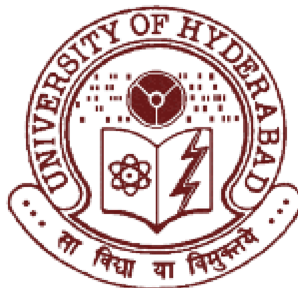
Studies on the Regulation of Keratinocyte Proliferation and Differentiation: Role of 12R-Lipoxygenase

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

Geetika Gupta



Department of Animal Biology
School of Life Sciences
University of Hyderabad
Hyderabad-500 046, INDIA

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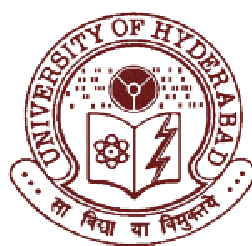

University of Hyderabad
(A Central University established in 1974 by act of parliament)
Hyderabad-500 046, INDIA

DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Studies on the regulation of keratinocyte proliferation and differentiation: Role of 12R-lipoxygenase."** has been carried out by me under the supervision of **Prof. P. Reddanna** and this has not been submitted for any degree or diploma of any other university earlier.

Prof. P. Reddanna
(Research Supervisor)

Geetika Gupta
(Research Scholar)

Prof. Aparna Dutta Gupta
(Research Co-Supervisor)

University of Hyderabad

(A Central University established in 1974 by act of parliament)

Hyderabad-500 046, INDIA

CERTIFICATE

This is to certify that **Ms. Geetika Gupta** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend her thesis **"Studies on the regulation of keratinocyte proliferation and differentiation: Role of 12R-lipoxygenase."** for submission for the degree of Doctor of Philosophy of this University.

Prof. P. Reddanna
Research Supervisor ______________________________

Prof. Aparna Dutta Gupta
Research Co-Supervisor ______________________________

Head
Department of Animal Biology ______________________________

Dean
School of Life Sciences ______________________________

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Table 1 : List of primers used for RT-PCR analysis

List of Abbreviations

^{0}C	:	Degree centigrade/ Degree Celsius
µM	:	Micro molar
AA	:	Arachidonic Acid
AD	:	Autosomal dominant
ARCI	:	Autosomal recessive congenital Ichthyosis
ASD	:	Autosomal subdominant
BSA	:	Bovine serum albumin
CCE	:	Cornified cell envelope
CE	:	Cornified envelope
CLE	:	Corneocyte lipid envelope
COX	:	Cyclooxygenase
DAPI	:	4',6-diamidino-2-phenylindole
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
ECL	:	Electrochemiluminescence
EDTA	:	Ethylene diamine tetra acetic acid
EDTA	:	Ethylenediaminetetraacetic acid
EFA	:	Essential fatty acids
EOS	:	Esterified ω-hydroxyacyl-sphingosine
FACS	:	Fluorescence activated cell sorter
FBS	:	Fetal bovine serum
FFA	:	Free fatty acids
FITC	:	Fluorescein isothiocyanate
FLAP	:	5-LOX activating protein
g	:	Gram
h	:	Hour(s)
HETE	:	Hydroxyeicosatetraenoic acid

HODE	:	Hydroxyoctadecadienoic acid
HPETE	:	Hydroperoxyeicosatetraenoic acid
HRP	:	Horseradish peroxidase
K1	:	Keratin 1
K5	:	Keratin 5
K10	:	Keratin 10
K14	:	Keratin 14
kDa	:	Kilo Dalton
l	:	Litre
LA	:	Linoleic acid
LBs	:	Lamellar bodies
LOX	:	Lipoxygenase
LT	:	Leukotriene
LX	:	Lipoxin
mg	:	Milligram
min	:	Minute(s)
ml	:	Milliliter
mM	:	Millimolar
MTT	:	3-(4, 5-dimethylthiazol-2-yl)-2,5- Diphenyl tetrazolium bromide
nm	:	Nanometers
OS	:	ω-hydroxyacyl-sphingosine
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PG	:	Prostaglandin
PI	:	Propidium iodide
PMSF	:	Phenylmethanesulphonylfluoride
PUFAs	:	Polyunsaturated fatty acids
RP-HPLC	:	Reverse phase- High pressure liquid chromatography

RT-PCR	:	Reverse transcriptase-polymerase chain Reaction
SB	:	Stratum basale
SC	:	Stratum corneum
SDS	:	Sodium dodecyl sulfate
SG	:	Stratum granulosum
SS	:	Stratum spinosum
TEMED	:	N,N,N’,N’-tetramethylene diamine
TEWL	:	Transepidermal water loss
TGM-1	:	Transglutaminase-1
Tris	:	Tris-(Hydroxymethyl) aminoethane
UV	:	Ultraviolet
X-LR	:	X-linked recessive

Chapter 1

General Introduction

1.1. Human Skin

The human skin is the largest and complex organ that shows heterogeneity in its origin from mesenchymal and epithelial cells. The major functions of human skin are to form a barrier against unwanted environmental influences like ultraviolet light, chemicals, pathogenic microorganisms and mechanical insult and to prevent the excessive loss of water and electrolytes from the body [1]. The skin comprises of the epidermis and dermis as well as the subcutaneous fat tissue [2]. The epidermis is the outermost layer of the skin consisting of several cell types including Langerhans cells, melanocytes and keratinocytes. The most abundant cell type is the keratinocyte, which synthesizes major structural components of the epidermal barrier through a programmed process of differentiation.

Epidermis is composed of four distinguished layers. Each layer represents one of the sequential differentiation stages in the terminal differentiation of keratinocytes. The layers display the superficial stratum corneum (SC) consisting of 10-25 layers of flat, dead corneocyte cells, stratum granulosum (SG) containing a high number of membrane-coating lipid contained granules called as the lamellar bodies (LBs), stratum spinosum (SS) comprising keratinocytes switched for differentiation, and the inner most stratum basale (SB) containing undifferentiated proliferating keratinocytes [3][4][5] (Figure 1). As keratinocytes escape from the SB after mitosis, they transiently migrate towards SC during terminal differentiation and finally adopt the characteristics of flattened corneocyte cells giving the structure of SC as “bricks in mortar” design where corneocytes are bricks and lipids are mortar. These corneocytes are filled with

water and microfibrillar keratin which is surrounded by cornified cell envelope (CCE) made up of lipids and proteins. The process of making of this CCE during terminal differentiation is referred as cornification which is primarily a step in establishing the permeability barrier function of skin.

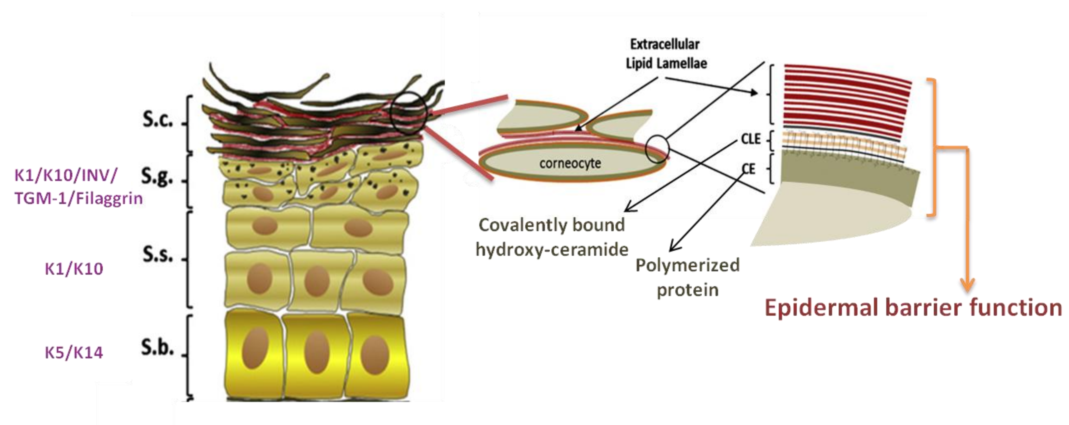


Figure 1: Structure of epidermis and epidermal barrier function. (Source: P Krieg et al, Biochimica et Biophysica Acta 1841, 2014)

1.2. Skin and barrier function

One of the most essential roles of the human skin is to perform the epidermal water barrier function [6]. Life on land would be impossible without permeability barrier. Impaired barrier function either in premature born infants or in patients with inherited skin disorder results in dehydration and increased susceptibility to infections [7][8][9]. The permeability barrier of skin localizes primarily to the outermost layer of the epidermis, stratum corneum (SC) and the lipids composition and organization in SC is the critical factor of this impermeability property [10][11]. Lipids are essential to this impermeability property through preventing the fluid loss, restricting the entry of allergens and toxic compounds and having antimicrobial properties [1][12][13]. Lamellar bodies of SG are the main precursors of the SC lipids which are enzymatically processed

into their final constituents: ceramides (50%), cholesterol (25%) and free fatty acids (FFAs) (15%). With the process of exocytose, the lipid content of the lamellar bodies is released together with hydrolytic enzymes into the intercellular space at the SG/SC interface [14].

Stratum corneum is a part of late terminal differentiation process of keratinocytes and accomplished by making its three major components- 1) matrix of intercellular lipids comprising mainly cholesterol, ceramides and free fatty acids organized in layers called lamellae, 2) cornified cell envelop (CCE) composed of a inner protein layer (of filaggrin, loricrin and Involucrin), cornified envelop (CE) and an outer lipid monolayer (of ω-hydroxylated ceramides and FFAs), the corneocyte lipid envelop (CLE) and 3) intracellular keratin-filaggrin degradation products [15][16][17][18] (Figure 1). In the establishment of CCE, the monolayer of non-polar lipids, CLE is esterified to the CE with the help of Transglutaminase-1 (TGM-1) mainly to glutamate residues of involucrin or filaggrin [19][20]. Making of this covalent bond in CCE enables it as a highly insoluble, sealing and extremely rigid structure formed beneath the cell membrane during terminal differentiation of keratinocytes to provide barrier function of skin.

1.3. Essential fatty acids in barrier function

Since 1929, when existence of essential fatty acids (EFAs) were reported by Burr and Burr, from several studies it is clear that there is some connection between EFAs and proper functioning of water impermeable barrier of skin [21]. Taking of EFA deficient diet consequences in an abnormal scaly skin disorder and accelerated transepidermal water loss (TEWL) that reflects defects in barrier

function [22]. Further, studies show special requirement of EFAs such as linoleate (C18: 2) to maintain and process the water impermeable barrier of epidermis [23]. Linoleate is being primarily incorporated in a specific class of ceramides, esterified ω-hydroxyacyl-sphingosine (EOS), unique to the epidermis through esterification to hydroxy of the amide-linked very long chain fatty acid (VLFA) [24]. EOS is further converted to ω-hydroxyacyl ceramides and ω-hydroxy-VLFA which covalently attach to the proteins of CE and constitute a CLE underlying CE to make essential structural integrity of the epidermal water barrier [19][25][26]. Substantial evidence reveals the involvement of lipoxygenase (LOX) enzymes in the oxidation of linoleate moiety of esterified structural lipids such as ceramides to form oxygenated derivatives and to provide ω-hydroxyacyl-sphingosine (OS) with free ω-hydroxy group, which plays an essential role in barrier function [27][28].

1.4. Polyunsaturated fatty acids and Eicosanoids

Polyunsaturated fatty acids (PUFAs) are associated with membrane phospholipids which are complexed with glycerol backbone. The phospholipases (A1, A2, C and D) get activated in response to either physiological or pathological stimuli to release PUFAs, which in turn get oxygenated by variety of pathways and lead to the formation of biologically active metabolites such as eicosanoids. Eicosanoids are a family of oxygenated metabolites of the 20-carbon fatty acids such as arachidonic acid (AA) which is released from phospholipids and diacylglycerol, principally, by the action of cytosolic phospholipase A2 (cPLA2) and diacyl glycerol lipase respectively to get available for oxidative metabolism by distinct enzymatic pathways [29][30].

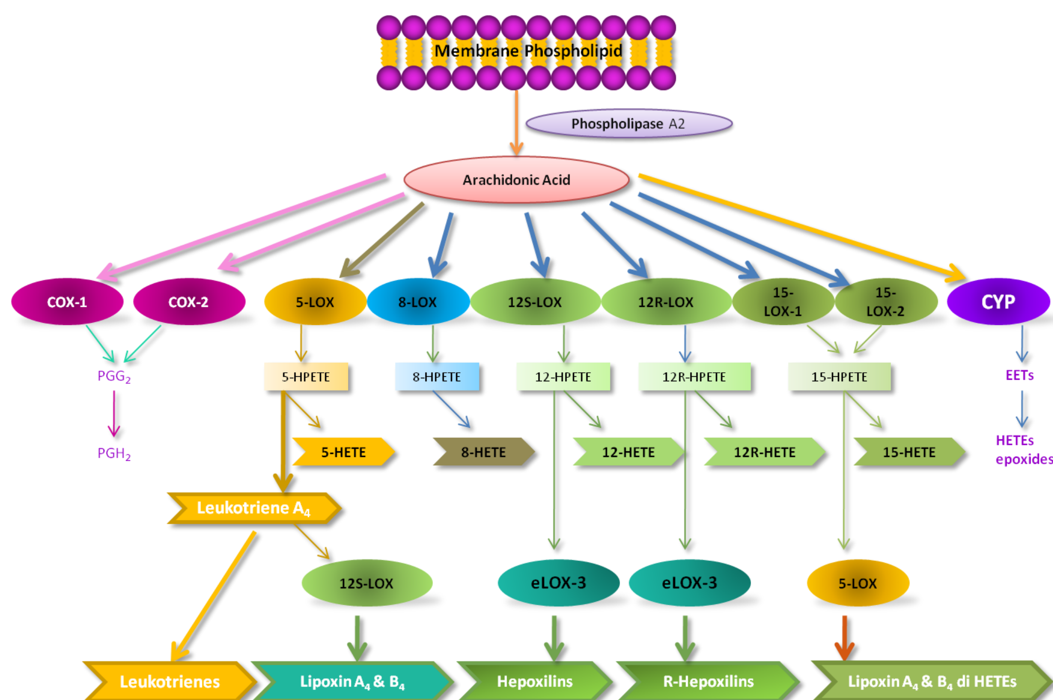


Figure 2: Arachidonic acid metabolism.

Eicosanoids are synthesized primarily by three enzymatic oxygenation pathways, each involving a distinct family of enzymes: cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome p450 (CYP) dependent monooxygenases (Figure 2). The COXs transform AA to prostaglandin H2 (PGH2), the precursor of prostaglandins, thromboxanes & prostacyclin [31] whereas LOXs metabolize AA in to hydroperoxy intermediates which are further transformed to hydroxyeicosatetraenoic acids (HETEs), leukotrienes & lipoxins [32]. Cytochrome p450 dependent monooxygenases metabolize AA to cis- epoxyeicosatrienoic acids (EETs) which have been shown to have major effects on physiological processes such as vascular & bronchial smooth muscle tone, peptide hormone secretion & ionic transport. COXs exist in two isoforms- COX-1, expressed constitutively in nearly all mammalian tissues is the source of prostaglandins associated with

"housekeeping" functions like vascular homeostasis and renal water re-absorption, and COX-2, expressed in most tissues at very low level unless induced by mitogenic or hormonal stimuli [31].

1.5. Mammalian lipoxygenases

LOXs represent a widespread family of non-heme, non-sulfur iron containing dioxygenases that incorporate molecular oxygen stereospecifically into PUFAs containing a (cis, cis)-1,4-pentadiene system [33][32]. From more than 70 years many studies reveal the existence of LOXs in plant kingdom [34] but in mid 1970s, the first report came on LOX activities in animal tissues. These studies established the existence of LOX activities in various hematopoietic cells like blood platelets [35][36], leukocytes (showing the primary step in leukotriene biosynthesis) [37] and reticulocytes [38]. Studies over the years showed the existence of these enzymes in many other mammalian tissues [39]. The distribution of LOXs appear widely throughout the plant and animal kingdoms including lower organisms like slime molds, corals, algae and recently in certain bacteria [40][41][42]. In plants, LOX metabolism generates signalling molecules like jasmonic acid [43][44].

The mammalian LOX enzymes prefer arachidonic acid (AA) as a substrate which is oxygenated to regioisomeric hydroperoxyeicosatetraenoic acids (HPETEs) which may be further metabolized to various tissue specific bioactive lipid mediators such as, leukotrienes, lipoxins, corresponding hydroxyeicosatetraenoic acids (HETEs), hydroxyoctadecadienoic acids (HODEs) and hepoxilins [32][39][45]. As endogenous signalling molecules, these LOX metabolites play an important role in blood clotting, vasoconstrictions, bronchocontrictions, inflammatory processes,

leukocytes chemotaxis [46][47], asthma [48], various inflammatory skin diseases like psoriasis [49][50], atherosclerosis [51][52] and cancer [53][54]. These products regulate cell growth and proliferation as well as survival. Thus arachidonic acid metabolism plays a central role in tumorogenesis [53].

1.5.1. Types of Mammalian lipoxygenases

Now it is clear from several studies the presence of six different functional lipoxygenase genes (*ALOX5*, *ALOX12*, *ALOX15*, *ALOX15B*, *ALOX12B* and *ALOXE3*) and two pseudogenes (*ALOX12P1*, *ALOX12P2*) in humans whereas total seven isoforms have been identified in mice. Classically mammalian LOXs are categorized based on the positional specificity of oxygen insertion into arachidonic acid (for e.g., 5-, 8-, 12- and 15-LOX) [55] and according to the prominent tissue of their existence - like platelet-type 12-LOX (*p*12-LOX), leukocyte-type 12-LOX (*l*12-LOX) and epidermis-type 12-LOX (*e*12-LOX) [32].

Currently, based on several studies, the phylogenetic linkage between all LOXs, mammalian LOXs are divided into four subgroups- (I) generalized 5-LOX [56], (II) platelet-type 12S-LOX (*p*12-LOX), (III) leukocyte-type 12/15-LOX (including both reticulocyte-type 15-LOX-1 and leukocyte-type 12S-LOX (*l*12-LOX) exhibiting a dual positional specificity) and (IV) with respect to their positional specificity and chirality a more heterogeneous group of epidermis-type LOX (12R-LOX, 15-LOX-2, 8-LOX, epidermis-type LOX-3 (eLOX-3)) [39][57][58][59] (Figure 3). Reticulocyte-type 15-LOX-1 is also present in eosinophils and trachea epithelium. In human, epidermis-type 15-LOX-2 of hair follicles represents the human homologue of mouse 8S-LOX [32]. Further, platelet-type 12S- LOX (p12-LOX) is also found in

epidermis [59]. Moreover, in epidermis-type of LOX, 12R-LOX represents the first mammalian LOX generating products with R chirality [60].

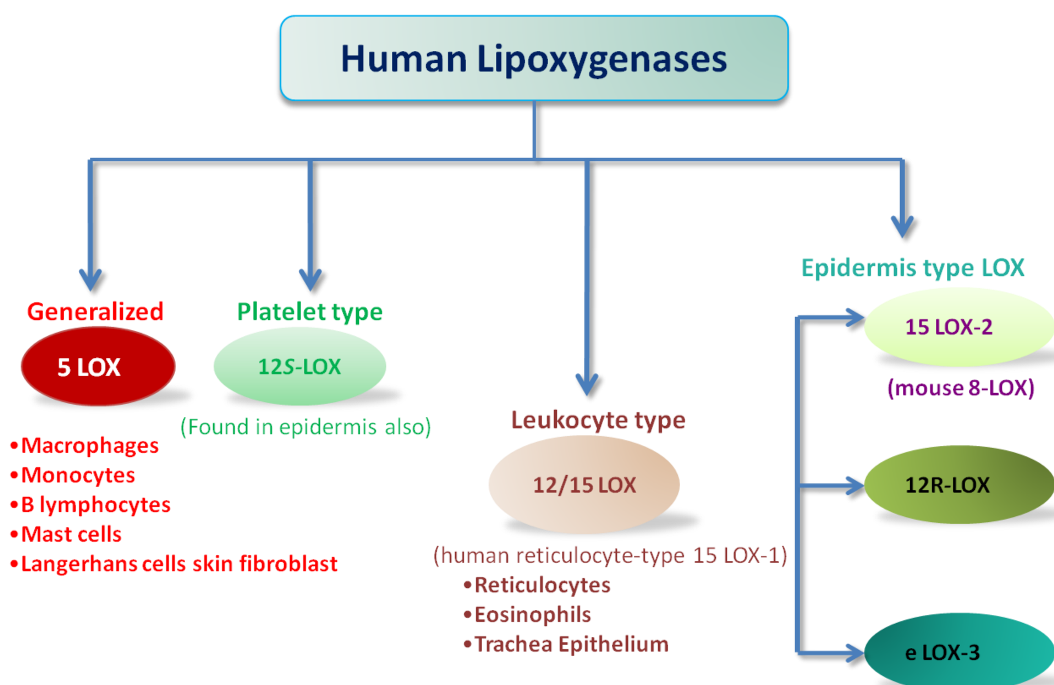


Figure 3: Subdivision of human lipoxygenases.

1.5.2. Structure of lipoxygenases

All the members of the LOX multigene family show very highly conserved gene and protein structures exhibiting 40–90% identical sequence and comprising well-conserved amino acid residues essential for the enzyme catalytic activity and the unique tertiary structure of the proteins. Entire human LOX genes are located at the human chromosome 17.p13 [61] and the mouse chromosome 11, respectively with the exception of the 5-LOX gene which is situated on chromosome 10 [62][63]. This reflects the common ancestor gene origin. In general, mammalian LOX genes comprising of 14 or 15 exons with exon/intron boundaries placed at highly conserved positions and all genes map close together at the chromosome. LOX proteins have a single polypeptide chain with a

molecular mass of ~75–81 kDa (662–711 amino acids) in mammals. The unique tertiary structure, that provides the ability to bind catalytic iron, is attributed to the highly conserved domains and sequence motifs of the polypeptide chain. Plant and mammalian LOXs, both exhibit same tertiary structure which displays two domains, an N-terminal β-barrel domain and a larger C-terminal catalytic domain consisting of a single non-heme iron atom in the ferrous (inactive) form and the substrate binding cavity. Oxidation to the active ferric enzyme is essential for the catalytic activity of LOXs [55]. The iron metal is liganded to conserved histidine and to the carboxyl group of a conserved isoleucine at the C-terminus of the protein. The positional specificity of the distinct isoforms of LOX is determined by the size of the substrate binding cavity and the alignment of the substrate [64].

1.6. Human lipoxygenases: Biological roles and functions

LOX metabolism leads to the biosynthesis of various lipid hormones such as leukotrienes, lipoxins, HETEs, HODEs and hepoxilins which are potent endogenous signaling molecules involved in regulation of cell growth and proliferation as well as survival and apoptosis [65][66][67]. However, some LOX subtypes like reticulocyte-type 15-LOX-1, leukocyte-type 12S-LOX, epidermis-type 15-LOX-2, 12R-LOX and eLOX-3 play a biological role outside the arachidonic acid cascade such as reticulocyte maturation, differentiation or participation in some structural changes. Further, several lines of studies have reported that overexpression of LOXs leads to progression of a variety of cancers like prostate, liver, colon, pancreas, skin, oral and epidermoid, suggesting a role for eicosanoids in carcinogenesis [68][69][70]. The involvement of LOXs in tumor formation and

cancer metastasis is also revealed from the studies on animal models and clinical observations [53][71]. Increasing new insights on connection of LOX metabolism and carcinogenesis lead to development of cancer chemoprevention based on novel molecular targets. These targets cover procarcinogenic LOXs like 5-LOX and 12S-LOX and anticarcinogenic LOXs like 15-LOX-1 and possibly 15-LOX-2.

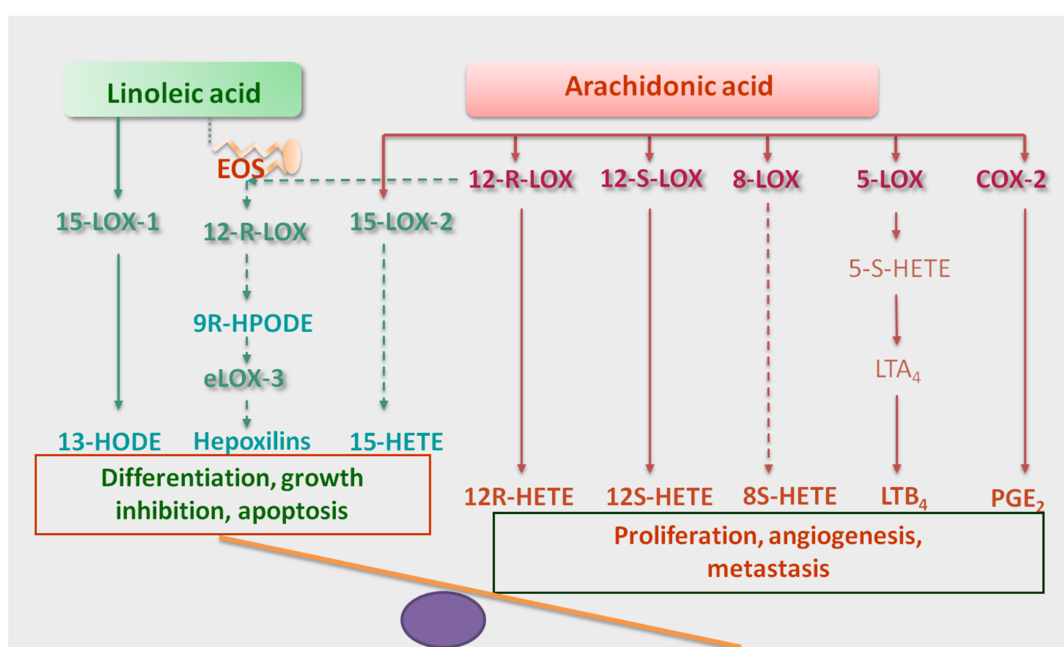


Figure 4: Dynamic balance among the PUFA metabolic pathways of arachidonic acid and linoleic acid through LOXs and COX-2 during tumorogenesis.

In cells, these LOX metabolic pathways occur in a dynamic balance that shifts during carcinogenesis toward 5-, 8-, and 12-LOX (and COX-2) and away from 15 LOX (Figure 4). Additionally, 12R-LOX appears to act as both procarcinogenic and anticarcinogenic (depends on availability of substrate- arachidonic acid or linoleic acid) [64]. So the development of LOX modulators, that can induce the anticarcinogenic and/or inhibit the procarcinogenic LOXs, thereby shifting the balance of LOX activities from procarcinogenic to anticarcinogenic metabolism of

polyunsaturated fatty acids, would be a novel approach to develop cancer chemotherapy [72]. Elsewhere, all LOXs metabolize AA in to HPETEs which further reduced to HETEs and the metabolism of linoleic acid (LA) preferentially results in the formation of hydroxyoctadecadienoic acids (HODEs) [73]. Therefore, the major AA metabolites formed by mammalian LOXs are 5-, 8-, 12- and 15-HETEs and the main reaction products of linoleic acid oxygenation are 9- and 13- HODEs [74]. Further, the pro- and anti-carcinogenic effects of LOX products, in turn might depend on the type of end product(s) formed in the tissue, as hydroperoxy (HPETEs and HPODEs) metabolites were shown to inhibit while the hydroxy (HETEs and HODEs) metabolites stimulate or inhibit the growth of cancer cells [67][75].

5-LOX converts AA into a pro-inflammatory metabolite, 5-HPETE, which further is converted to leukotrienes A_4 (LTA_4) and then to LTB_4 and cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) and as well as anti-inflammatory metabolites, lipoxin A_4 (LXA_4) and lipoxin B_4 (LXB_4) [76]. These leukotrienes constitute mediators of various anaphylactic and allergic reactions like hay fever, asthma and implicated in inflammatory disorders like psoriasis, inflammatory bowel disease and arthritis and in inflammation-associated carcinogenesis. So the agents targeting this pathway have been proposed for cancer chemoprevention [77]. 5-LOX has been found to be over expressed in human cancers of prostate [78], pancreas [79][80], colon [81], urinary bladder [82], testis [83], renal cell carcinoma [84], oral cavity [85] and esophagus [86]. Further, 5-HETE inhibits apoptosis induced by a specific FLAP inhibitor- MK-886 in prostate cancer cell lines [87]. 5-LOX stimulation affects the migration and invasion of breast cells by producing

metalloproteases [88]. On the other hand, lipoxins represent a group of endogenous anti-inflammatory metabolites of AA, formed by the action of 5- and 15-LOXs, and analogs of these metabolites show very promising results in treatment of inflammatory diseases. Moreover, LXA_4 antagonizes the proinflammatory effects of LTB_4 and LTD_4 [89][90].

The biological role of cytosolic leukocyte-type 12/15-LOX in atherogenesis has been discussed during past few years [91][92]. The *l*12-LOX transforms AA into both 12S-HETE and 15S-HETE [54]. The possible role of 12/15-LOX in cell differentiation and atherogenesis is attributed to its capability of oxygenating polyenoic fatty acids esterified to complex lipid/protein assemblies (biomembranes, lipoproteins). The studies show the role of reticulocyte-type 15-LOX-1 in cell development and differentiation [93][94] where this enzyme has been implicated in monocyte/macrophage transitions and/or in macrophage functions. 15-LOX-1 is the only 15-LOX isoform found in the epithelium of human colon [95]. In colon cancer cells and human tracheobronchial epithelial cells, induction of differentiation causes the expression of 15-LOX-1 [96][97]. Further, a reduction in the level of 13-HODE and 15-LOX-1 expression has been shown in human colorectal cancers and human esophageal cancers. Moreover, treatment with 13-HODE and restoration of 15-LOX-1 expression has resulted in induced apoptosis and cell cycle arrest in colorectal cancer cells and human esophageal cancer cells respectively [65][98].

The microsomal platelet-type 12-LOX (*p*12-LOX) metabolizes AA exclusively into 12S-HETE, a profound modifier of tumor cell function and this transformation

was first demonstrated in human and bovine platelets [35] and this was the very first documented evidence of a lipoxygenase in the animal kingdom. The *p*12-LOX is generally absent in normal epithelia and can be induced by pro-inflammatory stimuli. Many studies reveal the constitutive expression of *p*12-LOX in various epithelial cancers including breast, prostate, colon, lung and in human epidermoid carcinoma A431 cells [53][99][100][101]. Further, *p*12-LOX and its metabolites have been shown to play a key mediator role in inducing tumor growth in animal model and increasing metastasis of breast cancer [102] and prostate cancer [103]. In addition, 12S-HETE has been shown as an important regulator of human cancer development. Many studies indicate that level of 12S-HETE biosynthesis in tumor cells can be used to know their metastatic potential [104]. Moreover, nordihydroguiaretic acid (NDGA), a non-specific inhibitor of lipoxygenases, has been demonstrated to bring down the adhesion of breast cancer cells to endothelial cells [105].

Further, 12-LOX of either type has been implicated in the biosynthesis of hepoxilins and lipoxins. Many hepoxilins are potent endogenous signaling molecules involved in the control of vasoconstriction, bronchoconstriction, blood clotting, as well as granulocyte chemotaxis and other inflammatory reactions [59].

1.7. Lipoxygenases functions in skin

In both human and mice skin, LOXs expressions are detected by immunohistochemistry, activity and polymerase chain reaction (PCR) of respective mRNA. From several studies, it is revealed that these LOXs play an important role in the modulation of epithelial proliferation and differentiation as well as in

wound healing, inflammation, inflammatory skin diseases and cancer [64][106]. In human skin, the 5-LOX metabolic pathway is mainly attributed to the myeloid cells including Langerhans cells which are identified as sole source of mRNA and protein of 5-LOX [107]. In addition, leukotrienes have been shown to play a crucial role in inflammatory skin disease like psoriasis, atopic dermatitis and UV-induced erythema [108][109][110]. In skin, 12/15-LOX activity is accountable for abundant production of 13-HODE using linoleic acid as a substrate and the studies reveal the counterbalancing role of 13-HODE by down regulating *p*12-LOX expression on the epidermal proliferation and inhibition of differentiation after treatment with 12-HETE [111][112].

The *p*12-LOX has been exhibited to be the predominant LOX isoenzyme in human and murine epidermis. Human epidermis displays *p*12-LOX expression in the germinal layer where its overexpression has also been reported in psoriatic skin [113], whereas, the expression of *p*12-LOX exists in both basal and suprabasal germinal epidermal layers of neonatal mouse skin [114]. The expression of *p*12-LOX during mouse embryonic epidermal development arises at embryonic day E13.5 and attains its maximum level of expression around E14.5 and E15.5 and this is the time when commitment to terminal differentiation happens [115]. The minor role of *p*12-LOX in epidermal barrier function has been revealed from the study in which *p*12-LOX-deficient mice showed slightly increased transepidermal water loss (TEWL) compared to a normal skin phenotype [116]. In differentiating keratinocytes, the expression of *p*12-LOX is regulated by p63, a key modulator of terminal differentiation of keratinocytes [115]. On the other hand, the exclusive product of *p*12-LOX, 12-HETE has also been shown to provoke proliferation and to

suppress terminal differentiation of keratinocytes [117]. Thus in epidermis, *p*12-LOX and its product 12-HETE display both proliferating and differentiating impacts depending on its onset of expression in either basal or suprabasal compartments of skin epidermis [64]. Further, in mouse, induced benign and malignant skin tumors by tumor promoter phorbol ester, TPA show elevated *p*12-LOX expression and activity (12-HETE production) [54], whereas *p*12-LOX deficient mice are less prone to develop tumor with the same protocol [118].

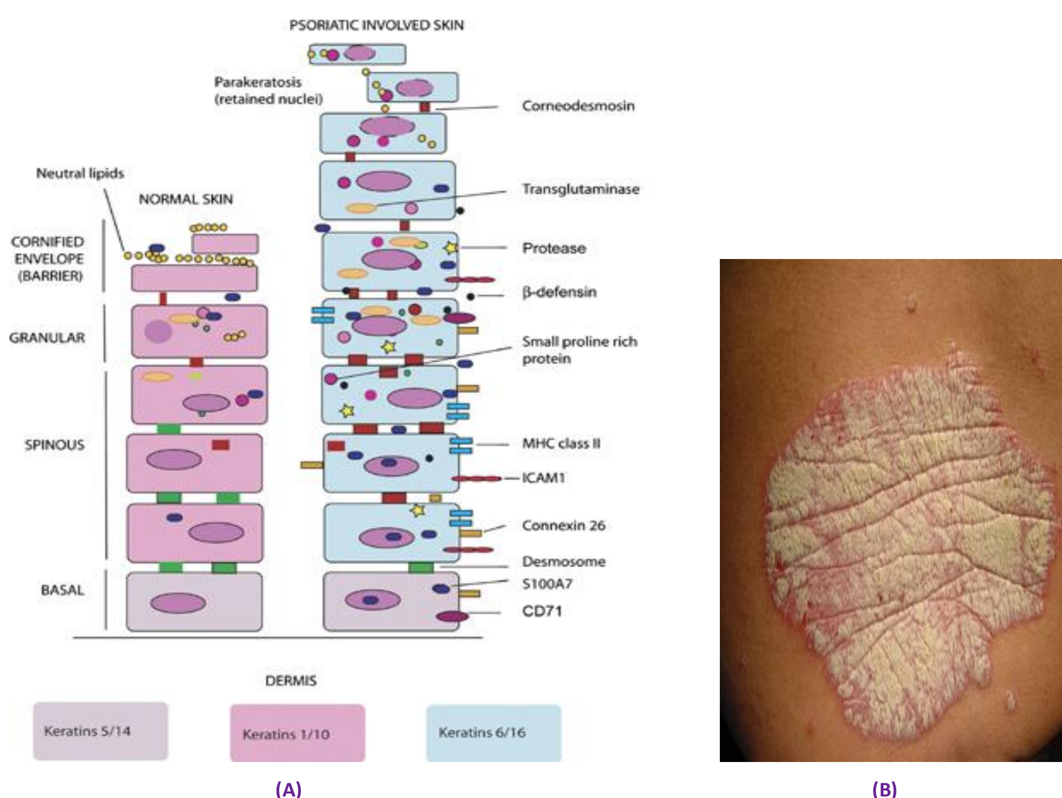


Figure 5: Psoriatic skin. (A) Structure of psoriatic epidermis (B) Patient with psoriasis. (Source: Y Liu et al, Genes and Immunity 8, 2007)

From previous studies, it is clear that both 12-LOXs- *p*12S-LOX and *l*12S-LOX have been involved specifically in the formation of 12S-HETE. Further, in 1975, the accumulation of 12-HETE in psoriasis has been reported by Hammarstrom *et al*. [119] which is the first indication for a role of PUFA

metabolism in human skin. So, it is therefore of interest that the 12-HETEs in psoriatic scales and other proliferative dermatoses are not the expected LOX derived 12S-HETE but instead consist predominantly of 12R-HETE [120] (Figure 5). From this stereochemical difference, it may indicate that 12-HETE is synthesized by different enzymes. Initially thought to involve only cytochrome P450 [121], 12R-HETE production also involves a 12R-LOX, which has been cloned from human skin [60]. This 12R-LOX together with 15-LOX-2 and eLOX-3 constitute a more heterogeneous group called epidermis-type of lipoxygenases. In epidermis, under physiological conditions, however, LOXs are known to regulate normal proliferation and differentiation of epithelial cells and keratinocytes [122].

1.8. Epidermis-type of lipoxygenases

Epidermis-type lipoxygenases, a distinct subgroup within the multigene family of mammalian LOXs, comprise novel isoenzymes isolated from human and mouse skin including human 15-LOX-2, human and mouse 12R-LOX, mouse 8S-LOX (human 15-LOX-2 homologue) and epidermis type LOX-3 (e-LOX-3) [45][122]. All genes of epidermis-type of lipoxygenases exist as a gene cluster of approximately 100 kb on human chromosome 17p13.1 containing gene encoding human e-LOX-3, *ALOXE3*, the 12R-LOX gene, *ALOX12B,* the 15-LOX-2 gene, *ALOX15B,* and a novel 15-LOX pseudo gene, *ALOX15P*. In this unique gene cluster, *ALOXE3* and *ALOX12B* are arranged in a head-to-tail fashion separated by 8.5 kb and both genes are divided into 15 exons and 14 introns spanning 22 and 15 kb, respectively. Whereas, *ALOX15B* occurs on the opposite DNA strand 25 kb downstream of *ALOX12B* and split into 14 exons and 13 introns spanning 9.7 kb of genomic sequence [122].

From several studies it is revealed that together with this property of existence as gene cluster, epidermis-type of lipoxygenases render mammalian skin as a tissue with an extensive and most diverse LOX metabolism. Thus, besides the conventional LOX isozymes- 5-LOX, 12/15-LOX and *p*12-LOX, seven novel epidermis-type LOX isozymes have been identified and cloned so far from skin of human and mice. These include a human 15-LOX-2 and its murine homologue 8S-LOX, a murine epidermis type *e*12-LOX, human and murine 12R-LOX and eLOX-3 [59]. The second 15-LOX isoform, 15-LOX-2 was cloned from human hair follicles [58]. 15-LOX-2 convert AA almost exclusively into 15-HETE. Both 15-LOX-2 and mouse homologue 8-LOX expression mainly attributed to hair roots, secretory cells of cutaneous adnexa and most abundantly to sebaceous glands [123]. Thus, the expression of 15-LOX-2 in secretory bodies and in psoriatic secretory cells reflects a role in regulating secretory differentiation or secretory process. In addition, 15-LOX-2 expression has also been shown in a variety of other human epithelia including cornea, esophagus, head and neck, prostate, lung and pancreas [58]. The biological functions of 15-LOX-2 and its product are generally contributed to growth suppression and modulation of differentiation through action of PPARγ [124][125]. Similarly, human 15-LOX-2 expression has also been demonstrated to be lost during development of prostate neoplasia and malignant progression of oral keratinocytes revealing the role of the enzyme as negative cell cycle regulator or tumor suppressor [126][127].

The characterization of atypical AA metabolites, 12R-HETE, in proliferative psoriatic scales reveals the existence of 12R-oxygenase which was later (about after two decades) shown to belong to the 12R-LOX. From the past study it has

been indicated that 12R-HETE promotes the proliferation of colon cancer cells [71]. Moreover, previously, we also have shown the pathological role and overexpression of 12R-LOX in human skin cancer cell line [128]. However, the role of 12R-LOX in normal skin development is implied from its spatial and temporal expression patterns in the mouse embryo [61].

Mutation in LOX gene causes disease

An important breakthrough in understanding the role of epidermis-type LOX enzymes in epidermis was accomplished by a genetic study in 2002 [129]. This study is the first report revealing linkage between mutations in coding region of any LOX gene and development of a disease and this loss of function mutations in LOX genes *ALOX12B* (12R-LOX) and *ALOXE3* (eLOX-3) on chromosome 17p13.1 are linked to the genesis of autosomal recessive congenital ichthyosis (ARCI), disease associated with defective skin barrier function (Figure 6). In addition, one recent study also suggests the structural role of the 12R-LOX/eLOX-3 pathway through the processing of ω-hydroxy acylceramides in the making of CLE [28]. Thus, in general, for all tissues individual LOX isozymes are thought to play a role as key enzymes in the modulation of proliferation and/or differentiation as well as in wound healing, inflammation and cancer by producing of a variety of oxidized signaling lipid metabolites. Nevertheless, skin shows the relation of LOX metabolism with signaling consequences as well as structural changes in the development and maintenance of skin permeability barrier.

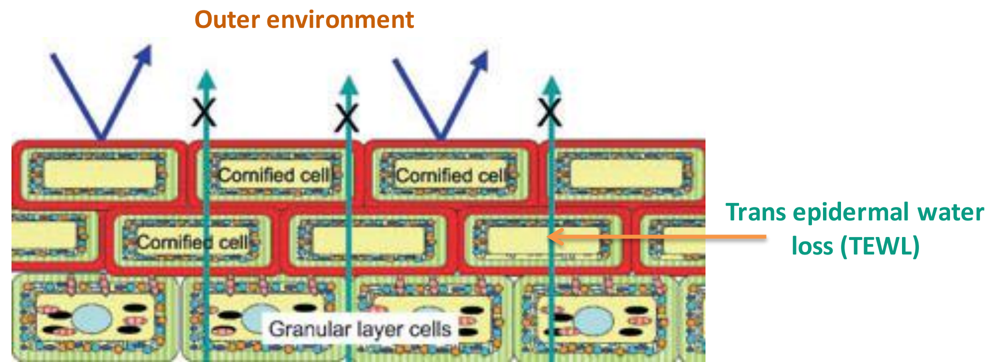


Figure 6: Skin barrier of cornified cells and intercellular lipid layers (red fillings) mimic the transepidermal water loss (TEWL). (Source: M Akiyama et al, Experimental Dermatology 17, 2008)

1.9. Skin disorder: Ichthyosis

Ichthyoses comprise a generalized group of mostly genetic skin disorders featured by thickened, dry and scaly or flaky skin. On the basis of mode of inheritance, ichthyoses can be recognized in a number of subgroups including autosomal recessive (AR), autosomal dominant (AD), autosomal subdominant (ASD) and X-linked recessive (X-LR) and these subgroups cover a number of subtypes from congenital severe form to non- congenital mild form of ichthyosis [130]. One of the ichthyosis phenotype, ARCI represent a clinically and genetically heterogeneous group of cornification disorders of skin and is characterized by skin desquamation and hyper keratosis, associated with erythema [131][132]. The disease covers a severe condition with an estimated incidence of one in 300,000 newborns in the European and northern American populations [133]. The affected newborn patients are mostly born as collodion babies those acquire a generalized scaling over the body after losing encasement of colloidal membrane in the first week of life [132]. Histological phenotypes of the epidermis in ichthyosis patients are non-specific, but typically include marked hyperkeratosis and epidermal

hyperplasia (Figure 7). Now it is conceded that the ARCI phenotype consequences from a physical compensatory thickening of the stratum corneum for the impaired cutaneous permeability barrier associated with all ichthyosis disorders.

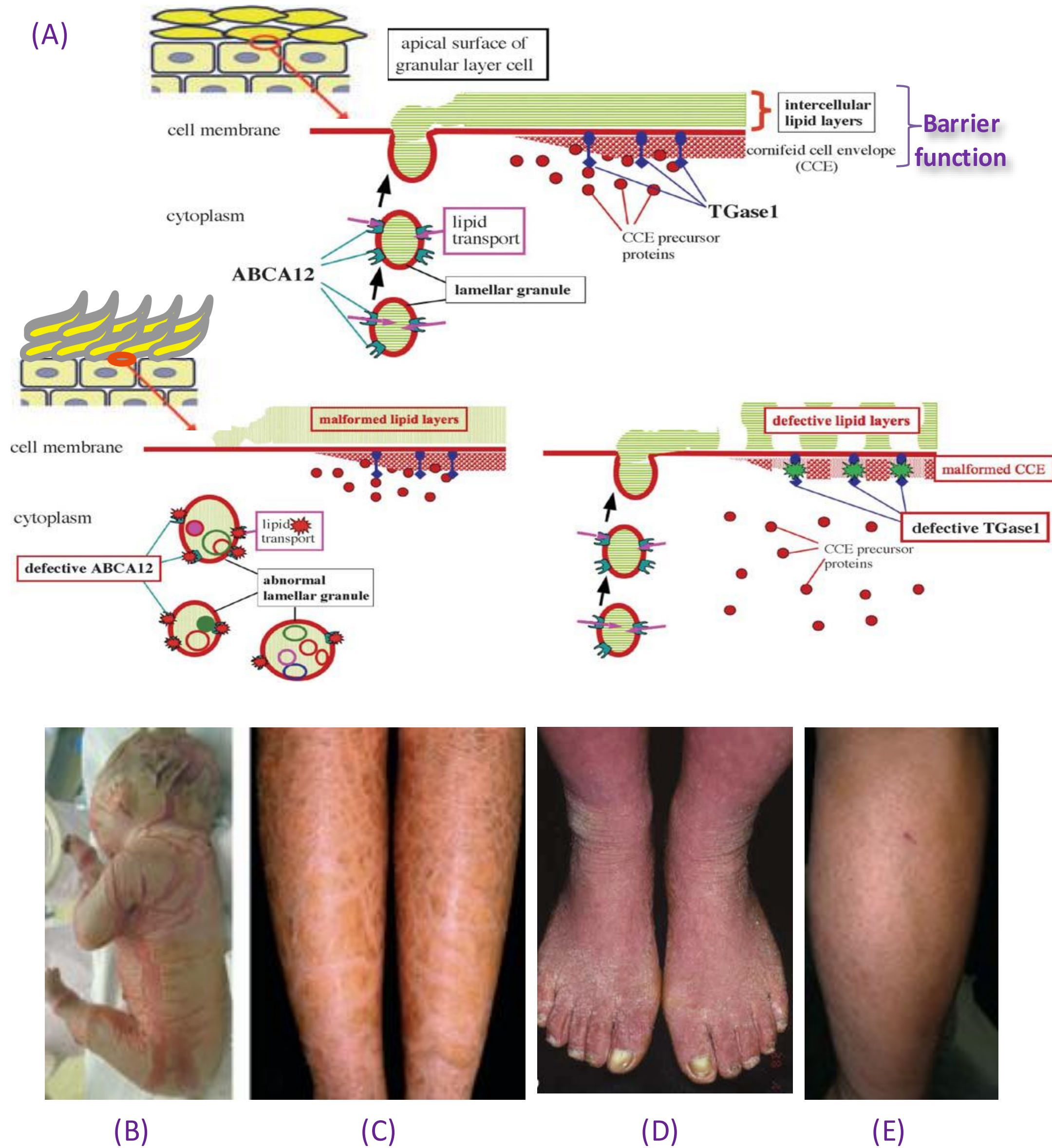


Figure 7: (A) Hypothetical pathomechanisms of ARCI due to ABCA12 and TGM1 mutations (B) Patient with Harlequin ichthyosis (HI) (C) Lamellar ichthyosis (LI) (D) Non-bullous congenital ichthyosiform erythroderma (NBCIE) and (E) Ichthyosis vulgaris (IV). (Source: M Akiyama, Journal of Dermatological Science 42, 2006, M Akiyama et al, Experimental Dermatology 17, 2008, M Akiyama et al, Clinical and Experimental Dermatology 28, 2003)

1.9.1. Subtypes of ichthyoses

The hereditary non-syndromic ichthyoses can be distributed in six major distinct clinical subtypes that are starting with –

- The most severe forms: Harlequin ichthyosis (HI)
- Lamellar ichthyosis (LI)
- Non-bullous congenital ichthyosiform erythroderma (NBCIE)
- Bullous congenital ichthyosiform erythroderma (BCIE)
- A mild and non-congenital form- X-linked ichthyosis (XLI)
- The mildest form- Ichthyosis vulgaris (IV) (covers almost 95% of cases)

Further, an additional phenotype similar to BCIE is also distinguished and named as ichthyosis bullosa of Siemens (IBS). Several studies reveal the underlying genetic defects and pathomechanism on the molecular basis of the human epidermal keratinization processes.

1.9.2. Genes involved in ichthyoses

To date, a number of genes have been recognized and appeared to cause ichthyoses. The genes identified for AD phenotypes include keratin 1 gene *KRT1*, keratin 2 gene *KRT2* and keratin 10 gene *KRT10* [134][135] and genes responsible for ASD and X-LR phenotypes are filaggrin gene *FLG* and steroid sulphatase gene *STS*, respectively [136][137]. Further, total nine different genes have been identified that are associated with ARCI phenotype. These are- *TGM1* encoding transglutaminase 1, *ABCA12* encoding a lipid transporter, a cytochrome P450 gene *CYP4F22*, a receptor gene *NIPAL4/ichthyin*, the LOX genes *ALOX12B* and *ALOXE3*, and recently identified, two lipase genes *LIPN* and *PNPLA1* with a ceramide synthase 3 gene *CERS3* [129][138][139][140][141].

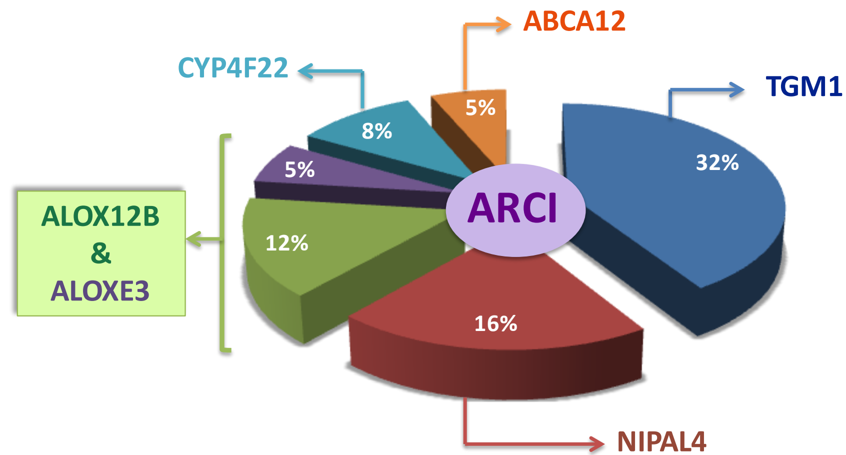


Figure 8: Mutational spectrum analysis among autosomal recessive congenital ichthyosis (ARCI) patients.

For ARCI, mutational spectrum analysis among these genes has been studied in 520 affected families that exhibits a mutation distribution of 32% for *TGM1*, 16% for *NIPAL4*, 12% for *ALOX12B*, 8% for *CYP4F22*, 5% for *ALOXE3*, and 5% for *ABCA12* [138] (Figure 8). Another study also displayed a 30% rate of incidence among ARCI patients for the LOX genes [142]. Thus, mutations in the LOX genes have been indicated as the second most common cause of ARCI cover about 10% of the cases. The patients with mutations in LOX genes showed palmoplantar hyperlinearity with or without mild keratoderma, heat intolerance because of a reduced sweating ability (hypohidrosis) and mild to moderate scaling with no or moderate erythema. However, it is observable that *ALOX12B* mutation displays a less and more discrete scaling compared to *ALOXE3* mutation with moderately adherent scaling [143][144].

1.9.3. Stratum corneum barrier: Molecules for Ichthyosis

Coming to the pathogenesis of ichthyosis, the primary phenomenon found in a majority of cases is defect in barrier function of stratum corneum which consist three main components. In any type of ichthyosis, the mechanism of pathogenesis of the disease associated with specific defect, at least, is on one of these components.

1.9.3.1. Intercellular lipid layers

The causative molecules of intercellular lipid layers in the stratum corneum underlie ichthyosis subtypes like- harlequin ichthyosis (HI), lamellar ichthyosis (LI), non-bullous congenital ichthyosiform erythroderma (NBCIE) and X-linked ichthyosis (XLI) and these include-

- ABCA12, a keratinocyte lipid transporter
- 12R-lipoxygenase
- Epidermis-type lipoxygenase-3
- Cytochrome P450 (family 4, subfamily F)
- Ichthyin
- Steroid sulphatase

1.9.3.2. Cornified cell envelope

Cornified cell envelope, assembly of several proteins, is the major component of barrier. The defect in molecule(S) which is essential for proper formation of this component causes the development of lamellar ichthyosis or non-bullous congenital ichthyosiform erythroderma (NBCIE) and this molecule is TGM-1.

1.9.3.3. Keratin network and keratohyalin granules

The imperfect molecules of keratin-filaggrin degradation products cause the genesis of bullous congenital ichthyosiform erythroderma (BCIE), ichthyosis bullosa of Siemens (IBS) and mildest form ichthyosis vulgaris (IV) and these include-

- Keratin 1
- Keratin 10
- Keratin 2
- Filaggrin

1.10. Human 12R-LOX and eLOX-3

After the identification of atypical AA metabolite 12R-HETE in psoriatic scales, it took a decade to discover the “accused” 12R-LOX enzyme which is responsible for this unique R-oxygenase activity in skin. In 1998, Brash and his colleagues, for the first time, cloned the 12R-LOX cDNA from the human keratinocytes and provided mechanistic evidence for the synthesis of 12R-HETE by 12R-LOX in psoriatic tissues [60]. Another, previously unknown LOX isoform was also cloned form mouse and human epidermis and named eLOX-3 [145]. Both enzymes, human 12R-LOX and eLOX-3, are the members of epidermal subfamily of human LOXs and preferentially express in human skin with little expression in other epithelial tissues like prostate, tongue and tonsil [59][146].

1.10.1. Unique structural and enzymatic features

12R-LOX and eLOX-3 have unique structural and enzymatic features when compared to all other mammalian LOXs [60][147]. Both proteins contain a proline-rich extra subdomain of 31 and 41 amino acid residues, respectively

between amino acids Leu150 and Pro182 (or Pro154 and Ile186, depending on the alignment) located at the surface of the catalytic subunit. This subdomain is critically involved in enzymatic activity of the enzymes [148]. It might be hypothesized that this amino acid insert, located on the surface of the protein, is also involved in subcellular localization of the enzyme or interaction with other proteins. For both human and mouse, the protein size of 12R-LOX is 701 amino acids in length and eLOX-3 is of 711 amino acids.

Human 12R-LOX encoded by one of the six functional human LOX genes, the *ALOX12B* gene, is located on chromosome17p13.1, where it forms a cluster of epidermis type LOXs together with two other LOX genes, the 15-LOX-2 gene *ALOX15B* and e-LOX-3 gene *ALOXE3*. The intron/exon boundaries in all human LOX genes are highly conserved. The *ALOX12B* and *ALOXE3* genes differ from other LOXs in their gene structure by having an additional intronic sequence in the coding region of exon 4 for the extra subdomain. As a result, the *ALOX12B* and *ALOXE3* genes are split into 15 exons and 14 introns and spans about 15 kb and 22 kb, respectively [122].

12R-LOX is the only mammalian LOX that forms product with R-chirality with arachidonic acid and few other PUFAs at the ω-9 position (mouse enzyme prefer methyl ester of fatty acid rather than free AA [149]), whereas, eLOX-3 does not show any dioxygenase activity but acts as a hydroperoxide isomerase that converts hydroperoxide, the primary product of LOX, to the epoxyalcohol and keto-derivatives [147][150][151]. Thus, in skin both enzymes act in sequence to metabolize fatty acid substrates via R-hydroperoxides to their specific R-

epoxyalcohol derivatives of hepoxilin family. The members of hepoxilin family are well characterized to function as signaling molecules [152].

1.10.2. Physio- & Pathological expression

In epidermis, the expression of 12R-LOX and eLOX-3 follow a similar pattern with the highest expression in the differentiated layer, stratum granulosum implying the functional linkage and a role in late terminal differentiation process [153]. Further, from the study it is revealed that 12R-LOX expression is induced in embryonic skin at embryonic day 15.5, time for the onset of skin development in mouse [154]. Moreover, in mice, expression of both 12R-LOX and eLOX-3 mRNA was occurred in additional tissues like tongue, trachea and forestomach and with very less abundance in testis and brain. The expression of 12R-LOX is also found in colon, kidney and liver [114]. Study also reveals the expression and activity of eLOX-3 in adipose tissue [148].

In human, 12R-LOX mRNA is also detected in tonsils, hair follicle and prostate and in B lymphoma cell line [146][154] whereas a less expression level of eLOX-3 mRNA is attributed to testis, ovary, placenta, brain, pancreas and some secretory epithelia [122]. Further, in humans, the 12R-LOX was pathologically expressed during the inflammatory and proliferative skin disease, psoriasis and other proliferative skin dermatoses [119][155] where 12R-HETE production is strongly elevated [156]. It has been speculated that its elevated synthesis is a result of keratinocyte hyper proliferation in psoriasis (Figure 5). Furthermore, our study clearly showed the differential expression of 12R-LOX, 12S-LOX and COX-2 in A431 (human epidermoid carcinoma cell line) cells compared to skin

fibroblasts, suggesting their significance in the pathology of skin cancer. This was the first report on the expression of 12R-LOX in epidermoid carcinoma cells [128].

1.11. 12R-LOX/eLOX-3 pathway in permeability barrier function

The atypical enzymatic features of 12R-LOX and eLOX-3 inculcate new insights into the role of a unique LOX metabolic pathway in formation and maintenance of epidermal permeability barrier. Further, differentiation dependent tissue expression and spatial and temporal co-localization of 12R-LOX and eLOX-3 in epithelial tissues indicate a pivotal physiological role of 12R-LOX and eLOX-3 in skin barrier function [64]. As already mentioned, this implication has paved the way by a major breakthrough provided by a genetic study of Fischer and colleagues in 2002 which showed for the first time involvement of mutations of a LOX gene in genesis of a disease. The study reveals that mutations in LOX genes *ALOX12B* and *ALOXE3* on chromosome 17p13.1 underlie the development of ARCI, disease associated with defective skin barrier function [129] and thus confirm the previously presumed role of 12R-LOX and eLOX-3 in the formation and maintenance of epidermal permeability barrier. To date, a number of studies have extended the findings on mutations reporting total 40 mutations in *ALOX12B* and 13 mutations in *ALOXE3* genes and revealing these mutations as second most common cause of ARCI found in about 10% of the cases [129][143][144][157]. Further it has been shown that these mutations led to completely loss of catalytic activity of the LOX enzymes indicating the concept that loss of function of either 12R-LOX or eLOX-3 is causally linked to the pathogenesis of LOX- dependent form of ARCI phenotype [144][143][158]. Because mutations in one or other LOX genes

resulting similar phenotype, it has been speculated that both enzymes belong to same metabolic pathway involved in epidermal differentiation [129].

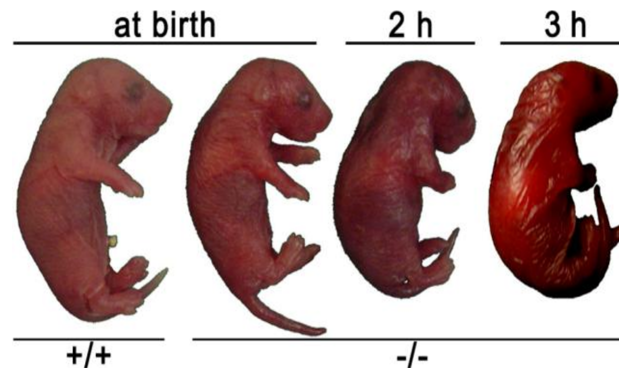


Figure 9: Macroscopic appearance of wild-type and 12R-LOX–/– mice at birth, and 2 and 3 h after birth. Note the red, shiny skin and the dehydrated appearance of 12R-LOX–/– mice. (Source: N Epp et al, The Journal of Cell Biology 177, 2007)

Furthermore, the critical role of 12R-LOX/eLOX-3 pathway on skin barrier function is supported by mouse knockout studies. The targeted disruption of both LOX genes in mice resulted in neonatal death due to excessive transepidermal water loss (TEWL) owing to impaired permeability barrier function [153][159]. These studies demonstrate more severe TEWL in 12R-LOX knockout mice, which died within 3 hours of birth compared to eLOX-3 deficient mice that survived up to 12 hours of birth (Figure 9). Another study has also showed same phenotype owing to impaired barrier function in ethylnitrosurea-induced 12R-LOX deficient mice [160]. In addition, skin grafting studies revealed the development of ichthyosiform appearance with thickening of the epidermis, severe hyperkeratosis, hyper proliferation and hypergranulosis in adult 12R-LOX deficient mouse skin transplants as the compensatory responses to impaired barrier function [161]. Moreover, cornified cell envelope from skin of 12R-LOX- deficient mice showed increased fragility and lipid analysis in these mice demonstrated a

disordered composition of ceramides, especially a decrease of ester-bound ceramide species. Thus, these mouse knockout studies reveal a structural role of 12R-LOX/eLOX-3 pathway in epidermal barrier function. From the recent study, this structural role of the 12R-LOX/eLOX-3 pathway is also suggested through the processing of ω-hydroxy acylceramides in the making of CLE [28]. Alternatively, 12R-LOX display an increased catalytic activity in acidic pH and elevated calcium-concentration (conditions as in keratinocytes of upper differentiated layers) and prefer esterified substrate (methyl ester) [60][149]. These findings imply the nature of the endogenous substrate to an esterified molecule found in differentiated upper layers. However, the study demonstrates that linoleate-containing EOS ceramide (O-linoleoyl-ω hydroxyacyl-sphingosine) is the natural substrate for the 12R-LOX/eLOX-3 pathway.

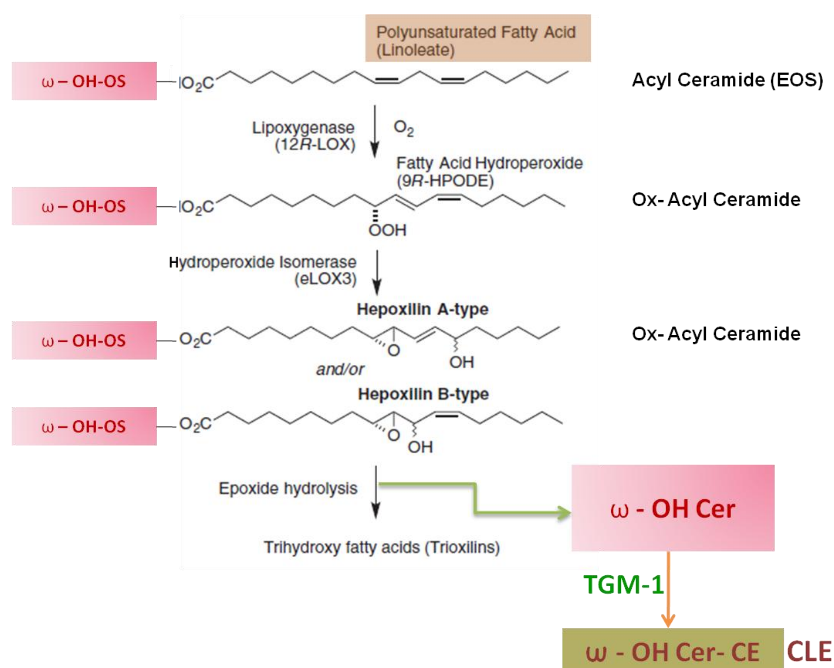


Figure 10: 12R-Lipoxygenase catalyzed oxidation of acyl ceramide to hepoxilins and trioxilins in the maintenance of epidermal lipid barrier. (Source: A Munoz-Garcia et al, Biochimica et Biophysica Acta 1841, 2014)

12R-LOX oxygenates linoleate moiety of esterified ω-hydroxyacyl-sphingosine (EOS) to the 9R- hydroperoxy lineoyl-ω-hydroxyceramide (9R-HPODE-EOS, a hydroperoxide derivative of EOS) which is subsequently converted by eLOX-3 to a specific epoxyalcohol, 9(R),10(R)-trans-epoxy-11E-13(R) hydroxylineoyl-ω-hydroxy-ceramide (9R(10R),13R-EpHOE-EOS) [28] (Figure 10). It is suggested that subsequently hydrolysis of epoxyalcohol derivative of EOS generates hepoxilins like derivatives, trioxilins and free ω-hydroxyl on ceramide (ω- OH Cer) and the LOX-catalyzed oxygenation is prerequisite to facilitate the hydrolysis of the oxidized linoleate moiety resulting in a free ω-hydroxyl group on the ceramides. This free ω-hydroxyl group on the ceramides is required for coupling to the cross-linked proteins of the CE [129][162] (Figure 11).

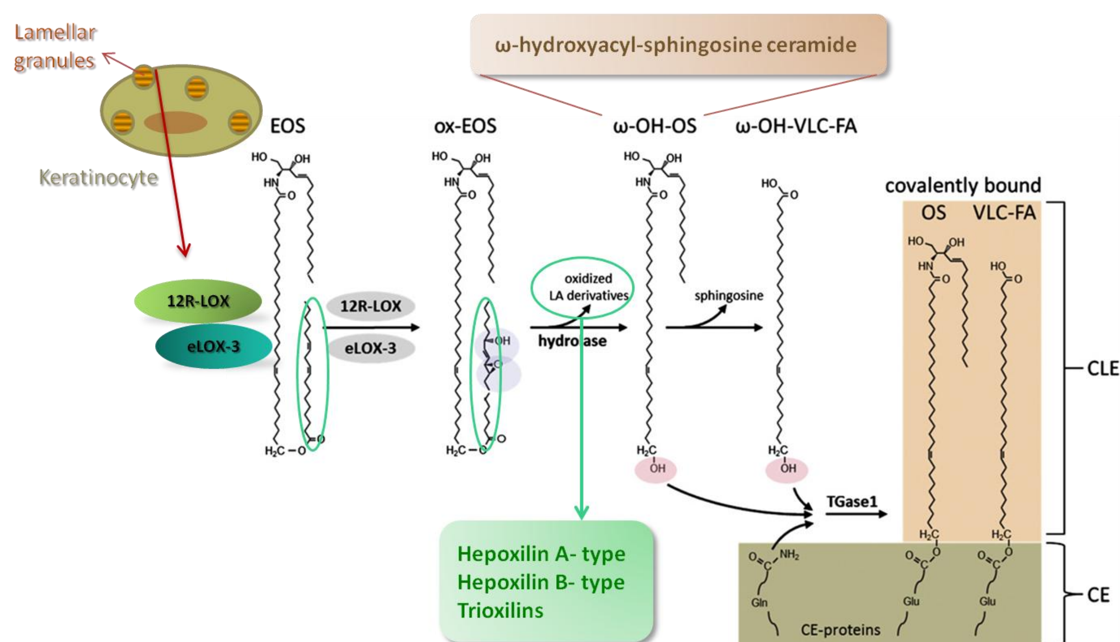


Figure 11: The proposed role of 12R-LOX/eLOX-3 pathway in the formation of the cornified lipid envelope to maintain skin barrier function. (Source: P Krieg et al, Biochimica et Biophysica Acta 1841, 2014)

Several previous studies reinforce this working concept for role of two LOX enzymes in the oxygenation of linoleate-containing ceramides and making of CLE of epidermal barrier. The oxygenated products of linoleate moiety in the ceramides are recognized as chiral, predominantly 9R-HODE, confirming involvement of 12R-LOX [28]. The covalently bound ω-hydroxyacyl-sphingosine ceramide species are strictly reduced in the epidermis of 12R-LOX knockout mice while it contains about 2-fold elevated amounts of unoxidized EOS, revealing accumulation of the LOX substrate. As a result of this, the CLE is largely absent in the epidermis of 12R-LOX knockout mice [153]. eLOX-3 knockout mice, which show less severe phenotype than the 12R-LOX knockouts, also demonstrate a reduction in covalently bound ceramides to about half of the normal levels [159]. These observations indicate that loss of ceramide binding owing to defective CLE may be the main cause of impaired epidermal barrier in the mutant mice.

Moreover, as described above, eLOX-3 transforms a variety of hydroperoxides (prefer R-hydroperoxides), the products of 12-LOX to generate epoxyalcohol derivatives of hepoxilin family. *In-vitro,* human eLOX-3 favorably transforms 12R-HPETE to 8R-hydroxy-11R,12R-epoxyeicosa-5Z,9E,14Z-trienoic acid which is one of the isomers of hepoxilin A3 while 12S-HPETEs are also efficiently converted by eLOX-3 to the R-stereoisomers of hepoxilin A3 and B3 [162]. In fact, eLOX-3 acts as a hepoxilin synthase, *in-vivo,* as demonstrated by eLOX-3 knockout study where a strongly diminished level of different hepoxilins in mutant skin revealed the pivotal role of eLOX-3 in making of these oxylipins in mammalian skin [159]. The occurrence of these hepoxilins and corresponding

trioxilins is greatly increased, may be because of higher levels of AA and 12-LOXs, in psoriatic lesions of skin [163]. Further, hepoxilins and their trioxilin derivatives have been unveiled to play a signaling role in several physiological processes including adipocyte differentiation, inflammation and neurotransmission [59][164].

Thus the 12R-LOX/eLOX3 pathway derived linoleate-related hepoxilins or trioxilin, which are released during hydrolysis of epoxyalcohol derivatives in making of free ω-hydroxyl-ceramides, might exert a signaling function in several processes related to components of the epidermal permeability barrier [165] (Figure 11). This is also the case for the arachidonate-derived hepoxilins as already demonstrated in the study [159][166]. Additionally, in 1895, the study from Nugteren and coworkers also provide the evidence for the first time that LOX-derived epoxyalcohol and their trioxilin derivatives are crucial in the formation of epidermal permeability barrier [27]. The findings do, present a conundrum for the proposed signaling role of linoleate-related hepoxilins (product of 12R-LOX/eLOX-3 pathway) in maintaining the epidermal barrier function of terminally differentiated keratinocytes. Thus, it is useful to know whether 12R-LOX/eLOX-3 pathway may have a role in the signaling mechanisms for terminal differentiation of keratinocytes, and through these signaling mechanisms may regulate the expression of other genes related to the components of the epidermal permeability barrier.

Scope and Objectives of the study

Human epidermis expresses multiple forms of lipoxygenases (epidermis type-lipoxygenases) which metabolize arachidonic acid in to specific eicosanoids. Among these epidermis-type lipoxygenases, 12R-LOX is crucial to the proper functioning of epidermal permeability barrier of skin. Stratum corneum barrier formation is part of late differentiation process and accomplished by making its three major components-intercellular lipids, cornified cell envelop and keratin-filaggrin degradation products [15][16][17][18]. In epidermis, linoleic acid esterified in ω-hydroxy-ceramide is oxidized into a specific hepoxilins related derivative via the consecutive actions of 12R-LOX and eLOX3 facilitating hydrolysis of the oxidized linoleate moiety from the ceramide [28]. This emanated free ω-hydroxyl of the ω-hydroxy-ceramide is covalently bound to the surface proteins to make the corneocyte lipid envelope, a key component of the epidermal barrier. Thus, the blocking of either of these two metabolic enzymes disrupts the normal functioning of epidermis [64].

However, under physiological conditions, LOXs are known to have a key role in regulation of normal proliferation and differentiation of epithelial cells [54][115][117][118]. Moreover, the overexpression of 12R-HETE, a product of 12R-LOX, has been reported in psoriasis and skin cancer, and this elevated synthesis of 12R-HETE is a result of the keratinocyte hyper proliferation in psoriasis [119][120][128][156]. On the other side, inactivating mutations of either ALOX12B or ALOXE3 gene have been found to result in a severe type of ichthyosis (skin disorder) associated with disruption of epidermal barrier function of

terminally differentiated keratinocytes, result in the loss of epidermal water [129][143][157][158]. Further, a 12R-LOX knockout mice study shows an impairment of epidermal barrier function and a severe loss of trans-epidermal water, leading to the death of the pups [153][160]. These studies suggest an essential role for 12R-LOX in the maintenance of epidermal barrier function of terminally differentiating keratinocytes. With this, the present study was designed to elucidate the role of 12R-LOX in terminal differentiation of human keratinocytes. For these studies, normal keratinocyte cell line, HaCaT and 12R-LOX overexpressing human epidermoid carcinoma cell line, A431 were taken. Based on these studies, the signaling role of 12R-LOX was undertaken to evaluate the regulation of expression of other components of the epidermal permeability barrier in terminal differentiation process of keratinocyte.

Thus the specific objectives of the present study are:

- Characterization of A431, human epidermoid carcinoma cell line and HaCaT, human keratinocyte cell line as models for differentiation study.
- To understand the role of 12R-LOX in differentiation of human keratinocytes.
- To understand the role of 12R-LOX in proliferation and differentiation of human epidermoid carcinoma cells.

Chapter 2

Characterization of A431, human epidermoid carcinoma cell line and HaCaT, human keratinocyte cell line as models for differentiation study

2.1. Introduction

The 12R-LOX/eLOX3 pathway represents a recently identified unique LOX metabolic pathway of skin that plays an essential role in the formation and maintenance of epidermal permeability barrier function [64]. This barrier function is furnished by the outermost layer of the epidermis, stratum corneum which is a part of terminal differentiation process of keratinocytes. Epidermal terminal differentiation is accomplished by sequential morphological and biochemical changes which result in highly specialized and organized four distinguished layers- the superficial stratum corneum (consisting dead corneocyte cells), stratum granulosum, stratum spinosum and the inner most replicative stratum basale containing undifferentiated proliferating keratinocytes [3][4][5]. Keratinocytes proliferate in the stratum basale and transiently migrate into the suprabasal layers towards stratum corneum upon a tightly controlled terminal differentiation program. Each layer represents one of the sequential differentiation stages of the terminal differentiation of keratinocytes and can be identified by distinguished differentiation markers [167] (Figure 1).

The expression of keratin markers K5 and K14 is strictly restricted to the lower most undifferentiated basal layer [168][169]. When transit amplifying cells of basal layer migrate to suprabasal spinous layer initiate expression of early differentiation markers like K1 and K10 indicating commence for terminal differentiation [168]. The expression of K1 and K10 increases with the suprabasal differentiated layers. Onset of late differentiation markers like loricrin, filaggrin, TGM-1 and involucrin indicate the differentiation of spinous cells into granular cells [170][171][172][173]. Additionally, in normal epidermis, the expression

pattern and spatial and temporal co-localization of 12R-LOX and eLOX-3 is found in nucleated stratum granulosum of upper epidermis indicating differentiation dependent expression of both enzymes [64]. Further, studying the signaling mechanism and regulatory pathway of 12R-LOX during the process of keratinocyte differentiation can provide the worthy potential to understand the pathomechanism of skin diseases like psoriasis, epidermal cancers and ichthyoses and for treatment of these diseases [165]. For understanding the differentiation process of keratinocytes, primary cell culture shows some stringent culture limitations like requirement of supplementary growth factors and a finite life span. Indeed, the spontaneously immortalized HaCaT human epithelium cell line from adult skin, showing phenotype similar to normal keratinocyte, exhibits unlimited growth potential and has been widely used as keratinocyte model [174]. Despite its ease of propagation, HaCaT cells reform an orderly structured and differentiated epidermal tissue upon transplanting onto nude mice [174] and can be maintained in basal-like state for efficient differentiation [175].

Several studies unveil that carcinogenesis in a variety of tumors is influenced with irregular expression of LOXs/COXs [68][69][176] and tumors of distinct histogenesis show considerably different profile of AA metabolism [177]. So, the expression and pathological role of AA metabolizing enzymes is considerably different in various cancers. Moreover, our study also reveals the pathological role of 12R-LOX in the growth of epidermoid carcinoma cells, A431 [128]. The A431 cells has originated from tumor of squamous layer of epidermis [178] and this implies the improper differentiation of keratinocytes which lead to tumorogenesis. On the other hand, the expression pattern of LOXs/COXs in

normal keratinocyte HaCaT cells which display proper differentiation is unknown. Thus, it is very important to know the expression profile of AA metabolizing enzymes and differentiation markers in human epidermoid carcinoma cells, A431 and normal human keratinocyte cells, HaCaT to know the role of LOXs in differentiation or tumorogenesis. Therefore, our first question was to investigate the expression of various AA metabolizing enzymes and differentiation markers in A431 cells and HaCaT cells. Thus, the aim of the present study is to analyze the expression profile of the LOXs/COXs and differentiation markers in both cell lines before going for differentiation study in these cells.

2.2. Materials and Methods

2.2.1. Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, phosphate buffered saline (PBS), fetal bovine serum (FBS) and 0.25% Trypsin-EDTA were purchased from Gibco and Invitrogen Life Technologies (Carlsbad, CA, U.S.A). HRP conjugated secondary antibodies, enhanced chemiluminescence (ECL) kit, Acrylamide, N, N'-Methylene-bis-acrlylamide, sodium dodecyl sulfate (SDS), ammonium persulfate, β-mercaptoethanol and bromophenol blue were from Bio-Rad Laboratories (Richmond, USA). Cell lysis buffers, Protease inhibitor cocktail and polyclonal antibody of human-12R-LOX were from Sigma (St Louis, MO, U.S.A.). Nitrocellulose and 0.22µm filter membranes were from Millipore (Bangalore, India). Monoclonal antibodies against human cytokeratin1, cytokeratin5, filaggrin and involucrin were obtained from Abcam (Cambridge, USA). COX-2, 5-LOX, 15-LOX and β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc., (CA, USA). TRizol was procured from Invitrogen Life

Technologies (Carlsbad, CA, U.S.A.). RT-PCR kit was procured from MBI Fermentas (Maryland, U.S.A.). All the other chemicals and reagents were purchased from local companies and are of molecular biology grade.

2.2.2. Cell culture: Growth and maintenance of cells

HaCaT, a naturally immortalized normal human keratinocyte cell line, was a gift from Prof. Kondaiah, IISc, Bangalore, India. A431 cell line is used most often as the model of human epidermoid carcinoma and has been procured from NCCS, Pune, India. The cells were grown in 90% Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 µg/ml). Exponentially growing cultures were maintained in a humidified atmosphere of 5% CO_2 at 37^0C. Sub culturing of cells were done twice a week. Cell viability was determined by the Trypan Blue dye exclusion method before seeding for each experiment.

2.2.3. RNA isolation and RT-PCR analysis

Total RNA was isolated from A431 cells and HaCaT cells using TRizol according to manufacturer's instructions. Reverse transcription of 2µg of total RNA isolated was achieved by mixing the RNA with 1µl of oligodT followed by incubations at 70^0C for 5 min and then at 4^0C for another 5 min, subsequently adding RT-PCR buffer (containing 4µl of 5X RT buffer, 0.25µl of RNAase inhibitor (10 units/µl), 1µl of deoxynucleotides) followed by incubation at 37^0C for 5 min and addition of 1µl of Reverse Transcriptase (200 units/µl) in a 20µl volume and then running the reaction at 42^0C for 60 min, and then for 10 min at 72^0C. 2µl of the product from the RT reaction was used for PCR run. The PCR reactions were

performed by initial denaturation at 94^0C for 2 min and then the samples were allowed to undergo 34 PCR cycles with temperature profile of denaturation at 94^0C for 30 sec, annealing at 52 - 60^0C for 40 sec and extension at 72^0C for 40 sec and then final extension was allowed to reactions by incubating at 72^0C for 2 min. The primer sequences for the human genes and annealing temperatures are listed in Table 1. The GAPDH primers served as control. The PCR products were visualized on 1% agarose gels under UV light.

Table 1: List of primers used for RT-PCR analysis

Target Gene	Primer Sequences	Orientation	Length (bp)	Annealing Temp (^0C)
12R-LOX	5'-CCA CTG GCG AGT CTT TCT TCC-3' 5'-GTC TGT GAC GGG GAA CTT GT-3'	Sense Antisense	442	55^{0}C
12S-LOX	5'-GAA GGG TTA CCC CTG ACC AT-3' 5'-GCA GCC AGG TAT TGC TTC TC-3'	Sense Antisense	458	55^{0}C
5-LOX	5'-ACT TCG CCG ACT TTG AGA AA-3' 5'-AGA AGG TGG GTG ATG GTC TG-3'	Sense Antisense	499	52^{0}C
15-LOX-2	5'-ACA TCT GCT GCC TGA GGT CT-3' 5'-TGA CAT CAC ATG TGG CAT TG-3'	Sense Antisense	458	52^{0}C
COX-1	5'-GCC AGT GAA TCC CTG TTG TT-3' 5'-GCA ACT GCT TCT TCC CTT TG-3'	Sense Antisense	422	52^{0}C
COX-2	5'-CCA CTT CAA GGG ATT TTG GA-3' 5'-CAG CAA ACC GTA GAT GCT CA-3'	Sense Antisense	581	60^{0}C
FLAP	5'-AGG CAA TGT TGT CCT GTT GG-3' 5'-TGG TGG TGG AGA TCG TCT TT 3'	Sense Antisense	443	53^{0}C
INV	5'-ATG TCC CAG CAA CAC ACA CT-3' 5'-GGA GCT CCA ACA GTT GCT CT-3'	Sense Antisense	466	55^{0}C
FLG	5'-ACC TCA ACA TCG GGT GCT AC-3' 5'-AGG CTG TCT GTT CGG AGG TA-3'	Sense Antisense	444	55^{0}C
TGM1	5'-GTC CCG GAC CTA TGA ATC CT-3' 5'-GTG GTC AAA CTG GCC GTA GT-3'	Sense Antisense	427	55^{0}C
KRT1	5'-TGG ATT TGG AAG TCG GAG TC-3' 5'-AGG AGG CAA ATT GGT TGT TG-3'	Sense Antisense	401	52^{0}C
KRT5	5'-CTT GTG GAG TGG GTG GCT AT-3' 5'-TCT GCC TCA CAG TCT TGG TG-3'	Sense Antisense	480	55^{0}C
KRT14	5'-GGC CTG CTG AGA TCA AAG AC-3' 5'-CAT ACT GGT CAC GCA TCT CG-3'	Sense Antisense	411	55^{0}C
GAPDH	5'-AGG TCA TCC CAG AGC TGA ACG-3' 5'-CAC CCT GTT GCT GTA GCC GTA T-3'	Sense Antisense	272	55^{0}C

2.2.4. Western blot Analysis

A431 and HaCaT Cells were grown and after getting 80% confluence, cells were harvested. To prepare the whole cell extract, the protocol was based on Sambrook et al [179]. Cells were washed with PBS and suspended in a lysis buffer with 1mM PMSF, 10 µg/ml leupeptin, 20 µg/ml aprotinin and phosphatase inhibitor cocktail. After 30 min of shaking at 4^0C, the mixtures were centrifuged (10,000 x g) for 30 min at 4^0C, and the supernatants were collected as the whole-cell extracts. The protein content was determined according to the Bradford method [180]. An equal amount of total cell lysate was resolved on 8-12% SDS-PAGE gels along with protein molecular weight standards, and then transferred onto Nitrocellulose membranes. The membranes were blocked with 5% w/v non-fat dry milk and then incubated with the primary antibodies against human 12R-LOX, 5-LOX, 15-LOX, COX-2, cytokeratin1, cytokeratin5, filaggrin, involucrin and β-actin in 10ml of antibody-dilution buffer (1:1000) (Tris buffered saline and 0.05% Tween-20 with 1% milk) with gentle shaking at 4^0C for 8 – 12 h and then incubated with respective conjugated secondary antibodies (HRP-conjugated). Signals from immunocomplexes were visualized by chemo-luminescence using ECL kit according to the manufacturer’s protocol. The band intensities were measured by Image J software.

2.2.5. Statistical analysis

All the data analysis was completed using the Sigma Plot 10 software. Data were presented as the Mean ± S. E. M. of three independent experiments and

statistical analysis was carried out by Student's t test using Excel (Microsoft). A p-value of less than 0.05 was considered as statistically significant over control.

2.3. Results

2.3.1. Growth and maintenance of human epidermoid carcinoma cell line, A431 (a malignant keratinocyte) and HaCaT (normal human keratinocyte cell line)

Phase-contrast microscopic pictures (20X) of A431 and HaCaT were taken to observe morphological features. Cells grown were very healthy with no vacuolation. Malignant keratinocyte, A431 (doubling time 24 h) were not with well defined boundaries in comparison to normal keratinocytes, HaCaT (doubling time 20 h). A well differentiated cornified cell boundary was observed in HaCaT cells. A431 cells were larger in size with prominent nucleus compared to HaCaT cells (Figure 12).

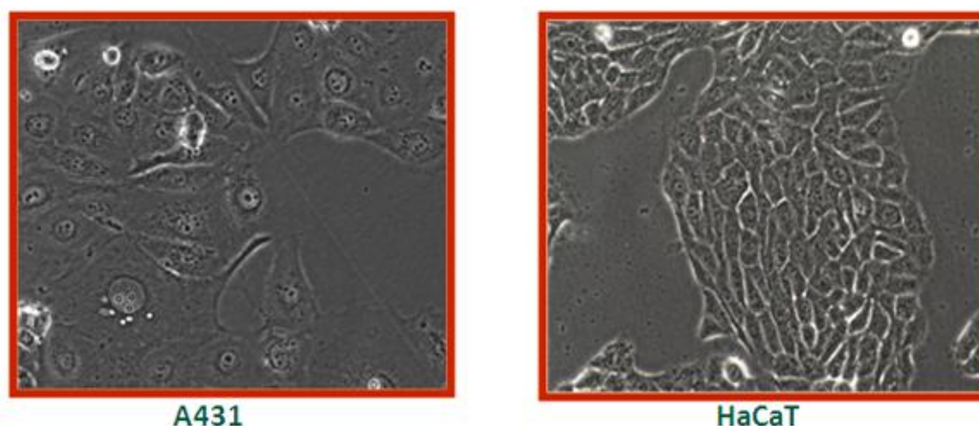


Figure 12: Phase Contrast microphotographs of A431 and HaCaT cells in culture.

2.3.2. Expression pattern of cyclooxygenase and lipoxygenase genes in A431 and HaCaT cells

Expression level of LOXs/COXs genes was analyzed by RT-PCR in normal human keratinocytes, HaCaT and epidermoid carcinoma cell line, A431. As shown in the figure 13, the level of expression of COX-1, COX-2, 12S-LOX, 12R-LOX, 15-LOX and 5-LOX activating protein (FLAP) was much higher in A431 compared to HaCaT cells. However, 5-LOX showed a reverse pattern with higher level of expression in HaCaT cells.

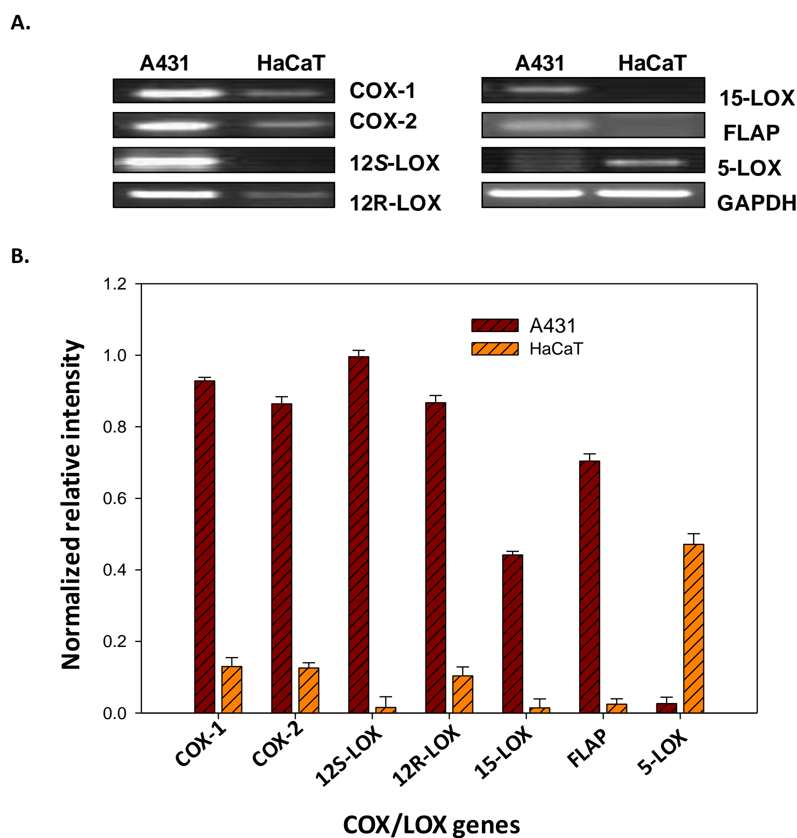


Figure 13: Expression levels of LOX and COX mRNA in A431 and HaCaT cells. ***A)*** *RT-PCR analysis of LOX/COX genes in human epidermoid carcinoma cell line (A431) and its comparison with normal keratinocyte cell line (HaCaT).* ***B)*** *Bar diagram showing the intensities of the RT-PCR bands measured by Image-J Analysis.*

2.3.3. Expression pattern of LOX/COX proteins in A431 cells and HaCaT cells

Cell lysate proteins (60µg) were resolved on SDS-PAGE and analyzed for 5-LOX, 15-LOX, and COX-2 and 12R-LOX protein expression by Western blotting. β-actin was used as internal control to monitor equal loading. Western analysis confirmed that A431 cells showed higher level of expression of COX-2, 15-LOX and 12R-LOX. On the other hand, 5-LOX was prominently seen in HaCaT cell line only (Figure 14).

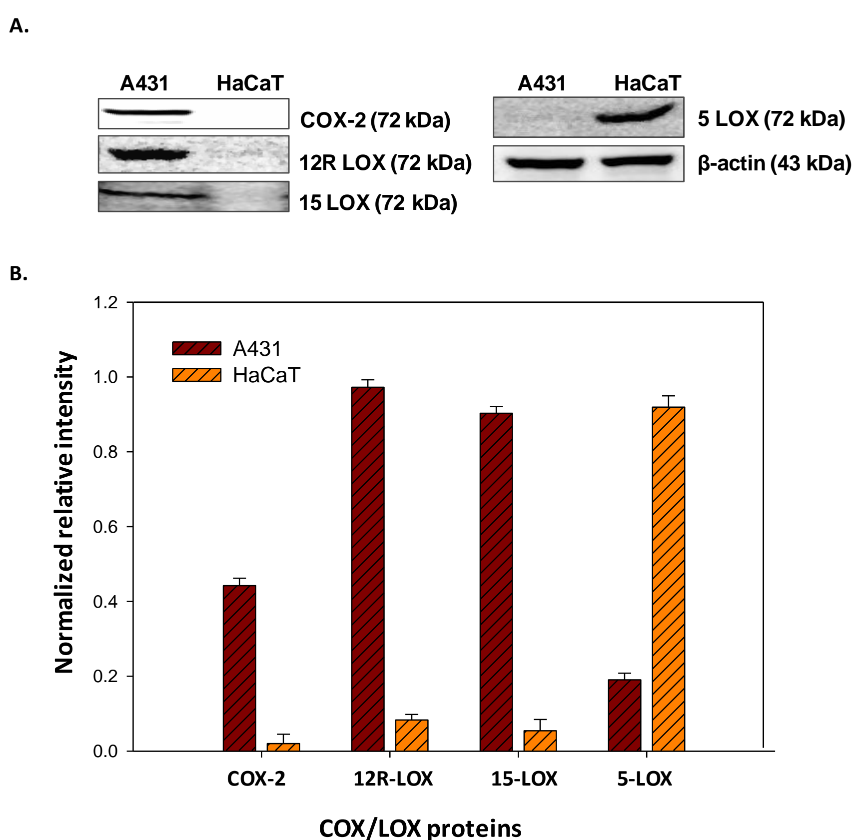


*Figure 14: Expression level of LOX/COX proteins in A431 & HaCaT cells. **A)** Immunoblot analysis of LOX/COX proteins expression in human epidermoid carcinoma cell line (A431) and its comparison with normal keratinocyte cell line (HaCaT). **B)** Bar diagram showing the intensities of the protein bands measured by Image-J Analysis.*

2.3.4. Expression level of keratinocyte specific differentiation marker genes in HaCaT and A431 cells

Expression level of genes required for terminal keratinocyte differentiation or corneocyte formation (e.g. involucrin, TGM-1 and filaggrin) and differentiation markers (K1, K5 and K14) was analyzed by RT-PCR in normal human keratinocytes, HaCaT and epidermoid carcinoma cell line, A431.

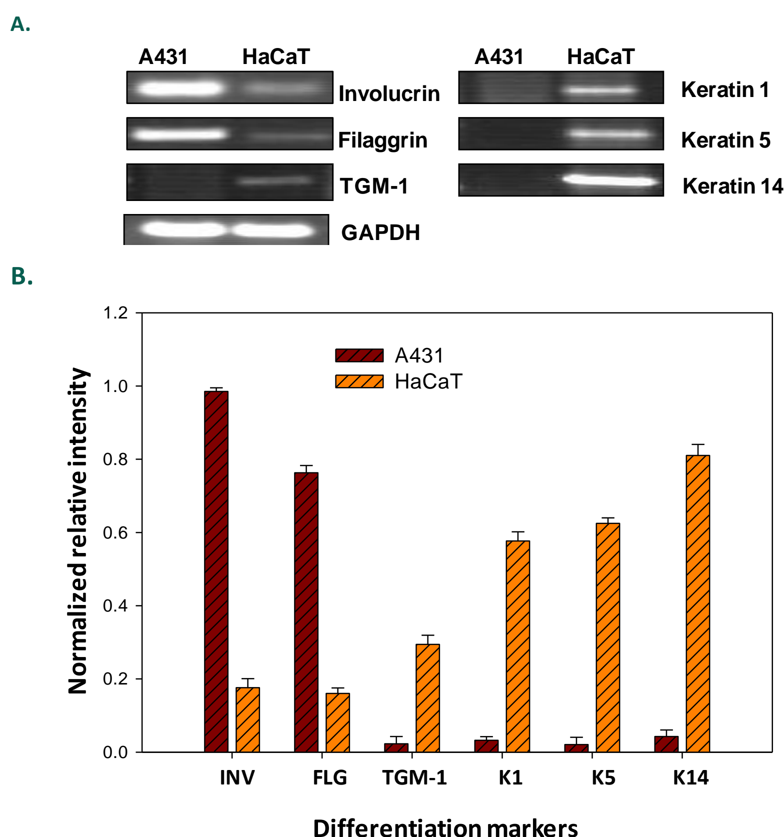


Figure 15: Level of differentiation marker gene expression in A431 & HaCaT cells. ***A)*** *RT-PCR analysis of expression of differentiation marker genes in human epidermoid carcinoma cell line (A431) and its comparison with normal keratinocyte cell line (HaCaT).* ***B)*** *Bar diagram showing the intensities of the RT-PCR bands measured by Image-J Analysis.*

As shown in figure 15, the expression levels of keratinocyte specific early differentiation markers like K1, K5 and K14 studied were several fold higher in the normal human keratinocytes (HaCaT), compared to malignant keratinocyte (A431)

cells, while, the late differentiation markers like involucrin and filaggrin showed higher level of expression in A431 cells. These studies indicate the loss of various early differentiation markers due to dedifferentiation in A431 cells.

2.3.5. Expression pattern of keratinocyte specific differentiation markers in A431 and HaCaT cells at protein level

Total proteins (60µg) were resolved on SDS-PAGE and analyzed for K1, K5, involucrin and filaggrin protein expression by Western blotting. β-actin was used as internal control to monitor equal loading. Western analysis has confirmed the elevated expression of K1 and K5 in HaCaT cell line. On the other hand, late differentiation markers like filaggrin and involucrin were prominently seen in A431cells only (Figure 16).

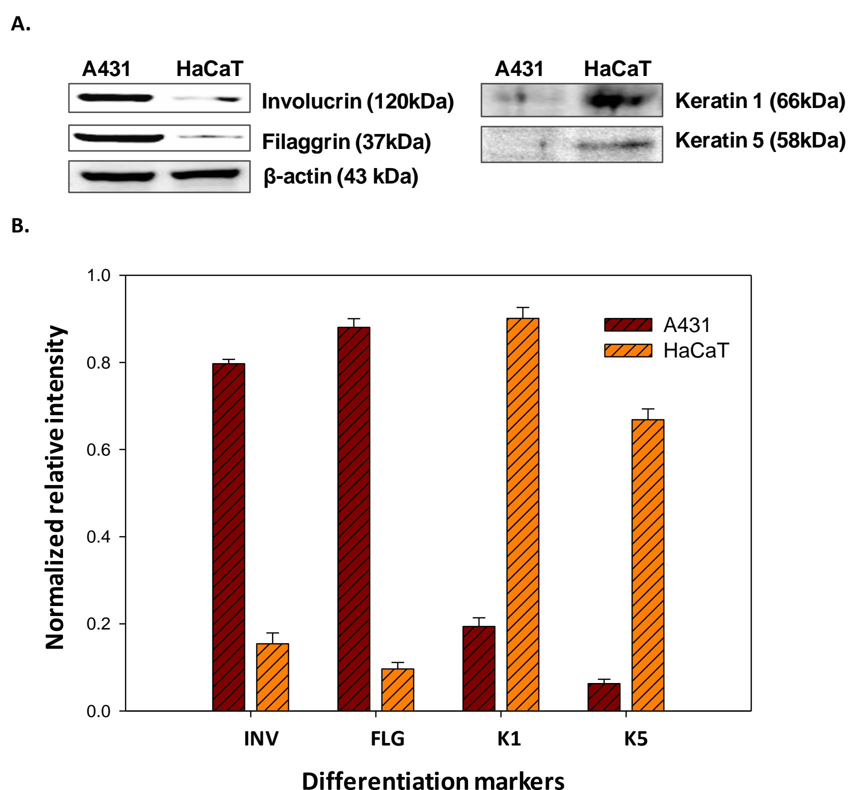


Figure 16: Expression level of different primary keratinocyte differentiation markers in A431 & HaCaT cells. ***A)*** *Immunoblot analysis of markers protein expression in human epidermoid carcinoma cell line (A431) and its comparison with normal keratinocyte cell*

line (HaCaT). ***B)*** *Bar diagram showing the intensities of the protein bands measured by Image-J Analysis.*

2.4. Discussion

AA metabolism leads to the biosynthesis of various lipid hormones such as prostaglandins, leukotrienes, lipoxins, HETEs, HODE and hepoxilins which are potent endogenous signalling molecules involved in regulation of cell growth and proliferation as well as survival and apoptosis [66][67][176]. From the previous studies, it is clear that progression of a variety of tumors is influenced with irregular and/or over expression of LOXs/COXs suggesting a role of eicosanoids in carcinogenesis [68][69][176]. Tumors of distinct histogenesis show considerably different profile of AA metabolism [177] and so, the expression and pathological role of AA metabolizing enzymes is considerably different in various cancers. Moreover, studies also reveal these LOXs/COXs as playing an important role in the modulation of epithelial proliferation and differentiation as well as in wound healing, inflammation, inflammatory skin diseases and cancer [64][106]. Additionally, our study also unveil the pathological role of 12R-LOX in the tumorigenesis of epidermoid carcinoma cells, A431 [128] which have originated from tumor of spinous layer of epidermis [178], indicating improper differentiation of keratinocytes which lead to tumorigenesis.

As far as normal human keratinocyte HaCaT cells, the spontaneously immortalized cells but showing phenotype like primary keratinocyte with the ability to differentiate, no study has reported about any changes in the expression pattern of LOXs/COXs after spontaneous immortalization. The speculative effect of immortalization of HaCaT cells on LOXs/COXs expression is still to investigate.

Thus, it is very important to know the expression profile of AA metabolizing enzymes and differentiation markers in A431 and HaCaT cells to evaluate the role of LOXs in differentiation or tumorigenesis. Therefore, our first aim was to analyze the expression profile of the LOXs/COXs and differentiation markers in both cell lines before used as models for differentiation. The present study clearly showed higher level of expression of 12R-LOX, 12S-LOX, 15-LOX, COX-1 and COX-2 in A431 cells compared to very low expression in HaCaT cells indicating their significant role in tumorogenesis of A431 cells. Further, 5-LOX activating protein (FLAP) was much higher in A431 while, 5-LOX showed a reverse pattern with higher level of expression in HaCaT cells. One the other hand, HaCaT cells showed a little expression of 12R-LOX while expression of 12S-LOX was not detectable in the cells.

Skin of both human and mice exhibit multiple forms of LOXs and both forms of COX [114][60][154][181]. The expression of 12S-LOX has been reported to be the predominant LOX isoenzyme in human and murine epidermis [54][182]. Human epidermis displays 12S-LOX expression in the germinal layer where its overexpression has also been reported in psoriatic skin [113]. Study also shows the predominant expression of 12S-LOX in neoplastic epidermal preparation [54]. Moreover, 12S-LOX-deficient mice have been shown to be less sensitive for tumour induction [183]. Study reports the existence of 15-LOX in the proliferative psoriatic secretory cells [127]. Studies on skin carcinogenesis demonstrate that overexpression of COX-2 contributes to the development of skin cancers [184]. Further, targeted disruptions of the genes encoding either COX-1 or COX-2 lead to altered epidermal differentiation and reduced skin tumorigenesis [185]. All these

findings suggest a lead role of LOXs/COXs in normal skin physiology and skin tumour development. Thus, in the present study, the predominant expression of LOX multiforms and COXs in human epidermoid carcinoma cell line, A431 suggests a possible role for AA metabolizing enzymes in regulating the growth and tumorigenesis of A431 cells. Whereas, a very low or no expression of LOXs/COXs in normal human keratinocyte cell line, HaCaT reveal the immortalization of HaCaT cells without prompting any tumorigenesis excepting some elevated level of 5-LOX. Indeed, this elevated expression of 5-LOX is attributed to the differentiated phenotype of HaCaT cells in culture revealing the expression of 5-LOX as an intrinsic property of human skin keratinocytes [186].

In the present study, a detectable level of expression of 12R-LOX was observed in HaCaT cells, which is a feature of normal keratinocytes, which demonstrates the unaltered property of these cells for the expression of 12R-LOX. So, these HaCaT cells can be used to analyze the physiological role of 12R-LOX in keratinocytes. Atypically, in epidermis, the expression of 12R-LOX follow the pattern of highest expression in the differentiated layer, stratum granulosum implying a role in late terminal differentiation process [153]. Further, psoriatic scales which are symbolized with an abundance of inappropriately differentiated spinous cells and granular cells resulted from incomplete terminal differentiation [187] (Figure 5), showed a very high level of 12R-HETE, a product of 12R-LOX. It has been speculated that its elevated synthesis is a result of keratinocyte hyper proliferation in psoriasis and co-occurrence of free AA [188] (not present normally, but a facet of the inflammation in psoriasis) [165] which is, in general, not a substrate for 12R-LOX in proper differentiated granular cells (linoleic acid

esterified in ceramide acts as a natural substrate for 12R-LOX in upper differentiated cells [28]). Similarly, although A431 cells have originated from spinous layer of epidermis, in the present study it demonstrated a very high expression of 12R-LOX. This may be generalized because of an improper terminal differentiation and/or tumorigenesis of A431 cell line and so, it shows the increased expression of 12R-LOX before being differentiated. 12R-LOX expression is induced in embryonic skin at embryonic day 15.5, time for the onset of skin development in mouse and thus, in balanced manner it is necessary for development of epidermis [154]. However, this enhanced expression of 12R-LOX in the undifferentiated cells of stratum spinosum with co-occurrence of free AA [188][23] may attributed to more tumorigenesis in A431 cells and thus play a role in regulation of growth of these epidermoid carcinoma cells [128].

The present study showed absence of keratin markers K5 and K14 in A431 cells giving affirmation of its origin from spinous layer of epidermis. Together with this, the study also showed a much diminished level of early differentiation marker like K1 in A431 cells even though its development from spinous layer which markedly expresses K1. This loss of expression of early differentiation marker K1 in A431 cells, further, indicates the irregular differentiation as in the proliferative keratinocytes of upper epidermal layers of psoriatic scales [187]. Nevertheless, the significance of the elevated expression of filaggrin and involucrin found in the present study has to be evaluated. However, this much less or no expression of early differentiation markers like K1, K5 and K14 with the higher expression of late differentiation markers like involucrin and filaggrin suggest the dedifferentiation in A431 cells. This constitutes the first report on

evaluation of differentiation state of A431. Moreover, higher expression of K1, K5 and K14 in HaCaT cells reveals its properly differentiated phenotype with a low expression level of 12R-LOX as in a primary keratinocyte model. Additionally, HaCaT cell line can be dedifferentiated after growing them in low Ca^{2+} medium and can be further differentiated with high Ca^{2+} level [189]. HaCaT and A431 cell lines are, therefore, better *in-vitro* models to study the role of 12R-LOX in terminal differentiation process of keratinocytes.

So, in the light of this dedifferentiated state of skin cancer cells, A431 with a dominant expression of 12-R-LOX and properly differentiated state of normal human keratinocytes HaCaT showing a detectable level 12R-LOX expression, further studies were undertaken to analyse the role of 12R-LOX in the regulation of keratinocyte proliferation and terminal differentiation.

Chapter 3

Role of 12R-LOX in differentiation of human keratinocytes

3.1. Introduction

One of the most essential roles of the human skin is to perform the epidermal water barrier function [6]. The lipid composition and deposition in outer layers of skin, stratum corneum is the critical factor of this impermeability property of the skin [10]. Stratum corneum barrier formation is a part of late terminal differentiation process of keratinocytes. Several studies show special requirement of EFAs such as linoleate (C18:2) to maintain and process the water impermeable barrier of epidermis [23]. Linoleate is being primarily incorporated in a specific class of ceramides, esterified ω-hydroxyacyl-sphingosine (EOS), unique to the epidermis [24].

Substantial evidence reveals the involvement of LOX enzymes in the oxidation of linoleate moiety of esterified structural lipids such as ceramides [27][28]. Moreover, the atypical enzymatic features of 12R-LOX promote new insights into the physiological role of a unique LOX metabolic pathway in formation and maintenance of epidermal permeability barrier and this is also evidenced by differentiation dependent tissue expression of 12R-LOX in epithelial tissues [64]. This implication has paved the way by an important breakthrough accomplished by a genetic study in 2002 [190]. The study reported, for the first time, linkage between mutations in coding region of any LOX gene and development of a disease and this loss of function mutations in LOX genes *ALOX12B* and *ALOXE3* on chromosome 17p13.1 is linked to the genesis of autosomal recessive congenital ichthyosis (ARCI), disease associated with defective skin barrier function. As mutations in one or other LOX genes resulting

in similar impaired barrier function phenotype, it has been speculated that both enzymes belong to same metabolic pathway involved in epidermal differentiation [190].

The role of 12R-LOX on skin barrier function is also revealed by mouse knockout studies. The targeted disruption of 12R-LOX gene in mice resulted in neonatal death due to severely impaired permeability barrier function [153][159]. The CE from skin of these 12R-LOX- deficient mice displayed increased fragility and a disordered composition of ceramides, especially a reduction of ester-bound ceramide species in lipid analysis. So, these studies on mouse knockout reveal a structural role of 12R-LOX which is also accordance to the previous study showing the processing of ω-hydroxy acylceramides in the making of CLE [28]. The study demonstrates that 12R-LOX oxygenates linoleate moiety of esterified ω-hydroxyacyl-sphingosine (EOS) to the 9R- hydroperoxy lineoyl-ω-hydroxyceramide (9R-HPODE-EOS, a hydroperoxide derivative of EOS) which is subsequently converted by eLOX-3 to a specific epoxyalcohol, 9(R),10(R)-trans-epoxy-11E-13(R) hydroxylineoyl-ω-hydroxy-ceramide (9R(10R),13R-EpHOE-EOS) [28]. Moreover, it is suggested that subsequently hydrolysis of epoxyalcohol derivative of EOS generates hepoxilins like derivatives, trioxilins and free ω-hydroxyl on ceramide (ω- OH Cer). This free ω-hydroxyl group on the ceramides is required for coupling to the cross-linked proteins of the CE [129][162].

As described above, both enzymes act in sequence to metabolize fatty acid substrates via R-hydroperoxides to their specific R- epoxyalcohol derivatives of hepoxilin family. The members of hepoxilin family are well characterized to

function as signaling molecules in several physiological processes including adipocyte differentiation, inflammation and neurotransmission [59][164]. In addition, one study, for the first time provides the evidence that LOX-derived epoxyalcohol and their trioxilin derivatives are crucial in the formation of epidermal permeability barrier [27]. Thus, in epidermis, 12R-LOX derived linoleate-related hepoxilins or trioxilins might exert a signaling function in several processes related to components of the epidermal permeability barrier [165].

In order to perform differentiation studies several strategies can be employed to induce basal keratinocyte to differentiate. Among these calcium, which acts as a trigger for the switch between epithelial cell growth and differentiation is the most physiologically employed agent that triggers differentiation *in-vitro* [191]. In epidermis, keratinocytes are exposed to Ca^{2+} gradient maintained throughout the layers, increasing from the basal to the uppermost differentiated layers. Perceiving a high Ca^{2+} concentration, proliferative basal keratinocytes are induced to way out the cell cycle and to command for terminal differentiation [192][193]. Further, cells in culture are induced for terminal differentiation by adding Ca^{2+} concentration in similar way as observed in the upper differentiated layers with withdrawal of cell cycle, specific structural changes and expression of differentiation related genes [194]. The impact of Ca^{2+} in culture can be observed within minutes like cell to cell junctions, formation of desmosomes and following to time duration of Ca^{2+} switch, cells commence to express differentiation markers like K1, K10, involucrin, filaggrin and TGM-1 [195] through calcium signaling in keratinocytes (Figure 17).

Further, cultured human keratinocytes like HaCaT cells, retaining normal morphogenesis and differentiation features, provide well-established differentiation model to study many aspects of terminal differentiation [174][196]. In addition, HaCaT cells have the capability to revert back and forth between the differentiated and the basal phenotype depending upon Ca^{2+} concentration, and therefore are usually employed as model for keratinocyte differentiation in culture [189][196]. The present study was undertaken to study the role of 12R-LOX in the keratinocyte terminal differentiation employing Ca^{2+} induced differentiation model in HaCaT cells.

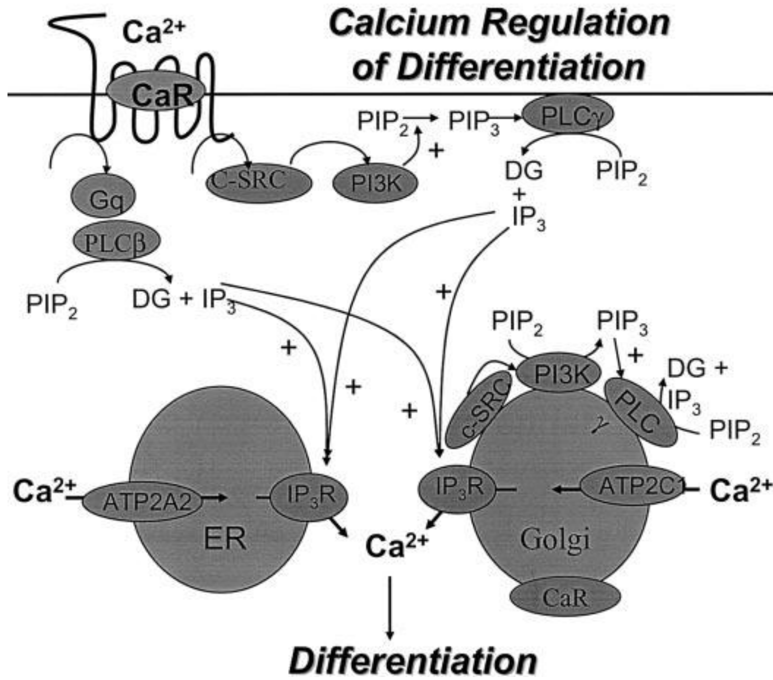


Figure 17: Calcium signaling in the keratinocyte. The keratinocyte contains a CaR that when activated increases PLC activity (both PLC β and γ1), resulting in the production of 2 important intracellular messengers, IP3 and DG. IP3 stimulates the release of calcium from intracellular stores (ER and Golgi). The rise in intracellular calcium leads to the induction of genes required for the differentiation process. (Source: D D Bikle, Vitamin D: Role in Skin and Hair in: Vitamin D, 2nd ed. Academic Press, SanDiego, 2004)

3.2. Materials and Methods

3.2.1. Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM) without calcium (# AL790A) was purchased from Himedia laboratories (Mumbai, India). Chelex 100 resin (#142–2832) was from Bio-Rad Laboratories (Richmond, USA). Cell culture grade calcium chloride, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide], dimethyl sulfoxide (DMSO), protease inhibitor cocktail and polyclonal antibody of human-12R-LOX were obtained from Sigma (St Louis, MO, U.S.A.). Monoclonal antibodies against human cytokeratin1, cytokeratin5, filaggrin, transglutaminase-1 and involucrin were purchased from Abcam (Cambridge, USA). Texas Red® and fluorescein–isothiocyanate (FITC) conjugated antibodies, 4', 6-diamidino-2-phenylindole (DAPI), TRizol and lipofectamine 2000 were procured from Invitrogen Life Technologies (Carlsbad, CA, U.S.A). 12R-LOX siRNA was obtained from IDT (U.S.A.). pCMV6-Neo-12R-LOX plasmid was from Origene (Rockville, U.S.A.). SYBR® Green PCR Master Mix was from Applied Biosystems, Invitrogen Life Technologies (Carlsbad, CA, U.S.A). All the other chemicals and reagents for the experiments were obtained as described before in previous chapter.

3.2.2. Cell culture

Cell culture and growth of HaCaT cells was maintained as described in previous chapter. For induction of differentiation, HaCaT cells were replaced and cultured in calcium-free DMEM medium, with 10% chelexed FBS and calcium chloride at 0.03mM (Low calcium) or 2.8mM (High calcium) final concentration.

FBS was calcium-depleted by incubation with Chelex 100 resin for 1 h at 4^0C according to the manufacture's protocol. The Chelex was subsequently removed using a 0.22µm filter membrane. Sub culturing of cells were done twice a week. To obtain cells exhibiting a basal keratinocyte phenotype, HaCaT cells were cultured in low Ca^{2+} (0.03mM) medium for at least 4 weeks and were maintained in the same culture conditions thereafter. Low Ca^{2+} HaCaT cultures were never allowed to exceed 85% confluency. Cell viability was determined by the Trypan Blue dye exclusion method before seeding for each experiment. For 12R-LOX knock down study, cells were introduced with 12R-LOX siRNA at every 48 h.

3.2.3. Cell proliferation assay

Cell proliferation of HaCaT cells grown in low calcium medium was assessed by MTT assay which is a colorimetric assay based on the ability of viable cells to change from soluble yellow tetrazolium salt to blue formazan crystals. Cells maintained in normal medium (control) and cells maintained in low calcium medium) at 5×10^3 cells per well were seeded in 96 well plates and then next day further incubated in fresh normal DMEM and low calcium concentration (Ca^{2+} ,0.03mM) DMEM medium for 24 h, 48 h and 72 h in a final volume of 100µl. After incubation, 20µl of MTT solution (5mg/ml in PBS) was then added to each well and incubated for an additional 4 hrs at 37^0C. The purple-blue formazan crystals were dissolved in 100µl of DMSO before measuring optical density at 570 nm with a plate reader. This colorimetric absorbance which refers to activity of the mitochondrial energy metabolism was shown as cell viability (% of proliferative cell).

3.2.4. Phase contrast microscopic analysis of cell morphology

HaCaT cells (1.5 X 10^6 cells/plate) were seeded and allowed to grow overnight and then treated with high calcium media for 3, 12, 24, 48, 72, 96, 120 and 144 h. After each time points cells were observed for morphological changes under phase contrast microscope.

3.2.5. Immunoblotting

HaCaT cells were grown under low Ca^{2+} (0.03mM) medium in 100 mm culture dishes and harvested weekly after getting 80% confluence for markers analysis. For induction of differentiation studies, cells grown in low Ca^{2+} condition were seeded at a density of 5 X 10^6 in 100 mm dishes and incubated with high Ca^{2+} (2.8mM $CaCl_2$) medium for 3, 6 and 9 days. For protein analysis, the whole cell extracts of the cells grown in low Ca^{2+} or high Ca^{2+} medium were prepared and protein contents were determined as described in chapter 1. 100μg of total cell lysate was resolved on 8-12% SDS-PAGE gels along with protein molecular weight standards, and then transferred onto Nitrocellulose membranes. The membranes were blocked with 5% (w/v) BSA and then incubated with the primary antibodies- 12R-LOX (1:100), cytokeratin1 (1:2000), cytokeratin5 (1:500), filaggrin (1:500), transglutaminase-1 (1:500), involucrin (1:1000) and β-actin (1:2000) in 10ml of antibody-dilution buffer (1 X Tris buffered saline and 0.05% Tween-20 with 1% BSA) with gentle shaking at 4^0C for 8–12 h and then incubated with respective conjugated secondary antibodies (HRP-conjugated). Signals from immunocomplexes were visualized by chemo-luminescence using ECL kit

according to the manufacturer's protocol. The band intensities were measured by Image J software.

3.2.6. 12R-LOX gene knockdown

The *ALOX12B* gene knockdown in HaCaT cells was carried out by delivering siRNA against 12R-LOX with lipofectamine 2000 as per the manufacture's protocol. In 60 mm tissue culture plate, 1.5 x 10^6 HaCaT cells (low Ca^{2+}) were taken in 4ml. The cells were incubated at 37^0C in a CO_2 incubator until the cells are 60-80% confluent. Cells were pelted down and washed with 2ml of PBS. Then cells were treated with or without validated 12R-LOX siRNA at a final concentration of 10nM and Lipofectamine 2000 (1: 2.5) mixture in antibiotic-free and FBS-free medium either with low Ca^{2+} (0.03mM) or high Ca^{2+} (2.8mM) condition. 4 h after transfection of 12R-LOX siRNA, medium was replaced by fresh low Ca^{2+} (0.03mM) DMEM or high Ca^{2+} (2.8mM) DMEM medium supplemented with 10% FBS. Then cells were incubated at 37^0C in a CO_2 incubator for 6 days following repeated transfection of 12R-LOX siRNA at 48 h intervals. Untransfected and Universal negative control1 siRNA transfected cells served as controls.

3.2.7. Immunofluorescence microscopy analysis

HaCaT cells (normal cells and dedifferentiated cells cultured in low Ca^{2+} (0.03mM) condition) were seeded at a density of 2 X 10^5 per well on glass cover slips in 6 well plates and were grown for a period of 18 h. For 12R-LOX knockdown during differentiation, cells cultured in low or high calcium medium were repeatedly (every 48 h) transfected with 12R-LOX siRNA for 6 days and then seeded on glass cover slips and were grown for 12 h. After fixing (with 4%

paraformaldehyde in PBS for 10 min), permeabilizing (with 0·2% Triton X-100 in PBS for 5 min), washing (PBS) and blocking (with 5% FBS for 1 h), cells were incubated with primary antibodies (cytokeratin1, cytokeratin5, filaggrin, transglutaminase-1, involucrin and 12R-LOX) for overnight followed by several washings with PBS, exposing with FITC- conjugated secondary antibodies for 1 h in PBS containing 1% FBS. The cells cover slips were mounted on glass slides with a drop of Prolong gold antifade reagent with DAPI (Invitrogen). The slides were examined under confocal laser-scanning microscope.

3.2.8. Transfection of pCMV6-Neo-12R-LOX mammalian vector

For over expression of 12R-LOX in HaCaT cells, transfection of pCMV6-Neo-12R-LOX mammalian vector with Lipofectamine 2000 was performed according to the manufacturer's protocol. Cells (1.5 x 10^6) were seeded in 60 mm culture dishes day before transfection. Liposome and DNA were incubated in DMEM without antibiotics and FBS for 20 min at room temperature to allow transfection complex formation. The liposome-DNA mixture was added to culture dishes containing HaCaT cells in 2ml of DMEM without antibiotics and FBS. After 4 h of transfection, 4ml of complete DMEM media (with 10% FBS) was added to each dish to allow differentiation. Fresh medium was added the next day and after 72 and 96 h post-transfection, for transient transfection, cells were assayed for the expression of the transfected gene by western blot.

3.2.9. RNA isolation and real time PCR

Total RNA was isolated from HaCaT cells transfected with pCMV6-Neo-12R-LOX plasmid using TRizol according to manufacturer's instructions. Reverse

transcription of 2μg of total RNA isolated was achieved by the protocol as previously described. After cDNA synthesis, for real time PCR quantitations, cDNA equivalent to 10ng total RNA were used for all the PCR reactions. All the PCR reactions were carried out in 25μl reactions mixture containing SYBR® Green Master mix, 1.5μl of diluted template (1:5 dilution), and 10pmol of each primer (human *ALOX12B*, 5'-TGA TCC GGA ATT GCA GTC TT-3' (sense) and 5'-CAT ATC GGA TCA GCT CAG GC-3' (antisense); human *TGM-1*, 5'-TGC CAC ACC CCA AGA GAC-3' (sense) and 5'-TCA GCA AAA ATG AAA GGC GT-3' (antisense); human *FLG*, 5'-TGT CTC ATC CTC ATT CAG GTG TTA-3' (sense) and 5'-TGG CTC CAA GTT GTC TGG AT-3' (antisense); human *IVL*, 5'-GGT CCC ATC AAA GCA AGA GG-3' (sense) and 5'-GCT GCT GAT CCC TTT GTG TT-3' (antisense); human *KRT1*, 5'-TCA ACA ACC AAT TTG CCT CC-3' (sense) and 5'-AGC AGC TCC CAT TTT GTT TG-3' (antisense)) in ABI Prism H7500 fast thermal cycler (Applied Biosystems, CA, USA) and results normalized to GAPDH. The analysis was done using SDS 2.1 software (Applied Biosystems, USA) and the relative gene expression of 12R-LOX and markers were calculated using the comparative $2^{-\Delta\Delta CT}$ method [197].

3.2.10. Statistical analysis

All the data analysis was completed using the Sigma Plot 10 software. Data were presented as the Mean ± S. E. M. of three independent experiments and statistical analysis was carried out by Student's t test using Excel (Microsoft). A p-value of less than 0.05 was considered as statistically significant over control.

3.3. Results

3.3.1. Standardization of methods for inducing dedifferentiation in HaCaT cell line at low Ca^{2+} levels

HaCaT cells grown in DMEM with 10% FBS were transferred to low Ca^{2+} (0.03mM Ca^{2+}) DMEM with 10% chelexed FBS and cultured for 1 month. Phase-contrast microscopic pictures (20X) of these cells were taken to observe morphological features. A well-differentiated cornified cell boundary was observed in HaCaT cells grown in normal DMEM, where as in low Ca^{2+} medium, cells were observed with their loose junctions within 4 h. Further when cultured in low Ca^{2+} medium for 1 month, HaCaT cells acquired morphological features of primary human keratinocytes (Figure 18). These HaCaT cells, acquiring morphological features of primary human keratinocytes, were further analyzed for biochemical features of primary keratinocytes.

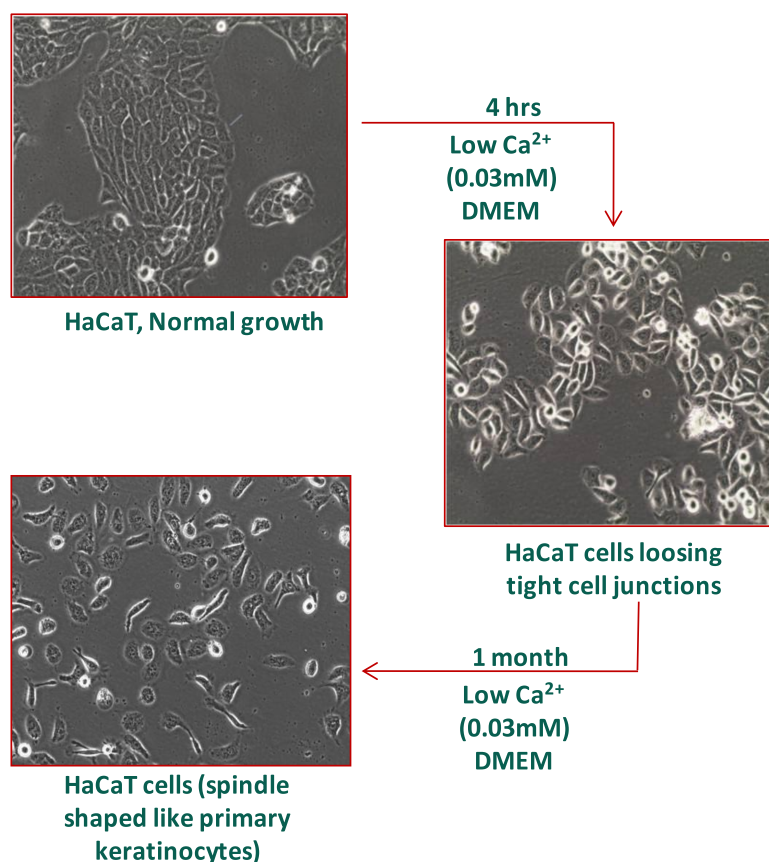


Figure 18: Dedifferentiation model of HaCaT cells. Dedifferentiation (basal-like state) of HaCaT cells had carried out by culturing them in low Ca^{2+} (0.03mM Ca^{2+}) DMEM medium from conventional DMEM medium. Above phase contrast images showing the morphological changes in HaCaT cells in response to low Ca^{2+}concentration medium at different time points.

3.3.2. Effect of low Ca^{2+} condition on expression of different primary keratinocyte differentiation markers in HaCaT cells

In skin, during the terminal differentiation, the differentiation state can be distinguished by the expression of differentiation markers which show an increasing pattern as differentiation is proceeded. So, HaCaT cells, cultured in low Ca^{2+} (0.03mM Ca^{2+}) DMEM with 10% chelexed FBS and showing features as primary keratinocytes, were analyzed for expression of keratinocyte differentiation markers at protein level for confirmation of differentiation state.

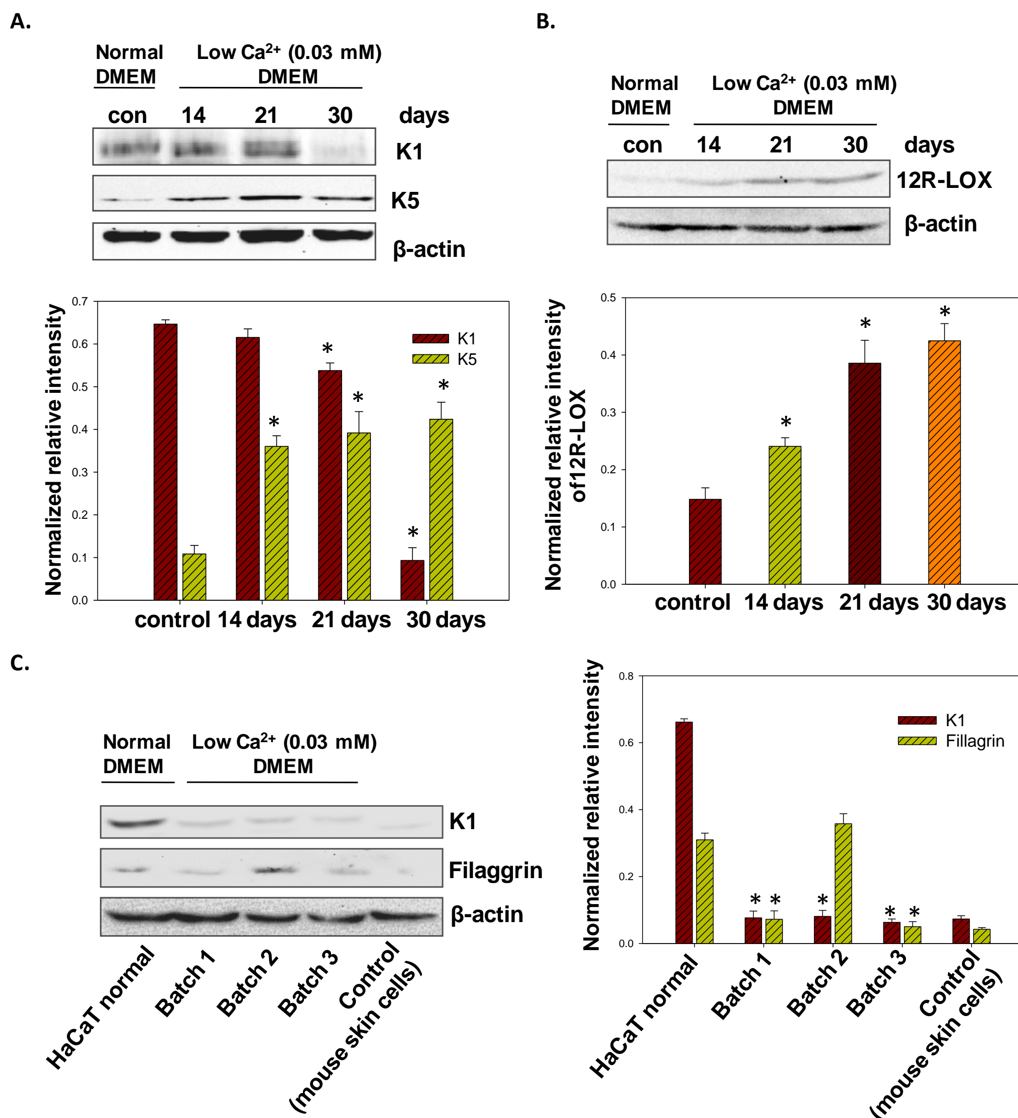


*Figure 19: Effect of low Ca2+ condition on expression of different primary keratinocyte differentiation markers in HaCaT cells. Above figure showing immunoblot analysis of protein extracts from HaCaT cells grown under normal DMEM medium or low Ca^{2+} (0.03mM Ca^{2+}) DMEM for one month (samples were collected every 7 days for immunoblot analysis) for detection of **A)** keratinocyte differentiation markers- keratin1 and keratin5, **B)** 12R-lipoxygenase. **C)** Detection of K1 and filaggrin in HaCaT cells grown under low Ca^{2+} (0.03mM Ca^{2+}) DMEM in different batches for one month (HaCaT cells grown in normal medium and mouse skin cells were used as control to show the basal like state of HaCaT cells. β-actin was used as an internal loading control. The relative band intensities were measured by quantitative scanning densitometry, bars indicate the mean ± S. D. (n = 3); *P < 0.05 compared with control cells.*

Cell lysates (100µg) were resolved on SDS-PAGE and analyzed for K1, K5, filaggrin and 12R-LOX protein expression by Western blotting. β-actin was used

as internal control to monitor equal loading. Western analysis had confirmed the dedifferentiation of HaCaT cells with decreasing K1 & filaggrin and increasing K5 marker levels in cells grown up to 30 days in low Ca^{2+} condition compared with normal HaCaT cells (Figure 19A&C). After confirming the much diminished expression of K1 & filaggrin in HaCaT cells, batch 3 was used for further experiments (Figure 19C). Apparently, these cells showed some higher expression of 12R-LOX as compared with normal HaCaT cells (Figure 19B).

3.3.3. HaCaT cells are able to revert back to a basal-like state both morphologically and biochemically

HaCaT cells, cultured in low Ca^{2+} condition, that attributed primary keratinocytes characteristics, were further analyzed for expression of keratinocyte differentiation markers like K1, involucrin, TGM-1, filaggrin, K5 and 12R-LOX using confocal microscopy. A much diminished expression of K1, involucrin, TGM-1 and filaggrin confirmed the result obtained from western blot. Further higher expression of K5 and 12R-LOX in cells cultured in low Ca^{2+} condition as compared with normal HaCaT cells were observed. As shown in figure 20, HaCaT cell line was dedifferentiated by growing in low calcium media and these dedifferentiated cells showed characteristics of basal-like state of primary keratinocytes (very low or no level of expression of differentiation markers like K1, involucrin, TGM-1 and filaggrin and high level of expression of K5). In contrast to the primary cells, these undifferentiated HaCaT cells showed elevated level of 12R-LOX as compared with normal HaCaT cells. These dedifferentiated cells like primary keratinocytes were further differentiated with high calcium medium.

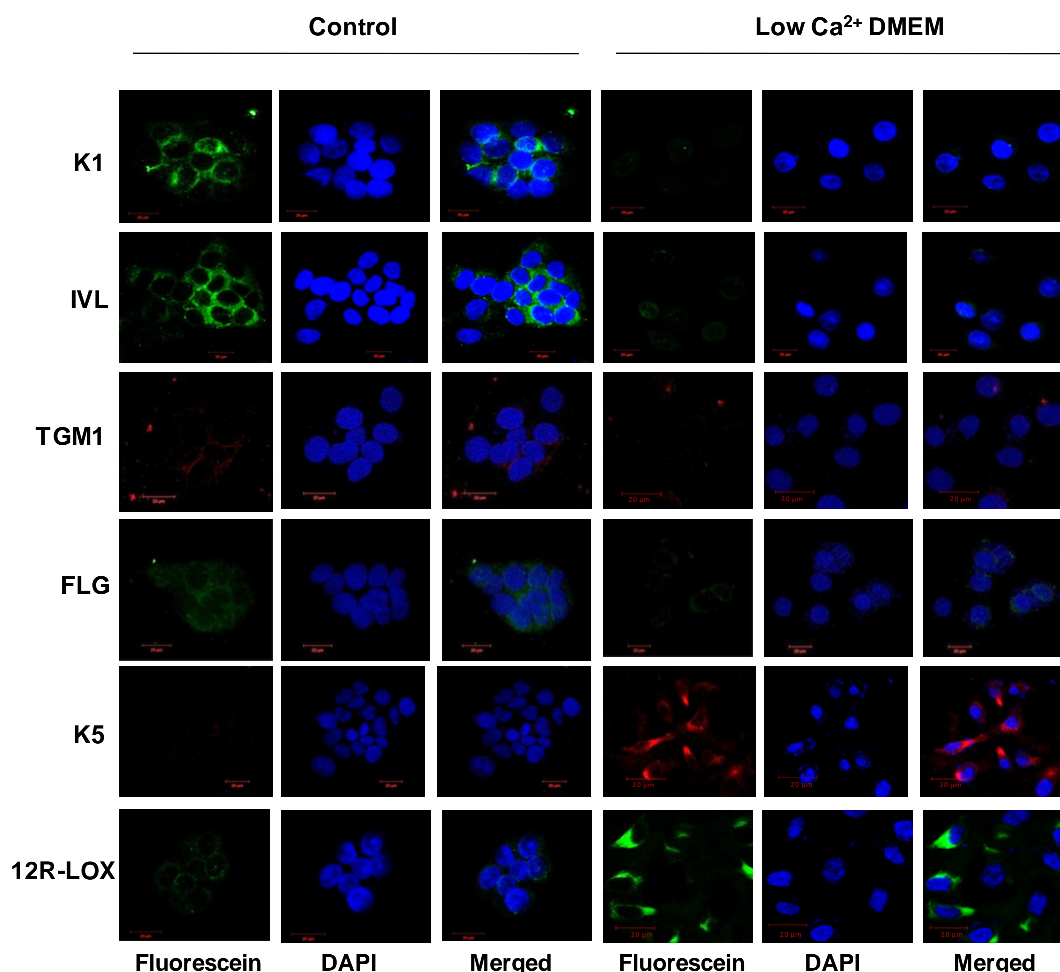


Figure 20: HaCaT cells are able to revert back to a basal-like state both morphologically and biochemically at low Ca^{2+} condition. Confocal micrograph showing expression of primary keratinocyte differentiation markers in HaCaT cells maintained in low Ca^{2+} condition for one month compared with HaCaT cells maintained in normal DMEM. After one month growth in low calcium medium, cells acquired characteristics of basal-like state of primary keratinocytes. Cells showed very low or no level of expression of differentiation markers like K1, involucrin (IVL), transglutaminase1 (TGM-1) and filaggrin (FLG). Again the higher expression of K5 in low Ca^{2+} cells confirmed the basal-like state of these cells. At this state cells were also showing some little higher expression of 12R-LOX. DAPI were used to stain nucleus. Bar 20µm.

3.3.4. Effect of low calcium concentration (Ca^{2+}, 0.03mM) on the proliferation of HaCaT cells (MTT assay)

HaCaT cells (both, cells maintained in normal medium and cells maintained in low calcium medium) were seeded in 96-well plate and further grown in normal DMEM medium and low calcium concentration (Ca^{2+} ,0.03mM) DMEM medium

for 24 h, 48 h and 72 h. Cell proliferation of these cells was estimated by the MTT assay. Cells, maintained in low calcium medium and having characteristics of primary keratinocytes, exhibited increased proliferation rate compared with cells grown in normal DMEM in time dependent manner (Figure 21). In 72 hour, proliferation rate of dedifferentiated cells increased more than two times of proliferation rate of normal cells.

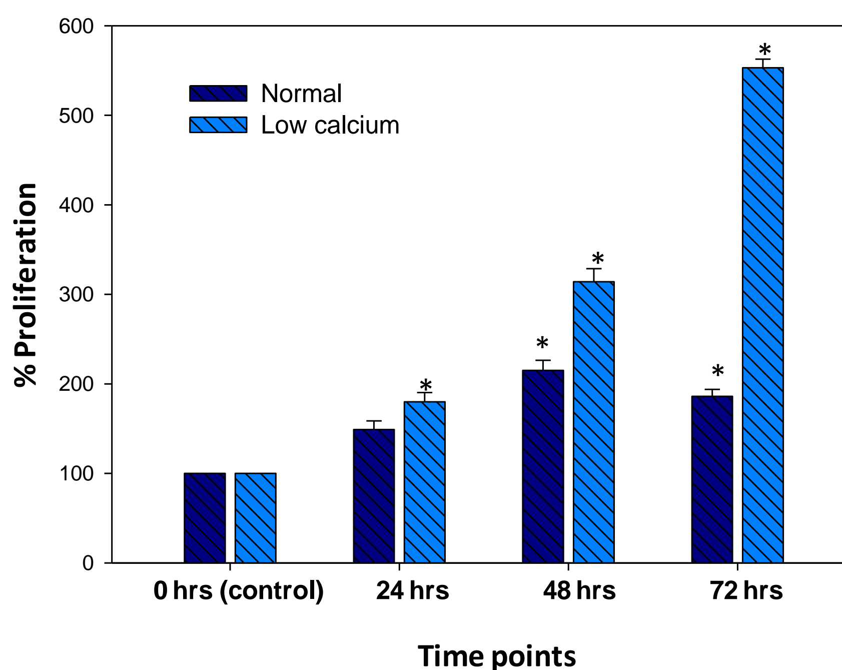


Figure 21: Effect of low Ca^{2+}concentration on the proliferation of HaCaT cells. Cells were incubated with normal DMEM and low calcium DMEM (as shown in graph) for 24h, 48 h and 72h then cell viability was examined by MTT assay. Time dependent growth encouragement was observed in low Ca^{2+} media. The number of cells in the control was taken as 100%. The values represent the mean ±SD of three independent experiments ($P < 0.05$).

3.3.5. Induction of differentiation in the normal human keratinocyte cell line (HaCaT)

To study the role of 12R-LOX in the differentiation of keratinocytes, the dedifferentiated HaCaT cells were induced for differentiation by growing them in high calcium medium. Phase-contrast microscopic pictures (20X) of cells grown in

high Ca^{2+} medium (2.8mM Ca^{2+}) were taken to observe morphological features. A well differentiated cornified cell boundary was observed in HaCaT cells grown in high Ca^{2+} medium compared to cells grown in low Ca^{2+} medium (Figure 22).

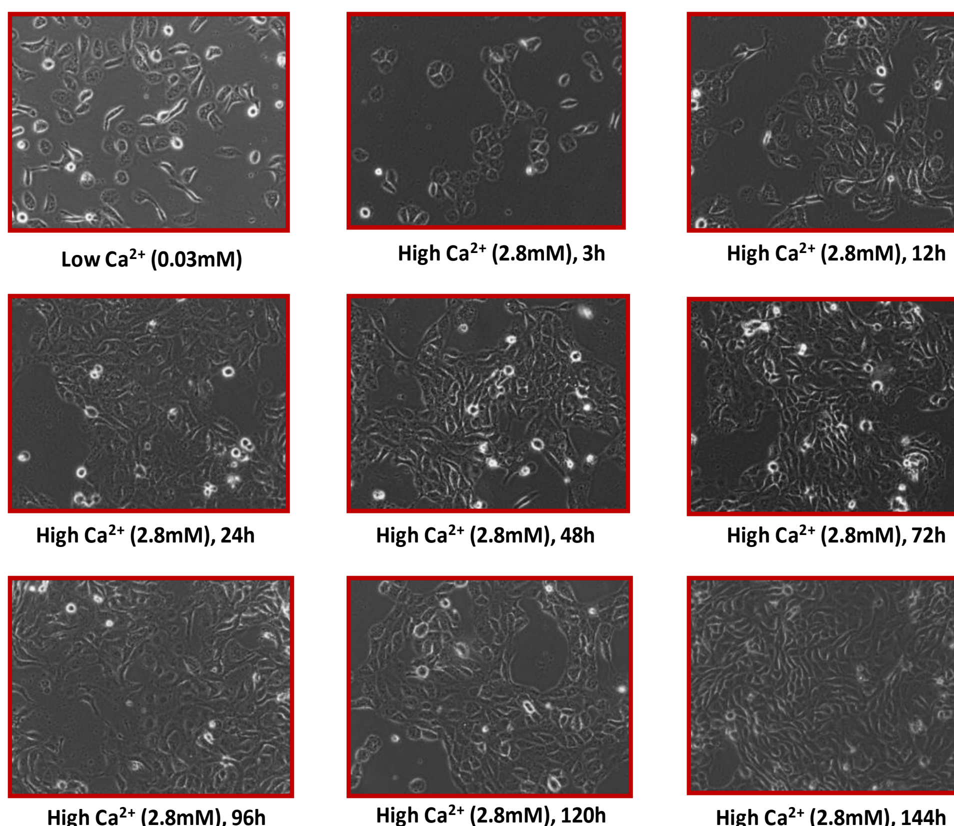


Figure 22: Phase contrast microscopic pictures during induction of differentiation in HaCaT cells grown in low Ca^{2+} (0.03mM) condition with high Ca^{2+} (2.8mM). Cells grown in low calcium media (control), or in high calcium media for various time points (up to 144 h), showing time--dependent alteration in cellular morphology. Magnifications 20 X.

3.3.6. 12R-LOX expression increases during differentiation process

The induction of differentiation was confirmed by studying the expression levels of K1, filaggrin, TGM-1 and involucrin by immunoblot analysis. The results showed an increase in the levels of expression of K1, filaggrin, TGM-1 and involucrin with affirmation of late stage of differentiation (up to 9 days of differentiation) and a further elevation in 12R-LOX expression as compared with

expression in low Ca^{2+} in time dependent manner with the progression of differentiation (Figure 23).

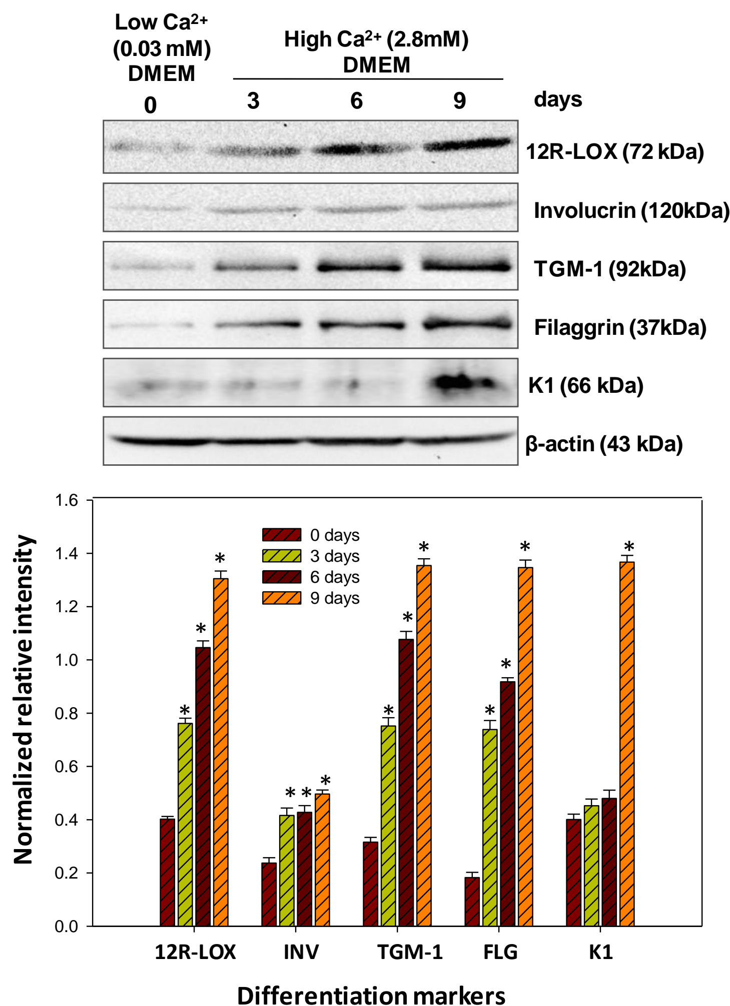


*Figure 23: 12R-LOX expression increases during differentiation process. Expression of 12R-LOX and keratinocyte differentiation markers- involucrin, TGM-1, filaggrin & K1 after induction of differentiation in HaCaT cells grown in low Ca^{2+} (0.03mM) condition with high Ca^{2+} (2.8mM). Figure showing immunoblot analysis of protein extracts from HaCaT cells grown under low Ca^{2+} (0.03mM Ca^{2+}) DMEM or high Ca^{2+} (2.8mM Ca^{2+}) DMEM for 9 days (samples were collected every 3 days for immunoblot analysis) with antibodies for 12R-LOX, involucrin, TGM-1, filaggrin & K1. β-actin was used as an internal loading control. The relative band intensities were measured by quantitative scanning densitometry, bars indicate the mean ± S. D. (n = 3); *P < 0.05 compared with control cells.*

3.3.7. Down regulation of 12R-LOX expression affects the onset of TGM-1 and filaggrin in terminal differentiation

To demonstrate the relation of 12R-LOX to differentiation markers we employed a siRNA strategy to deplete 12R-LOX expression and re-examined the effect on these markers. HaCaT cells, cultured in low Ca^{2+} condition, that showed features of primary keratinocytes, were induced to differentiate with high Ca^{2+} (2.8mM Ca^{2+}) DMEM in presence or absence of siRNA targeting 12R-LOX. Then cells from both sets (with or without 12R-LOX siRNA) were analyzed for expression of keratinocyte differentiation markers like TGM-1, filaggrin, K1, involucrin and 12R-LOX at day 6 of differentiation using confocal microscopy and using DAPI to stain nucleus. The stage of differentiation after 6 days was confirmed by immunostaining with antibodies that recognize markers- TGM-1, filaggrin, K1, and involucrin where an increased expression pattern of differentiation markers was observed. The results clearly showed that the expression of late differentiation markers like TGM-1 and filaggrin were completely diminished even at late stage of differentiation (6 days) with high calcium when cells were introduced with 12R-LOX siRNA at undifferentiated stage and absence of 12R-LOX siRNA did not have any effect on expression of TGM-1 and filaggrin (Figure 24).

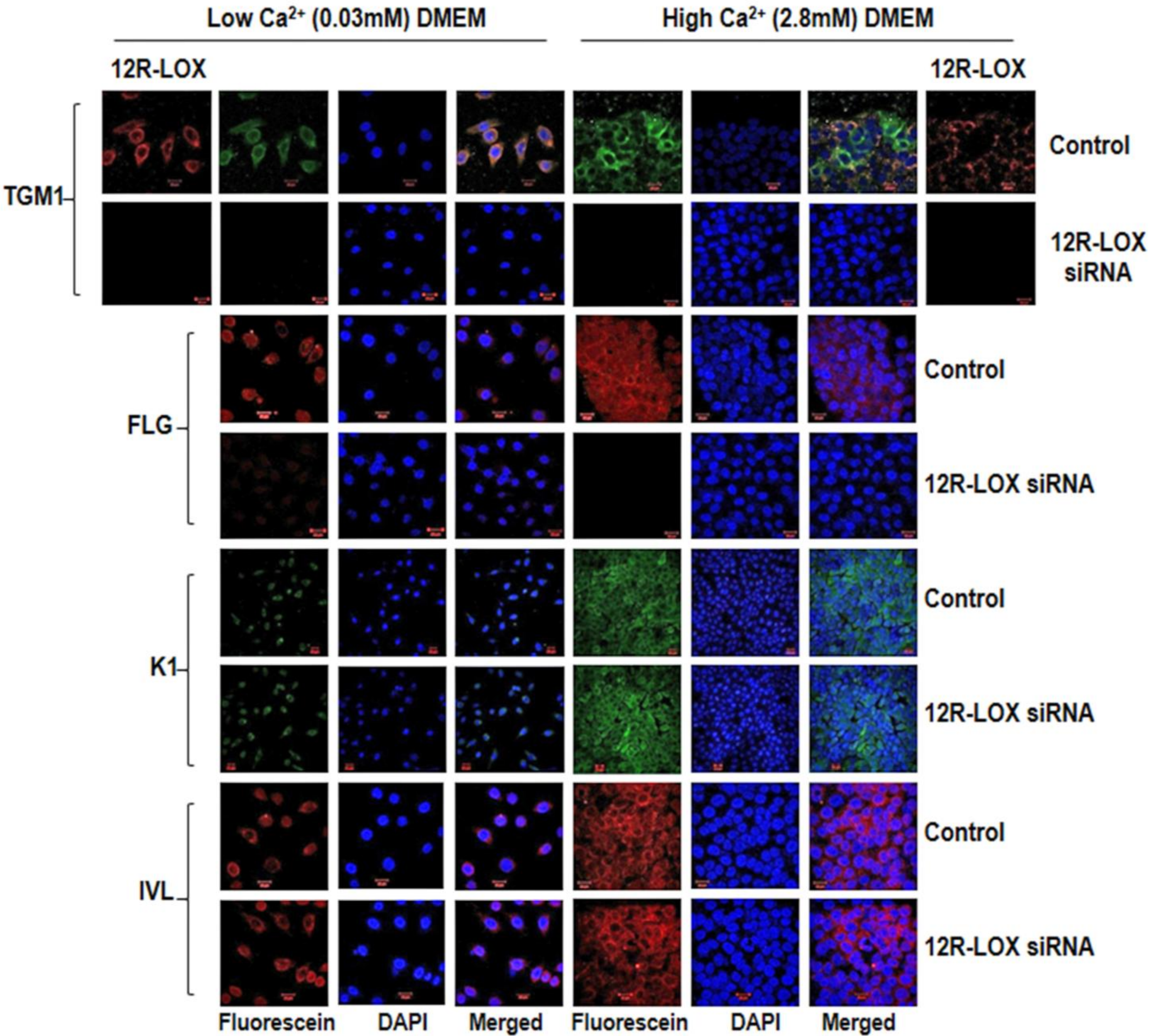


Figure 24: Down regulation of 12R-LOX expression affects the onset of TGM-1 and filaggrin in terminal differentiation. Confocal micrograph showing expression of primary keratinocyte differentiation markers- TGM-1, filaggrin, K1, involucrin and 12R-LOX after induction of differentiation in HaCaT cells grown in low Ca^{2+} (0.03mM) condition with high Ca^{2+} (2.8mM) in the presence or absence of 12R-LOX siRNA for 6 days . After 6 days of calcium induced differentiation, HaCaT cells were characterized as well differentiated with higher expression of TGM-1, filaggrin, K1 and involucrin in the presence of 12R-LOX. But when calcium induced differentiation was carried out in the absence of 12R-LOX (12R-LOX siRNA treaded cells), there was no expression of late differentiation markers like TGM-1 and filaggrin during differentiation. DAPI were used to stain nucleus. Bar 20µm.

3.3.8. Up regulation of 12R-LOX in HaCaT cell line is associated with enhanced expression of TGM-1 and filaggrin

HaCaT cells were cultured overnight in culture plate and transfected with pCMV6-Neo-12R-LOX recombinant expression vector, expressing 12R-LOX under the control of a CMV promoter, using lipofectamine 2000 according to manufacturer's recommendations. An 80% transfection efficiency was observed in HaCaT cells when transfected with pEGFP plasmid for 48 h (Figure 25A). 72 h after transfection, up regulated expression of 12R-LOX mRNA was observed as shown by amplification of 12R-LOX cDNA in PCR method. Similarly, a remarkable transient expression of 12R-LOX protein in transfected cells compared with control was confirmed with immunoblotting after 96 h using antibody against 12R-LOX (Figure 25B). These 12R-LOX over-expressive HaCaT cells were analyzed for expression of keratinocyte differentiation markers like TGM-1, filaggrin, involucrin and K1 following 96 h transfection. Late differentiation markers- TGM-1 and filaggrin are also involved in the formation of epidermal permeability barrier, a key feature of terminal differentiation. Real time PCR analysis demonstrated a time dependent increase in level of mRNA transcripts of TGM-1 and filaggrin in 12R-LOX over expressing cells. In contrast, study with early differentiation markers indicated a little reduction in K1 expression. In keratinocytes, expression of involucrin increases with the differentiation and death of the cells. In transfected cells, level of involucrin first elevated (as cells died upon transfection) and then decreased over 96 h time course (Figure 25C). These studies thus reveal that the expression of late differentiation markers- TGM-1 and filaggrin is associated with 12R-LOX in differentiating keratinocytes to facilitate and perform

the epidermal barrier formation efficiently during late terminal differentiation process.

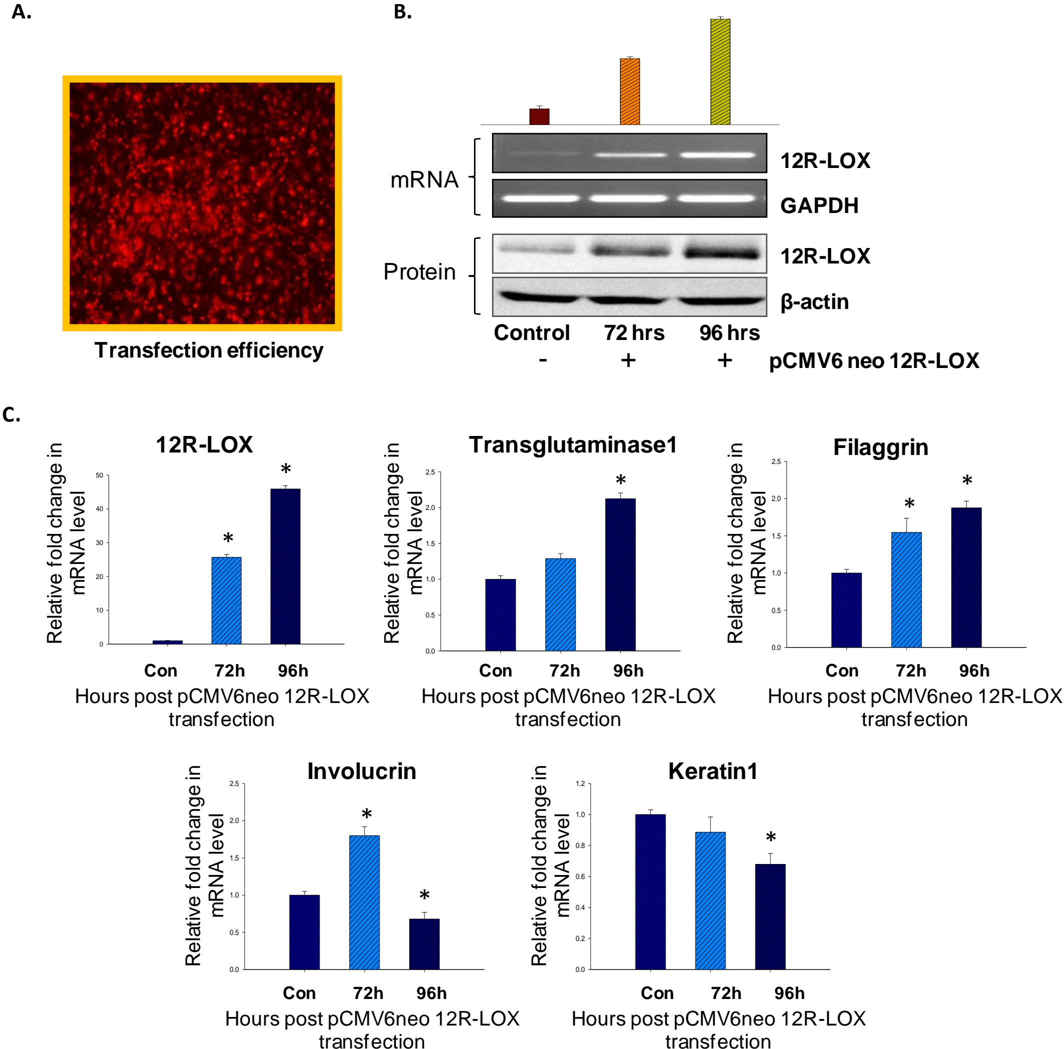


Figure 25: Up regulation of 12R-LOX in HaCaT cell line is associated with enhanced expression of TGM-1 and filaggrin. Real-time RT-PCR study to analyze the relative expression of differentiation markers in HaCaT cells under 12R-LOX over expression. ***A)*** *Transfection efficiency (80-85%) with reference transfection reagent (lipofectamine 2000) on HaCaT cells. pEGFP plasmid was transfected to HaCaT cells at 90% confluency in absence of serum and antibiotics. GFP fluorescence was detected with florescence microscopy 48 h post transfection.* ***B)*** *RT-PCR and western blot analysis of mRNA and protein extracts respectively from HaCaT cells transfected with pCMV6-Neo-12R-LOX plasmid for 72, 96 h in comparison to control (only lipofectamine without any vector) for confirmation of over expression of 12R-LOX protein. GAPDH and β-actin were used as internal loading control respectively. The up regulation in expression of 12R-LOX in HaCaT cells following 96 h post transfection with pCMV6-Neo-12R-LOX plasmid relative to control was significant. Data represent the result from one of three similar experiments.*

C) *Bar graphs representing the real-time RT-PCR analysis to examine the relative expression of keratinocyte differentiation markers- TGM-1, filaggrin, involucrin, K1 and 12R-LOX in HaCaT cells after transfection with pCMV6-Neo-12R-LOX plasmid for 72 h and 96 h. Control: cells without any transfection. The values are expressed as the mean ±SD of three independent experiments. * denote P < 0.05 compared with control cells.*

3.4. Discussion

Several studies, since mechanistic evidence of 12R-LOX in epidermis became evident, suggest a pivotal role of it in development and maintenance of epidermal permeability barrier function and so in the process of keratinocyte terminal differentiation [153][160][129][157]. Recent studies on formation of permeability barrier indicate not only a structural function of 12R-LOX using linoleate moiety of ceramide as a substrate [28] but also a signaling role of 12R-LOX which may be affected and mediated through linoleate-related hepoxilins [64][165]. The present study, in this regard, was undertaken to study the role of 12R-LOX in terminal differentiation process using immortalized HaCaT cells with Ca^{2+} induced differentiation approach. HaCaT cells can be dedifferentiated and have the capacity to revert back to a basal-like state after growing them in low Ca^{2+} medium [189]. Accordingly, in the present study, first, we practiced the approach to achieve the HaCaT cells as in basal-like states (cells showing features of stratum basale) growing them in low Ca^{2+} medium (0.03mM), so that these basal-like cells can be further differentiated as primary keratinocytes [189]. In the study, a much diminished or no expression level of differentiation markers like K1, filaggrin, involucrin and TGM-1, spindle shape with no cell to cell tight junctions in the dedifferentiated HaCaT cells revealed the basal-like state of these cells both morphologically and biochemically. Similar observations were shown in primary human keratinocytes cultured in submerged conditions with non-delipidized

serum [198]. Since, the expression of K5 and K14 is strictly restricted to the proliferative cells of stem cell layer and stratum basale where the Ca^{2+} concentration is very low [168][169], a remarkably increased expression of K5 in these dedifferentiated HaCaT cells gives the affirmation of a state similar to cells of stratum basale of epidermis. The characteristic feature of cells of basal layer especially an increased proliferation rate [199] is also unveiled by these basal-like HaCaT cells showing two fold increase in proliferation rate compared with normal HaCaT cells.

In the present study, apparently, a little elevated expression of 12R-LOX in basal-like HaCaT cells grown in low Ca^{2+} condition might be due to calcium depletion in medium which provides similar environment as in disruption of epidermal barrier function and so the proteins which are necessary for epidermal barrier formation might be slightly up regulated as a consequence under permeability barrier homeostasis [200]. Similarly, a decrease in Ca^{2+} level in the upper epidermis as a result of passive loss of Ca^{2+} through the damaged stratum corneum after permeability barrier disruption signals the upper granular cells to secrete their pool of lamellar bodies [201][202] and prevention of this decrease in Ca^{2+} level after barrier disruption outcome in failure of lamellar body secretion with no initiation of barrier repair process. Conversely, a decrease in Ca^{2+} level in the outer epidermis without disrupting permeability barrier by sonophoresis or ionophoresis leads to stimulation of lamellar body secretion [203][204].

Interestingly, a differentiation dependent further increment in 12R-LOX expression level was noticed in the present study when cells showing the

characteristics of basal-like primary keratinocytes were induced for differentiation with high Ca^{2+} level. Here, the progress of differentiation was evident from the changes in the expression of differentiation markers which follow rising prototype of expression during terminal differentiation of keratinocytes [168][171][172][173]. The absence of 12R-LOX enzyme activity in primary cultures of human keratinocytes which produce only traces of 15-HETE [183], pave the way to its association with late stage of terminal differentiation process [165]. Similarly, it is now understandable that the increasing pattern of expression of 12R-LOX during differentiation of HaCaT cells is attributed to its association with the late stages of terminal differentiation, a condition that demands a long term treatment to mimic in culture. Furthermore, study on localization pattern of 12R-LOX also reveals the differentiation dependent tissue expression and spatial and temporal co-localization of 12R-LOX in upper differentiated epithelial tissues (stratum granulosum) and thus, implying the functional linkage of 12R-LOX in late terminal differentiation process [64]. Additionally, in embryonic skin of mouse, expression of Alox12b begins at embryonic day 15.5, time for the onset of skin development and after the epidermal development it shows a differentiation dependent expression pattern with highest expression in stratum granulosum [154]. In a similar way, onset of Alox12 expression, found at E14.5 and E15.5 (same time ΔNp63α is predominantly co-expressed) further enhances during epidermal differentiation and consistent with the known role of 12S-LOX in epidermal barrier formation. This 12S-LOX expression is directly induced by ΔNp63α at around E15.5, the time when commitment to terminal differentiation occurs nevertheless, the ΔNp63α

expression declines as differentiation progresses [115]. ΔNp63α also promotes the IKKα expression necessary for taking out cell cycle in cells of stratum spinosum [205][206] together with activation of components of Notch signalling to induce expression of differentiation marker K1 [207]. Thus, elevation in 12R-LOX expression during differentiation of keratinocytes strengthens the presumed role of 12R-LOX in late differentiation process and so in epidermal barrier formation.

In the present study, depletion of 12R-LOX expression by 12R-LOX siRNA treatment in keratinocytes during differentiation gives new prospects in the understanding of its role in late differentiation process. A diminished or no expression of TGM-1 and filaggrin in the absence of 12R-LOX (treatment with 12R-LOX siRNA) during differentiation process of keratinocytes indicates a regulatory effect of 12R-LOX in late stage of differentiation. In the study, this regulatory role of 12R-LOX is yet again revealed with induced expression of TGM-1 and filaggrin under 12R-LOX overexpression. These late differentiation markers are important components of epidermal permeability barrier. Filaggrin is the result of a complex proteolytic processing of profilaggrin by a number of enzymatic reactions [208][209]. After aggregation with keratin filaments cytoskeleton, the processed and mature filaggrin forms macrofibrils crisscrossing the cornified cells of stratum corneum [210]. This collapsed macrofibrils is cross-linked to CLE by TGM-1. Thus, filaggrin serves as an integral part of protein layer of CE beneath to lipid layer, CLE which contributes to structural integrity of corneocytes. Filaggrin also plays a role in maintaining moisture retention in the cornified layers with degradation products of filaggrin in keratohyalin granules

[211]. Further, a functional loss or reduction of filaggrin is linked to ichthyosis vulgaris having excessively dry skin and impaired barrier function [212]. Thus filaggrin is a key epidermal protein and essential for the formation of a normal, intact and protective skin barrier. In addition, studies on the molecular mechanisms involved in the disruption of the barrier function in 12R-LOX-deficient mice reveal severely affected CE proteins together with lipid components of lamellar bodies in the stratum corneum. The epidermis of 12R-LOX-deficient mice demonstrated a disturbed processing of filaggrin, which is a crucial component of the CE, resulting in increased fragility of corneocytes (decreased mechanical strength of CE) of mutants and therefore causative to early neonatal death of the mutant mice [153][160]. However, this defect in filaggrin processing was limited to the neonatal 12R-LOX knockout phenotype, while epidermis of eLOX-3 knockout with impaired barrier function did not display any default in processing in filaggrin protein [159]. From the present study, it is now evident that there is a functional linkage between regulatory signaling mechanism of filaggrin and 12R-LOX in the formation of barrier function during terminal differentiation of keratinocytes.

TGM-1, the other late differentiation marker, which is found to associated with 12R-LOX in the study, is essential for the formation of CCE compartment of permeability barrier in stratum corneum. On the inner surface of cell membrane of corneocytes, a protein layer, CE is assembled by accumulation of several precursor proteins including filaggrin, loricrin and Involucrin [213]. Previous studies revealed that these precursor proteins are cross-linked by the action of TGM-1 [214]. Further, TGM-1 is, primarily, reported to cross-link (making covalent

bond) proteins of CE to the hydroxyceramide (which is released or freed by consequence actions of 12R-LOX/eLOX-3 pathway) of CLE making the CCE of permeability barrier in stratum corneum [19][215][25][26]. Formation of CCE, upon which intercellular lipid layer can be formed, is essential to maintain structural integrity of the epidermal water barrier [216]. In addition, mutations in TGM-1 underlie the NBCIE and LI phenotypes of ichthyosis [217][216]. Further, in the present study, an induced expression of TGM-1 under 12R-LOX over expression and *vice-versa* with knockdown of 12R-LOX in HaCaT cells evidently revealed the regulation of TGM-1 expression by 12R-LOX in differentiating keratinocytes.

Previous studies demonstrate the structural role of 12R-LOX as it is involved in oxygenation of linoleate moiety of esterified ceramides to release the hydroxyceramide (with free –OH group) to make the covalent bond with proteins of CE by TGM-1 for the formation of CCE of epidermal barrier [28][64][165]. In addition, the present study markedly showed a regulatory effect of 12R-LOX in the expression of filaggrin and TGM-1 during late terminal differentiation process. Moreover, in keratinocytes of granular layer, oxidation of linoleate moiety of esterified ceramide results in the generation of the 12R-LOX/eLOX3 pathway derived linoleate-related hepoxilins or trioxilin, which are released during hydrolysis of epoxyalcohol derivatives in making of free ω-hydroxyl-ceramides. Hepoxilins and their trioxilin derivatives have been unveiled to play a signaling role in several physiological processes including adipocyte differentiation, inflammation and neurotransmission [59][164]. Additionally, one study also provides the evidence for involvement of LOX-derived epoxyalcohol and their

trioxilin derivatives in the formation of epidermal permeability barrier [27]. Thus, the findings showed a regulatory signaling role of 12R-LOX, which may be mediated by linoleate-related hepoxilins (product of 12R-LOX/eLOX-3 pathway), in the expression of filaggrin and TGM-1 for maintaining the epidermal barrier function of terminally differentiated keratinocytes. In conclusion, 12R-LOX along with its structural role thus plays a crucial role in the signaling mechanisms for expression of permeability barrier components and thus facilitate the barrier function properly. Nevertheless, the intermediate molecules and mechanisms have to be identified for further understanding of the role of 12R-LOX in the maintenance of epidermal barrier function.

Chapter 4

Role of 12R-LOX in proliferation and differentiation of human epidermoid carcinoma cells

4.1. Introduction

In epidermis, the keratinocyte stem cells, beneath stratum basale, proliferate to the basal layer where they undergo for differentiation and transiently migrate into the suprabasal layers towards stratum corneum upon a tightly controlled terminal differentiation program [167]. Interestingly, terminally differentiating keratinocytes display some features that resemble to cellular changes in programmed cell death implying the terminal differentiation as a specialized type of apoptosis in epidermis [218]. In human epidermal keratinocytes, terminal differentiation process involves mitochondria and caspase-dependent cell death pathway [219]. Further, it is very comprehensible that irregular differentiation causes to over proliferation and leads to development of proliferative diseases including psoriasis and skin cancers. In addition, differentiation stage of tumors is an essential feature in the histopathological classification of various cancers. The differentiation phase is associated with tumor behavior, and generally an immature tumor is more aggressive than its more differentiated counterpart.

Several studies unveil that carcinogenesis in a variety of tumors is influenced with irregular expression of LOXs [68][69][176] and tumors of distinct histogenesis show considerably different profiles of AA metabolism [177]. These LOXs play an important role in the modulation of epithelial proliferation and differentiation as well as in wound healing, inflammation, inflammatory skin diseases and cancer [64][106]. Leukotrienes, the downstream metabolites of LOX pathway, have been shown to play a crucial role in inflammatory skin diseases like psoriasis, atopic dermatitis and UV-induced erythema [108][109][110]. In addition, the *p*12-LOX has

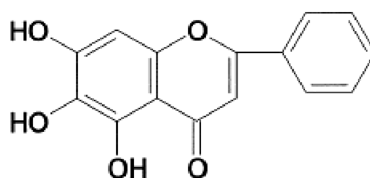
been exhibited to be the prime LOX isoenzyme in human and murine epidermis. Human epidermis displays *p*12-LOX expression in the germinal layer where its overexpression has also been reported in psoriatic skin [113], whereas, the expression of *p*12-LOX exists in both basal and suprabasal germinal epidermal layers of neonatal mouse skin [114]. It is reported that *p*12-LOX is involved in both cancer cell proliferation and survival [220]. Inhibition of *p*12-LOX either with *p*12-LOX inhibitors or a *p*12-LOX antisense oligonucleotides inhibits proliferation and induces apoptosis in cancer cells. Also, adding back the *p*12-LOX metabolite, 12S-HETE prevents *p*12-LOX inhibitor-induced apoptosis [221]. In addition, 12S-HETE has been shown as one of the main eicosanoids formed by the epidermis and, with the discovery of large quantities of 12R-HETE in human psoriatic lesions, the epidermal 12-LOXs have gained considerable interest [222]. Expression of *p*12-LOX is also correlated with tumor cell metastasis. In metastatic prostate cancers, *p*12-LOX expression level is higher than non metastatic ones. Clinically, the degree of *p*12-LOX expression in human prostate cancer correlates with the tumor grade and stage [69].

In normal epidermis, the expression pattern and spatial and temporal co-localization of 12R-LOX and eLOX-3 is found in nucleated stratum granulosum of upper epidermis indicating differentiation dependent expression of both enzymes [64]. Moreover, our previous study also reveals the pathological role of 12R-LOX in the growth of epidermoid carcinoma cells, A431 [128]. The A431 cells have originated from tumor of stratum spinosum layer of epidermis [178] and this implies the improper differentiation of keratinocytes which lead to tumorogenesis.

Thus, studying the signaling mechanism and regulatory pathway of 12R-LOX during the process of keratinocyte proliferation and differentiation can lead to understanding of the pathomechanism of skin diseases like psoriasis, epidermal cancers and ichthyoses and potentially in the treatment of these diseases [165]. Further, in proliferative disorders like psoriasis and skin cancer, inhibition of these LOX pathways can control the growth of proliferative tissues, giving a therapeutic prospectus.

Baicalein

The IUPAC name of baicalein is 5, 6, 7-Trihydroxyflavone. Baicalein is a key flavonoid from *Scutellaria baicalensis*, the herb which has been extensively used in Chinese herbal medicine. It has been known that baicalein is a specific inhibitor of *p*12S-LOX with an IC_{50} value of 0.12µM. Previous studies showed the various biological effects of baicalein including a role in anti-inflammation, antiviral, anti-hepatotoxicity and anti-tumor [223][224][225][226]. It was shown that baicalein causes an S-phase arrest in cell cycle and induces apoptosis in many human cancer cell lines e.g. lung squamous carcinoma cells, CH27 [227], hepatoma cells, Hep3B [228], prostate cancer cell lines, LNCaP and JCA-1 [229], pancreatic cancer cells, MiaPaCa-2 and AsPC-1 [230], breast cancer cells- MCF-7 [231].



Baicalein

In the present study, we employed epidermoid carcinoma cells, A431 (dedifferentiated and showing a higher expression of 12R-LOX) for inhibition/knockdown of 12R-LOX with baicalein/12R-LOX siRNA to understand the role of 12R-LOX in proliferation and differentiation of human epidermoid carcinoma cells.

4.2. Materials and Methods

4.2.1. Chemicals and Reagents

All chemicals and solvents for chromatography were of HPLC grade and purchased from Merck. Baicalein, arachidonic acid methyl ester and LOXs metabolites used as standards (5-HETE and 15-HETE) in the study were purchased from Cayman Chemicals Co. Inc, (U.S.A.). Propidium Iodide (PI) was obtained from Gibco and Invitrogen Life Technologies (Carlsbad, CA, U.S.A). All the other chemicals and reagents for the experiments were obtained as described before in previous chapters.

4.2.2. Cell culture studies

A431 cells were grown and maintained in 90% DMEM supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37^0C. For 12R-LOX activity assay, A431 cells were grown up to 90% confluency in 100 mm tissue culture plates and a huge pellet of these cells was collected from a number of culture plates to prepare the whole cell extract.

4.2.3. 12R-LOX enzyme activity assay on High pressure liquid chromatography (HPLC)

The whole cell extract of A431 cells (to examine the activity of 12R-LOX present in A431 cells) or bacterial cell lysate (to analyze the 12R-LOX activity inhibition with baicalein) was added to 1X phosphate buffered saline (pH 7.4) making the final volume to 500µl with methylated AA (met-AA) or free AA as the substrate at 0.1mM final concentration and then reaction was vortexed and incubated at 37^{0}C for 15 min. After the incubation, a 100µl of saturated solution of sodium borohydride was added to the reaction to reduce the hydroperoxy products, formed in the reaction, to its hydroxy derivatives and incubated for another 5 min. After 5 min, the reaction was stopped by acidification to pH 3 using 50 µl of acetic acid and then the proteins were precipitated with the addition of 0.5 ml of ice cold methanol. The total mixture was then centrifuged (14000 rpm for 15 min) to remove the precipitated proteins and the clear supernatant was analysed on Reverse phase HPLC (RP-HPLC). High-pressure liquid chromatography was performed on a Shimadzu system equipped with Hewlett-Packard diode array detector 1040A and HP-Chemstation program. The solvents, methanol: water: acetic acid (ratio-73: 27: 0.1) were used for RP-HPLC run at a flow rate of 1 ml/min. The chromatograms were followed by monitoring the absorbance for 12R-HETE-Me at 235nm. Free or Methylated 15-HETE and 5-HETE (15-HETE, 5-HETE, 15-metHETE and 5-metHETE) were used as standards. Methylation of HETE products was achieved by incubating HETEs with ice cold diazomethane for 30 min at 4^{0}C, evaporating and finally dissolving in HPLC solvent. For 12R-LOX activity inhibition study, different concentrations of baicalein (made from the stock solution of

baicalein, 100mM which was achieved by dissolving 2.5mg of baicalein in 100µl of DMSO) were added to the reactions and products were analyzed on RP-HPLC.

4.2.4. Baicalein preparation and cell viability assay (MTT assay)

The stock solution of baicalein, 100mM, was used in the study. The experimental concentrations were prepared by serial dilutions in DMEM media. Final concentration of vehicle in the cells did not exceed 0.01 %. Cell viability was determined by MTT assay which is a colorimetric assay based on the ability of viable cells to change from soluble yellow tetrazolium salt to blue formazan crystals. Cells at 4×10^3 cells per well were seeded in 96 well plates and incubated in the presence or absence of various concentrations of baicalein for 48 h in a final volume of 100µl with DMEM media. After treatments, 20µl of MTT solution (5mg/ml in PBS) was then added to each well and incubated for an additional 3 hrs at 37^0C. The purple-blue formazan crystals were dissolved in 100µl of DMSO and the optical density was quantified at 570 nm on µ Quant Bio-tek Instruments, Inc. Micro titer plate reader. Activity of the mitochondrial energy metabolism was shown as cell viability (% of control) and the percentage inhibition of cell viability was calculated as a fraction of control. Each concentration was tested in triplicates.

4.2.5. 12R-LOX gene knockdown in A431 cells

The gene knockdown was carried as per the manufacture's protocol (IDT, U.S.A.). In 60 mm tissue culture plate, 1.5×10^6 cells (A431) per plate were taken in 4ml antibiotic-free normal growth medium supplemented with FBS. The cells were incubated at 37^0C in a CO_2 incubator until the cells are 60-80% confluent.

Cells were pelted down and washed with 2ml of PBS. For 12R-LOX gene knockdown, the cells were transfected with or without validated 12R-LOX siRNA at a final concentration of 10nM, and Lipofectamine 2000 (1: 2.5) mixture in antibiotic-free and FBS-free medium. After transfection, the cells were incubated at 37^0C in a CO_2 incubator for 24, 48, 72 and 96 h following repeated transfection of 12R-LOX siRNA at 48 h intervals. Untransfected and Universal negative control1 siRNA transfected cells served as controls.

4.2.6. Cell cycle analysis by flow cytometry

A431 cells were treated with baicalein (with IC_{50} conc.) or transfected with 12R-LOX siRNA (10nM conc.) for 24, 48, 72 and 96 h and washed with PBS, trypsinized with 0.5% Trypsin-EDTA and then were centrifuged at 2000 rpm for 2 min. Cells were then washed twice with PBS and fixed with 70% chilled ethanol overnight. After washing with PBS cells were incubated with RNAase A (0.1 mg/ml), stained with propidium iodide (PI) (50 mg/ml) and resuspended in 100µl of sheath fluid. Flow cytometric analyses were performed by using a program Cell Quest of FACS Calibur (Becton Dickinson, CA, U.S.A.). The fluorescence intensity of PI was detected with FL2-H detector.

4.2.7. Western blot Analysis

For baicalein (a 12R-LOX inhibitor) inhibition studies, A431cells were seeded in 100 mm tissue culture plates and after achieving 60-70% confluency, cells were treated with baicalein at a final concentration of 40µM (IC_{50} = 100µM) for 3, 6 and 9 days. To confirm the knocking down of 12R-LOX with 12R-LOX siRNA, cells were seeded in 100 mm tissue culture plates and transfected with

12R-LOX siRNA as described above for 48, 72 and 96 h. After harvesting the treated cells, the whole cell extracts were prepared and protein contents were determined as described in previous chapters for protein analysis. An equal amount of total cell lysate was resolved on 8-12% SDS-PAGE gels along with protein molecular weight standards, and then transferred onto Nitrocellulose membranes. The membranes were blocked with 5% w/v non-fat dry milk and then incubated with the primary antibodies against- human 12R-LOX, cytokeratin1, filaggrin, TGM-1, involucrin and β-actin in 10 ml of antibody-dilution buffer (1:1000) (Tris buffered saline and 0.05% Tween-20 with 1% milk) with gentle shaking at 4^0C for 8–12 h and then incubated with respective HRP-conjugated secondary antibodies. Signals were detected using western blot detection reagent (ECL kit). The band intensities were measured by Image J software.

4.2.8. RNA isolation and Real time PCR

Total RNA was isolated from 12R-LOX siRNA transfected A431 cells using TRizol according to manufacturer's instructions. Reverse transcription of 2μg of total RNA isolated was achieved by the protocol as previously described. The PCR reactions were performed using 2μl of the product from the RT reaction and primers (*ALOX12B* sense 5'-CCA CTG GCG AGT CTT TCT TCC-3' and antisense 5'-GTC TGT GAC GGG GAA CTT GT-3') as described in chapter 1. The PCR products were visualized on 1% agarose gels under UV light. The GAPDH primers served as control. For real time PCR quantisation, cDNA equivalent to 10ng total RNA were used and reactions were carried out as described in previous chapter.

4.2.9. Statistical analysis

All the data analysis was done using the Sigma Plot 10 software. Data were presented as the Mean ± S. E. M. of three independent experiments and statistical analysis was carried out by Student's t test using Excel (Microsoft). A p-value of less than 0.05 was considered as statistically significant over control.

4.3. Results

4.3.1. Activity identification of 12R-LOX present in A431 cell lysate

RP-HPLC analysis of the incubation of the whole cell extract with methyl ester of arachidonic acid (met-AA) showed the peak of methyl ester of 12R-HETE (12-metHETE) formation at 235 nm revealing the 12R-LOX activity in A431 cell extract. In RP-HPLC run, the reaction product peak was separated at the retention time, ~42 min prior to the 5-metHETE peak (retention time, ~47 min) which was used as standard. The retention time of product peak (~42 min) revealed the peak as 12-metHETE when compared with standards peaks (15-metHETE, ~40 min and 5-metHETE, ~47 min). The pattern of HPLC chromatograms including RTs, for both, standard (Figure 26A) and experimental (where methyl ester of AA was incubated with A431 cell extract) (Figure 26B), were similar. Further, confirmation of the peaks was based on the co-chromatography of the standard and sample peaks and GC-MS analysis (data not shown).

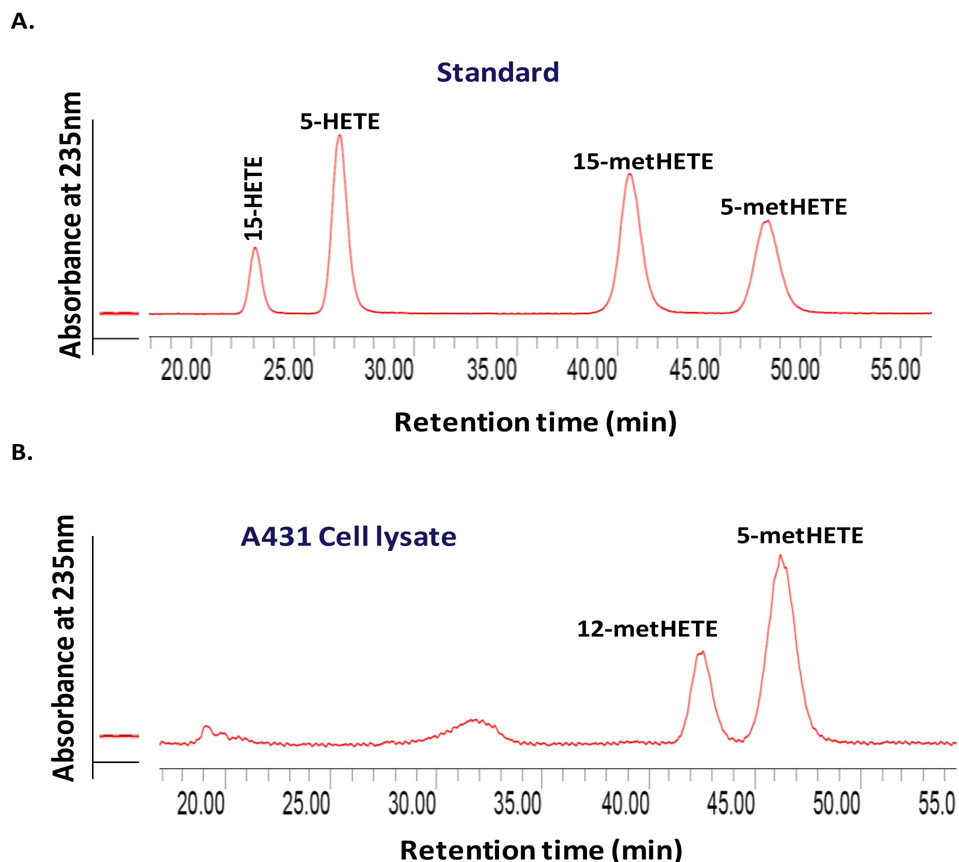


Figure 26: HPLC analysis of the reaction product formed from substrate by human 12R-LOX present in A431 cell extract. HPLC chromatogram analyses of ***A)*** *only standards run* ***B)*** *reaction products run along with one standard (when A431 cell extract was incubated with methyl ester of AA). Note that 5-metHETE standard peak follow the same RT in both chromatograms.*

4.3.2. Effect of baicalein on 12R-LOX activity

For *in-vitro* inhibitory assay of 12R-LOX with baicalein, enzyme reactions were performed using bacterial cell lysate (expressing recombinant human 12R-LOX enzyme) with or without (control) different concentrations of baicalein (0.4, 4, 40 and 400µM). 100mM stock solution (in DMSO) of baicalein was used for serial dilution to make different concentrations. Only DMSO in reaction was used as vehicle control. RP-HPLC analysis of the reactions revealed the inhibition of 12R-LOX with baicalein as absorbance of 12R-HETE peak of the reaction was

significantly reduced in the presence of baicalein at 235 nm. In RP-HPLC analysis, a concentration dependent inhibition of 12R-LOX with baicalein was observed (Figure 27A). A 50 % decrease in 12R-LOX enzyme activity was perceived at about 6.5μM (IC_{50}) (Figure 27B).

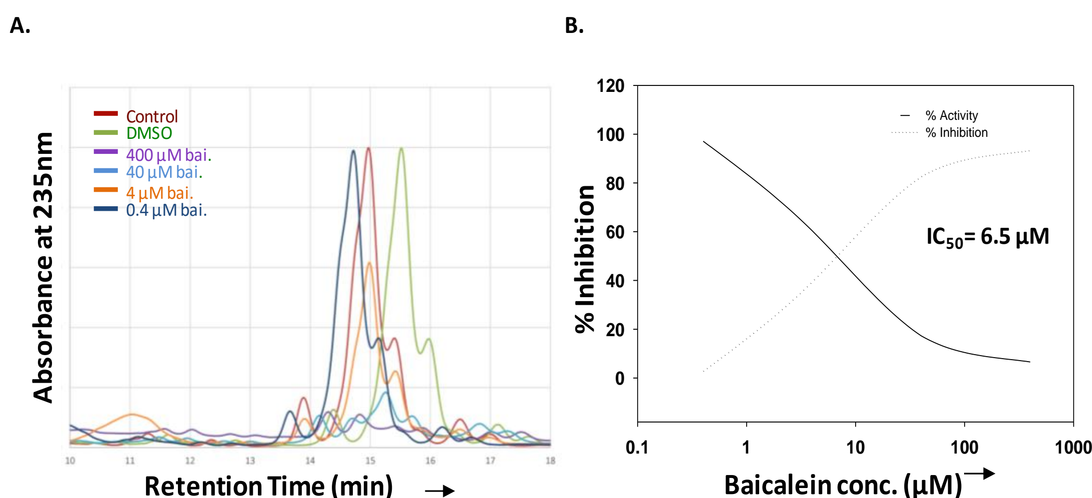


Figure 27: Baicalein inhibit the activity of human 12R-LOX. ***A)*** *RP-HPLC chromatogram analysis of the reaction product, 12R-HETE formed from AA by human 12R-LOX (in bacterial cell lysate) in the absence (control) or presence of different concentrations (0.4, 4, 40 and 400μM) of baicalein.* ***B)*** *The area of the peak was measured and the percentage of activity inhibition was plotted with baicalein concentrations to calculate the IC_{50} value. The values represent the mean ±SD of three independent experiments.*

4.3.3. Effect of baicalein on proliferation of A431 cells (MTT assay)

Role of 12R LOX on keratinocyte differentiation was also studied by inhibiting 12R LOX in A431 cells (over expressing 12R LOX) with baicalein. A431 cells were treated with different doses of baicalein (0.1, 1, 10, 100 and 1000 μM) for 48 h and cell viability was determined by the MTT assay. Under these conditions, a dose-dependent decrease in the cell viability was observed in A431 cells. A 50 % decrease in cell growth was observed at about 100μM (GI_{50}) baicalein (Figure 28) following 48 h of treatment. Further experiments for differentiation

studies were carried out on A431 cells at lower (40µM) to GI_{50} concentration as 50% inhibition of A431 cells proliferation was observed at this concentration.

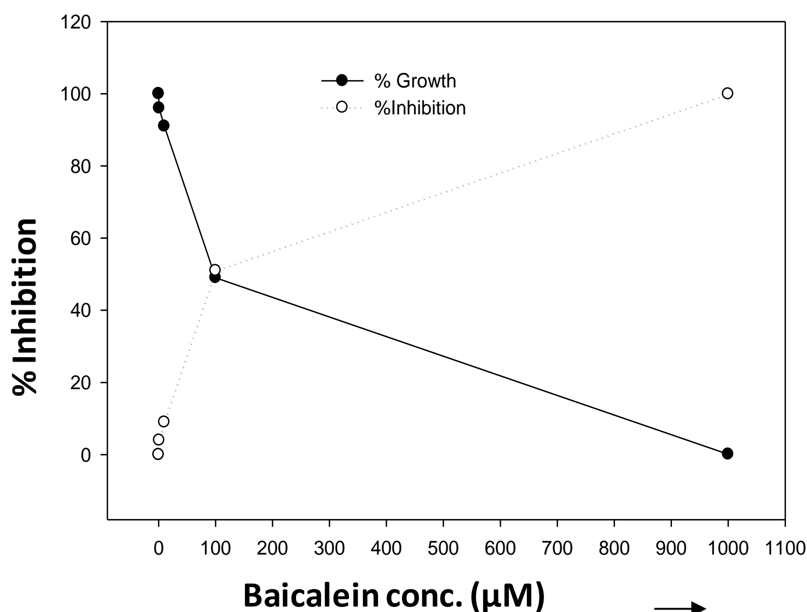


Figure 28: Effect of baicalein on the proliferation of A431 cells. A431 cells were treated with different concentrations (0.1, 1, 10, 100, and 100 µM) of baicalein for 48 h. The percentage of cell viability with treatment was examined by MTT assay. The number of cells in the control was taken as 100%. A significant dose dependent growth inhibition was observed in treated cells in comparison with untreated control cells. The values represent the mean ±SD of three independent experiments.

4.3.4. Inhibition of 12R-LOX with baicalein elicited in G1 phase cell cycle arrest prior to terminal differentiation

Induction of differentiation in baicalein treated A431 cells was quantified by flowcytometric analysis of DNA content. The proliferative cells show variation in its DNA quantity at different stages of cell cycle (G1<S<G2/M). So cells, when, are arrested in G0/G1 phase of cell cycle (switched for terminal differentiation), have less DNA. The P1 represents the cells before G0/G1 peak, indicating the cells that are undergoing cell death and thus having less DNA (hypodiploid) than G0/G1 phase cells. As shown in the figure 29, there is clear increase in number of cells in

G1 phase and before G1 peak (hypodiploid cells) when cells were treated with baicalein (40µM) compared to untreated cells. Increase in hypodiploid cells and G1 phase cells demonstrate that baicalein treatment arrested the cells in G1 stage of cell cycle and also switched terminal differentiation.

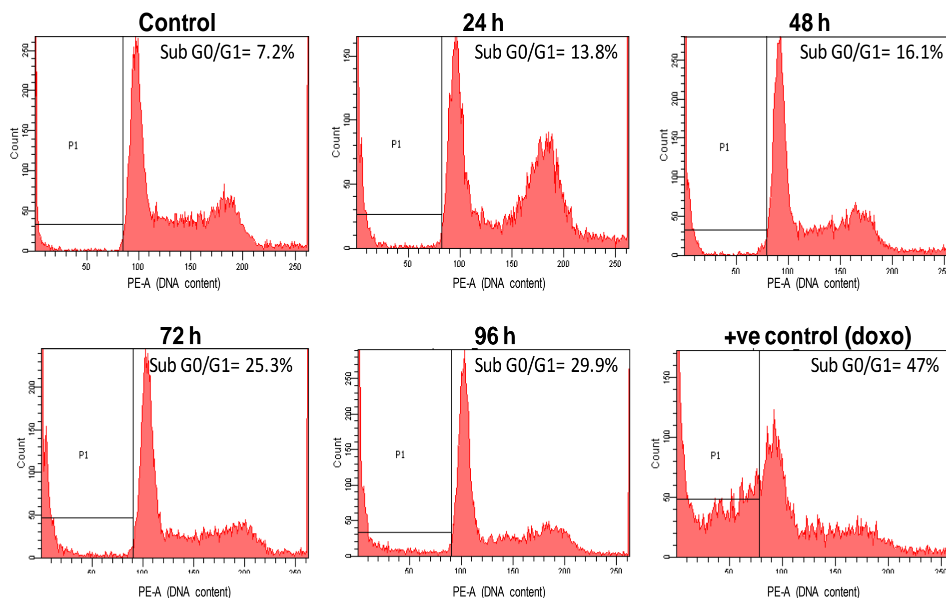


Figure 29: Effect of baicalein on the cell cycle of A431 cells (Cell cycle analysis). Cells were treated with medium alone (control), or with baicalein (40 µM) for 24, 48, 72 and 96 h, fixed and stained with propidium iodide solution for 1 h and analyzed for DNA content by flow cytometry. The number of cells in hypo diploid (subG0/G1) phase is expressed as a percentage of the total number of cells. Data represent the results from one of three similar experiments.

4.3.5. 12R-LOX inhibition with baicalein affects expression of late differentiation markers like TGM-1 and filaggrin

The keratinocyte differentiation markers increase with the stage of terminal differentiation. At the end of differentiation these markers are highly expressed in the cells of stratum granulosum and stratum corneum (upper most layers). A431 cells treated with baicalein (40µM) showed a time dependent increase in expression of involucrin and K1 but decrease in expression of filaggrin

and TGM-1 until 9 days (Figure 30). Untreated cells showed no effect on expression of filaggrin and TGM-1. From the results it is cleared that 12R-LOX inhibition with baicalein affects the expression of TGM-1 and filaggrin even though differentiation has been progressed (increased involucrin and K1).

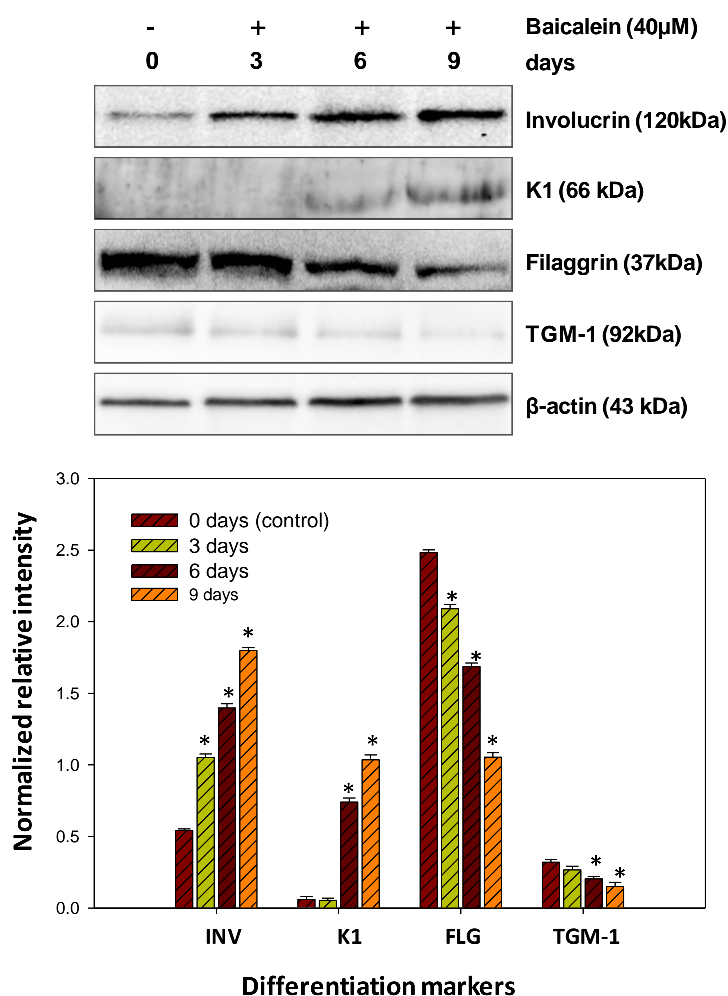


*Figure 30: 12R-LOX inhibition with baicalein affects expression of late differentiation markers like TGM-1 and filaggrin. Expression of keratinocyte differentiation markers-involucrin, K1, filaggrin & TGM-1 after treatment with baicalein, 12R-LOX inhibitor. Figure showing immunoblot analysis of protein extracts from A431 cells treated with baicalein (40µM) for 3, 6 & 9 days with antibodies for involucrin, K1, filaggrin & TGM-1. β-actin was used as an internal loading control. The relative band intensities were measured by quantitative scanning densitometry, bars indicate the mean ± S. D. (n = 3); *P < 0.05 compared with control cells.*

4.3.6. 12R-LOX knockdown with 12R-LOX siRNA results in G1 cell cycle arrest: Cell cycle analysis

Knockdown of 12R-LOX was also achieved by introduction of 12R-LOX siRNA in A431 cells. RT-PCR and immunoblot studies confirmed a remarkable decrease or no expression of 12R-LOX in cells introduced with 12R-LOX siRNA (10 nM) (Figure 31). Number of cells in G0/G1 phase increases with terminal differentiation. Induction of differentiation in 12R-LOX siRNA treated A431 cells was quantified by flowcytometric analysis of DNA content. X axis represents FL2-H, detector for propidium iodide (PI) a DNA binding dye, in the flow cytometer. Y axis represents cell counts, showing number of cells at particular FL2-H. The proportion of fluorescence emission of PI is based on quantity of DNA in the cell. Cells when arrested in G0/G1 phase of cell cycle (switched for terminal differentiation), are having less DNA. In the figure 31, we can clearly observe, the increase in number of cells in G1 phase and before G1 peak (hypodiploid cells) with 12R-LOX siRNA (10 nM) compared to control scrambled siRNA. These results indicate that transfection of 12R-LOX siRNA in A431 cells arrested the cells in G1 stage of cell cycle for induction of terminal differentiation.

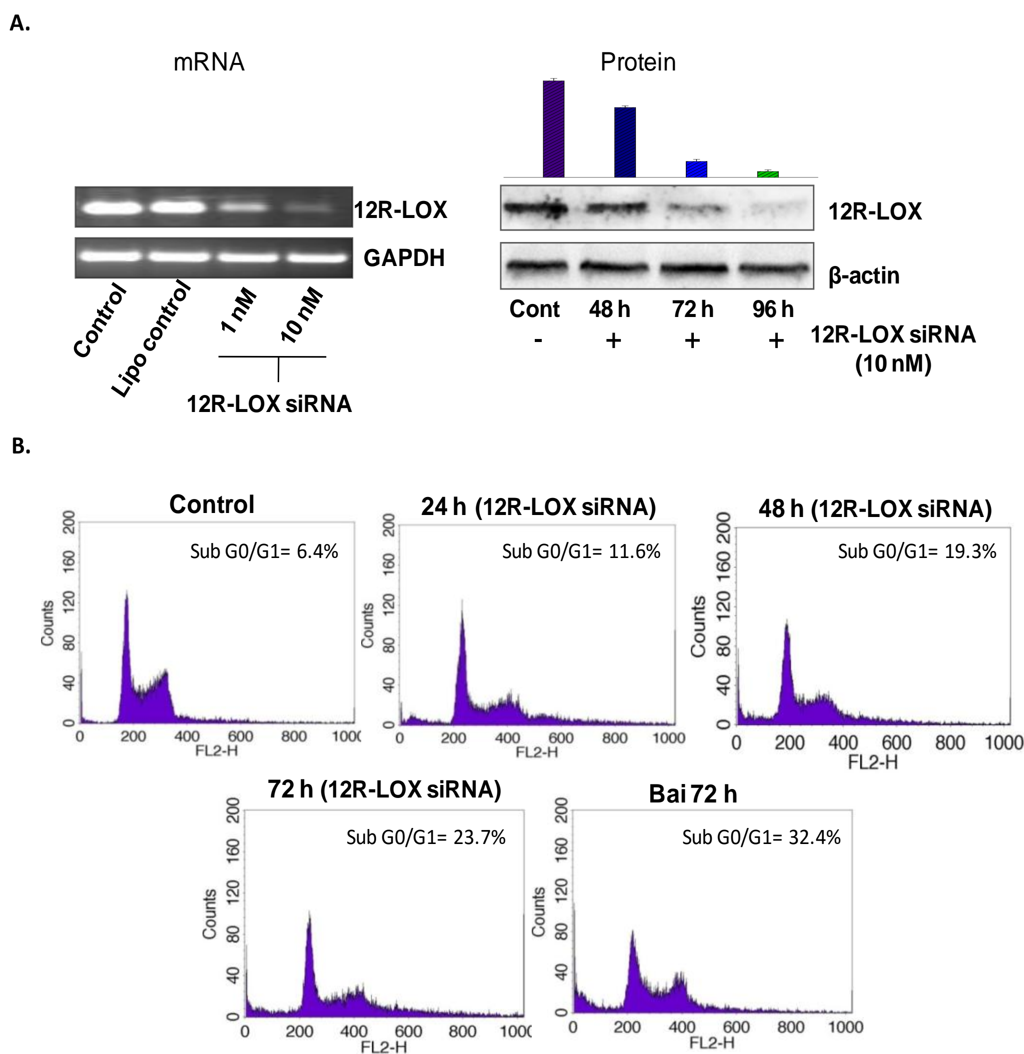


Figure 31: 12R-LOX knockdown results in G1 cell cycle arrest. Effect of inhibition of 12R-LOX by siRNA on cell cycle of A431 cells. ***A)*** *RT-PCR and western blot analysis of mRNA and protein extracts respectively from A431 cells introduced with either control scrambled siRNA or 12R-LOX siRNA with 1 nM and/or 10 nM concentrations for 48, 72, 96 h for confirmation of knockdown of 12R-LOX expression. GAPDH and β-actin were used as an internal loading control respectively.* ***B)*** *Flow cytometric analysis of cell cycle of A431 cells introduced with either control scrambled siRNA or 12R-LOX siRNA (10 nM) for 24, 48 and72 h. cells were fixed and stained with propidium iodide solution for 1 h and analyzed for DNA content by FACS. The number of cells in hypo diploid (subG0/G1) phase is expressed as a percentage of the total number of cells. Data represent the result from one of three similar experiments.*

4.3.7. 12R-LOX knockdown in A431cells is associated with remarkable reduced expression of TGM-1 and filaggrin

To confirm a regulatory activity of 12R-LOX on the expression of late differentiation markers- TGM-1 and filaggrin, A431 cells (showed higher level of

12R-LOX and filaggrin) were introduced with either control scrambled siRNA or 12R-LOX siRNA (10 nM) for 72 and 96 h according to manufacturer's instructions. Real time PCR was used to analyze the transcript levels of the differentiation markers. A time dependent reduction in mRNA transcript levels of TGM-1 and filaggrin was observed in response to 12R-LOX knock down compared with control scrambled siRNA. In contrast, study with involucrin indicated an elevation in its expression, may be due to a significant cell death upon introduction of siRNA for 96 h, where as 12R-LOX knock down showed no or little change on transcript level of early differentiation marker- K1 following 96 h post treatment (Figure 32).

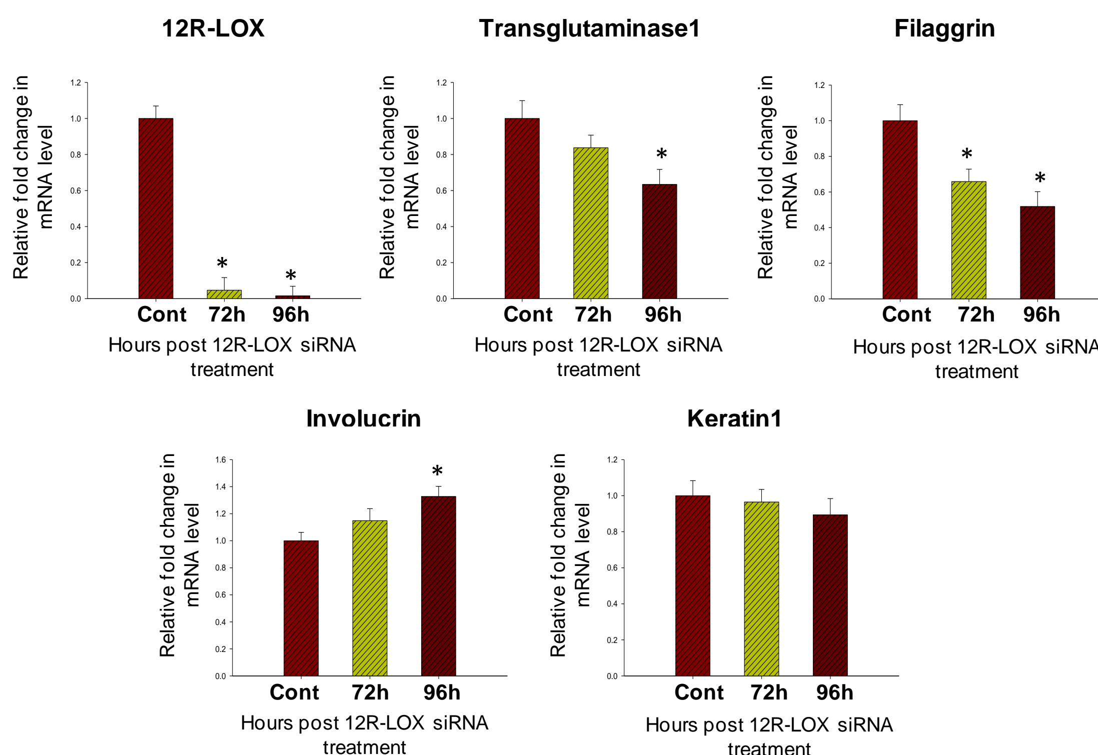


Figure 32: 12R-LOX knockdown in A431cells is associated with remarkable reduction in expression of TGM-1 and filaggrin. Real-time RT-PCR analysis to examine the relative mRNA expression of keratinocyte differentiation markers- TGM-1, filaggrin, involucrin, K1 and 12R-LOX in A431cells following treatment either with control scrambled siRNA or 12R-LOX siRNA (10 nM) for 72 h and 96 h. Real-time RT-PCR was performed with total mRNA isolated from cells post treatment. Bar graph representing the fold changes of mRNA levels quantified by normalization to the GAPDH as an internal loading control.

*Data are expressed as the mean ±SD of three independent experiments. * denote P < 0.05 compared with control cells.*

4.4. Discussion

Epidermis, a continuously renewing multilayered epithelium from cell birth to cell death, is formed as a consequence of well organized process of terminal differentiation. However, an irregular differentiation can leads to over proliferation of keratinocytes in epidermis and thus cause to development of proliferative diseases like psoriasis and skin cancers. Studies suggested that in the course of this tumorigenesis or carcinogenesis, a variety of tumors are influenced with irregular expression of LOXs [68][69][176]. These LOXs which can modulate the epithelial proliferation and differentiation, play an important role in development during wound healing, inflammation, inflammatory skin diseases like psoriasis and cancer [64][106]. Interestingly, when coming to the expression pattern of these LOXs during terminal differentiation in normal epidermis, the expression pattern and spatial and temporal co-localization of 12R-LOX and eLOX-3 is found in nucleated stratum granulosum of upper epidermis indicating differentiation dependent expression of both enzymes [64]. Further, in humans, the 12R-LOX was pathologically expressed during the psoriasis and other proliferative skin dermatoses [232][155]. Earlier studies also reveal the pathological role of 12R-LOX in the growth of epidermoid carcinoma cells, A431 [128]. It is praiseworthy to know that the A431 cells have originated from tumor of stratum spinosum layer of epidermis [178] and so, this implies the improper differentiation of keratinocytes which lead to tumorogenesis. Thus, studying the regulatory signaling role of 12R-LOX during the process of keratinocyte proliferation and differentiation can provide

vital information to understand the potential pathomechanisms of skin diseases like psoriasis, epidermal cancers and ichthyoses and thus in treatment of these diseases [165]. In the view of this, proliferative disorders like psoriasis and skin cancer can be controlled by inhibition of these LOX pathways and thus, a new therapeutic prospectus can be provided. Therefore, in the present study, we performed inhibition of 12R-LOX either with baicalein or 12R-LOX siRNA in A431 cells to understand the role of 12R-LOX in proliferation and differentiation.

In human epidermoid carcinoma A431 cells, identification of an endogenous inhibitor of arachidonate metabolism has been reported. Phospholipid hydroperoxide glutathione peroxidase (PHGPx) plays functional role in down regulating the arachidonate metabolism catalyzed by 12S-LOX and COX-1 in A431 cells [233]. So, the inhibitory effect of baicalein (inhibitor of 12-LOXs) in A431 cells can be attributed to inhibition of 12R-LOX, another form of 12-LOX present in epidermis, rendering baicalein as a selective inhibitor of 12R-LOX in A431 cell model. Nevertheless, for inhibition/knockdown study in A431 cells, it is essential to know the presence of an active 12R-LOX first. 12R-LOX activity identification, in the present study, is achieved by incubation of whole cell extracts with methyl ester of AA (met-AA) and analysis of the products on RP-HPLC. This active 12R-LOX in A431 cells, thus, may have a primary role in tumorigenesis and dedifferentiation. In order to understand this role of 12R-LOX in proliferation and differentiation of keratinocytes, inhibition of 12R-LOX was performed by 12R-LOX siRNA or baicalein. Further, an *in-vitro* inhibitory assay showed the inhibition of 12R-LOX activity with baicalein at IC_{50} value of 6.5μM. (Figure 27) which was achieved in A431 cells (*in-*

vivo) at 100µM revealing a remarkable inhibition of the enzyme in both systems. This is the first study reporting baicalein as an *in-vitro* inhibitor of 12R-LOX.

Keratinocyte terminal differentiation (denucleation), a specialized type of cell death, is the process in which cells lose their nuclei but remain functional and where total destruction and removal of dead cells (corneocytes) is not desirable, as they fulfill a specific function such as formation of the skin barrier provided by corneocytes [219]. Human keratinocytes suppress DNA replication and cell cycle arrest in G0/G1 before they initiate terminal differentiation and the number of cells in G0/G1 phase increases with differentiation [219]. Also during 3T3-L1 cell differentiation, control of the cell cycle arrest is not effected with pocket proteins [234] and cell fate decisions are tightly associated with the cell-cycle machinery and the mechanisms synchronizing differentiation and proliferation in developing tissues [235]. Our previous study also shows a cell cycle arrest and growth inhibition in A431 cells when treated with baicalein [128]. Further, in this study, the increase in hypodiploid cells and G1 phase cells after treatment either with baicalein or 12R-LOX siRNA evidently indicates arrest of the cells in G1 stage of cell cycle and so, switches to terminal differentiation.

Thus inhibition of the LOX pathway, which may be improperly regulated during differentiation and thus lead to dedifferentiation and tumorogenesis of the cells, can lead to proper differentiation of A431 cells. In chapter 1, the study based on evaluation of differentiation state of A431 cells showed the loss of expression of early differentiation marker K1 in A431 cells indicating, so, the irregular differentiation as in the proliferative keratinocytes of upper epidermal layers of

psoriatic scales [187]. However, in the present study, a much less or no expression of early differentiation markers like K1, K5 and K14 with the higher expression of late differentiation markers like involucrin and filaggrin suggest the dedifferentiation in A431 cells. Further, the stage of a terminally differentiating keratinocyte can be explained by knowing the expression level of keratinocyte differentiation markers like K1 (early differentiation marker), involucrin, filaggrin and TGM-1 (late differentiation markers). These differentiation markers follow an increasing prototype of expression during terminal differentiation of keratinocytes with highest expression in the cells of stratum granulosum and stratum corneum [168][171][172][173]. An elevated expression of K1 and involucrin in A431 cells upon 12R-LOX inhibition with baicalein clearly indicates restoration of proper differentiation which had been deregulated towards tumorigenesis. Moreover, this study showed that 12R-LOX inhibition with baicalein or knockdown with 12R-LOX siRNA reduces the expression of TGM-1 and filaggrin even though differentiation has been proceeded (increased involucrin). The late differentiation markers, filaggrin and TGM-1 are the major components of two barrier compartments and are very important for the formation of permeability barrier. TGM-1 is essential for the formation of CCE compartment of permeability barrier in stratum corneum[214]. On the other hand, filaggrin aggregates with keratin filaments of cytoskeleton to make macrofibrils crisscrossing the cornified cells of stratum corneum [210]. This collapsed macrofibrils is cross-linked to CLE by TGM-1. Thus, along with these keratohyalin granules, filaggrin serves as an integral part of protein layer of CE beneath the lipid layer, CLE which contributes to structural integrity of corneocytes[213]. Thus, it is also clear from the depleted expression of TGM-1 and

filaggrin with 12R-LOX inhibition/knock down that 12R-LOX regulates the expression of late differentiation markers- TGM-1 and filaggrin to facilitate the permeability barrier formation and perform the terminal differentiation process in keratinocytes efficiently. However, the intermediate molecules and mechanisms involved in this process, need to be identified. This study forms the first report showing a functional linkage between 12R-LOX expression and the components of late differentiation process. In conclusion, 12R-LOX along with its structural role plays a crucial role in the signaling mechanisms for permeability barrier components expression and to facilitate the barrier function properly.

Chapter 5

Summary and Conclusions

The human skin, the largest tissue in the body, performs several major functions and one necessary role is to accomplish the epidermal water barrier function [6]. The lipid composition and deposition in outer layers of skin, stratum corneum, is the important factor of this impermeability property of the skin [10]. Studies show special requirement of EFAs such as linoleate (C18:2) to maintain and process the water impermeable barrier of epidermis [23]. Linoleate is being primarily incorporated in a specific class of ceramides, esterified ω-hydroxyacyl-sphingosine (EOS), unique to the epidermis [24]. EOS is further converted to ω-hydroxyacyl-ceramides which covalently attaches to the proteins of CE and constitute a CLE underlying CE to make essential structural integrity of the epidermal water barrier [19][25][26] (Figure 11).

Substantial evidence reveals the involvement of LOX enzymes in the oxidation of linoleate moiety of esterified structural lipids such as ceramides to form oxygenated derivatives and to provide ω-hydroxyacyl-sphingosine (OS) with free ω-hydroxyl which makes CLE of permeability barrier [27][28]. An important breakthrough in understanding the role of LOX enzymes in epidermis was accomplished by a genetic study in 2002 [190]. The study reported, for the first time, linkage between mutations in coding region of LOX genes, *ALOX12B* (12R-LOX) and *ALOXE3* (eLOX-3) on chromosome 17p13.1 and development of a disease and this loss of function mutations in LOX genes is linked to the genesis of autosomal recessive congenital ichthyosis (ARCI). ARCI disease is associated with defective skin barrier function. Mutations in one or other LOX genes were found to result in similar phenotype and it is speculated that both enzymes belong to same metabolic pathway involved in epidermal differentiation [190]. Further,

differentiation dependent tissue expression of 12R-LOX and eLOX-3 in epithelial tissues indicates a pivotal physiological role of 12R-LOX and eLOX-3 in skin barrier function [64]. In addition, the important role of 12R-LOX/eLOX-3 pathway on skin barrier function is supported by mouse knockout studies where targeted disruption of both LOX genes in mice resulted in neonatal death due to severely impaired permeability barrier function [153][159]. Moreover, CCE from skin of 12R-LOX-deficient mice showed increased fragility with a disordered composition of ceramides, especially a decrease of ester-bound ceramide species, revealing a structural role of 12R-LOX/eLOX-3 pathway in epidermal barrier function.

From the recent study, structural role of the 12R-LOX/eLOX-3 pathway is also suggested through the processing of ω-hydroxy acylceramides in the making of CLE [28]. It is suggested that subsequently hydrolysis of epoxyalcohol derivative of EOS generates hepoxilins like derivatives, trioxilins and free ω-hydroxyl on ceramide (ω- OH Cer) and the LOX-catalyzed oxygenation is a prerequisite to facilitate the hydrolysis of the oxidized linoleate moiety resulting in a free ω-hydroxyl group on the ceramides. This free ω-hydroxyl group on the ceramides is required for coupling to the cross-linked proteins of the CE [129][162]. Further, hepoxilins and their trioxilin derivatives have been unveiled to play a signaling role in several physiological processes including adipocyte differentiation, inflammation and neurotransmission [59][164]. Thus, the 12R-LOX/eLOX3 pathway derived linoleate-related hepoxilins or trioxilin, which are released during hydrolysis of epoxyalcohol derivatives in making of free ω-hydroxyl-ceramides, might exert a signaling function in several processes related to components of the epidermal permeability barrier function of keratinocytes [165].

This is also evidenced by the study indicating a crucial role of LOX-derived epoxyalcohol and their trioxilin derivatives in the formation of epidermal permeability barrier [27]. In contrast to the essential role played by LOXs in epidermal barrier function, the 12R-LOX was pathologically expressed during the psoriasis and other proliferative skin dermatoses [232][155]. These divergent roles of 12R-LOX point to the importance of the studies on its regulation in physiological and pathological process. Studying regulatory pathway of 12R-LOX during keratinocyte differentiation process may provide mechanisms underlying the skin diseases like psoriasis and epidermal cancers. Also, it is worth to know whether 12R-LOX/eLOX-3 pathway may have a role in the signaling mechanisms for terminal differentiation of keratinocytes. The present study was designed to elucidate the signaling role of 12R-LOX in proliferation and terminal differentiation of human keratinocytes.

Analysis of LOXs/COXs and differentiation markers expression unveils the dedifferentiated state of A431 cells and properly differentiated state of HaCaT cells

In this study, the expression profile of the LOXs/COXs and differentiation markers in both cell lines were analyzed before using them as models for differentiation. The progression of a variety of tumors is influenced with unbalanced expression of LOXs/COXs suggesting a role of eicosanoids in carcinogenesis [68][69] and tumors of distinct histogenesis show significantly different profiles of AA metabolism [177]. In addition, our earlier studies demonstrate the pathological role of 12R-LOX in the growth of epidermoid carcinoma cells, A431 [128] which have originated from tumors of spinous layer of epidermis [178], indicating improper differentiation of keratinocytes during

tumorigenesis. Further, the role of LOXs/COXs in immortalization of HaCaT cells is not known. Thus, the evaluation of expression profile of AA metabolizing enzymes and differentiation markers in A431 (epidermoid carcinoma cells) and HaCaT (normal human keratinocytes) cells may provide clues on the role of LOXs in differentiation or tumorigenesis.

In the present study, the predominant expression of LOX multiforms and COXs in human epidermoid carcinoma cell line, A431 suggests a significant role for AA metabolizing enzymes in regulating the growth and tumorigenesis of A431 cells. Whereas, a very low or no expression of LOXs/COXs in normal human keratinocyte cell line, HaCaT, reveal the immortalization of HaCaT cells without prompting any tumorigenesis. Further, HaCaT cells demonstrated a detectable level of expression of 12R-LOX, which is a feature of normal keratinocytes, showing the unaltered property of these cells as normal keratinocytes for the expression of 12R-LOX. So, these HaCaT cells can be used to analyze the physiological role of 12R-LOX in keratinocytes. Despites its origin from spinous layer of epidermis, A431, in the present study, demonstrated a very high expression of 12R-LOX which is atypically expressed in differentiated granulosum layer of epidermis. This may be generalized because of an improper terminal differentiation and/or tumorigenesis of A431 cell line and so, it shows the increased expression of 12R-LOX before being differentiated. 12R-LOX expression is induced in embryonic skin at embryonic day 15.5, time for the onset of skin development in mouse and thus, in balanced manner it is necessary for development of epidermis [154]. However, this enhanced expression of 12R-LOX

in the undifferentiated cells of stratum spinosum with co-occurrence of free AA [188][23] may attribute to more tumorigenesis in A431 cells and thus for a role in regulation of growth of these epidermoid carcinoma cells [128].

In the present study, absence of keratin markers K5 and K14 in A431 cells gives the affirmation of its origin from spinous layer of epidermis. Together with this, the study also showed a much diminished level of early differentiation marker like K1 in A431 cells even though its development is from spinous layer which markedly expresses K1. This loss of expression of early differentiation marker K1 in A431 cells, further, indicates the irregular differentiation as in the proliferative keratinocytes of upper epidermal layers of psoriatic scales [187]. However, this much less or no expression of early differentiation markers likes K1, K5 and K14 with the higher expression of late differentiation markers like involucrin and filaggrin suggest the dedifferentiation in A431 cells. This constitutes the first report on evaluation of differentiation state of A431. On the other hand, the higher expression of K1, K5 and K14 in HaCaT cells reveals its properly differentiated phenotype with a low expression level of 12R-LOX as in a primary keratinocyte model. In the light of this dedifferentiated state of skin cancer cells, A431 with a dominant expression of 12R-LOX and properly differentiated state of normal human keratinocytes HaCaT showing a detectable level 12R-LOX expression, further studies were undertaken on A431 and HaCaT cells to analyse the role of 12R-LOX in the regulation of keratinocyte proliferation and terminal differentiation.

HaCaT cells are able to revert back to a basal-like state both morphologically and biochemically: Development of HaCaT cell line as primary keratinocyte cells under low Ca^{2+} condition

In this study, we established HaCaT cell line showing primary keratinocytes features under low Ca^{2+} condition for studying the role of 12R-LOX in the keratinocyte terminal differentiation. HaCaT cells have the capability to revert back and forth between the differentiated and the basal phenotype depending upon Ca^{2+} concentration, and therefore are usually employed as a model for keratinocyte differentiation in culture [189][196]. Accordingly, in the present study, first, our approach is to transform the HaCaT cells as in basal-like states (cells showing features of stratum basale) by growing them in low Ca^{2+} medium (0.03mM), so that these basal-like cells can be further differentiated as primary keratinocytes [189]. In the study, a much diminished or no expression level of differentiation markers like K1, filaggrin, involucrin, TGM-1 and spindle shape with no cell to cell tight junctions in the HaCaT cells grown under low Ca^{2+} conditions (dedifferentiated HaCaT cells) reveals the basal-like state of these cells both morphologically and biochemically. Since, the expression of K5 and K14 is strictly restricted to the proliferative cells of stem cell layer and stratum basale where the Ca^{2+} concentration is very low [168][169], a remarkably increased expression of K5 in these dedifferentiated HaCaT cells gives the affirmation of a state similar to cells of stratum basale of epidermis. The characteristic feature of cells of basal layer especially an increased proliferation rate [199] is also unveiled by these basal-like HaCaT cells showing two fold increase in proliferation rate compared with normal HaCaT cells. These dedifferentiated HaCaT cells, further,

were used for Ca^{2+} induced differentiation to evaluate the role of 12R-LOX in terminal differentiation.

The increment of 12R-LOX with Ca^{2+} induced differentiation in HaCaT cells indicates its role in late differentiation process of keratinocytes

Studies show that proliferative basal keratinocytes are induced to arrest the cell cycle and initiate terminal differentiation perceiving a high Ca^{2+} concentration [192][193]. So, in order to understand the role of 12R-LOX in differentiation, HaCaT cells showing primary keratinocytes features were induced for differentiation after growing them in high Ca^{2+} medium in this study. Interestingly, a differentiation dependent increment in 12R-LOX expression level was noticed when cells showing the characteristics of basal-like primary keratinocytes were induced for differentiation with high Ca^{2+} level. The progress of differentiation was evident from the changes in expression of differentiation markers which follow a rising prototype of expression during terminal differentiation of keratinocytes [168][171][172][173].

The study on localization pattern of 12R-LOX also reveals the differentiation dependent tissue expression and spatial and temporal co-localization of 12R-LOX in upper differentiated epithelial tissues (stratum granulosum) and thus, implying the functional linkage of 12R-LOX in late terminal differentiation process [64]. Additionally, in embryonic skin of mouse, expression of Alox12b begins at embryonic day 15.5, time for the onset of skin development and after the epidermal development it shows a differentiation dependent expression pattern with highest expression in stratum granulosum [154].

Similarly, it is now understandable that the increasing pattern of expression of 12R-LOX during differentiation of HaCaT cells is attributed to its association with the late stages of terminal differentiation. Thus, elevation in 12R-LOX expression during differentiation of keratinocytes strengthens the presumed role of 12R-LOX in late differentiation process and so in epidermal barrier formation.

12R-LOX affects the expression of late differentiation markers- TGM-1 and filaggrin during differentiation of HaCaT cells

The enhanced expression of 12R-LOX during differentiation of HaCaT cells clearly indicates its potential role in late differentiation process of keratinocytes. Recent studies on formation of permeability barrier indicate not only a structural role of 12R-LOX using linoleate moiety of ceramide as a substrate [28] but also a signaling role of 12R-LOX which may be affected and mediated through linoleate-related hepoxilins [64][165]. So, in order to demonstrate the relation of 12R-LOX with late differentiation process we employed either a siRNA strategy to deplete 12R-LOX expression during Ca^{2+} induced differentiation or transfection method to over express the 12R-LOX in normal HaCaT cells and re-examined the effects on late differentiation markers. A diminished or no expression of TGM-1 and filaggrin in the absence of 12R-LOX (treatment with 12R-LOX siRNA) during differentiation process of keratinocytes indicates a regulatory effect of 12R-LOX in late stage of differentiation. In the present study, the regulatory role of 12R-LOX is yet again revealed with induced expression of TGM-1 and filaggrin with overexpression of 12R-LOX. Thus, in this study, an induced expression of TGM-1 and filaggrin under 12R-LOX over expression and *vice-versa* with knockdown of 12R-LOX in HaCaT cells evidently reveals the regulation of TGM-1 and filaggrin expression by 12R-

LOX in differentiating keratinocytes to facilitate the epidermal barrier formation effectively during late terminal differentiation process.

Inhibition of 12R-LOX with baicalein in epidermoid carcinoma A431 cells leads to proper differentiation but affects the late differentiation markers expression

Terminally differentiating keratinocytes display some features that resemble to cellular changes in programmed cell death implying the terminal differentiation as a specialized type of apoptosis in epidermis [218]. Further, it is very comprehensible that irregular differentiation may result in over proliferation and leads to development of proliferative diseases including psoriasis and skin cancers. The LOXs play an important role in the modulation of epithelial proliferation and differentiation as well as in wound healing, inflammation, inflammatory skin diseases and cancer [64][106]. Moreover, our previous study also reveals the pathological role of 12R-LOX in the growth of epidermoid carcinoma cells, A431 [128]. The A431 cells have originated from tumor of stratum spinosum layer of epidermis [178] and this implies the improper differentiation of keratinocytes which lead to tumorogenesis. In view of this, proliferative disorders like psoriasis and skin cancers can be controlled by inhibition of these LOXs and thus, a new therapeutic prospectus can be provided. Therefore, in this study, we performed inhibition of 12R-LOX with baicalein in A431 cells to understand the role of 12R-LOX in proliferation and differentiation.

For inhibition study, it is essential to know the presence of an active 12R-LOX first. In the present study, we identified 12R-LOX in A431 cells in an active form. This active 12R-LOX in A431 cells, thus, may have a primary role in tumorigenesis and dedifferentiation. Further, in the study, *in-vitro* inhibitory assay showed the

inhibition of 12R-LOX activity with baicalein. This is the first study reporting baicalein as an *in-vitro* inhibitor of 12R-LOX. Further, the increase in hypodiploid cells and G1 phase cells after treatment either with baicalein evidently indicates arrest of the cells in G1 stage of cell cycle and so, switches to terminal differentiation. An elevated expression of K1 and involucrin in A431 cells upon 12R-LOX inhibition with baicalein clearly indicates restoration of proper differentiation which had been deregulated towards tumorigenesis. Moreover, the present study showed that 12R-LOX inhibition with baicalein reduces the expression of TGM-1 and filaggrin even though differentiation has been progressed. From this study it is clear that inhibition of 12R-LOX, which is over expressed and may cause for tumorigenesis in A431 cells, causes to bring back cells for differentiation process, nevertheless, late differentiation is affected. This regulation of 12R-LOX on expression of late differentiation markers was further evaluated with 12R-LOX knockdown studies in A431 cells.

Down regulation of 12R-LOX results in reduction of late differentiation markers- TGM-1 and filaggrin in A431 cells

In order to evaluate the regulatory role of 12R-LOX in late differentiation process of keratinocytes, 12R-LOX expression in A431 cells was knocked down with 12R-LOX siRNA. In this study, from the depleted expression of TGM-1 and filaggrin with 12R-LOX knock down it is clear that 12R-LOX regulates the expression of late differentiation markers- TGM-1 and filaggrin to perform the terminal differentiation process in keratinocytes efficiently.

During terminal differentiation of keratinocytes, stratum corneum barrier formation is a part of late differentiation process and accomplished by making its three major components- 1) intercellular lipids comprising mainly cholesterol, ceramides and free fatty acids, 2) CCE composed of a inner protein layer, CE and a outer lipid monolayer, the CLE and 3) intracellular keratin-filaggrin degradation products [15][16][17][18]. The late differentiation markers, filaggrin and TGM-1 are the major components of two barrier compartments and are very important for the formation of permeability barrier. Filaggrin aggregates with keratin filaments cytoskeleton to make macrofibrils crisscrossing the cornified cells of stratum corneum [210]. This collapsed macrofibrils is cross-linked to CLE by TGM-1. So, along with these keratohyalin granules, filaggrin serves as an integral part of protein layer of CE beneath the lipid layer, CLE which contributes to structural integrity of corneocytes [213]. In addition, the 12R-LOX knockout studies showed that the epidermis of 12R-LOX-deficient mice demonstrated a disturbed processing of filaggrin, which is a crucial component of the CE, resulting in increased fragility of corneocytes (decreased mechanical strength of CE) of mutants and therefore causative to early neonatal death of the mutant mice [153][160]. From the present study, it is now evident that there is a functional linkage between regulatory signaling mechanism of filaggrin and 12R-LOX in the formation of barrier function during terminal differentiation of keratinocytes.

TGM-1, which is also found to be associated with 12R-LOX in the present study, is essential for the formation of CCE compartment of permeability barrier in stratum corneum [214]. On the inner surface of cell membrane of corneocytes,

a protein layer, CE is assembled by accumulation of several precursor proteins including filaggrin, loricrin and Involucrin [213]. Previous studies revealed that these precursor proteins are cross-linked by the action of TGM-1 [214]. Further, TGM-1 is, primarily, reported to cross-link (making covalent bond) proteins of CE to the hydroxyceramide (which is released or freed by consequence actions of 12R-LOX/eLOX-3 pathway) of CLE making the CCE of permeability barrier in stratum corneum [19]. Formation of CCE, upon which intercellular lipid layer can be formed, is essential to retain structural integrity of the epidermal water barrier [216]. In the present study, an induced expression of TGM-1 under 12R-LOX over expression and *vice-versa* with knockdown of 12R-LOX in HaCaT and A431 cells evidently revealed the regulation of TGM-1 expression by 12R-LOX in differentiating keratinocytes. This study forms the first report showing a functional linkage between 12R-LOX expression and the components of late differentiation process.

The present study markedly showed a regulatory effect of 12R-LOX in the expression of filaggrin and TGM-1 during late terminal differentiation process. Moreover, in keratinocytes of granular layer, oxidation of linoleate moiety of esterified ceramide results in the generation of the 12R-LOX/eLOX3 pathway derived linoleate-related hepoxilins or trioxilin, which are released during hydrolysis of epoxyalcohol derivatives in making of free ω-hydroxyl-ceramides. Hepoxilins and their trioxilin derivatives have been unveiled to play a signaling role in several physiological processes including adipocyte differentiation, inflammation and neurotransmission [59][164]. Additionally, one study also

provides the evidence for involvement of LOX-derived epoxyalcohol and their trioxilin derivatives in the formation of epidermal permeability barrier [27]. Thus, the findings, showed a regulatory signaling role of 12R-LOX, which may be mediated by linoleate-related hepoxilins (product of 12R-LOX/eLOX-3 pathway), in the expression of filaggrin and TGM-1. By regulating these late differentiation components, 12R-LOX plays a crucial role in maintaining of two compartments of permeability barrier of terminally differentiated keratinocytes (Figure 33).

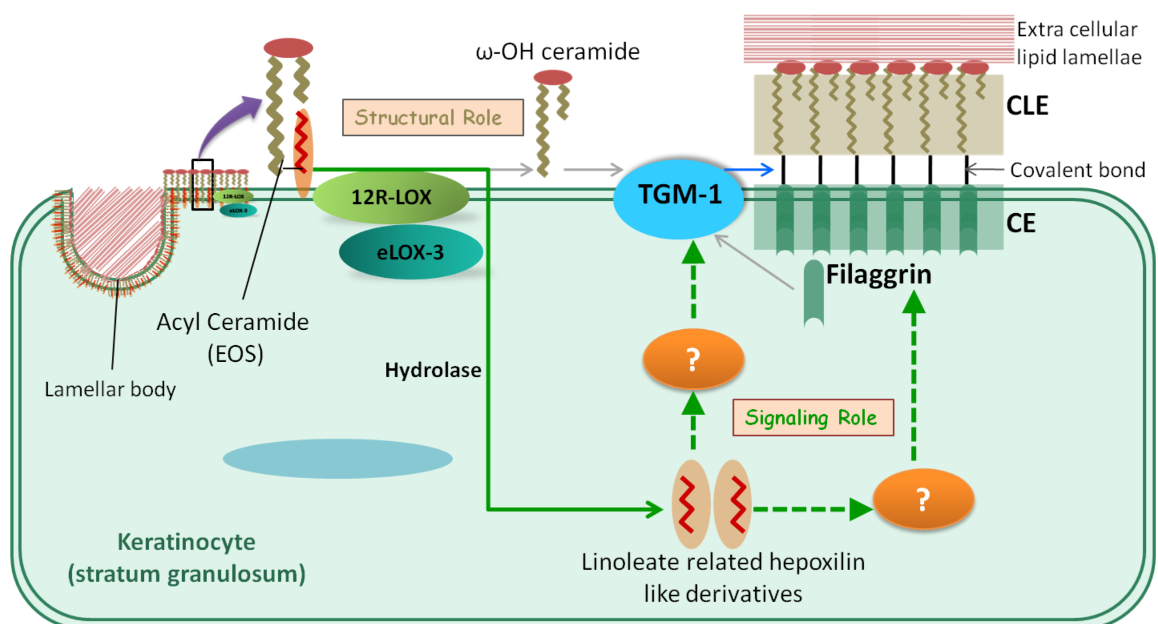


Figure 33: Schematic representation of the proposed signaling role of 12R-LOX/eLOX-3 pathway in the formation of epidermal permeability barrier of skin.

In conclusion, 12R-LOX along with its structural role plays a crucial role in the signaling mechanisms for expression of permeability barrier components and thus facilitates the barrier function properly. Nevertheless, the intermediate molecules and mechanisms have to be identified for further understanding of the role of the 12R-LOX in the maintenance of epidermal barrier function (Figure 33).

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List of Publications

1. **Geetika Gupta,** Naresh Kumar, Venkat, R. Karnati, Aparna Dutta Gupta and Pallu Reddanna. A pivotal role of 12R-Lipoxygenase in late terminal differentiation of human keratinocytes (Manuscript).

2. **Geetika Gupta,** K. Kumar, Naresh Kumar, R. Karnati, Aparna Dutta Gupta and Pallu Reddanna. 12R- Lipoxygenase knockdown affects expression of TGM-1 and Filaggrin epidermoid carcinoma cell line, A431 (Manuscript).

3. **Geetika Gupta,** Naresh Kumar, R. Karnati and Pallu Reddanna. Synergistic effects of COX-2 and 5-LOX dual inhibitor, chebulagic acid and 12S- LOX inhibitor, baicalein on growth inhibition and induction of apoptosis in human breast cancer cell line (MCF-7) (Manuscript).

4. Naresh Kumar, **Geetika Gupta**, Kotha Anilkumar, Naireen Fatima, Roy Karnati and Pallu Reddanna 15-Lipoxygenase metabolites of α-linolenic acid, [13-(S)-HPOTrE and 13-(S)-HOTrE], mediate anti-inflammatory effects by inactivating NLRP3 inflammasome in RAW 264.7 cells. *Nat. Scientific Report* (Revised).

5. Naresh Kumar**, Geetika Gupta**, Kotha Anilkumar and Pallu Reddanna. Isolation, purification and characterization of Lipoxygenase from Linseed seedlings (Manuscript).

6. Chandramohan Reddy, D. Bharat Reddy, A. Aparna, K.M. Arunasree, **Geetika Gupta**, C. Achari, et al., Anti-leukemic effects of gallic acid on human leukemia K562 cells: downregulation of COX-2, inhibition of BCR/ABL kinase and NF-κB inactivation., Toxicol. In Vitro. 26 (2012) 396–405.

7. A. Bhujade, **Geetika Gupta**, S. Talmale, S.K. Das, M.B. Patil, Induction of apoptosis in A431 skin cancer cells by Cissus quadrangularis Linn stem extract by altering Bax-Bcl-2 ratio, release of cytochrome c from mitochondria and PARP cleavage., Food Funct. 4 (2013) 338–46.

8. A. Bhujade, S. Talmale, N. Kumar, **Geetika Gupta**, P. Reddanna, S.K. Das, et al., Evaluation of Cissus quadrangularis extracts as an inhibitor of COX, 5-LOX, and proinflammatory mediators., J. Ethnopharmacol. 141 (2012) 989–96.