APPENDIX A

List of chemicals and materials used in this study

Chemicals and reagents

All chemicals and reagents used in this study are analytical grade and are listed as Follows

Name of chemicals	Cat. No.	Companies
<u>Tissue culture materials</u>		
Dulbecco's modified eagle's medium(DMEM)	12800-017	Gibco BRL (Grand Island, N.Y., USA)
Keratinocyte -SFM (serum-free keratinocyte medium for culture of human keratinocytes, without hydrocortisone, insulin and T-3)	10726-016	Gibco BRL
Keratinocyte -SFM (serum-free keratinocyte medium for culture of human keratinocytes)	10724-011	Gibco BRL
Dispase	17105-032	Gibco BRL
Penicillin-streptomycin	15140-148	Gibco BRL
Supplements for keratinocyte-SFM -Bovine pituitary extract(BPE) -Recombinant epidermal growth factor (rEGF)	37000-015	Gibco BRL
Trypsin-EDTA	25300-062	Gibco BRL
Trypsin inhibitor	T-6522	Sigma (St Loius, Mo,USA)
FNC coating mix™	AF-10	BRAFF (USA)
Fungizone (Amphoteracin B)	15295-017	Gibco BRL

Name of chemicals	Cat. No.	Companies
<u>Carcinogen/mitogen</u>		
12-O-tetradecanoyl-phorbol-13-acetate (TPA)	P-8139	Sigma
<u>Diet chemopreventive agent</u>		
Curcumin	C-1386	Sigma
<u>Buffer</u>		
Tris (hydroxymethyl aminomethane (tris-base)	T-8524	Sigma
<u>Chelating agent</u>		
Ethylenediaminetetraacetic acid(EDTA)	E-5134	Sigma
Ethyleneglycol-bis(β-aminoethylether)-N,N,N',N'tetraaceticacid (EGTA)	E-4378	Sigma
<u>Protease inhibitors</u>		
Dithiothreitol	D-6032	Sigma
Aprotinin	A-6279	Sigma
Luepeptin	L-2884	Sigma
Phenylmethyl sulfonyl fluoride(PMSF)	P-7626	Sigma
<u>Homoginizing agent</u>		
TritonX-100	T-8787	Sigma
<u>Reducing agent</u>		
2-mercaptoethanol	M-7154	Sigma

Name of chemicals	Cat. No.	Companies
Electrophoresis and estern blotting materials		
Ammonium persulfate	A-3678	Sigma
Acrylamide (Eastman)	A-3553	Sigma
Sodium dodecyl sulphate (SDS)	L-4390	Sigma
N,N,N,N,-tetramethyl ethylene-diamine (TEMED)	T-9281	Sigma
Western blotting detection reagents		
-Luminol/enhancer solution	1856151	PIERCE (Illinois, USA)
-Stable peroxidase solution	1856150	PIERCE (Illinois, USA)
Skim milk	0032-17-3	Difco, USA
Organic solvents		
Absolute ethanol	UN 1170	E. Merck, Germany
Methanol	UN 1230	E. Merck
Propanol	UN 1219	E. Merck
Acid		
Acetic acid	UN2789	E.Merck
Trichloroacetic acid (TCA)	UN 1839	E.Merck
Dyes		
Coomassie brilliant blue R-250	B-0149	Sigma
Amido black	H-0763	Sigma

Name of chemicals	Cat.No.	Companies
Miscellaneous		
HEPES	S-5011	Sigma
Monobasic sodium phosphate	S-0876	Sigma
Dibasic sodium phosphate	S-9625	Sigma
Sodium chloride	G-5516	Sigma
Glycerol	G-8898	Sigma
Glycine	A-5633	Sigma
Antifoam A	D-8779	Sigma
Dimethyl sulfoxide	S-4019	Sigma
Protein determination reagents		
Sodium potassium tartrate	S-2377	Sigma
Folin&clocalteu's phenol reagent.	F-9252	Sigma
Copper sulfate	Art. 2790	E-Merck
Sodium hydroxide	S-5881	Sigma
Protein		
Bovine serum albumin (BSA)	05480	Fluka (Switzerland)

APPENDIX B

List of equipment and instruments used in this study

Name	Companies
Larminar flow biological cabinet	NUAIR 2000 Fernbrook Lane Plymouth, MN 55447
Carbondioxide incubator	Forma Scientific
Light microscope	Olympia Tokyo
Inverted microscope	Nikon
Spectrophotometer	MILTON ROY spectronic 1001 plus
Autoclave	Tomy autoclave SS-240
Analytical balance AC 100	Satorius
pH meter	Hanna Instruments 8417
Ultracentrifuge	Ivan Sorval Inc., USA.
Microcentrifuge, bench-topped	Clay
Water bath 37 °C	GFL 1083
Mini protein II slab gel	Biorad
Mini-PROPTEANTII electrophoresis cell	Biorad
Trans-blot® electrophoretic transfer cell	Biorad
X-rays photographic film, Kodak-X-OMAT	Kodak
ECL-hyper film	Amersham
Automatic pipette	GIBCO BRL
Serological pipette	Pyrex
Pasture pipette	Pyrex
25 or 75 cm^3 T-flask	Nunc, Costa
Glassware	Pyrex
Freezer (-80 °C)	Forma Scientific
Refrigerator	Sanyo, Hitachi

Name	Companies
Freezer (-20 °C)	Sanyo
Hood	British Klockner Switchgear (BKS)
Hot air oven	Haraeus
Speed vacuum SC 110	Savant
Magnetic stirrer	Sybron/Thermolyne
Vortex	Scientific industries
Power supply	E-C Apparatus corporation
Shaker bath	Unitronic 320 OR
Harvard trip balance (2 kg-5lb capacity)	OHAUS
Distilled water machine	Hamilton
Deionized water machine	Barnstead
Liquid nitrogen tank	Taylor-wharton

APPENDIX C

Preparation of some reagents and buffers

Human epidermal keratinocytes culture

1. Incomplete DMEM medium

DMEM (cat.No.12800-017)	1	package (13.5 g)
HEPES	3.57	g
$NaHCO_3$	3.7	g
Mercaptoethanol	1	ml
Deionized water	800	ml

Adjusted pH to 7.2-7.4 then adjusted volume to 1000 ml and sterilized by suction filter (membrane No. 0.2).

2. Incomplete keratinocyte-SFM (cat.No.10726-016), 500 ml per bottle stock solution

Incomplete keratinocyte-SFM	490	ml
Pen/strep	5	ml
Fungizone	5	ml
Total	500	ml

Placed 100 ml per bottle and stored at -20 °C.

3. Incomplete keratinocyte-SFM (cat.No.10724-011), 500 ml per bottle stock solution

Incomplete keratinocyte-SFM	490	ml
Pen/strep	5	ml
Fungizone	5	ml
Total	500	ml

Placed 100 ml per bottle and stored at -20 °C.

4. Bovine pituitary extract (BPE)

Each vial contain 25 mg / 2.75 ml

600 µl aliqout, stored frozen at -20 °C.

5. recombinant Epidermal growth factor (rEGF)

Each vial contain 2.5 µg

Dissolved 2.5µg rEGF in sterilized deionized water 1 ml.

Stored frozen in 250 µl aliquot at -20 °C.

6. Complete keratinocyte-SFM (BPE 50 µg/ml, rEGF 5 ng/ml)

Incomplete keratinocyte	100	ml
BPE	550	µl
rEGF	200	µl

Stored at 4 °C.

7.Dispase

Stock solution (20 units/ml) preparation.

Dispase (0.9 units/mg)	1	g
HBSS	45	ml

Sterilized by filtration, placed 4.5 ml per conical tube and stored frozen at - 20 °C.

Working solution (10 units/ml) preparation.

Complete keratinocyte-SFM	4.5	ml
Penicillin/streptomycin	1.0	ml
Dispase	4.5	ml

8. 20% Penicillin/streptomycin

Penicillin/streptomycin	2	ml
Complete keratinocyte-SFM	8	ml
Total	10	ml

9. **10 % Penicillin/streptomycin**

Penicillin/streptomycin	1	ml
Complete keratinocyte-SFM	9	ml
Total	10	ml

10. **Commercial Trypsin EDTA (0.05 % EDTA) preparing from 0.5 g Trypsin (1:250) EDTA.4Na/l in HBSS without Ca $^{2+}$and Mg^{2+} and 0.53 mM EDTA.4 Na.**

Each bottle contained 500 ml. Placed 100 ml per bottle and freezed at -20 °C.

Before using, thawed at room temperature and stored at 4 °C.

11. **Commercial Trypsin-Inhibitor (Type I-S:from soybean,cell culture tested);250 mg**

1 mg will inhibit 2.0 mg trypsin with approx. 10,000 BAEE units/mg protein

Need 147.06 mg TI to make 500 ml.

Calculate how much is needed to neutralize trypsinize trypsin 1:1

Mix measured TI with PBS as following

PBS	500	ml
Trypsin-Inhibitor	147.06	mg

Sterilized by filtration, placed 100 ml per bottle and stored at -20 °C.

Before using, thawed at room temperature and stored at 4 °C.

12. **Commercial FNC collagen-coating mix**

Each bottle contain 50 ml, keep at 4 °C.

Preparation of cytosolic and membrane fractions of PKC

1. Phosphate buffer saline (PBS)

KH_2PO_4	0.24	g
Na_2HPO_4	1.44	g
NaCl	8	g
KCl	0.2	g

Dissolved in 800 ml deionized water, adjusted pH to 7.4 then top up to 1000 ml.

2. 0.05% of trypsin-EDTA

3. Trypsin-inhibitor

4.Buffer A : homoginizing buffer

25 mM tris-HCL, pH 7.5

2mM EDTA

2mM EGTA

0.05 mM dithiotreitol (DTT)

0.02% triton X-100 for cytosolic fraction

0.2% triton X-100 for membrane fraction

10 µg/ml leupeptin

25 µg/ml Aprotinin

2mM PMSF

Buffer B : washing buffer

25 mM tris-HCl pH7.5

2 mM EDTA

2 mM EGTA

2 mM PMSF

Protein determinations

1. Reagent A

2% (w/v) Na_2CO_3 in 0.1 N NaOH

NaOH	2	g
Na_2CO_3	10	g
Distilled ionized water	500	ml

2. Reagent B

0.5%(w/v) $CuSO_4$. $5H_2O$ and 1% $NaKC_4H_4O_6$. $2H_2O$(Na-k Tatrate)

2 reagents of $CuSO_4$ and Na-k Tatrate were prepared as followed

$CuSO_4$	0.5 g
Distilled ionized water	50 ml

Stored in container

Na-k Tatrate	1 g
Distilled ionized water	50 ml

Stored in container

Before using 0.5 ml of $CuSO_4$. and Na-k Tatrate were mixed with the final concentration 0.5%(w/v) $CuSO_4$ and 1% $NaKC_4H_4O_6.2H_2O$(Na-k Tatrate).

3. Solution C

Working solution, was freshly prepared by mixing solution A 50 ml and soution B 1 ml (50:1)

4. SolutionD (Folin- Ciocaltea Pheno reagent 1N)

Folin- Ciocaltea Pheno reagent 2 N from Sigma company was diluted into 1N

5. Protein standard ; BSA

Working standard protein solution = 1 mg/ml of BSA in distilled water

SDS-PAGE analysis

1. Stock solution A : separating gel buffer 1.5 mM Tris HCl pH 8.8

Tris base	18.15	g
Deionized water	80.0	ml

Adjusted volume to 100 ml and sterilized by filtration, collected in dark Container.

2. Stock solution C : Stock acrylamide solution (30 %T, 2.7 %)

Acrylamide (Eastman)	29.2	g
Bis (Eastman)	0.8	g
Deionized water	70	ml

Adjusted volume to 100 ml and sterilized by filtration, collected in dark Container, shelf-life 1 month.

3. Stock solution D: Stacking gel buffer (0.5 mM Tris-HCl pH 6.8)

Tris-Base	6.05	g
Deionized water	70	ml

Adjusted pH to 6.8 with 5N HCl then adjusted volume to 100 ml and sterilized by filtration, collected in dark container.

4. Stock ammonium persulfate solution (10 % v/v APS in deionized water)

ammonium persulfate	0.1	g
Deionized water	1	ml

Sterilized by filtration then 100 µl aliquot, stored at - 20 $^{\circ}$ C.

5. Electrode buffer

Tris-base	3.0	g
Glycine	14.4	g
SDS	1.0	g

Dissolved in deionized water 1000 ml then filtrated by suction filter and stored at 4 °C

6. 5X Nonreducing buffer(sample solubilization solution)

1.0 M Tris-HCl pH6.8	0.625	ml
Glycerol	1.0	ml
1% Bromphenol blue	0.125	ml

Adjusted volume to 10 ml with distilled water

7. 5Xreducing buffer

5X Nonreducing buffer	475	µl
2-mercaptoethanol	25	µl

8. Coomassie blue

Coomassie blue	0.25	g
Methanol	20	ml
Acetic acid	10	ml
Deionized water was top up to	100	ml

9. Coomassie blue destining solution

Methanol	100	ml	(20 %)
Acetic acid	50	ml	(10 %)
Deionized water was top up	to	500	ml

Protein Western blot analysis.

1. Blotting Buffer

Tris-base	3.0	g
Glycine	14.4	g
Methanol	200	ml

Top up with deionized water to 1000 ml then sterilized by filtration.

2. Amido black

Amido black	0.25	g	(0.1%)
Isopropanol	62.5	ml	(25 %)
Acetic acid	25.0	ml	(10 %)

Top up with deionized water to 250 ml.

3. Amido black destain solution

Isopropanol	125	ml
Acetic acid	50	ml

Top up with deionized water to 500 ml.

4. PBS pH7.4

9.1 mM dibasic sodium phosphate (Na_2HPO_4)	1.3	g
1.7 mM monobasicsodium phosphate (Na_2HPO_4)	0.204	g
150 mM NaCl	7.28	g

Adjusted pH to 7.4 then adjusted volume to 1000 ml and sterilized by filtration.

5. Blocking reagent

skim milk	5	g
anti foam	20	µl
Dissolved in PBS pH 7.4	100	ml

6. Washing buffer

PBS pH 7.4	500	ml
Tween 20	500	µl

VITA

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