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RESEARCH ARTICLE

Role of gap junctions between keratinocyte and melanocyte in melanogenesis

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Abstract **The process of melanogenesis in melanocytes and the transport of melanin in the form of melanosomes to the neighboring keratinocytes are the key steps in human skin pigmentation. Keratinocytes and melanocytes interact in intricate manner to maintain the homeostasis. The present study was designed to understand the role of cell-cell interaction through the gap junctions between melanocytes and keratinocytes on melanogenesis. We show that, inhibition of the gap junctional activity between human keratinocytes and melanocytes in a coculture system using gap junction blocker lowers the expression of key regulatory genes of melanogenesis such as tyrosinase and microphthalmia-associated transcription factor (MITF). This was followed by concurrent decrease in tyrosinase protein levels and activity. Our results show the preliminary evidence for the regulation of melanogenesis in melanocytes through direct gap junctional communication by keratinocytes. Deciphering the mechanism and factors involved in the process would uncover the significance of gap junctions in melanogenesis.**

Keywords melanocyte, keratinocyte, melanogenesis, gap junctions, cell communication

Introduction

In human epidermis, melanin-producing melanocytes are regulated by several factors including the signals from neighboring keratinocytes. Melanocytes synthesize and package the melanin in melanosomes and transfer the melanosomes to the neighboring keratinocytes (Park et al., 2009). Survival of melanocytes depends on the growth factors like GM-CSF, SCF, bFGF, α-MSH produced by neighboring keratinocytes (Hirobe 2005). To understand the pigmentation of the skin, intricate cellular and molecular interactions between melanocytes and keratinocytes should be implicit, which together compose the epidermal melanin unit. Melanocyte and keratinocyte unit, responds to stress and other environmental factors by communicating through paracrine factors (Costin and Hearing, 2007), but direct communication through gap junctions between melanocytes and keratinocytes is poorly understood.

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Gap junctions are intercellular communicating channels, made up of connexins (Evans and Martin, 2002). Through these channels, cells selectively exchange ions, second messengers and small metabolites (Goldberg et al., 2004). Gap junctional communication plays key roles in tissue homeostasis by involving in events such as cell synchronization (Sherman and Rinzel, 1991), cell differentiation (Gu et al., 2003) and metabolic coordination (Suadicani et al., 2004), with stringent regulation in permeability and communication (Dbouk et al., 2009). The role of connexins in pigmentation was mentioned in the context of pigment pattern in zebra fish (*Danio rerio*), where mutations in connexin 41.8 gene were responsible for different spot patterns (Watanabe et al., 2006; Watanabe and Kondo, 2012).

In human epidermis, it has been shown that, gap junctional communication has vital role in keratinocytes growth and differentiation (Mese et al., 2007) and melanocytes communicate with keratinocytes through gap junction *in vitro* (Hsu et al., 2000). However, the role of gap junctions in human pigmentation is less explored. Recognizing the significance of gap junctions in the tissue, present study is to address the possible role of gap junctional communication between keratinocytes and melanocytes on melanocyte survival and

its functions using gap junction blocker oleamide (Boger et al., 1998).

Methodology

Cell culture

After obtaining the institutional ethical clearance and consent from the parents/guardian of the subjects, foreskin samples were collected from two children below 10 years of age, who had undergone circumcision for various purposes. Cells were isolated from the skin samples and grown separately as two sets of pure cultures. To isolate the cells skin samples were dipped in 70% ethanol for 30 s and washed extensively with phosphate buffered saline (PBS) pH 7.4. The skin samples were cut into small pieces and incubated in dispase (BD Biosciences, USA) for 16–18 h at 4°C. After the incubation, epidermis was peeled off from the dermis. Epidermis was then incubated with 0.25% trypsin (Himedia, India) and 0.5 mM EDTA (SRL, India) for 5–10 min and shaken until cell suspension was obtained. Then cells were pelleted and washed with PBS. These mixtures of cells were divided and plated onto two different selective media separately. Keratinocytes were cultured in defined keratinocytes-serum free medium (DK-DFM) (Gibco, USA) and melanocytes were cultured using melanocyte growth medium (Nutrient mixture Ham F10 (Himedia, India) supplemented with 5%FBS (PAN-Biotech, Germany), 85nM PMA (Sigma Aldrich, USA), 0.1 mM IBMX (Sigma Aldrich, USA) and 0.25nM cholera toxin (Sigma Aldrich, USA)) at 37°C in 5% CO_2incubator. DK-SFM is a specialized medium, which allows the growth of keratinocytes but not melanocytes. Melanocyte growth medium is selective for the proliferation of melanocytes meanwhile inhibiting the survival of keratinocytes. Thus from the mixture of epidermal cells isolated from skin samples, pure cultures of keratinocytes and melanocytes were established by using specialized media.

Cell toxicity assay

1×10^4 per well melanocytes or keratinocytes were plated onto 96 well plates with melanocyte growth medium and Defined Keratinocytes-Serum Free Medium respectively for 24 h. After the incubation, cells were added with different concentration of oleamide dissolved in respective growth media. After 24 h of incubation with oleamide, 20 µL of MTT (Sigma Aldrich, USA) 5 mg/mL solution was added to each well and incubated for additional 4 h. Then the medium was removed and formazan crystals were dissolved in DMSO and optical density (O.D.) was read at 570 nm with reference wavelength 650 nm. The readings were converted to percent of readings obtained from the control/untreated group. Mean and standard deviation were calculated and plotted on the graph. All experiments were repeated to confirm the results.

Gap junction assay

Gap junction activity was assessed by using dye transfer (preloading) method explained earlier with slight modification (Hsu et al., 2000). The dyes used in these experiments were Calcein AM (Molecular Probes, USA) and 1,1'-Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perchlorate (Dil) (Molecular Probes, USA). Calcein AM is a live cell labeling dye, which is permeable to cell membrane. After entering the cells, the acetoxymethyl (AM) group of Calcein AM is cleaved by esterases present in the live cells. This reaction converts non-fluorescent Calcein AM to fluorescent Calcein, which is impermeable to cell membrane and thus does not leach out from live cells but is transferred from one cell to another through gap junctions. Dil is a fluorescent lipophilic dye usually used as long-term cell tracer. Dil is highly lipophilic and thus strongly stains the cells and does not get transfer to another cell through contact or through gap junctions. Hence, it serves as a marker of recipient cells, which can be easily differentiated in flow cytometry, from Calcein stained donor cells. In two separate sets of experiments, dye transfer activity was studied between keratinocytes (donor) and melanocytes (recipient) as well as between melanocytes (donor) and melanocytes (recipient). The donor cells were labeled with 5µM Calcein AM and recipient cells with 15µM Dil (in serum-free medium for 1 h. Cells were washed and donor cells were incubated in optimal growth medium for 1 h for Calcein AM to Calcein conversion. Labeled cells were washed thoroughly and mixed in 1:1 ratio and cocultured in defined keratinocyte serum free medium (Invitrogen, USA) for 20 h, in the presence or absence of nontoxic concentrations of gap junction blocker oleamide (Sigma Aldrich, USA). Cells were observed under fluorescence microscope. Subsequently the cells were trypsinized, inactivated using DMEM with 10%FBS, washed, re-suspended in PBS and analyzed using flow cytometry (BD FacsCalibur, USA).

Dye transfer from donor cells (calcein stained; green) to recipient (Dil stained; red) cells, was observed as double stained population in flow cytometry analysis. Percentage of recipient population gaining double positivity (Quadrant 2) was taken as equivalent to gap junction activity between these cells. The cells, which were inhibited to gain Calcein from donors, remained as Dil stained cells occupying quadrant 1 in dot blots. Data was presented as percentage of control in which well without oleamide was used as control. All experiments were repeated to confirm the results.

qRT-PCR

RNA was isolated from the cells using PureLink RNA isolation kit (Invitrogen, USA) and cDNA was prepared using Superscipt III First strand synthesis kit (Invitrogen, USA) according to the manufacturer's instructions. mRNA level of TYR and MITF was assessed using comparative CT method

and Taqman probes; TYR (Assay ID Hs01099965_m1), MITF (Assay ID Hs00165156_m1) and GAPDH (Assay ID Hs99999905_m1) (Applied bio systems, USA) according to the manufacturer's instructions in Applied Biosystems 7500 Fast Real-Time PCR System (Applied bio systems, USA). GAPDH was taken as endogenous control. Level of expression of TYR and MITF in untreated cells were taken as control to calculate the fold change. All experiments were repeated to confirm the results.

Tyrosinase assay

Cells were washed on plate with PBS and lysed with lysis buffer having 30 mM Tris-HCl (SRL, India) pH 7.4 and 0.1% Triton X 100 (Sigma Aldrich, USA). Protein concentration was estimated by Bradford method. 50μl of lysate was then treated with 100μL of L-DOPA (Himedia, India) (0.1% in PBS) at 37°C for 1 h. Formation of DOPA chrome was quantified by absorbance at 475 nm and expressed as percent activity per milligram of protein. All experiments were repeated to confirm the results.

Western blot

Cells were washed and lysed using lysis buffer containing 30 mM Tris-HCl pH 7.4 and 0.1% Triton X 100 (Sigma Aldrich, USA). Protein concentration was quantified using Bradford method. Equal amount of protein was separated on SDS-PAGE and transferred to nitrocellulose membrane. Proteins were identified using anti-beta actin (Cell Signaling Technology, USA), anti-tyrosianse (Abcam, USA) antibodies, and secondary antibodies conjugated with HRP (Cell Signaling Technology, USA). Chemiluminescence was quantified using Image Quant LAS 4000 (GE healthcare, UK). Intensity of chemiluminescence of tyrosinase was normalized to beta actin and plotted as fold change compared to control. All experiments were repeated to confirm the results.

Statistical analysis

Data was presented as mean±standard deviation or standard error. One-way analysis of variance (ANOVA) followed by post Dunnett's test was used to compare the data and determine the statistical significance of difference in measured parameters with control in all assays. Difference was considered significant at $p < 0.05$.

Results

Toxicity of oleamide in melanocytes and keratinocytes

When treated with oleamide in broad range of concentration i.e.1000 μM to 15.63 μM, both melanocytes and keratinocytes showed toxicity above125 μM concentration (Fig. 1). Therefore, we have used non-toxic concentration of oleamide for both cell types i.e. 25 μM, 50 μM and 100 μM to minimize the cell death and its interference in the analysis of cellular functions.

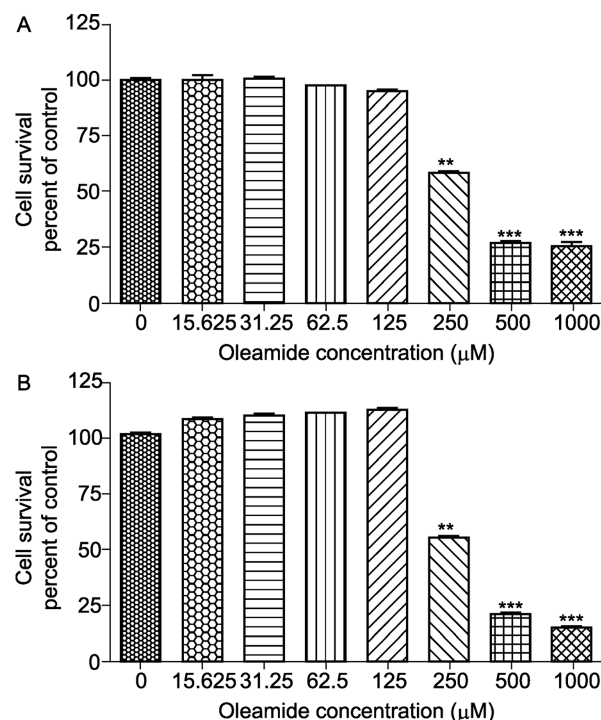

Figure 1 Cytotoxicity of oleamide. Oleamide toxicity in (A) keratinocyte and (B) melanocytes. A concentration of oleamide above 125 μM was found to be toxic. **, $p < 0.01$; ***, $p < 0.001$ in comparison with untreated group.

Effect of oleamide on gap junctional communication between keratinocyte and melanocyte

In coculture of stained keratinocytes and melanocytes, calcein was observed under fluorescence microscope using blue excitation filter (450–480nm) (Fig. 2A) and Dil was observed using green excitation filter (510-550 nm) (Fig. 2C). The quantification of dye transfer i.e. quantitatively detecting the double positive cells was not possible using fluorescence microscopy because of the low intensity of the signals (Fig. 2E and 2F). Thus, we switched to flow cytometry method in which cells were quantitatively analyzed using FL1 (530/30nm) and FL2 (585/40 nm) channels to detect Calcein and DIL stained cells respectively.

On addition of gap junction blocker oleamide, a dose depended inhibition in transfer of dye Calcein from keratinocytes to melanocytes was found. However, statistically significant inhibition was found in oleamide at 50μM concentration, with ~ 25% of inhibition and at 100μM concentration with ~ 40% inhibition in dye transfer (Fig. 3).

RNA isolated from the cells from the coculture with

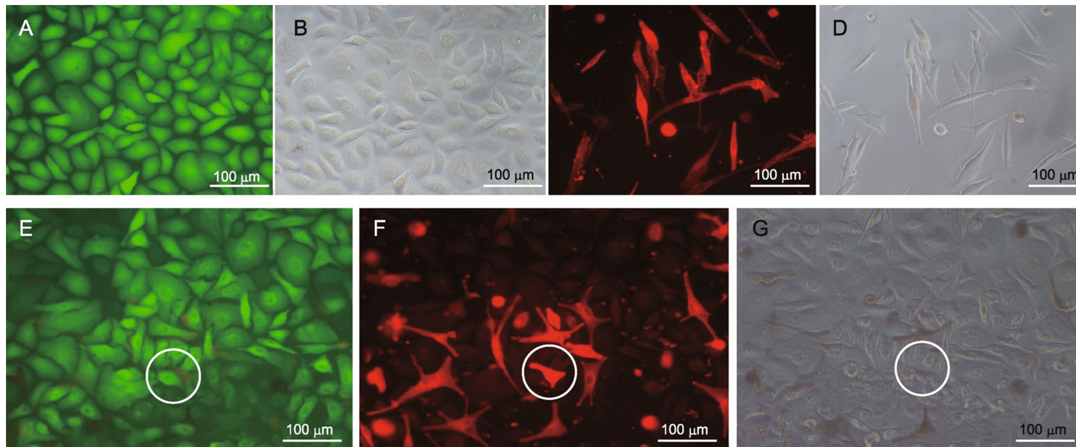

Figure 2 Micrographs of stained keratinocytes and melanocytes. Human keratinocytes stained with Calcein (A) Fluorescence, (B) bright-field image. Human melanocytes stained with Dil (C) Fluorescence (D) bright-field image. Coculture of keratinocytes and melanocytes (E) stained with Calcein (fluorescence) (F) stained with Dil (fluorescence) and (G) bright-field image. White circles are marking Dil stained melanocyte interacting with Calcein stained keratinocytes, observed under (E) blue excitation filter (450-480nm) (F) green excitation filter (510–550nm) and (G) bright-field in fluorescence microscope.

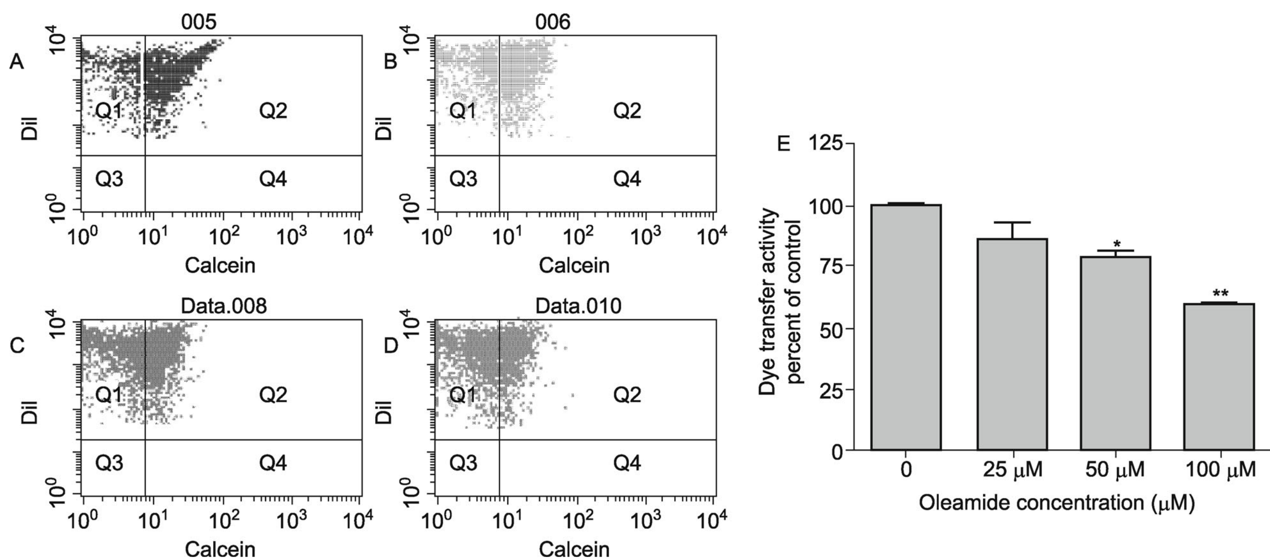

Figure 3 Gap junctional activity between keratinocyte and melanocyte–flow cytometry assay. Quadrant2 (Q2) cells are double positive (positive for both Calcein and Dil) indicating the dye transfer from donor (keratinocytes) to recipient (melanocytes) cells. (A) Control, (B) + 25μM of oleamide, (C) + 50μM of oleamide, (D) + 100μM of oleamide. (E) Dose dependent decrease in the double stained cells i.e. decreased gap junctional activity was observed in oleamide treated cocultures (F). *, $p < 0.05$ and **, $p < 0.01$

oleamide inhibition was subjected to qRT-PCR and western blot. qRT-PCR was done to assess the level of expression of tyrosinase the key enzyme for melanogenesis and MITF the master regulator of melanogenesis. Results showed that, treating the keratinocytes-melanocytes coculture with different concentrations of oleamide resulted in dose depended decrease in expression of MITF. Nearly 50% reduction at 50μM concentration of oleamide and 75% reduction at 100μM concentration of oleamide was observed.

Further, 50% reduction in the expression of tyrosinase was found when cells in the coculture was treated with 50μM and 100μM of oleamide (Fig. 5). Further, confirming the result of the reduction in tyrosinase gene expression, the western blot analysis showed 30% reduction in the tyrosinase protein level (Fig. 6). Interestingly the increased amount of tyrosinase enzyme in coculture compared to only melanocyte in western blots, endorses the probable interactions between keratinocyte and melanocytes in coculture, which induced melanogenesis. Additionally, decreased tyrosinase enzyme activity was also found when gap junctional activity was blocked in keratinocyte-melanocyte coculture using oleamide (Fig. 7A).

Effect of oleamide on gap junctional communication between melanocytes themselves

To evaluate the specificity of the action of oleamide we treated the coculture of Dil and Calcein stained melanocytes alone with oleamide. Remarkably, oleamide did not block the dye transfer between melanocytes (Fig. 4). In addition, there were no significant reduction in MITF and tyrosinase expression as well as tyrosinase protein level and activity when melanocytes alone treated with oleamide (Figs. 5, 6 and 7A). Therefore, we concluded that oleamide did not block the gap junctional activities between melanocytes themselves. Therefore, the inhibitory effect of oleamide on melanogenesis in melanocyte-keratinocyte coculture was the result of block in communication specifically between keratinocytes and melanocytes.

Effect of oleamide on tyrosinase enzyme

Oleamide is a known gap junction blocker, however reduction in tyrosinase enzyme activity on its treatment as observed in our study could be due to direct inhibition of tyrosinase enzyme activity. To address this issue, we performed the tyrosinase enzyme activity assay in melanocyte lysates in the presence of oleamide keeping the amount of cells used, concentration of oleamide and time of exposure similar to that of gap junction assay. As a positive control Kojic acid, a known tyrosinase inhibitor was used. Results showed that, oleamide did not inhibit the tyrosinase enzyme activity in melanocyte lysate at the concentration of 50–100μM, whereas Kojic acid significantly inhibited the tyrosinase activity (Fig. 7B). The results indicated that, inhibition of tyrosinase enzyme activity by the oleamide

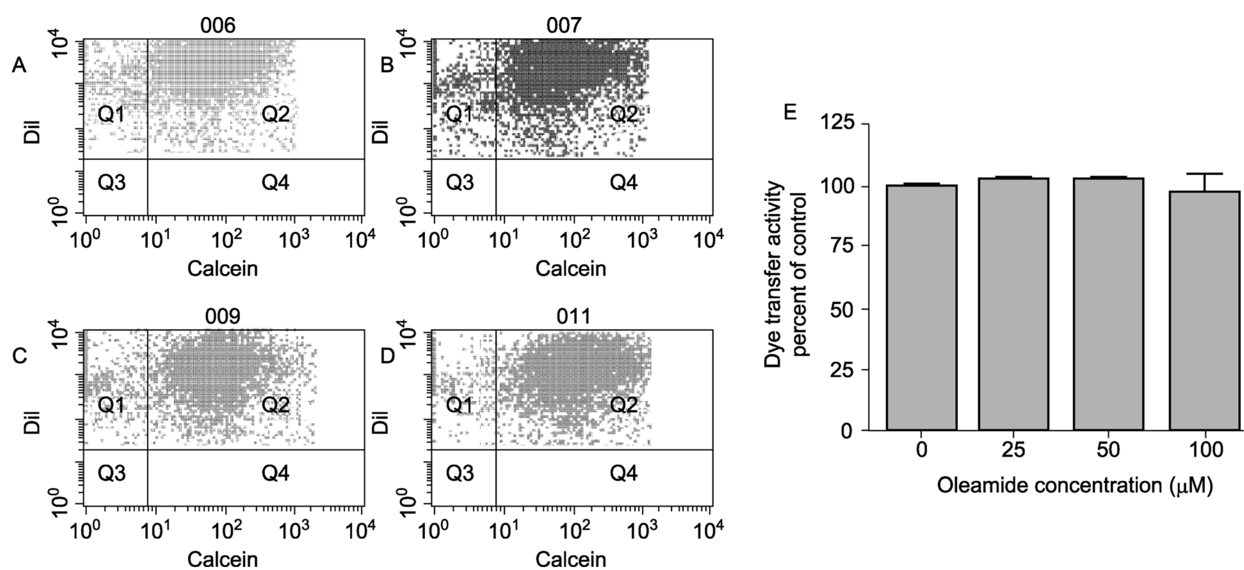

Figure 4 Gap junctional activity between melanocytes—flow cytometric analysis. Quadrant 2 (Q2) represents the cells which aredouble positive (positive for both Calcein and Dil) which in turn indicates the dye transfer from donor melanocytes (Calcein stained cells) to recipient melanocytes (Dil stained cells). (A) Control, (B) + 25μM of oleamide, (C) + 50μM of oleamide, (D) + 100μM of oleamide. (E) Addition of oleamide to the culture containing Calcein stained andDil stained melanocytes did not show any significant changes in the gap junctional activity.

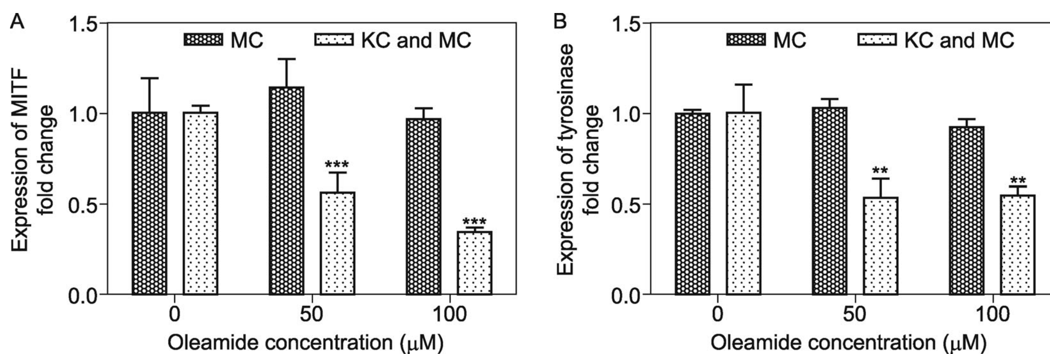

Figure 5 Effect of oleamide on transcriptional level of MITF (A) and TYR (B) in melanocytes and coculture of keratinocytes and melanocytes. No significant change was observed in theexpression level of MITF and TYR in melanocytes when treated with oleamide but in coculture of keratinocytes and melanocytes, there was a significant decrease in expression level of MITF (***, $p < 0.001$) and tyrosinase (**, $p < 0.01$) in comparison with untreated control group.

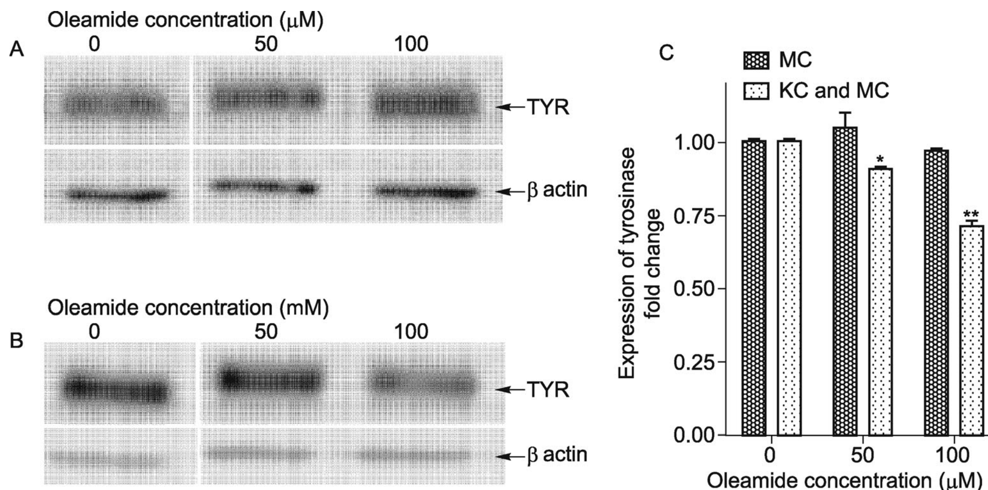

Figure 6 Western blot analysis to show the tyrosinase protein level. Level of tyrosinase protein in the cell lysate (A) from melanocytes alone (B) from coculture of melanocyte and keratinocytes with or without addition of oleamide. (C) Graphical representation to show the reduction in the expression of tyrosinase protein. Addition of oleamide significantly reduced the expression of tyrosinase protein in coculture of keratinocytes and melanocytes and not in the culture containing only melanocytes. The data represents the mean of two experiments with standard error (*, $p < 0.05$ and **, $p < 0.01$).

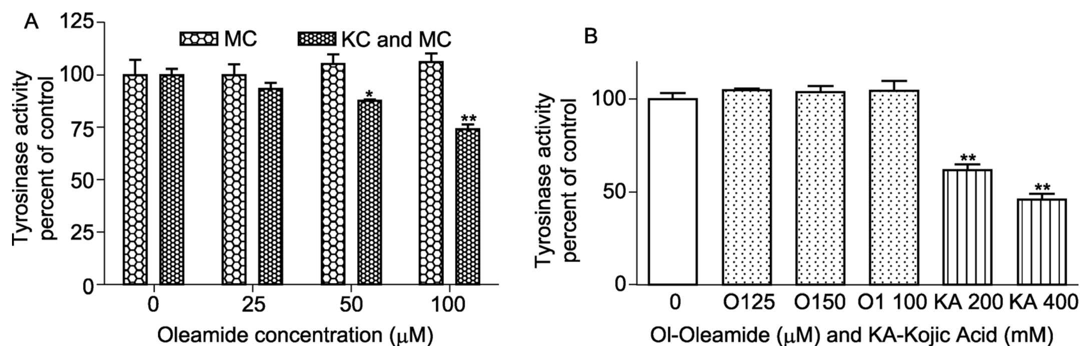

Figure 7 Effect of oleamide on tyrosinase enzyme activity. (A) No significant change in tyrosinase enzyme activity was found when melanocytes alone were treated with oleamide. However, significant reduction in the tyrosinase enzyme activity was found when coculture of keratinocytes and melanocytes were treated with oleamide. (B) Addition of oleamide to the melanocyte lysate did not alter the tyrosinase enzyme activity indicating no direct inhibitory effect of oleamide on tyrosinase enzyme activity. However, when the known inhibitor of tyrosinase activity, the Kojic acid was added to the melanocyte lysate we observed significant reduction in the tyrosinase activity.*, $p < 0.05$, **, $p < 0.01$ in comparison with control group.

observed in gap junctional assay was not due to direct inhibition of oleamide on tyrosinase enzyme activity.

Discussion

Gap junctions were previously considered as channels for passive flow of metabolites and ions, and have gained importance in many areas of cell signaling and communication (Zhou and Jiang, 2014). Our results showed preliminary evidence for the role of direct communication between keratinocyte and melanocytes in melanogenesis. We studied the gap junction activity using dye transfer method and flow cytometry. The flow cytometry method of quantification of gap junction activity assures the measurement of whole population as a unit unlike other methods such as scrap loading or microinjection method, which examines the efficacy of the gap junction blocker in a few cells (Fonseca et al., 2006). Our results showed that functional gap junction channels between keratinocytes and melanocytes facilitated the communication of signals continuously between them. When these cells were treated with oleamide a gap junction blocker in coculture, the process of melanogenesis was altered whereas no effects were seen when melanocytes alone were treated with oleamide. It was also evident that oleamide did not have any effect on tyrosinase enzyme activity directly. Thus the effect of decrease in melanogensis in coculture of keratinocytes and melanocytes upon treatment of oleamide, was probably due to the blocking of the specific and critical factors transferred from keratinocytes to melanocytes through

gap junctions. As gap junction channels are selectively permeable (Dbouk et al., 2009) we can assume that the keratinocytes and melanocyte interactions also could be selective. Oleamide selectively blocked dye transfer between keratinocytes and melanocytes but not between melanocytes. This selective nature of oleamide could not be explained but only reported by us. However, it indicated towards two separate systems of gap junctions between keratinocytes and melanocytes as well as between melanocytes, which are quite different from each other. We hypothesize that the cells which oleamide blocked, received less factors from keratinocytes compared to controls, through gap junctions. These factors may have melanogenesis inducing properties in melanocytes. Although our results are very preliminary and involve only one gap junction blocker, the concept is theoretically rational as a few factors, which are capable of being transported through gap junctions, do induce melanogenesis and are commonly discussed in pigmentation. The factors which have the potential to pass through gap junctions and may modulate the melanogenesis include cAMP, cGMP, NO and Ca^{2+} (Roméro-Graillet et al., 1996; Otreba et al., 2012). However, it has not yet proven that these factors travel through gap junctions and affect the melanin synthesis as a part of regulation. The present study shows that some type of gap junctions that could be blocked via oleamide has significant influence on melanogenesis. However, as there are different types of gap junctions, testing more number of specific gap junction blockers would confirm the role of direct cell-cell communication in melanogenesis.

In conclusion, oleamide a gap junction blocker inhibited the intercellular gap junctional communication between keratinocytes and melanocytes and altered the melanin synthesis with no effects on melanocytes individually. This suggests a less known pathway of regulation of melanogenesis by keratinocytes via direct communication, apart from the known paracrine pathways. Further, the role of connexins and types of connexins involved along with the factors that are transmitted through gap junctions between keratinocytes and melanocytes remain to be fully elucidated.

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Compliance with ethics guidelines

Divya Padma, K. Satyamoorthy and Kumar M.R. Bhat declare that they have no conflict of interest.

This project has obtained the institutional ethical clearance before conducting the experiments. In this project, foreskin samples were obtained from children who were undergoing circumcision surgery for various purposes. Informed consent from the parents/guardian of the subjects were taken before collecting the samples.

Abbreviations

bFGF: Basic fibroblast growth factor
Calcein AM: Calcein Acetoxymethyl
cAMP; Cyclic adenosine mono phosphate
cGMP: Cyclic guanosine mono phosphate
Dil: 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate
DMEM: Dulbecco's Modified Eagle Medium
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF: Granulocyte macrophage colony-stimulating factor
HRP: Horseradish peroxidase
IBMX: 3-isobutyl-1-methylxanthine
L-DOPA: L-3, 4-dihydroxyphenylalanine
MITF: Microphthalmia-associated transcription factor
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NO: Nitric oxide
PMA: Phorbol 12-myristate 13-acetate
qRT-PCR: Quantitative real time polymerase chain reaction
SCF: Stem cell factor
SDS-PAGE: Sodium dodecyl sulfate- Polyacrylamide Gel Electrophoresis
α-MSH: alpha-Melanocyte-stimulating hormone

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