCMF for 24 hours. Result showed that DCMF-induced cell migration was abolished when Src was inhibited, indicating that Src is involved in DCMF-promoted cell migration **(Fig. 6B).**

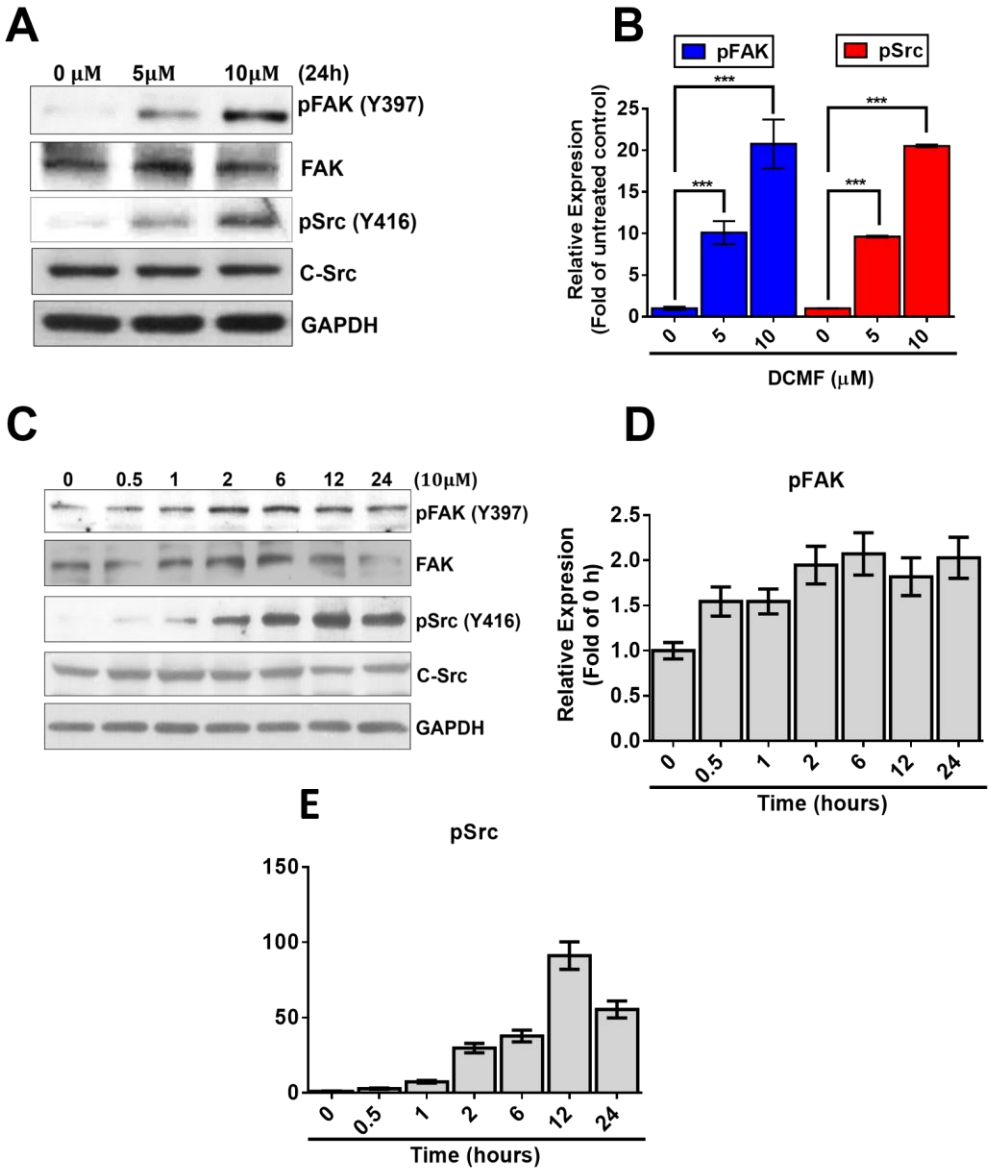

Figure 5: DCMF promotes phosphorylation of FAK (Y397) and Src (Y416).

(A) HaCaT cells were incubated with different doses of DCMF (5–10μM) for 24h. (B) Representative graph of relative expression from (A). (C) Cells were incubated with 10μM of DCMF for various time periods (0–24 hours). (D) Representative graph of relative expression from (C). (A and C) Total proteins were extracted and blotted with FAK (Y397) and Src (Y416) antibodies. Data represent means±S.E. ***P<0.001 versus untreated control.

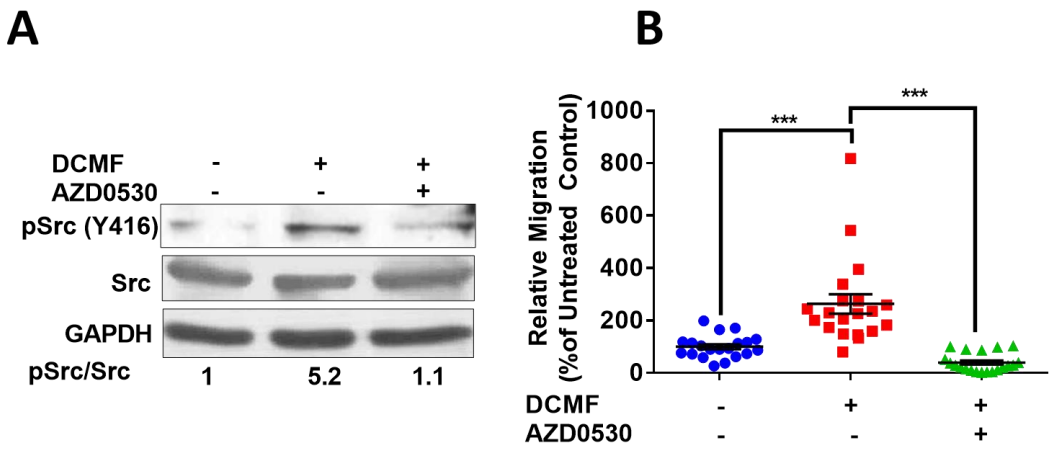

Figure 6: DCMF promotes cell migration via Src.

(A) Cells were pre-treated with Src inhibitor AZD0530 for 1 h and then incubated with 10μM of DCMF for 2 h and the levels of phospho-Src were examined by western blotting with total cell lysates. Ratio of relative expression versus untreated control is shown. (B) Relative migration of Scratch wound healing assay of cells pre-treated with 1μM of AZD0530 for 1 h were incubated with 10μM of DCMF for 24h. Data represent means±S.E. ***P<0.001 versus untreated control.

4.2. DCMF activates Src to mediate phosphorylation of ERK, AKT and p38 MAPK

To increase our knowledge into molecular mechanism of DCMF-mediated cell migration, we investigated expression of ERK, p38 MAPK and AKT that were previously reported as major regulators of cell migration (Chen et al., 2009; Huang, Jacobson, & Schaller, 2004; Kakinuma, Roy, Zhu, Wang, & Kiyama, 2008). To determine the influence of DCMF on phosphorylation of ERK, AKT and p38 MAPK, phosphorylated ERK1/2, AKT and p38 MAPK were evaluated by western blotting. As compared non-treated cells, DCMF greatly increased phosphorylation of ERK1/2, AKT and p38 MAPK with a dose dependent manner **(Fig. 7A and B)**. Additionally, DCMF induced phosphorylation of ERK1/2 at 30 min and dramatically decreased until 24h while phosphorylation of AKT peaked at 2 h and p38 did at 6 h **(Fig. 7C and D)**.

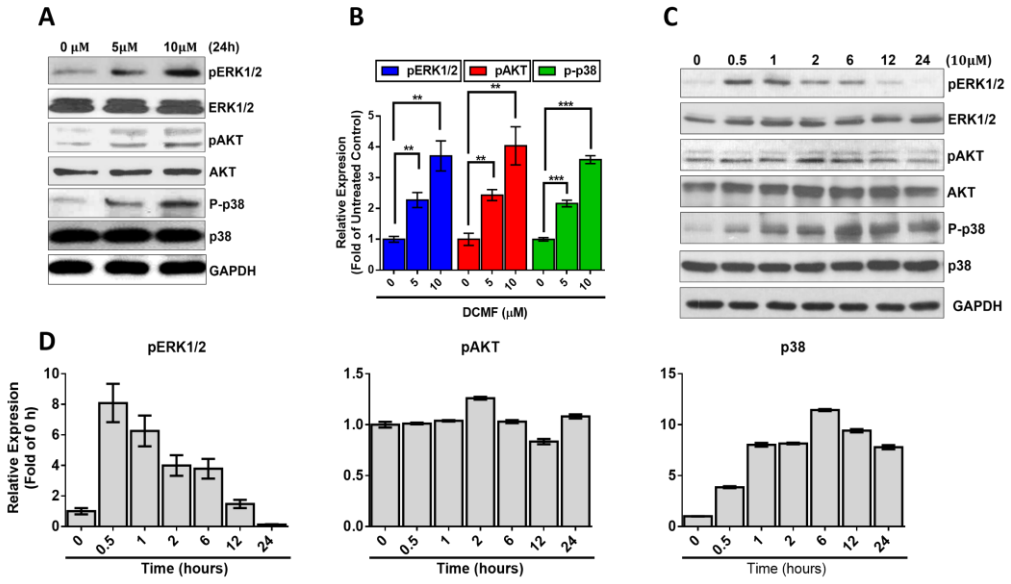

Figure 7: DCMF activates ERK, AKT, p38 MAPK.

HaCaT cells were incubated with different doses of DCMF (5–10μM) for 24h. (B) Representative graph of relative expression from (A). (C) Cells were treated with 10μM of DCMF for various time periods (0–24 hours). (D) Representative graph of relative expression from (C). (A and B) phopho-ERK1/2, AKT, and p38 were examined by western blot of total proteins from cell lysates. Data represent means±S.E. **P<0.01 and ***P<0.001 versus untreated control.

Previous reports showed that Src is necessary for the activation of ERK, p38 MAPK and AKT pathway as its downstream factors to regulate cell motility (Frey, Golovin, & Polk, 2004; Kim et al., 2008; Scapoli, Ramos-Nino, Martinelli, & Mossman, 2004; Vindis, Cerretti, Daniel, & Huynh-Do, 2003). To provide a clear evidence that phosphorylation of ERK1/2, AKT and p38 MAPK were involved in DCMF-mediated cell migration through activation of Src, cells were pre-treated with AZD0530 for 1 hours and then incubated with DCMF for 2 h. We found that the DCMF-induced phosphorylation of ERK1/2, AKT and p38 MAPK was significant abrogated by AZD0530 treatment **(Fig. 8)**. We also found DCMF induced cell migration was abolished by treatment with ERK1/2, Pi3K and p38 inhibitors **(Fig. 9)**. Taken together, the activation of ERK1/2, AKT and p38 MAPK are involved in DCMF-induced cell motility through the activation of Src.

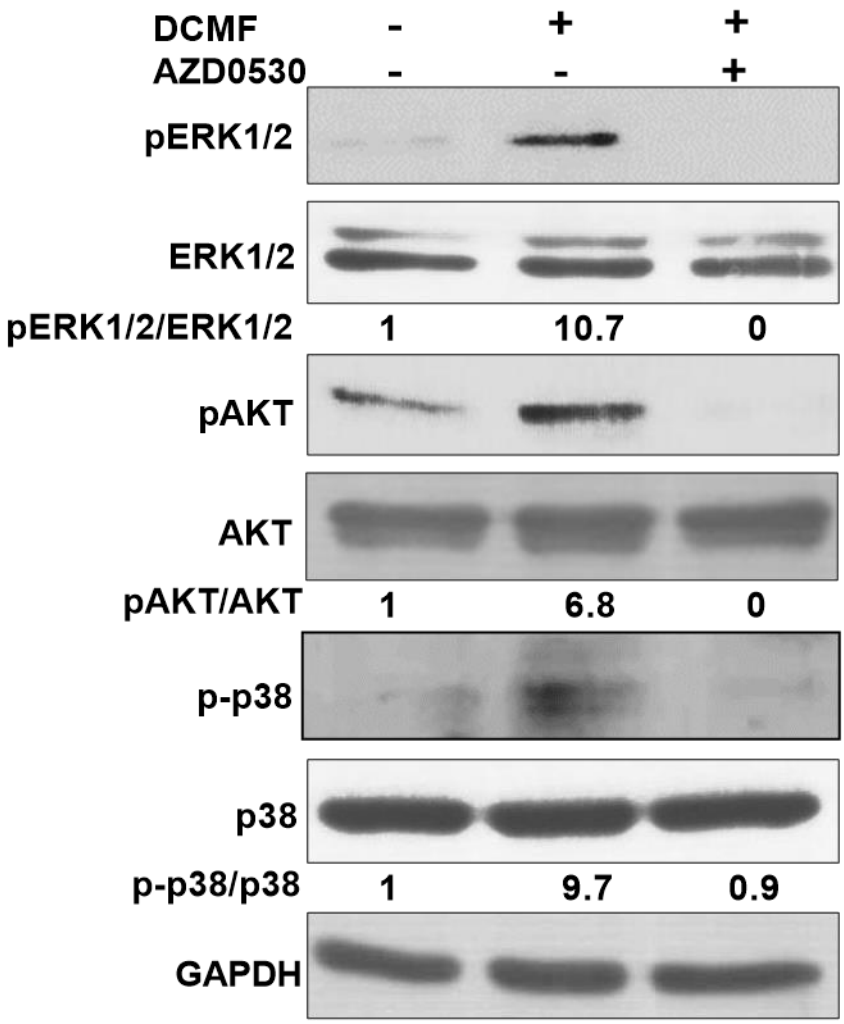

Figure 8: DCMF activates ERK, AKT, and p38 MAPK via Src.

Cells pre-treated with 1μM of AZD0530 for 60 min were incubated with 10μM of DCMF for 2 h. Total cell lysate were analyzed using phopho-ERK1/2, AKT, and p38 antibodies. Ratio of relative expression versus untreated control is shown.

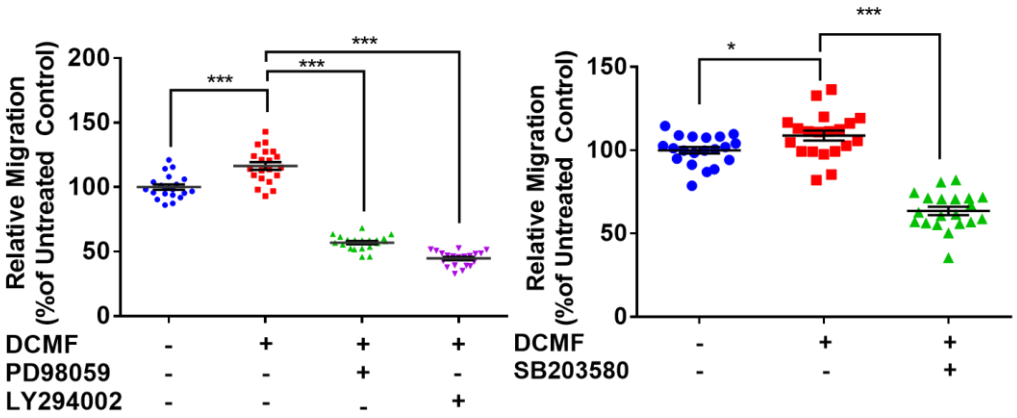

Figure 9: DCMF induces cell migration via ERK, AKT and p38 MAPK.

10μM of PD98059 (ERK inhibitors), LY294002 (PI3K inhibitor), and SB203580 (p38 inhibitor) were pretreated to HaCaT cells for 30 min before incubating 10μM of DCMF for 24h, and then their inhibitory effect on the DCMF-induced HaCaT cell migration was examined by scratch assay. Data represent means±S.E. *P<0.05, and ***P<0.001 versus untreated control.

4.3. DCMF promotes MMP-2 and MMP-9 Secretion

Matrix metalloproteinases (MMPs) are a large family of calcium-dependent zinc-containing endopeptidases that play crucial role in tissue remodeling and degradation of the extracellular matrix (ECM) proteins, including collagens, elastins, gelatin, matrix glycoproteins, and proteoglycan (Verma & Hansch, 2007). MMPs family is a key regulator of migration and invasion of cancer cells (Nabeshima, Inoue, Shimao, & Sameshima, 2002). We thought the secretion of MMPs protein into culture medium maybe involved in DCMF-mediated cell migration. To prove this, we investigated the secretion of MMPs induced by DCMF. HaCaT cells were treated with different concentration of DCMF and conditional media were concentrated as described in the method. The results showed that DCMF significantly induced secretion of MMP-2 and 9. Collagen type I is known as substrate of MMP-2. We thought increasing of MMP-2 level in the media may affect to collagen I. To check this, we concentrated conditional media from cells treated with DCMF for 24 hours. Interestingly, the level of collagen I in the medium was decreased in DCMF treated cells compared to untreated cells **(Fig. 10).** This finding could explain that the high level of MMPs secreted into the medium resulting in degradation of EMC molecules such as collagen I to regulate cell movement.

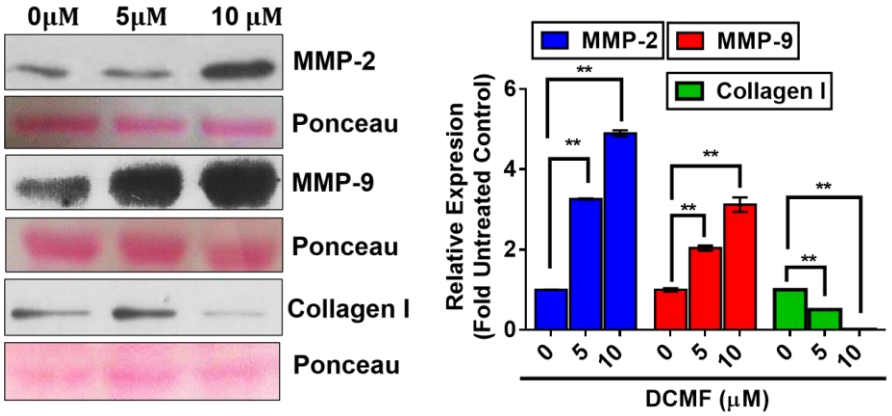

Figure 10: DCMF induces MMPs secretion.

Cells were treated with different doses of (5-10μM) of DCMF in serum-free media. Conditioned media were removed and concentrated using Amicon centrifugation. Protein in the medium was analyzed by Western blot for MMP-2, MMP-9 and collagen I. Representative graph of relative expression is shown. Data represent means±S.E. **P<0.01 versus untreated control.

4.4. DCMF induces change EMT regulatory molecules via Src

The epithelial-to-mesenchymal transition is involved in loss of cell-cell adhesion and enhancing cell migration (Byles et al., 2012). We investigated the effect of DCMF on EMT process. We found that epithelial marker E-cadherin was down-regulated in DCMF treated cells compared to untreated control, whereas mesenchymal markers, such as Vimentin, Snail and Slug were increased with a dose dependent manner **(Fig. 11A and B)**. Interestingly, the expression of E-cadherin was increased by inhibition AZD530, a Src inhibitor, whereas EMT transcription factors Snail and Slug were decreased, indicating that Src is involved in DCMF-induced EMT changes (**Fig. 11C)**.

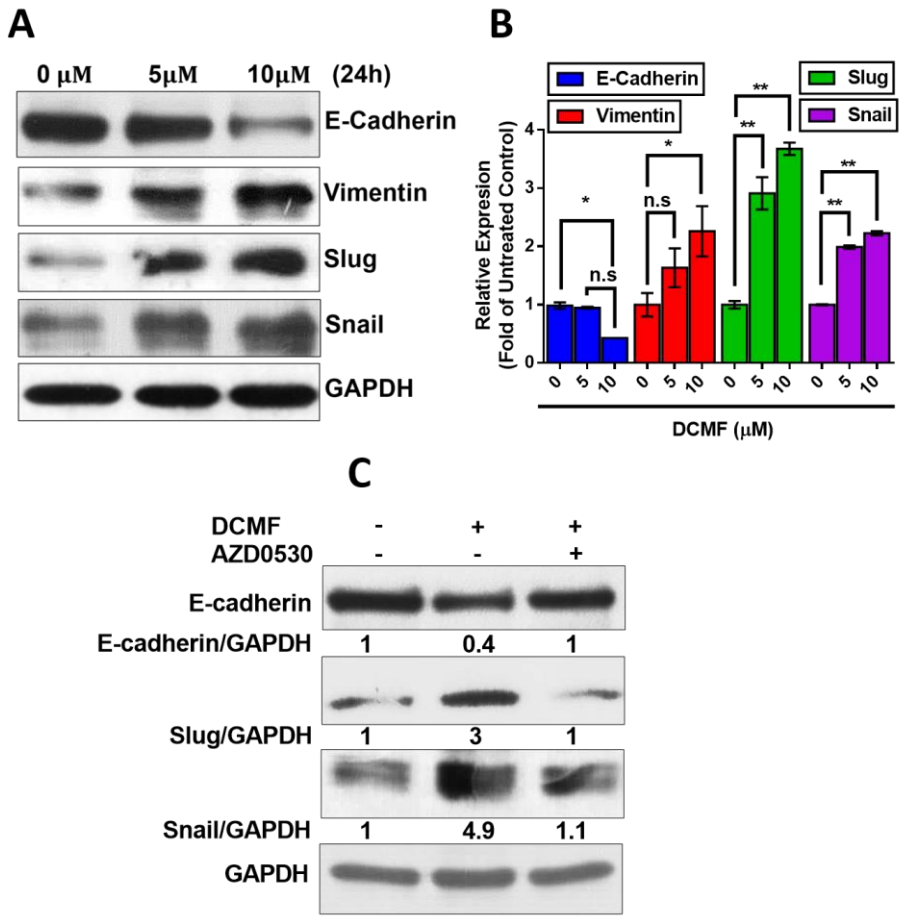

Figure 11: DCMF triggers Src to mediate EMT related gene expression.

(A) Protein level of EMT related proteins of HaCaT cells incubated with different doses of DCMF (5–10μM) for 24h. (B) Relative expression from (A) is shown. (C) Cells were pretreated with AZD0530 1 μM for 1 h and then co-treated with 10μM of DCMF 24 hours. (A and C) Total proteins were extracted and blotted with indicated antibodies. Ratio of relative express versus untreated control is shown. All Data represent means±S.E. N.S: no significant, *P<0.05, and **P<0.01 versus untreated control.

4.5. DCMF improves wound healing and re-epithelialization

Our in vitro data demonstrated that DCMF has a good effect in promotion of keratinocyte migration. This led us to ask whether effect of DCMF on keratinocyte cell migration contributed to *in vivo* wound healing. To prove this, we performed in vivo mouse-wound healing model. Full-thickness excisional wound was made on the dorsal of ICR mice using 5 mm punch biopsy. DCMF or DMSO or Madecassol were daily applied to wound sites topically. To confirm the effect of DCMF in promotion of wound healing and re-epithelialization, wound tissues were isolated with paraffin-embedded for histological study. We observed that DCMF treatment group exhibited significant reduction of wound diameter (determined by measuring the lengths between the wound margins) and increased re-epithelialization (determined by measuring the lengths newly formed epidermis) compared to control. In addition, there is no significant difference on both wound closure and re-epithelialization between DCMF and positive control Madecassol treated wounds **(Fig. 12 A-C).** This result indicates that DCMF may induce wound healing by promotion keratinocyte migration.

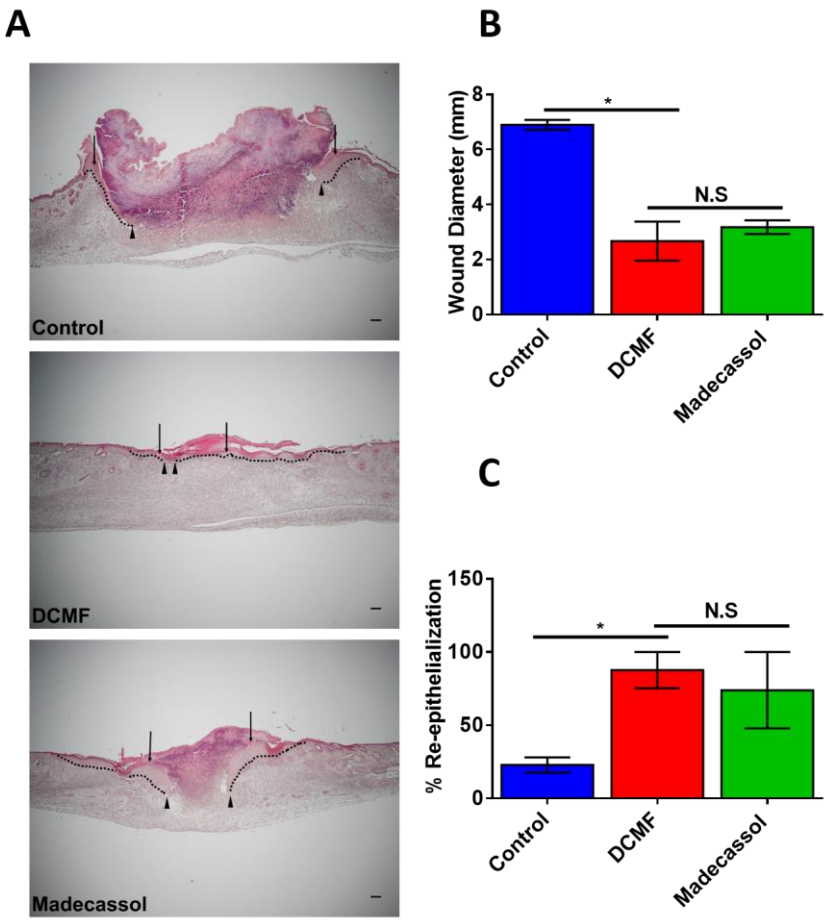

Figure 12: DCMF improves wound healing

(A). Representative H&E–stained sections of incisional wounds at day 7. Arrows: wound margins, arrow head: epithelial leading edge, dotted line: newly formed epidermis. (B-C) Representative graphs of wound diameter and re-epithelialization respectively. ImageJ program was used to analyses both re-epithelialization and wound closure. Scale bar: 200μM. The values were expressed as means ± S.E., N.S: no significant, *P<0.05 versus untreated control.

5. Discussion

In this study, we characterized the dynamic change of the cell migration induced by a synthetic compound from flavonoid. We screened several derivatives of TMF to find a better candidate on promotion of cell migration and wound healing. We found that DCMF is on one of tested compounds that provided the most upregulated cell migration. Our results showed that DCMF highly induced cell migration with a dose dependent manner. On the one hand, DCMF induced activation of Src, ERK, AKT, and p38 MAPK to regulate cell migration. We also found DCMF induced wound healing in *in vivo* study.

Our previous reports found that flavonoid induced HaCaT cell migration and wound healing with several signaling pathways (Cho et al., 2014; Ho et al., 2014; Seo et al., 2015) However, in these studies, the mechanism involved on cell migration such as activation of Src or FAK were not yet studied. In the present study, we demonstrated that DCMF may enhance skin wound healing through the induction of keratinocyte migration, in which DCMF stimulates Src, ERK, AKT, and p38 MAPK signaling to regulate cell motility.

Src and Src-family protein-tyrosine kinases are regulatory proteins that play a crucial role in cell differentiation, motility, proliferation, and survival (reviewed in ref. Roskoski, 2005)). *Li et al., 2005* reported the Src-activating and signaling molecule mediated differentiation of keratinocytes in linking EGF receptor and SFK-dependent signaling. Another study by *Zhenlian Wang et al., 2010* also found c-Src was involved in GPR48 mediates EGFR-induced cell keratinocyte proliferation and migration. Here we found that DCMF has an ability to induce cell migration with a dose dependent manner. The phosphorylation of Src was induced by treatment with DCMF in a dose and time dependent manner. Furthermore, the DCMF-promoted cell migration was abolished by Src inhibitor AZD0530. Src and FAK are known to cross-activation proteins. Inhibition of FAK/Src complex showed reduced cancer cell migration (Schaller, 2001; Slack et al., 2001). We found DCMF induced phosphorylation FAK (Y397). Y397 FAK phosphorylation is responsible for recruitment of Src and the generation of an activated FAK–Src complex *reviewed by Mitra & Schlaepfer, 2006*. This auto-phosphorylation of FAK Y397 is necessary for

its activity, Src phosphorylation of FAK Y576/Y577 is important in enhancing downstream signaling pathways (Parsons, 2003). Our finding showed that inhibition of Src by AZD0530 had no effect on phosphorylation of FAK (Y397) induced by DCMF (data not shown). This is consistent with previous study that AZD0530 targets Src but not FAK (Chang et al., 2008). Indicating that DCMF induced activation of FAK/Src to regulate cell migration.

ERK, AKT and p38 MAPK signaling pathways are known as major mediators of cell migration in many cell types (Du et al., 2010; Hoq et al., 2011b; Ryu et al., 2010; Segarra, Balenci, Drenth, Maina, & Lamballe, 2006). *Shibata et al., 2012* reported that ERK signaling was involved in adiponectin regulated keratinocytes migration and proliferation (Sayaka Shibata et al., 2012). *Stoll, Kansra, and Elder 2003* also reported the inhibition of p38 MAPK resulted in the impairment of the formation of keratinocyte outgrowth in human skin explant cultures, as well as the migration of keratinocytes in an in vitro wound assay. In corneal wound healing model, the activation and prompt nuclear accumulation of phospho-p38 (p-p38) and -ERK1/2 (p-ERK1/2) were rapidly activated by hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF). Inhibition of either the ERK1/2 or p38 pathway resulted in delayed corneal epithelial wound healing by impairment cell migration and proliferation (Sharma, He, & Bazan, 2003). Our result showed that DCMF-incubated cells led to significantly increase phosphorylation of ERK1/2, AKT and p38, whereas inhibition of ERK, AKT and p38 MAPK by specific inhibitors led to impairment of cell migration induced by DCMF, indicating that ERK1/2, AKT and p38 MAPK are required in DCMF-mediated HaCaT cell migration.

Activation of c-Src is known as upstream signaling cascade of ERK, AKT and p38 activation (Frey et al., 2004; Zhang et al., 2012). We found that treatment with AZD0530 led to decreasing phosphorylation of ERK, AKT and p38 induced by DCMF, suggesting that Src to mediates phosphorylation of ERK, AKT and p38 MAPK in DCMF-induced cell migration. MMPs is a family of proteolytic enzymes that is responsible for degradation of extracellular matrix proteins as well as non-matrix proteins, including cadherins to modulate tissue remodeling, cell

migration and proliferation (George & Dwivedi, 2004). MMP-13 plays a role in keratinocyte migration and contraction in wound healing, whereas MMP-9 is required for keratinocyte migration. Knockout MMP-9 and MMP-13 and double knockout MMP-9/13 showed delaying of wound closure and re-epithelialization compared to wile-type mice (Hattori et al., 2009). MMP-2 and MMP-9 were reported to be upregulated during TGF-β1-inuced keratinocyte migration (Seomun, Kim, & Joo, 2008) . In our data, MMP-2 and MMP-9 were upregulated in the DCMF treatment compared to untreated control. Interestingly, extracellular matrix, collagen I was significantly decreased in DCMF treated cells. This could explain that the upregulation of MMP-2 in the culture medium maybe affected to the degradation of collagen I and mediate cell motility (Nagase, 2001).

Activation Src/FAK complex is a key regulator of EMT (Wilson et al., 2014). Transcription factors, such as Snail, Slug, and Twist, are known as pivotal activators of EMT (Sanchez-Tillo et al., 2012). Our finding showed that incubation of HaCaT cells with DCMF led to significantly increased expression of Snail, slug and Vimentin, whereas epithelial marker E-cadherin was down-regulated. We also found up-regulation of Slug and Snail were inhibited by AZD0530 and showed up-regulation of E-cadherin. Indicating that Src is required for DCMF-mediate EMT related gene expression.

The migration of keratinocyte is one the most important factors that responsible for regulation of wound healing and re-epithelialization mechanism (Raja et al., 2007; Yang et al., 2011). Our finding suggested that DCMF has a high effect in regulation of Keratinocyte migration in scratch wound healing model. This led us to think that that the migratory effect of DCMF on Keratinocyte maybe contributed to *in vivo* wound healing. As expected, typically treatment of DCMF was found to be enhanced wound healing by inducing wound contraction as well as wound re-epithelialization in *in vivo* mouse incisional wound healing model. This finding indicates that the DCMF may induce wound healing and improve re-epithelialization via promotion of Keratinocyte migration.

Taken together, our finding suggests that the effect of DCMF on cell migration and wound healing may provide a new method to the future of drug development. In conclusion, DCMF promotes keratinocyte migration via activation of Src, ERK, AKT, p38 MAPK and may affect to MMPs activity and EMT to regulate cell migration that this mechanism maybe implicated in wound healing and re-epithelialization.

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