Supplemental Material
Materials and Methods

1. *Cell cultures*

Normal adult epidermal keratinocytes, melanocytes and dermal fibroblasts were either purchased from Cascade or primary cultures were established from the foreskin as described previously (Janjetovic *et al.*, 2009; Slominski *et al.*, 2005a; Slominski *et al.*, 2005b). The culture conditions and media were previously described for commercially obtained normal skin cells (Slominski *et al.*, 2005a; Slominski *et al.*, 2005b), primary skin cell culture established in this laboratory (Janjetovic *et al.*, 2009), immortalized normal melanocyte line PIG-1(gift of Dr Le Poole, Loyola University) (Le Poole *et al.*, 1997), immortalized HaCaT keratinocytes, and melanoma lines that derived from tumors at different stages of progression (SKMELK-188, SBCE2, WM35, WM98, WM164 and WM1341; gift of Dr. Menhardt Herlyn, Wistar Institute) (Coburn *et al.*, 2003; Pisarchik and Slominski, 2001; Slominski *et al.*, 2006).

To test the effects of UVB, lipopolysaccharide (LPS; catalog number: L 4516, derived from *Escherichia coli* 0127:B8, cell culture tested, γ-irradiated Sigma, St Louis, MO) or PAM3CSK4, human adult epidermal keratinocytes or human epidermal melanocytes from moderately pigmented skin were grown in EpiLife medium with Human Keratinocyte Growth Supplement Human or Melanocyte Growth Supplement, respectively and gentamycin and amphotericin B solution (Cascade Biologics, Inc., Portland, OR). Cells were seeded into multiwell plates in growth medium that was changed after 24 h to Epilife medium with EpiLife Defined Growth Supplement free of serum and pituitary extract (EDGS; Cascade Biologics). After incubation for further 24 h, the cells were washed with PBS, exposed to UVB as described (Fischer *et al.*, 2006a) or LPS (TLR-4 agonist, Sigma, St Louis, MO) or PAM3CSK4 (TLR-2 agonist, EMC microcollections, Tuebingen, Germany), and then incubated in EpiLife medium with EDGS. Thereafter, supernatants were collected and RNA extracted from the cells.

2. *Flow cytometry*

After the appropriate treatment the cells were fixed with cold 2% paraformaldehyde, incubated with mouse monoclonal antibody NOC 1 (catalog number MA1-83139, 0.5 µg; Abcam, Cambridge, MA) with following staining with goat anti-mouse secondary FITC-conjugated antibody (1:50; Caltag Laboratories, Burlingame, CA) as described previously (Cuello *et al.*, 1984). Samples were read with a FACS Calibur flow cytometer. Signal intensities were analyzed with Cell Quest (BD Biosciences, San Diego, CA) and graphical representations of the FL-1 signal were prepared with WinMdi 2.8 (shareware from Joseph Trotter, The Scripps Research Institute, San Diego, CA).

3. *Reverse transcription polymerase chain reaction (RT-PCR) and Real-time PCR (qPCR).*

The RNA from tissue and cells was isolated using the Absolutely RNA Miniprep Kit (Stratagen La Jolla, CA). All PCR products were purchased from Applied Biosystems, Foster City, CA. Reverse transcription was performed using High Capacity cDNA Archive Kit (melanocytes) or Taqman® Reverse Transcription Reagents (keratinocytes). RT-PCR detection of PENK mRNA was performed using primers P352 GCCGAATGCAGCCAGGATTG from exon 1and P353 TTCCGGTTTGCTATTTTCTCTG from exon 2 of the PENK gene with following program: 95^{o}C – 3 min; 30 cycles: 94^{o}C 30sec, 55^{o}C 40sec, 72^{o}C 40sec (Pisarchik *et al.*, 2004). The cDNA was separate on agarose and photographed under UV. The final product of 204 kb was sequenced (Pisarchik and Slominski, 2001) and showed perfect match with the expected sequence. RNA without RT was run and was negative control and showed as expected no amplification (not shown)

To measure by real-time PCR (qPCR) PENK mRNA expression in human epidermal melanocytes we used the primers described above. The sequences of primers used for 18S rRNA were as follows: forward, 5'-TTC GGA ACT GAG GCC ATG AT; reverse, 5'-TTT CGC TCT GGT CCG TCT TG. The reaction was performed with Sybr Green PCR Master Mix. In case of keratinocytes the following ABI PCR reagents were used: for pro-enkephalin: Hs00175049_1; for 18S rRNA: Hs99999901_s1. The reaction was performed with Taqman® Universal PCR Master Mix. Data were collected on an ABI Prism 7700 and analyzed on Sequence Detector 1.9.1. Specific gene amounts were related to 18SrRNA by comparative C_T method.

4. *Western Blotting*

Western blot detection was performed using standard protocol as described previously (Zmijewski and Slominski, 2009). Shortly, cells were collected using trypsinization, lysed with 0.2% Triton X100, centrifuged for 10 minutes at 16000 *g* and supernatants (50µg of proteins) were subjected to SDS-Page electrophoretical separation and transferred to PVDF membrane. PENK immunoreactivity was detected using mouse anti-PENK antibody (NOC-1, ab23503, 1:100; Abcam, Cambridge, MA), also available from Pierce (part of Thermo Fisher; catalog number, MA1-83139), and anti-mouse secondary antibody conjugated with horseradish peroxidase (1:200, Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

5. *Time-of-flight liquid chromatography mass spectrometry (TOF-LCMS) analysis*

Peptides extractions followed protocols previously described with some modification (Slominski *et al.*, 2000b). Human skin was deep frozen (liquid nitrogen), crashed into powder, homogenized at ratio of 400 mg per 1 mL of 0.1% trifluoroacetic acid (TFA) + 1mM PMSF (buffer A) and then centrifuged for 30 min at 16000 g at 4 ^{0}C. The supernatant were applied on pre-equilibrated SEPCOL columns (activated by equilibration 1x 1 ml of Buffer B (60% acetonitrile in buffer A) followed by washing 3x3 mL of buffer A). After passing the supernatant the columns were washed 3x 2mL with buffer A, eluted with buffer B (3ml) and dried on Speedvac and kept at -70^{0}C.

Actual identification of Met- and Le-enkephalin peptides was accomplished by LC-MS system using an Agilent 6520 TOF/Q-TOF mass spectrometer and 1200 HPLC system (Agilent, Wilmington, Delaware). For mass determination, the experimental samples and peptide standards were dissolved in 200 µL of 0.1% TFA and separated through Agilent eclipse plus C18 column (2.1 x 50, mm; particle size, 1.8 µm). with a mobile phase A: 0.1% TFA. Separation was performed with acetonitrile plus 0.1% TFA (mobile phase B) with 8 min gradient from 5% to 50%, while the flow rate was maintained at 0.2 mL/min. The effluent from the HPLC system was routed to the MS through electrospray interphase. The ESI conditions were as follow: gas temp: 350 ^{0}C; gas flow 10 l/min; nebulizer 50 psi. The scan was performed at positive ion polarity with acquisition scan range 100 to 1000 m/z, reference mass enable and scan source parameters: VCap 4000; fragmentor 165; skimmer 1 65; octopoleRFPeak 250.

6. *Construction of pPENK-luc construct*

The PENK promoter region covering sequence -772 to +11 from ATG starting codon was introducted into a pLuc plasmid containing the promotorless firefly luciferase gene in similar manner as described previously (Pisarchik and Slominski, 2004). This construct enables effective expression of luciferase only upon activation of PENK promoter. The construct was created as follow:

A backbone of pLinker vector was amplified from plasmid pGL3-basic (Promega, Madison, WI) by primers P762 (TCGAATTCCCTAGGGCCGCTTCGAGCAGACATGA) and P763 (TTCTCGAGACGCGTTATCGATAGAGAAATGTTCTGGC). The insert was synthesized as a pair of complimentary primers P764 (AACTCGAGGCTAGTCTGCAGGAGCTCAAGCTTTCTAGAGAATTCA) and P765 (TGAATTCTCTAGAAAGCTTGAGCTCCTGCAGACTAGCCTCGAGTT). The vector backbone and the insert were digested by EcoRI and XhoI restriction enzymes, purified by Gel-band purification kit (Qiagen, Germantown, MD) and ligated by T4 DNA ligase.

The luciferase gene was amplified from pGL3-basic vector (Promega, , Madison, WI) by primers P766 (AAAAGCTTCCCGGGCATTCCGGTACTGTTGGTAAA) and P767 (GGGAATTCGACTCTAGAATTACACGGCGA), purified by Gel-band purification kit (Qiagen), cut by HindIII and EcoRI and ligated with pLinker plasmid, which was also cut by HindIII and EcoRI restriction enzymes.

The PENK promoter covering sequence -772 to +11 (from ATG start codon) was amplified from the genomic DNA by nested PCR. The first PCR reaction was performed by primers P817 (TTCCTAACTGCCTTGGGTTTG) and P818 (AGCAGCCAAGTGCAAAGTGTC). This PCR was diluted 40 times and used for the second round of PCR by primers P819 (AACTCGAGGTTTGGGGCTAATTATAAAGTG) and P820 (AAGAGCTCCATGGACTGCGAGGAGAGA). The resulting fragment was purified by Gel-band purification kit (Qiagen, Germantown, MD), cut by XhoI and SacI restriction enzymes and ligated with pLuc plasmid, cut by the same restriction enzymes.

All amplifications were carried out using Pfu DNA polymerase (Stratagene). The PCR reaction (20 µL) was initially heated at 95^{o}C for 2.5 min followed by 30 cycles of denaturation at 94^{o}C for 15s, annealing at 55^{o}C for 15s and extension at 72^{o}C for 1 min. Each PCR amplification reaction contained 30 pmol of each primer and 100 ng of the template DNA.

7. *PENK promoter luciferase activity assay*

Mouse anterior pituitary-derived AtT-20 cells were grown in F10 medium (5% FBS and antibiotics) until 70-80% confluent and then incubated for 24 h in medium without FBS and antibiotics. Reporter gene activity was assayed on AtT-20 cells transfected with firefly luciferase reporter gene plasmids pPENK-Luc (described above) and phRL-TK plasmid (coding *Renilla* luciferase and used as normalization control; Promega, Madison, WI) using lipofectamine and PLUS reagents (Invitrogen, Carlsbad, CA). 24 h after transfection, the cells washed with PBS and subjected to ultraviolet irradiation as indicated (Fischer *et al.*, 2006b; Pisarchik and Slominski, 2001), followed by incubation in F10 medium (5% FBS and antibiotics) for 24 h. The firefly luciferase and *Renilla* luciferase signals were recorded with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) using Dual-Luciferase Reporter Assay System (Promega). After subtracting background luminescence, the ratio of firefly signal to Renilla signal was calculated. The values obtained were divided by the mean of control (untreated) cell.

8. *Immunohistochemistry/immunofluorescence*

Normal human haired scalp tissue obtained after elective plastic surgery was cryosectioned (6 μm) and processed for immunocytochemistry as previously described (Kauser *et al.*, 2006; Slominski *et al.*, 2004). Briefly, sections were are dried, fixed in ice-cold acetone, blocked with 10% normal goat serum. After quenching of the endogenous peroxidase activity sections were incubated with monoclonal antibodies NOC 1 (ab23503, Abcam Inc., Cambridge, MA, UK) at dilutions 1:25 to 1:1,000 for 2 h at room temperature (RT). While this antibody was discontinued from Abcam, it is currently available from Millpore (MAB350), and it recognizes both Leu- and Met-enkephalin (Cuello *et al.*, 1984). In majority of stain dilution 1:500 was used. Specificity of the PENK mouse monoclonal antibody was assessed by preabsorbing the antibody (dilution 1:500) with a 5-times excess (by weight) of a 1:1 mixture of [Leu5]-enkephalin and [Met5]-enkephalin (Cambridge Biosciences, Cambridge, UK) overnight at 4º C. Following blocking the antibody/peptide mixture was incubated with the skin tissue sections and processed immunohistochemically as above.

Secondary antibody incubations were with goat anti-mouse antibodies for 30 min (LSAB2 kit, DAKO, Carpinteria, CA), followed by streptavidin for 20 min and the immunoreactions detected using the AEC substrate/chromogen system (DAKO). Negative controls included omission of primary antibodies and their replacement with pre-immune serum from the host species of the secondary antibody. Sections were mounted under the glycerogel and viewed under Nikon eclipse 80i light microscope, (Nikon instruments Europe B.V., Badhoevedorp, The Netherlands) and photo-documented using a Nikon DS digital camera and the ACT-2U graphics program (Nikon)**.**

Samples of pathological skin were obtained from 16 patients (supplemental table 1). The immunofluorescence studies were performed as described previously (Slominski *et al.*, 2000a; Zmijewski and Slominski, 2009). Briefly, formalin-fixed paraffin-embedded tissue sections were dewaxed with xylene, and gradually hydrated. Antigen retrieval was achieved by microwave heating in 0.01M citrate buffer (pH 6.0) for 5 min. Sections were washed and then blocked by 1% bovine serum albumin, Sigma-Aldrich Inc., St. Louis, MO, USA) in TBS pH 7.6 for 2h at room temperature. Thereafter, sections were incubated with NOC 1 primary antibody (1:100, Abcam Inc., Cambridge, MA, USA) in TBS pH 7.6 with 1% BSA overnight at 4°C and then incubated with fluorescein-conjugated secondary antibody goat anti-mouse (1:200, H+L, Caltag/Invitrogen Corporation, Carlsbad, CA, USA) for 2h at room temperature, washed with TBS (pH 7.6) with 0.1% Triton X-100 and mounted in Vectashield mounting medium containing propidium iodide (PI) (Vector Laboratories Inc., Burlingame, CA, USA). Positive control represented detection of PENK in adrenal gland (supplemental figure 2).

Cultured human skin cells were seeded in 8 well Lab-Tek II chamber slides (Nalge Nunc, Inc.,Naperville, IL) and processed as described previously (Zmijewski *et al.*, 2007). The immunodetection of PENK was carried out with mouse anti-PENK antibody (NOC-1, 1:50; Abcam, Cambridge, MA) and anti-mouse secondary antibody conjugated with FITC (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). Cells incubated with corresponding non-immune serum were used as negative controls.

The results of staining was visualized with a Leica DigitalMicroscope DM4000B fluorescence microscope (Leica Microsystems Inc., Bannockburn, IL), equipped with Retiga EXi camera and QCapture Software for Windows (QImaging Corp., Burnaby, BC, Canada). The images produced with the two different fluorochromes rhodamine (red) and fluorescein (green) were subsequently merged together using the Paint Shop Pro 7 graphics program (JascSoftware, Oxon, UK). Staining intensity for enkephalin was scored as negative (-), weak (+), moderate (++) and strong (+++).

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Supplemental table 1. Patient and lesions characteristic.

Sample	Gender	Age (years)	Anatomic site	Histopathologic diagnosis of lesion
1	F	53	neck	granuloma annulare
2	M	45	arm	cellular hemorrhagic dermatofibroma
3	F	83	wrist	squamous cell carcinoma
4	F	39	leg	lichenoid keratosis
5	F	39		seborrheic keratosis
6	F	58	ND*	psoriasis
7	F	56	thumb	psoriasis
8	F	30	back	intradermal melanocytic nevus
9	F	30	back	intradermal melanocytic nevus, neurotized
10	F	66	foot	intradermal melanocytic nevus
11	F	42	back	melanocytic nevus, compound lentiginous type
12	F	42	back	intradermal melanocytic nevus
13	F	66	arm	melanoma malignant, VGF, tumor thickness >2 mm
14	F	68	neck	melanoma with regression changes, at least *in situ*
15	F	58	shoulder	melanoma malignant, VGF, tumor thickness >2 mm
16	F	39	neck	nodular melanoma, tumor thickness >2 mm

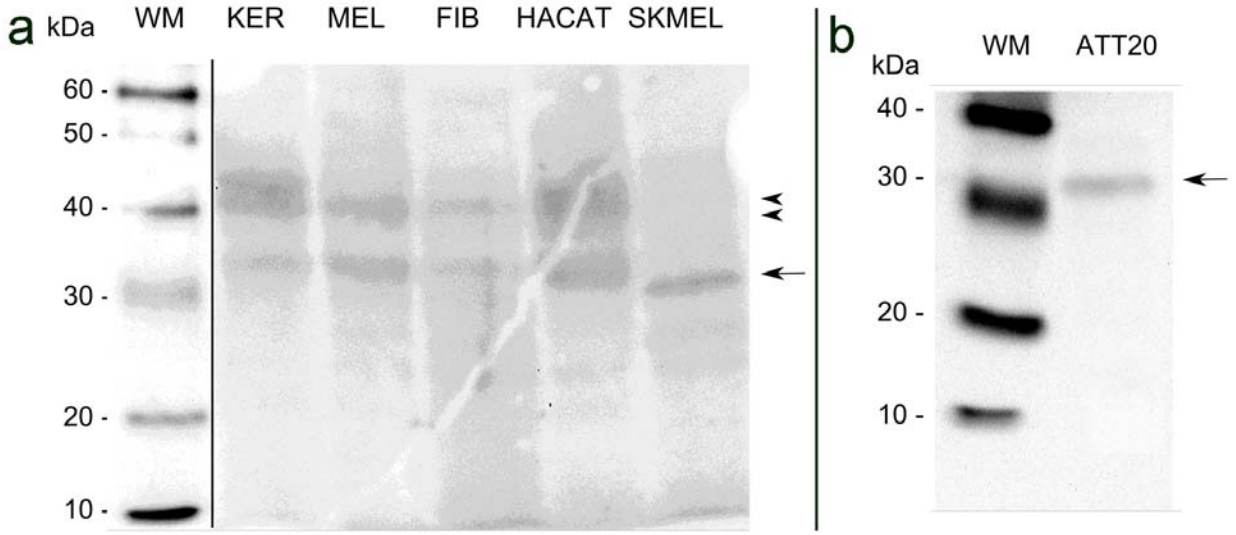
ND-no information; VGF: vertical growth phase

Supplemental Figures

Supplemental Figure 1. Western blot detection of PENK in human skin cells (A) and in AtT-20 cells transiently overexpressing PENK (B).

a. WM – Molecular weight marker. KER- neonatal epidermal keratinocytes, MEL – melanocytes, FIB – dermal fibroblasts, HaCaT – immortalized human HaCaT keratinocytes, SKMEL – melanoma SKMEL-188 cells, ATT20 - AtT-20 cells. Dividing vertical line separates WM from experimental samples.

b. AtT-20 cells were transiently tranfected with 4 µg of plasmid DNA containing coding sequence of PENK (SC321732, OriGene) using Lipofectamine reagent (Invitrogen). After 24 hours the cells were harvested and expression of PENK was assayed using WB technique as described under Materials and Methods.



Supplemental Figure 2. **Localization of PENK antigens in adrenal gland (positive control).**

Skin sections were stained with anti-PENK antibody, followed by secondary antibody linked to fluorescein. Cell nuclei were stained with propidium iodide (A). Arrows indicate PENK staining in medulla cells. In negative control (B) primary antibodies were omitted. Bar: 50 µm. Two different sections are shown that are separated by white vertical line.