IN VITRO AND *IN VIVO* FUNCTIONAL STUDIES ON HUMAN 12*R*-LIPOXYGENASE

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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DECLARATION

I hereby declare that the work embodied in this thesis entitled **"*In vitro* and *in vivo* functional studies on human 12*R*-Lipoxygenase"** has been carried out by me under the supervision of Prof. P. Reddanna and that this work has not been submitted for any degree or diploma of any other University earlier.

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CERTIFICATE

This is to certify that Ms. Naireen Fatima has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the PhD ordinance of this University. We recommend her thesis **"*In vitro* and *in vivo* functional studies on human 12*R*-Lipoxygenase"** for submission for the degree of Doctor of Philosophy of this University.

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TO My
FAMILY

In the name of Allah, the Most Gracious, the Most Merciful

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CONTENTS

CONTENTS 1-5

LIST OF ABBREVIATIONS 6-7

CHAPTER 1: INTRODUCTION 8-27

1.1 Polyunsaturated fatty acids (PUFA) and Arachidonic acid (AA) 9

1.2 Arachidonic acid cascade 10-11

1.3 Eicosanoids 12-13

1.3.1 Eicosanoids Biosynthesis

1.3.2 Eicosanoids and carcinogenesis

1.4 Lipoxygenases 14-16

1.4.1 Introduction

1.4.2 Nomenclature

1.4.3 Lipoxygenase structure

1.4.4 Catalytic reaction

1.5 R-Lipoxygenases 17

1.6 12R-Lipoxygenases 18-19

1.6.1 Human 12*R*-Lipoxygenase

1.6.2 Importance of 12*R*-Lipoxygenase

1.7 Pathophysiology of lipoxygenases and chemopreventive approaches 20-21

1.8 Models to study gene functions 22

1.9 Transgenic technology 23-24

1.10 Scope and Objectives 25-27

CHAPTER 2: MATERIALS AND METHODS **28-50**

2.1 DNA manipulation **29-33**

2.1.1 Plasmid DNA isolation

2.1.1.1 Plasmid miniprep

2.1.1.2 Endotoxin free plasmid maxiprep

2.1.2 Preparation of ultra competent cells and transformation

2.1.3 Restriction digestion of plasmid DNA

2.1.4 Isolation of DNA from agarose gel

2.1.5 End filling reactions

2.1.6 Ligation Reactions

2.1.7 Screening of recombinant clones by cracking method

2.2 Vector Designing **34**

2.2.1 Construct preparation for transgenic mice

2.2.2 Construct preparation for over expression in bacteria.

2.3 Protein Isolation and purification **35**

2.4 Antibody generation **36**

2.5 Protein Analysis **37-38**

2.5.1 Western blot analysis

2.5.2 Immunocytochemistry

2.6 HPLC Assay **39**

2.7 Pronuclear microinjection of DNA into the mouse embryos **40**

2.7.1 Preparation of DNA

2.7.2 Restriction digestion of the plasmid DNA

2.7.3 Collection of pronuclei

2.7.4 Microinjection of the DNA and transfer of the injected embryos

2.7.5 Screening of the progeny for transgene integration

2.8 Isolation of genomic DNA 41

2.8.1 Isolation of genomic DNA from mouse-tail

2.8.2 Isolation of genomic DNA from cell line

2.9 PCR analysis of DNA 42

2.9.1 Primer designing

2.9.2 PCR amplification

2.10 Southern analysis of DNA 43-44

2.10.1 Electrophoresis of DNA

2.10.2 Transfer of DNA onto hybond-N^+ membrane

2.10.3 Preparation of radiolabeled DNA probe

2.10.4 Purification and measurement of radio labeled probe

2.10.5 Hybridization

2.11 Phenotypic analysis of mice 45-46

2.11.1 Breeding of h*12R*-LOX transgenic mice

2.11.2 Sperm analysis of mice

2.11.2.1 Isolation of sperm from epididymis

2.11.2.2 Sperm motility analysis by computer assisted sperm analysis (CASA)

2.11.3 Histopathological examination of various tissues

2.12 Transfection of mammalian cells 47

LIST OF PRIMERS 48

LIST OF ANTIBODIES 49

LIST OF VECTORS 50

CHAPTER 3: Human 12R-Lipoxygenase enzyme- expression, purification and characterization 51-62

3.1 INTRODUCTION 52-54

3.2 RESULTS 55-58

3.2.1 Expression of h-12*R*-Lipoxygenase in eukaryotic expression system.

3.2.2 Activity assay for h-12*R*-LOX using cell lysate from the stable cell line.

3.2.3 Expression of h-12*R*-Lipoxygenase in prokaryotic expression host.

3.2.4 Purification of active recombinant His-tagged h12*R*-LOX.

3.2.5 Standardization of activity assay for purified h-12*R*-LOX enzyme

3.2.6 Extracts of *Acalypha indica* plant significantly inhibits enzyme activity of h-12*R*-LOX

3.3 DISCUSSION 58-62

CHAPTER 4: Generation and characterization of h-12R-Lipoxygenase transgenic mouse.

4.1 INTRODUCTION 64-65

4.2 RESULTS 66-70

4.2.1 Generation of human-12*R*-Lipoxygenase transgenic mice

4.2.1.1 Generation of human-12*R*-LOX transgenic mice using pCMV6-12*R*-LOX vector

4.2.1.2 Generation of mice using pG3Z-K14 BetaGlobin-h12*R*-LOX vector

4.2.2 Identification of insertion site of the transgene in mouse genome

4.2.3 Phenotypic analysis of the transgenic mice.

4.3 DISCUSSION **71-72**

CHAPTER 5: OVERVIEW **73-78**

LIST OF REFERENCES **79-86**

APPENDIX **89**

LIST OF ABBREVIATIONS

%	Percent
Amp	Ampicillin
µg	Microgram
µL	Microliter
µM	Micromolar
ß-globin-PolyA	Beta Globin polyadenylation
12-H(p)ETE	(5Z, 8Z, 10E, 14Z)-12-Hydro (pero)xyeicosa-5,8,10,14-tetraenoic acid
15-H(p)ETE	(5Z, 8Z, 11Z, 13E)-15-Hydro (pero)xyeicosa-5,8,11,13-tetraenoic acid
bp	Base pair
BSA	Bovine Serum Albumin
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Delbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
ES	Embryonic stem
FITC	Fluorescein isothiocyanate
GC/MS	Gas chromatography/Mass spectrometry
HPLC	High Performance Liquid Chromatography
h	Hour
HCl	Hydrochloric acid
HRP	Horse raddish peroxidase
RP-HPLC	Reverse phase HPLC
IPTG	Isopropyl-ß-thiogalactopyranoside

Kb	Kilo base
Kg	Kilo gram
kDa	Kilo Dalton
Kan	Kanamycin
LB-Medium	Luria Bertani Medium
LT	Leukotriene
LX	Lipoxin
MCS	Multiple cloning site
Min	Minutes
mg	Milligram
mL	Millilitre
mm	millimetre
mM	Millimolar
mRNA	Messenger Ribonucleic acid
NaoAc	Sodium Acetate
Ni-NTA	Nickel-Nitrilotriacetic acid
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSG	Pregnant mare's serum gonadotropin
PUFA	Polyunsaturated fatty acid(s)
PVDF	Polyvinylidine difluoride
RT	Room temperature
RT PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
Tm	Melting temperature
Tris	Tris-hydroxymethylaminomethane

Chapter 1

Introduction

CHAPTER 1: INTRODUCTION

From the ongoing studies, it is clear that eukaryotic cells invest considerable part of the resources in producing different lipids. About 5% of our genes are responsible for lipid synthesis. The fundamental rule in biology that "structure serves function" also explains the requirement of such repertoire of lipids. Lipids fulfil many general but important functions. Lipids encompass many types like: Triglycerides (Fats), Fatty acids, Phospholipids, glycerophospholipids, sphingolipids, and Sterol-containing metabolites. Fatty acids are made up of an aliphatic tail (hydrocarbon chain) that terminates in a carboxylic acid group. This hydrocarbon chain in different fatty acids varies in number of carbon atom and may be saturated or unsaturated (carbon-carbon double bond). Fatty acids not only act as the metabolic fuel but also confer structural flexibility and integrity to biomembranes, depending upon their degree of unsaturation. This flexibility allows cell to alter their shapes, which is an important requirement for normal cell functions. Additionally, they produce first and second messengers for signal transduction. Genetic, epidemiological and animal studies have been providing clear evidence that the aberrant metabolism of fatty acids contributes to variety of abnormal cellular functions.

1.1 PUFA and Arachidonic acid

Polyunsaturated fatty acids (PUFA) comprises of a family of lipids including lipids with more than one double bond in their structure. They are classified into various groups according to their chemical structures.

Mainly divided in three classes: Methylene-Interrupted (-C-C=C-C-C=C-) (Omega-3, Omega-6, Omega-9), Conjugated fatty acids (-C=C-C=C-) and other polyunsaturates. Omega-3 (n-3) and Omega-6 (n-6), defined as "essential fatty acids" are not synthesized in the human body, but are taken from outside sources. PUFA n-3 includes alpha linolenic acid (ALA), eicosapentaenoic acid (EPA), and decosahexaenoic acid (DHA). PUFA n-6 includes linoleic acid (LA) and arachidonic acid (AA). Mutual interaction and balance between PUFA n-3 and PUFA n-6 fatty acids strongly affect cellular processes like inflammation and cellular signalling and have long term effects on many diseases like cardiovascular disease, cancer,

inflammatory and autoimmune diseases (**Fig. 1.1**). Higher amounts of n-3 polyunsaturated fatty acids and very high ratio of n-6 to n-3 promotes the risk factors for such diseases, where as increased levels of n-3 PUFA exerts the suppressive effect (Taha et al., 2014).

Arachidonic acid (AA) (5,8,11,14-eicosatetraenoic acid, 20:4), a polyunsaturated fatty acid, constitutes the major component of the fatty acids in cell membrane and is predominantly found esterified in membrane phospholipids in all mammalian cells. Various classes of enzymes metabolize it and give rise to the eicosanoids, which is a family of inflammatory mediators (prostaglandins, leukotrienes and other lipid mediators) (Sun et al., 2014).

1.2 Arachidonic Acid Cascade

Phospholipids present in the cell membranes are not only the structural components but also comprise an important functional domain. Enzymatic processing of these phospholipids by phospholipases results in the release of lipid mediators or second messengers, which include arachidonic acid, phosphatidate, diacylglycerol and phosphotidyl inositols (IPs). These mediators play important roles in membrane trafficking, signal transduction, and regulation of variety of physiological and pathological processes. Structurally, phospholipids (glycerophospholipids) consist of glycerol -3-phosphate molecule esterified at carbon-1 (sn-1) and carbon-2 (sn-2) positions to non-polar fatty acids. On the basis of the ester bond that is cleaved within a phospholipid molecule, phospholipases are grouped into four families: A, B, C, and D. Phospholipase A enzymes cleave the acyl ester bond at either sn-1 or at sn-2 positions, so are named as phospholipase A1 and phospholipase A2 respectively. The sn-2 positions of phospholipids contain unsaturated fatty acids such as arachidonic acid (AA).

Phospholipases (PLA2) hydrolyse these phospholipids and release free arachidonic acid (**Fig. 1.3**). 4 types of PLA2 are known to occur in the cell: a secretory, a cytosolic, a Ca^{2+} independent PLA2 and the platelet activating factor acetyl-hydrolase (PAF-AH). Among these, cytosolic PLA2 (cPLA2) is selective to AA and is expressed in most cells including keratinocytes and fibroblasts. In response to stimuli

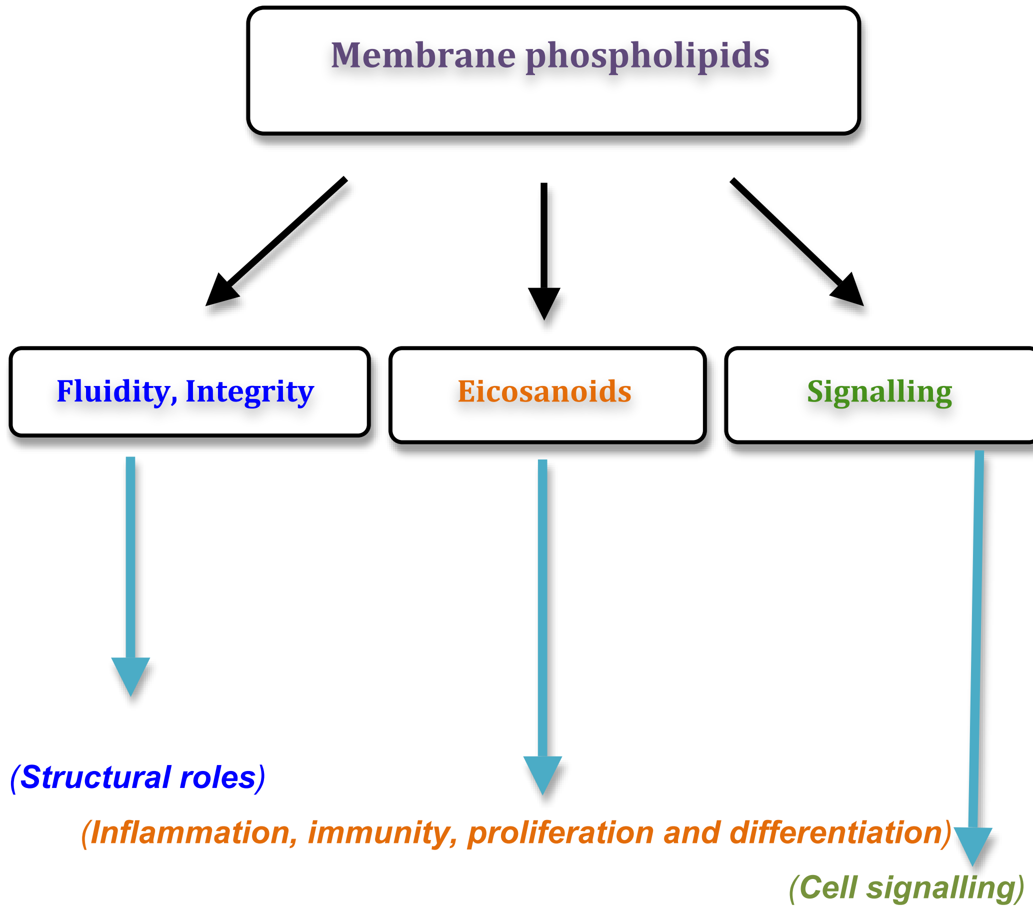
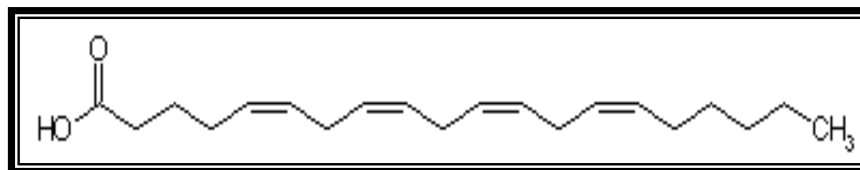


Fig. 1.1 Polyunsaturated fatty acids present in the membrane of the cells play important role in various cellular processes.



Chemical structure of Arachidonic acid.

Formula: C_{20} O_2 H_{32} ; =bond between $C_{5\text{-}6}$, $C_{8\text{-}9}$,$C_{11\text{-}12}$, $C_{14\text{-}15}$

Fig. 1.2 Pictorial representation of the chemical structure of Arachidonic acid.

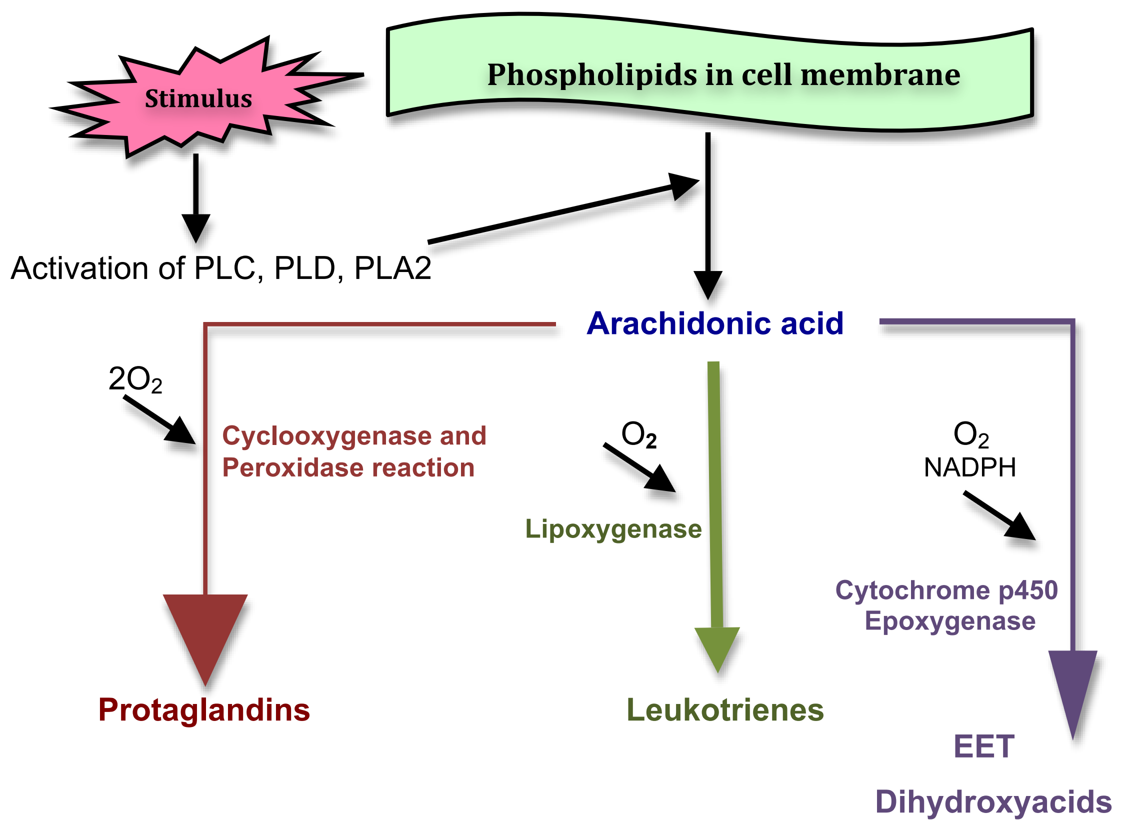


Fig. 1.3 Schematic representation of the metabolism of Arachidonic acid via different enzymatic pathways.

(interferon's, lipopolysaccharides, cytokines, growth factors, Ca2+ ionophore), cPLA2 is translocated from the cytosol to intracellular membrane compartments i.e; Golgi apparatus, endoplasmic reticulum and nuclear envelope. Apart from above phosopholipase A2 enzymes another class of enzymes known as Phospholipase C (PLC) can also generate free AA. PLC first breaks down the phospholipid (PIP2) to diacylglycerol (DAG) and inositol-triphosphate (IP3). DAG-lipase then converts DAG to free AA and monoacylglycerol (MAG). The dominant pathway that releases AA, however, is via the activity of PLA2 (Smith et al., 1992).

In chemical structure, arachidonic acid is a carboxylic acid with a 20- carbon chain and four cis-double bonds at 5-, 8-, 11-, 14- carbon atom positions. The double bonds of AA (polyenoic fatty acids) provide the possibility to react with molecular dioxygen (Brash, 2001) (**Fig. 1.2**).

Biologically active lipids (eicosanoids) are generated from arachidonic acid when oxygenated by enzymes like cyclooxygenases (COX), lipoxygenases (LOX) and P450 monooxygenases. The products of these enzymatic and non-enzymatic lipid peroxidation are potent regulatory substances, which are synthesized intracellularly (are not stored) and then released into the extracellular spaces to act locally (local hormones or autocoids) or to be transported with the blood to other tissues and organs. They regulate many cellular functions and play crucial roles in a variety of physiological and pathophysiological processes, such as inflammation, regulation of vasodilation, vascular permeability, pain, recruitment of leukocytes etc.

So, the structures and functions of these oxygenated derivatives are quite diverse. The critical difference in the biological properties of each of these molecules is the number, position and stereochemistry of oxygen insertion. Living cells regulate the balance of each of these molecules to respond appropriately to the internal/external stimuli and maintain homeostasis. Eicosanoids, thus, are extremely potent biologically active molecules with bewildering variety of actions on divergent processes. Their uncontrolled production, however, is associated with many inflammatory and neuro-degenerative diseases. As a result the enzymes involved in the production of these eicosanoids have become the targets for the development of anti-inflammatory drugs.

1.3 Eicosanoids

1.3.1 Eicosanoid biosynthesis

The metabolism of arachidonic acid (AA) by cyclooxygenases, lipoxygenases and P450-epoxygenase pathways generate eicosanoids, which include prostanoids, leukotrienes, hydroperoxyeicosatetraenoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs), and epoxyeicosatrienoic acids (EETs).

(a) Cyclooxygenases are the enzymes exhibiting both cyclooxygenase and peroxidase activity and are involved in the formation of prostaglandins, prostacyclin and thromboxane. Cyclooxygenase activity on arachidonic acid (AA) produces cyclic hydroperoxy endoperoxide, PGG_2, which is reduced by the peroxidase activity of the enzyme to the hydroxy endoperoxide PGH_2 (Smith and Marnett , 1991). PGH_2 undergoes further enzymatic modifications to produce prostaglandins (PGD_2, PGE_2, PGF_2), prostacyclin (PGI_2) or thromboxaneA_2 (Smith, 1992). There are two isoforms of COX enzymes: COX-1 is widely distributed in different tissues and COX2 is either absent or expressed in low levels in most tissues. COX-1 is a housekeeping gene whereas COX-2 is induced by growth factors, hormones, cytokines, tumor promoting agents and mechanical stress. Low concentrations of AA can be catalysed by COX-2 whereas COX-1 catalyzes preferably at high concentrations of AA (>10micromolar) (Yamamoto, 1999).

(b) Lipoxygenases are the enzymes that act on AA (or other fatty acids such as linoleic acid) and produce fatty acid hydroperoxides, which are rapidly reduced to the corresponding hydroxy compounds by glutathione peroxidases. LOXs contain one mole of non-heme iron per mole of enzyme, which is involved in initial hydrogen abstraction followed by the introduction of molecular oxygen into the fatty acid chain. The position of oxygen insertion depends on the positional specificity of the enzyme (Kuhn et al., 1986; Yamamoto, 1992).

(c) Cytochrome P-450s are the monooxygenases belonging to membrane bound hemoprotein family, which are located in the endoplasmic reticulum of most of the eukaryotic cells. These enzymes participate in the oxidative metabolism of various lipophilic molecules, cholesterol, steroids, vitamins and fatty acids. Skin, liver, kidney, heart and pituitary tissues have been found to express these monooxygenases. Enzymatic reactions involve breakdown of molecular O_2, which is NADH-dependent, and insertion of one atom of oxygen into the substrate and the release of water (Capdevila et al., 1992).

1.3.2 Eicosanoids and Carcinogenesis

Pro-inflammatory eicosanoids are abundantly produced by cancer cells and also by the cells surrounding these cells. There are several mechanisms by which these lipid molecules modulate tumor progression. These molecules: **(i)** regulate cellular proliferation by directly activating receptors on tumor epithelial cells **(ii)** support and spread tumor growth by directly inducing epithelial cells to secrete growth factors, proinflammatory mediators and angiogenic factors **(iii)** maintain stem cell growth, maturation and homeostasis (giving a clear indication of their role in regulating tumor formation) and **(iii)** promote angiogenesis (mediate neovessel assembly, and increase the proliferation of endothelial cells, which provide continuous blood supply to the tumor cells and thus help them to proliferate) (Wang and Dubois , 2010).

Prostaglandins act in an autocrine or paracrine manner. They bind to their cognate cell surface receptors that belong to G-protein-coupled receptor family (GPCR). In some cases certain prostaglandins directly bind to nuclear receptors such as peroxisome proliferator-activated receptors (PPARs). The functions of prostaglandins in a particular tissue depend on the type of prostaglandin produced in a tissue and the expression of their cognate receptors as well.

Leukotrienes also exert their biological effects through activation of GPCRs. The leukotrienes are primarily produced by stimulated leukocytes. These stimulated leukocytes express the set of enzymes required for the synthesis of leukotrienes. Leukotrienes are also generated by epithelial and endothelial cells at the inflammatory sites by transcellular metabolism in which epithelial or endothelial cells use

leukotrienes (LTA_4) released from immune cells. Also, leukocytes can use arachidonic acid as a substrate, secreted by the epithelial cells. Thus, transcellular biosynthesis between epithelial, endothelial and immune cells leads to overproduction of leukotrienes, which amplifies inflammatory response.

1.4 Lipoxygenases

1.4.1 Introduction:

Lipoxygenases are stereo- and regiospecific dioxygenases which catalyze the addition of molecular oxygen into polyunsaturated fatty acids containing at least one or more (Z, Z)- 1,4-pentadiene structural unit to produce unsaturated fatty acid hydroperoxides. Lipoxygenases comprise of a structurally related non-heme, iron-containing enzymes found ubiquitously in all plants and animal systems. Recently, a research group has reported their occurrence even in bacteria (Porta and Rocha-Sosa , 2001). Interestingly, bacterial amino acid sequence reveals their close resemblances to mammalian lipoxygenases as compared to plant lipoxygenases. There have been speculations of horizontal transfer of lipoxygenase genes to bacteria. They were discovered in lower marine organisms i.e., algae, sea urchin, starfish, clams and corals (Hawkins and Brash, 1987; Brash et al., 1987; Hada et al., 1997). They were found to be present in fungi too (Bisakowski et al., 1997; Su and Oliw, 1998).

1.4.2. Nomenclature:

Lipoxygenases are named based on their positional specificity of dioxygenation of their substrates e.g., 8-, 12- and 15-LOX. Stereoconfiguration is also specified, e.g., 12S-LOX and 12R-LOX. In mammals, additionally they are named after the prototypical tissue of their occurrence. For e.g., platelet-type, leukocyte-type and epidermis-type 12S-LOX. Based on phylogenetic relatedness mammalian LOXs have been categorised as: i) 5-LOX, ii) platelet-type 12-LOX, iii) 12/15-LOX (reticulocyte-type 15-LOX-1 and leukocyte-type 12-LOX, both exhibiting a dual positional

specificity and iv) epidermis-type LOX (12R-LOX, 15-LOX-2, 8-LOX, epidermis-type LOX-3).

1.4.3 Lipoxygenase structure:

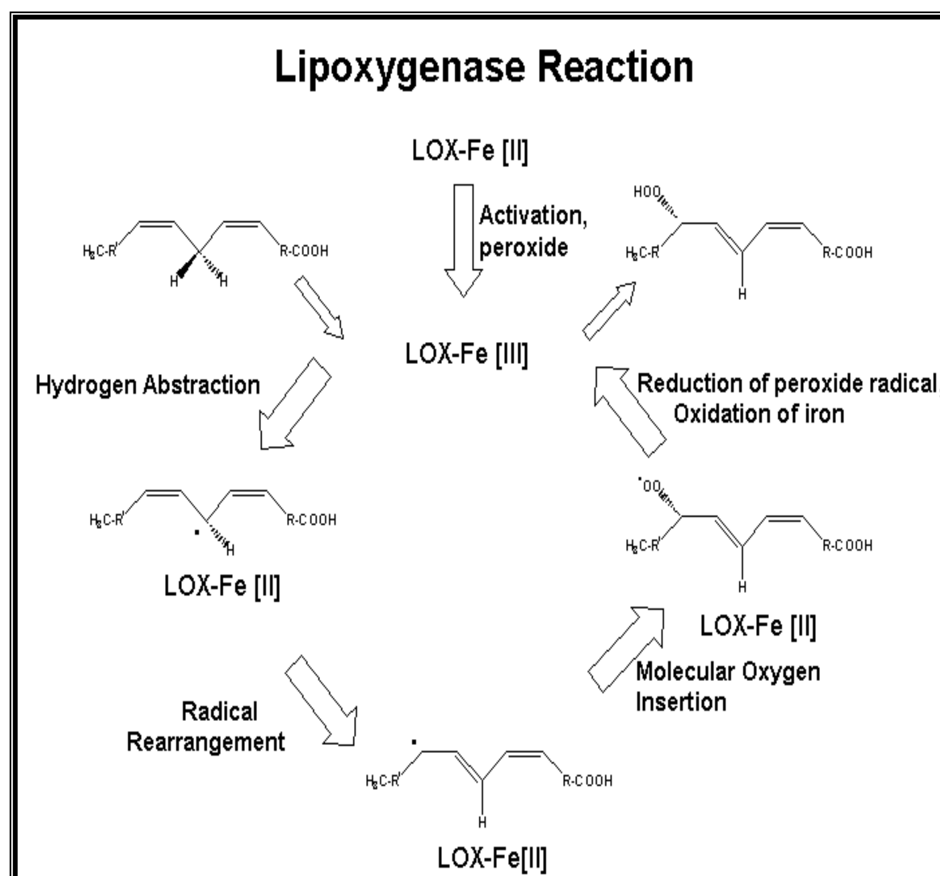
It took more than five decades for the investigators to study the structure of lipoxygenases. The complete crystal structure for mammalian LOX (rabbit reticulocytes-type 15-LOX) was described in 1990 (Sloane,1990). Using this structural data as the template, structures of other mammalian lipoxygenase proteins are being deduced by employing homology modelling and other bioinformatics tools (Aparoy et al., 2009). Generally, LOX protein structure comprises of a single polypeptide chain with molecular weight of 75-80 kDa in animals and 94-104 kDa in plants. Although the enzymes in the LOXs family have related structures but they form specific products with unique functions. LOXs, multigene family of enzymes, show sequence identity of 20-40%. LOX proteins contain highly conserved domains, which are responsible for their distinct structure and their binding to the catalytic iron. Lipoxygenases generally consist of two domains: Smaller N-terminal domain consisting of antiparallel ß-barrel which is similar in size, sequence and structure to C-terminal-barrel domain of mammalian lipases and is thought to play role in localising the enzyme near the substrate. Larger domain is the C-terminal catalytic domain. It consists of alpha helices interrupted by small beta sheet sub-domain and contains catalytic non-heme iron. The metal is liganded to conserved histidines and to the carboxyl group of a conserved isoleucine at the C-terminus of the enzyme. Since the metal ligand is non-heme iron so the lipoxygenases appear colorless to the human eye. Iron is in the ferrous (inactive) state when isolated and needs to be in the ferric state to become active for the catalysis (Sloane et al., 1991).

1.4.4 Catalytic reaction:

Lipoxygenases contain one non-heme iron per mole of the enzyme. The mechanisms proposed for lipoxygenases catalysis involves three steps:

1. In the **first step**, there is an abstraction of hydrogen from the arachidonic acid, in the form of a proton, from double allelic methylene group and the electron is

transferred to the ferric lipoxygenases so that it gets reduced to ferrous state. This abstraction occurs in a regio-specific and stereo-selective manner and depends on the enzyme specificity.



2. **Second step** involves radical rearrangement accompanied by Z, E-diene conjugation. It has been hypothesised that the direction of the radical rearrangement depends on the conformation, which the fatty acid adopts at the active site.
3. **Third step** leads to the formation of an oxygen-centered fatty acid hydroperoxide radical, which occurs due to insertion of a molecular dioxygen in a stereo-specific manner, to the pentadienyl system. It has been proposed that the oxygen entry to the fatty acid binding site is diffusion controlled. This oxygen-centered radical intermediate formed is reduced to corresponding anion and the enzyme is oxidised back to the ferric form.

1.5 *R*-Lipoxygenases

Biosynthesis of *S*-configuration of lipoxygenase products was thought to be rule until 1980, when it was unexpectedly discovered that there is the presence of enzymes in marine invertebrates that can convert arachidonic acid to products with *R*-configuration. Both enzyme types *R*- and *S*- catalyse stereo-selective removal of a hydrogen atom from the methylene group positioned between two *cis* double bonds. Both follow "antarafacial relationship" between hydrogen abstraction and the addition of molecular oxygen. With the cloning and purification of 8*R*-LOX from corals, two classes i.e; ***R***- and ***S***- enzymes were viewed as the members of the same gene family but with opposite stereospecificity for the oxygenation reactions (Brash AR *et al.,* 1996). *R*-LOX is widespread in aquatic and marine invertebrates, in algae, in fungi, in higher plants and in humans.

8*R*-LOX was first discovered to be present in corals (Bundy GL et al, 1986). Later **8*R***-LOX activity was also found to be present in fresh water species of starfish where it helps in maturation of oocytes (Meijer *et al.,* 1986). **5*R***- and **8*R***- LOXs were found in surf clam (Hada *et al.,* 1997) and **11*R***- and **12*R***- were found in sea urchin (Hawkins and Brash, 1987). **11*R*-**HETE in aquatic invertebrate *Hydra* is involved in tentacle regeneration. A highly glycosylated enzyme, secreted by the fungus of wheat, displays lipoxygenase activity with linoleic acid as its natural substrate. This enzyme, however, contains manganese as the catalytic metal instead of iron (Su and Oliw, 1998). **5*R*-**LOX was found to be present in temperate red algae (Jiang *et al.,* 2000). In plants, formation of ***R*-**configuration hydroperoxides is accompanied by the formation of ***S*-**hydroperoxides as well. Change of pH optimum for the reaction of the enzymes (from the seeds of leguminosae like soybeans and peas) controls the ratio of the type (***R***- or ***S***-) of the product produced. Both human and murine **12*R***-LOX have been shown to produce the product of ***R***-specificity (Siebert M *et al.,* 2001).

1.6 12*R*-Lipoxygenase (12*R*-LOX)

Human and mouse 12*R*-LOX proteins are 701 amino acids in length and share 86% identity. The first report of 12-HETE in human skin came in 1975, where it was reported from the psoriatic areas of the skin (Hammerstorm *et al.,* 1975). Chiral

analysis of the product showed that the major enantiomer was 12*R*-HETE.12-*R*-LOX gene is unique among all animal lipoxygenases in that it is divided into 15 exons and 14 introns. All human lipoxygenase genes including 12(*R*)-Lipoxygenase gene have been localised to chromosome 17p13 except 5-lipoxygenase gene, which is situated on chromosome 10. By reverse transcription–polymerase chain reaction analysis 12(*R*)-lipoxygenase mRNA was detected in B cells and adult skin.

The ortholog of the human ALOX12B gene in mice (Alox12b) encodes 12*R*-LOX. In mouse, 12-R-LOX is localized on the central region of mouse chromosome 11. Murine Alox12b gene and protein both share structural features of human 12*R*-LOX. Mice have a total of seven functional LOX genes, one more than human. All of them encode *S*-type except Alox12b and Aloxe3 genes. Mouse 12-R- lipoxygenase mRNA was highly expressed in epidermis of newborn mice (Sun *et al.,* 1998). In adult mice, it is strongly expressed in stratifying epithelia of skin (Heidt *et al.,* 2000).

Northern analysis of human keratinocytes using 12*R*-lipoxygenase-specific probe gave a single band of 2.5Kb compatible with the predicted size of the mRNA comprising 260bp of 5'UTR, 2.103bp ORF and 150 bp 3' UTR. 12*R*-LOX cDNA is unusual in having 260bp of 5' UTR and a short sequence of 150bp of 3'UTR. The ORF encodes a protein having characteristics of all typical lipoxygenases. It also encodes an extra sequence of approximately 5kDa of extra sequence accounted for by an insert of 31 amino acids (also present in murine 12*R*-LOX). The close relationship in primary structure between human 12*R*-LOX and human 15*S*-LOX argues for similarities in the architecture of the active sites of these two enzymes. With reference to crystal structure of the rabbit reticulocyte 15*S*-lipoxygenase, 5kDa extra sequence in 12*R*-lipoxygenase is located after the first alpha-helix region of the main C-terminal domain. So, it can be accommodated on the outside of the protein without disruption of the overall tertiary structure. This 31-aminoacid domain is Proline and Arginine rich and is proposed to have the characteristics of SH3- binding domain and thus is thought to play role in regulatory functions such as in sub cellular localization of the enzyme or interaction with other proteins.

Human 12*R*-LOX uses arachidonic acid and produces 12*R*-HETE. Different from the human 12*R*-LOX, enzymatic activity of the mouse 12*R*-LOX has not been detected on free arachidonic acid. Murine enzyme reacts only with arachidonic acid methyl esters and exhibits a minor activity with free arachidonic acid (Krieg *et al.,* 1999; Siebert *et al.,* 2001).

A decrease in the pH, calcium concentration and DMSO in the incubation medium was reported to strongly increase the enzymatic activity of human 12*R*-LOX towards arachidonic acid. There was only slight increase in the enzymatic activity of mouse 12*R*-LOX towards arachidonic acid methyl ester on decreasing the pH of the incubation medium (Siebert *et al.,* 2001).

1.6.1 Human 12*R*-LOX

In humans there are six functional LOX genes. *ALOX12B* encodes 12*R*-LOX and this enzyme produces 12*R*-HpETE from arachidonic acid (Boeglin *et al.,* 1998). *ALOX12B* is located on chromosome 17p13.1 where it forms a cluster of epidermis-type LOX together with other LOX enzymes 15-LOX-2 and eLOX-3 (Yu *et al.,* 2003). Human 12*R*-LOX has very limited tissue distribution. To date, only human normal and psoriatic skin and tonsils have been found to express the enzyme and to convert exogenous arachidonic acid to 12*R*-HETE. The levels of 12*R*-LOX and its oxygenation products appear to be elevated in psoriasis and other proliferative skin dermatoses (Baer *et al.,* 1991; 1995). In tonsils, the enzyme is expressed in the stratified squamous epithelium, and is absent from the underlying lymphatic tissue including the germinal centres as determined by *in situ* hybridization (Schneider *et al.,* 2001). Using RT-PCR techniques, Krieg *et al.,* (1999) detected expression of RNA in additional tissues like lung, testis, adrenal gland, ovary, prostate, and skin; lower abundance was detected in salivary and thyroid glands, pancreas, brain, and plasma blood leukocytes.

1.6.2 Importance of human 12*R*-Lipoxygenase.

Human enzyme 12*R*-LOX enzymatically oxidizes arachidonic acid and leads to biosynthesis of pro-inflammatory lipid mediators. Since, in psoriatic skin, there was an abundance of 12*R*-HETE, so it was thought that there is a strong association of such eicosanoids and skin inflammation and immunity. It was quite natural to equate the synthesis of 12*R*-HETE in psoriasis with the inflammatory disorders in skin (Baer *et al.,* 1991; 1995). On the other hand, many *in vitro* studies have proven modest pro-inflammatory activity (Fretland *et al.,* 1989). Also in normal skin 12*R*-HETE is barely detectable (Schneider *et al.,* 2004). So, the activity of 12*R*-LOX in normal healthy skin is quite different from that observed in psoriasis. Recently, an established study of gene inactivation in mice revealed that the biological role of 12*R*-LOX is important to seal the permeability barrier of outer epidermis (Kreig *et al.,* 2013). It was also established that the natural substrate of 12*R*-LOX is linoleate ester rather than arachidonic acid.

Two epidermal lipoxygenases, 12*R*-LOX and eLOX3 act in tandem to oxidize linoleic acid molecules of ceramide EOS (esterified omega-hydroxyacyl sphingosine) (Krieg *et al.,* 2013). Linoleate, is the most abundant essential fatty acid (EFA) present in epidermis of the skin. As a result of oxidation, very important hepoxilin intermediates are produced, which act as signals for recognition and hydrolysis of the oxidized linoleate ester to produce OS (omega-hydroxyacyl sphingosine) (A. Munoz-Gracia, *et al.,* 2013). The omega-hydroxy fatty acid chain of the resulting ceramide OS, covalently binds to proteins present in the corneocytes (containing polymerised proteins), with the help of transglutaminase, to form the cornified lipid envelop (CLE). This process is necessary to form a competent skin barrier.

1.7 Pathophysiology of Lipoxygenases and chemoprevention approaches

Leukotrienes (LTs), HETEs and Lipoxins (LXs) produced by the action of lipoxygenases, are small lipid molecules, involved in many diseases such as inflammatory responses, cancer, cardiovascular, neurodegenerative, renal diseases and metabolic syndromes (Kuhn and O’Donnell, 2006).

a) Inflammatory diseases comprise of many disorders that include atherosclerosis, arthritis, asthma, diabetes and hypertension. Lipoxygenase generated HETEs have been observed to be implicated in vascular pathogenesis and renal injury associated with diabetes as well. For eg., 5-LOX is abundantly expressed in atherosclerotic lesions. An increased 12/15-LOX mRNA and protein expression parallels the increases of fibronectin and other factors responsible for diabetic nephropathy. Additionally, 12/15-LOX enzymes oxidise LDL and appear to contribute to the pathogenesis of atherosclerosis (Ezaki M, *et al.,* 1995).

b) Disturbance in the critical balance controlling cellular proliferation and cell death is the hallmark of cancer. The LOX derived products act on different growth factor receptors and induce signalling cascade, which stimulate tumour cell growth. Leukotrienes and HETEs exert profound biological effects on development and progression of human cancers e,g., 12-LOX mRNA expression has been documented in many tumor cells like prostate, colon, and epidermoid carcinoma (Honn *et al.,*1994; Chen *et al.,*1994). 5-HETE directly stimulates prostate cancer cell growth (Ghosh and Myers, 1997). Expression of 5-LOX mRNA in lung cancer tissues, links its involvement in lung cancer (Avis *et al.,* 1996). In breast cancerous tissue, 12-LOX mRNA expression was observed to be increased by 3-30 fold when compared to normal tissue, which barely showed any levels (Natarajan *et al.,* 1997) Similarly, higher amounts of mRNA were documented in breast cancerous cell lines (MCF-7 and COH-BR1) compared to non-cancerous breast epithelial cell line (MCF-10). There have been considerable evidences which support role of lipoxygenase products in development of epidermal tumors. 12(*S*)-HETE levels were reported to be (50-60) fold greater when compared with the normal epidermis (Krieg *et al.,* 1995). Additionally, 12- and 15-HETE are reported as the predominant metabolites in squamous epithelial carcinoma of head and neck (Liu, et al 1994).

c) Lipoxygenase metabolites influence renal haemodynamics and have been recognized to contribute to development of hypertension (Fulton, *et al.,* 1995). In vascular tissue, the expression of LOX enzymes is highly localized to platelets (12-LOX), monocytes/macrophages (12/15-LOX) and neutrophils (5-LOX) (Brash, 1999). Glomerular mesangial cells and endothelial cells express LOX enzymes and produce leukotriene A_4 (LTA_4), 12-HETE and 15-HETE (Imig, 2000). These metabolites have

inflammatory actions, affect growth and regulate renal haemodynamics. 12-HETE and 15-HETE constrict renal vessels (Li *et al.,* 2005).

d) In animal models for diabetes and also in diabetic patients the generation of HETEs and LOX metabolites have been implicated in vascular pathogenesis and renal injury. 12-HETE, product of 12/15-LOX is increased in the urine of diabetic patients (Kang *et al.,* 2001).

All these observations put together clearly imply that, arachidonic acid metabolites produced by the action of lipoxygenases contribute to oxidative stress and inflammatory responses thus modulating inflammatory, proliferative and metastatic activities of the cells. Understanding the role of these metabolites will help for the theoretical basis for developing new cancer chemoprevention approaches targeted on LOX activity. In the last decade, an extensive research has shown that targeted inhibition of LOX can suppress the proliferation of a variety of tumor cells e.g., prostate, breast, colon, lung and bladder. A number of compounds that inhibit the metabolism of arachidonic acid by LOXs or its isoforms have been produced, however they need clinical settings (Pidgeon *et al.,* 2007).

1.8 Models to study gene functions

Model organisms are the species about which a lot of information is available with respect to their biological functions, ecology and behaviour, which makes them an attractive target to be used for experimental manipulations. Animal models have greatly improved our understanding to study human genetic diseases and for discovering targets for therapeutic drugs. Animal models are employed in the study of human disease, because of their similarity to humans in terms of genetics, anatomy, and physiology. Commonly used model organisms in research are *Escherichia coli*, *Saccharomyces cerevesiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio*, *Arabidopsis thaliana*, and *Mus musculus*. The success of a scientific study does not only depend upon the choice of the right biological question but also on the choice of a suitable model system. The choice for a model organism, for an experimental disease research, in turn depends on their unlimited supply and ease of manipulation.

For example, to obtain statistically significant data, the conditions associated with an experiment must be closely controlled. This means changing only one variable while keeping others constant, and then observing the effects of that change. Also, an adequate number of subjects must be used to statistically test the significance of the results of experiment.

Rodents are the most common type of mammals employed in laboratory studies and exhaustive research is being conducted on these rodents like rats, mice, guinea pigs, and hamsters. However, most of the studies, especially those involving diseases, have used mice as the model organisms. This is because their genomes are very much similar to that of humans and also because their litter size is more; can be handled easily, and their maintenance is relatively cost efficient.

Mus musculus- the laboratory mouse is the most commonly used mammalian model system. Mouse is an attractive mammalian model because of its small size, short life cycle, high fecundity, and availability of various inbred and out-bred strains. Laboratory mice have large number of inbred strains in which individuals are genetically identical and homozygous at all loci. The coat colour genetics of mice is one of the most well studied fields in mouse genetics, which is utilized in breeding experiments. The laboratory mice have gestation period of 18 to 20 days depending on the strains. By three weeks of age the pups are developed completely and independent enough to be weaned away from the mother. Females attain sexual maturity by 6 weeks and males by 8 weeks.

1.9 Transgenic technology.

Transgenic technology is one of the most important tools to investigate the genome of an organism. It was “Gordon and Ruddle”, who introduced the term transgenic in 1981 to describe mice whose germline had been genetically modified by the injection of the transgenes into one of the pronuclei of a zygote.

The first mouse gene transfers were performed in 1980 (Hardouin & Nagy, 2000). Presently transgenic term is used to characterize an organism whose genome has been altered by the stable integration of a recombinant DNA sequence.

Early embryonic stage is the best-suited stage for manipulating a genome when the cells have not yet differentiated into the precursor body cells and germ cells. There are three different types of methods, which are routinely carried out to generate transgenic animals. These include: 1) Transfection of the germ cells or the early embryos with recombinant viral vectors carrying a foreign gene, 2) Microinjection of the transgene into one of the pronuclei of the zygote, and 3) Transfection of pluripotent embryonic stem cells.

Microinjection of the transgene into one of the pronuclei involves the introduction of a small amount of defined genetic material directly into the location where a haploid set of the zygote's DNA is sequestered. This method of DNA transfection has proven to be very efficient in stable integration and germ-line transmission of the transgenic offsprings. This method thus has broad applications for laboratory and farm animals. It also offers a route for several applications in biomedical research.

In mammals, with exception of humans, the mouse is genetically the most thoroughly analysed species. Most transgenic mice used in biomedical research are generated by pronuclear microinjection of the recombinant transgene into one of the pronuclei of a zygote. The successful microinjection of embryonic cells of mammals has been developed with oocytes and zygotes of mice. Mammalian geneticists recognised both the biological and economical importance of mice strains. High fertility under laboratory conditions, short-generation span, relatively low cost, plenty of mouse genome information and the availability of appropriate, well characterized inbred strains and mutants are the advantages, which have been playing crucial role for the rapid development of transgenic technology.

1.10 Scope and Objectives

Scope:

Reports on lipoxygenases indicate that lipoxygenases (LOXs) and the signaling pathways that modulate the expression of LOXs are involved in various pathological implications. Although rapid and time-limited expression of lipoxygenase products is required for cells to fight injury and infection but a constitutive LOX activity leads to pathogenesis of several diseases, including asthma (Drazen , *et al.,*1994), Alzheimer (Piomelli, *et al.,*1987), arthritis (Jira *et al.,*1997), ulcerative colitis (Cole *et al.,*1996), psoriasis (Iversen at al., 1997), atherosclerosis (Natarajan and Nadler, 2004), renal failures, inflammatory diseases (Drazen JM, 1999) and cancer (Shureiqi and Lippman, 2001).

One of the important classes of lipoxygenase products are the HETEs. 5-LOX leads to formation of 5(S)-HETE and LTs (Leukotrienes). The 12- and 15-LOXs can form 12(S)- and 15(S)-HETE from arachidonic acid. HETEs including 5-HETE appear to enhance cancer cell growth by activating certain isoforms of Protein Kinase C (Natarajan and Nadler, 2004). 12-HETE is seen overexpressed in many epidermoid tumor tissues including breast, prostate, colorectal and lung cancers. 12(S)-HETE has potent effects on cell adhesion and metastasis in prostate carcinoma (Piao *et al.,* 2008). Other HETEs such as 8-HETE, the product of 8-LOX, act as the ligands for nuclear receptors (Yu *et al.,* 1995).

Nevertheless, LOX catalyze the peroxidation of membrane lipids, thus inducing structural and physical changes in the cell, which are important for differentiation and maturation. In this regard, LOX have been discussed to play a potential role in red cell maturation (Rapoport S *et al.,* 1986), as well as in the maturation of erythroid cells (Thorburn *et al.,* 1991), keratinocytes (Muga *et al.,* 2000), lens epithelium (Lysz *et al.,* 1994), macrophages and adipocytes (Yuk *et al.,*2007).

While more studies are needed to completely understand the mechanistic link between inflammation and tumorogenesis, several lines of evidences suggest that up-regulation of specific LOX in different type of carcinomas is related to increased tumor growth. Therefore, inhibition of these enzymes may represent a promising approach to

halt or reverse the progression of malignancies. Simultaneously, it becomes important to understand the molecular mechanism underlying the anti-tumor effect of specific, or general inhibitors against LOX, which will lead to the design of biologically and pharmacologically targeted therapeutic strategies. Additionally, an enzyme inhibitor acts as a pharmacological tool to elucidate the biological significance of the enzyme. To further investigate the role of these enzymes, genetically altered model organisms need to be analyzed.

Genetic evidences suggested that mutations in 12*R*-LOX have been associated with various skin diseases as in Non-bullous congenital ichthyosiform erythroderma (NCIE) and autosomal recessive congenital icthyosis (ARCI) (Yu *et al.,* 2005; Eckl *et al*., 2005; Moran *et al*., 2007). In addition to this, there was an earlier observation regarding increased amount of product of 12*R*-LOX enzyme, in skin diseases called psoriasis and other inflammatory conditions (Woolard *et al.,* 1986; Baer *et al*., 1991, 1995). These observations gave a strong indication of theoretical basis of involvement of 12R-LOX activity in skin barrier functions. Epp *et al.,*generated 12R-LOX knockout mice, which were deficient for water barrier functions and died within 3-4 hrs after birth (Epp *et al.,*2007).

In-vitro biochemical analyses have been used as a tool to investigate the hypothesis proposed based on observations obtained from genetic studies. Considering the importance of 12*R*-LOX enzyme, we decided to express this enzyme for biochemical studies. Since, the enzyme has been observed to be over expressed in psoriasis and proliferative dermatosis, the inhibitors of this enzyme may have therapeutic role. So we intended to screen inhibitors against this enzyme.

In addition to this, present study also undertakes to investigate the role of h-12*R*-LOX in proliferation and differentiation and thereby understanding its role in pathophysiology. In order to study the role of h-12R-LOX we resolved to generate the transgenic mice over expressing h-12-*R*-LOX by microinjection of DNA in pronuclear stage of mouse embryos. The h-12-R-LOX transgenic mice, thus generated, were characterized for phenotypic analysis.

Aim and Objectives:

The primary aim of the present study is to characterize h12*R*-LOX biochemically and to dissect out its role in pathophysiology. The specific objectives include:

1- Cloning, expression, purification and standardization of an assay system for 12*R*-LOX enzyme,

2- screening of natural compounds against 12*R*-LOX using the standardized assay system, and

3- generation of h12*R*-Lipoxygenase (LOX) transgenic mouse and its phenotypic analysis.

Chapter 2

Materials and Methods

CHAPTER 2: Materials and Methods

2.1 DNA manipulation

2.1.1 Plasmid DNA isolation from bacteria

2.1.1.1 Plasmid miniprep

Plasmid DNA was prepared by alkali lysis method. Single bacterial colony of DH5α strain was inoculated in 5 mL of LB broth containing appropriate antibiotics and incubated overnight at 37°C in a shaking incubator (Innova) at 180 rpm. Further QIAprep Spin Miniprep Kit (Qiagen) was used for isolation of plasmid DNA as per the instructions of the manufacturer. Briefly, overnight culture was transferred to a 15 mL centrifuge tube and centrifuged for five minutes at 6000*g*. Supernatant was poured off; pelleted bacterial cells were re-suspended by vortexing in 250 µL buffer P1 and the suspension was transferred into 1.5 mL micro centrifuge tubes. 250 µL buffer P2 was added and mixed well by inverting tubes gently 4-6 times. 350 µL buffer N3 was added and tubes were inverted immediately 4-6 times. The tube was centrifuged for 10 minutes at 13000*g*. The supernatant was loaded on to the QIAprep column in a two mL collection tube by careful pipetting with cut tips and centrifuged for one minute at 10000*g*. The flow through was discarded; 750 µL of buffer PE was applied to the column and centrifuged for one minute at 10000*g*. The flow through was discarded and the column was centrifuged for two minutes at 10000*g* to remove the residual liquid. The column was transferred into a fresh clean sterile 1.5 mL micro centrifuge tube. DNA was eluted by addition of 50µl ddH_2O and centrifuging for one minute.

2.1.1.2 Endotoxin free plasmid maxiprep

Endotoxin free plasmid DNA from large scale bacterial culture was prepared using QIAGEN **-** EndoFree Plasmid Maxi Kit (Qiagen) except that the inoculation was done in 250 mL LB broth instead of 100 ml LB broth containing appropriate antibiotics and incubated overnight at 37°C in a shaking incubator (Innova) at 180 rpm.

Bacterial cells were harvested by centrifugation at 6000*g* for 15 minutes at 4°C and supernatant was discarded. Bacterial pellet was resuspended in 10 mL buffer P1 by

vortexing. 10 mL buffer P2 was added and mixed gently but thoroughly by inverting 4–6 times and the tubes were incubated at room temperature for two minutes. During the incubation QIAfilter cartridges was prepared by first screwing the cap onto the outlet nozzle of the QIAfilter Maxi Cartridge. QIAfilter cartridge was placed into a tube. 10 mL chilled buffer P3 was added to the lysate, and mixed immediately by inverting 4–6 times. The lysate was poured into the barrel of the QIAfilter cartridge and incubated at room temperature for 10 minutes. Cap from the QIAfilter outlet nozzle was removed and the plunger was inserted into the QIAfilter maxi cartridge. The cell lysate was filtered into a 50 mL tube. 2.5 mL of buffer ER was added to the filtered lysate, mixed by inverting the tube 10 times and incubated on ice for 30 minutes.

During the incubation QIAGEN-tip 500 was equilibrated by applying 10 mL buffer QBT, and allowing the column to empty by gravity flow. After 30 minutes incubation the filtered lysate was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. QIAGEN-tip was washed with 2 x 30 ml buffer QC. DNA was eluted with 15 mL buffer QN and precipitated by adding 10.5 mL (0.7 volumes) room temperature isopropanol to the eluted DNA. The contents were mixed well and centrifuged immediately at 13000*g* for 30 minutes at 4°C. The supernatant was carefully decanted and DNA pellet was washed with 5 ml endotoxin-free 70% ethanol (prepared by adding 40 mL of 96–100% ethanol to the endotoxin-free water supplied with the kit) at room temperature. Ethanol was removed by centrifugation at 13000*g* for 10 minutes. Supernatant was discarded without disturbing the pellet. The DNA pellet was air-dried for 5 -10 minutes and the DNA redissolved in 1 mL of endotoxin-free H_2O.

2.1.2 Preparation of ultra competent cells and transformation

E.coli DH5α ultra competent cells were prepared as described by Inoue *et al.*, (Inoue *et al.,*1990). Inoue transformation buffer was prepared as described in appendix; filter sterilized through a pre-rinsed 0.22 μm filter and stored at –20°C.

A single bacterial colony of *E.coli* strain *DH5*α was inoculated and cultured for 6–8 hours at 37°C in 25 mL of LB medium in a 250 ml flask with vigorous shaking (250–300 rpm). The starter culture was used to inoculate three one liter flasks, each

containing 250 mL of LB broth. The first flask received 10 mL of starter culture, the second received four ml and the third received two ml. All three flasks were incubated for 14 hours at 18–22°C with moderate shaking. Optical Density (OD) was read at 600 nm. OD of all three cultures was monitored every 45 minutes. Once the OD_{600} of one of the cultures reached exactly 0.55, the flask was transferred to an ice-water bath for 10 minutes. The other two cultures were discarded. Cells were harvested by centrifugation at 2500*g* (3900 rpm in a Sorvall SLC-1500 rotor) for 10 minutes at 4°C. The cells were gently resuspended in 80 ml of ice-cold transformation buffer. The cells were harvested by centrifugation at 2500*g* (3900 rpm in a Sorvall SLC-1500 rotor) for 10 minutes at 4°C. The transformation buffer was poured off and centrifuge tube was stored open on a stack of paper towels for 2 minutes. The cells were gently resuspended in 20 mL of ice-cold transformation buffer. 1.5 ml DMSO was added; the bacterial suspension was mixed by swirling and then stored in ice for 10 minutes. The competent cell suspension was dispensed as aliquots of 70 µl into chilled, sterile micro centrifuge tubes and snap-frozen in liquid nitrogen. Competent cells were stored at -70°C until used.

In a typical transformation reaction 4-5 µl of ligation mix or 1-2 ng of plasmid DNA was incubated on ice with 70 µl ultra competent cells for 25-30 minutes, followed by a heat shock at 42°C for 1.5 minutes. 900 µl of plane LB media was added and allowed to grow at 37°C for 45 minutes. The cells were plated on to LB agar plate containing appropriate antibiotics and incubated overnight at 37°C. For blue-white colony selection forty µl of 2% X-gal (Sigma) solution and 7 µl of 20% IPTG (Sigma) solution were spread on to the LB plate containing appropriate antibiotics.

2.1.3 Restriction digestion of plasmid DNA

Plasmid DNA was digested with various restriction endonucleases (New England Biolabs) as per the manufacturer's instructions, except that the enzymes were used 2 to 3 fold excess of the recommended units.

2.1.4 Isolation of DNA from agarose gel

Fragments of DNA (From PCR or restriction digestion reactions) were isolated from the gel using Gel extraction kit (Qiagen) as per the manufacturer's instructions. Briefly, gel fragments containing the DNA band were excised with clean sharp scalpel

and weighed in micro centrifuge tube. Three volumes of buffer QG was added to one volume of gel and incubated at 50°C until the gel slice was completely dissolved. One gel volume of isopropanol was added to the sample and mixed by inverting the tube. The sample was applied on to the QIAquick/minelute column placed in two ml collection tube and centrifuged for one minute at 10000*g* to allow the DNA to bind to the column. Flow-through was discarded and the column was washed with 0.75 ml of buffer PE and centrifuged for one minute at 13000*g*. Flow-through was discarded and the column was for centrifuged for an additional one minute to remove residual liquid. QIAquick/minelute column was placed in a clean 1.5 ml micro centrifuge tube. To elute DNA 50 µl (for QIAquick column) or 20 µl (for minelute column) of pre-warmed (65°C) ddH_2O was added to centre of the membrane in the column and centrifuged for one minute at 13000*g*.

2.1.5 End filling reactions

Plasmid DNA digested with appropriate restriction enzymes was precipitated with 0.1 volume 3M NaOAc and two volumes of absolute alcohol. DNA was pelleted by centrifugation at 13000*g* for 30 minutes and was washed with 70% ethanol. The DNA pellet was dissolved in sterile ddH_20. The end filling was performed as described below:

Digested DNA	X µl (~ 1 µg)
NEB Buffer 1,2,3,4	2.5 µl
dNTPs (2.5 mM each)	1.0 µl
Klenow	0.2 µl (10U/ µl)
ddH_2O	Up to 25 µl

The above components were mixed well in micro centrifuge tube and the reaction was carried out at 25°C for 30 minutes.

2.1.6 Ligation Reactions

Ligation reaction was carried out at 22°C for 2 hours with following components:

Vector DNA	~25-40 ng
Insert DNA	3-5 times molar concentration of vector DNA
10X NEB ligation buffer	1 µl
T4 DNA ligase	0.4 µl (50U/ µl)
ddH_2O	Up to 10 µl

2.1.7 Screening of recombinant clones by cracking method

The colonies were screened for the recombinant clones by a modified cracking method, which was adapted from protocol described by Maniatis *et al.,* (1982). Individual colonies were picked with a sterile toothpick and smeared at the bottom of a micro centrifuge tube containing 200 µl LB media with appropriate antibiotics and allowed it to grow for 12 hours at 37°C in shaking condition. 50 µl of freshly prepared cracking buffer (refer to appendix) was added and the contents were vortexed for 1-2 minutes, incubated for 5 minutes at 70°C on a water bath and allowed to cool to room temperature. 50 µl of chloroform and 10µl 6X loading dye were added to the tube and vortexed for a brief period. The contents were centrifuged at 10000*g* for 5 minutes and to analyze for shift, upper aqueous layer was loaded in 0.8% agarose gel along with the control plasmid and DNA size marker. The clones showing slow mobility were presumed to be recombinant and were cultured overnight in five ml LB broth containing appropriate antibiotics. Plasmids were isolated and quantified spectrophotometrically.

2.2 Vector Designing

2.2.1 Construct preparation for transgenic mice

pCMV6-Neo-h12R-LOX plasmid got form OriGene (USA) containing *ALOX12B* complete cDNA along with 3' and 5'UTR was initially used for microinjection experiments. pCMV6-Neo-h12R-LOX plasmid contains CMV promoter which will drive the expression of h12R-LOX product in all tissues. For skin specific overexpression of h12R-LOX another vector was constructed which was named as pGE.3Z-K14-h12R-LOX. This vector contains full length cDNA of h12R-LOX under Keratin14 promoter, a betaglobin intron and keratin14 polyA tail. pGE.3Z-K14 beta globin cassette, a gift from (Elian Fusch from Rockfellar University, USA), was used as the basic vector for cloning of h12R-LOX under K14 promoter. Human12R-LOX was cloned in this cassette at XbaI site under Keratin14 promoter. Full length cDNA of h12R-LOX, along with 3' and 5'UTRs from pCMV6-Neo-h12R-LOX was released by NotI digestion and was first subcloned in pBKS plasmid by blunt end ligation at SmaI site. Now from this plasmid pBKS-h12R-LOX, whole cDNA was released by XbaI digestion and was ligated into pGE3Z.K14 linearised by XbaI. For generation of transgenic mice for h12R-LOX with overexpression only in skin, pGE3Z.K14-h12R-LOX vector was double digested with SmaI/HindIII to release the whole cassette containing promoter, betaglobin intron.cDNA and polyA tail. This fragment was then purified and injected into mouse embryos.

2.2.2 Construct preparation for over expression in bacteria.

For overexpression of h-12R-LOX in bacteria and purification of the recombinant protein h12R-LOX cDNA was cloned in pET 28(b) (Novagen) with 6XHis-affinity tag placed at the N-terminus of the protein. The cDNA fragment with the coding region was amplified from pCMV6-Neo-h12R-LOX plasmid using primers containing overhangs for restriction sites (NdeI and HindIII). Proofreading enzyme Pfu (Invitrogen) was used to amplify the fragment. Amplified product was then blunt end ligated into EcoRV enzyme site of pBKS plasmid. pBKS – h12R-LOX plasmid was then digested with NdeI and HindIII to release the fragment containing h12R-LOX cDNA which was ligated with pET28(b) at NdeI/HindIII sites. The clone positive for insertion of the h-12R-LOX was transformed into Rossetta cells (BL21) and checked for the expression of the recombinant protein.

2.3 Protein Isolation and purification

The selected recombinant clone was grown in LB media containing the antibiotics for selection. It was allowed to grow at 37^0 C with shaking till it reached O.D of 0.5-0.6. At this O.D, IPTG (1mM) was added to the culture to induce expression of the recombinant protein. The culture was shifted to 18^0C for protein after IPTG induction. After 10-12 hrs., the cells were harvested by centrifugation at 6000 rpm in centrifuge at 4^0C, for 10 min. The cells were washed with PBS (Buffer) and were lysed in PBS containing lysozyme (conc.) and protease inhibitor cocktail (Rosch). The lysed cells were centrifuged at high speed (SS34 Rotor) at 13000 rpm for 30 min at 4^0C, to get a clear supernatant containing the soluble recombinant protein.

Purification of the recombinant protein was performed using nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography as per manufacturer's protocol. Briefly, Ni-NTA slurry (500 µl for 1 ltr culture) was added to the cleared bacterial lysate and mixed gently by shaking at 4° C for 60 min. Then the lysate- Ni-NTA slurry was loaded into a column and washed twice with wash buffer containing 20 mM imidazole and 300 mM NaCl. The imidazole ring is a part of the structure of histidine so imidazole itself can also bind to the nickel ions. At low imidazole concentrations, non-specific, low affinity binding of background proteins is prevented. In contrast, the 6xHis-tagged protein binds strongly to the Ni-NTA matrix and thus is not removed under these conditions. After washing the column to remove non-specific proteins, the protein of interest was eluted with buffer containing 250 mM imidazole, which displaces the His-tag fusion protein from the matrix. The purified protein was fractionated and the fractions were analyzed further by SDS-PAGE and confirmed by Western blotting analysis.

2.4 Antibody generation

10 months old rabbit weighing 2.5 kg was injected with 600-700 µg (~0.5 ml) of purified h-12R-LOX protein expressed in E.*coli* BL21 (DE3) strain, mixed and emulsified with 0.5 ml of Freund's complete adjuvant (Santacruz Biotechnology Inc). 5 ml blood was collected from the rabbit as pre-immune serum before antigen injection. 250 µl of the emulsified antigen was injected at 4 different sites on the thoracic region on either sides of the vertebral column, subcutaneously. After 14 days, booster dose of the antigen was given. For this, 200 µg (~0.5 ml) of the antigen was mixed and

emulsified with Freund's incomplete adjuvant (0.5 ml) (Santacruz Biotechnology Inc). 250 µl each of the emulsified antigen was injected at 4 different sites on the thoracic region on either sides of the vertebral column, below the skin. The mouse was bled 1 week after the booster dose and the titer of the antibody was examined by western blotting. About 30 ml of blood was collected from the rabbit. The collected blood was kept in slanting position for about 30 minutes followed by centrifugation at 2200g for 10 minutes. The serum that was in the supernatant was collected, mixed with sodium azide and stored at -30°C until use.

2.5 Protein Analysis

2.5.1 Western blot analysis

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was carried out by the method described by Laemmli (Laemmli, 1970) in a discontinuous buffer system. Gel of 1.5 mm thickness was cast and run on a vertical gel apparatus (Broviga, India). The resolving gel (6%-12% acrylamide, 0.375 M Tris.HCl pH 8.8, 0.1% SDS) and stacking gel (5% acrylamide, 0.125 M Tris.HCl pH 6.8, 0.1% SDS) were polymerized by the addition of TEMED (12 µl for a 15 ml gel) and freshly prepared ammonium persulphate solution (150 µl of a 10% solution for 15 ml of gel mixture). The protein samples were boiled in 1X Laemmli buffer (50 mM Tris.HCl pH 6.8, 100 mM DTT, 7% SDS, 0.1% bromophenol blue, 10% glycerol) for 5 minutes and loaded on the gel. Electrophoresis was carried out in electrophoresis buffer (0.025M Tris.HCl, 0.250 M glycine pH 8.3, 0.1% SDS) at a constant current of 20 mA till the samples entered the stacking gel and then at 40 mA through the resolving gel. After the run, the gels were stained with 0.25% Coomassie Brilliant Blue R250 in 100 ml of methanol: acetic acid: water (40:10:50) for 2-3 hours and destained in 200 ml of methanol: acetic acid: water (40:10:50) for 3-4 hours on a shaker, with several changes of the destaining solution.

For silver staining gel was fixed in methanol: acetic acid: water (40:10:50) for 1 hour, followed by 2 washes of 50% and 30% methanol for 30 minutes each. To remove methanol gel was washed with water. The gel was sensitized with 250 mg/L of

$Na_2S_2O_3$ for one minute and washed with water. Gel was impregnated (2g/L $AgNO_3$, 750 µl of 37% Para formaldehyde) for 20 minutes. Colour was developed in developer (60g/L Na_2CO_3, 500 µl of 37% Para formaldehyde) for 10-20 minutes. Colour development was stopped by adding methanol: acetic acid: water (40:10:50) mixture. Finally gel was stored in 7% glycerol.

Western transfer of proteins

The protein lysates were separated on SDS-PAGE and gels were equilibrated for 10 minutes in transfer buffer (192 mM glycine, 25 mM Tris base, and 20% methanol) and electro blotted onto a PVDF membrane by wet transfer apparatus (Bio-red). The PVDF membrane was pre-wetted in methanol, rinsed in distilled water and equilibrated in transfer buffer. The gels were placed in contact with the membrane and sandwiched between 6 pieces of Whatman No. 1 paper on each side. The sandwich was then placed between electrodes with the membrane facing the anode. The proteins were transferred for 1.5 hour using a current of 0.8 mA/ cm^2 blot. After transfer the proteins were visualized by staining with Ponceau-S dye (0.1% Ponceau-S dissolved in 5% acetic acid) and the positions of the protein molecular mass markers were marked on the membrane for immunodetection by chemiluminescence reaction.

The PVDF membrane containing electro blotted proteins was blocked with 5% BSA or 5% milk in TBS containing 0.05% Tween-20 (TBST) for one hour at room temperature. The blot was incubated in the primary antibody diluted in TBST for 3 hours at room temperature and washed for an hour with several changes of buffer. The blot was then incubated with HRP-conjugated anti-IgG antibody diluted in TBST for 1 hour and washed extensively as described above. Immunoreactivity was determined with the use of luminol as substrate and detected by chemiluminescence using an ECL detection kit as per the manufacturer’s instructions.

Deprobing of protein blots

To reprobe the western blots, the bound antibodies were stripped off from the PVDF membrane by incubating the membrane in stripping buffer (0.1 M β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH: 6.7) at 50°C for 30 minutes with constant shaking in the water bath. This step was repeated twice if necessary. The blot

was then washed thoroughly with distilled water initially and then with TBST with many changes till the β-mercaptoethanol smell disappeared.

2.5.2 Immunocytochemistry

Fixing of cells

The cells on cover slips were fixed directly in six well polystyrene plates. Cover slips containing cells were rinsed with PBS and incubated with 3.7% formaldehyde in PBS for 10 minutes at room temperature followed by permeabilisation with 0.5% Triton X-100 for 6 minutes at RT.

Blocking

The fixed cells were incubated with 3% BSA or 5% horse serum in PBS for one hour at room temperature in order to block non-specific binding during incubation with primary and secondary antibodies.

Antibody incubation

The blocked cells were incubated first with the primary antibody diluted appropriately in PBST or blocking buffer or 3% BSA in PBST for one hour at room temperature. The slides were washed for 5 minutes with PBST. The wash was repeated twice. This was followed by incubation with secondary antibody conjugated with fluorophor for 45 minutes to one hour at room temperature. Then slides were washed thrice with PBST for 5 minutes each. The cross-reactivity of the secondary antibody conjugates to non-specific proteins was tested prior to the experiments. Cover slips were mounted in Vectashield, a glycerol-based mounting medium containing anti-fade reagent, with DAPI added to a final concentration of 1 μg/ml.

Immunofluorescence microscopy

Confocal laser-scanning immunofluorescence microscopy (CLSM) was carried out with a Zeiss LSM510 META confocal microscope. Mercury arc lamp for Zeiss system was used for excitation and a 435-485 band pass filter for detection of the DAPI signal. The 488 nm laser was used for excitation and a 500-530 band pass filter was used for detection of FITC and Alexa-488, the 543 nm laser was used for excitation and a 565-615 band pass filter was used for detection of Cy3. The 633 nm laser was used

for excitation and a 650-670 band pass filter was used for detection of Cy5. Images were analyzed using LSM510 META software (Carl Zeiss, Germany) and assembled using Adobe Photoshop CS2.

2.6 HPLC Assay

Either purified protein or bacterial cell lysate, which expressed 12R-LOX protein, was added to a total volume of 0.5 ml of phosphate buffered saline (7.4). The substrate concentration used was 0.1 mM (final concentration). The mixture was vortexed and incubated for 15 min at 37° C. After the incubation time, hydroperoxy fatty acids formed in the reaction were reduced to their more stable hydroxy derivatives by addition of sodium borohydride. After 5 min the reaction was acidified to pH 3 with 50 µl of acetic acid and then the proteins were precipitated with the addition of 0.5 ml of methanol. The total mixture was centrifuged (14000 rpm for 15 min) to remove the 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 solvent system used was methanol: water: acetic acid in the ratio of 80: 20: 0.1 with a flow rate of 1 ml/min. The chromatograms were followed by monitoring the absorbance at different wavelengths:

-235 nm for conjugated diene [hydro (pero) xy fatty acids]

-242 nm for double conjugated dienes [DiH (p) ETEs]

-210 nm for polyenoic fatty acids

-270 nm for conjugated trienes

The product peaks from RP-HPLC were identified by comparing with authentic standards under identical experimental conditions.

2.7 Pronuclear microinjection of DNA into the mouse embryos

2.7.1 Preparation of DNA

The plasmid DNA was isolated using Endotoxin free maxi kit (Qiagen) from a 250 ml bacterial culture. See section 2.5.1.2 for the detailed protocol.

2.7.2 Restriction digestion of the plasmid DNA

2µg of the plasmid DNA was linearised by double digestion using SmaI and HindIII enzymes for 3hours. The linearised DNA was first checked on ethidium bromide agarose gel for its complete digestion. The remaining digested DNA was run on ethidium bromide free ultrapure agarose gel with 0.5X of the running buffer and at low voltage 50V for good resolution of the DNA fragments.The gel was stained using methylene blue to detect DNA bands. The DNA was eluted into microinjection buffer (5 mM TrisCl, 0.5mM EDTA, pH 8.0) using the gel elution kit (Qiagen) and the DNA content was quantified with a Nanodrop.

2.7.3 Collection of pronuclei

F_1 progeny of the cross between C57BL/6J and CBA mice were used for super ovulation. 5-6 F_1 females were injected intra-peritoneally with 5 injection units (IU) of Pregnant Mare's Serum Gonadotrophin (PMSG) in the afternoon (~2.30 pm) of day 1. After 46–48 hrs., at day 3 (~12.30 pm), these females were injected intra-peritoneally with 5 IU Human Chorionic Gonadotrophin (HCG) to induce ovulation 10–12 h later. Following the HCG injection, in the evening at 5:00 pm, the females were paired with F_1 males in a 1:1 ratio, overnight. To obtain pseudo-pregnant females for the purpose of transferring the embryos after microinjection, 5 CD-1 females in estrus (as evidenced by a swollen, moist and pink vagina) were selected and paired with vasectomized F_1 males overnight in order to induce hormonal stimulation. Next morning, the F_1 females were checked for vaginal plugs and were selected to be opened up for embryo collection. Similarly, the CD-1 females were also checked for the plugs and were left in the animal facility, which were later used for the re-implantation of the injected embryos. The plugged F_1 females were sacrificed by cervical dislocation. The animals were surgically exposed and the oviducts were collected into M_2 medium. The oviducts were cut open to release the embryos. These embryo clumps were given hyaluronidase treatment. Good quality embryos were selected and were transferred to M_{16} medium with the help of a mouth pipette and were left in CO_2 incubator until they were injected with the DNA.

2.7.4 Microinjection of the DNA and transfer of the injected embryos

The purified DNA fragment (mentioned in 2.5.2) was diluted to a standardized concentration of 4 ng/µl and was injected into the pronucleus (containing both male and female nucleus but are not yet fused) of the single cell embryo. The injections are done in M_2 medium using a micromanipulator. After the injection, the injected embryos which are not lysed are stored in the M_{16} medium at 37 ^{o} C and 5% CO_2. Following this, unlysed embryos were selected and 15 to 18 embryos were transferred into the uterus of each pseudo-pregnant CD-1 female mouse. These CD-1 females deliver the young ones after 20 days.

2.7.5 Screening of the progeny for transgene integration

After 21 days of birth of the young ones from CD-1 females, the pups were weaned and were numbered by punching their pinna. Less than 1cm of their tails were cut and genomic DNA was isolated. Using P.C.R screening strategy these DNA samples were checked for the transgene integration. Southern hybridization was finally carried out to confirm the integration and to know the copy number of the transgene.

2.8 Isolation of genomic DNA

2.8.1 Isolation of genomic DNA from mouse-tail

Mice were anesthetized with 1.25 % avertin (Sigma) according to their body weights to cut the tail tips for genomic DNA isolation. Tails were collected in micro centrifuge tubes kept on ice. Tails were lysed over night at 55°C in tail lysis buffer (20mM Tris- buffer pH8.0, 5mM EDTA, 400mM NaCl, 1% SDS and 400µg/ml proteinase K). Next morning one volume of phenol-chloroform-isoamyl mixture (25:24:1) was added to the lysed tails and mixed properly. Tubes were centrifuged at 10,000*g* for 5 minutes. The aqueous phase containing the DNA was transferred to separate tube and one volume of isopropanol was added to precipitate the DNA. Tubes were centrifuged at 10,000*g* for 5 min. DNA pellets were washed with 0.5ml of 70% alcohol once. Finally, the DNA was dissolved in 100 µl of ddH_2O and kept at 4°C till further use.

2.8.2 Isolation of genomic DNA from cell line

Genomic DNA was prepared by a simplified method described by Larid *et al.,*(Laird *et al.,*1991). Once the cells were confluent in 24 well plates the medium was removed and the cells were washed with PBS twice. 0.5 ml lysis buffer (100 mM Tris.CL pH8.0, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 μg/ml proteinase K) was added to the cells and the plates were incubated at 37°C overnight. Next day the lysate was transferred into fresh micro centrifuge tubes. The DNA was precipitated by addition of one volume of isopropanol. The DNA precipitates were collected by centrifugation at 10000*g* for 10 minutes. The DNA pellet was washed with 70% ethanol. About 100 μl of ddH$_2$O was added to the DNA pellet. The tubes were incubated at 4°C for 2-3 days for complete dissolution of DNA.

2.9 PCR analysis of DNA

2.9.1 Primer designing

All primers were designed by using Primer3 Software. The length of the amplicon was set as per the requirement in each case. The optimum Tm was set at 60°C, with minimum Tm at 55°C and maximum Tm at 65°C. The GC content of the primers ranged between 40%-60%. The optimal length of primers was set at 20 nucleotides for amplification.

2.9.2 PCR amplification

All PCR reactions were performed with *Taq* DNA polymerase (Invitrogen; Bioron) as per the guidelines of the manufacturer. A typical PCR reaction was carried out in 20 μl containing 1X PCR buffer, 2.0 mM MgCl$_2$, 0.1 μM of each primers, 50 ng of genomic DNA or 2-5 ng cDNA, 200μM dNTPs and one unit of *Taq* DNA polymerase. Typically the annealing temperature was set 5°C below the calculated Tm of the primers. For some primer sets, the annealing temperature was optimized by gradient PCR. PCR was carried out for 30-35 cycles with one minute/ kb elongation time and final extension time of 7 minutes in PTC-200 Peltier Thermal Cycler (MJ research). Amplicons exceeding 3kb length were amplified with Phusion Hot Start (Finnzymes) as per the instructions of the manufacturer. PCR reactions were set as 20

μl reactions containing 1X polymerase buffer, 0.5 μM each primer, 5- 10 ng of cDNA, 200μM dNTPs and 1.0 units of DNA polymerase.

2.10 Southern analysis of DNA

2.10.1 Electrophoresis of DNA

Genomic DNA samples (~ 10 μg) were digested with appropriate restriction enzymes and size fractionated on 0.8% agarose gel at 2 volt/cm overnight in a continuous buffer system (40 mM Tris-acetate, 1 mM EDTA pH 8.0, with 0.5 μg/ml ethidium bromide). Loading buffer (1/10 volume of a solution containing 40 mM EDTA, 0.1% SDS, 30% ficoll, and 0.25% bromophenol blue) was added to DNA samples before applying onto the agarose gels. After electrophoresis the gels were imaged in gel documentation system (Bio-Rad) at 302 nm.

2.10.2 Transfer of DNA onto hybond-N^+ membrane

The DNA was transferred from the agarose gel onto hybond-N^+ membrane (Amersham) using a VacuGene XL Vacuum Blotting System (Amersham) by modifying the method described by Southern (Southern, 1975) as per the instructions of the manufacturer. After gel documentation, high molecular weight DNA was depurinated by treatment of the gel in 0.2N HCl for 10 minutes with gentle shaking. The gel was washed at least twice with distilled H_2O to remove traces of HCl. Nylon membrane was pretreated with 2X SSC and placed on the porous screen. The plastic mask was placed on the membrane in a way that it overlaps on each side of the membrane by approximately 3mm. The top frame was fixed and secured by tightening the four locking clamps. The gel was loaded on VacuGene XL blotting unit by gradually sliding the gel on to the nylon membrane. Care was taken to avoid trapping of air bubbles. VacuGene XL pump was switched on and the vacuum was stabilized at 50-60 mbar. The gel was always covered with 0.4N NaOH solution. The transfer was carried out for 1.5 hours. After the transfer the gel was stained with ethidium bromide and visualized under trans-illuminator to confirm the transfer of DNA. The membrane was rinsed with excess of 2X SSC to wash away the NaOH, air dried and stored appropriately until used.

2.10.3 Preparation of radiolabeled DNA probe

Double-stranded DNA was radiolabeled using random primers using random labeling kit (BRIT) as per the manufacturer's instructions. About 50 ng of double-stranded DNA and 5µl of random primers solution were denatured in a volume of 25 µl by boiling for 5 minutes and quick chilling on ice. This was followed by the sequential addition of 5µl of 10X reaction buffer, 5µl each of dCTP, dGTP, dTTP, 40 µCi of α-[^{32}P]-dATP and 2 units of Klenow enzyme. The reaction volume was made up to 50 µl and the reaction was carried out at 37°C for 1 hours. The probe was separated from the unincorporated nucleotides by Sephadex G-50 spun column chromatography and denatured in boiling water for 5 minutes and rapidly chilled on ice.

2.10.4 Purification and measurement of radio labeled probe

Sephadex G-50 column chromatography was used to purify radio labeled probe. A sterile 2 ml disposable plastic syringe was plugged with sterile glass wool and filled with the Sephadex G-50 slurry previously equilibrated with TE pH 8.0. The column was packed by centrifugation at 1000*g* for 5 minutes in a Sorvall HB-4 rotor at room temperature. Then 100 µl of the DNA solution to be purified was loaded and the column was centrifuged at 1000*g* for 5 minutes at room temperature to elute the purified DNA. For routine checking of labeling efficiency, samples were counted by Cerenkov's method. An aliquot of 1 µl of labeled DNA was spotted on a Whatman 3 mm filter and activity was counted scintillation counter.

2.10.5 Hybridization

Pre-hybridisation was carried out at 65˚C for at least 2 hours in a hybridization bottle containing 0.5 M sodium phosphate buffer (pH 7.4), 1mM EDTA and 7% SDS. Then the membrane was hybridized at 65˚C for 16 hours by the addition of denatured radio labeled probe (prepared by random primer labeling as described above). Post-hybridization, membrane was washed in 50 ml of 40 mM sodium phosphate buffer (pH 7.4), 0.5% SDS and 50 mM EDTA at 65˚C for 10 minutes three washes each. The hybridized blot was wrapped in a saran wrap or a thin plastic bag, sealed and exposed for 4-12 hours in a Fuji phosphorimager cassette and the imaging plate was scanned using a phosphorimager from Fujifilm.

2.11 Phenotypic analysis of mice

2.11.1 Breeding of h*12R*-LOX transgenic mice

Transgenic mice h12R-LOX males were bred with wild type F1 females. Offsprings were analyzed by Southern blot as well as by PCR. For all experiment littermates were used.

2.11.2 Sperm analysis of mice

2.11.2.1 Isolation of sperm from epididymis

h-12R-LOX transgenic mice and their wild type littermate mice were anesthetized with 2.5% avertin (Sigma) according to their body weight and euthanized by cervical dislocation. After dissecting the mice Epididymis was separated and kept in 1ml of Krebs Ringer bicarbonate media (Tulsiani *et al.,*1997).

HTF(Human Tubal Fluid)-Media : For maintenance of the mouse sperms

NaCl	101.6mM
KCl	4.7mM
KH_2PO_4	0.37mM
$Mg_2SO_4.7H_2O$	0.2mM
$CaCl_2$	2mM
$NaHCO_3$	25mM
Glucose	2.78mM
Pyruvate	0.33mM
Lactate	21.4mM
Glucose	5.6mM

Penicillin G Streptomycin	100U/ml 0.1mg/ml
BSA	5mg/ml

Media was filter sterilized with 0.22μm filter and stored at 4°C. Epididymis was dissected in HTF bicarbonate media and sperm were allowed to release in media at 37°C and various parameters were analyzed in this media.

2.11.2.2 Sperm motility analysis by computer assisted sperm analysis (CASA)

Sperms were diluted in Krebs Ringer bicarbonate media in 1:5 ratios. The motile swim up spermatozoa were then collected and counted in a Makler chamber using a HTM-CEROS (Hamilton Thorne, Beverly, MA, USA) Computer Assisted Semen Analyzer (CASA). Motility of sperms was recorded at 0h, 1h, 2h, 3h and 4h.

2.11.3 Histo-pathological examination of various tissues

12*R*-LOX transgenic mice and their wild type littermate mice were anesthetized with 2.5% avertin and euthanized by cervical dislocation. At various age points tissues were collected and fixed in 4% Para formaldehyde in PBS overnight. Next morning samples were washed in PBS and dehydrated by 30%, 60%, 80%, 95% and 100% ethanol gradients in shaking condition, each for one hour. After dehydration tissues were kept in chloroform for 1 hour. Finally tissues were dipped in paraplast tissue embedding medium (Fisher Health care) at 56°C for one hour and tissues blocks were prepared. Using microtome (*Leica*) 4.0μm thickness sections were taken in for each tissue and allowed to attach on positively charged slides (Fisher Scientific).

The slides were warmed for 10 minutes on a slide warmer at 45°C. Then the slides were immersed and washed in xylene for 10 minutes. The xylene wash was repeated thrice. The sections were rehydrated by soaking the slides in the following ethanol series: 100%, 95%, 80%, 60%, and 30% ethanol, for a minute at each concentration. Finally the slides were washed in tap water for 2 minutes. The sections

were stained in Gill's Hematoxylin for 2-3 minutes followed by a wash in tap water for 4 minutes. The slides were immersed in acidic alcohol solution for 5-10 seconds to reduce background and then in tap water for 5 minutes. The sections were further stained in Eosin solution for 10-20 seconds. Then the sections were dehydrated by passing three times through 95% ethanol for one minute each and then twice through 100% ethanol for 2-3 minutes each. Finally the slides were passed through xylene twice for 5 minutes each. Cover slips were mounted on the sections in the slides with DPX mountant and allowed to air-dry overnight in a hood. The sections were imaged in inverted phase contrast microscope (Leica).

2.12 Transfection of mammalian cells

NIH3T3 cells were grown in complete media (13.3g/L DMEM, 3.7g/L $NaHCO_3$, 10% Serum, 50µg/ml ampicillin and 50µg/ml streptomycin). After reaching confluence, cells were washed with 7 mL PBS at least twice, and then cells were trypsinized using pre-warmed (37°C) trypsin/EDTA solution (one ml for 75cm^2 flask), the flask was swirled to cover the culture completely and incubated in an incubator (37°C) for 120 seconds. Flasks were gently tapped from the sides to lift off the cells from the surface; approximately 5 ml of complete medium was added to neutralize the Trypsin/EDTA solution. Cell suspension was pipetted up and down several times to dissociate into single cells. Cells were centrifuged in 15 ml tubes at room temperature for 5 minutes at 1000*g*. Supernatant was removed and cells were resuspended in fresh medium. The cells were seeded at desired splitting ratio (generally 1:9) and cultured until confluent. Cells were frozen and thawed as described in section 2.6.

Cells were transfected using Xfect^{TM} (Clontech) as described by manufacturer's instructions. Briefly cells were transfected at 60-80% confluence as described in table 2.1.

Table 2.1 - Transfection of various cell lines using X-fect^{TM}

Culture Vessel	Surface Area/Well	Complete Medium	DNA	DNA Dilution Volume	X-fect Polymer Volume	Polymer Dilution Volume
24-well plate	2 cm^2	250 µl	0.75 µg	25 µl	0.23 µl	25 µl
12-well plate	4 cm^2	500 µl	1.50 µg	50 µl	0.45 µl	50 µl
6-well plate	10 cm^2	01 ml	5.00 µg	100 µl	1.50 µl	100 µl
10-cm dish	60 cm^2	10 ml	30.00 µg	600 µl	9.00 µl	600 µl

DNA was diluted in dilution buffer, mixed with X-fect polymer and incubated for 10 minutes to form complex at room temperature. Finally DNA and X-fect mix was added to cells and swirled to mix with complete medium. The complex was incubated with cells for 3-4 hours and changed with fresh complete medium. The transfected cells were incubated at 37 ^{o}C for 48 to 72 hours.

Table 2.2-LIST OF PRIMERS

Primer	Sequence	Information
H12-(R) LOXF H12-(R) LOXR	CAACTTCCCAGCGTCCATGCGTAATCCA TGGTGTTTTGGTCTCTGAGGTTTTTGTGTT	Exon14 end and Exon 15 start of h12R-LOX cDNA
HLOXFNI HLOXRHIII	GCAGCCCATATGGCCACCTACAAAGTCAGG GCGCTCAAGCTTCTAAATAGAAATGCTGTT CTC	For cloning h12R-LOX cDNA in pET 28(b)at NdeI and HindIII
H12-(R)LOXFXbaI H12-(R) LOXRPstI	ATCAATTCTAGACCCAGACACCTGCTCACT CACCACCAGCTG ATCAATCTGCAGTGGTGTTTTGGTCTCTGA GGTTTTTGTGTT	For subcloning EGFP in pG3Z.K14
Wdr13E2F Wdr13E2R	AACGCCTACCGTACACCAAC TGCTATAGGCACGAGCACTG	Wdr13Exon2 primers used as endogenous control for screening the transgenic mice.
Beta actin F Beta actinR	TGTTACCAACTGGGACGACA CCATCACAATGCCTGTGGTA	Beta actin used as an endogenous control
OutloxF- OutloxR-	GTAGTCGGCCAGGTAAATGTTCCCC CTACTACTACCTGGACCCGGTGC	Inverse PCR for identification of the transgene integration

Table 2.3-LIST OF ANTIBODIES USED

Antibody	Source
Anti-Beta	Abcam, Cat No-8226
Anti-mouse HRP	Amersham, Cat No-NA9310V
Anti- rabbit HRP	Amersham, Cat No-NA9340V
Anti- mouse FITC	Sigma, Cat No-F-5262

Anti-His	Santacruz
h-12R-LOX	In-House (generated in rabbit)

Table 2.4- LIST OF VECTORS USED

Plasmids	Information
pCMV6-Neo-h12R-LOX	For generation stable cell line
pET28(b)	For cloning and expression of h12R-LOX in bacteria
pBluescript SK	Used for subcloning in Keratin14 expression vector.
pG3Z:K14	For designing of the h12R-LOX transgenic vector
pEGFP	Vector used as a control for transfection experiments

Chapter 3

Human 12R-Lipoxygenase enzyme- expression, purification and characterization

CHAPTER 3: Human 12R-Lipoxygenase enzyme- expression, purification and characterization.

3.1 INTRODUCTION

Lipoxygenases are a class of non-heme iron oxygenases that catalyze the conversion of arachidonic acid (AA) and other polyunsaturated fatty acids to their hydroperoxy derivatives. These may be metabolized further to various bioactive lipid mediators including leukotrienes, lipoxins, hydroperoxyeicosatetraenoic acids (HPETEs), and hepoxilins. The products of these reactions are involved in myriad of biological events ranging from inflammation to cell development and differentiation. Lipoxygenases (LOXs) stereoselectively and regioselectively oxygenate the substrate, arachidonic acid, and are thus named as 5-, 8-, 12 and 15-LOX. 12*R*-LOX is the only lipoxygenase that directs molecular oxygen insertion into 12*R* position of arachidonic acid.

Importance of 12-HETE came into existence with the discovery of high levels of 12-HETE in psoriatic skin cells (Hammarstom *et al*., 1975). Later, it was found out that major enantiomeric product was of *R*-configuration, 12*R*-HETE (Woolard, 1986). Further studies revealed that 12*R*-HETE constitutes only minor fraction in normal human skin cells (Baer and Green, 1993) but becomes a predominant product in case of psoriasis and other inflammatory dermatoses, suggesting its possible role in the regulation of proliferation and differentiation of keratinocytes (Woolard , 1986; Baer *et al*., 1991, 1995). The enzyme responsible for the production of 12*R*-HETE was characterized from human keratinocytes and identified as 12R-Lipoxygenase (Boeglin, *et al*., 1998). 12*R*-Lipoxygenase utilizes arachidonic acid as a substrate and produces 12*R*-hydroperoxyeicosatetraenoic acid (12*R*-HPETE), which is acted upon by another epidermal lipoxygenase known as eLOX-3 to generate hepoxilins, which play significant role as signaling molecules (Brash *et al*., 2007).

Inactivating mutations in 12*R*-LOX and eLOX-3 genes are linked to genetic defect of autosomal recessive congenital icthyosis (ARCI) and to other inflammatory skin disorders (Eckl *et al*., 2005). Moreover, knocking out 12*R*-LOX gene in mice showed role of 12*R*-LOX in normal skin development. 12*R*-LOX deficiency in mouse results in death of newborn mice due to severe impairment of epidermal barrier

functions (Epp *et al*., 2007). Similarly, eLOX-3 deficiency in mice leads to postnatal death, of the offsprings, with lesser severe skin barrier defects as compared to those mice deficient in 12*R*-LOX (Krieg *et al*., 2013). These studies indicate that 12*R*-LOX – eLOX-3 pathway plays a key role in the process of epidermal barrier organisation and for proper skin development.12*R*-LOX and eLOX3 cooperate to produce hepoxilin **(Fig. 3.1A).** Arachidonic acid is almost absent from the outermost cells of epidermis of skin. However, linoleate esterified in the epidermal-specific ceramides is abundant and forms the natural substrate for 12*R*-LOX and eLOX3 in skin. Hepoxilin like products synthesised from the ceramides have been hypothesised to activate peroxisome proliferator activated receptors (PPARs). 12*R*-HPETE- derived (archidonate origin) hepoxilin and other products (trioxilins) have been shown to activate PPAR alpha (Yu et *al.,* 1995). Although linoleate derived hepoxilin type molecules have not been tested for the same in relation to barrier functions, hepoxilins act as signals for proper organisation of lipid bodies and corneocytes to form cornified lipid envelope (A Munoz-Garcia, *et al.,* 2013). PPARs act as transcriptional regulators of transglutaminase (Hanley and Feingold *et al.,* 1998), which are important enzymes for polymerizing proteins of cornified envelope to help seal the skin barrier. Moreover, PPARs regulate a variety of genes related to skin functions like differentiation, proliferation, immune response, wound healing and lipid metabolism (Schmuth *et al.,* 2008) **(Fig. 3.1B).**

In-vitro biochemical analyses have been used as a tool to investigate the hypothesis proposed based on observations obtained from genetic studies. Different point mutations were identified in eLOX-3 and 12*R*-LOX genes in non-bullous congenital ichthyosiform erythroderma (NCIE), thereby suggesting that both belong to the same metabolic pathway (Jobard F *et al*., 2002). The observation was later confirmed biochemically by expressing human eLOX-3 enzyme in bacteria and showed that eLOX3 functions as a hydroperoxide isomerase (epoxyalcohol synthase) by using the product of 12R-LOX as the preferred substrate (Yu *et al*., 2003). Using cellular homogenates prepared from HEK cells, transiently expressing murine 12R-LOX enzyme, Krieg and coworkers showed that mouse 12*R*-LOX uses methyl ester of arachidonic acid and not arachidonic acid as the preferred substrate (Krieg P *et al*., 1999). In further characterization, murine 12*R*-LOX expressed either in HEK cells or in baculovirus system was also found to use methyl ester of arachidonic acid as the

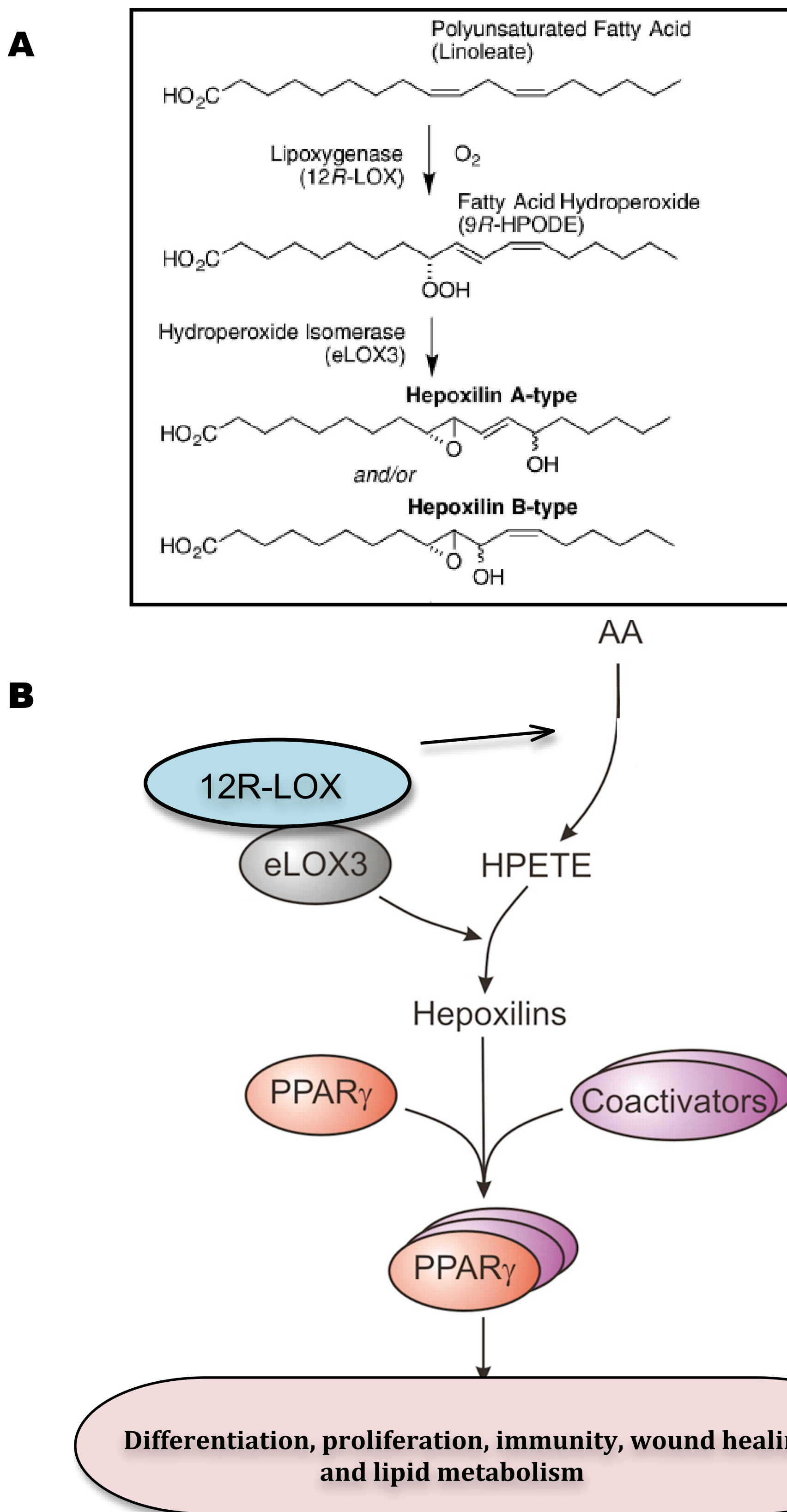


Fig. 3.1 (A) Lipoxygenase catalysis to hepoxilin 12R-LOX and e-LOX 3 cooperate each other in their activities. **(B)** Hepoxilins regulate PPARs, which is involved in various functions.

preferred substrate (Siebert *et al*., 2001; Meruvu *et al*., 2005). By mutational analysis, glycine residue at 441th position was proposed to be important for the positional specificity of 12*R*-LOX enzyme (Coffa and Brash, 2004; Meruvu *et al*., 2005). However, a recent study indicates that prediction of LOX specificity on the basis of primary structure is not always accurate and functional analysis is required to confirm product identity (Jansen C *et al*., 2011). 12*R*-LOX was believed to be present only in the skin cells but biosynthesis of 12*R*-HETE observed after incubation of arachidonic acid with homogenates of human tonsillar tissue gave mechanistic evidence for the presence of 12*R*-LOX enzyme in tonsils and its role in tonsillitis (Schneider C *et al*., 2001).

Although cell extracts have been widely used as enzyme source for biochemical assays of various LOX enzymes but have limitations in terms of protein abundance and less specific activities for the respective enzymes. The presence of the endogenous inhibitor of 12-lipoxygenase was identified in the cytosolic fraction of A431 cells, which can affect the activity of the enzyme when tested *in vitro*. To overcome such problems, heterologous expression systems are preferable, which help us to get high yields of protein that can be used for activity assays. However, difficulties have been reported in formation of the product, 12*R*-HETE, by using human12*R*-LOX protein even if expressed in higher amount in bacterial and eukaryotic expression systems (Sun *et al*., 1998; Krieg *et al*., 1998; Schneider *et al*., 2001). For structural studies, human 12*R*-LOX enzyme was purified in soluble fraction only when it was co-expressed with bacterial chaperones GroES and GroEL in an *Escherichia coli* expression system (Deb *et al*., 2011). Thus, there is a need of system that can express active h-12*R*-LOX enzyme, which can be used further for proper biochemical characterization of enzyme.

We report here the cloning and expression of human-12*R*-LOX gene in bacteria. Recombinant h-12*R*-LOX enzyme was expressed successfully as His-tagged protein in BL21-Rosetta strain. Further, we have purified this enzyme by Ni-NTA affinity chromatography and HPLC based assay was employed for measuring LOX activity. In our study we observed that h-12*R*-LOX enzyme utilizes methyl ester of arachidonic acid as a substrate and shows negligible activity towards arachidonic acid. The product of h-12*R*-LOX enzyme was confirmed by mass spectrometry. More importantly, this assay was employed for screening and identification of natural product based inhibitors of 12-*R*-LOX.

3.2 RESULTS

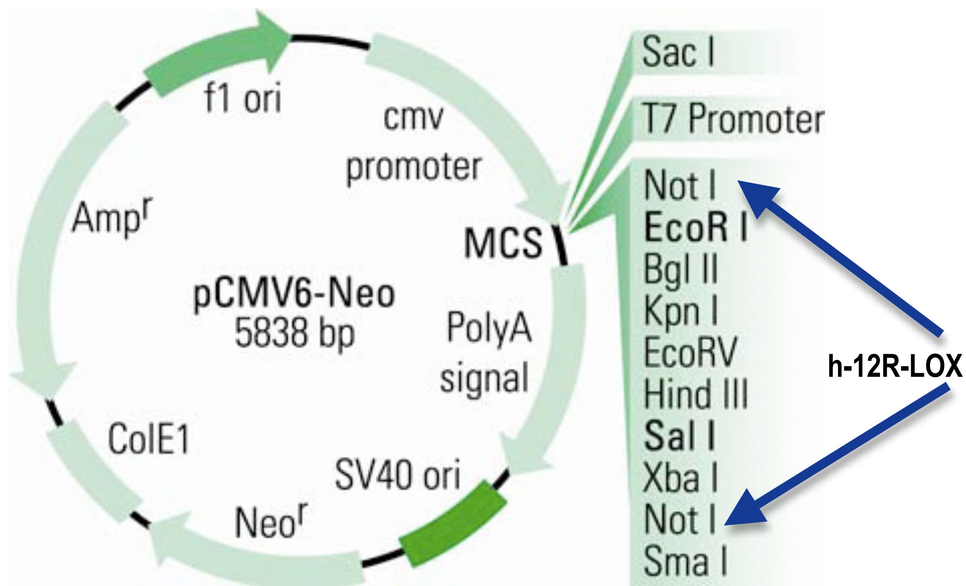
3.2.1 Expression of h-12*R*-Lipoxygenase in eukaryotic expression system.

pCMV6-Neo vector harbouring h-12*R*-LOX gene (with 5′ and 3′ UTR) was obtained commercially from OriGene, USA (**Fig. 3.2A**). The gene was sequenced and its expression was checked by immunofluorescence after transient transfection into NIH3T3 and HEK cell lines, as described in Material and Methods (**Fig 3.2B**). h-12*R*-LOX is cloned at NotI site in pCMV6-Neo vector and the expression of the cDNA is driven by CMV promoter and also has a SV40 ployadenylation signal. Neomycin (G418) is the eukaryotic resistance gene marker used for selection. This mammalian expression vector was used to express h-12*R*-LOX protein *in vivo* in fibroblast cell line (NIH3T3). The vector was first amplified using endotoxin free plasmid isolation kit from Qaigen as described in Materials and Methods. NIH3T3 cells were grown to confluence and then harvested by trypsinizing the cells. These cells were then electroporated (described in Materials and Methods) by the linearized plasmid pCMV6-Neo-h12*R*-LOX linearised at ScaI (present in ampicillin), a prokaryotic resistance gene marker. The electroporated cells were incubated at room temperature and then transferred to complete medium. After 24 hours, medium was changed to complete medium containing different concentrations of G418 (250 μg/ml, 500 μg/ml, 750 μg/ml and 1000 μg/ml) to select for neomycin resistance for selection of positive clones. After 15 days of selection, individual colonies were isolated for expansion and DNA analysis. After amplification of the individual colonies they were checked for the insertion of the cDNA by PCR using gene specific primers. These colonies were later checked for the expression of the protein by western analysis using polyclonal antibody anti-12*R*-LOX (**Fig. 3.3A**). Polyclonal antibody was raised in rabbits against unique 31-amino acid residues, which is specific only to 12*R*-LOX. We could see clear expression of h12*R*-LOX protein in the selected clones, which was absent in the normal NIH3T3 cell line.

3.2.2 Activity assay for h-12R-LOX using cell lysate from the stable cell line.

Cell lysate from h12*R*-LOX stably expressing clones were used as the protein source for the activity assay for h12*R*-LOX. Activity assay was performed in 1ml reaction with PBS (pH 7.2) buffer and substrate as arachidonic acid and cell lysate as

A



B

Untransfected

Transfected

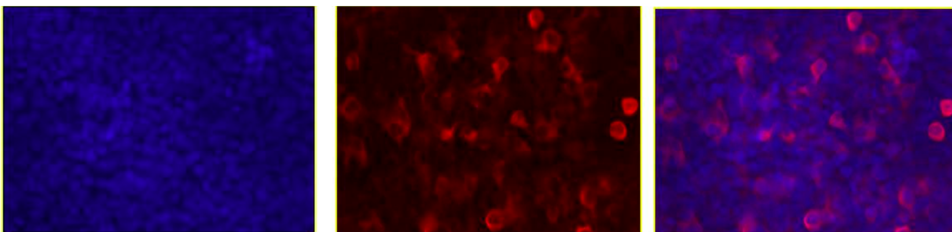
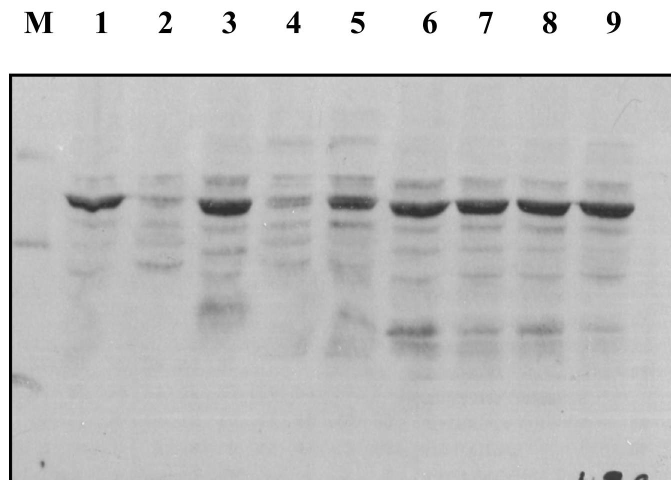


Fig. 3.2 Transient transfection of NIH3T3 cells for checking the expression of the cDNA (A) Map of pCMV6-Neo harboring h-12R-LOX gene along with 5' and 3' UTR's cloned at NotI site. **(B)** Immunofluorescence on NIH3T3 cells transiently transfected with the vector pCMV6-Neo-h12R-LOX using Lipofectamine. DAPI (Left), Cy3 (Middle), Overlay (Right).

A



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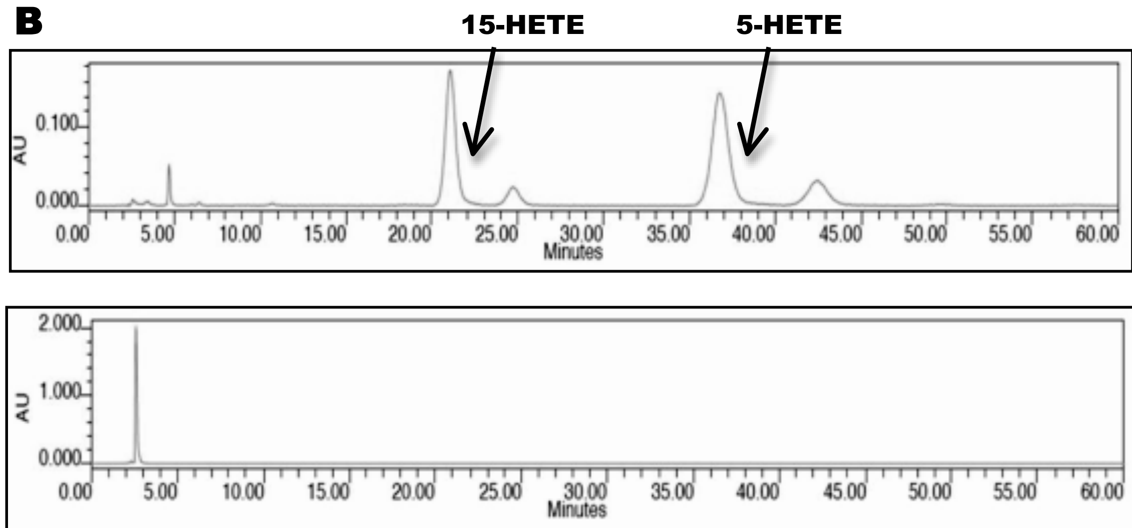


Fig 3.3 pCMV6-Neo- h12R-LOX was electroporated into NIH3T3 cells for production of stable cell line and positive clones were selected on G418 (Neomycin). (A) Western blot analysis of the cell lysates prepared from stable cell line expressing h12R-LOX. **M**-LMW, **1,3, 5-9**-+ve clones, **2&4-** –ve clones. **(B)** RP-HPLC Chromatogram**;** Upper panel shows the peaks for 15-HETE and 5-HETE which have been used as standards and Lower panel shows the chromatogram for the reaction product of h12R-LOX.

the protein source. After incubating the reaction at 37^0C, the reaction was stopped by acidification. The products formed were converted to their reduced form using sodium borohydride and then the extra proteins were precipitated by ice-cold methanol. All the contents of the reactions were centrifuged and the supernatant containing products were run on reverse phase HPLC. Unfortunately, we were not able to detect any product peak on HPLC (**Fig. 3.3B**)

3.2.3 Expression of h-12R-Lipoxygenase in prokaryotic expression host.

pCMV6 vector harboring h-12*R*LOX gene (with 5′ and 3′ UTR) was used to express the gene in bacteria, ORF of h-12*R*-LOX gene was amplified using suitable primer and cloned into pET28b vector to generate pET-12*R*-LOX (12*R*-LOX in-frame with 6X His tag), as described in Materials and Methods. pET-12*R*-LOX vector was initially transformed into BL21-pLys strain and induced with IPTG to check protein expression. To our surprise, we could not see induction in SDS-PAGE analysis (**Fig. 3.4B**), however, we could detect low amount of protein in western blot performed with anti-12*R*-LOX antibody (**Fig. 3.4D**). Analysis of nucleotide sequence of h-12*R*-LOX gene revealed presence of codon for few amino acids (Proline, Arginine etc.) which are rare in *E. coli* genome (**Fig. 3.4A**) and thus tRNA for these codons are depleted in *E. coli* cells. Hence, codon bias was suspected for the low protein production in the BL21-pLys strain. To circumvent this problem, pET-12*R*-LOX vector was transformed into BL21-Rosetta strain; which is engineered to have tRNA for these unique codons, and indeed protein of expected size (size~80 kDa) was expressed in considerable amount upon induction with 1 mM IPTG (**Fig. 3.4C**). The identity of protein was confirmed by western blot analysis using anti-12R-LOX antibody (**Fig. 3.4E**) and anti-His antibody (data not shown). The over-expressed protein band was further subjected to MALDI analysis to reconfirm human 12*R* -LOX protein's identity (data not shown).

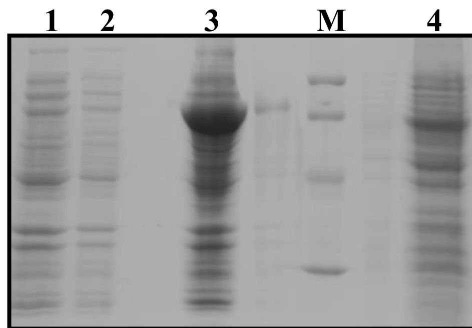
3.2.4 Purification of active recombinant His-tagged h12*R*-LOX.

Recombinant His-tagged h-12*R*-LOX enzyme was over expressed and purified using Ni-NTA affinity chromatography as described under the Materials and Methods. The protein was purified and analysed on SDS-PAGE. Two extra proteins of smaller sizes were observed on the coomassie stained SDS-PAGE gel (**Fig. 3.5A**). We tried to further purify the protein using Gel filtration column but we couldn't separate it out with the other proteins (**Fig. 3.5C** and **3.5D**). Overexpressed proteins tend to associate

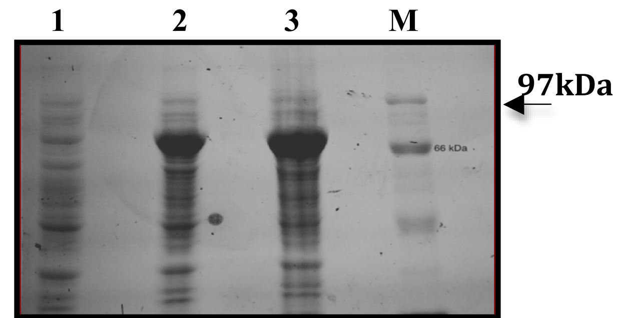
A

Amino acids	Major codon in *E. coli*	Major codon in h-12R LOX
Glycine	GGG/GGU/GGC	GGA/GGG
Proline	CCG/CCA/CCU	CCC

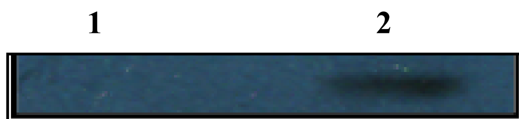
B



C



D



E

Fig. 3.4 Expression analysis of human 12R-LOX protein in *E. coli*. (A) Table showing codon usage for Glycine and Proline amino acids in *E.coli* compared to human 12R-LOX gene**. (B)** SDS PAGE analysis showing expression of 12R-LOX protein in BL21-pLYS strain (**1**-Induced, **2**-Uninduced) and in Rossetta strain (**3**-Induced, **4**-Uninduced) **(C)** The SDS-PAGE gel shows expression profile of h-12R-LOX protein after 1 mM IPTG induction for 8 hrs and 16 hrs in BL21-Rosetta strain (**1**-uninduced, **2**,**3**-Induced, **M**-Low molecular weight marker) (Amarsham Biosciences). **(D)** And **(E)** Western analysis of 12R-LOX proteins in BL21-Rossetta and in BL21 (pLys) strain respectively. Immunoblots were probed with anti-12R-LOX polyclonal antibodies, and detected by HRP conjugated secondary antibodies.

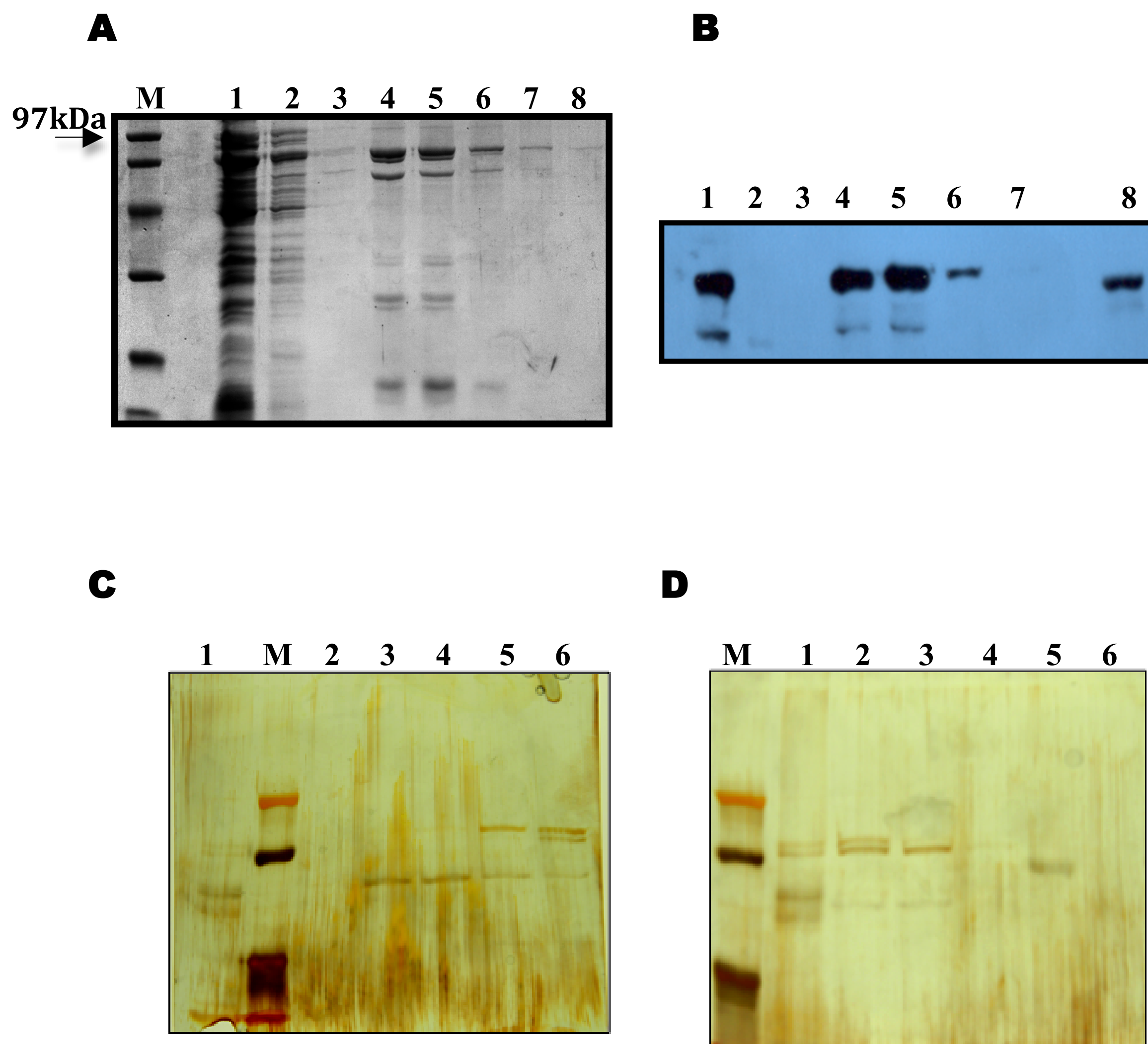


Fig. 3.5 Purification of recombinant h-12R-LOX protein by Ni-NTA agarose column chromatography. (A) SDS-PAGE analysis of protein fractions of h-12R-LOX protein following Ni-NTA agarose chromatography. **M-** Low molecular weight marker proteins.**1-** Flow through collected after cell lysate passed through Ni-NTA agarose column.**2-**Wash fraction collected after washing the Ni-NTA agarose column.**3-8-** are the eluted fractions of 6X his-tagged h-12R-LOX associated protein. **(B)** Western analysis of protein fractions of h-12R-LOX protein following Ni-NTA agarose chromatography. Immunoblots were probed with anti-12R-LOX polyclonal antibodies.1- Flow through, 2- Low Molecular Weight Marker, 3 to 7 – First five elution fractions, 8- protein lysate from A431 cell line which was used as the positive control for the western blot analysis. **(C)** and **(D)** FPLC fractions of the Ni-NTA purified protein h-12R-LOX. **M**-LMW marker, **1-** Ni-NTA purified protein and **2-6-** Fractions from the FPLC.

with the GroEL and other chaperones when expressed at low temperatures in order to avoid inclusion body formation (Vorderwulbecke *et al.,* 2003) and hence at this stage we speculate that co-purified proteins could be chaperones.

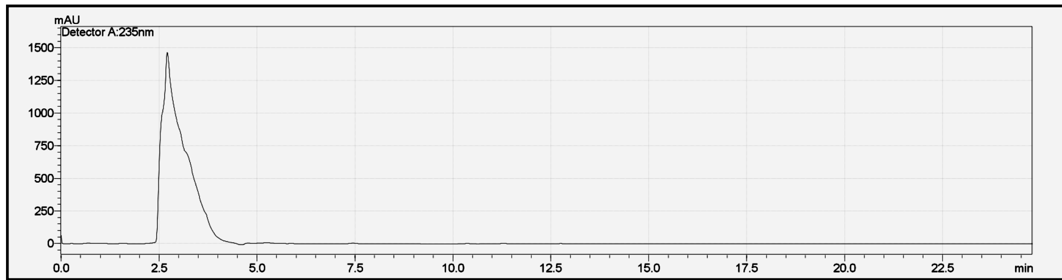
Western hybridization of purified proteins was performed with anti-12*R*-LOX antibody (**Fig. 3.5B**). Elution fragments E_2 and E_3 contained large amount of proteins as was observed in SDS-PAGE. Flow through (unbound proteins) also had large amount of 12*R*-LOX protein, which is possible in case of all Ni-NTA beads were already saturated with the proteins. Increased Ni-NTA beads only increase the non-specific protein binding to the beads thus we used the amount of Ni-NTA beads where we got maximum purity of proteins. As a control in the western analysis, we have also used A431 cell lysate; where 12*R*LOX protein is constitutively up regulated (Smita et al. 2009), and didn't notice any change in the migration pattern of both the proteins (**Fig. 3.5B**), suggesting absence of any major covalent modification of proteins expressed in A431 cells.

3.2.5 Standardization of activity assay for purified h-12R LOX enzyme

Though, different groups have expressed 12*R*-LOX protein in prokaryotic and eukaryotic expression vectors to obtain protein in large amount but have achieved minimal enzymatic activity (Sun *et al.,* 1998; Krieg *et al.*, 1998, Siebert *et al.,* 2001; Meruvu *et al*, 2005). Therefore, we decided to first check the activity of the purified 12*R*-LOX enzyme on its known substrate. Reverse-phase HPLC based assay was standardized to check the activity of h-12*R* LOX enzyme. The assay conditions and steps were used as described under Materials and Methods.

We started characterizing the enzyme for its activity using IPTG induced BL21-Rosetta cell extracts expressing h-12*R*-LOX enzyme (**Fig. 3.6C**). For all these assays saturating concentration of substrate (100µM) was incubated with protein concentrations of 3 mg/ml of cell extracts. RP-HPLC analysis of the product got from incubation of the cell extracts with arachidonic acid showed the formation of 12*R*-HETE peak (RT 5.3 mins) as only product with ~80 mAU at 235 nm (**Fig. 3.6B**) We didn't observe any HETE product peak when methyl ester of arachidonic acid was used as the substrate (**Fig. 3.6A**).

A



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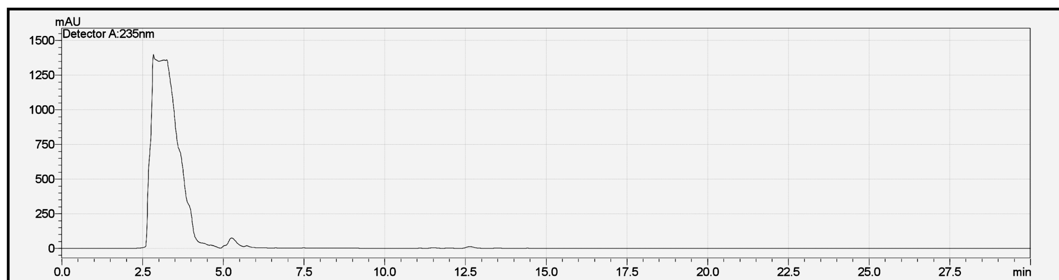
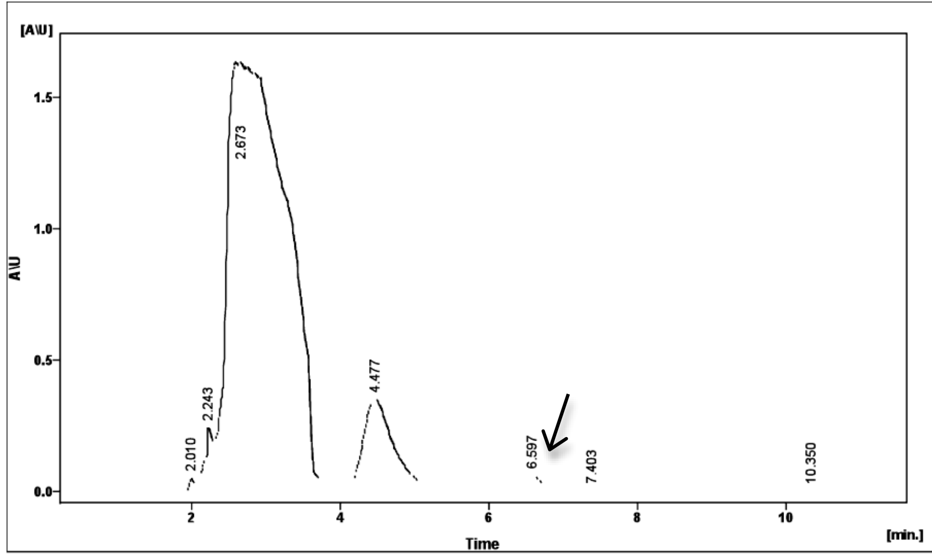


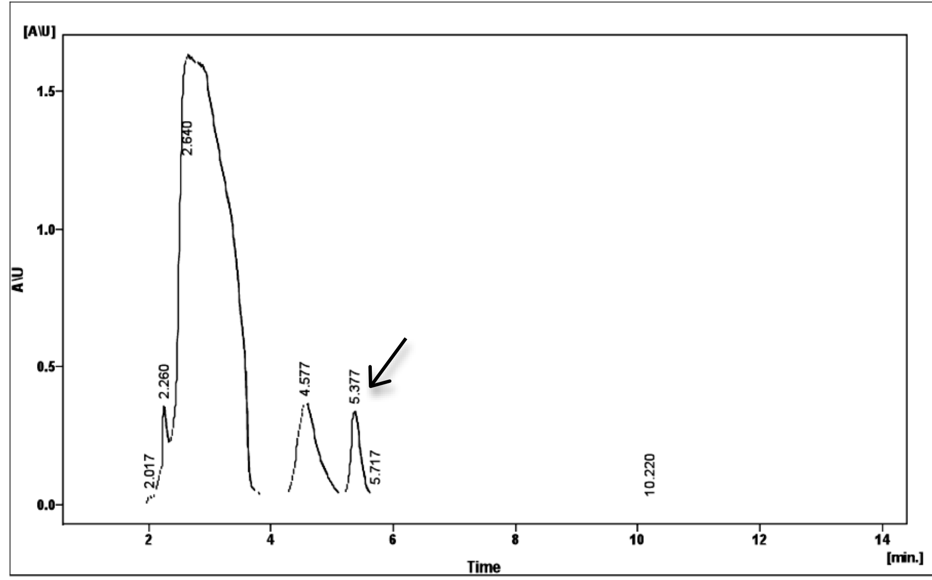
Fig. 3.6 Reverse phase HPLC analysis of the reaction products incubation of enzyme (source of enzyme being the Rosetta cell lysate expressing h12R-LOX) with AA or AA Me. (A) RP-HPLC chromatogram analysis when methyl ester of arachidonic acid was used as the substrate. **(B)** RP-HPLC chromatogram analysis when arachidonic acid was used as the substrate.

A

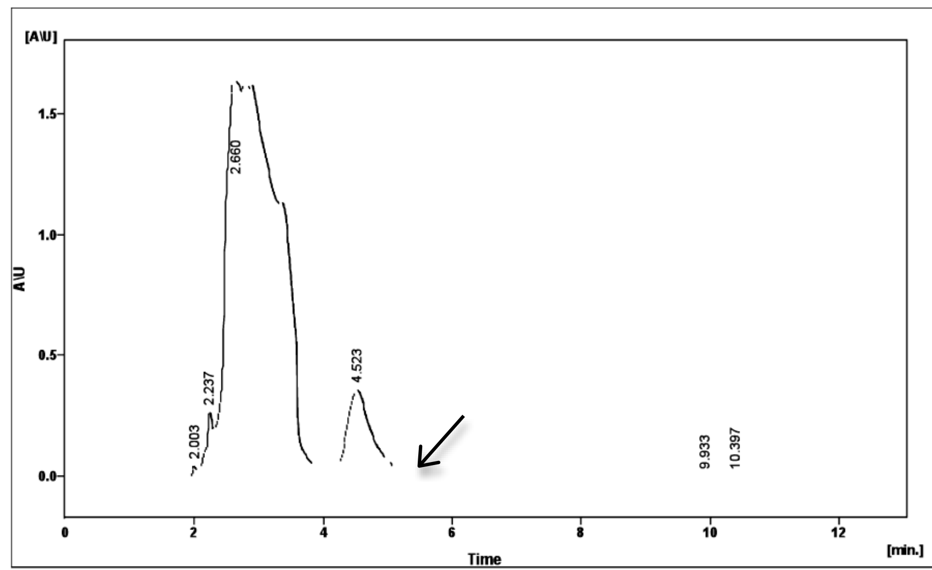
a)



b)



c)



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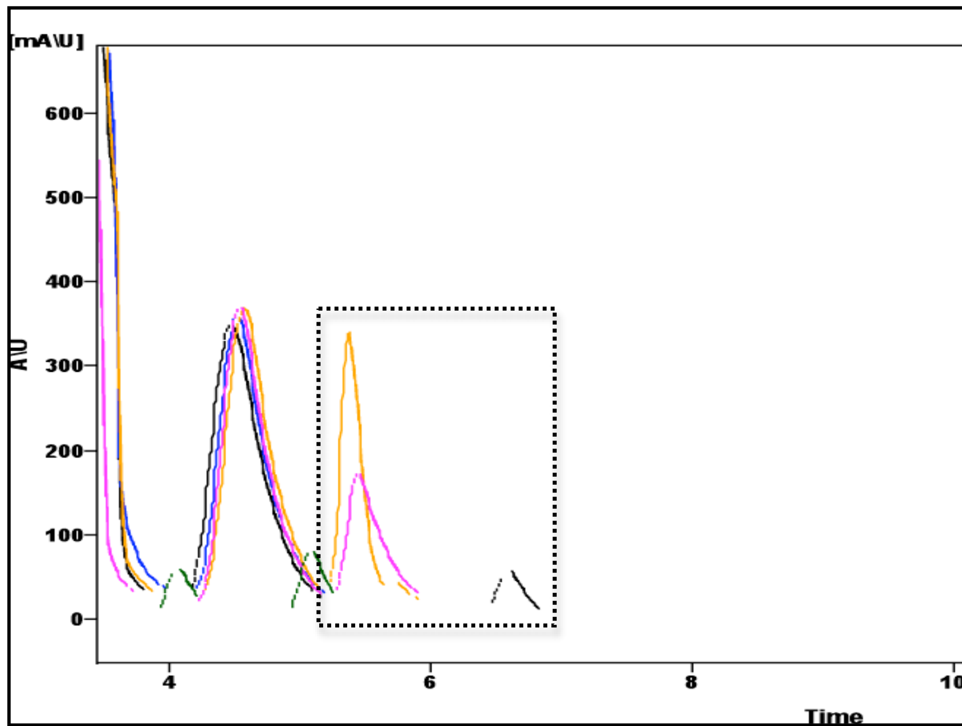


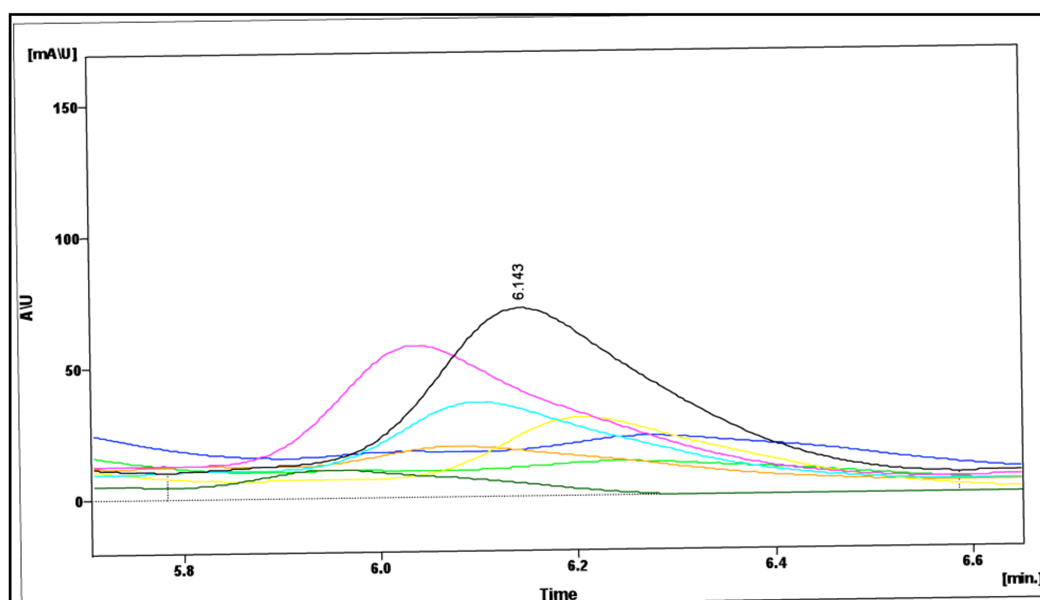
Fig. 3.7 HPLC analysis of the reaction products incubation of purified human 12R-LOX enzyme with AA or AA Me. (A) Chromatogram analysis when purified human 12R-LOX enzyme was either incubated **a)** with methyl ester of arachidonic acid (AA Me) **b)** with arachidonic acid (AA) **c)** purified human 12R-LOX enzyme was first incubated with NDGA (general LOX inhibitor) and then substrate (AA or AA Me) was added to the reaction mixture. **(B)** Overlay of the chromatogram peaks from individual assay reactions where purified h12R-LOX was used as an enzyme source; **yellow peak** –Standard 15-HETE product, **pink peak** - 12R-HETE product at RT-5.3, when arachidonic acid was used, **black peak** – 12R-HETE product when methyl ester of arachidonic acid was used.

Further, to increase specific activity of the enzyme, we purified h-12*R*-LOX enzyme by Ni-NTA affinity chromatography and purified enzyme was used for the enzyme assay (**Fig. 3.7B**). 200 µg/ml of purified h-12R-LOX enzyme was incubated with saturating concentration of substrates (100µM for AA and 100µM for AA Me) and products were analysed on RP-HPLC. In contrast to cell extracts, purified enzyme showed formation of a product peak with retention time ~6.5 minutes when methyl ester of arachidonic acid was used as the substrate, though absorbance of peak was limited to only~50 mAU (**Fig. 3.7A-a**). On incubation of arachidonic acid with purified h-12*R*-LOX enzyme, a prominent (12R HETE) peak with an absorbance of ~350-400 mAU was observed with retention time ~5.3 minutes (**Fig. 3.7A-b**), a pattern similar to that of cell extracts. After purification the activity of the enzyme increased by 637 fold. NDGA (nordihydroguaiaretic acid) is frequently used as a nonspecific inhibitor for LOXs. The addition of NDGA successfully inhibited the formation of 12*R*-HETE, further confirming that the product 12-HETE is in fact formed through enzymatic action of LOX (**Fig. 3.7-c**).

3.2.6 Extracts of *Acalypha indica* plant significantly inhibits enzyme activity of h-12*R* LOX

Acalypha indica herb is used for traditional medication in various parts of the world like India, Bangladesh, Sri Lanka, Africa and the Philippines. It possesses properties like diuretic, purgative and anthelmintic. The plant extracts are being used in the treatment of bronchitis, asthma, pneumonia, scabies and other cutaneous diseases (Kirtikar and Basu, 1999). In the present study seven extracts of *Acalypha indica* plant leaves were prepared with different solvents using soxhalation method. All these extracts were checked for h12*R*-LOX enzyme inhibition but only ethyl acetate and aqueous extracts of *Acalypha indica* showed potent inhibition of 12*R*-Lipoxygenase activity (**Fig. 3.8A**). Further we tested whether zileuton, a standard 5-LOX inhibitor being used as an anti-asthmatic drug, inhibits 12-*R*-LOX activity. As shown in the **Fig. 3.8B**, zileuton showed potent inhibition of 12-*R*-LOX at 100µM (70µg/µl). This forms the first report on the inhibition of 12-*R*-LOX by zileuton.

A



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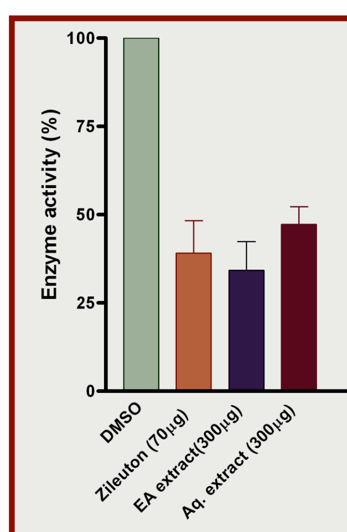


Fig. 3.8 (A) Overlay of the 12R-HETE peak in different reverse phase HPLC analysis of the reaction products formed when purified human 12R-LOX was incubated with AA as the substrate and in the presence or absence of extracts of *Acalypha indica*/zileuton. Black peak - the enzyme was pretreated with control DMSO (solvent used for dissolution of extracts); Pink peak- Methanol extracts of plant *Acalypha indica*; Orange peak- Zileuton (standard LOX inhibitor); Blue peak-Ethyl acetate extracts of plant *Acalypha indica*; Fluorescent green peak- Aqueous extracts of plant *Acalypha indica* **(B)** Area of the peak was measured and inhibition of 12R-LOX enzyme activity was plotted quantitatively. The values are the average of 3 individual experiments.

3.3 Discussion

12-*R*-Lipoxygenase is unique as it incorporates molecular oxygen in *R* stereospecific configuration. It is very essential for normal embryonic development of skin. At the same time it's over expression is associated with psoriasis, inflammatory dermatoses and skin cancers. In the light of the above there is need for identification of specific inhibitors 12-*R*-LOX, which could form potential drug candidates for the treatment of various skin disorders. A major constraint in the development of inhibitors for 12-*R*-LOX is the lack of abundant enzyme source and an easy assay system. The present study, therefore, is designed to develop an expression system and assay protocol for 12-*R*-LOX.

This system can then be used for the biochemical characterization of h12*R*-LOX and identification of its inhibitors. The plan of the work involved over expression of h12*R*-LOX, purification of the enzyme and to analyze its activity.

Cell lines form an ideal system for the expression of any eukaryotic protein as they have appropriate mechanisms to carry out all the post-translational modifications required for a eukaryotic protein. In the present study also we selected NIH3T3 cell line, which had no endogenous expression of h12*R*-LOX. Although we could select some of the colonies, which were positive for the expression of h12*R*-LOX protein, we could not use the protein generated from these stable clones for assaying the activity of the enzyme on HPLC based assay system. It might be because of the less abundance of the protein produced in the mammalian cell line or low specific activity of the protein.

We have successfully cloned, expressed and purified human 12*R*-LOX protein from bacteria. We further used RP-HPLC based assay to show that protein is active and uses arachidonic acid (AA) as a preferred substrate and show very less activity towards methyl ester of arachidonic acid. Using this assay system we have found out inhibitory activity of 12*R*-LOX enzyme in both aqueous and ethyl acetate extracts of medicinal plant *Acalypha indica*, which may be subsequently used for isolating potent inhibitor(s) against 12*R*-LOX protein.

12*R*-LOX protein belongs to the family of LOX proteins, which are widely distributed in eukaryotes and also reported to be present in bacteria (Porta *et al*, 2001). The enzyme has unique property amongst other LOXs as it has unique *R*-

stereospecificity of oxygen insertion into its substrate as opposed to *S*-stereospecificity present in other known LOXs. 12*R*-LOX along with other enzyme eLOX-3 utilizes arachidonic acid (or methyl ester of AA) and produces hepoxilins with potent biological activities. Mutation in 12*R*-LOX has been associated with various skin diseases as in Non-bullous congenital ichthyosiform erythroderma (NCIE) and autosomal recessive congenital icthyosis (ARCI) (Yu, 2005; Eckl *et al*., 2005; Moran *et al*., 2007). Increased amount of 12*R*-HETE, product of 12*R*-LOX enzyme, was reported in skin disease psoriasis and other inflammatory conditions (Woolard 1986; Baer *et al*., 1991, 1995). More importantly 12*R*-LOX deficient mouse exhibited water barrier dysfunction and died within 3-4 hrs after birth (Epp *et al*., 2007).

Considering the importance of 12*R*-LOX enzyme, we decided to purify and to do the biochemical characterization of human 12*R*-LOX enzyme. Bacterial protein expression systems are very convenient and economic to use hence we decided to express and purify human-12*R*-LOX enzyme from bacteria. Human 12*R*-LOX gene was cloned in bacterial expression vector pET28b and transformed in BL21-pLys strain to express His-tagged recombinant h-12*R*-LOX. We couldn't see much protein expression upon IPTG induction in BL21-pLys strain. The low amount of 12*R*-LOX protein expression in bacteria was also reported in earlier study. Meruvu *et al* cloned and expressed murine 12R-LOX in *E. coli* and observed that expression of gene was very low and thus they used baculovirus/insect cell system for further study (Meruvu *et al*., 2005). Recently for structural studies, human 12*R*-LOX was co-produced with two chaperones in *E. coli* that allowed the purification of 12*R*-LOX-chaperone complex (Deb *et al*. 2011). Codon bias is a general problem when eukaryotic proteins are expressed in bacteria. Hence, we used BL21-Rossetta strain to express human-12*R*-LOX protein and indeed we observed abundant expression of human-12*R*-LOX protein. Further we purified recombinant h-12*R*-LOX protein by Ni-NTA affinity chromatography method. Two extra protein bands of less intensity were also observed apart from h-12*R*-LOX protein and as we could not separate them by conventional techniques (**Fig. 3.4C** and **3.4D**) we proceeded further with these fractions for all activity assays.

Although many groups have expressed 12*R*-LOX gene both from murine and human in heterologous system but they have achieved either no or very less enzymatic activity. Human 12*R*-lipoxygenase was identified and cloned from human keratinocytes

and then expressed in HeLa cells. HeLa cells expressing h-12*R*-LOX showed very low catalytic activity with arachidonic acid (AA) as the substrate to produce 12*R*-HPETE (Boeglin, 1998). Subsequently, Sun and co-workers cloned both murine and human 12*R*-LOX and expressed in HEK293 and COS-M6 cells but were unable to detect any enzyme activity; however they could detect enzymatic activity of h-12*R*-LOX and not of murine, when expressed in baculovirus/insect system (Sun *et al*., 1998). They also reported that enzyme activity was very low and h-12*R*-LOX enzyme used arachidonic acid (AA) to produce 12*R*-HETE. Human epidermoid carcinoma cell line A431 cells were reported to have high level of expression of 12*R*-LOX enzyme (Agarwal *et al*., 2009). We failed to get any 12*R*-LOX enzymatic activity using A431 cell extracts, possibly because of presence of inhibitor reported earlier (Chang *et al*, 1992). On the other hand, murine 12*R*-LOX has been reported to use only esterified arachidonic acid (methyl ester of AA) as a substrate and it is completely inactive on non-esterified AA. Characterization of mouse 12*R*-LOX was done by expressing it in different systems like in HEK-293 cells and baculovirus/insect system (Krieg *et el*., 1999; Siebert *et al.,* 2001; Meruvu *et al.,* 2005). This poses a question about the nature of the endogenous substrate of 12*R*-LOX enzyme of mouse and human.

In the present study, which was carried out with BL21-Rosetta cells extracts, h-12*R*-Lipoxygenase showed activity with arachidonic acid only (and not methyl ester of arachidonic acid), generating 12*R*-HETE as the product. However, purified h-12*R*-LOX showed activity with methyl ester of arachidonic acid but with low catalytic activity compared to arachidonic acid. A good quantity of 12*R*-HETE was produced with arachidonic acid (AA) as the substrate when purified human 12*R*-LOX enzyme was used. We propose here that human-12*R*-LOX preferentially utilizes arachidonic acid as the substrate as compared to methyl ester of arachidonic acid. However, our results also suggest that using higher amounts (human 12*R*-LOX), this enzyme can metabolize methyl ester of arachidonic acid and generate 12*R*-HETE methyl ester (**Fig 4A-a**). These findings are consistent with earlier results where it has been reported that human 12*R*-LOX enzyme utilizes arachidonic acid (Sun *et al*., 1998). In addition to this, we are able to show that h12*R*-LOX can utilize methyl ester of arachidonic acid also but with low specific activity.

Additionally, in this study we made an attempt to identify a medicinal plant source for inhibition of human 12*R*-LOX enzyme activity. These studies revealed that

the aqueous and ethyl acetate extracts of *Acalypha indica* leaves inhibit 12-*R*-LOX. *Acalypha indica* is being used widely in traditional medicine for skin disorders in Asian countries (Hasan *et al.,* 2013; Mauli *et al.,* 2012). Extracts of this plant have been shown to possess anti-malarial and anti-bacterial activity (Govindrajan *et al*., 2008 (1,2)). Recent studies have attributed anti-inflammatory and wound healing properties to the extracts of this plant (Rahman *et al*., 2010, Ganesh kumar *et al*., 2012). High level of 12*R*-HETE has been reported (Woolard 1986; Baer *et al*., 1991, 1995) in case of psoriasis and other inflammatory dermatoses, suggesting enhanced activity of 12*R*-Lipoxygenase in these diseased conditions. To test, if the anti-inflammatory property observed in *Acalypha indica* extracts is related to inhibition of 12*R*-Lipoxygenase enzyme, we tested 7 different extracts of *Acalypha indica* leaves against 12*R*-lipoxygenase activity. Of all the extracts tested only two extracts, aqueous and ethyl acetate, inhibited human 12*R*-LOX enzyme activity up to 60-70% at 150ug/ul concentration. These studies reveal that the aqueous and ethyl acetate extracts of *Acalypha indica* leaves may form potential source for the development of drug candidates for the treatment of psoriasis and other inflammatory dermatoses. Further studies on isolation and characterisation of the candidate molecule(s) inhibiting 12-*R*-LOX, however, are required for proceeding further to the drug development.

Another important finding of the present study is that Zileuton, a 5-LOX inhibitor, also inhibits human 12*R*-LOX activity very potently. Zileuton, an orally active 5-LOX inhibitor, was introduced by Abbott Laboratories in 1996 and is being used in the maintenance treatment of asthma (Israel *et al*., 1996). Earlier studies reported little or no inhibition of several related enzymes, such as platelet 12-lipoxygenase, soybean and rabbit reticulocyte 15-lipoxygenase and sheep seminal vesicle cyclooxygenase (Carter *et al*., 1991). The present study forms the first report on the inhibition of 12-*R*-LOX by zileuton, suggesting the possible repositioning/repurposing of zileuton in the treatment of psoriasis and other inflammatory dermatoses. Further in-depth efficacy studies, however, required before repurposing zileuton for the treatment of psoriasis and other inflammatory dermatoses.

In summary, we have successfully expressed human 12*R*-LOX enzyme in bacterial expression system. The recombinant human 12*R*-LOX was purified in native conditions and was found to be fully active. It is evident from our results that human-12*R*-LOX preferentially utilizes arachidonic acid but it can also metabolize methyl

ester of arachidonic acid with very low specific activity. Further, we showed potent inhibition of h12-R-LOX by the aqueous and ethyl acetate extracts of *Acalypha indica* leaves, suggesting it as a potential source for the development of a drug candidate(s) for the treatment of psoriasis and other inflammatory dermatoses.

Chapter 4

Generation and characterization of h-12R-Lipoxygenase transgenic mice

CHAPTER 4: Generation and characterization of h-12R-Lipoxygenase transgenic mice.

4.1 INTRODUCTION

Human12*R*-LOX coded by *ALOX12B* gene, is an enzyme, which adds an oxygen molecule, into 12*R*-position of fatty acids like arachidonic acid, to make a product known as 12R-hydroperoxyeicosatetraenoic acid (12R-HPETE). Another LOX enzyme eLOX-3 uses 12*R*-HPETE as the preferred substrate to produce 8*R*-hydroxy, 11*R*, 12*R*-epoxy eicosatrienoic acid.12*R*-LOX and eLOX-3 are epidermis type LOX and are preferentially expressed in the skin of human and mice. Both proteins follow similar tissue expression pattern. Immunofluorescence analysis shows 12*R*-LOX and eLOX-3 proteins to colocalize in the stratum granulosum of mouse skin (Zheng *et al.,* 2011; Li, *et al.,* 2012). 12*R*-LOX expression in embryonic skin starts at 15.5 embryonic day. In human, 12*R*-LOX expression is most abundant in psoriatic scales, hair follicles, prostate and squamous epithelial cells (Lefevre *et al.,* 2004).

12*R*-LOX is expressed in differentiated layers of epidermis giving an indication of functional linkage with role in terminal differentiation. Keeping in view the expression pattern of 12*R*-LOX and eLOX-3, it was speculated that 12*R*-LOX enzyme function could be crucial in maintaining homeostasis and an aberrant expression of this enzyme may lead to deleterious consequences. Indeed various reports have confirmed its role in various diseases. Mutation in 12*R*-LOX and e-LOX3 has been associated with various skin diseases as in Non-bullous congenital ichthyosiform erythroderma (NCIE) and autosomal recessive congenital icthyosis (ARCI) (Yu et al, 2005; Eckl *et al*., 2005; Moran *et al*., 2007).

Critical role of 12*R*-LOX and eLOX-3 in barrier function was evident form the studies from knockout mice for these LOX proteins. Ablation of 12*R*-LOX lead to death of 12*R*-LOX-deficient mice within 3-4 hours of birth while eLOX-3 knockout mice survived up to 12 hours after birth. Electron microscopy revealed that cornified lipid envelope (CLE) was absent in 12*R*-LOX knockout mice. This study provides convincing evidence for action of LOX in barrier functions and links 12*R*-LOX/eLOX-3 pathway to ceramide processing and thereby formation of cornified lipid envelope. They were able to show that linoleate – containing esterified omega-hydroxyacyl-

sphingosine (EOS) ceramide is a natural substrate for the 12*R*-LOX/eLOX-3. This suggested that this LOX catalyzed oxygenation is the prerequisite for the coupling of the cross-linked proteins of the cornified envelope (CE), which becomes the preliminary step for barrier formation.

Increased amount of 12*R*-HETE, product of 12*R*-LOX enzyme, was reported in skin disease psoriasis and other inflammatory conditions (Woolard 1986; Baer *et al*., 1991, 1995). Some of the *in vitro* studies carried out in A431 cell line showed that 12*R*-HETE, 12*S*-HETE and PGE_2 up- regulated the p-ERK and p-AKT levels, suggesting involvement of 12*R*-HETE in the regulation of growth in epidermoid carcinoma cell line (A431) (Agarwal *et al.,* 2009). *In vitro* data on cell line revealed that 12*R*-HETE promotes the proliferation of colon cancer cell lines. In addition to this, the product formed from the enzymatic action of 12*R*-LOX, which is 12*R*-HPETE, acts as precursor molecule for the activation of PPARα pathway. PPARα in skin plays an important role in proliferation, differentiation, wound healing and lipid processing.

Present study undertakes to investigate the role of h-12*R*-LOX in proliferation and differentiation and thereby understanding its role in pathophysiology. In order to study the role of h-12*R*-LOX we resolved to generate transgenic mice over expressing h-12*R*-LOX by microinjection DNA in pronuclear stage of mouse embryos. The h-12*R*-LOX transgenic mice, thus generated, were characterized for phenotypic analysis. Through transgenic technology it is possible to generate animal models that will help to elucidate the precise functions of the candidate genes implicated in aberrant pathways, diseases and disorders.

4.2 RESULTS

4.2.1 Generation of human-12*R*-Lipoxygenase transgenic mice

4.2.1.1 Generation of human-12*R*-LOX transgenic mice using pCMV6-12*R*LOX vector:

pCMV6-Neo, a mammalian expression vector, was obtained form OriGene, USA. Human 12*R*-LOX full-length cDNA along with 5’ and 3’UTRs was cloned at NotI site in MCS. The expression of the cDNA is driven by CMV promoter, which expresses globally, nearly in all tissues. It harbors a SV40 PolyA signal. The

immunofluorescence study was performed for this vector to check the expression level of this vector by transient over expression of this vector *in vitro* in NIH3T3 cell line **(Chapter 3, Fig. 3.2B).** For generation of the transgenic mice, this vector was digested with PvuI and PstI and the whole fragment containing promoter, full-length cDNA, SV40 PolyA was injected into pronuclear stage of the F1 mice embryos **(Fig. 4.1A)**. The injected embryos were then transferred into pseudopregnant CD1 female mice. The protocol for the generation of the transgenic mice is presented in **Fig. 4.1B**. In total, seven rounds of microinjection experiments were performed. Although pregnancies were established in the CD1 females but most of the times abortions were observed with these pregnant females. In two of the seven rounds the females were seen to have resorption of the embryos. The offsprings, which were born, were found to be negative for the transgene integration. The results of the microinjection using CMV construct are shown in **Table 4.1**.

With most of the CD1 pregnant female mice, we observed either abortions of the embryos or the live pubs born out were -ve for insertion of the transgene. There might be a possibility that global expression through CMV promoter is deleterious. This urged us to think of using an endogenous tissue specific promoter. We chose keratin14 promoter, which is a well characterised endogenous promoter and expresses in squamous cell epithelia including the basal layer of epidermis of the skin. For this we used pG3Z-K14 Beta globin vector for cloning full-length cDNA of human12*R*-LOX. This vector has K14 promoter followed by beta globin intron a multiple cloning site and K14 polyA tail. This cassette was a kind gift from Dr. Elian Fuchs, from Rockefeller University, USA.

4.2.1.2 Generation of mice using pG3Z-K14 BetaGlobin-h12*R*-LOX vector:

Cloning of human-12*R*-LOX in pG3Z-K14 Beta globin vector.

pCMV6-Neo-h-12*R*-LOX was used as a source for full length cDNA of human 12*R*-LOX along with 5'UTR and 3'UTR. There were two restriction enzyme sites – BamHI and XbaI to clone in pG3Z-K14 Beta globin vector. Since BamHI is present internally in the cDNA of h-12*R*-LOX so we have selected XbaI to clone cDNA. For this it was needed to create XbaI sites on the flanking sides of the full-length cDNA.

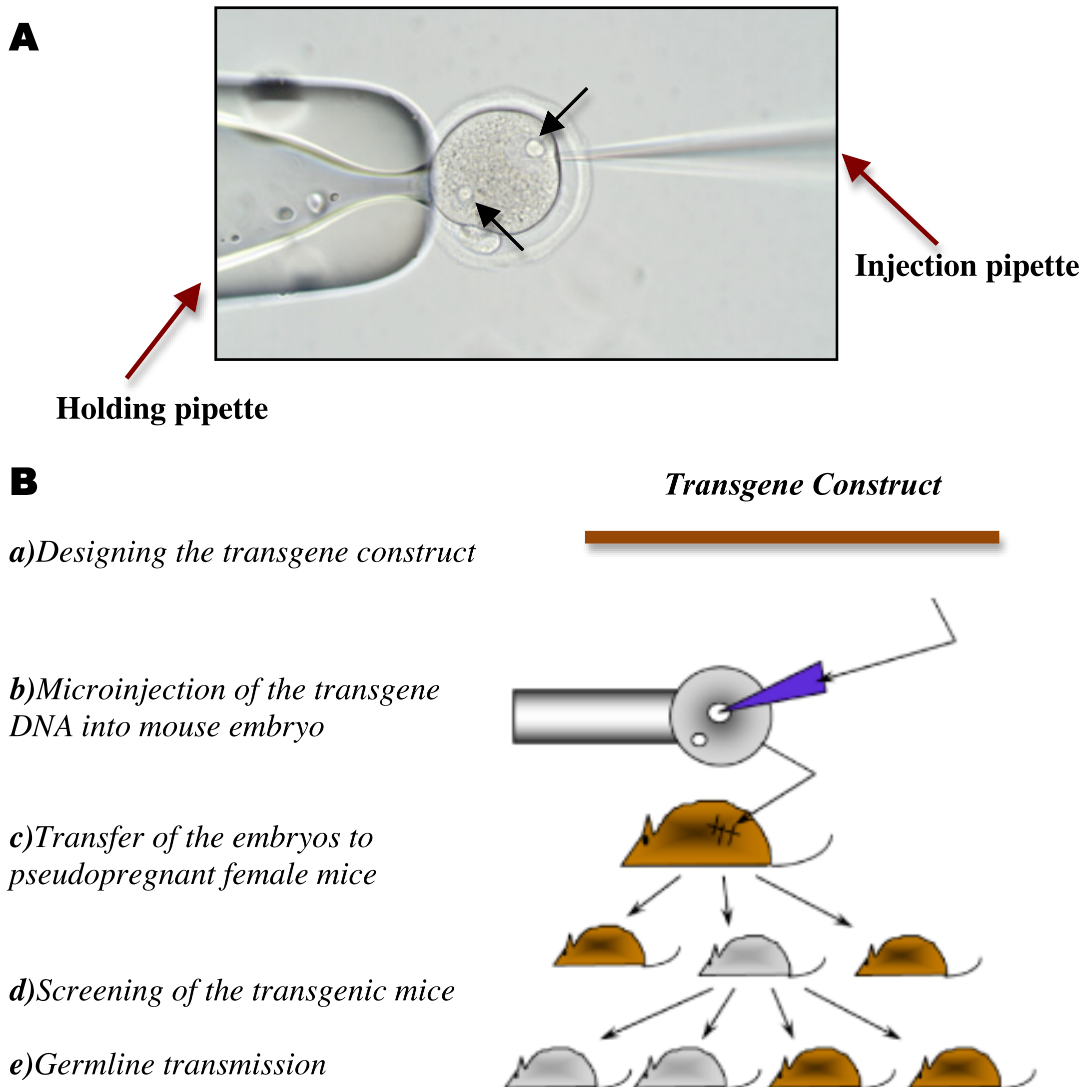


Fig. 4.1 Schematic representation for the generation of the transgenic mice (A) Pronuclear microinjection showing a holding pipette, holding the mouse embryo, where male and female nuclei (shown with black arrows) have not fused yet and an injection pipette containing DNA to be injected in one of the nuclei of the embryo **(B)** Steps for generation of the transgenic mice generation a) Generation of the transgenic construct b) Microinjection of the transgenic DNA into one of the pronuclei of the mouse embryo c) The successfully injected embryos are transferred to the uterus of the pseudopregnant female mice d) After the pups are born, they are screened for the transgene integration e) The mice found to be transgenic are further bred to wild type males/females for the germline transmission of the transgene.

The full-length cDNA was first released by NotI digestion of pCMV6-Neo-h-12*R*-LOX. It was then subcloned in pBKS vector by blunt end ligation so as to create XbaI sites on either sides of the cDNA. Later, it was released with XbaI and cloned in pG3Z-K14 vector at XbaI site. Orientation of the insert was checked by digestion with BamHI and also confirmed by sequencing. The cloning strategy is depicted in **Fig. 4.2**

Checking the expression of pG3Z-K14-h12*R*-LOX vector in vitro.

After construction of the vector it was important to check the expression of cDNA under Keratin14 promoter *in vitro* before using this construct for injecting in the mouse embryos for making the transgenic animal. Expression of the construct was checked by immunofluorescence by transient transfection of pG3Z-K14-h12*R*-LOX in NIH3T3 cell line using primary polyclonal antibody against h12*R*-LOX **(Fig. 4.3).** *In vitro* expression of the construct showed moderate expression of h-12*R*-LOX in the transfected cells.

Results of the microinjection experiments using pG3Z-K14-h12*R*-LOX.

After checking the expression of pG3Z-K14-h12*R*-LOX construct *in vitro* by transfection in NIH3T3 cells **(Fig. 4.3C)**, the plasmid DNA was amplified and isolated in Endotoxin-free conditions by using Endofree Qiagen Kit. The procedure has been described in Materials and Methods. The plasmid DNA (12ug) was then digested for three hours with SmaI and HindIII to release the whole cassette of 5.7Kb containing K14 Promoter followed by beta globin intron, the cloned full-length h-12R-LOX cDNA with 5' and 3'UTRs and Keratin14 ployA tail **(Fig. 4.3A),** leaving behind backbone of 2.7Kb **(Fig. 4.3B).** 5.7Kb fragment was separated from 2.7Kb fragment on EtBr free agarose gel. The bands were seen on the gel by staining the gel with methylene blue. The bigger 5.7 Kb fragment was eluted from the agarose gel in endotoxin-free conditions and diluted to the required concentration for injection in the pronuclear stage of mouse embryo. F1 females (which are hybrid strain of CBA and C57 Black) have been used to procure embryos. For transfer of the injected embryos to a pseudopregnant mother, CD1 female strain has been used.

In all, seven rounds of microinjection experiments were performed which yielded three transgenic lines. Results of the microinjection have been put in the form of table **(Table 4.2)**

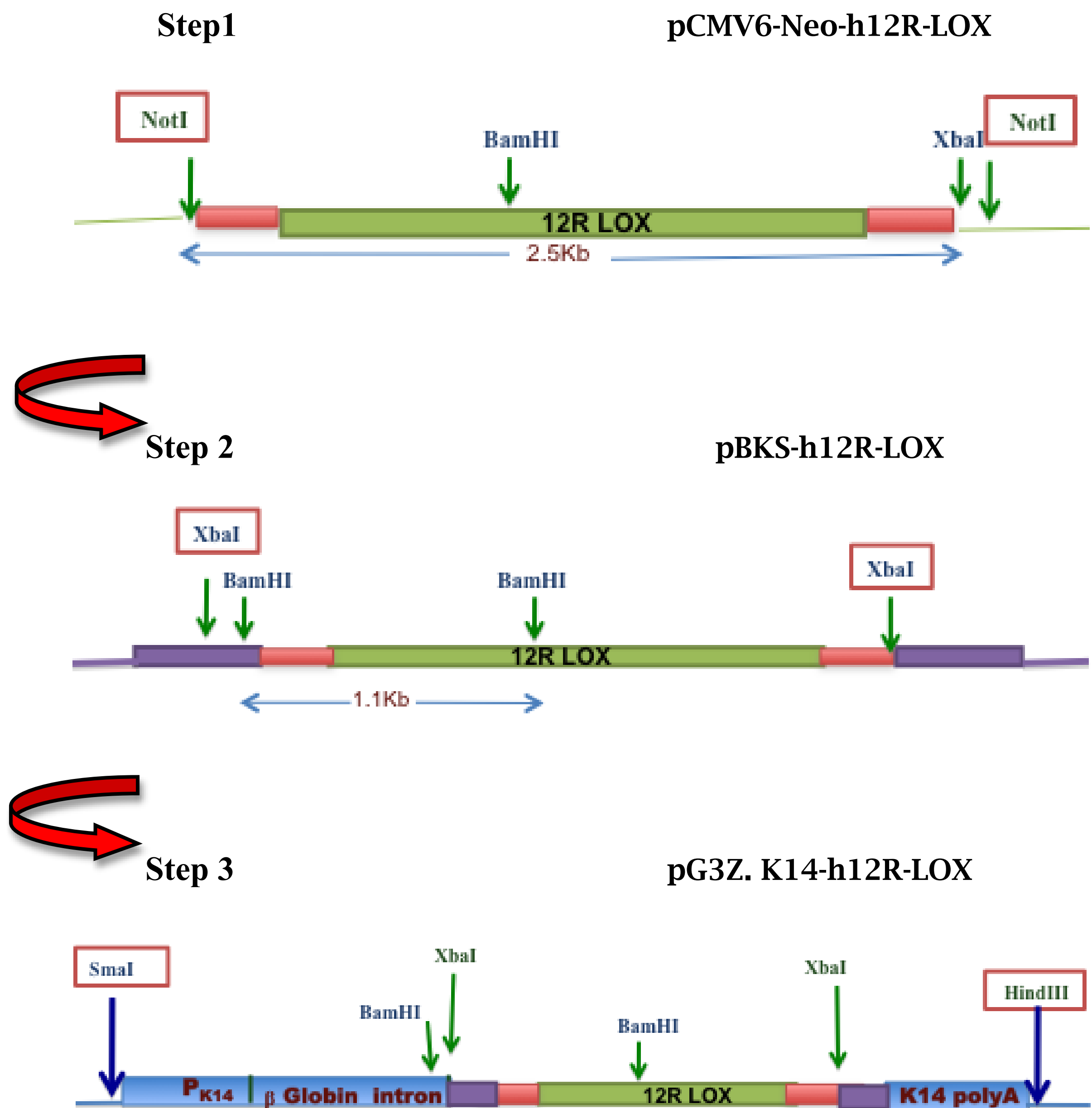


Fig. 4.2 Schematic representation of the strategy followed to clone human-12*R*-LOX in pG3Z-K14 Beta globin vector. Step1- Human 12R-LOX full-length cDNA along with UTR's was released by NotI digestion of pCMV6-Neo-h12R-LOX vector. **Step2-**Whole cDNA was then blunt-end ligated to the SmaI linearized pBKS. **Step3-** XbaI digestion was performed to release the cDNA from pBKS-h12R-LOX and ligate it into XbaI linearized pG3Z. K14 plasmid DNA to create whole construct pG3Z. K14-h12R-LOX.

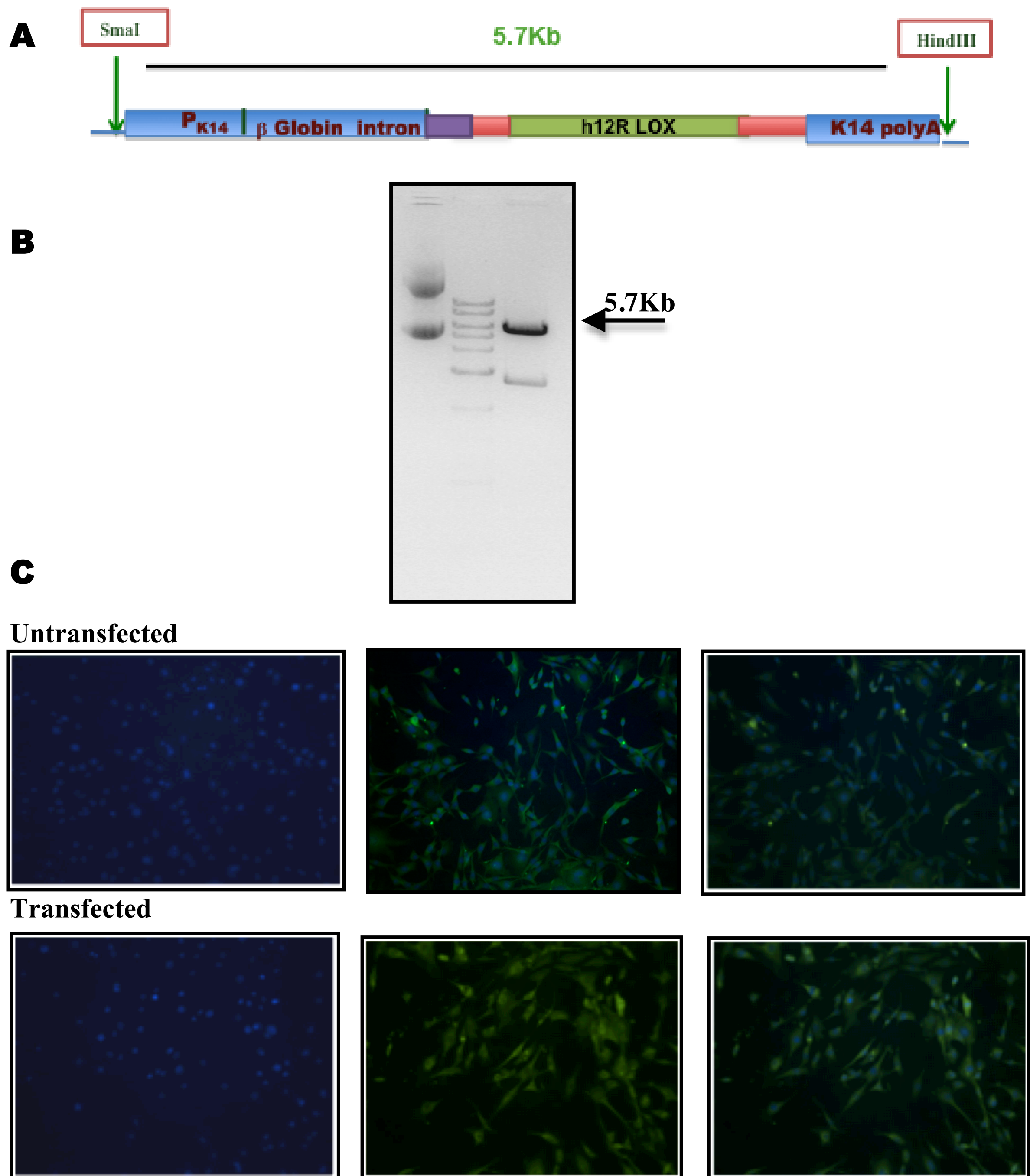


Fig. 4.3 (A) Schematic representation of the transgenic construct - K14-h12R-LOX, used for generation of the h12R-LOX transgenic mice. **(B)** Release of the whole construct (containing K14 Promoter, beta globin intron, cDNA with UTR's and K14 PolyA) by SmaI and HindIII double digestion of pG3Z.K14-h12R-LOX plasmid. This fragment is separated on the gel and used for microinjection for generation of the transgene. **(C)** Immunofluorescence study in NIH3T3 cells transiently transfected with the transgenic construct pG3Z. K14-h12R-LOX using Lipofectamine, DAPI (Left), FITC (Middle), Overlay (Right).

Screening of the transgenic animals

From all the microinjection experiments, pregnant (CD1) females delivered a total numbers of 105 offspring. After weaning, these animals were subjected to screening for the transgenic animals. Tail samples were taken, from the litter, for genomic DNA isolation. Genomic DNA was used as the template for PCR analysis using full-length h-12R-LOXcDNA primers **(Fig. 4.4A)** to screen the insertion of the transgene. As expected, a 2.1 Kb amplicon was observed in three animals, which were male in gender. This band was not seen in the sample for negative control **(Fig. 4.4C).** These three male mice were numbered as Tg4, Tg11 and Tg17.

Southern analysis.

To confirm the integration of the transgene, southern analysis was performed. For this, genomic DNA was digested completely with specific enzyme and the fragments were separated on the agarose gel. We have used two restriction enzymes – XbaI and BamHI. XbaI releases full length cDNA (2.5Kb) and BamHI produces two fragments - a shorter fragment of 1.1Kb and a longer fragment whose length will depend on the presence of BamHI site in mouse genome **(Fig. 4.5A).** The fractionated DNA was transferred to nylon membrane (hybond N^+) by capillary transfer as described in Materials and Methods. The membrane was then probed with radiolabelled probe. Double-stranded DNA was prepared by PCR using the full-length cDNA as template. The amplified fragment was then radiolabelled by random priming using random labelling kit (BRIT) as per manufactures' protocol and Southern analysis was performed on gDNA samples form both wild type and transgenic mice. When XbaI digested genomic DNA was probed, we could observe a band of 2.5Kb, which confirms the integration of the transgene **(Fig. 4.5B)**. BamHI digested genomic DNA revealed two bands –a smaller band of 1.1Kb and another bigger band of approximately 4.0Kb, further confirming the integration of the transgene (h-12*R*-LOX). No signal was seen in the wild type control littermates, suggesting that the bands observed from the transgenic mice sample were indeed because of the integrated transgene **(Fig. 4.5C).**

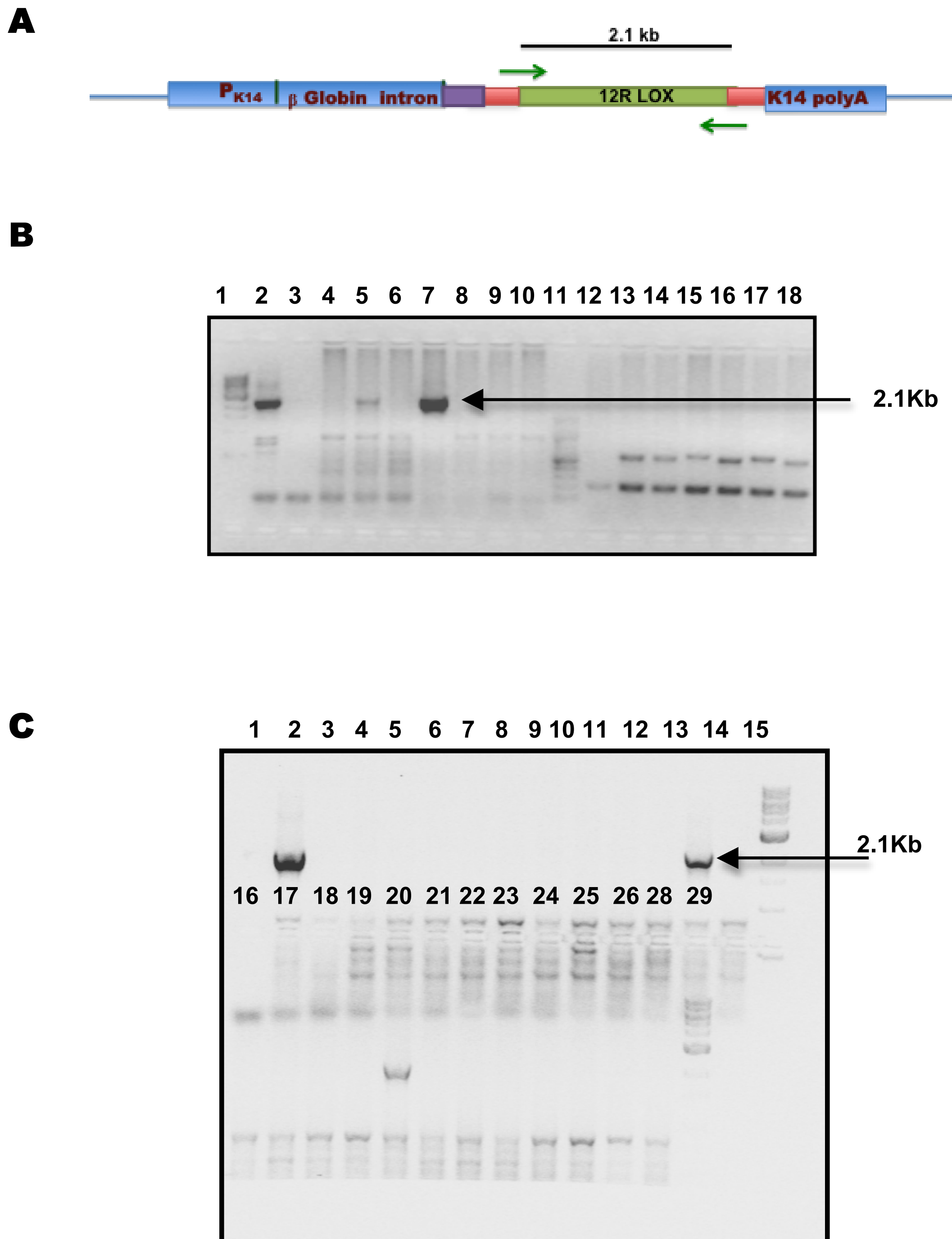


Fig. 4.4 Generation of h-12R-LOX transgenic mice. (A) Pictorial representation to depict the binding of the primers for PCR screening strategy. Full -length primers were used to amplify the cDNA, which gives an amplicon of 2.1Kb. **(B)** And **(C)** PCR screening of pups obtained after microinjection of transgene. lane no.**2** in both (**B** and **C**) are +ve control samples - ; lane no.**7** (**B**) and lane no. **13 (C)**, lane no. **20** (**C**) were found to be positive for transgene integration.

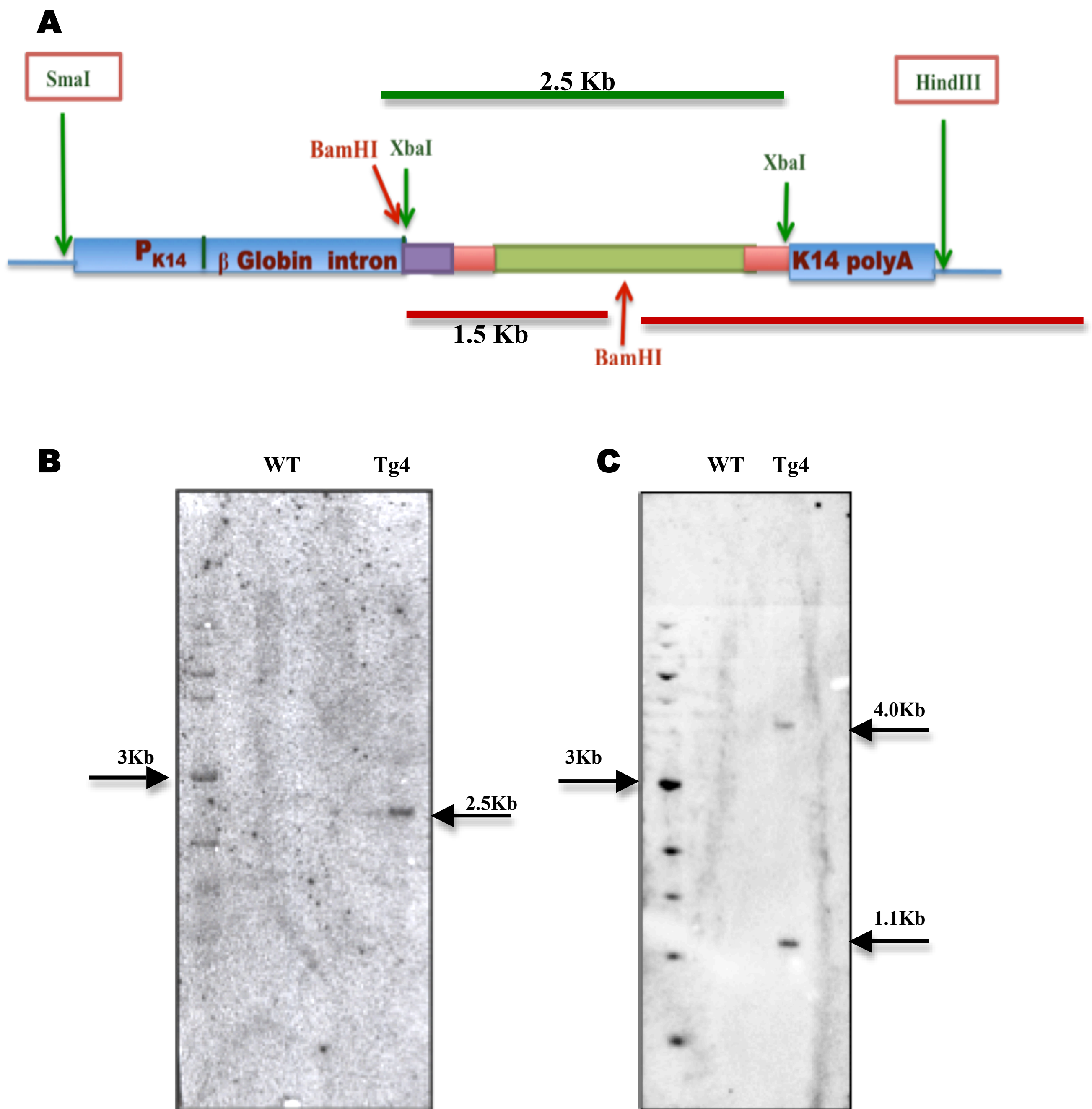


Fig. 4.5 Southern analysis of the mouse genomic DNA for transgene integration. (A) Schematic representation depicting the expected sizes of the fragments, when radiolabelled full-length cDNA probe (2.5Kb –green color) was hybridized to XbaI digested and BamH1 digested mouse genomic DNA from the transgenic mice. **(B)** Southern analysis done with the mouse genomic DNA digested with XbaI enzyme, which upon hybridization with the radiolabelled probe, gave the signal at the expected size as 2.5Kb. **(C)** Southern analysis done with the mouse genomic DNA digested with BamHI enzyme, which upon hybridization with the radiolabelled probe, showed two bands – 1.1Kb (expected- red color) and another approx. 4.0Kb.

Germline transmission of the transgene

For checking the ability of the transgenic male founder mice to transmit the transgene through germline, all the three transgenic male founder mice (G_0-Generation Zero) were bred to wild type females F_1 (C57 Black × CBA). Only Tg4 was able to produce sufficient litter size, however the other two lines Tg11 and Tg17 did not produce sufficient litter size due to subfertility. The next generation pups - G_1 (Fist generation) were again screened by PCR, using full-length primers as mentioned above.

Detection of the expression level of 12*R*-LOX protein in the transgenic mice

For checking the expression of the transgene, transgenic animals and control animals were sacrificed and tissues were collected for preparation of protein lysate. The protein lysate from both transgenic and wild type animals was estimated and equal quantity of protein was run on SDS-PAGE. The protein was then transferred to the membrane and it was subjected to western analysis to check expression of the transgene. Polyclonal antibody against human 12*R*-LOX was used to detect expression of the transgene (human12*R*-LOX) in transgenic mice. Beta actin antibody was also used as a control to calculate fold expression in transgenic mice. Transgenic mice showed 2.5-fold relative expression of the transgene **(Fig. 4.6)**.

4.2.2 Identification of insertion site of the transgene in mouse genome

Site of the h-12R-LOXtransgene integration was detected by inverse PCR. Briefly, 1-2 microgram of the genomic DNA was digested with BamHI in 30µl of reaction volume for 7-8 hrs. The restriction enzyme was then heat inactivated and ligation reaction for this digested DNA was set up in 300µl of reaction volume to promote self-ligation of digested fragments. This was followed by precipitation of the ligated product, which was resuspended in 20µl of TE buffer. This DNA was later used as the template to amplify the mouse genomic DNA region, flanking the integrated transgene, using cDNA specific primers (outloxF and outloxR).

In tg4 line, inverse PCR confirms that the transgene has got integrated in the euchromatic region of the mouse genome. h-12R-LOXtransgene was seen to be integrated into the third intron of an ORF annotated as “Down Syndome cell adhesion molecule – like protein1 homolog” (accession no. NC_000075.6, 45429495..45753713)

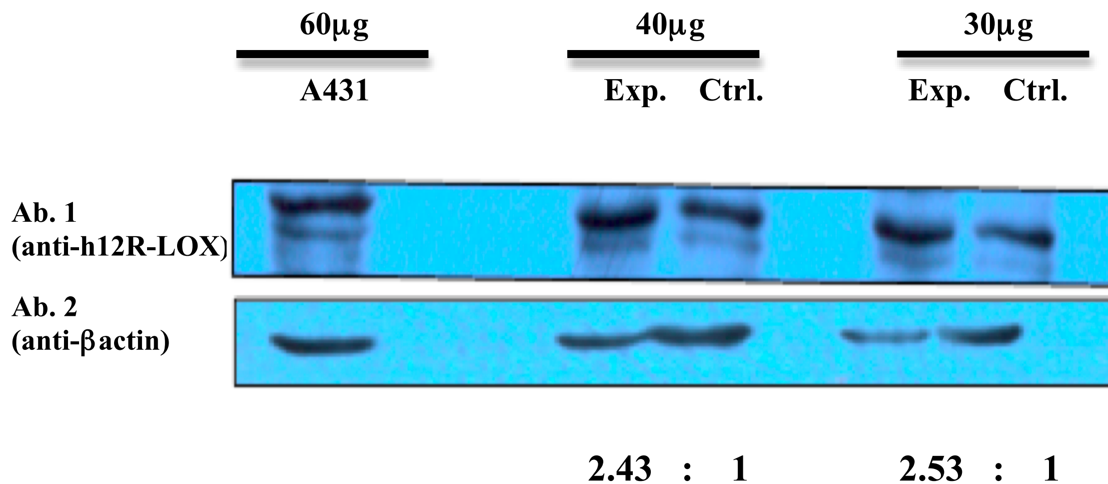


Fig. 4.6 Western analysis for checking the expression of the integrated transgene. The protein lysates were made from the skin of the transgenic mice and their wild type littermates in order to see the fold expression due to integrated transgene h12R-LOX. **A431**- Protein lysate from A431 cells, human epidermoid carcinoma cell line, endogenously expressing h12R-LOX and is used as the positive control. **Exp.** Protein lysate from the transgenic mice. **Ctrl.** Protein lysate from the wild type control littermates. **Ab.1** The blot is probed using polyclonal h12R-LOX antibody raised against a unique 31-aminoacid residue, present only in 12R-LOX. **Ab. 2** The blot is probed with beta actin antibody used as a loading control. The intensities of the bands were read on the densitometer. The values got from the Ab.1 (12R-LOX) were normalized with the values got from Ab.2 (Beta actin). These values were then used to calculate fold expression, which was 2.5 fold more than the WT.

on chromosome no. 9. The total size of this gene is 324.218 Kb. Since, this transgene integration is not disrupting any of its exon, it will not affect the expression of the gene.

4.2.3 Phenotypic analysis of the transgenic mice

Phenotypically, transgenic mice were indistinguishable from their wild type littermates. Since the expression of transgene was driven by Keratin14 promoter, it was obvious to examine the squamous cell epithelia where keratin14 promoter activity has already been reported. For further analysis we took out skin sections from the transgenic mice as well as from their wild type littermates for microscopic examinations. Haematoxylin and eosin staining of the skin sections revealed differences in the stratum corneum of epidermis. When compared to the skin of wild type mice, the skin of the transgenic mice showed many layers of the stratum corneum. These layers were not intact when compared to stratum corneum of WT mice. This difference was observed only in Tg4-12R-LOX **(Fig. 4.7)**.

The other two transgenic lines Tg11 and Tg17 were not able to produce sufficient litter size. These lines were analyzed for their fertility parameters. Sperms were collected from epididymis of both transgenic as well as wild type mice and their motilities were observed. Interestingly, the sperm motility factors of the wild type mice were normal whereas the sperms from the transgenic mice were abnormally immotile. The sperms were stained with eosin and were observed under microscope. Morphologically, the sperms of the transgenic mice and the wild type mice were different. The wild type sperm had normal hook-shaped head with clearly visible nucleus and a long whip-like tail whereas the sperm from the transgenic mice had abnormal head and kinky tail, which suggested improper development of sperms in transgenic mice. For further analysis, testis sections were taken from transgenic as well as from wild type animals. Haematoxylin and eosin staining of the transgenic mouse testis sections revealed an irregular arrangement of different types of cells in the seminiferous tubule.

Normally, the epithelia of the seminiferous tubules are lined by long columnar Sertoli cells (nurse cells) and spermatogonial cells (germ cells) arranged, parallel to

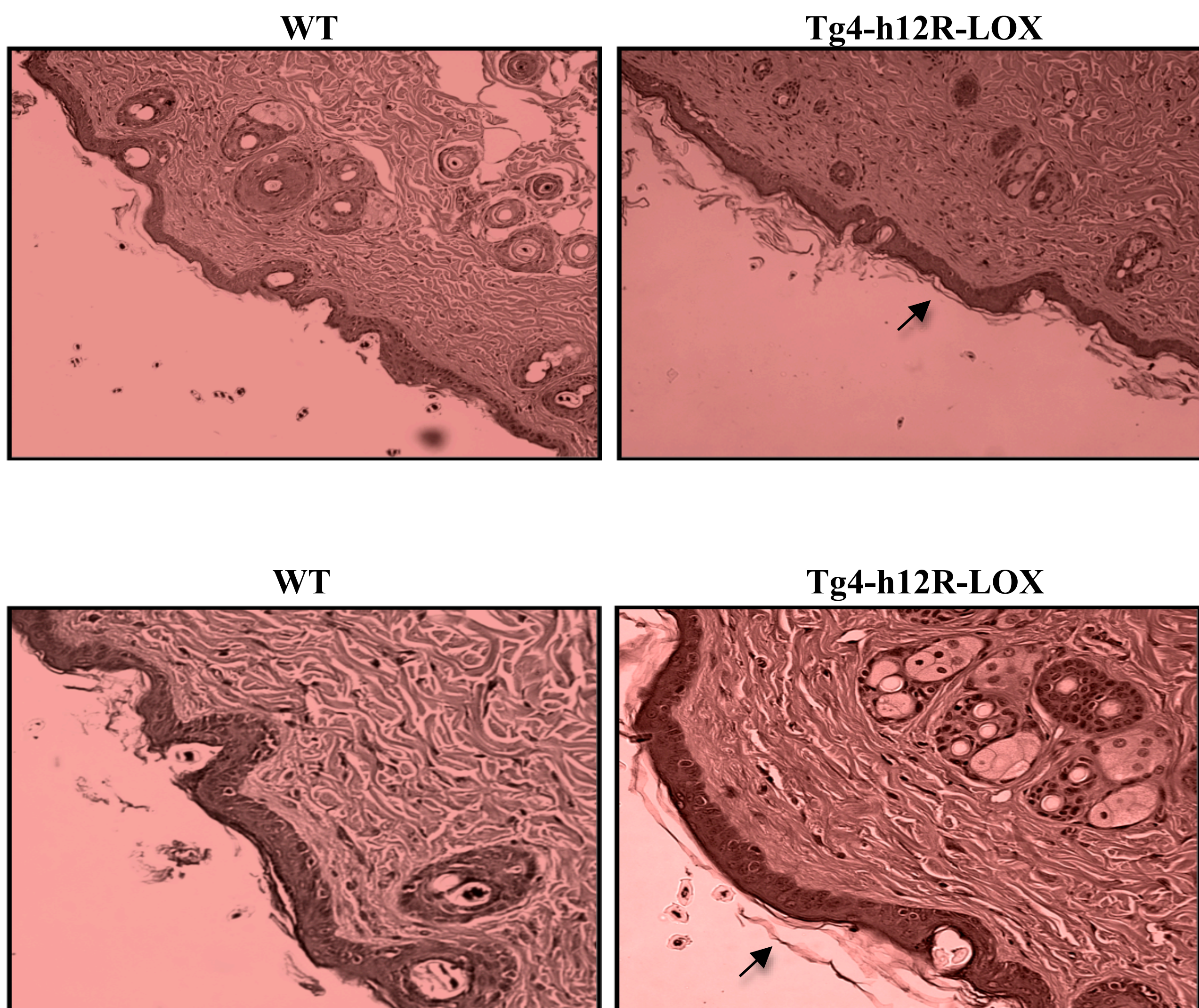
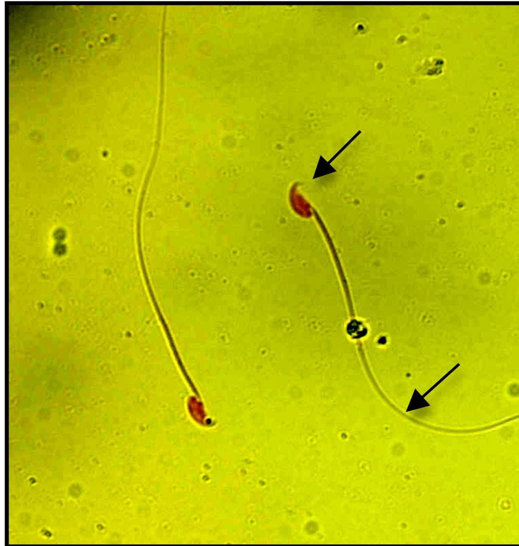


Fig. 4.7 Comparative analysis of the skin section of the wild type and transgenic mice Tg4-h12R-LOX mice Haematoxylin and eosin staining of skin sections revealed differences in the stratum corneum of epidermis. Stratum corneum was intact in case of wild type, which was not seen in case of Tg4-h12R-LOX.

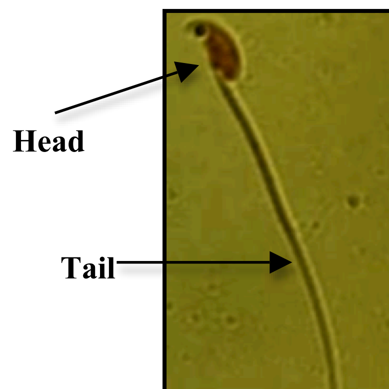
A

WT Tg11-12RLOX



B

WT Tg11-12RLOX



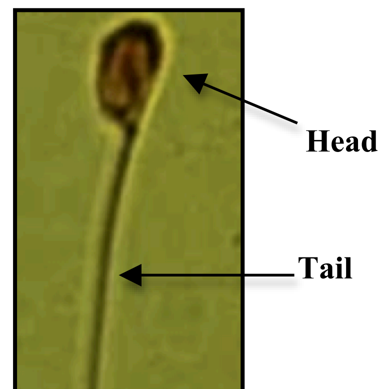
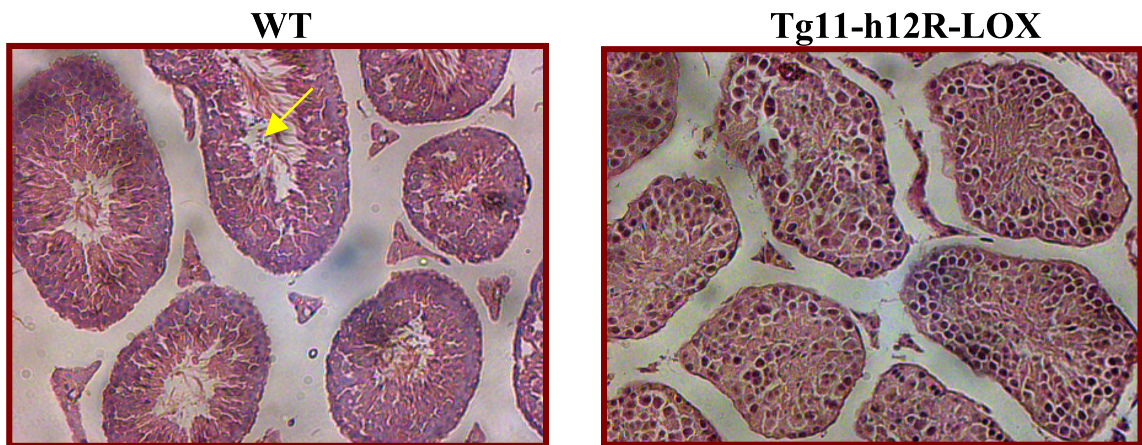


Fig. 4.8 Comparative study of the sperm morphology between wild type and transgenic Tg11-h12R-LOX mice (A) Spermatozoa collected form the wild type were normal in shape and motility whereas 70% of the spermatozoa collected from the transgenic mice were distinctly different from that of the sperms of the transgenic mice and were less motile **(B)** The head of the wild type mice showed clear nucleus and was hook shaped while the head of the sperms from the transgenic mice were irregular in shape and showed kinky tails.

A



B

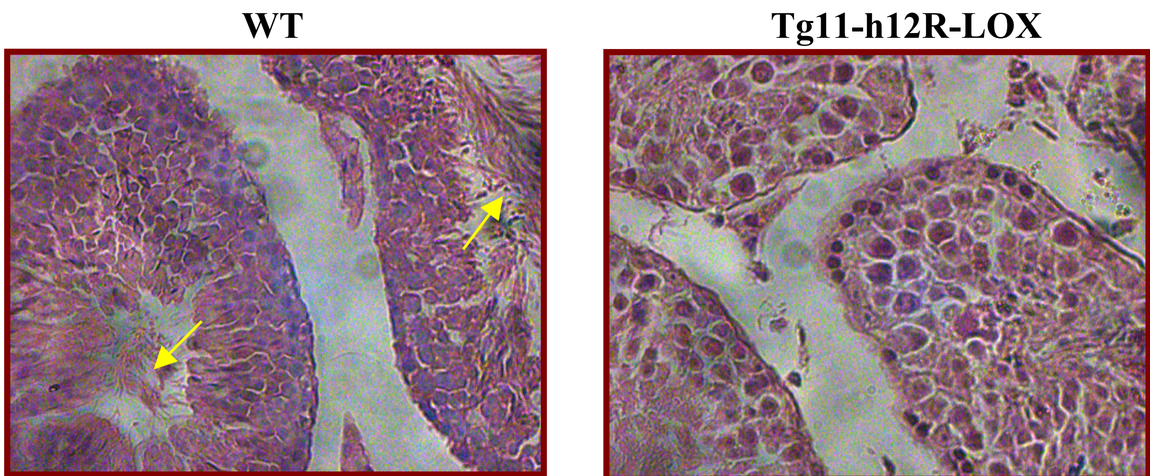


Fig. 4.9 Comparative analysis of the testis sections between wild type and transgenic mice Tg11-h12R-LOX mice (A) H&E staining of the testis sections revealed the arrangement of the seminiferous tubules from both wild type and 12RLOX-transgenic mice. The seminiferous tubules in the wild type testis sections revealed distinct outer tubular part and central lumen whereas the seminiferous tubules of the transgenic mice testis did not show any such distinction. **(B)** Higher magnification of the wild type testis sections revealed the presence of spermatogonial cells in different stages in a concentric manner with mature spermatozoa inside the lumen. The testis sections in the 12RLOX-transgenic mice however revealed an aberrant arrangement of the cells with no clear lumen and rarely any mature spermatozoa were observed.

each other. The spermatogonial cells undergo meiosis and finally mature into spermatozoa. During this maturation process, they change their shapes and translocate from periphery to the lumen of the tubule. The mature spermatozoa are seen in the lumen with long tails.

In wild type seminiferous tubules of mouse testis section, the arrangement of the cells was typical as seen in the testis sections. There is a clear-cut distinction between the outer layer containing dividing spermatogonial cells and inner lumen containing mature spermatozoa. However this arrangement was not at all observed in the seminiferous tubules of the transgenic mice testis. The cells were not found to be aligned in the normal fashion but were seen scattered all over the tubular surface. No clear lumen was visible in the seminiferous tubules of the transgenic mice testis **(Fig. 4.9A)**. Only few mature spermatozoa were seen in the tubules, giving enough evidence for the sub-fertility of the male transgenic mice **(Fig. 4.9B)**. The shape of the sperm head from the wild type was normally sickle-shaped with flagellated tails whereas sperms from transgenic mice had round-headed sperm with kinky tails **(Fig. 4.8A,B).**

4.3 Discussion.

12R-LOX exhibited a tissue specific pattern with the highest expression in differentiated layers of epidermis suggesting functional linkage and its role in differentiation process. Mutations in h-12R-LOXgene have been seen to be associated with abnormalities of the skin with impaired lipid membrane formation of the cells in the epidermal region (NBCIE) (Jobard *et al.,* 2002). Complete ablation of the gene in mouse indicates much more severe consequences with complete loss of barrier functions of the skin leading to the death of the neonates (Epp *et al.,* 2007). 12*R*-HPETE, product of 12R-LOX, acts as an activator of PPARα, which regulates various functions, including differentiation, proliferation, wound healing and lipid metabolism (Yu *et al.,* 1995). Interestingly, 12*R*-HETE has also been seen to be accumulated in scales of the psoriatic skin (Hammerstrom *et al.,* 1975). These observations clearly state that regulated expression of 12*R*-LOX is important for the maintenance of tissue homeostasis.

The present study has been undertaken to generate the transgenic mice model, over-expressing human 12*R*-LOX, for *in vivo* characterization. We started with

generation of h-12R-LOXtransgenic mouse where expression of the transgene was driven by CMV promoter. We were not able to get any transgenic mice. Most of the time we observed abortions of the embryos with least number of live births, which were negative for the transgene integration. This could be due to the reason that huge and global expression of the transgene, under CMV promoter, which may be proving lethal. During embryonic development, 12*R*-LOX expression starts very late, (15.5 dpc) the onset of skin development, in mice and is also spatially regulated, signifying the importance of its regulated expression for normal development (Sun *et al.,* 1998). To circumvent this problem we chose an endogenous Keratin 14 promoter, which is well-characterized promoter (and had been used for transgene expression) and its expression is restricted to squamous cell epithelia. For this we cloned h-12R-LOX downstream of K14 promoter in pG3Z-Betaglobin-K14 PolyA cassette that was a kind gift from Dr. Elian Fuchs, Rockefeller University, USA.

We were able to generate three independent transgenic lines for 12R-LOX by pronuclear microinjection of the DNA, i.e., Tg4, Tg11 and Tg17. Tg4 line showed an overexpression of 12R-LOX protein by 2.5 fold and the transgene was found to be integrated inside the intron of a gene “Down syndrome like cell adhesion protein”. There were no morphological differences between the transgenic and wild type mice. However, H&E stained skin sections revealed intact epidermal layers in wild type as compared to that of transgenic mice skin. Although, detailed examination is required yet the difference was clear and significant.

The other two independent founder transgenic lines (Tg11 and Tg17), which were male mice, did not produce sufficient litter size. This observation prompted us to look for the fertility parameters. Interestingly, the sperms from the transgenic mice were abnormal in shape and less motile. Testis sections of the transgenic mice revealed eccentric arrangement of different cell types in the seminiferous tubules. Since, transgenic line Tg4 did not show any problem with the fertility parameters and because the integration of the transgene by microinjection is random, it might be possible that integration of the transgene has disrupted an important gene related to male fertility parameters. Disrupted gene can be identified by inverse PCR and will help to illustrate the function of that gene in great detail.

Chapter 5

Overview

CHAPTER 5: OVERVIEW

In last two decades there has been a significant rise in the number of reports examining the functional links between various LOX enzymes and the mechanisms controlling cancer development and its progression. Examination of knockout and transgenic animals revealed important roles for lipoxygenases in inflammatory diseases. These studies have revealed diverse functions for lipoxygenases, which include cellular proliferation, differentiation and apoptosis. They form an important class of enzymes, which utilize PUFAs with 1,4-cis, cis pentadiene structures such as arachidonic acid (or linoleic acid) and produce various lipid mediators such as hydro(pero)xidies (H(P)ETEs), leukotrienes, hepoxilins, etc. *In vitro* and *in vivo* studies have revealed that these molecules have been observed to play critical roles in various cellular functions. The lipoxygenase enzyme with which I have dealt with in my thesis is categorized as 12*R*-LOX, owing to its tendency to add molecular oxygen at 12th position of arachidonic acid and produce the product with R-stereospecificity. This is a unique lipoxygenase, as its product is of R-stereospecificity, i.e., 12R HPETE.12R-LOX enzyme is highly essential as it plays an important role in barrier formation and prevention of transepidermal water loss. Its up regulation has been observed in psoriasis and other inflammatory dermatoses.

5.1 Development of an *in vitro* assay system for human 12R-LOX enzyme and identification of its inhibitors

This section deals with the designing of an *in vitro* assay system to assay the enzymatic activity of the purified 12R-LOX enzyme. For this I have cloned human12R-LOX cDNA in bacterial expression vector pET28 (b) and expressed it in bacteria. Production of this enzyme in bacterial system is advantageous as the yield of the protein is high and is also cost efficient. Though people have earlier tried to express human 12R-LOX in bacteria with a little achievement, but I have been able to overcome such problem. I have used an engineered strain of BL21 Rossetta strain, which takes care of codon bias, normally observed in any heterologous expression system. HPLC based assay system was developed to assess the activity of 12RLOX enzyme *in vitro*. We showed that human 12R-LOX enzyme utilizes AA over AA Me as

a substrate, which is similar to what was observed with mouse 12R-LOX enzyme. Purified enzyme showed higher specific activity compared to crude cell lysate.

Further, using this assay system we have screened several plant extracts to check inhibition of this enzyme. Natural product sources from traditionally used medicinal plants have proved to be better alternatives to the synthetic chemicals, with little or no side effects. Our lab has earlier reported the anti-cancer effects of natural compounds like c-Phycocyanin (COX-2 inhibitor) and Betanin (5-LOX inhibitor). Chebulagic acid (COX-2/5-LOX dual inhibitor) has been shown to have potent anti-inflammatory and anti-cancer effects in pre-clinical studies. *Acalypha indica* extracts have been attributed with anti-inflammatory and wound healing properties (Rahman *et al*., 2010, Ganeshkumar *et al*., 2012).

Interestingly, aqueous extracts and ethyl acetate extracts of plant *Acalypha indica* showed potent inhibition against 12R-LOX activity. We propose here that further purification of different compounds of these extracts may lead to the identification of potent molecules inhibiting 12R-lipoxygenase. The lead molecules thus identified and their further optimization may result in the development of a drug candidate for the treatment of psoriasis and other inflammatory skin pathologies. Further the present study resulted in showing potent inhibition of 12R-LOX by Zileuton, a standard 5-LOX inhibitor being used as an anti-asthmatic drug.

In conclusion, we have expressed and purified h-12R-LOX in bacterial expression system. Further we have developed an assay system for human 12R LOX enzyme, which was employed successfully in inhibitor screening assays.

5.2 Generation of transgenic mice model expressing human 12R-LOX.

Transgenesis has proven to be significant for both physiological as well as pathophysiological studies. Animal models with conditional expression of a transgene can be generated where the expression of the transgene can be controlled. Such approaches are helpful for the generation of models suitable for physiological analysis or for mimicking disease state.

Knocking out 12R-LOX gene in mice showed role of 12R-LOX in normal skin development. 12R-LOX deficiency in mouse resulted in severe impairment of epidermal barrier function and led to death of newly born mice (Epp *et al*., 2007).

Similarly eLOX-3, which acts on the product of 12R-LOX, deficiency in mice led to postnatal death but with lesser severe skin barrier defects than for the mice deficient in 12R-LOX (Kreig *et al*., 2013). These studies indicate that 12R-LOX – eLOX-3 pathway plays a key role in the process of epidermal barrier organisation and for proper skin development. Inactivating mutations in 12R-LOX and eLOX-3 genes are linked to genetic defect of autosomal recessive congenital icthyosis (ARCI) and to other inflammatory skin disorders (Eckl *et al*., 2005).

The background information thus clearly indicates that the enzyme 12R-LOX is indispensable for maintenance of the barrier functions. In order to get an insight about its role in pathophysiology of inflammatory skin diseases, we have designed our study to generate a transgenic mice model overexpressing h-12R-LOX. We first tried to generate the 12R-LOX transgenic mice using CMV promoter construct but to our surprise, most of the time the female mice had abortions and the live pups borne were all negative for the transgene integration We speculated that global and strong expression from CMV promoter must have led to loss of the transgenic embryos.

It has been speculated that it may be disadvantageous that the transgene is expressed in several organs rather than one of interest. The toxic effect or lethality related to early expression, in some tissues or organs, of the transgene during embryonic development thus impede analysis of the phenotype (Jaisser, 2000). To overcome such hurdles, several strategies have been developed to allow the regulated control on the expression of the transgene. Restriction on the transgene expression is also linked to the properties of the promoter used to make the construct. So the protein will be expressed when or where the promoter used in the transgenic construct is turned on.

For precise analysis of the function of human 12R-LOX, I cloned human 12R-LOX full-length cDNA under keratin K14 promoter and then using this construct transgenic mouse was generated. The construct harbours K14 promoter, which is a well- characterized promoter and has been used to generate transgenic mice for various genes (Wang *et al.,* 1996). The cassette contains a beta globin intron, which acts like an enhancer for the expression of the transgene and a keratin14 poly A tail which helps in stabilizing the mRNA transcript produced from the transgene. We were successful in generating h-12R-LOX transgenic mice by using Keratin14 promoter construct, which

restricts the expression of the transgene specifically to the stratified squamous epithelia. We could generate three independent transgenic founder lines. In Tg4 we could clearly observe the increased number of the stratum corneum layers in the epidermal regions of transgenic mice skin when compared to wild type skin. Further confirmation for this hyperproliferation can be done using some proliferation markers. The other two transgenic lines, which were males, exhibited compromised fertility and did not produce sufficient litter size. Examination of the fertility parameters for these male founders, revealed 70% immature sperms with reduced motility and abnormal shapes of their heads and tails. Testis sections clearly showed aberrant shaped spermatogonial cells in the seminiferous tubules. The appearance of such phenotype could possibly be due to the fact that an important gene responsible for maturation or differentiation of the male spermatogonial cells in the testis has been disrupted because of the transgene integration in the mouse genome. Further studies employing inverse PCR, however, are required to precisely identify the location of the transgene integration in the genome.

5.3 Conclusions

The present study is an attempt to characterize 12R-LOX protein *in-vitro* as well as *in-vivo*. I have expressed and purified active human 12R-LOX protein in bacteria and assessed its biochemical activity. Using this assay system, we have identified *Acalypha indica,* used in the Traditional System of Medicine for the treatment of various skin ailments, as a potential source for candidate molecules inhibiting h12R-LOX. Further identification and validation of the potential compound from these extracts will help in the development of potential drug candidate(s) for inflammatory skin ailments, which are characterized with huge expression of the 12R-LOX.

For *in vivo* studies, I have generated transgenic mouse expressing human 12R-LOX. In all, I could get three transgenic founder lines of male mice with integration of the h12R-LOX transgene. One of the transgenic lines, which showed 2.5-fold protein expression of the transgene exhibited differences in the outermost layer of the skin sections (stratum corneum) when compared to the skin from wild type mice. By using inverse PCR, for Tg4 line, I was able to detect the site of integration of the transgene which was in one of the introns of a gene named as “Down syndrome like cell adhesion

protein". However, this line needs to be characterized further for detailed phenotypic analysis. The transgenic mice can be used to decipher the precise pathways affected due to the overexpression of 12R-LOX. This will further help us to dig out useful information regarding the role of 12R-LOX.

Other two lines, Tg11 and Tg17, showed compromised fertility parameters. However, the inverse PCR analysis for knowing the site of integration for the transgene in these two lines is not complete. There is a possibility that the transgene integration might have disrupted some important genes related to male fertility parameters. Further characterization on the precise location of integration may unravel the function of an important gene, which has been disrupted by the transgene integration.

References

Agarwal S, Achari C, Praveen D, Roy KR, Reddy GV, *et al*. (2009) Inhibition of 12-LOX and COX-2 reduces the proliferation of human epidermoid carcinoma cells (A431) by modulating the ERK and PI3K-Akt signalling pathways. Exp Dermatol 18: 939-946.

Aparoy P, Leela T, Reddy RN, Reddanna P (2009) Computational analysis of R and S isoforms of 12-lipoxygenases: homology modeling and docking studies. J Mol Graph Model 27: 744-750.

Avis IM, Jett M, Boyle T, Vos MD, Moody T, *et al*. (1996) Growth control of lung cancer by interruption of 5-lipoxygenase-mediated growth factor signaling. J Clin Invest 97: 806-813.

Baer AN, Costello PB, Green FA (1991) Stereospecificity of the products of the fatty acid oxygenases derived from psoriatic scales. J Lipid Res 32: 341-347.

Baer AN, Green FA (1993) Fatty acid oxygenase activity of human hair roots. J Lipid Res 34: 1505-1514.

Baer AN, Klaus MV, Green FA (1995) Epidermal fatty acid oxygenases are activated in non-psoriatic dermatoses. J Invest Dermatol 104: 251-255.

Bisakowski B, Perraud X and Kermasha S. (1997). "Characterization of hydroperoxides and carbonyl compounds obtained by lipoxygenase extracts of selected microorganisms." Biosci Biotechnol Biochem. 61: 1262-9.

Boeglin WE, Kim RB, Brash AR (1998) A 12R-lipoxygenase in human skin: mechanistic evidence, molecular cloning, and expression. Proc Natl Acad Sci U S A 95: 6744-6749.

Brash AR. (1999). "MINIREVIEW - Lipoxygenases: Occurrence, Functions, Catalysis, and Acquisition of Substrate." J Biol Chem. 274: 23679-82.

Brash AR, Baertschi SW, Ingram CD and Harris TM. (1987). "On non-cyclooxygenase prostaglandin synthesis in the sea whip coral *Plexaura homomalla*: an 8(*R*)-lipoxygenase pathway leads to formation of α-ketol and a racemic prostanoid". J Biol Chem 262:15829-39.

Brash AR, Boeglin WE, Chang MS, Shieh BH (1996) Purification and molecular cloning of an 8R-lipoxygenase from the coral Plexaura homomalla reveal the related primary structures of R- and S-lipoxygenases. J Biol Chem 271: 20949-20957.

Brash AR (2001) Arachidonic acid as a bioactive molecule. J Clin Invest 107: 1339-1345.

Brash AR, Yu Z, Boeglin WE, Schneider C (2007) The hepoxilin connection in the epidermis. FEBS J 274: 3494-3502.

Bundy GL, Nidy EG, Epps DE, Mizsak SA and Wnuk RJ. (1986). "Discovery of an arachidonic acid C-8 lipoxygenase in the gorgonian coral *Pseudoplexaura porosa*". J Biol Chem 261:747-51

Capdevila JH, Falck JR, Estabrook RW (1992) Cytochrome P450 and the arachidonate cascade. FASEB J 6: 731-736.

Carter GW, Young PR, Albert DH, Bouska J, Dyer R, *et al*. (1991) 5-lipoxygenase inhibitory activity of zileuton. J Pharmacol Exp Ther 256: 929-937.

Chang WC, Ning CC, Lin MT, Huang JD (1992) Epidermal growth factor enhances a microsomal 12-lipoxygenase activity in A431 cells. J Biol Chem 267: 3657-3666.

Chen W, Li Z, Zhang J (1994) Treating osteoarthritis of the knee joint by traditional Chinese medicine. J Tradit Chin Med 14: 279-282.

Coffa G, Brash AR (2004) A single active site residue directs oxygenation stereospecificity in lipoxygenases: stereocontrol is linked to the position of oxygenation. Proc Natl Acad Sci U S A 101: 15579-15584.

Deb G, Boeshanes K, Idler WK, Ahvazi B (2011) Cloning, expression, purification, crystallization and preliminary X-ray diffraction studies of a 12R-LOX-chaperone complex. Acta Crystallogr Sect F Struct Biol Cryst Commun 67: 903-906.

Drazen JM (1999) Asthma therapy with agents preventing leukotriene synthesis or action. Proc Assoc Am Physicians 111: 547-559.

Drazen JM, Lilly CM, Sperling R, Rubin P, Israel E (1994) Role of cysteinyl leukotrienes in spontaneous asthmatic responses. Adv Prostaglandin Thromboxane Leukot Res 22: 251-262.

Eckl KM, Krieg P, Kuster W, Traupe H, Andre F, *et al*. (2005) Mutation spectrum and functional analysis of epidermis-type lipoxygenases in patients with autosomal recessive congenital ichthyosis. Hum Mutat 26: 351-361.

Epp N, Furstenberger G, Muller K, de Juanes S, Leitges M, *et al*. (2007) 12R-lipoxygenase deficiency disrupts epidermal barrier function. J Cell Biol 177: 173-182.

Ezaki M, Witztum JL, Steinberg D (1995) Lipoperoxides in LDL incubated with fibroblasts that overexpress 15-lipoxygenase. J Lipid Res 36: 1996-2004.

Fretland DJ, Widomski DL, Zemaitis JM, Tsai BS, Djuric SW, *et al*. (1989) 12(R)-hydroxyeicosatetraenoic acid is a neutrophil chemoattractant in the cavine, lapine, murine and canine dermis. Prostaglandins Leukot Essent Fatty Acids 37: 79-81.

Fulton D, Mahboubi K, McGiff JC, Quilley J (1995) Cytochrome P450-dependent effects of bradykinin in the rat heart. Br J Pharmacol 114: 99-102.

Ghosh J, Myers CE (1997) Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-lipoxygenase. Biochem Biophys Res Commun 235: 418-423.

Govindarajan M, Jebanesan A, Pushpanathan T, Samidurai K (2008) Studies on effect of Acalypha indica L. (Euphorbiaceae) leaf extracts on the malarial vector, Anopheles stephensi Liston (Diptera:Culicidae). Parasitol Res 103: 691-695.

Govindarajan M, Jebanesan A, Reetha D, Amsath R, Pushpanathan T, *et al*. (2008) Antibacterial activity of Acalypha indica L. Eur Rev Med Pharmacol Sci 12: 299-302.

Hada T, Swift LL and Brash AR. (1997). "Discovery of 5R-lipoxygenase activity in oocytes of the surf clam, *Spisula solidissima*." Biochim Biophys Acta 1346: 109-19.

Hammarstrom S, Hamberg M, Samuelsson B, Duell EA, Stawiski M, *et al*. (1975) Increased concentrations of nonesterified arachidonic acid, 12L-hydroxy-5,8,10,14-eicosatetraenoic acid, prostaglandin E2, and prostaglandin F2alpha in epidermis of psoriasis. Proc Natl Acad Sci U S A 72: 5130-5134.

Hanley K, Feingold KR, Komuves LG, Elias PM, Muglia LJ, *et al*. (1998) Glucocorticoid deficiency delays stratum corneum maturation in the fetal mouse. J Invest Dermatol 111: 440-444.

Hardouin SN, Nagy A (2000) Mouse models for human disease. Clin Genet 57: 237-244.

Hassan-Abdallah A, Merito A, Hassan S, Aboubaker D, Djama M, *et al*. (2013) Medicinal plants and their uses by the people in the Region of Randa, Djibouti. J Ethnopharmacol 148: 701-713.

Hawkins DJ and Brash AR. (1987) "Eggs of the sea urchin, *Strongylocentrotus purpuratus*, contain a prominent (11R) and (12R) lipoxygenase activity." J Biol Chem. 262: 7629-34.

Heidt M, Fürstenberger G, Vogel S, Marks F and Krieg P. (2000). “Diversity of mouse lipoxygenases: identification of a subfamily of epidermal isozymes exhibiting a differentiation-dependent mRNA expression pattern”. Lipids 35: 701-7.

Honn KV, Tang DG, Gao X, Butovich IA, Liu B, *et al*. (1994) 12-lipoxygenases and 12(S)-HETE: role in cancer metastasis. Cancer Metastasis Rev 13: 365-396.

Imig JD (2000) Eicosanoid regulation of the renal vasculature. Am J Physiol Renal Physiol 279: F965-981.

Israel E, Cohn J, Dube L, Drazen JM (1996) Effect of treatment with zileuton, a 5-lipoxygenase inhibitor, in patients with asthma. A randomized controlled trial. Zileuton Clinical Trial Group. JAMA 275: 931-936.

Inoue, H., Nojima, H., and Okayama, H. (1990). High efficiency transformation of Escherichia coli with plasmids. Gene 96, 23-28.

Jansen C, Hofheinz K, Vogel R, Roffeis J, Anton M, *et al*. (2011) Stereocontrol of arachidonic acid oxygenation by vertebrate lipoxygenases: newly cloned zebrafish lipoxygenase 1 does not follow the Ala-versus-Gly concept. J Biol Chem 286: 37804-37812.

Jaisser F (2000). Inducible gene expression and gene modification in transgenic mice. J Am Soc Nephrol 11:S95-S100.

Jiang ZD, Ketchum SO and Gerwick WH. (2000). "5-Lipoxygenase-derived oxylipins from the red alga *Rhodymenia pertusa"*. Phytochemistry 53: 129-33.

Jira W, Spiteller G, Richter A (1997) Increased levels of lipid oxidation products in low density lipoproteins of patients suffering from rheumatoid arthritis. Chem Phys Lipids 87: 81-89.

Jobard F, Lefevre C, Karaduman A, Blanchet-Bardon C, Emre S, *et al*. (2002) Lipoxygenase-3 (ALOXE3) and 12(R)-lipoxygenase (ALOX12B) are mutated in non-bullous congenital ichthyosiform erythroderma (NCIE) linked to chromosome 17p13.1. Hum Mol Genet 11: 107-113.

Kang SW, Adler SG, Nast CC, LaPage J, Gu JL, *et al*. (2001) 12-lipoxygenase is increased in glucose-stimulated mesangial cells and in experimental diabetic nephropathy. Kidney Int 59: 1354-1362.

Kirtikar KR, Basu BD (1999) *In*: Indian Medicinal Plant, vol. III, International Book Distributors, Dehradun, India, pp. 2262-2263.

Krieg P, Kinzig A, Ress-Loschke M, Vogel S, Vanlandingham B, *et al*. (1995) 12-Lipoxygenase isoenzymes in mouse skin tumor development. Mol Carcinog 14: 118-129.

Krieg P, Kinzig A, Heidt M, Marks F, Furstenberger G (1998) cDNA cloning of a 8-lipoxygenase and a novel epidermis-type lipoxygenase from phorbol ester-treated mouse skin. Biochim Biophys Acta 1391: 7-12.

Krieg P, Rosenberger S, de Juanes S, Latzko S, Hou J, *et al*. (2013) Aloxe3 knockout

mice reveal a function of epidermal lipoxygenase-3 as hepoxilin synthase and its pivotal role in barrier formation. J Invest Dermatol 133: 172-180.

Krieg P, Siebert M, Kinzig A, Bettenhausen R, Marks F, *et al*. (1999) Murine 12(R)-lipoxygenase: functional expression, genomic structure and chromosomal localization. FEBS Lett 446: 142-148.

Kuhn H, O'Donnell VB (2006) Inflammation and immune regulation by 12/15-lipoxygenases. Prog Lipid Res 45: 334-356.

Kuhn H, Schewe T, Rapoport SM (1986) The stereochemistry of the reactions of lipoxygenases and their metabolites. Proposed nomenclature of lipoxygenases and related enzymes. Adv Enzymol Relat Areas Mol Biol 58: 273-311.

Kuhn H, Schewe T, Rapoport SM (1986) The stereochemistry of the reactions of lipoxygenases and their metabolites. Adv Enzymol Relat Areas Mol Biol 58: 273-311.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Laird, P.W., Zijderveld, A., Linders, K., Rudnicki, M.A., Jaenisch, R., and Berns, A. (1991). Simplified mammalian DNA isolation procedure. Nucleic Acids Res *19*, 4293.

Lefevre C, Bouadjar B, Karaduman A, Jobard F, Saker S, *et al*. (2004) Mutations in ichthyin a new gene on chromosome 5q33 in a new form of autosomal recessive congenital ichthyosis. Hum Mol Genet 13: 2473-2482.

Li H, Lorie EP, Fischer J, Vahlquist A, Torma H (2012) The expression of epidermal lipoxygenases and transglutaminase-1 is perturbed by NIPAL4 mutations: indications of a common metabolic pathway essential for skin barrier homeostasis. J Invest Dermatol 132: 2368-2375.

Li SL, Dwarakanath RS, Cai Q, Lanting L, Natarajan R (2005) Effects of silencing leukocyte-type 12/15-lipoxygenase using short interfering RNAs. J Lipid Res 46: 220-229.

Lysz TW, Arora JK, Lin C, Zelenka PS (1994) 12(S)-hydroxyeicosatetraenoic acid regulates DNA synthesis and protooncogene expression induced by epidermal growth factor and insulin in rat lens epithelium. Cell Growth Differ 5: 1069-1076.

Mouli KC, Vijaya T, Dattatreya Rao S (2012) Effectiveness of flavonoid-rich leaf extract of Acalypha indica in reversing experimental myocardial ischemia: biochemical and histopathological evidence. Zhong Xi Yi Jie He Xue Bao 10: 784-792.

Meijer L, Brash AR, Bryant RW, Ng K, Maclouf J and Sprecher H. (1986). "Stereospecific induction of starfish oocyte maturation by 8(*R*)-hydroxyeicosatetraenoic acid". J Biol Chem 261:17040-7.

Meruvu S, Walther M, Ivanov I, Hammarstrom S, Furstenberger G, *et al*. (2005) Sequence determinants for the reaction specificity of murine (12R)-lipoxygenase: targeted substrate modification and site-directed mutagenesis. J Biol Chem 280: 36633-36641.

Moran JL, Qiu H, Turbe-Doan A, Yun Y, Boeglin WE, *et al*. (2007) A mouse mutation in the 12R-lipoxygenase, Alox12b, disrupts formation of the epidermal permeability barrier. J Invest Dermatol 127: 1893-1897.

Muga SJ, Thuillier P, Pavone A, Rundhaug JE, Boeglin WE, *et al*. (2000) 8S-lipoxygenase products activate peroxisome proliferator-activated receptor alpha and induce differentiation in murine keratinocytes. Cell Growth Differ 11: 447-454.

Munoz-Garcia A, Thomas CP, Keeney DS, Zheng Y, Brash AR. (2013) The importance of the lipoxygenase-hepoxilin pathway in the mammalian epidermal barrier. Biochim Biophys Acta 1841: 401-408.

Natarajan R, Esworthy R, Bai W, Gu JL, Wilczynski S, *et al*. (1997) Increased 12-lipoxygenase expression in breast cancer tissues and cells. Regulation by epidermal growth factor. J Clin Endocrinol Metab 82: 1790-1798.

Natarajan R, Nadler JL (2004) Lipid inflammatory mediators in diabetic vascular

disease. Arterioscler Thromb Vasc Biol 24: 1542-1548.

Piao YS, Du YC, Oshima H, Jin JC, Nomura M, *et al*. (2008) Platelet-type 12-lipoxygenase accelerates tumor promotion of mouse epidermal cells through enhancement of cloning efficiency. Carcinogenesis 29: 440-447.

Pidgeon GP, Lysaght J, Krishnamoorthy S, Reynolds JV, O'Byrne K, *et al*. (2007) Lipoxygenase metabolism: roles in tumor progression and survival. Cancer Metastasis Rev 26: 503-524.

Piomelli D, Volterra A, Dale N, Siegelbaum SA, Kandel ER, *et al*. (1987) Lipoxygenase metabolites of arachidonic acid as second messengers for presynaptic inhibition of Aplysia sensory cells. Nature 328: 38-43.

Porta H, Rocha-Sosa M (2001) Lipoxygenase in bacteria: a horizontal transfer event? Microbiology 147: 3199-3200.

Rapoport S, Schmidt J, Prehn S (1986) Fe-dependent formation of a protein that makes mitochondria lipoxygenase-susceptible during maturation of reticulocytes. FEBS Lett 198: 109-112.

Schmuth M, Jiang YJ, Dubrac S, Elias PM, Feingold KR (2008) Thematic review series: skin lipids. Peroxisome proliferator-activated receptors and liver X receptors in epidermal biology. J Lipid Res 49: 499-509.

Schneider C, Keeney DS, Boeglin WE, Brash AR (2001) Detection and cellular localization of 12R-lipoxygenase in human tonsils. Arch Biochem Biophys 386: 268-274.

Schneider C, Strayhorn WD, Brantley DM, Nanney LB, Yull FE, *et al*. (2004) Upregulation of 8-lipoxygenase in the dermatitis of IkappaB-alpha-deficient mice. J Invest Dermatol 122: 691-698.

Siebert M, Krieg P, Lehmann WD, Marks F and Fürstenberger G. (2001). “Enzymic characterization of epidermis-derived 12-lipoxygenase isozymes". Biochem J 355:97-104.

Sloane DL, Browner MF, Dauter Z, Wilson K, Fletterick RJ, *et al*. (1990) Purification

and crystallization of 15-lipoxygenase from rabbit reticulocytes. Biochem Biophys Res Commun 173: 507-513.

Sloane DL, Leung R, Craik CS and Sigal E. (1991). "A primary determinant for lipoxygenase positional specificity." Nature **354**: 149–52.

Smith DM, Waite M (1992) Phosphatidylinositol hydrolysis by phospholipase A2 and C activities in human peripheral blood neutrophils. J Leukoc Biol 52: 670-678.

Smith DM, Waite M (1992) Phosphatidylinositol hydrolysis by phospholipase A2 and C activities in human. J Leukoc Biol 52: 670-678.

Smith WL (1992) Prostanoid biosynthesis and mechanisms of action. Am J Physiol 263: F181-191.

Smith WL, Marnett LJ (1991) Prostaglandin endoperoxide synthase: structure and catalysis. Biochim Biophys Acta 1083: 1-17.

Su C and Oliw EH (1998). "Manganese lipoxygenase. Purification and Characterization." J Biol Chem 273: 13072-9.

Sun D, McDonnell M, Chen XS, Lakkis MM, Li H, *et al*. (1998) Human 12(R)-lipoxygenase and the mouse ortholog. Molecular cloning, expression, and gene chromosomal assignment. J Biol Chem 273: 33540-33547.

Sun GY, Chuang DY, Zong Y, Jiang J, Lee JC, *et al*. (2014) Role of Cytosolic Phospholipase A in Oxidative and Inflammatory Signaling Pathways in Different Cell Types in the Central Nervous System. Mol Neurobiol.

Taha AY, Cheon Y, Faurot KF, Macintosh B, Majchrzak-Hong SF, *et al*. (2014) Dietary omega-6 fatty acid lowering increases bioavailability of omega-3 polyunsaturated fatty acids in human plasma lipid pools. Prostaglandins Leukot Essent Fatty Acids 90: 151-157.

Thorburn DR, Beutler E (1991) The loss of enzyme activity from erythroid cells during maturation. Adv Exp Med Biol 307: 15-27.

Tulsiani DR, Yoshida-Komiya H, Araki Y (1997) Mammalian fertilization: a carbohydrate- mediated event. Biol Reprod. 57(3):487-94.

Wang D, Dubois RN (2010) Eicosanoids and cancer. Nat Rev Cancer 10: 181-193.

Wang X, Zinkel S, Polonsky K, and Fuchs (1996) Transgenic studies with a keratin promoter-driven growth hormone transgene: prospects for gene therapy. Proc. Natl. Acad. Sci. USA 94.

Woollard PM (1986) Stereochemical difference between 12-hydroxy-5, 8, 10, 14-eicosatetraenoic acid in platelets and psoriatic lesions. Biochem Biophys Res Commun 136: 169-176.

Yamamoto S (1992) Mammalian lipoxygenases: molecular structures and functions. Biochim Biophys Acta 1128: 117-131.

Yamamoto S (1999) [Essential unsaturated fatty acids]. Nihon Rinsho 57: 2242-2246.

Yu K, Bayona W, Kallen CB, Harding HP, Ravera CP, *et al*. (1995) Differential activation of peroxisome proliferator-activated receptors by eicosanoids. J Biol Chem 270: 23975-23983.

Yu Z, Schneider C, Boeglin WE, Marnett LJ, Brash AR (2003) The lipoxygenase gene ALOXE3 implicated in skin differentiation encodes a hydroperoxide isomerase. Proc Natl Acad Sci U S A 100: 9162-9167.

Yu Z, Schneider C, Boeglin WE, Brash AR (2005) Mutations associated with a congenital form of ichthyosis (NCIE) inactivate the epidermal lipoxygenases 12R-LOX and eLOX3. Biochim Biophys Acta 1686: 238-247.

Zheng Y, Yin H, Boeglin WE, Elias PM, Crumrine D, *et al*. (2011) Lipoxygenases mediate the effect of essential fatty acid in skin barrier formation: a proposed role in releasing omega-hydroxyceramide for construction of the corneocyte lipid envelope. J Biol Chem 286: 24046-24056.

APPENDIX

A.1 1M Phosphate buffer (pH 7.2)

684 ml 1M Na_2HPO_4 and 316 ml 1M NaH_2PO_4

A.2 Avertin

A stock of 100% avertin was prepared by mixing 10 gram of 2,2,2-tribromomethyl alcohol with 10 ml of *tert*-amyl alcohol by heating at 50°C overnight. The solution was diluted to 1.25% in saline for final use.

A.3 Luria-Bertani Medium (LB Medium)

LB Medium was prepared by dissolving 10g tryptone, 5g Yeast extract, 10g NaCl in one litter MilliQ H_2O and pH was adjusted with 5N NaOH.

For LB agar 2% agar-agar was added to LB Medium.

A.4 TBST

50mM Tris.Cl (pH 7.5), 150mM NaCl, 0.05% Tween-20

A.5 Inoue Transformation Buffer (TFB)

Inoue transformation buffer was prepared by dissolving all of the solutes listed below in 800 ml of MilliQ H_2O and then 20 ml of 0.5 M PIPES (pH 6.7) was added.

Reagent Amount per Liter Final Concentration

$MnCl_2.4H_2O$ 10.88 g (55 mM)

$CaCl_2.2H_2O$ 2.20 g (15 mM)

KCl 18.65 g (250 mM)

PIPES (0.5 M, pH 6.7) 20 ml (10 mM)

The final volume of the transformation buffer was adjusted to 1 liter with MilliQ H_2O.