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Transcriptional Regulation and the Role of

Murine 8*S*-Lipoxygenase in Mouse Skin Carcinogenesis

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Transcriptional Regulation and the Role of Murine 8*S*-lipoxygenase in Mouse Skin Carcinogenesis

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Dedication

To my loving family;

father, Bookil Kim,

mother, Bomi Kim,

and brother, Woochan Kim

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8*S*-lipoxygenase (8*S*-LOX), the murine homolog of human 15*S*-LOX-2, has unique expression features in mouse skin, i.e., it is not expressed at a detectable level in normal skin but is strongly expressed in several stages of skin tumorigenesis, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treated skin or in skin tumors. In this study, we studied a specific mechanism by which 8*S*-LOX gene expression is regulated by TPA in keratinocytes and the functional role of 8*S*-LOX during mouse skin carcinogenesis. In the first study, we found TPA-induced 8*S*-LOX mRNA expression in SSIN primary mouse keratinocytes was the result of increased transcription. Therefore, transcriptional regulation of 8*S*-LOX expression was further studied by cloning its promoter. The cloned 8*S*-LOX promoter (~ 2 kb) has neither a TATA box nor a CCAAT box, however, it was

highly responsive to TPA in SSIN primary keratinocytes. We then identified a Sp1 binding site (-68/-77) as a TPA responsive element (TRE) in the promoter and showed that Sp1, Sp2, and Sp3 proteins bind to the TRE. Binding of these proteins to the TRE was significantly increased by TPA treatment and 8*S*-LOX transcription was decreased when the binding of these proteins was inhibited. We thus concluded that increased binding of Sp1, Sp2, and Sp3 to the TRE of the 8*S*-LOX promoter is a mechanism by which TPA induces 8*S*-LOX expression in keratinocytes. In the second study, we found 8*S*-LOX is closely associated with keratinocyte differentiation and forced expression can inhibit mouse skin tumorigenesis. Targeted C57BL/6J transgenic mice overexpressing the 8*S*-LOX gene under control of the loricrin promoter exhibited more differentiated epidermal phenotypes as well as reduced papilloma development in a two-stage skin carcinogenesis protocol. Forced expression of the 8*S*-LOX gene in tumor-derived cell lines, MT1/2 (papilloma) and CH72 (carcinoma), also caused increased differentiation and inhibition of cell proliferation *in vitro* as well as in *in vivo* xenografts, respectively. Moreover, histochemical analyses showed 8*S*-LOX expression was strictly confined to the differentiated region of the skin in the course of skin tumorigenesis. Collectively, these findings suggest that 8*S*-LOX plays a role as a prodifferentiating, an anticarcinogenic, and a tumor suppressing gene in mouse skin.

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5-bromo-2’-deoxyuridine	BrdU
12-*O*-tetradecanoylphorbol-13-acetate	TPA
Arachidonic acid	AA
Cyclooxygenase	COX
Diacylglycerol	DAG
Dimethylbenz[*a*]anthracene	DMBA
Epidermal growth factor	EGF
Eagle’s minimal essential medium	EMEM
Electrophoretic mobility shift assay	EMSA
Fetal bovine serume	FBS
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
Hematoxylin and eosin	H&E
Hydroperoxyeicosatetraenoic acid	HPETE
Hydroperoxyoctadecadienoic acid	HPODE
Hydroxyeicosatetraenoic acid	HETE
Hydroxyoctadecadienoic acid	HODE
Keratin	K
Linoleic acid	LA
Lipoxygenase	LOX

Liquid chromatography/tandem mass spectrometry	LC/MS/MS
Leukotriene	LT
Mithramycin A	MMA
Mitogen-activated protein kinase	MAPK
Peroxisome proliferator-activated receptor	PPAR
Phosphate buffered saline	PBS
Phosphatidyl choline	PC
Phosphatidyl inositol	PI
Phospholipase A_2	PLA_2
Phospholipase C	PLC
Polyvinylidene difluoride	PVDF
Prostacyclin	PGI
Prostaglandin	PG
Protein kinase C	PKC
Reactive oxygen species	ROS
Reverse transcriptase-polymerase chain reaction	RT-PCR
Simple sequence repeat	SSR
Sodium dodecyl sulfate	SDS
Standard saline citrate	SSC
Transgenic	Tg
Transglutaminase	TG

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling	TUNEL
Thromboxane	TX
TPA responsive element	TRE
Tween 20-Tris-buffered saline	TTBS
Ultraviolet	UV
Wild type	WT

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1. Skin

1.1.1. Structure of the Skin

The skin of man or other animals is a large organ covering the entire body (reviewed in Odland 1983). It protects internal organs from various environmental hazards, mechanical stress, and water loss. At the same time, its vasculature and sweating system contributes to the regulation of body temperature as well as of excretion of metabolic waste. In addition, the remarkably sensitive nervous system in the skin promptly transduces environmental information to the central nervous system.

The skin is composed to two major compartments (Fig. 1.1). One is the dermis, a dense fibroelastic connective tissue layer, and the other is the thin stratified epithelial layer lying above the dermis, called epidermis. These distinct layers are physically separated by the basement membrane located between them. All of the vasculature and nervous system as well as various specialized glands

and hair follicles are located in the dermis. Therefore, the dermal layer is responsible for maintaining homeostasis of the skin as well as for neural transduction. The epidermal layer is primarily composed of epidermal cells and plays a role in protection of the skin and in conducting environmental information to responding elements in the dermis. Beneath the skin is the subcutaneous tissue which is composed of loose or fatty connective tissue.

1.1.2. Structure and Differentiation of the Epidermis

The epidermis has a continuous sheet-like cellular organization in which the integrity of the sheet is only disrupted by the pores of glandular structures and of hair follicles (reviewed in Odland 1983). Probably because of the innate function of the epidermis in protecting important body organs from the environment, the epidermal cells are primarily composed of keratinocytes, which produce the most rigid intermediate filament protein, keratin, as the major cytoskeletal protein. Moreover, the epidermis consists of multiple layers of keratinocytes and continuously repeats the renewing process in which old, fully differentiated cells are sloughed off and are replaced by young daughters of progenitor cells.

The self-renewing process of the epidermis is a result of an elaborate orchestration of epidermal differentiation (Fig. 1.1). The process of keratinocyte

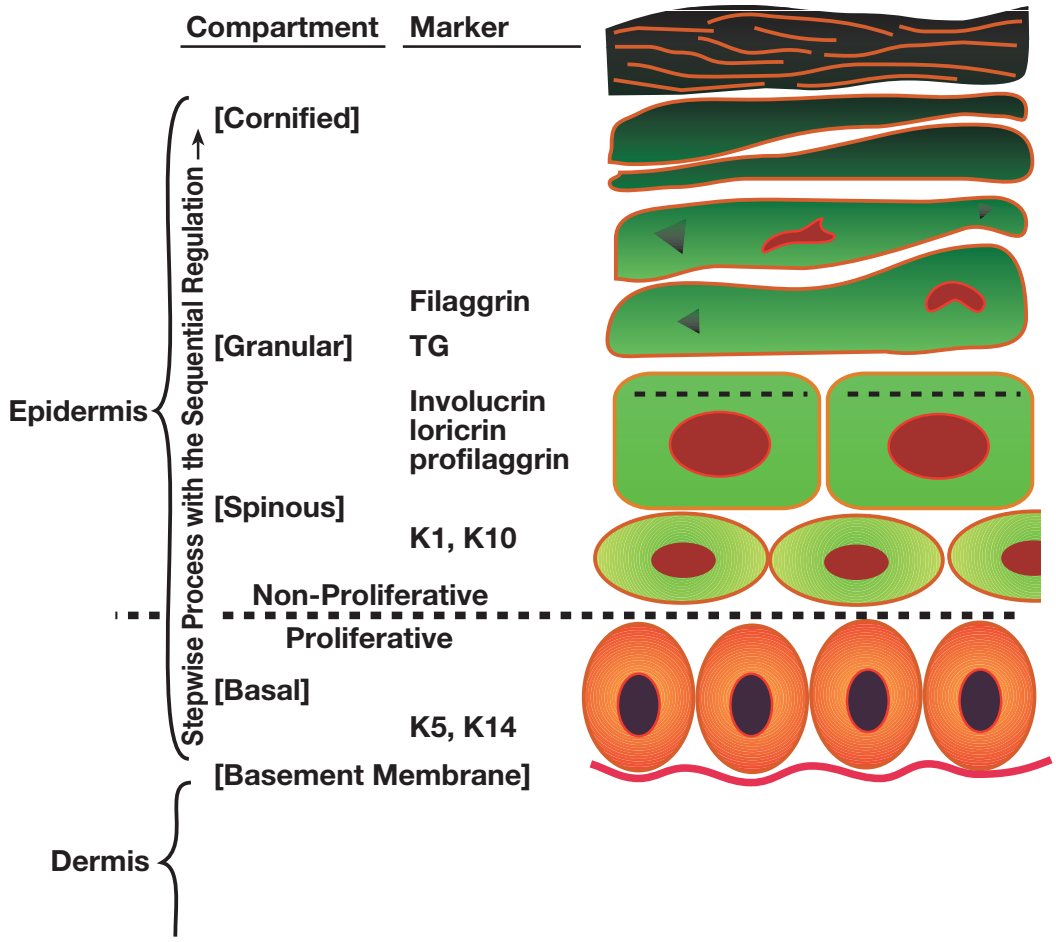


Fig. 1. 1. Mammalian skin structure.

Simplified schematic illustrating skin layers with representative differentiation-specific markers and epidermal compartments. Mammalian skin is composed of two different layers, dermis and epidermis, which are physically separated by a basement membrane (illustrated as a waved line). When the proliferative basal cells cease their mitotic activity, they start to migrate from the basement membrane to the suprabasal cell layers and to differentiate. The epidermal cells at each stage of differentiation express specific marker proteins and compose specific compartments of epidermis.

differentiation is accomplished by migration of epidermal cells from the basement membrane to the skin surface and is marked by specific marker proteins that are characteristic of each stage of differentiation (Eckert 1989). The very inner layer of the epidermis lying on top of the basement membrane is composed of a proliferative epidermal cell population refeffed to as basal cells. These undifferentiated basal cells express keratin (K) 5 and K14 as the cytoskeletal proteins (Fuchs and Green 1980; Woodcock-Mitchell et al. 1982). When the basal cell ceases its mitotic activity and migrates to the suprabasal layer, it starts to differentiate, which is marked by an alteration of the major cell products from K5 and K14 to K1 and K10 (Roop et al. 1983). The basal cells moving outward assume a polyhedral and spinous shape. Thus, the first cell type seen in the suprabasal cell layer is called the stratum spinosum (Odland 1983). The shape of the spinous cells, however, becomes progressively more flattened as it moves toward the skin surface. As the spinous cell further matures and differentiates, the cytoplasm of the cell is increasingly filled with filament proteins including involucrin (Banks-Schlegel and Green 1981) and loricrin (Mehrel et al. 1990), which results in aggregation of those proteins. Since the aggregation is seen as lamellar granules under the electron microscope, this layer of cells is called the stratum granulosum (Odland 1983). A subsequent differentiation of the granular cell is associated with several remarkable events in which mitochondria and ribosomes are degraded, the nuclear envelope disappears, and ultimately the

nucleus is degraded (Odland 1983). Additionally, the interstices of the cell are filled with a filament aggregating protein, filaggrin (Rothnagel et al. 1987), which results in a dense marginal band adjacent to the inner face of the plasma membrane (Odland 1983). Later on, the marginal band is attached to the plasma membrane by formation of ε-(γ-glutamyl) lysine bonds by transglutaminase (Buxman and Wuepper 1978). Since this dense marginal band makes up the thickened cell envelop, this terminally differentiated cell layer is called the stratum corneum and provides resistance of the epidermis to mechanical and chemical destruction. Subsequently, filaggrin in the cornified cell is degraded into free amino acids, which raises osmolarity in the cell and contributes to the retention of moisture and the maintenance of flexibility of the cornified cell (Scott et al. 1982).

1.2. Two-stage Mouse Skin Carcinogenesis

The development of a malignant neoplasm is a multistage process that usually starts from a single mutated cell that begins to proliferate abnormally in a limitless manner. In terms of multistage carcinogenesis, the mouse skin model provides an excellent model since it shows distinct physiological and molecular events in each process (DiGiovanni 1992; Slaga et al. 1980a; Slaga et al. 1980b). The multistage carcinogenesis process in skin has been subdivided into three

distinct stages termed initiation, promotion, and progression (Fig. 1.2) (Kulesz-Martin 1997).

Initiation of carcinogenesis is generally accomplished by a single, topical application of subcarcinogenic dose of chemical (e.g., 7,12-dimethylbenz[*a*]anthracene (DMBA), tobacco tar, etc.) or physical (e.g., X-rays, ultraviolet (UV) radiation) initiating agents to the dorsal mouse skin (Mukhtar et al. 1995). These initiating agents have the property of binding to DNA, introducing methylation, or causing damage to chromosomal DNA, which leads to genetic changes such as mutations, deletions, translocations, or loss of regions of chromosomes. Since every cell has a DNA damage repair system, however, application of an initiating agent itself does not always generate an "initiated" cell (Farber 1984). It needs several rounds of DNA synthesis and cell division in order to "fix" the mutation permanently (Pitot and Dragan 1991). Therefore, proliferating cell populations including basal cells and epidermal stem cells residing in hair follicles are prime targets for initiation. Once the mutation is fixed, it is not reversible. The most frequently mutated gene in the initiation stage is the *ras* oncogene (Balmain and Brown 1988; Bowden et al. 1995; Brown et al. 1990; Quintanilla et al. 1986). It is activated by specific point mutations (Quintanilla et al. 1986) and many of the commonly used skin carcinogens are in fact strong point mutagens. With chemical and UV irradiation as an initiator, a specific point mutation is introduced to Ha-*ras* and N-*ras*, respectively (Bowden

et al. 1995), which leads to constitutive activation of the proteins. On the other hand, ionizing radiation as an initiator activates distinct non-*ras* transforming genes because it induces larger genomic alterations, including DNA strand breaks, deletions, and chromosomal rearrangements rather than point mutations (Bowden et al. 1995). In any case, these genetic changes may produce constitutive growth signals in the initiated cells. Initiation alone at the subcarcinogenic dose is, however, not sufficient for the development of visible tumors during the life span of the animal. The selective clonal expansion of the initiated cells follows in the promotion stage, which is possible because the initiated cells are unable to differentiate. This is called two-stage skin carcinogenesis in which tumor development is accomplished through the stages of both initiation and promotion (Slaga 1984a). On the other hand, in the complete carcinogenesis protocol, application of the initiating agent at a carcinogenic dose alone is sufficient to cause tumors (Slaga 1984a).

Unlike the initiation stage, tumor promotion is accomplished by repetitive application of tumor promoting agents to the initiated skin (Bowden et al. 1995). Upon repeated treatment, mouse skin develops benign tumors, papillomas, which are characterized as a contained, differentiated lesion of uncoordinated growth, and lacking vascularization. It is generally accepted that tumor promoters do not bind to DNA and are not mutagenic like a carcinogen. Therefore, normal mouse skin usually does not develop tumors with application of the promoter alone

(Slaga 1984a). However, the promoting agents bring about several important epigenetic changes (e.g., alteration of gene expression, signal transduction, differentiation, and intercellular communication) to the cell, which is thought to help in the selective growth of the initiated cell (Winberg et al. 1995). These epigenetic changes are reversible in the early stages of promotion and are not inheritable like a carcinogen-induced DNA mutation in the initiation stage. Therefore, continued and repetitive promoter treatment is required to maintain the promoting activity of the agents and a cessation of promoter treatment causes regression or disappearance of benign tumors at least up to some point. The epigenetic changes induced by the promoter elicit many diverse genetic and physiological changes in the skin, including induction of epidermal hyperplasia, inflammation and ornithine decarboxylase expression (Winberg et al. 1995). Although the mechanism of tumor promotion has not been fully elucidated, induction of sustained hyperplasia of the skin has been the best correlated with the tumor promoting activity of the promoter (Slaga 1984a). In fact, regardless of type of tumor promoter, it has been reported that all known tumor promoters induce epidermal hyperplasia to some extent (Slaga et al. 1981). 12-*O*-tetradecanoylphorbol-13-acetate (TPA), the most potent and frequently used tumor promoter in mouse skin carcinogenesis, elicits those events mainly through activation of protein kinase C (PKC) (Castagna et al. 1982; Niedel et al. 1983). An endogenous ligand for PKC is diacylglycerol (DAG). However, TPA binds to

the same cysteine-rich zinc fingers in PKC with much higher potency since it has a longer half-life then DAG (Kuroki et al. 1995). Activated PKC exerts its major effect in skin via various mitogen-activated protein kinase (MAPK) cascades and stimulates expression of genes involved in hyperproliferation, tissue remodeling, and inflammation such as transforming growth factor α and cyclooxygenase-2 (Marks and Fürstenberger 2000). In the multistage model, many skin tumors remain as papillomas but some go further into irreversible progression stage, depending on strain and protocol.

In the tumor progression stage, benign papillomas convert into malignant carcinomas which are characterized by rapidly growing, invasive, and vascularized phenotypes. The conversion process occurs spontaneously in the absence of further tumor promoter treatment. Although how this process progresses is not understood very well, a number of gene and chromosome alterations are associated with tumor progression in mouse skin, which are thought to be responsible and/or permissive for this stage of carcinogenesis. Those alterations include trisomy of chromosome 7, loss of the normal Ha-*ras* allele, loss of E-cadherin, loss of K1 and K10, and stable expression of certain proteases (Bowden et al. 1995). In particular, increased expression of proteases is thought to be responsible for the acquisition of an invasive phenotype of highly aggressive tumor cells (Matrisian et al. 1986; Ostrowski et al. 1988). On the other hand, application of a progressing agent to the papilloma-bearing mice can

increase carcinoma incidence (Hennings et al. 1983; O'Connell et al. 1986). In this case the application doesn't need to be repetitive. The progressing agents include genotoxic carcinogens (e.g., benzo[*a*]pyrene diol epoxide, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, etc.), free radical generators (e.g., benzoyl peroxide, hydrogen peroxide, etc.), and others (e.g., acetic acid, ionizing radiation, etc.) (Winberg et al. 1995). In general, these agents do not affect the multiplicity of papillomas but only affect the conversion rate of preexisting papillomas to carcinomas. In this context, it has been thought that there is a genetic lesion(s) other than H-*ras* in the papillomas (Winberg et al. 1995). That is, occurrence of such a lesion(s) would be at a low rate in papillomas, leading to low incidence of carcinoma. Application of the progressor to the papillomas, however, increases the rate of the occurrence either directly or indirectly and leads to carcinoma development. Additionally, selective toxicity to the nonmalignant cells is another potential mechanism for the tumor progressing activity of the agents. That is, progressor treatment leads to elimination of those cells and allows malignant cells to further expand (Rotstein and Slaga 1988). Further progression of the tumors allows them to invade other tissues through blood and lymphatic vessels. Metastasis from skin squamous cell carcinomas, however, is uncommon.

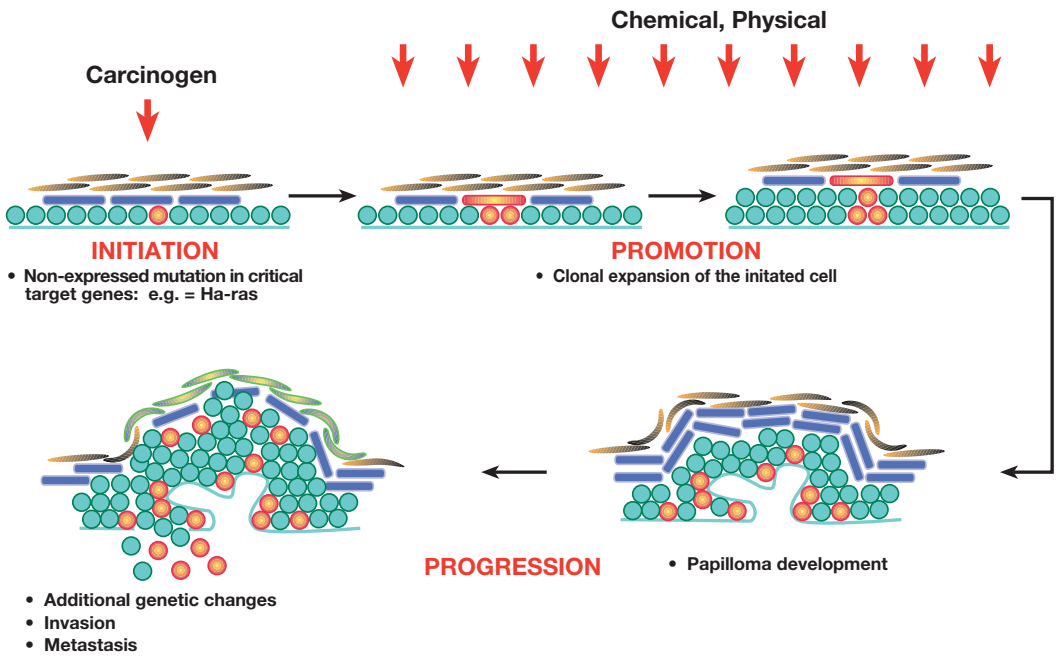


Fig. 1. 2. Mouse skin multistage carcinogenesis.

A multistage process of mouse skin tumor development is demonstrated. In the initiation stage, a critical target gene in epidermal cells is mutated by a single application of subcarcinogenic dose of carcinogen to mouse skin. In the promotion stage, clonal expansion of initiated cells occurs by repetitive treatment of chemical or physical tumor promoters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to the initiated skin. During this process, the initiated cells may develop into papillomas. In the progression stage, additional genetic mutations occur within rapidly proliferating cells, which convert benign papillomas to malignant carcinomas. Further progression allows them to invade and metastasize to other tissues.

1.3. Arachidonic Acid Metabolism in Mouse Skin

Arachidonic acid (AA) (5,8,11,14-eicosatetraenoic acid, 20:4, n-6) is a polyunsaturated fatty acid with 20 carbon atoms and 4 double bonds. It is commonly distributed to all tissues of animals and it can be obtained from either dietary sources or an endogenous biosynthetic pathway of desaturation and elongation of linoleic acid (18:2, n-6) (Iversen and Kragballe 2000). For humans, fats of land animals including brain, liver, muscle, glandular, and egg lipids are the dietary sources of AA. Synthesis of AA is however limited due to the absence of both Δ6-desaturase and Δ5-desaturase in certain tissues including human and mouse epidermis (Chapkin et al. 1986). Therefore, AA in these tissues must come from dietary intake or from transportation to the tissues from other endogenous sources such as liver, which is capable of synthesizing AA. Arachidonic acid exists in an esterified form in the *sn*-2 position of membrane phospholipids, primarily in phosphatidylcholine (PC) or phosphatidylinositol (PI), and rarely exists in a free form. Upon a variety of stimuli, for instance wounding, growth factors, hormones and TPA treatment, AA can be released by two distinct kinds of enzymes, phospholipase A_2 (PLA_2) and phospholipase C (PLC). PLA_2 was so named since it hydrolyzes the ester linkage in the *sn*-2 position, which allows AA to be released. Unlike PLA_2, PLC hydrolyzes PC or PI in the *sn*-3 position first, and generates free phosphocholine or phosphoinositol, and DAG

moieties which are further hydrolyzed by DAG lipase and finally release AA. This free, nonesterified AA can be further metabolized by one of three distinct metabolizing enzyme pathways including cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 monooxygenase (reviewed in Ruzicka and Printz 1984) (Fig. 1.3). Thus the amount of free AA is a rate-limiting step in AA metabolism. In the development of some tumors these AA releasing phospholipases become permanently activated allowing AA to be a constant substrate for COX and LOX (Fischer et al. 1982; Marks and Fürstenberger 2000). Moreover, PLA_2 inhibitors have been reported as some of the most potent inhibitors of skin tumor promotion (Fischer et al. 1982; Fischer et al. 1983; Nakadate et al. 1982). In addition to this, excessive activation of AA metabolism by COX and LOX was also found in a variety of tumors both in man and in experimental animals. Since these tumors could be effectively inhibited by specific inhibitors for the AA metabolic pathway as well (Fischer et al. 1987; Fischer et al. 1982; Kato et al. 1983; Nakadate et al. 1982), the entire AA cascade has been an intense focus of research in the cancer field.

Cyclooxygenase, also known as prostaglandin H_2 synthase, is a 75 kDa hemeprotein which has two different enzyme functions. Free AA is first converted to prostaglandin (PG) G_2 resulting from oxygen incorporation and the creation of a cyclopentane ring by the COX moiety of the enzyme. PGG_2 is then reduced to PGH_2 by the peroxidase activity of the same enzyme. PGH_2 now

serves as a substrate for diverse PGH_2-metabolizing enzymes and is transformed to PGs (PGD_2, PGE_2, and $PGF_{2\alpha}$), prostacyclin (e.g., PGI_2), and thromboxanes (e.g., TXA_2, and TXB_2). There are two forms of COX that are encoded by different genes. One is COX-1 which is a constitutively expressed form, and the other is COX-2 which is transiently and highly induced by tissue stimulation (e.g., hormonal or growth factor stimulation, tissue damage, irritation, and TPA treatment) (Goppelt-Struebe 1995; Herschman 1994). Important roles for the PG metabolites of COX have been documented in mouse skin carcinogenesis (reviewed in Fischer 1997). Highly increased levels of PG production have been observed in mouse skin or cultured murine keratinocytes after TPA treatment (Fürstenberger et al. 1989). Moreover, TPA-induced tumor promotion and tumor development were effectively inhibited by COX-specific inhibitors, including non-steroidal anti-inflammatory drugs (e.g., indomethacin, and ibuprofen) (Fischer et al. 1999; Loprinzi and Verma 1985) and the inhibition was overcome by add-back of PGs (Fischer et al. 1987). Constitutively upregulated COX-2 expression was also found in mouse skin papillomas and carcinomas along with a significant amount of PG production by those tumors (Müller-Decker et al. 1995). In particular, PGE_2 has shown to play an important role in proliferation, angiogenesis, anti-apoptosis, and immunosuppression of mouse keratinocytes (Fischer 1997) and to be essential for the initial epidermal hyperproliferation (Marks 1990). On the other hand, $PGF_{2\alpha}$ appears to be needed for the

maintenance of sustained hyperplasia and for tumor promotion (Fürstenberger et al. 1989).

Lipoxygenase, which is described in more detail in the following section, is a non-heme iron protein that dioxygenates polyunsaturated fatty acids to hydroperoxy derivatives in a regio- and stereo-selective manner (Kühn et al. 1986). At least 5 different LOXs exist in mouse skin and AA is metabolized into biologically active hydroperoxyeicosatetraenoic acid (HPETE) by these LOXs. Subsequently, HPETE is rapidly reduced to hydroxyeicosatetraenoic acid (HETE) by glutathione peroxidase, and to leukotrienes (LTs) and lipoxins in some cases (Funk 1996; Samuelsson et al. 1987; Spector et al. 1988). Some of the LOX products are known to be required for the inflammatory (Chan et al. 1989; Dowd et al. 1985) and angiogenic response (Nie et al. 1998; Tang et al. 1995) to tumor promoters and for tumor growth (Chan et al. 1989).

Finally, AA can also be metabolized by cytochrome P450 monooxygenase. The enzyme inserts a single oxygen atom into the double bonds at various positions of AA and produces a number of hydroxy and carboxy products, including the 19-hydroxy, 19-oxo, 20-hydroxy, and 20-carboxy metabolites (Capdevila et al. 1982). However, the involvement of these metabolites in mouse skin carcinogenesis is probably of minimal importance, because extremely low levels of the cytochrome P450 are found in normal skin and the level is further suppressed by TPA treatment (Reiners et al. 1992).

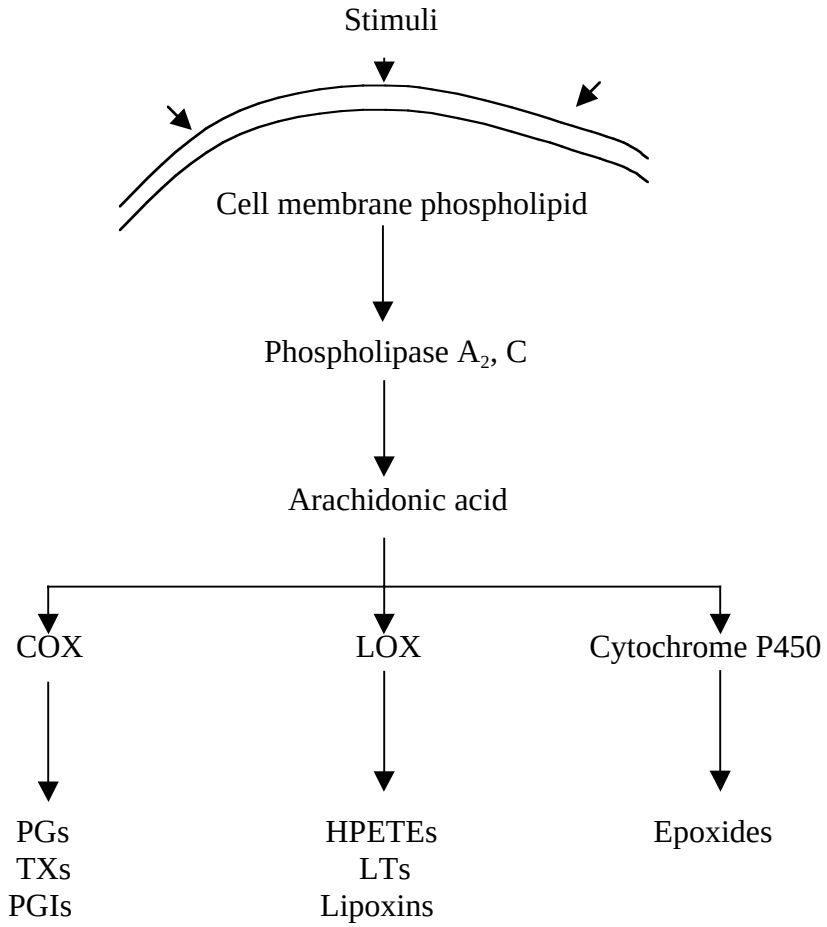


Fig. 1. 3. Arachidonic acid metabolism.

Upon variety of stimuli, arachidonic acid is released from cell membrane phospholipid by the action of phospholipase A_2 or phospholipase C. Free arachidonic acid undergoes enzymatic oxygenation by the COX, LOX, or cytochrome P450 monooxygenase pathway, and is converted to biologically active metabolites, called eicosanoids.

1.4. Lipoxygenase

1.4.1. Overall Structure and the Catalytic Action of Lipoxygenase

Lipoxygenase proteins are long, single polypeptide chains with a molecular mass of ~75-80 kDa in animals and ~94-104 kDa in plants. From the three dimensional structure of soybean LOX 1 which is the most studied enzyme in both mechanistic and spectroscopic aspects, it was revealed that the polypeptide chain is organized into two three-dimensional domains (Boyington et al. 1993; Prigge et al. 1997). Those are β–barrel (domain I) and α–helical (domain II) domain in N-terminal and C-terminal of the peptide, respectively. Domain I is composed of a much smaller portion of the protein compared to domain II (e.g., in the case of soybean LOX 1; 146 and 693 amino acid residues for N-terminal and C-terminal domains, respectively, out of a total 839 residues). It also stands quite separate from and only loosely connects with the rest of the molecule. Domain II, which is the major domain of the protein, contains a single atom of non-heme iron in the center of the domain and executes the catalytic function of the enzyme (Prigge et al. 1997).

Although the mechanism for the catalytic reaction of the enzyme is not completely understood, it appears that the iron atom in domain II composes a central part of the catalytic activity of a lipoxygenase. It has been observed that

when the metal exists in Fe^{2+} (ferrous) form, the enzyme is inactive, whereas, as the ferrous ion is oxidized to Fe^{3+} (ferric), the enzyme becomes active and can oxidize substrates such as arachidonic acid or linoleic acid (LA) (Prigge et al. 1997). From this observation, many studies have proposed a model for the iron-mediated catalytic reaction of LOX. One of the commonly proposed models is as follows (Prigge et al. 1997): ① Molecular oxygen reaches the iron center from outside the enzyme and oxidizes iron from Fe^{2+} to Fe^{3+}; ② The Fe^{3+} ion abstracts one electron from the 1,4 diene system of the unsaturated fatty acid substrate, which produces a free radical form of the 1,4 diene system, and is reduced to Fe^{2+}; ③ Now, O_2 reacts with this substrate radical and forms a peroxyl radical. The peroxyl radical subsequently abstracts an electron from the metal, which regenerates Fe^{3+}, and produces a peroxidate anion; ④ The peroxidate anion receives the proton from the base, which finally results in hydroperoxide products. Notably, all these reactions occur in a regio-and stereo-selective manner. Most known LOXs form hydroperoxide products with '*S*' stereochemistry (Kühn et al. 1986). Recently, however, several LOXs forming the mirror image '*R*' configuration have also found in coral (Brash et al. 1996), mouse (Krieg et al. 1998), and human (Boeglin et al. 1998).

1.4.2. Occurrence

Wide distribution of lipoxygenases has been found in plants, fungi, and animals (De Petrocellis and Di Marzo 1994; Funk 1996; Gerwick 1994; Grechkin 1998; Yamamoto et al. 1997). However, it has been reported that most bacteria, yeast, and other prokaryotes do not contain LOX genes (Brash 1999), partly because these organisms do not have proper substrates for the LOXs (Watanabe et al. 1997). In the case of yeast, lack of desaturases, which are necessary for the synthesis of polyunsaturated fatty acids, was reported (Brash 1999). On the other hand, higher plants as well as animals have multiple LOXs in various tissues. At least eight different LOXs were identified in soybean and seven and five different LOXs are found in mouse and in humans, respectively (Brash 1999).

1.4.3. Nomenclature

Typically, lipoxygenases are classified according to the specific carbon position of oxygen insertion in the substrate and if it is necessary, the stereoconfiguration is specified. For example, 8*S*-LOX incorporates an oxygen molecule onto the carbon-8 from the carboxyl end of arachidonic acid and produces the '*S*' configuraion of hydroperoxide products, 8*S*-HETE. However, since this is based on a single property of LOXs and does not take into account

other enzymatic, protein-chemical, and molecular biological characteristics of the enzyme, this type of nomenclature has several inherent problems. Those problems typically occur in the following cases: 1) When the carbon length of substrate is changed; 8*S*-LOX produces 8*S*-HETE from AA (C20:4), but produces 9*S*-hydroxyoctadecadienoic acid (9*S*-HODE) from LA (C18:2). 2) When more than one isozyme is present in the same species; mouse has three different 12-LOXs that differ in tissue distribution, sequence homology, substrate affinity, and biological function (Yamamoto et al. 1997). To solve this problem, currently prototypical tissues of their predominant occurrence are specified for the mammalian LOXs such as platelet, leukocyte, or epidermal type of 12-LOX. 3) When LOX has dual activity on a specific substrate; leukocyte type 12*S*-LOX can oxidize both C-12 and C-15 of AA although C-12 is the preferred site (Yamamoto et al. 1997). Despite these problems, classical nomenclature is still popularly used because it conveys the useful message in a most simple way.

1.4.4. The Importance of Lipoxygenase Pathway in Mouse Skin Tumor Development

Although various parts of the AA cascade have been known to be involved in tumor development, the COX pathway of AA metabolism has been a prime target for cancer prevention or inhibition. This is because excessively activated

COX pathway was one of the most prominent biological changes in many tumors and because inhibition of COX-2 activity by COX-2 selective inhibitors effectively reduced tumor burden (Fischer et al. 1987; Fischer et al. 1982; Kato et al. 1983; Nakadate et al. 1982; Steele et al. 1999). With this booming interest in COX, the LOX pathway in AA metabolism has been relatively underestimated and understudied. However, the importance of LOX metabolites in human cancer development has also been gradually acknowledged (Fischer and Klein 2003; Steele et al. 1999).

In mouse skin, at least 5 different LOXs , 5*S*- (Chen et al. 1995), platelet-type (*p*) 12- (Krieg et al. 1995), leukocyte-type (*l*) 12- (Krieg et al. 1995), epidermal type (*e*) 12- (Kinzig et al. 1997), and 8*S*-LOX (Fürstenberger et al. 1991; Gschwendt et al. 1986; Jisaka et al. 1997), are expressed. In the course of mouse skin tumor development, it has been found that the expression of some of these LOXs is constitutively upregulated (Bürger et al. 1999; Krieg et al. 1995). Moreover, inhibition of LOX enzyme activity was at least as effective as that of COX in the inhibition of tumorigenesis (Fischer et al. 1987; Fischer et al. 1982; Fischer et al. 1983; Kato et al. 1983; Nakadate et al. 1982; Steele et al. 1999), which suggests a critical role for LOX in mouse skin tumor development as well. The function of individual LOXs and their metabolites in mouse skin carcinogenesis is, however, only slowly being elucidated. Some of the reported functions for the LOX are described below.

Of all the LOX family members, 5*S*-LOX is the only enzyme involved in leukotriene (LT) synthesis (Samuelsson and Funk 1989). It oxidizes AA to 5*S*-HPETE, which is either reduced to 5*S*-HETE or further metabolized to LTA_4 by the LTA_4 synthase activity of 5S-LOX. The LTA_4, an unstable C-5 epoxide of AA, can be converted to the glutathione-conjugated LTC_4 and its metabolites, LTD_4 and LTE_4. These leukotrienes are well known participants in host defense reactions and in pathophysiological conditions such as immediate hypersensitivity and inflammation (Samuelsson et al. 1987). Since the immediate response of mouse skin to external stimuli, such as wounding or TPA treatment, is an inflammatory reaction, which is an important component of tumor promotion, the implication of LTs in the reaction as well as tumor promotion was expected. In fact, the presence of LTB_4, LTC_4, LTD_4, and LTE_4 was identified in mouse skin (Fürstenberger et al. 1994) and in particular the level of the latter three LTs were strongly increased by TPA (Fürstenberger et al. 1994). Many studies also reported the effective inhibition of two-stage as well as complete skin carcinogenesis by application of specific 5*S*-LOX inhibitors (Jiang et al. 1994; Yamamoto et al. 1991). The production of 5*S*-HETE by epidermal keratinocytes has been controversial. Fischer et al. reported that the levels are nearly undetectable (Fischer et al. 1988a).

12-LOX is the most abundant LOX expressed in mouse skin (Siebert et al. 2001). It occurs in 3 isoforms, platelet (*p*)-, leukocyte (*l*)-, and epidermis (*e*)-

types, which are encoded by different genes and converts AA primarily to 12-HETE (Yamamoto et al. 1997). The function of *p*12S-LOX in normal epidermis appears to be involved in normal permeability barrier function of the skin (Johnson et al. 1999). However, constitutive overexpression of *p*12S-LOX in papillomas, squamous cell carcinomas (Krieg et al. 1995), and several epithelial tumor cell lines (Chang et al. 1993) suggest its involvement in mouse skin tumorigenesis as well. When the *p*12S-LOX gene was disrupted by gene targeting, the papilloma and carcinoma incidence was in fact significantly reduced in two-stage carcinogenesis, although there was variability depending on the genetic background of the mice (Virmani et al. 2001). Many reported studies on the procarcinogenic functions of 12*S*-HETE further support a critical role of *p*12S-LOX in mouse skin tumor development. Those functions include stimulation of epidermal proliferation (Chan et al. 1989), infiltration of neutrophils and monocytes to the skin (Dowd et al. 1985), angiogenesis (Tang et al. 1995), metastasis (Tang and Honn 1994), or repression of K1 expression (Fischer et al. 1996).

The presence of *l*12*S*-LOX in keratinocytes has been controversial. The transcripts of *l*12*S*-LOX were not detectable in normal epidermis and were detectable only in a few tumors (Krieg et al. 1995). Since the increased level of *l*12*S*-LOX mRNA coincided with the infiltration of granulocytes to the epidermis in the course of TPA-induced inflammation, it has been suggested that *l*12*S*-LOX

specific mRNA found in tumor samples may originate from non-epithelial cells such as infiltrated inflammatory cells or Langerhans' cells residing in the epithelium (Krieg et al. 1995).

Epidermis type 12-LOX was recently found in mouse skin and has not been found in humans yet. So far, three isoforms of *e*12-LOX have been cloned, *e*-LOX-1 (Funk et al. 1996), *e*-LOX-2 (12*R*-LOX) (Krieg et al. 1998), and *e*-LOX-3 (Kinzig et al. 1999). The biological functions of each individual *e*12-LOX are not known yet; however, observations on the constitutive expression of *e*12-LOX in differentiated stratified epithelia (Funk et al. 1996; Krieg et al. 1998) suggest its potent role in keratinocyte differentiation. It has been reported that the *e*12-LOX expression is not further induced by TPA (Funk et al. 1996; Krieg et al. 1998) and is transcriptionally downregulated in the course of skin tumorigenesis (Müller et al. 2002), which suggest this enzyme may not produce 12-HETE and may have an anticarcinogenic effect.

Besides *e*12-LOX, mouse skin expresses another unique epidermal type LOX, 8*S*-LOX, which is described in detail in the following section. This enzyme is distinguished from other LOXs expressed in mouse skin because it is not expressed in normal mouse skin, however, is highly induced by a single topical treatment of TPA (Fürstenberger et al. 1991; Gschwendt et al. 1986; Jisaka et al. 1997). Since constitutively overexpressed 8*S*-LOX was found in papillomas

(Bürger et al. 1999), it has been hypothesized that 8*S*-LOX also plays an important role in skin tumor development.

Another important feature of the LOX pathway in skin tumorigenesis is the generation of reactive oxygen species (ROS) as byproducts of LOX metabolism. Since LOX reactions are carried out by hydrogen abstraction, radical rearrangement and oxygen insertion processes (Prigge et al. 1997), besides the hydroperoxide products of the substrate, many ROS can be generated during these processes (Kühn and Borngraber 1999). In fact, it has been shown that either LOX inhibitors (nordihydroguaiaretic acid and benoxaprofen) or inhibitors for both COX and LOX (eicosatetraynoic acid and phenidone) were effective in inhibiting ROS production, whereas, COX inhibitors (indomethacin and flurbiprofen) were not good inhibitors (Fischer et al. 1988b). The excess level of oxygen radicals has been shown to be involved in lipid peroxidation, enzyme activation or inactivation, and DNA strand breaks, which can contribute to tumorigenesis. Interestingly, many tumor promoters and progressors are free-radical generating compounds such as benzoyl peroxide. Considering that antioxidants in general are effective inhibitors of mouse skin tumor development (Katiyar et al. 1996; Nakadate et al. 1984; Singh and Agarwal 2002) and that many LOX inhibitors are in fact antioxidants, it is very likely that LOX contributes to the skin tumor development through ROS by-production as well.

1.5. Murine 8*S*-Lipoxygenase

The possible involvement of 8*S*-LOX in mouse skin carcinogenesis originated in a reported finding that the most highly elevated LOX metabolite in TPA treated mouse skin was 8-HETE (Gschwendt et al. 1986). Since this 8-HETE was subsequently shown to be the 8*S* enantiomer (Hughes and Brash 1991), which is an indication of an enzyme-mediated reaction product, the presence of 8-HETE synthesizing enzyme in mouse skin was expected. Later on, 8*S*-LOX was identified as the enzyme responsible for the synthesis of 8*S*-HETE (Fürstenberger et al. 1991). Of note is that 8*R*-HETE occurs only as an autoxidation product of AA.

1.5.1. Molecular Biology of Murine 8*S*-LOX

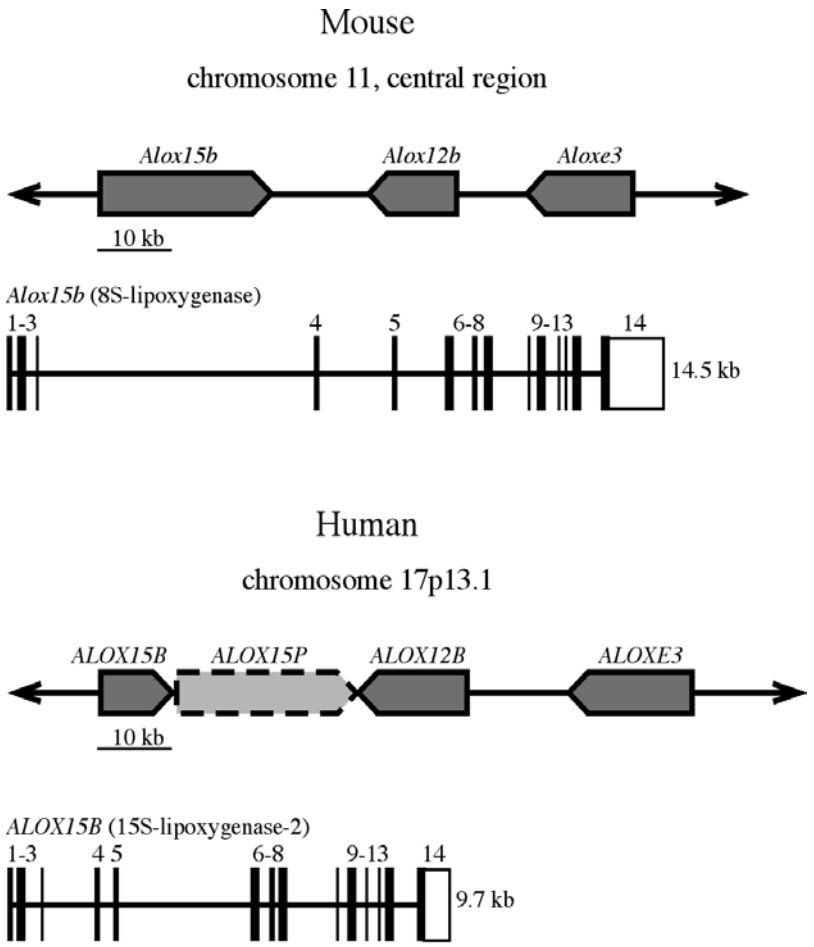
Cloning of 8*S*-LOX cDNA was accomplished accidentally in the course of searching for a murine homolog of human 15*S*-LOX-2 in mouse skin (Jisaka et al. 1997). The cloned cDNA is 3.2 kb in length and encodes a protein of 677 amino acids with a calculated molecular weight of 76 kDa. The amino acid sequence has 78% identity with human 15*S*-LOX-2 and approximately 40% identity with other mammalian LOXs. A phylogenetic tree of mammalian LOXs showed that 8*S*-LOX and its human and bovine orthologue 15*S*-LOX-2 are placed close to

other epidermal-type LOXs, including 12*R*-LOX (*e*-LOX-2) and *e*-LOX-3 (Fürstenberger et al. 2002).

Gene structure as well as chromosomal localization of the 8*S*-LOX gene was only recently reported (Fürstenberger et al. 2002). Approximately 14.5 kb-long, the 8*S*-LOX gene (designated *Alox15b* referring to the previously annotated human 15*S*-LOX-2 gene, *ALOX15B*) is composed of 14 exons and is located in the central region of mouse chromosome 11 (Fig. 1.4). It was also identified that the *Alox15b* gene is adjacent to the genes encoding 12*R*-LOX (*Alox12b*) and *e*-LOX-3 (*Aloxe3*). Interestingly, these genetic findings of 8*S*-LOX, such as gene organization and LOX gene cluster in the chromosome, is very similar to those of human 15*S*-LOX-2 gene (*ALOX15B*) except that the latter gene is located on human chromosome 17 (Krieg et al. 2001).

1.5.2. Enzymatic Properties of 8*S*-LOX

8*S*-LOX can metabolize both AA and LA to 8*S*-HPETE and to 9*S*-hydroperoxyoctadecadienoic acid (9*S*-HPODE), respectively, however, it uses AA as a preferred substrate (Fig. 1.5) (Bürger et al. 1999; Jisaka et al. 1997). The enzyme has an optimum pH of 8.0 and does not require Ca^{2+} or ATP for the activity (Fürstenberger et al. 1991). Most enzyme activity of 8*S*-LOX is detected in the cytosolic fraction of suprabasal keratinocytes (Fürstenberger et al. 1991).



Taken from Fürstenberger et al. Prostaglandins Other Lipid Mediat. 68-69, 235-243, 2002

Fig. 1. 4. Gene structure and chromosomal localization of murine 8*S*-LOX and human 15*S*-LOX-2.

(A) A mouse gene cluster encoding 8*S*-LOX (*Alox15b*), 12*R*-LOX (*Alox12b*), and e-LOX-3 (*Aloxe3*) is shown in the linear arrangement. (B) A human gene cluster encoding 15*S*-LOX-2 (*ALOX15B*), 12*R*-LOX (*ALOX12B*), and e-LOX-3 (*ALOXE3*) and the 15*S*-LOX pseudogene (*ALOX15P*) is depicted. The orientation of the genes is illustrated as arrows. The exon/intron organization of the 8*S*-LOX and 15*S*-LOX-2 genes is drawn to scale and numbered. The closed boxes indicate exons and the open boxes indicate 5'- and 3'-untranslated regions.

Of particular interest is that the enzymatic activity is very low in normal mouse skin, however, it is strongly induced by a single, topical treatment of TPA to mouse skin (Fürstenberger et al. 1991; Gschwendt et al. 1986). This increased enzyme activity was found to depend on 8*S*-LOX protein biosynthesis (Fürstenberger et al. 1991). In NMRI mice, the activity became apparent 3 h after TPA treatment, reached a maximum between 18 and 36 h and disappeared 3-5 days after treatment (Fürstenberger et al. 1991). However, the enzyme sensitivity to TPA is largely mouse age-dependent. Newborn pups could not be induced to generate 8*S*-HETE in response to TPA, whereas 6-7-day-old mice showed the most prominent response (Fürstenberger et al. 1991). TPA, however, could not induce the 8*S*-HETE production in a cell free system prepared from either the rat tongue epithelial cell line (RTE2) or the murine epidermal cell line (HEL30), although both systems were active in metabolizing AA to 12- and 15-HETE (Gschwendt et al. 1986). Other than the TPA, 12-*O*-retinoylphorbol-13-acetate was also effective to produce 8-HETE in mouse skin (Gschwendt et al. 1986). However, non-promoting phorbol esters including 12-*O*-ethacrynyl-phorbol-13-acetate and 4-*O*-methyl TPA and Ca^{2+}-ionophore were inefficient in causing 8-HETE production (Gschwendt et al. 1986).

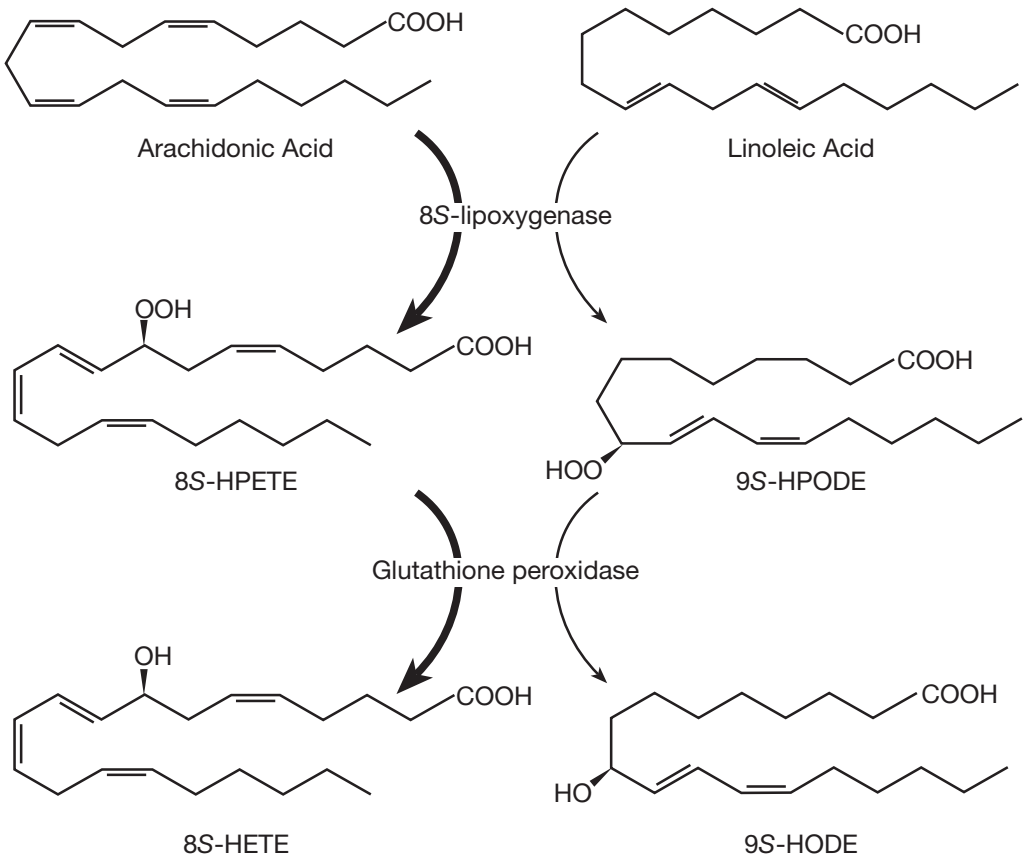


Fig. 1. 5. Metabolic action of murine 8*S*-LOX.

8*S*-LOX metabolizes arachidonic acid and linoleic acid to 8*S*-HPETE and 9*S*-HPODE, respectively. The 8*S*-HPETE and 9*S*-HPODE are subsequently reduced to 8S-HETE and 9*S*-HODE, respectively, by glutathione peroxidase. Arachidonic acid metabolism occurs more efficiently than linoleic acid metabolism by 8*S*-LOX, which is illustrated by a thicker line.

1.5.3. Tissue and Cellular Localization of 8*S*-LOX

The presence of 8-HETE was first identified in human neutrophils (Goetzl and Sun 1979), mouse peritoneal macrophages (Rabinovitch et al. 1981), mouse and rat liver (Capdevila et al. 1986), rat kidney glomeruli (Jim et al. 1982), human psoriatic skin (Camp et al. 1983), human tracheal cells (Hunter et al. 1985), and human primary squamous cell carcinomas of head and neck (El Attar et al. 1985). However, 8*S*-LOX enzyme expression has not yet been detected in those tissues or cell types.

So far, only limited tissues of mice have been reported to express 8*S*-LOX transcripts and/or protein. In black Swiss mice, strong constitutive 8*S*-LOX mRNA expression was detected in brain but not in heart, spleen, lung, liver, skeletal muscle, kidney, or testis (Jisaka et al. 1997). Immunohistochemical analyses showed constitutive 8*S*-LOX protein expression in the hair follicles of the mice as well (Jisaka et al. 1997). In NMRI mice, low levels of constitutive 8*S*-LOX mRNA expression were detected in the footsole (Krieg et al. 1998). Recently, the possibility of the presence of 8*S*-LOX protein in rat cornea has been suggested (Yamada and Proia 2000).

In normal mouse skin, including dorsal and tail skin, 8*S*-LOX is not expressed at a detectable level. However, the expression is highly induced by a single, topical treatment of TPA (Jisaka et al. 1997). This TPA-induced

expression is however highly mouse strain-dependent (Fischer et al. 1988a; Fischer et al. 1987). That is, using 6-10-day-old mice, low constitutive 8*S*-LOX expression and strong TPA-induced expression was observed in black Swiss and NMRI mice (Gschwendt et al. 1986; Jisaka et al. 1997). In the SENCAR strain, high constitutive 8*S*-LOX activity with little extra induction by TPA was observed (Jisaka et al. 1997), while very low constitutive expression was observed in C57BL/6J mice and little induction was seen with TPA treatment (Fischer et al. 1988a). Notably, 8*S*-LOX protein expression in skin was limited to the differentiated keratinocyte compartment, the stratum granulosum (Jisaka et al. 1997). Since this compartment is highly expanded after TPA treatment, it has been suggested that TPA-induced 8*S*-LOX expression is correlated with an increase in the number of cells that produce 8*S*-LOX in skin.

1.5.4. Biological Functions of 8*S*-LOX

1.5.4.1. Implication in Skin Tumor Development

Despite several reports showing that LOX metabolites are critically involved in skin inflammation as well as tumor promotion, and that LOX inhibitors effectively inhibit mouse skin carcinogenesis, 8*S*-HETE is so far the only highly induced LOX metabolite in mouse skin after TPA treatment.

Although 12*S*-HETE is the most abundant LOX metabolite in the skin, the level of 12*S*-HETE is not significantly increased by TPA (Fischer et al. 1988a). This observation led to the hypothesis that 8*S*-HETE/8*S*-LOX may contribute to mouse skin tumor development. Supporting this idea, constitutively upregulated 8*S*-LOX expression as well as activity was also observed in early stage of papilloma development (reversible papillomas) (Bürger et al. 1999). Interestingly, however, in the course of malignant progression of papillomas to carcinomas, it was observed that the level of 8*S*-LOX expression and activity decreased (Nair et al. 2000). That is, the level was reduced in irreversible papillomas, which are precursor lesions of carcinomas, and in carcinomas returned completely back to the level of normal epidermis. This profile of 8*S*-LOX expression is quite different from that of COX-2 and *p*12*S*-LOX, which were found to be constitutively upregulated in carcinomas as well (Krieg et al. 1995; Müller-Decker et al. 1995). Since conversion of papillomas to carcinomas occurs spontaneously without any other treatment, it has been thought that endogenous genotoxic agents in the papillomas are responsible for further genetic mutations or instability, which contribute to the malignant progression. In this context, strongly increased 8*S*-HETE in papillomas was suspected to be one of the genotoxic agents contributing to tumor progression (Fürstenberger et al. 2002). In fact, an ability of 8*S*-HETE to induce chromosomal damage, predominantly gaps and breaks, in primary keratinocytes was reported (Bürger et al. 1999).

Moreover, an observation that the level of 8*S*-HETE was closely correlated with promutagenic etheno-DNA adduct formation in skin tumors (Nair et al. 2000) appears to further support a critical role of 8*S*-HETE in mouse skin tumor development as well as in malignant tumor progression.

1.5.4.2. Induction of Terminal Differentiation in Keratinocytes

On the other hand, histochemical analyses showed that 8 *S*-LOX protein in mouse skin was localized in a terminally differentiated epidermal cell compartment, the stratum granulosum (Jisaka et al. 1997). Since TPA-induced 8*S*-LOX expression as well as enzyme activity was closely related with expansion of this compartment after TPA treatment, a potential role of 8*S*-LOX in keratinocyte differentiation has also been suggested. In fact, targeted FVB transgenic mice overexpressing 8*S*-LOX gene under control of the loricrin promoter exhibited a highly differentiated and keratinized epidermal phenotype (Muga et al. 2000). Moreover, K1 staining in the transgenic epidermis was not only more intense, but also occured in many of the basal cells as well as in the suprabasal layers, whereas the staining in wild type epidermis was primarily in suprabasal layers. Interestingly, the transgenic mice also showed increased rates of proliferation in inter- and intra-follicular keratinocytes without exhibiting significant hyperplasia. This phenomenon was explained in the context of a

compensatory mechanism in which loss of suprabasal cells resulting from rapid migration of epidermal keratinocytes from the basement membrane and terminal differentiation was compensated for by increased proliferation of basal cells. More direct evidence for the ability of 8*S*-HETE to induce K1 expression was also demonstrated in primary keratinocytes and the induction of K1 was a peroxisome proliferator-activated receptor (PPAR)-α mediated process (Muga et al. 2000). These observations suggest that 8*S*-LOX is not only closely related to, but also actively participates in the process of keratinocyte differentiation as well.

1.6. Specific Aims

The involvement of LOX in the development of particular tumors in humans has gradually been acknowledged and LOX has emerged as a novel target to prevent or treat human cancers (Fischer and Klein 2003; Steele et al. 1999). In the mouse skin carcinogenesis model, which provides an excellent model to study multistage nature of human cancer development, many studies have shown that some of the LOXs are constitutively upregulated in their expression (Bürger et al. 1999; Krieg et al. 1995). Moreover, application of LOX inhibitors effectively reduced tumor burdens (Fischer et al. 1987; Fischer et al. 1982; Kato et al. 1983; Nakadate et al. 1982; Steele et al. 1999), which implicates the involvement of LOX in mouse skin tumor development as well.

8*S*-LOX is a recently cloned LOX, which is specifically expressed in mouse skin after TPA treatment but not in normal skin (Fürstenberger et al. 1991; Gschwendt et al. 1986; Jisaka et al. 1997). Unlike other members of the LOX "family" expressed in mouse skin, this TPA-induced expression of 8*S*-LOX is prominent only in the skin of the TPA tumor promotion-sensitive strains of mice (SENCAR, CD-1, and NMRI) but not in the promotion-resistant C57BL/6J mice (Fischer et al. 1988a; Fürstenberger et al. 1991; Gschwendt et al. 1986). This is a very unique phenomenon among strains of mice. Constitutive upregulation of 8*S*-LOX was also found in early stage papillomas and the expression was gradually reduced as the tumors became malignant (Bürger et al. 1999). Based on these observations, it has been thought that 8*S*-LOX is involved in TPA-induced tumor promotion as well as in tumor conversion from papillomas to carcinomas. In accordance with this hypothesis, several studies have suggested possible roles of 8*S*-HETE, a AA metabolite of 8*S*-LOX, in mouse skin tumor development. A clastogenic activity of 8*S*-HETE was demonstrated in primary keratinocytes (Bürger et al. 1999) and a close correlation between the levels of etheno-DNA adducts and 8*S*-HETE during skin carcinogenesis was also reported (Nair et al. 2000). On the other hand, it has been reported that 8*S*-LOX protein expression is restricted to a differentiated keratinocyte compartment (Jisaka et al. 1997). Moreover, reported findings on the ability of 8*S*-HETE to cause keratinocyte differentiation (Muga et al. 2000) appear to be contrary to the procarcinogenic

features of the 8*S*-LOX expression, presenting a question as to the role of 8*S*-LOX during mouse skin carcinogenesis.

Based on these observations, we **hypothesized that 8*S*-LOX is highly regulated in normal keratinocyte proliferation, whereas, aberrantly upregulated gene expression, i.e., increased 8*S*-HETE synthesis, occurs and likely plays a functional role during skin tumor development.** Thus, the goal of this study was to elucidate a specific mechanism through which 8*S*-LOX expression is regulated and to investigate the function of the 8*S*-LOX/8*S*-HETE during mouse skin tumor development. The specific aims to achieve this goal were:

Specific Aim 1: How is 8*S*-LOX expression regulated by TPA in normal murine keratinocytes? It was determined whether the enhanced 8*S*-LOX mRNA levels following TPA treatment in SSIN primary keratinocytes were due to increased transcription rates or altered mRNA stability by treating the cells with the transcription inhibitor, actinomycin D. To further investigate transcriptional regulation of 8*S*-LOX gene expression by TPA, the 8*S*-LOX promoter was cloned using a PCR-based DNA "Walking" method and sequenced. The promoter activity was tested by transfection of a 8*S*-LOX promoter-driven reporter construct into SSIN keratinocytes with/without TPA treatment. To identify regulatory DNA sequences on the 8*S*-LOX promoter and transcription factors

involved in TPA-induced 8*S*-LOX expression in SSIN keratinocytes, various deletion assays and gel shift assays were conducted.

<u>Specific Aim 2:</u> What is the functional role of 8*S*-LOX/8*S*-HETE in mouse skin tumor development?

1) Will 8*S*-HETE confer sensitivity to TPA promotion in C57BL/6J mice?

The contribution of 8*S*-HETE to skin tumor development was determined by backcrossing the 8*S*-LOX/FVB transgenic mice onto the C57BL/6J mouse background to make congenic mice. These 8*S*-LOX transgenic and wild type congenic C57BL/6J mice were subjected to two-stage chemical carcinogenesis protocols.

2) Will 8*S*-HETE promote malignant progression of papillomas to carcinomas? To determine the functional role of increased 8*S*-HETE synthesis in papillomas, MT1/2, a murine papilloma cell line, was stably transfected with 8*S*-LOX cDNA and subsequently examined for the effect of 8*S*-LOX expression on cell proliferation and differentiation *in vitro*.

3) What would be the functional consequence of forced 8*S*-HETE synthesis in carcinomas? To determine the role of 8*S*-HETE in carcinomas, CH72, a murine carcinoma cell line, was stably transfected with 8*S*-LOX cDNA and subsequently examined for the effect of 8*S*-LOX expression on cell proliferation *in vitro* and in *in vivo* xenografts.

CHAPTER II

IDENTIFICATION AND CHARACTERIZATION OF A PHORBOL ESTER-RESPONSIVE ELEMENT IN THE MURINE 8*S*-LIPOXYGENASE GENE

2.1. Summary

Murine 8*S*-lipoxygenase (8*S*-LOX) is a 12-*O*-tetradecanoylphorbol-13-acetate (TPA) inducible lipoxygenase. That is, it is not detected in normal mouse skin, however, a significant increase in expression is detected in the skin of TPA promotion-sensitive strains of mice after TPA treatment. In this study, we found TPA-induced 8*S*-LOX mRNA expression is a result of increased transcription in SSIN primary keratinocytes and further investigated transcriptional regulation of 8*S*-LOX expression by cloning its promoter. The cloned 8*S*-LOX promoter (~ 2 kb) in which a transcription initiation site was mapped at -27 from the ATG has neither a TATA box nor a CCAAT box. However, the promoter was highly responsive to TPA in TPA promotion-sensitive SSIN but not in TPA promotion-resistant C57BL/6J primary keratinocytes. We then identified a Sp1 binding site located -77 to -68 from the ATG that is a TPA responsive element (TRE) of the

promoter, and that Sp1, Sp2, and Sp3 proteins bind to the TRE. We also found that the binding of these proteins to the TRE is significantly increased by TPA treatment and inhibition of the binding by mithramycin A decreased TPA-induced promoter activity as well as 8*S*-LOX mRNA expression. These data suggest that increased binding of Sp1, Sp2, and Sp3 to the TRE of the 8*S*-LOX promoter is a mechanism by which TPA induces 8*S*-LOX expression in keratinocytes.

2.2. Introduction

Tumor promotion is a critical step in two-stage skin carcinogenesis (Slaga 1984b). Cells initiated with a subthreshold dose of carcinogen usually do not further develop into visible and detectable tumors without promotion. 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which is the most commonly used tumor promoter in skin carcinogenesis, has strong effects on mouse skin, causing such events as hyperplasia, inflammation, and ornithine decarboxylase induction (Kato et al. 1983; Nakadate et al. 1982; Slaga 1984b). Substantial evidences suggest that some lipoxygenase (LOX) metabolites of arachidonic acid are involved in TPA-induced epidermal hyperplasia and tumor promotion and that LOX inhibitors can effectively inhibit skin carcinogenesis (Fischer et al. 1987; Fischer et al. 1982; Kato et al. 1983; Nakadate et al. 1982; Steele et al. 1999).

Lipoxygenases are non-heme iron proteins that dioxygenate polyunsaturated fatty acids such as arachidonic or linoleic acid to hydroperoxy derivatives, hydroperoxyeicosatetraenoic acid (HPETE) or hydroperoxyoctadecadienoic acid (HPODE) respectively (Kühn et al. 1986). These metabolites are subsequently reduced to hydroxyeicosatetraenoic acid (HETE) or hydroxyoctadecadienoic acid (HODE) by glutathione peroxidase. Lipoxygenases are classified according to the position of oxygen insertion onto specific carbons. For example, 8*S*-LOX oxygenates arachidonic acid at carbon-8. At least 5 different LOXs exist in mouse skin including 5*S*- (Chen et al. 1995), platelet-type (*p*) 12- (Krieg et al. 1995), leukocyte-type (*l*) 12- (Krieg et al. 1995), epidermal type (*e*) 12- (Kinzig et al. 1997), and 8*S*-LOX (Fürstenberger et al. 1991; Gschwendt et al. 1986; Jisaka et al. 1997). However, 8*S*-LOX is the lipoxygenase most strongly induced by TPA treatment of mouse skin (Fürstenberger et al. 1991; Gschwendt et al. 1986; Jisaka et al. 1997).

8*S*-lipoxygenase is a recently cloned murine lipoxygenase which has 78% homology with human 15*S*-LOX-2 (Jisaka et al. 1997). It metabolizes arachidonic acid as its preferred substrate and produces 8*S*-HETE. In addition, it was shown that 8*S*-LOX can convert linoleic acid to 9*S*-HODE, although with a much lower efficiency (Bürger et al. 1999). So far constitutive enzyme expression has been detected only in mouse brain, footsole, tail, forestomach, and hair follicles (Fürstenberger et al. 1991; Heidt et al. 2000; Jisaka et al. 1997). In

mouse skin, however, the level of 8*S*-LOX message, protein, as well as enzyme activity are highly induced by a single topical treatment of TPA (Fürstenberger et al. 1991; Gschwendt et al. 1986; Jisaka et al. 1997). Notably, the protein expression was shown to be restricted to the post-mitotic, terminally differentiated epidermal compartment, stratum granulosum (Jisaka et al. 1997). This characteristic of 8*S*-LOX protein expression implies a causal relationship between enzyme expression and terminal differentiation in keratinocytes. In fact, we have recently reported that 8*S*-LOX transgenic mice have a highly differentiated and keratinized epidermis along with abnormally high expression of a differentiation marker, keratin-1 (Muga et al. 2000). In addition, the ability of 8*S*-HETE to induce keratinocyte differentiation through peroxisome proliferator-activated receptor (PPAR) α has been shown *in vitro* (Muga et al. 2000). On the other hand, increased 8*S*-LOX expression was also detected in the culture of calcium-induced differentiated keratinocytes (unpublished data). Interestingly, this prominent TPA-induced 8*S*-LOX expression is observed only in TPA promotion-sensitive mice (SENCAR, NMRI and CD-1) but not in the promotion-resistant C57BL/6J mice (Bürger et al. 1999; Fischer et al. 1988a). The responses of SSIN and C57BL/6J mice to a TPA treatment have been reported to be similar with respect to the induction of ornithine decarboxylase and to the synthesis of other arachidonic acid metabolites from cyclooxygenases or lipoxygenases pathway. However, TPA does not induce hyperplasia, edema or oxidant generation in

C57BL/6J mice (Fischer et al. 1988a). Considering the amount of evidence showing an association of arachidonic acid metabolites with these events in TPA treated mouse skin (Fischer et al. 1987; Fischer et al. 1982; Nakadate et al. 1982; Verma et al. 1980), 8*S*-HETE, the only different arachidonic acid metabolite that is different between SSIN and C57BL/6J mice, may be critical to these events and to tumor promotion.

Despite these potentially important roles of 8*S*-LOX in mouse skin, the mechanism by which it is regulated by TPA has not been previously reported. In an early study, Fürstenberger et al. proposed that TPA-induced 8*S*-LOX enzyme activity depends on protein biosynthesis, based on the observation that treatment of mouse skin with cycloheximide before or during TPA treatment prevented an increase in 8*S*-LOX activity (Fürstenberger et al. 1991). Thereafter, Jisaka et al. found that the 8*S*-LOX protein expression was restricted to the stratum granulosum compartment and that increased expression of 8*S*-LOX by TPA was associated with an expansion of this compartment in TPA-induced hyperplastic skin (Jisaka et al. 1997). Given this observation, they suggested an increase in the number of cells which produce 8*S*-LOX is one of the mechanisms of the TPA-induced enzyme activity. However, the fact that 8*S*-LOX message induction occurs as quickly as 3 h after TPA treatment suggests that TPA regulates 8*S*-LOX gene expression at the message level.

In this study, we demonstrate that 8*S*-LOX is transcriptionally regulated by TPA in SSIN primary keratinocytes and further studied the mechanistic basis of the regulation by cloning and characterizing its promoter. A TPA responsive element (TRE) of the 8*S*-LOX promoter was mapped to a Sp1 transcription factor binding site located -77 to -68 of the promoter and Sp1, Sp2, and Sp3 were identified as the transcription factors binding to this site. Finally we showed an increased binding of these factors to the TRE by TPA treatment and propose this as a mechanism of TPA-induced 8*S*-LOX expression in SSIN primary keratinocytes.

2.3. Materials and Methods

Cloning of the Murine 8S-LOX Promoter- The murine 8*S*-LOX promoter was cloned using the Mouse GenomeWalker kit (Clontech, Palo Alto, CA) following the manufacturer's protocol. The primary PCR reaction for isolating Clone A (Fig. 2.1) was performed with the gene-specific primer 8*S*-LOXA128 (Table 2.1) and the secondary PCR reaction was performed with the nested gene-specific primer 8*S*-LOXA122 (Table 2.1). To isolate Clone B (Fig. 2.1), the procedure described in the Mouse GenomeWalker kit was repeated using the gene-specific primers 8*S*-LOXA(-)364 and 8*S*-LOXA(-)441 (Table 2.1) in the primary and secondary PCR reactions, respectively. Gel purified products from

the secondary PCR reaction of each cloning experiment were inserted into pCR2.1 (Invitrogen, Carlsbad, CA) for automated sequencing, and the sequencing results were analyzed by MacVector 7.0 (Oxford Molecular Ltd., Madison, WI). The cloned sequences were then scanned with MatInspector 2.1 (Quandt et al. 1995) for transcription factor binding motifs.

Assembly of Reporter Constructs- The parent reporter vector for all 8*S*-LOX promoter constructs in this paper was pGL2 basic-m (Mar et al. 1995), a generous gift from Dr. Andrew P. Butler (U.T.M.D. Anderson Cancer Center, Smithville, TX). This plasmid was created by disrupting a cryptic AP1 site in the Simian Virus 40 segment downstream of the luciferase gene in pGL2 basic (Promega, Madison, WI). The mutation was made to eliminate any spurious response to TPA. Clones A and B were combined into a contiguous, non-overlapping fragment for use in a reporter construct by first subcloning them into pGL2 basic-m separately. A Not I/Hind III fragment from the pCR2.1 construct of Clone A was inserted into pGL2 basic-m digested with Sma I and Hind III to create the plasmid pGL2m-CloneA, and a Not I/Kpn I fragment from the pCR2.1 construct of Clone B was inserted into pGL2 basic-m digested with Hind III and Kpn I to create pGL2m-CloneB. An EcoR V digestion fragment of pGL2m-CloneA containing all of Clone A that did not overlap with Clone B was then ligated into the EcoR V digestion fragment of pGL2m-CloneB containing both the parent vector and all of Clone B that was unique from Clone A. The segment

created from Clones A and B in this new construct was then amplified by PCR with primers 8*S*-LOXA(-)1 and 8*S*-LOXS(-)2248 (Table 2.1). That PCR product (Clone C in Fig. 2.1) was inserted into pCR 2.1 (pCR2.1CloneC), from which it was subcloned into pGL2 basic-m digested with Kpn I and Hind III. Truncated promoters for the deletion constructs were generated by PCR amplification or restriction enzyme digestion of pCR 2.1CloneC and by ligation of the desired product into pGL2 basic-m. The insert in each reporter construct was verified by automated sequencing.

For the assembly of pLuc-8*S*-LOX(-81/-65) construct, two complementary oligonucleotides spanning -81 to -65 of the cloned 8*S*-LOX promoter region to which a noncomplementary protruding Xho I site is linked at each 5' end (5'-tcgaCTGAT***GGGCGGG***GCATC -3' and 5'- tcgaGATGC***CCCGCCC***ATCAG -3') were synthesized (Integrated DNA Technologies, Coralville, IA). After a process of annealing, the resulting double stranded oligonucleotides were ligated into the pLuc-MCS reporter vector (Stratagene, La Jolla, CA) digested with Xho I.

Rapid Amplification of cDNA Ends (5' RACE)- 5'RACE was performed with the SMART^{TM} RACE cDNA Amplification kit (Clontech) as described by the manufacturer. First-strand cDNA was synthesized from TPA-treated mouse epidermis total RNA using 8*S*-LOXA483 (Table 2.1) as the gene-specific primer. The same gene-specific primer was used to amplify the resulting 5' RACE ready-

cDNA, and the desired fragment was further amplified by PCR using 8*S*-LOXA297 (Table 2.1) as the nested gene-specific primer. The nested products were gel purified and cloned into pCR 2.1 for automated sequencing. To confirm the results of 5' RACE, 5' RACE ready-cDNA from the 8*S*-LOXA483 primer was amplified by PCR using 8*S*-LOXA297 paired with each of the following 8*S*-LOX gene specific primers: 8*S*-LOXS(-)32, 8*S*-LOXS(-)51, 8*S*-LOXS(-)149, 8*S*-LOXS(-)370, 8*S*-LOXS(-)627, 8*S*-LOXS(-)1126, and 8*S*-LOXS(-)2195 (Table 2. 1).

Site-directed Mutagenesis- Seven nucleotides, GGGCGGG, in the Sp1 binding motif in the -121 deletion construct was mutated to TTTATTT by PCR-based site-directed mutagenesis (Ho et al. 1989). The initial overlapping fragments were generated by PCR amplification of the wild type -121 deletion construct, using the upstream outer primer 5'- CAACACTCAACCCTATCTCG -3' with the mutant primer 5'- CCTCAGCGATGC**AAATAAA**ATCAGACCAGG TTAAG -3' and using the downstream outer primer 5'- ATAGCCTTATGCAGT TGCTC -3' with the mutant primer 5'- CTTAACCTGGTCTGAT**TTTATTT**GC ATCGCTGAGG -3' (IDT). The product of annealing between the initial overlapping fragments was amplified by PCR using the upstream and downstream outer primers. The final product was digested with Pvu I and Xba I, and ligated into corresponding sites in pGL2 basic-m. The resulting deletion construct (-121m) was sequenced to confirm the desired mutation.

Cell Culture- Primary keratinocytes were isolated from 1-2 day old SSIN or C57BL/6J mouse skin by trypsinization as described previously (Ristow 1982; Yuspa and Harris 1974) and grown in Eagle's minimal essential medium supplemented with 8% chelexed fetal bovine serum at 37°C under 5% CO_2.

Transient Transfections and Luciferase Assays- The assembled 8*S*-LOX promoter reporter constructs or corresponding parent vector were co-transfected with the pCMV-β-galactosidase expression vector (Clontech) into primary keratinocytes, 24 h after plating at 1 X 10^6 cells/35 mm dish, using FuGENE^{TM}6 Transfection Reagent (Roche, Indianapolis, IN) as described by the manufacturer. After 16 h, transfected cells were treated for 24 h with either acetone vehicle or TPA. For one set of experiments, cells were treated with 1 μM of mithramycin A (MMA) (Sigma, St. Louis, MO) 1 h before acetone or TPA treatment. Luciferase activity was measured using the Luciferase Assay System (Promega) and β-galactosidase activity was measured using the Galacto-Light^{TM} assay kit (Tropix, Bedford, MA). Light from either assay was detected by a luminometer (Tropix). The protein concentration of each cell lysate was quantified by the BCA protein assay (Pierce, Rockford, IL). Luciferase activity was normalized to β-galactosidase activity and protein concentration and then expressed as relative luciferase activity.

Electrophoretic Mobility Shift Assay (EMSA)- Nuclear extracts were prepared as previously described (Kim and Fischer 1998) from SSIN primary

keratinocytes treated with acetone or TPA for 6 h. For one set of experiments, cells were treated with 1 μM of MMA (Sigma) 1 h before acetone or TPA treatment. A synthetic probe (IDT), 5'- CTTAACCTGGTCTGAT***GGGCGGG***G CATCGCTGAGG-3' (-92/-58), 5'-CTTAACCTGGTCTGAT***TTTATTT***GCATC GCTGAGG-3' (-92/-58m), or 5'- tcgaCTGAT***GGGCGGG***GCATC -3' (Xho I(-81/-65)) was end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (Amersham Pharmacia Biotech, Piscataway, NJ), and 15,000 cpm of the labeled probe was incubated with 2 μg of nuclear extracts and 1 μg of poly(dI-dC) in binding buffer (20 mM Tris-HCl, 60 mM HEPES-KOH (pH 7.9), 300 mM KCl, 60% glycerol, 2.5 mM EDTA, and 5 mM DTT) for 25 min at room temperature. The probe was then electrophoresed on a 5% nondenaturing polyacrylamide gel and viewed by autoradiography. Binding specificity was tested in parallel assays through the addition of unlabeled probe or consensus oligonucleotides for Sp1, AP1, CREB or NF-I (Santa Cruz Biotechnology, Santa Cruz, CA) at 100-fold molar excess over the labeled probe. Supershifting was assayed by incubating 2 μg nuclear extracts on ice for 30 min with 2 μl of Sp1, Sp2, Sp3, or Sp4 antibody (Santa Cruz Biotechnology) before inclusion of the extracts in the binding mixture.

Northern Analysis- Total RNA was isolated from mouse whole skin or primary keratinocytes treated with acetone, TPA, or a combination of TPA and actinomycin D (Sigma) for various time points using Tri-reagent (Molecular

Research Center, Cincinnati, OH) according to the manufacturer's protocol. Ten μg of RNA was separated on a formaldehyde-containing 1% agarose gel, transferred onto nylon membrane (Micron Separation, Westboro, MA), and UV cross-linked onto the membrane with a Stratalinker (Stratagene). cDNAs for 8*S*-LOX, Sp1, and GAPDH was labeled with [α-^{32}P]dCTP by using Random Primed DNA Labeling kit (Roche) and hybridized to the blot by using the QuickHyb (Stratagene) solution. Specific bands were detected by autoradiography.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)- RT-PCR was performed with the SuperScript™ First-Strand Synthesis System for RT-PCR kit (Invitrogen) following the manufacturer's protocol. First strand cDNA was synthesized from total RNA extracted from SSIN primary keratinocytes after treatment with acetone, TPA (30 ng/ml) or a combination of MMA (1 μM) plus TPA (30 ng/ml) for 6 h using the oligo dT primer. MMA was added 1 h before TPA treatment. Resulting cDNA was then amplified with the gene-specific primers 8*S*-LOXS3 and 8*S*-LOXA483 (see Table 2.1). GAPDH sense (5'-ACCACAGTCCATGCCATCAC -3') and antisense (5'- TCCACCACCCTGTTG CTGTA -3') primers were used in the separate reaction mixtures to control for amplification.

Western Analysis- Nuclear extracts prepared from SSIN primary keratinocytes were separated on a 8% SDS-PAGE and transferred onto a PVDF membrane (Pierce). The blot was blocked with 5% non-fat dried milk in 0.1%

Tween 20-Tris buffered saline and probed with antibodies against Sp1, Sp2, Sp3 and β-actin (Santa Cruz Biotechnology). After washing, the blot was then probed with horseradish peroxidase-conjugated secondary antibody. The specific bands were detected by enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

2.4. Results

Transcriptional Regulation of 8S-lipoxygenase by TPA - TPA-induced 8*S*-LOX expression in SSIN primary keratinocytes occured quickly and with a pattern that is similar to many immediate early genes, i.e., increased message was observed by 3 h, peaked at 9 h and started to decline thereafter (Fig. 2.2A). We then treated cultures of the cells with acetone, TPA (30 ng/ml) or the combination of TPA (30 ng/ml) plus actinomycin D (5 μg or 10 μg/ml, a transcription inhibitor) for 9 h to see whether TPA induces 8*S*-LOX transcriptionally. 8*S*-LOX mRNA was readily detected after treatment with TPA but not detected after treatment with acetone or after a combined treatment of actinomycin D and TPA (Fig. 2.2B). This data strongly suggest that TPA-induced elevation of the 8*S*-LOX message in SSIN primary keratinocytes occurs through transcriptional activation.

Cloning and characterization of the murine 8S-LOX promoter- To better understand the transcriptional regulation of 8*S*-LOX by TPA treatment, we cloned the 2248 bp of genomic sequence immediately upstream of the translation start site, using a PCR-based method (see Materials and Methods and Fig. 2.1). Fig. 2.3 shows the cloned sequence. Unlike regions that have been shown to serve as the promoter for other lipoxygenases (Hoshiko et al. 1990; Kritzik et al. 1997; Silverman et al. 2002; Yoshimoto et al. 1992), this sequence is not G+C rich. The mono-nucleotide frequencies are T (28.1%), C (25.8%), A (27.5%), and G (18.5%) and these frequencies are observed even near the downstream end of the fragment. The sequence presented in Fig. 2.3 has neither a TATA box nor a CCAAT box, however, this sequence contains several putative binding sites for transcription factors such as AP1 (five sites), C/EBP (two sites), GATA (three sites), NF-κB (one site), Oct-1 (three sites), and Sp1 (one site).

Mapping the transcription start site- The previously described 8*S*-LOX cDNA contains 27 bp of 5' untranslated region (Jisaka et al. 1997) and this was confirmed in our hands by 5' RACE. Performing 5' RACE with a 30 bp adaptor oligonucleotide and an antisense primer that binds from 483 to 456 bp downstream of the translation start codon (see Materials and Methods and Fig. 2.4A) abundantly produced a 540 bp fragment (Fig. 2.4B, *lane 2*) containing only the previously reported sequence. This result was verified by 5' RACE with a

nested primer that binds 186 bp closer to the predicted cDNA end (data not shown).

Since the 5’ RACE method is highly dependent on the efficiency of adaptor ligation at the 5’ end of the cDNA, we sought another approach to rule out the presence of a transcription start site farther upstream. We therefore determined which of the primers shown in Fig. 2.4A would permit PCR amplification of the cDNA template from 5' RACE. A fragment of approximately 320 bp length was strongly amplified with a sense primer that bound within the known 27 bp of 5’ untranslated region (Fig. 2.4C, *lane 3*), and that fragment contained the expected sequence (data not shown). No fragment was generated using any other sense primer except for a fragment of approximately 150 bp length amplified with a primer that bound 370 bp upstream from the translation start site (Fig. 2.4C, *lane 6*). However, sequencing of the 150 bp fragment showed it to be the product of non-specific amplification (data not shown). Taken together, we concluded that the 8*S*-LOX gene transcription starts 27 bp upstream from the translation start site.

TPA responsiveness of the 8S-LOX promoter- Since the transcription start site for the 8*S*-LOX gene appears to fall within the region we cloned, we wanted to determine whether the cloned region contains elements responsive to TPA. A reporter construct containing the entire cloned fragment (-2248) was transfected into SSIN primary keratinocytes, and the level of luciferase activity after

treatment with various concentrations of TPA was measured. Basal luciferase activity was detected from the -2248 construct and the activity was clearly increased by TPA, whereas luciferase activity from a promoter-less control vector was not (Fig. 2.5A). Induction of the reporter increased with increasing doses of TPA up to 30 ng/ml, however, it decreased thereafter, likely due to the observed toxicity of TPA to SSIN primary keratinocytes.

TPA-induced 8*S*-LOX mRNA expression was very strong in TPA promotion-sensitive SSIN mouse skin, whereas the expression was quite weak in TPA promotion-resistant C57BL/6J mouse skin even when higher doses of TPA are used (Fig. 2.5B). To determine whether this relationship was retained in cultured keratinocytes, we transfected the -2248 construct into both SSIN and C57BL/6J primary keratinocytes and compared the level of luciferase activity after TPA treatment. Consistent with the expression profile, TPA-induced luciferase activity was also much lower in C57BL/6J primary keratinocytes compared to that in SSIN primary keratinocytes (Fig. 2.5B). Taken together, these data suggest that the cloned region of 8*S*-LOX promoter contains at least one TPA responsive element and it mimics, at least in part, the normal regulation of the endogenous gene.

Isolation of a TPA responsive region in the 8S-LOX promoter- To locate TPA responsive elements in the isolated region of the 8*S*-LOX promoter, we inserted progressively shortened segments of the cloned promoter in front of a

luciferase gene and transfected the resulting constructs into SSIN primary keratinocytes. Fig. 2.6 shows the structure of the deletion constructs and the corresponding luciferase activities with or without TPA treatment. Although fold induction of the luciferarse activity by TPA was variable to some extent, deletions of the promoter upto -93 bp from the translation start site did not affect the TPA response of the promoter. However, deletion from -92 to -70 led to the complete loss of both basal and TPA-induced activity similar to that seen with a promoter-less control vector (pGL2m). It therefore appears that the response of the 8*S*-LOX promoter to TPA is mediated within a -92 to -70 segment of the promoter that is also critical to basic promoter activity.

Interaction between nuclear proteins and the TPA responsive region of the 8S-LOX promoter- Since the segment between -92 and -70 seems to be critically important to the 8*S*-LOX promoter function, we looked for the binding of factors within that segment (Fig. 2.7). The nuclear extracts prepared from TPA treated SSIN primary keratinocyte were incubated with radiolabeled oligonucleotides spanning from -92 to -58 of the 8*S*-LOX promoter. This binding reaction generated three retarded protein-DNA complexes (complexes I, II, and III, *lane 2*). To determine the specificity of these binding complexes, we added 100-fold molar excess of unlabeled 8*S*-LOX oligonucleotide (-92/-58) to the binding reaction. Complexes I and II were entirely competed away, however, complex III was not significantly affected (*lane 3*). Based on the putative transcription factor

binding site information presented in Fig. 2.3, a Sp1 binding motif located between -77 and -68 of the promoter was the only predicted site in the fragment between -92 and -70. We therefore tried to determine if this putative Sp1 binding motif was responsible for producing these protein-DNA complexes. Interestingly, when nuclear extracts were incubated with radiolabeled -92/-58m probe in which the putative Sp1 binding motif was point mutated, complexes I and II were no longer detected (*lane 10*). Moreover, when 100-fold molar excess of the unlabeled -92/-58m probe was added to the binding reaction, it could not compete away complexes I and II (*lane 4*). The critical role of the putative Sp1 binding site to generate complexes I and II was further confirmed when we chased labeled -92/-58 probe with unlabeled Sp1, AP1, CREB, and NF-I consensus oligonucleotide. Only Sp1 consensus oligonucleotide competed away complexes I and II (*lane 5*), whereas AP1, CREB, and NF-I consensus oligonucleotides did not reduce complex formation (*lanes 6 to 8*). These data collectively demonstrate that a specific interaction between at least one nuclear protein and the Sp1 binding motif generated complexes I and II, whereas non-specific binding gave rise to complex III.

Identification of a Sp1 binding site as a TPA responsive element in the 8S-LOX promoter- To verify that this Sp1 binding site plays a critical role in promoter activity, we mutated the Sp1 binding site in the -121 construct and transfected the resulting mutant construct (-121m) into SSIN primary

keratinocytes. In these cells, the -121 construct retained basal activity and responded to TPA as previously shown in Fig. 2.6. However, the -121m construct could not generate either basal or TPA-induced luciferase activity (Fig. 2.8A). This data distinctly proves that the Sp1 binding site encompasses a functionally essential segment of the 8*S*-LOX promoter.

The inability of TPA to induce luciferase activity from the -121m construct presents a strong possibility that the Sp1 binding site may also mediate the TPA responsiveness of the promoter, however, we could not further test it in the absence of basal transcription activity. We therefore generated a new reporter construct based on a TATA box containing reporter vector (pLuc-MCS). In the construct, a single copy of the 8*S*-LOX promoter segment (-81/-65) which just includes the Sp1 binding motif (-77/-68) was inserted in front of the TATA box in the reporter vector. We then transfected the resulting construct, pLuc-8*S*-LOX(-81/-65), into the SSIN primary keratinocytes and treated the cells with vehicle or TPA to see if the insertion of the Sp1 binding motif could exert TPA-responsiveness on the reporter vector. As shown in Fig. 2.8B, basal luciferase activity from the pLuc-8*S*-LOX(-81/-65) construct was significantly increased compared to that of the control vector and the activity was further increased by TPA treatment (about 2- to 3- fold induction), whereas the activity from control vector was not increased by TPA treatment. This observation clearly demonstrates that the single Sp1 binding site alone can mediate TPA response of

the promoter and further suggests that this Sp1 binding site is a TRE of the 8*S*-LOX promoter.

A mechanism of the TPA-induced 8S-LOX gene transcription- To understand how a single Sp1 binding site can mediate TPA responsiveness of the promoter, we first compared nuclear protein binding to the Sp1 binding site between acetone and TPA treated SSIN primary keratinocytes. We therefore prepared nuclear extracts from 6 h acetone or TPA treated cells and incubated the extracts with radiolabeled oligonucleotides spanning -81 to -65 of the 8*S*-LOX promoter. From this reaction, three retarded protein-DNA complexes were generated, as shown in Fig. 2.7, and the migration pattern of these complexes was not different between acetone and TPA treated cells (Fig. 2.9A). However, it was obvious that the formation of these binding complexes, especially complexes I and II, were clearly increased by TPA treatment (Fig. 2.9A). This experiment was repeated with more than five independent nuclear extract preparations, all yielding the same results.

We then tried to identify the nuclear protein(s) composing these complexes in acetone or TPA treated SSIN primary keratinocytes. Since the segment -81/-65 encodes the consensus binding site for Sp1, the effect of antibodies against Sp1, Sp2, Sp3, and Sp4 upon the complexes was first tested (Fig. 2.9B). Incubating the Sp1 antibody with keratinocyte nuclear extracts led to the disappearance of most of complex I (*lanes 3 & 7*), the Sp2 antibody

supershifted a portion of complex I (*lanes 4 & 8*), and the Sp3 antibody supershifted some of complex II (*lanes 5 & 9*). However, incubating the Sp4 antibody with the same extracts did not supershift any of the complexes but rather generated even stronger binding complexes than the original complexes in the absence of the antibody (compare *lanes 1 & 6*, *lanes 2 & 10*). This phenomenon can occur with some antibodies which stabilize protein-DNA interactions (Carey and Smale 2000). Since complex II was not significantly affected by Sp1, Sp2, or Sp3 antibody in our supershift assay conditions, we explored the possible existence of other transcription factor(s) in this complex. Antibodies against c-Jun, c-fos, Jun B, Fra1, Fra2, Ap2, TFIIF, ets-1/ets-2, CBP, CREB, c-myc, C/EBPα, C/EBPβ, C/EBPδ, PPARα, PPARβ, PPARγ, Smad2/3, USF1 or USF2 were incubated with the nuclear extracts and none of the antibodies could supershift either complex I or complex II (data not shown). However, as we increased the amount of antibody against Sp1, Sp2 or Sp3 in the binding mixture, a supershifted band grew more intense and most of complex II disappeared (data not shown). These data demonstrate that complexes I and II include Sp1, Sp2, and Sp3 and that complex II, in particular, includes these factors in a stable, tightly-bound state. On the other hand, we did not detect any differences in the level of Sp1, Sp2, and Sp3 protein expression between acetone and TPA treated cells (Fig. 2.9C). Taken together, these observations suggest that enhanced binding of Sp1, Sp2 and Sp3 to the Sp1 binding site between -77 and -68 within

the 8*S*-LOX promoter mediates TPA-induced expression of the 8*S*-LOX message in keratinocytes.

Functionality of the Sp1 binding site as a TRE- Our evidence for Sp1, Sp2 and Sp3 binding to their cognate site of the 8*S*-LOX promoter prompted us to explore whether such binding has a functional consequence. Treating SSIN primary keratinocytes with mithramycin A (MMA), an antibiotic which has a GC base specific binding property (Goldberg and Friedmann 1971), before TPA treatment inhibited TPA-induced formation of complexes I and II (Fig. 2.10A, compare *lanes 2, 3, & 4*). Basal complex formation was also reduced by MMA treatment, although to a much lower extent (Fig. 2.10A, compare *lanes 2 & 5*). Consistent with the result shown in Fig. 2.10A, MMA treatment before TPA treatment significantly decreased TPA-induced luciferase activity from the -121 construct in primary keratinocytes (Fig. 2.10B). Further confirming this, MMA pre-treatment reduced TPA-induced 8*S*-LOX mRNA expression as well (Fig. 2.10C). These findings suggest that modulation of Sp1, Sp2, and Sp3 binding to the TRE of the 8*S*-LOX promoter is a mechanism for regulating 8*S*-LOX expression.

2.5. Discussion

The most notable difference between murine 8*S*-LOX and other lipoxygenases is that the levels of 8*S*-LOX message, protein and activity are very weak in normal mouse skin but are strongly increased after a single topical treatment of TPA (Fürstenberger et al. 1991; Gschwendt et al. 1986; Jisaka et al. 1997). In NMRI mouse skin, for example, the activity of 8*S*-LOX is dramatically increased by TPA treatment, yet the activity of 12*S*-LOX, which is constitutively expressed, is not, and the activity of 5*S*-LOX is increased only slightly (Gschwendt et al. 1986). Recent studies have proposed that the induction of 8*S*-LOX activity by TPA is a result of protein biosynthesis (Fürstenberger et al. 1991; Jisaka et al. 1997), however, the specific mechanism through which TPA induces 8*S*-LOX expression has not been previously demonstrated.

Since we found that TPA-induced expression of 8*S*-LOX mRNA in SSIN primary keratinocytes was completely blocked by actinomycin D, we pursued the idea that TPA induction of 8*S*-LOX expression occurs at the transcription level. We began by cloning the 2248 bp region immediately upstream of the 8*S*-LOX translation start codon. This region contains a major transcription start site 27 bp upstream from the translation start site, and when inserted into a luciferase reporter construct, it promotes luciferase activity. It thus appears to include the 8*S*-LOX proximal promoter region. To date, the promoter of three human

lipoxygenases (5*S*-LOX (Funk et al. 1989), 12*S*-LOX (Yoshimoto et al. 1992), and 15*S*-LOX-1 (Kritzik et al. 1997)) and the promoter of one mouse lipoxygenase (5*S*-LOX (Silverman et al. 2002)) have been cloned. Each of these promoters also has a predominant transcription start site within 100 bp of the translation start site, and, like the cloned region from 8*S*-LOX, each lacks TATA and CCAAT boxes.

The 8*S*-LOX promoter not only drives activity of a reporter gene but also displays strong induction by TPA. From deletion and mutation analyses of the 8*S*-LOX promoter in SSIN primary keratinocytes, we identified the Sp1 binding motif between positions -77 and -68 of the promoter as being critical for basal and TPA-induced transcription. We then presented strong evidence that this site is a functional Sp1, Sp2, and Sp3 binding element.

At least one Sp1 binding site has been identified in the promoters of other lipoxygenase, and Sp1 has been shown to play a critical role in the basal or induced expression of such promoters in various cell types. The mouse 5*S*-LOX promoter has one Sp1 site at -189 to -184, and the human 5*S*-LOX promoter has five Sp1 binding sites located at -179 to -145 from ATG (Hoshiko et al. 1990). Disrupting the single Sp1 binding site in the mouse 5*S*-LOX promoter dramatically reduces basal activity from a luciferase reporter plasmid transfected into mouse monocyte-macrophage cells (Silverman et al. 2002). Adding or deleting Sp1 sites within the human 5*S*-LOX promoter greatly affects

transcription from a CAT reporter in Schneider cells (Silverman and Drazen 2000), and the Sp1 binding sites in the human promoter are important regulatory regions for TPA induced expression (Hoshiko et al. 1990). Notably, mutations in the Sp1 sites in the human 5*S*-LOX promoter are related to development of asthma (Silverman and Drazen 2000) and breast cancer (Wang et al. 2001). Sp1 binding sites are also important in the human 12*S*-LOX promoter. That promoter has five Sp1 binding sites, and two of them (located at -158 to -150 and -123 to -114) are essential for basal and epidermal growth factor (EGF) induced transcription in human epidermal carcinoma A431 cells (Liu et al. 1997).

If Sp1, Sp2 and Sp3 participate in the induction of 8*S*-LOX transcription by TPA, it will obviously be important to understand how this occurs. TPA treatment has been observed to increase the mRNA and protein expression of Sp1, as well as to enhance the binding of Sp1, in Chinese hamster ovary cells (Noe et al. 2001) and chronic myelogenous leukemia cells (D'Angelo et al. 1996). So, we examined whether Sp1, Sp2 or Sp3 might participate in TPA induction of the 8*S*-LOX promoter through one of these mechanisms. In fact, we found a significant increase of these proteins binding to the Sp1 binding site of the 8*S*-LOX promoter in the SSIN primary keratinocytes after TPA treatment. We did not measure a significant change in the endogenous level of Sp1, Sp2, or Sp3 protein, however, when keratinocytes were treated with TPA. Therefore, it appears likely that TPA transduces its effect on 8*S*-LOX expression through increased binding of Sp1, Sp2

and Sp3 to the Sp1 binding site of the promoter rather than through increased expression of these proteins in SSIN primary keratinocytes. Data showing TPA-induced 8*S*-LOX gene transcription is inhibited by treating cells with Sp1 binding inhibitor, MMA, further supports this conclusion. However, this is not in agreement with related observations of others. Binding of Sp1 to the human 5*S*-LOX promoter in HL-60 cells and binding of Sp1 to the rat ornithine decarboxylase promoter in Reuber H35 rat hepatoma cells are both unaffected by TPA treatment although TPA activates both promoters (Hoshiko et al. 1990; Kumar et al. 1995). Sp1 binding to the 12*S*-LOX promoter in human epidermal carcinoma A431 cells is also unchanged after EGF treatment although EGF induces 12*S*-LOX promoter activity through Sp1 sites (Liu et al. 1997). Thus, it appears that an induction of gene transcription by Sp1 can occur in a gene specific- as well as cell type specific-manner through different mechanisms.

The functional consequences of elevated 8*S*-LOX expression by TPA in mouse skin are still not known. Considering previous studies which suggest 8*S*-LOX is associated with differentiation (Jisaka et al. 1997; Muga et al. 2000), however, our finding of transcriptional activation of 8*S*-LOX by TPA provides a deeper insight into a function of this gene, at least in part, in the process of keratinocyte differentiation. That is, since the protein expression was found prominently in differentiated keratinocytes, it has been an unresolved question as to whether 8*S*-LOX induces keratinocyte differentiation or differentiated

keratinocytes produce 8*S*-LOX. However, the fact that 8*S*-LOX is transcriptionally activated by TPA in a proliferating basal keratinocyte population indicates a more active participation of this gene in the process of keratinocyte differentiation. Interestingly, forced overexpression of 8*S*-LOX in C57BL/6J mice caused a highly differentiated as well as thinner epidermis and, moreover, resulted in fewer tumors than in wild type mice in a two stage skin carcinogenesis protocol. Considering that the failure to fully differentiate is a defining characteristic of malignant cells, it is possible that the 8*S*-LOX gene could be a novel target for skin cancer prevention by modulating its expression.

In summary, our results demonstrate that TPA regulates 8*S*-LOX expression at the transcriptional level through an increased Sp1, Sp2 and Sp3 binding to the Sp1 binding site in the 8*S*-LOX promoter. However, this finding raises another question of how TPA alters DNA binding ability of those factors. Considering recent reports showing that protein kinase C (PKC), a cellular receptor for TPA, -mediated Sp1 phosphorylation increases Sp1 binding to a Sp1 binding site (Pal et al. 1998; Pal et al. 2001; Zheng et al. 2000), it is possible that phosphorylation of Sp1, Sp2, and Sp3 following the activation of PKC may enhance their DNA binding activity to the 8*S*-LOX promoter. On the other hand, we can not exclude the possibility of PKC-independent post-translational activation of Sp1. Torgeman et al. reported that TPA-stimulated Sp1 DNA binding activity was not diminished by a PKC-specific inhibitor (Torgeman et al.

1999). Furthermore, they showed the Sp1 binding stimulation was mediated by formation of Sp1-p53 protein complex following TPA treatment (Torgeman et al. 2001). This observation suggests TPA may modulate DNA binding activity of Sp1 by regulating its interaction with other transcription factors or co-factors. Therefore, pursuit of this question will require extensive work in the future and may well provide significant insight into how TPA promotes tumorigenesis on a global level.

Table 2. 1. Primers for cloning 8*S*-LOX promoter and mapping a transcription initiation site.

Primer Name	Sequence (5'-3')	Position (bp)[a]
8*S*-LOXA483	GCG AGG CCA ACC TTC AAT GTA AGT CTT C	483 to 456
8*S*-LOXA297	AGG TAG CCA CTC CAG CTC GAA CCA G	297 to 273
8*S*-LOXA128	TTG CCC AGA TGG TCC AGA GG	128 to 109
8*S*-LOXA122	AGA TGG TCC AGA GGT ACT AAG	122 to 102
8*S*-LOXA76	TGC TGA CAG ACA CTT TGT CCC ATG TGC	76 to 50
8*S*-LOXA32	CCC GTG GAT ACT CTC ACC CTG CAT TTC	32 to 6
8*S*-LOXA(-)1	CCT CCT GAC CAG TTT AGC TCT CTA C	-1 to -25
8*S*-LOXA(-)364	CAC AAG GCT GCT TTC ACT TC	-364 to -383
8*S*-LOXA(-)441	GGG TCC AGG CAA TAC TCT AGC	-441 to -461
8*S*-LOXS3	GGC GAA ATG CAG GGT GAG AGT ATC C	3 to 27
8*S*-LOXS(-)32	GCT TGC AGT AGA GAG CTA AAC TGG TCA G	-32 to -5
8*S*-LOXS(-)51	TCA CCC TGC CAG GTA GTC CAG CTT G	-51 to -27
8*S*-LOXS(-)149	TCT TCC TGA TGG CAA GGA AGC TGA AGG	-149 to -123
8*S*-LOXS(-)370	GCC TTG TGG CGT GGG AAG AGT TAA AGG	-370 to -344
8*S*-LOXS(-)627	TCT GGG TCT CTG AGG GCC TTT CTC TCG	-627 to -601
8*S*-LOXS(-)1126	CCA CTG CCC TTT TCC AGC GAT CTT CTC	-1126 to -1100
8*S*-LOXS(-)2195	CCA GGA GGC CTC AAC CCT ACA CAA AGA AC	-2195 to -2167
8*S*-LOXS(-)2248	AAC ATG AGC TGA ACC AGA AC	-2248 to -2229

[a]Numbering is relative to the translation start codon (adenosine is designated +1). Positions upstream of the translation start site are assigned negative numbers.

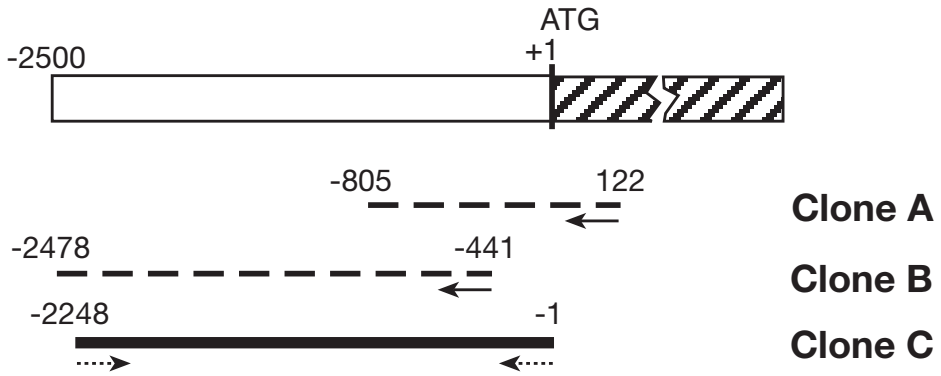


Fig. 2. 1. Cloning of the murine 8*S*-lipoxygenase.

The genomic region surrounding the translation start site of the murine 8*S*-LOX gene. The open box depicts the segment immediately upstream of the translation start, and the hatched box represents the protein-coding region (not to scale). Solid arrows indicate the nested gene-specific primers (8*S*-LOXA122 and 8*S*-LOXA(-)441; Table 2.1) used to isolate Clones A and B (dashed lines), respectively. Dotted arrows represent the PCR primers (8*S*-LOXS(-)2248 and 8*S*-LOXA(-)1; Table 2.1) used to synthesize Clone C (thick black line) from Clones A and B. Numbers denote base pair position relative to the translation start site (+1).

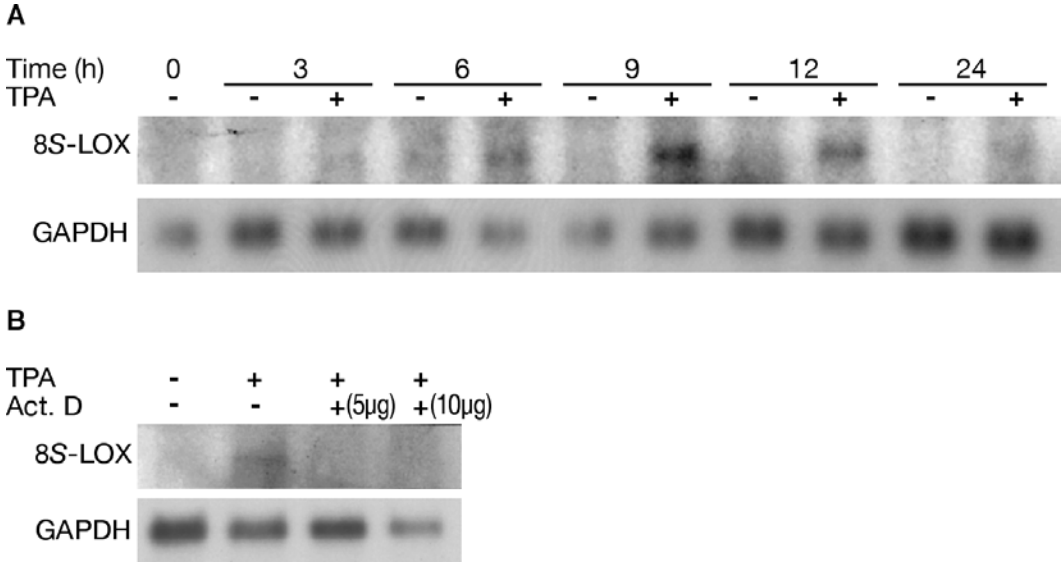


Fig. 2. 2. Inhibition of TPA-induced 8*S*-LOX message expression by actinomycin D in SSIN primary keratinocytes.

(A) A northern blot of total RNA (10 μg) isolated from keratinocytes treated for various time periods with acetone or TPA (30 ng/ml). (B) A northern blot of total RNA (10 μg) isolated from keratinocytes treated for 9 h with acetone, TPA (30 ng/ml), or a combination of TPA (30 ng/ml) plus actinomycin D (5 or 10 μg/ml). Each blot was hybridized with a radiolabeled 8*S*-LOX cDNA probe and thereafter re-hybridized with a radiolabeled GAPDH cDNA probe to control for loading.

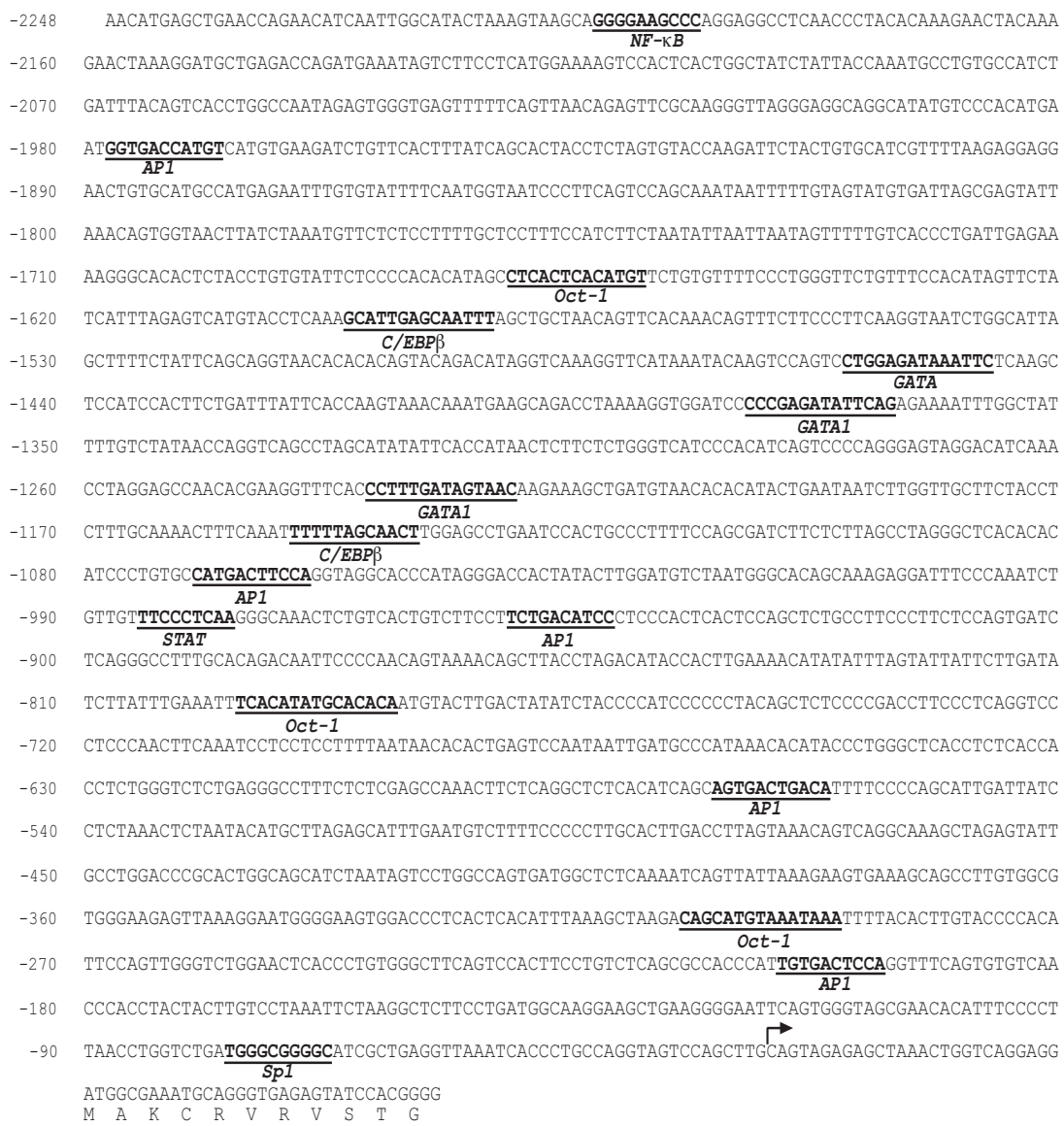


Fig. 2. 3. Murine 8*S*-LOX upstream genomic sequence and putative transcription factor binding sites.

The sequence of the 2248 bp genomic region immediately upstream of the murine 8*S*-LOX translation start site (+1). A transcription start site at -27 is indicated by the bent arrow. The beginning of the 8*S*-LOX open reading frame is also included for reference. Transcription factor binding motifs that were identified by computer analysis are shown in boldface, underlined and labeled.

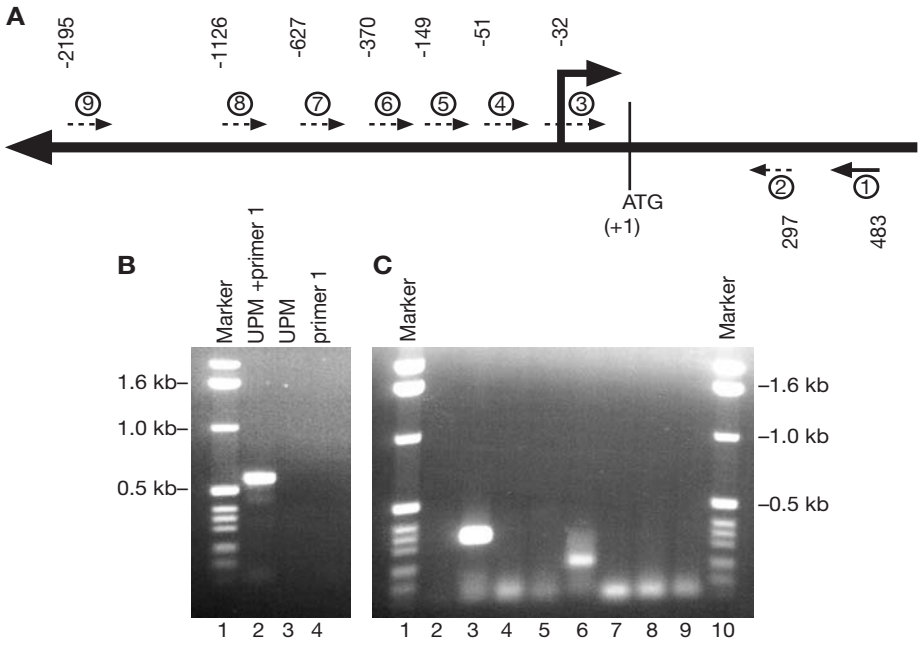


Fig. 2. 4. Mapping of the 8*S*-LOX transcription start site.

(A) The primer used in 5'RACE (solid arrow) and primers used in PCR (dotted arrows) to determine the 5' end of 8*S*-LOX mRNA. Primers 1 (8*S*-LOXA483) and 2 (8*S*-LOXA297) were used as the gene specific outer and nested primers, respectively, in the 5'RACE experiment (see Results). Primers 3 (8*S*-LOXS(-)32), 4 (8*S*-LOXS(-)51), 5 (8*S*-LOXS(-)149), 6 (8*S*-LOXS(-)370), 7 (8*S*-LOXS(-)627), 8 (8*S*-LOXS(-)1126) and 9 (8*S*-LOXS(-)2195) were individually paired with primer 2 (8*S*-LOXA297) for PCR amplification of 8*S*-LOX cDNA to confirm 5' RACE results. The transcription start site at -27 that was mapped by these combined approaches is indicated by a bent arrow. (B) The results of 5' RACE conducted upon mouse total RNA, using Primer 1 for reverse transcription and using UPM (the 5' RACE adapter primer) and Primer 1 (*lane 2*), or UPM alone (*lane 3*) or Primer 1 alone (*lane 4*) to perform PCR upon the reverse transcription products. Length markers are provided in *lane 1*. Presented in (C) are the results of PCR carried out on 8*S*-LOX cDNA used in 5'RACE, using Primer 2 alone (*lane 2*) or using Primer 2 with one of the following: Primer 3 (*lane 3*), Primer 4 (*lane 4*), Primer 5 (*lane 5*), Primer 6 (*lane 6*), Primer 7 (*lane 7*), Primer 8 (*lane 8*) or Primer 9 (*lane 9*). Length markers are included in *lanes 1 & 10.* A band seen in *lane 6* is a product of non-specific amplification and weak bands seen on the bottom of the gel are primer dimers.

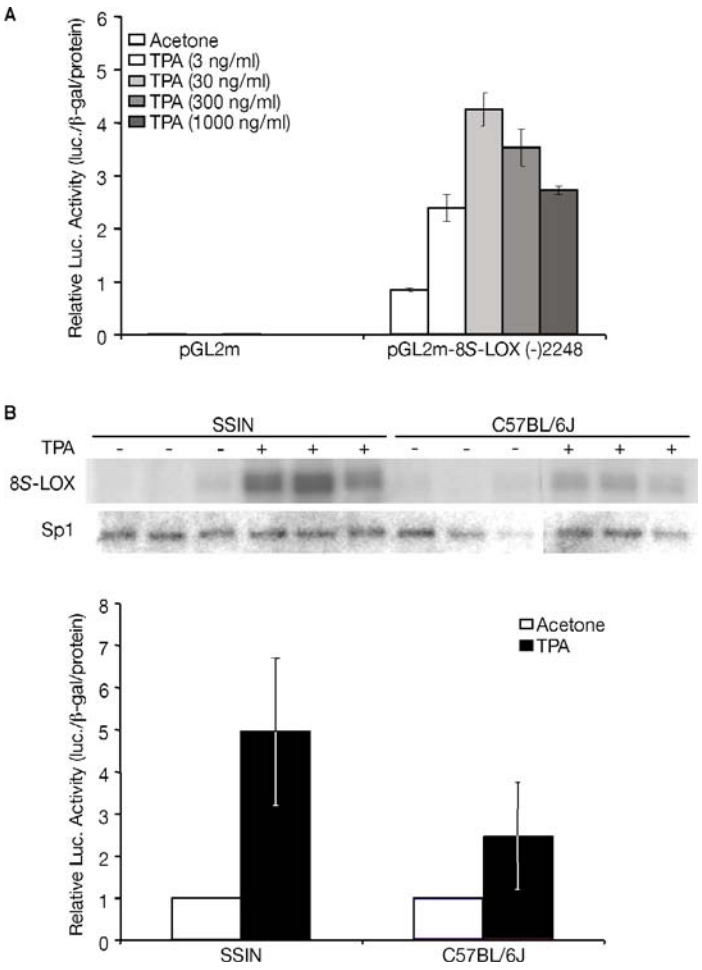


Fig. 2. 5. TPA induction of the mouse 8*S*-LOX promoter in primary keratinocytes.

(A) The relative luciferase activity of whole cell extracts from SSIN primary keratinocytes transiently transfected with 2 μg of pGL2 basic-m vector or pGL2m-8*S*-LOX(-)2248 plasmid for 16 h and then treated for another 24 h with acetone or with 3, 30, 300, or 1000 ng /ml of TPA. The data were obtained from a single experiment repeated two more times with similar results. Each bar displays the mean ± S.D. of relative luciferase activity from triplicate wells. (B) Shown above in (B) is a northern blot of total RNA (10 μg) isolated from SSIN and C57BL/6J mouse whole skin after a single, topical treatment with acetone or TPA (1 μg and 4 μg for SSIN and C57BL/6J mice, respectively) for 9 h. The blot was hybridized with a radiolabeled 8*S*-LOX cDNA probe and thereafter re-hybridized with a radiolabeled Sp1 cDNA probe to control for loading. Shown below in (B) is the relative luciferase activity of whole cell extracts from SSIN or C57BL/6J primary keratinocytes transiently transfected with 2 μg of pGL2m-8*S*-LOX(-)2248 plasmid for 16 h and then treated for another 24 h with acetone or TPA (30 ng/ml). Each value is the mean ± S.D. of at least three independent experiments.

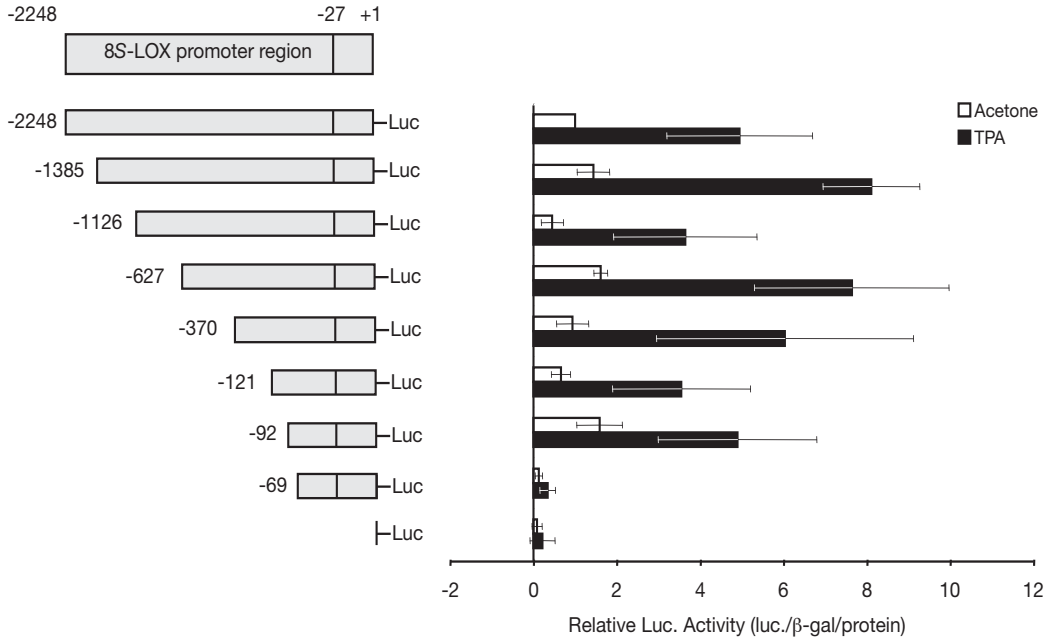


Fig.2. 6. Isolation of a TPA responsive region within the 8*S*-LOX promoter.

Illustrated on the left are various deletion constructs tested for TPA induction. Each construct is named according to the distance in nucleotides of its upstream end from the translation start site (+1). A transcription start site (-27) is indicated by a vertical line. Two μg of each construct or pGL2 basic-m, along with 0.125 μg of an expression vector for β-galactosidase (pCMV-β-gal; an internal control), were transfected into SSIN primary keratinocytes. After 16 h of transfection, the cells were subsequently treated with acetone or TPA (30 ng/ml) for 24 h. Shown on the right is the relative luciferase activity from each construct in response to acetone or TPA. Luciferase activity was normalized to both β-galactosidase activity and protein concentration and then standardized to the normalized activity from pGL2m-8*S*-LOX(-)2248 after acetone treatment. Each value is the mean ± S.D. of at least three independent experiments.

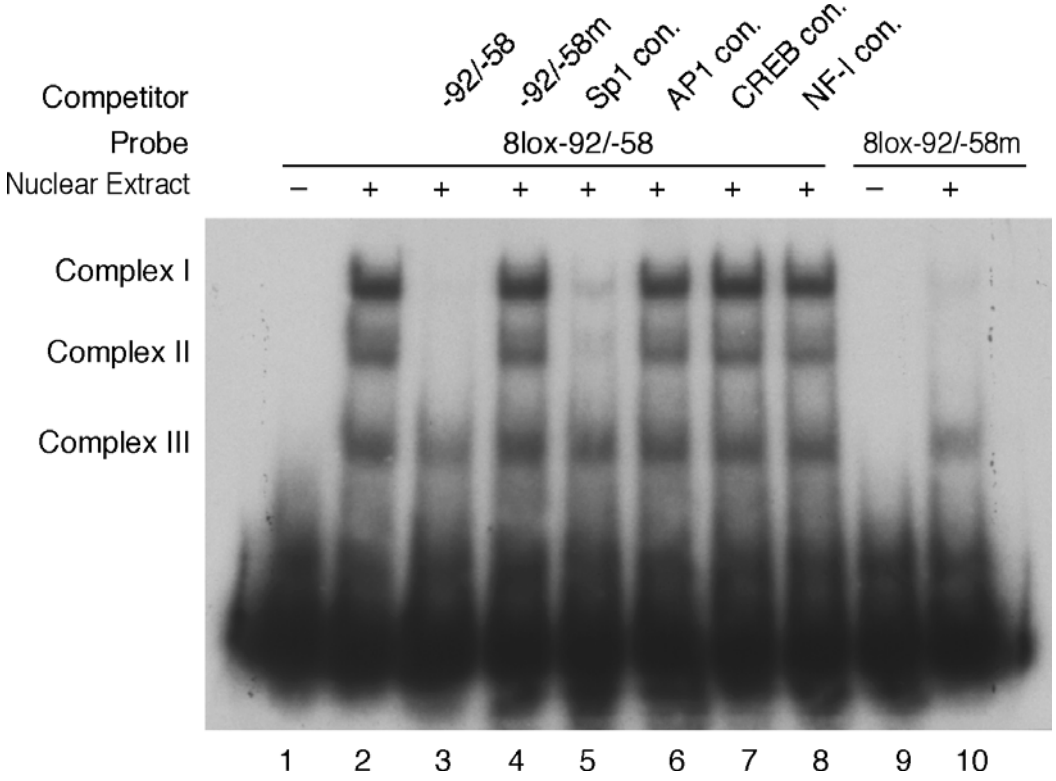


Fig.2. 7. Specific interaction between nuclear proteins and Sp1 binding motif in the TPA responsive region of the 8*S*-LOX promoter.

Nuclear extracts were prepared from SSIN primary keratinocytes after treatment with TPA (30 ng/ml) for 6 h. Two μg of extracts were incubated with a ^{32}P-end labeled oligonucleotide spanning the 8*S*-LOX promoter segment from -92 to -58 (-92/-58; *lanes 2 thru 8*) or incubated with a similarly-labeled oligonucleotide covering the same segment but containing TTTATTT in place of the Sp1 binding motif, GGGCGGG (-92/-58m; *lane10*). Binding specificity was confirmed by chasing labeled -92/-58 with a 100-fold molar excess of unlabeled -92/-58 (*lane 3*), -92/-58m (*lane 4*) or consensus oligonucleotides for Sp1, AP1, CREB, and NF-I (*lanes 5 thru 8*). Protein-DNA complexes were resolved on a 5% nondenaturing polyacrylamide gel and the positions of three protein-DNA complexes (complexes I, II, and III) were noted. Labeled probe in the absence of nuclear extract migrated as shown in *lanes 1 & 9*.

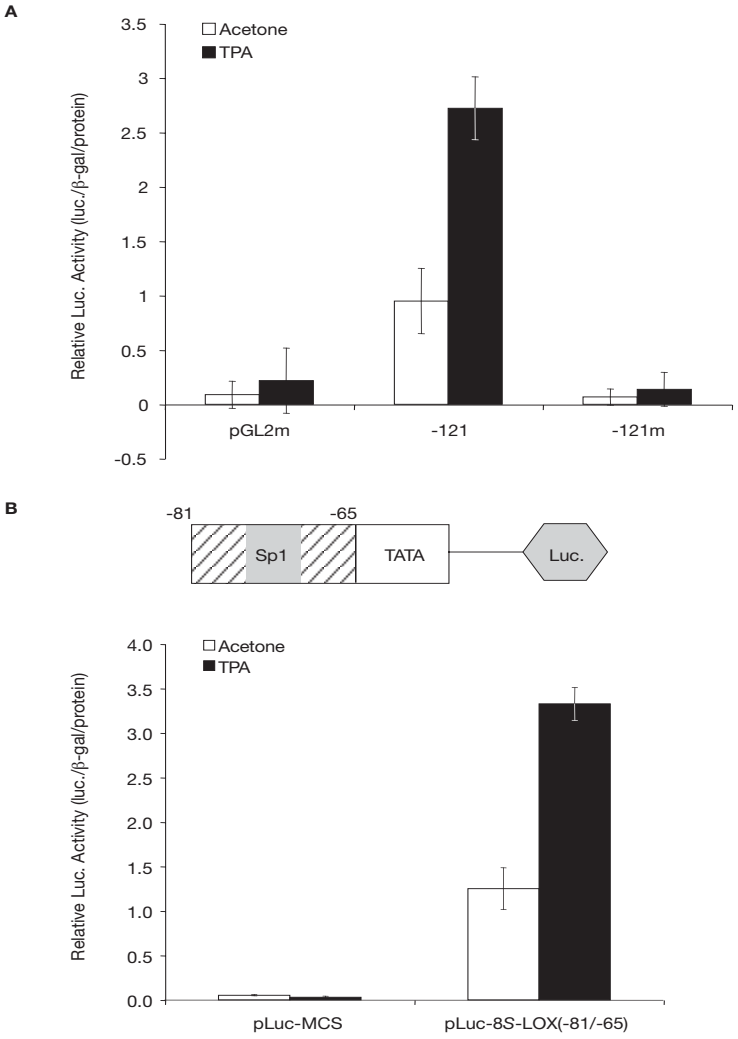


Fig.2. 8. Identification of a Sp1 binding site as a TPA responsive element in the 8*S*-LOX promoter.

(A) Relative luciferase activities from deletion constructs and the pGL2 basic-m in response to acetone or TPA. The 7 nucleotides of Sp1 binding motif (GGGCGGG) in the -121 construct were mutated to TTTATTT in the -121m. (B) A structure of the pLuc-8*S*-LOX(-81/-65) construct and the relative luciferase activities from the construct and from its parent vector, pLuc-MCS, in response to acetone or TPA. A 8*S*-LOX promoter segment between -81 and -65 inserted in front of the TATA box (open box) in the pLuc-MCS vector is illustrated as a hatched box and the Sp1 binding motif located -76 to -70 is depicted as a solid box. The relative luciferase activities in (A) and (B) were obtained from SSIN primary keratinocytes transfected with 2 μg of each construct along with its parent vector for 16 h and thereafter treated with acetone or TPA (30 ng/ml) for another 24 h. The data are the mean ± S.D. of at least three independent experiments.

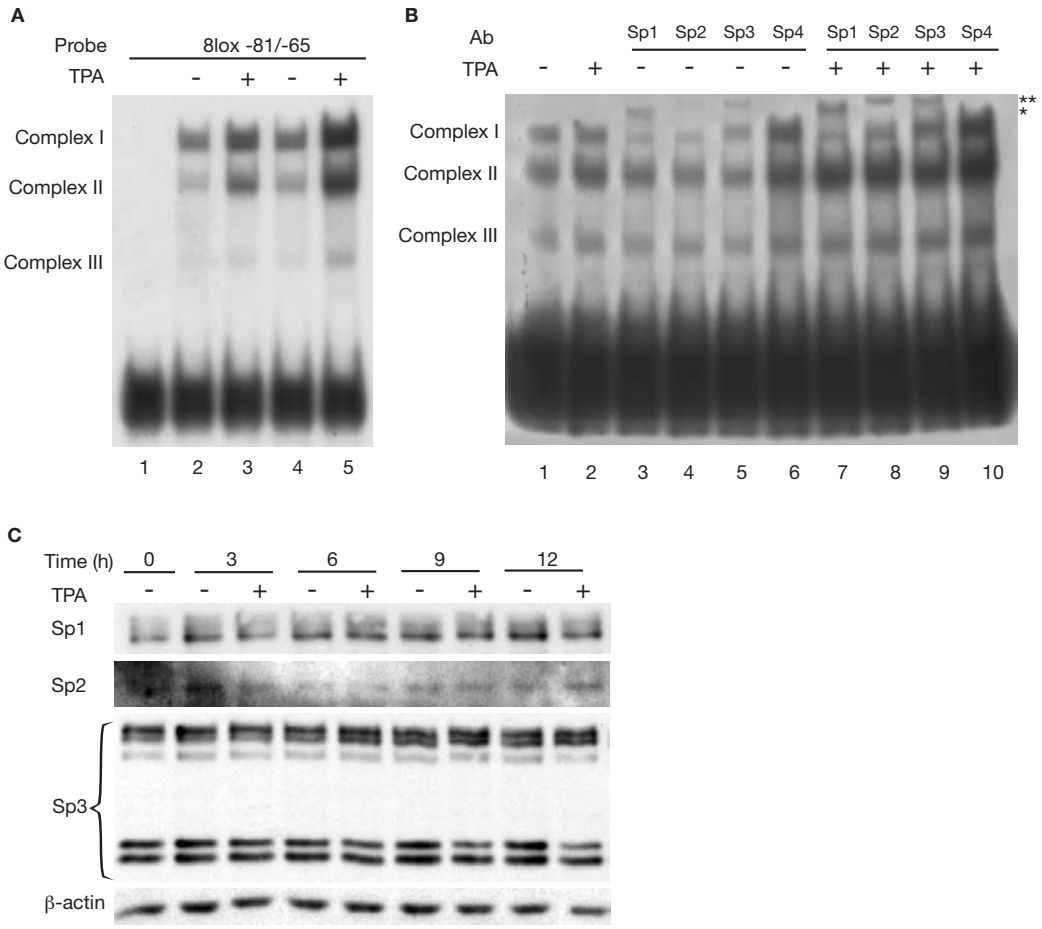


Fig. 2. 9. Increased binding of Sp1, Sp2, and Sp3 to the TRE of the 8*S*-LOX promoter after TPA treatment.

(A) An EMSA showing an effect of TPA on protein-DNA complex formation. Two independent nuclear extract preparations were made from SSIN primary keratinocytes after treatment with acetone (*lanes 2 & 4*) or TPA (30 ng/ml; *lanes 3 & 5*) for 6 h. Two μg of extracts were incubated with a ^{32}P-end labeled oligonucleotide spanning the 8*S*-LOX promoter segment from -81 to -65. Labeled probe in the absence of nuclear extract migrated as shown in *lane 1*. In (B), the proteins complexed to the labeled probes were identified by pre-incubating 2 μg of nuclear extracts with 2 μl of anti-Sp1 (*lane 3 & 7*), -Sp2 (*lane 4 & 8)*, -Sp3 (*lane 5 & 9*) or -Sp4 (*lane 6 & 10)* antibody before addition to the binding reaction. Protein-DNA complexes were resolved on a 5% nondenaturing polyacrylamide gel. The positions of three protein-DNA complexes (complexes I, II, and III) are noted and the positions of supershifted bands are indicated by one (*) or two asterisks (**). (C) A western blot of nuclear proteins (20 μg) prepared from SSIN primary keratinocytes after treatment with acetone or TPA (30 ng/ml) for various time period. The blot was hybridized with an antibody against Sp1, Sp2, or Sp3 and thereafter re-hybridized with a β-actin antibody to control for loading.

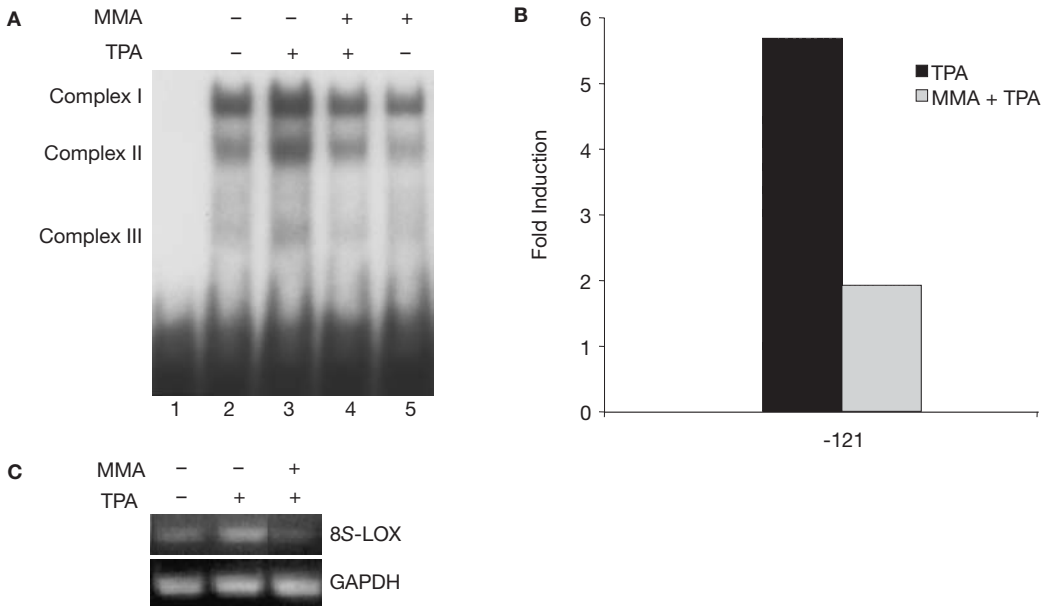


Fig. 2. 10. Inhibition of TPA-induced 8*S*-LOX gene expression by MMA in SSIN primary keratinocytes.

(A) An EMSA showing the effect of mithramycin A (MMA) on protein-DNA complex formation. Nuclear extracts were prepared from keratinocytes after treatment with acetone (*lane 2*), TPA (30 ng/ml; *lane 3*), MMA (1 μM) plus TPA (30 ng/ml; *lane 4*) or MMA (1 μM) plus acetone (*lane 5*) for 6 h. Keratinocytes were treated with MMA 1 h before acetone or TPA treatment. Two μg of extracts were incubated with a ^{32}P-end labeled oligonucleotide spanning the 8*S*-LOX promoter segment from -81 to -65. Labeled probe in the absence of nuclear extract migrated as shown in *lane 1*. The positions of three protein-DNA complexes (complexes I, II, and III) are noted. (B) The effect of MMA on TPA-induced 8*S*-LOX promoter activity. Keratinocytes were transiently transfected with 2 μg of -121 reporter construct for 16 h and then treated for another 24 h with acetone, TPA (30 ng/ml), MMA (1 μM) plus acetone or MMA (1 μM) plus TPA (30 ng/ml). MMA was treated 1 h before acetone or TPA treatment. Data are expressed as fold induction of the luciferase activity by TPA relative to acetone treatment. The data is a representative of three independent experiments with similar results. (C) An assay by RT-PCR to measure the inhibitory effect of MMA on TPA-induced 8*S*-LOX mRNA expression relative to GAPDH expression. cDNA synthesis was carried out on total RNA extracted from SSIN primary keratinocytes after treatment with acetone (*lane 1*), TPA (30 ng/ml; *lane2*), or MMA (1 μM) plus TPA (30 ng/ml) (*lane 3*) for 6 h. MMA was added 1 h before TPA treatment.

CHAPTER III

INCREASED DIFFERENTIATION CONFERRED BY MURINE 8*S*-LIPOXYGENASE INHIBITS MOUSE SKIN TUMOR FORMATION

3.1. Summary

8*S*-lipoxygenase (8*S*-LOX) is a recently cloned murine lipoxygenase that metabolizes arachidonic acid to 8*S*-hydroxyeicosatetraenoic acid (8*S*-HETE). Although the level of 8*S*-HETE is highly elevated in the early stages of mouse skin carcinogenesis, it is still not clear whether 8*S*-HETE is a procarcinogenic or anticarcinogenic lipid. Several reports have shown that 8*S*-HETE is also closely associated with keratinocyte differentiation. To determine the role of 8*S*-HETE in mouse skin carcinogenesis, we generated skin-targeted C57BL/6J transgenic mice overexpressing the 8*S*-LOX gene under control of the loricrin promoter. The transgenic mice showed a more differentiated epidermal phenotype as well as a 64% reduced papilloma development in a two-stage skin carcinogenesis protocol. We then stably transfected tumor-derived cell lines, MT1/2 (papilloma) and CH72 (carcinoma), with the 8*S*-LOX cDNA to determine the effect of the gene on tumor cell growth *in vitro* and *in vivo*. Interestingly, forced expression of the gene in

MT1/2 cells caused a more differentiated appearance as well as keratin 1 (K1) expression. Forced expression of 8*S*-LOX in CH72 carcinoma cells did not induce K1 expression, however, it inhibited by 30% cell proliferation in culture. Subcutaneous injection of CH72-8*S*-LOX clones into athymic nude mice showed an 86% reduced tumor growth *in vivo* as well. In accordance with this observation, exogenous addition of 5 μM 8*S*-HETE to the parental CH72 cells also significantly inhibited cell proliferation and caused cell cycle arrest at the G1 phase. Finally we localized 8*S*-LOX protein expression in mouse skin at various stages of skin tumor development by immunohistochemical analyses. The expression of 8*S*-LOX was strictly confined to the differentiated compartment of mouse skin. Collectively, these data suggest that 8*S*-LOX plays a role as a pro-differentiating, an anti-tumorigenic, and a tumor suppressing gene in mouse skin carcinogenesis.

3.2. Introduction

Upon a variety of stimuli such as wounding, growth factors, and hormones, arachidonic acid (AA, 5,8,11,14 eicosatetraenoic acid, 20:4, n-6) is promptly released from cell membrane phospholipids by the action of phospholipases and is metabolized to bioactive lipid mediators by cyclooxygenases (COXs) and lipoxygenases (LOXs). These metabolites,

including prostaglandins (PGs), thromboxanes (from COXs), leukotrienes, and hydroxyeicosatetraenoic acids (HETEs) (from LOXs) are known to execute a number of physiologically important functions (Ruzicka and Printz 1984). The AA cascade is tightly regulated in the cell and aberrant metabolism has been implicated in the pathogenesis of many human diseases such as cancer (Marks et al. 2000).

A large body of evidence has shown an excessively activated COX pathway in a variety of human cancers (Fischer 1997). Protumorigenic roles of PGs have been documented (Fischer 1997) and moreover, some of the COX-specific inhibitors are already in clinical use for the prevention of colon cancer (Steinbach et al. 2000). On the other hand, research on the role of LOX metabolites in tumorigenesis has lagged behind that of the COX metabolites. This is partly because: 1) most of the LOX isozymes have only recently been cloned; 2) LOXs are a more complex system in terms of number of isozymes, tissue specificity as well as substrate usage than the COXs; and 3) only a few specific LOX inhibitors are available. However, various reports suggesting that LOX metabolites also exert a substantial impact on the development of animal as well as human cancers have gradually increased (Fischer and Klein 2003; Shureiqi and Lippman 2001; Steele et al. 1999).

LOXs are a family of dioxygenases that insert an oxygen molecule onto specific carbons of polyunsaturated fatty acids in a regio- and stereo-selective

manner (Prigge et al. 1997). So far, at least 6 different isozymes have been identified in mammals and the enzymes convert AA to 5*S*-, 8*S*-, 12*S*-, 12*R*-, or 15*S*- hydroperoxyeicosatetraenoic acid (HPETE) and convert linoleic acid to 9*S*- or 13*S*- hydroperoxyoctadecadienoic acid (HPODE) (Brash 1999). These hydroperoxy derivatives are subsequently reduced to the corresponding HETE and hydroxyoctadecadienoic acid (HODE) by glutathione peroxidase. Although not all of the molecular functions of these LOX metabolites in carcinogenesis have been elucidated, unlike the procarcinogenic role of COX metabolites, LOX metabolites appear to play a role as either pro- or anticarcinogenic lipid mediators in a metabolite- and tumor type-specific manner (Shureiqi and Lippman 2001). For example, 13*S*-HODE induces apoptosis in human colon cancer cells (Shureiqi et al. 2000; Shureiqi et al. 1999; Slaga 1984a) or in esophageal cancer cells (Shureiqi et al. 2001) whereas, it accelerates growth of human prostate cancer cells (Kelavkar et al. 2001). In the case of 5*S*-HETE and 12*S*-HETE, they are mainly procarcinogenic mediators and stimulate the growth of cancer cells via inflammation (Chan et al. 1989; DuBois 2003), angiogenesis (Nie et al. 1998), or metastasis (Tang and Honn 1994). On the other hand, 15*S*-HETE has been shown to be a critical negative cell cycle regulator in human prostate cancer (Tang et al. 2002). Therefore, clear understanding of the roles of each LOX metabolite is indispensable to further understand their contribution to carcinogenesis as well as their potential as preventive or therapeutic targets.

One of the LOX products for which the role in tumorigenesis has not yet been clearly elucidated is 8*S*-HETE. It is a major metabolite of 8*S*-LOX and is produced primarily during mouse skin carcinogenesis (Bürger et al. 1999; Fürstenberger et al. 1991; Gschwendt et al. 1986; Jisaka et al. 1997). However, a report showing the production of 8-HETE in human primary squamous cell carcinomas of head and neck (El Attar et al. 1985) suggests that 8-HETE may be involved in the development of human cancer as well.

In normal mouse skin, the level of 8*S*-HETE is nearly undetectable. However, the level is highly elevated after a single topical treatment of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Fürstenberger et al. 1991; Gschwendt et al. 1986; Jisaka et al. 1997) and is even further increased in papillomas (Bürger et al. 1999; Nair et al. 2000). In support of a relationship between 8*S*-HETE and skin tumor development, a good correlation between the level of 8*S*-HETE and the level of promutagenic etheno-DNA adduct formation in mouse skin tumors has been reported (Nair et al. 2000). Additionally, a clastogenic activity of 8*S*-HETE has also been demonstrated (Bürger et al. 1999). Since accumulation of chromosomal alterations accompanies malignant conversion of papillomas to carcinomas, Fürstenberger et al. (2002) suggested that 8*S*-HETE could be an endogenous factor that is responsible for the genotoxicity of the premalignant lesions. On the other hand, we have recently found that 8*S*-HETE has an ability to induce keratinocyte differentiation through a selective activation of peroxisome

proliferator-activated receptor (PPAR) α (Muga et al. 2000). In fact, skin targeted 8*S*-LOX transgenic mice showed a highly differentiated and keratinized epidermal phenotype (Muga et al. 2000). Although a high degree of differentiation can stimulate keratinocyte proliferation in skin through a compensatory mechanism, considering that differentiation in general inversely correlates with the malignancy of tumors, the ultimate consequence of 8*S*-HETE production during mouse skin carcinogenesis remained to be clarified.

To that end, we conducted a series of gain-of-function studies on 8*S*-LOX/8*S*-HETE *in vitro* and *in vivo*. Our results show that a forced increase of 8*S*-HETE production in normal and tumorigenic keratinocytes resulted in increased differentiation and cell cycle arrest of the keratinocytes.

3.3. Materials and Methods

Generation of C57BL/6J Transgenic Mice Overexpressing the 8S-LOX Gene- Previously established homozygous loricrin 8*S*-LOX transgenic mice on the FVB background (Muga et al. 2000) were backcrossed for 6 generations onto C57BL/6J mice (Jackson Laboratory, Bar Harbor, MA). All progeny were genotyped for the expression of the 8*S*-LOX transgene using primers 5'-CTCTTCCAGCTCTGTTGTCTCCG -3' and 5'- GTATGCCCTCCTTGTTGG -3'with the following PCR cycling parameters: 94°C, 1 min; 39 cycles: 94°C, 1

min; 50°C, 2 min; 72°C, 3 min; 1 cycle: 94°C, 1 min; 50°C, 2 min; 72°C, 10 min. All of the mice were handled and housed in Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities and in accordance with USPHS and institutional guidelines.

Two-Stage Skin Carcinogenesis- Groups of twenty 7-week-old female F6 8*S*-LOX/C57BL/6J transgenic mice or corresponding wild type littermates were housed four animals/cage and were fed *ad libitum* under an artificial 12 h day-night cycle. Dorsal skins of the mice were shaved 2 days prior to initiation and only those mice in the resting phase of the hair cycle were used in the tumor experiments. Initiation was accomplished by a single topical application with 200 nmol (50 μg) 7,12-dimethylbenz[*a*]anthracene (DMBA) under subdued light. Two weeks after initiation, the mice were treated with 6.5 nmol (4 μg) of TPA in 200 μl of acetone twice a week for 15 weeks. The mice were then treated with same dose of TPA five times a week for another 14 weeks. The tumor incidence (number of mice bearing tumors/total number of mice) and the tumor multiplicity (number of papillomas/mice) were recorded weekly.

Cell Culture- A murine papilloma cell line, MT1/2 (Conti et al. 1988), was grown in Eagle's minimal essential medium (EMEM) supplemented with 4% fetal bovine serum (FBS). Stable transfectants of MT1/2 cells were grown in the same media containing 100 μg/ml of G418 (Invitrogen, Carlsbad, CA). CH72 (Conti et al. 1988), a murine squamous carcinoma cell line, was grown in EMEM

supplemented with 1% FBS. Stable transfectants of CH72 cells were grown in the same media containing 200 μg/ml of G418. Cells were grown in 37°C humidified incubator under 5% CO_2

Generation of Cell Lines Stably Expressing 8S-LOX - The 2034 bp EcoR I fragment of 8*S*-LOX cDNA from pGEM3Z.8*S*-LOX vector (Muga et al. 2000) was inserted into the corresponding site in a pCI-neo mammalian expression vector (Promega, Madison, WI). The resulting pCI-neo.8*S*-LOX expression vector or pCI-neo empty vector was transfected into the MT1/2 or CH72 cells at 40-50% confluence using FuGENE^{TM}6 Transfection Reagent (Roche, Indianapolis, IN) as described by the manufacturer. When cultures were 85% to just confluent, cells were trypsinized and subcultured at a cell density of 2×10^5 cells/60 mm dish. G418 (100 and 200 μg/ml for MT1/2 and CH72 cells, respectively) was added to the media at the time of subculture and fresh media with G418 was added every 2-3 days until resistant colonies arose. The colonies were then individually collected or pooled together and expanded into cell lines.

Northern Analysis- Total RNA was isolated from cells or tissues using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. Ten μg of RNA was separated on a formaldehyde-containing 1% agarose gel, transferred onto nylon membrane (Micron Separation, Westboro, MA), and UV cross-linked onto the membrane with Stratalinker (Stratagene, La Jolla, CA). cDNAs for 8*S*-LOX, keratin 1 (K1), and

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were labeled with [α-^{32}P]dCTP (Perkin Elmer, Boston, MA) by using Random Primed DNA Labeling kit (Roche). Labeled probes were hybridized to the blot using QuickHyb (Stratagene) solution for 2 h at 65°C. Blots were washed twice in 2X standard saline citrate (SSC; 1X=0.15 M NaCl, 15 mM sodium citrate, pH 7), 0.1% sodium dodecyl sulfate (SDS) for 15 min at room temperature and then washed once in 0.1X SSC, 0.1% SDS for 40 min at 68°C. Specific bands were detected by autoradiography.

Western Analysis- Total cellular proteins were separated by 8% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Pierce, Rockford, IL). The blot was blocked with 5% non-fat dried milk in 0.1% Tween 20-Tris-buffered saline (TTBS) and probed with antisera against human 15*S*-LOX-2 (a generous gift from Dr. Alan R. Brash, Vanderbilt University School of Medicine, Nashville, TN) at 1:500 dilution for 1 h at room temperature. This antibody also reacts with murine 8*S*-LOX (Jisaka et al. 1997). After washing three times with 0.1% TTBS, the blot was then probed with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) at 1:5000 dilution. The specific bands were detected by an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

8S-LOX Enzyme Activity Assay- Frozen mouse skin was pulverized and then homogenized with 150 μl ice-cold PBS buffer containing 0.1% butylated

hydroxytoluene (BHT, Sigma Chemical Co. St. Louis, MO) and 1 mM EDTA using the ultrasonic sample processor (Misonix Inc., Farmingdale, NY). Subsequently, 8-HETE and 9-HODE were extracted from the homogenates following a modified method of Kempen et al. (Kempen et al. 2001). Briefly, aliquots of 1 N citric acid (20 μl) as well as of 2.5 μl of 10% BHT were added to the homogenates and an aliquot (10 μl) of 9(S)-HODE-d4 (100 ng/ml, Cayman Chemical Co., Ann Arbor, MI) was also added to each sample as an internal standard. Samples were then extracted with 1 ml of hexane:ethyl acetate (1:1, v/v) by vortex mixing for 2 min. After centrifugation at 1800 X g for 10 min at 4 °C, the upper organic layer was collected, and dried under nitrogen gas. Samples were reconstituted in 100 μl of methanol: 10 mM ammonium acetate buffer, pH 8.5 (70:30, v:v) prior to liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis. LC/MS/MS was performed using a Quattro Ultima tandem mass spectrometer (Micromass, Beverly, MA) equipped with an Agilent HP 1100 binary pump HPLC inlet. The protein concentration was determined by the method of Bradford (Bio-Rad, Hercules, CA) and final concentration of 8-HETE and 9-HODE in the tissues were expressed as ng/ mg of protein.

CH72-neo or CH72-8*S*-LOX clones were cultured in 75-cm^2 tissue culture flasks. When cells reached 70-75% confluency, cells were incubated with 50 μM of arachidonic acid (Sigma Chemical Co.) for 30 min, 1 h, and 2 h. The conditioned medium was collected at each time point and subjected to 8-HETE

analysis. Ten μl of 10% BHT was added to 1 ml of the collected medium before application to a Sep-Pak C18 cartridge (Waters Corp., Milford, MA). HETEs were eluted with 1 ml of methanol and dried under a stream of nitrogen. Samples were reconstituted in 200 μl of methanol:10 mM ammonium acetate buffer, pH 8.5 (70:30, v/v) prior to LC/MS/MS anlysis. LC/MS/MS was performed under the same conditions as described above. The results were expressed as ng of 8-HETE/ 5×10^6 cells.

Cell Proliferation Assays- CH72 cells were plated at a density of 8×10^3 cells/well in 96-well plates. Twenty-four hours after plating, cells were fed with fresh media containing ethanol (vehicle), 1, 5, or 10 μM of 8*S*-HETE (Cayman Chemical Co., Ann Arbor, MI). Since the volume of the desired amount of 8*S*-HETE differed from one dose to the other, a compensatory amount of ethanol was included in each experimental group to provide the same volume of vehicle. The cell proliferation rate was determined every 24 h using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay Kit (Promega), following the manufacturer's protocol. The absorbance was measured at 490 nm using the ELx 800 uv Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT).

CH72-neo and CH72-8*S*-LOX stable clones were plated at 5×10^4 cells/60 mm dish and were fed with fresh media with G418 every two days. At day 1, 3, and 5 after plating, cells were washed twice with phosphate buffered saline (PBS)

and trypsinized with 0.28% trypsin, 0.02% EDTA. Cell numbers were counted with a hemacytometer.

Flow Cytometry Analysis- CH72 cells were plated in 60 mm dishes (7×10^5 cells/dish) and fed with fresh media containing 0.1% ethanol vehicle, 5, or 10 μM 8*S*-HETE at approximately 80% cell confluency. After 24 h of treatment, cells were trypsinized, filtered through a 70 μm mesh, and centrifuged. Cell pellets were then resuspended in PBS and fixed in ethanol on ice for 1 h. Fixed cells were collected by centrifugation and stained with 40 μg/ml of propidium iodide. Single cell suspensions were then analyzed for DNA content using a Coulter EPICS Elite flow cytometer (Beckman Coulter, Miami, FL). DNA histograms were further analyzed with MultiCycle software (Phoenix Flow Systems, Inc., San Diego, CA) to quantitate the cell cycle phases.

In Vivo Xenografts- Cultured CH72-neo and CH72-8*S*-LOX stable clones were trypsinized, centrifuged, and re-suspended in PBS at a concentration of 5×10^7 cells/ml PBS. Aliquots (100 μl: 5×10^6 cells) of suspended cells were then subcutaneously injected into each of four different sites (two shoulders and two flanks) of 2 female athymic nude mice each (Jackson Laboratory). Tumor diameters (length and width) were measured once a week with a caliper for total of 9 weeks and tumor volume was estimated as 1/2 (length × width^2). Mice were sacrificed after 9 weeks, and the tumors were collected for further analyses.

Histological Analyses- Dorsal skin or tumors were isolated from mice, fixed in formalin, and processed for paraffin embedding. Tissue sections were stained with hematoxylin and eosin (H&E) and immunostained with antibodies against K1, Ki67, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) or 8*S*-LOX.

For measuring the rate of epidermal cell proliferation, 100 mg/kg body weight of 5-bromo-2'-deoxyuridine (BrdU, Sigma, St. Louis, MO) was injected i.p. into mice 1 h before they were sacrificed. Isolated skin or tumors were fixed in formalin, processed for paraffin embedding and immunostained with anti-BrdU antibody. The number of BrdU positive cells in the interfollicular epidermis or in the tumors was counted in several random tissue sections from several mice in each experimental group. The labeling index was calculated as the percentage of BrdU positive basal cells to total number of basal cells in the interfollicular epidermis

Inter-simple Sequence Repeat (SSR) PCR- Genomic DNA from CH72 stable clones as well as matched xenograft tumors was analyzed by the inter-SSR PCR method (Zietkiewicz et al. 1994) using equimolar mixture of $(CA)_8RY$, $(CA)_8RG$, and $(AGC)_4Y$ primers (where R = purine, and Y = pyrimidine). Amplifications were carried out in 25 μl reactions containing 50-100 ng of genomic DNA, 2.5 μl 10X PCR buffer (15 or 20 mM $MgCl_2$), 2% formamide, 0.2 mM dNTP, and 1 μM primer, using the following cycling parameters: 94°C, 3

min; 30 cycles: 94°C, 30 sec; 54°C, 45 sec, 72°C, 2 min; 72°C, 7 min. PCR products were analyzed by Sequi-Gen Sequencing Cell (BioRad Laboratories, Hercules, CA) and Criterion pre-cast 10% polyacrylamide gels (BioRad Laboratories). The products were visualized by silver staining (Amersham-Pharmacia Biotech) and with Vistra Green staining (Amersham International, Buckinghamshire, UK).

3.4. Results

Generation of 8S-LOX/C57BL/6J transgenic mice- To determine the role of 8*S*-HETE in mouse skin carcinogenesis, we generated skin targeted 8*S*-LOX transgenic mice on a C57BL/6J background under the control of loricrin promoter. Of note is that unlike in the skin of TPA tumor promotion-sensitive strains (SSIN, CD-1, and NMRI), 8*S*-LOX expression is only weakly induced by TPA treatment in the skin of promotion-resistant C57BL/6J mice (Bürger et al. 1999; Fischer et al. 1988a). Fig. 3.1A shows 8*S*-LOX mRNA expression in wild type and in 8*S*-LOX/C57BL/6J transgenic mice. A slight induction of 8*S*-LOX mRNA by TPA treatment was detected in wild type mice whereas strong and constitutive expression of 8*S*-LOX mRNA was detected in transgenic mouse skin. This transgene expression was not increased much further by TPA treatment. Notably, 8*S*-LOX transgene message was detected as a shorter fragment than the

TPA-induced endogenous 8*S*-LOX mRNA (3.2 kb) in wild type mice. This is probably because the transgene does not contain the entire 3' untranslated region. We also detected strong 8*S*-LOX protein expression from the transgenic mouse skin (Fig. 3.1B) and we confirmed that the protein has active enzyme activity by LC/MS/MS analysis (Table 3.1). The transgenic mouse skin produced 3-fold more 8*S*-HETE compared to wild type skin (Table 3.1). However, the level of 9*S*-HODE, a linoleic acid metabolite of 8*S*-LOX, was not different between transgenic and wild type mice (Table 3.1).

Characterization of 8S-LOX/C57BL/6J transgenic mice- Overexpression of 8*S*-LOX in C57BL/6J mouse skin did not create a macroscopic skin phenotype. However, histological analysis showed that the epidermis of 8*S*-LOX transgenic mice was much thinner and more stretched than that of wild type mice (Fig. 3.2A). Moreover, aberrant K1 expression was detected in some of the basal cells and stronger K1 expression was detected in the suprabasal layers of transgenic mouse skin compared to wild type mice (Fig. 3.2B). Interestingly, when the 8*S*-LOX transgenic and the wild type mice were subjected to the two-stage skin carcinogenesis protocol, shaved dorsal skin hair did not grow back in wild type mice whereas it completely grew back in 8*S*-LOX transgenic mice after 8 weeks of multiple TPA treatments (Fig. 3.2C). Since hair is another differentiated structure of the epidermis, we speculate that increased hair growth also arises from enhanced differentiation stimulated by 8*S*-LOX. Despite the observed

phenotypic characteristics of increased differentiation in transgenic mice, the rate of basal and TPA-induced cell proliferation in the interfollicular epidermis was not significantly different between wild type and 8*S*-LOX transgenic mice (Fig. 3.2D).

Two-stage skin carcinogenesis study- From previous experience, we knew that twice a week treatment with TPA would not induce tumor formation in C57BL/6J mice (Fischer et al. 1989). Nevertheless, we used this protocol to determine whether 8*S*-LOX transgene expression could restore TPA promotion sensitivity to the C57BL/6J mice. However, no tumors arose in either wild type or transgenic mice with this protocol (Fig. 3.3A). We therefore switched the protocol from twice a week to five times a week TPA treatment at 17 weeks after initiation and examined tumor formation. This protocol was previously shown to elicit tumors in C57BL/6J mice (Fischer et al. 1989). As shown in Fig. 3.3A, both wild type and transgenic mice started to develop papillomas at 4 weeks from the point of protocol switch (i.e., 21 weeks after initiation). Interestingly, tumor multiplicity (number of papillomas/mouse) kept increasing in wild type whereas it reached an early plateau in transgenic mice (average 3.5 papillomas/mice). At 29 weeks of promotion, tumor multiplicity in the 8*S*-LOX transgenic mice was 64% lower than that of wild type mice. To verify these results, we conducted another independent tumor experiment and similar results were obtained (data not shown). On the other hand, there was no significant difference in tumor incidence or

carcinoma formation between wild type and transgenic mice (Fig. 3.3B and data not shown).

Forced 8S-LOX transgene expression in MT1/2 papilloma cells- To determine the role of 8*S*-HETE in skin tumor cells, an expression vector with 8*S*-LOX cDNA was transfected into MT1/2 murine papilloma cells and the effect of 8*S*-LOX expression on proliferation and differentiation was examined. Of note, although MT1/2 cells form skin papillomas when grafted onto nude mice (Conti et al. 1988), the cells do not express the endogenous 8*S*-LOX gene, similar to that observed in other established murine keratinocyte cell lines. Twelve and 23 individual neomycin resistant clones from the vector control and the 8*S*-LOX transfected MT1/2 cells, respectively, were selected and screened for expression of the 8*S*-LOX message by northern analysis. Five 8*S*-LOX transfected clones expressed various levels of 8*S*-LOX, whereas none of the clones selected from the vector control transfectants expressed 8*S*-LOX (Fig. 3.4A and data not shown). Interestingly, the 8*S*-LOX expressing cells showed a much more flattened cell morphology, which is a phenotypic characteristic of differentiated keratinocytes, compared to the parental MT1/2 or MT1/2-neo cells (Fig. 3.4B). In accordance with this observation, although there was variability depending on the clones, various levels of K1 expression were also detected only in the 8*S*-LOX transfected clones but not in the any of MT1/2-neo clones (Fig. 3.4A and data not shown). Despite these interesting observations, however, we could not proceed

further with these clones because the MT1/2-8*S*-LOX clones started to lose 8*S*-LOX transgene expression as well as their differentiated cell morphology after 2 to 3 passages. We attempted two more times to generate MT1/2-8*S*-LOX stable clones and also tried to generate 8*S*-LOX stable clones in another papilloma cell line, 308, but consistently found a loss of 8*S*-LOX transgene expression after a few passages (data not shown). This suggests that 8*S*-LOX expression in papilloma cells may induce terminal differentiation such that when passaged, only those cells which do not express the transgene are able to proliferate.

Effects of exogenous 8S-HETE on the proliferation of CH72 carcinoma cells- We then wanted to determine whether 8*S*-HETE could inhibit carcinoma cell proliferation as well. CH72 murine squamous carcinoma cells were treated with 1, 5, and 10 μM of 8*S*-HETE and the effect of exogenous 8*S*-HETE on cell proliferation was determined. As shown in Fig. 3.5A, 1 μM of 8*S*-HETE did not significantly affect the proliferation of CH72 cells compared to the vehicle control cells. However, 5 and 10 μM of 8*S*-HETE appeared to inhibit cell proliferation at 48 and 72 h. We confirmed that this 8*S*-HETE-mediated inhibition was not due to increased apoptosis of the cells but rather due to cell cycle arrest at G1 phase by flow cytometry (Fig. 3.5B and C).

Generation of a CH72 carcinoma cell line constitutively expressing 8S-LOX- To assess the possible inhibitory effect of 8*S*-HETE on CH72 carcinoma cell proliferation *in vivo*, we next generated CH72-8*S*-LOX stable clones. Fig.

3.6A shows various levels of 8*S*-LOX mRNA expression in the CH72-8*S*-LOX stable clones and the absence of 8*S*-LOX expression in the CH72-neo clones. We further screened these clones on the basis of 8*S*-LOX protein expression as well as enzyme activity and chose two vector control (CH72-neo #1 and #2) and two 8*S*-LOX expressing clones (CH72-8*S*-LOX #7 and #9) for further studies. The CH72-8*S*-LOX #7 and #9 clones clearly expressed 8*S*-LOX protein (Fig. 3.6B) and converted exogenously added arachidonic acid to 9- and 13-fold more 8*S*-HETE, respectively, than the CH72-neo clones (Fig. 3.6C). We confirmed that the 8*S*-LOX transgene expression in the CH72-8*S*-LOX #7 and #9 clones was maintained at least up to passage 10 (data not shown).

Characterization of CH72-8S-LOX stable clones in vitro- Unlike MT1/2-8*S*-LOX stable clones, CH72-8*S*-LOX clones did not express K1 and also did not exhibit a differentiated cell morphology (data not shown). However, we noticed that CH72-8*S*-LOX #7 and #9 clones looked much more irregular as well as more stressed and in addition, cell proliferation was also poorer than the CH72-neo clones (data not shown). Therefore, a rate of cell proliferation over 5 days between CH72-8*S*-LOX #7 and #9 clones and CH72-neo #2 clones was compared by counting cell number. Interestingly, 8*S*-LOX#7 and #9 clones proliferated similarly to the CH72-neo #2 clone when the cells were in a sub-confluent state. However, the number of cells/dish of the 8*S*-LOX clones was significantly reduced (approximately 30%) compared to the neo clone as the cells became more

confluent (Fig. 3.7A). These data suggest a possibility of contact inhibition or senescence conferred by 8*S*-LOX expression in CH72 cells. Genomic instability in CH72-8*S*-LOX and -neo stable clones was also assessed by inter-SSR PCR, however, no difference was detected between 8*S*-LOX and neo clones (data not shown).

Xenograft studies- CH72-neo #1, -neo #2, -8*S*-LOX #7, and -8*S*-LOX #9 clones were then injected subcutaneously into athymic nude mice and tumor formation was examined for 9 weeks. As shown in Fig. 3.7B, xenografts of all of the clones resulted in tumors. However, the tumors arising from the 8*S*-LOX clones were slower growing (mean doubling time: 71.4 vs. 17.4 days for 8*S*-LOX and neo clones, respectively) and were much smaller (mean volume ± SD: 0.05 ± 0.04 vs. 0.35 ± 0.06 cm^3 for 8*S*-LOX and neo clones, respectively) compared to the tumors arising from the CH72-neo clones. Since slow and poor tumor growth could be due to either increased apoptosis or decreased proliferation of tumor cells, BrdU was injected into all of the mice before sacrifice and tumor sections were immunostained for TUNEL or BrdU. There was no difference in the number of TUNEL positive cells between the tumors derived from either 8*S*-LOX or neo clones (data not shown). However, much lower BrdU incorporation was detected in 8*S*-LOX xenografts compared with the neo xenografts (mean BrdU positive cells ± SD: 23.63 ± 16.88 vs. 111.42 ± 66.73 for 8*S*-LOX and neo clones, respectively). The tumor sections were also stained with antibody against 8*S*-

LOX or K1, however, none of the tumors expressed significant amount of 8*S*-LOX or K1 protein (data not shown). We also compared the genomic instability by inter-SSR PCR of neo- and 8S-LOX xenografts, however, we could not detect any differences between them (data not shown).

Immunohistochemical findings- It has been reported that 8*S*-LOX protein expression is restricted to the differentiated keratinocyte compartment in normal and TPA-treated mouse skin (Jisaka et al. 1997). To localize 8*S*-LOX expression in the course of skin tumor development, tissue sections from normal, TPA-treated skin, papillomas, and carcinomas from wild type C57BL/6J mice were immunostained for 8*S*-LOX, as well as for K1 and the proliferation marker Ki67. Interestingly, proliferating, undifferentiated cells were negative for 8*S*-LOX staining, whereas, differentiated keratinocytes were positive for 8*S*-LOX staining even in the tumors (Fig. 3.8). This observation suggests that 8*S*-LOX is closely associated with keratinocyte differentiation and that loss of 8*S*-LOX expression accompanies loss of differentiation during progression of malignant tumors.

3.5. Discussion

Despite much evidence suggesting a procarcinogenic activity of 8*S*-HETE in mouse keratinocytes (Bürger et al. 1999; Gschwendt et al. 1986; Nair et al. 2000), here we provide strong evidence that 8*S*-HETE is in fact playing an

anticarcinogenic role in mouse skin carcinogenesis, mainly through induction of keratinocyte differentiation. The evidence is; 1) highly differentiated epidermal phenotype and reduced tumor development in 8*S*-LOX/C57BL/6J transgenic mice, 2) induction of K1 as well as a differentiated cell morphology in MT1/2-8*S*-LOX clones, 3) inhibition of CH72 carcinoma cell proliferation conferred by 8*S*-HETE *in vitro* and *in vivo*, and 4) close association of 8*S*-LOX expression with the differentiated epidermal compartment and the absence of 8*S*-LOX expression in proliferating, undifferentiated cells in normal skin as well as in tumors.

C57BL/6J mice develop only mild inflammatory and hyperplastic responses to TPA treatment and are resistant to TPA tumor promotion using the classical twice weekly protocol (Fischer et al. 1988a; Naito and DiGiovanni 1989; Sisskin et al. 1982). Since TPA-induced inflammation as well as hyperplasia are events most closely associated with TPA tumor promotion (Slaga 1984a), the absence of these events after chronic TPA treatment has been thought to be one of the critical reasons for the TPA tumor promotion resistance in C57BL/6J mice. In fact, forced expression of ornithine decarboxylase in C57BL/6J mouse skin induced hyperplasia of the epidermis and converted C57BL/6J from resistant to sensitive to TPA-induced tumor promotion (Guo et al. 1999). On the other hand, since arachidonic acid metabolites have also been shown to play an important role in inflammation as well as hyperplasia in mouse skin (Fischer et al. 1987; Fischer et al. 1982; Nakadate et al. 1982; Verma et al. 1980), the profiles of major COX

and LOX products after TPA treatment were compared between SSIN and C57BL/6J mouse skin. The only significant difference found in the products was 8*S*-HETE, which was highly increased by TPA in SSIN but not in C57BL/6J skin (Fischer et al. 1988a). For this reason, enforced production of 8*S*-HETE in C57BL/6J mouse skin was expected to increase the hyperplastic response and sensitivity to tumor promotion by TPA treatment. However, 8*S*-LOX/C57BL/6J transgenic mice instead exhibited several indications of increased differentiation, including thinner epidermis, faster hair growth and K1 expression in basal as well as suprabasal cells. Significantly, they were even more resistant to TPA tumor promotion. Moreover, the fast regrowth of shaved hair after multiple TPA treatments in the transgenic mice suggests an anti-inflammatory effect of 8*S*-HETE as well, since hair loss has been reported as one of the secondary effects elicited from TPA-induced inflammation (Mahé 1998; Rückert et al. 2000). A number of reports have shown anti-inflammatory properties of PPARα coupled with anti-proliferative and pro-differentiating effects in keratinocytes (Hanley et al. 1998; Kömüves et al. 2000; Sheu et al. 2002; Thuillier et al. 2000). Considering 8*S*-HETE is the most potent endogenous ligand of PPARα (Muga et al. 2000; Yu et al. 1995), it is reasonable to speculate that the observed hair phenotype, increased differentiation, and reduced tumorigenesis in the 8*S*-LOX transgenic mice resulted from a PPARα-mediated effect of 8*S*-HETE in the keratinocytes. Of note is that despite all these anticarcinogenic features in 8*S*-

LOX transgenic mice, basal cell proliferation was not affected. A possible explanation is that the total number of proliferating cells was not affected, since only a subpopulation of basal cells expressed K1 caused by 8*S*-LOX transgene expression.

Considering that established keratinocyte cell lines are not as sensitive as primary keratinocytes to differentiation signals such as Ca^{2+}, induction of K1 as well as the differentiated cell morphology seen in MT1/2-8*S*-LOX stable clones also strongly supports a role of 8*S*-LOX in keratinocyte differentiation. Moreover, the fact that this phenomena was seen only in early passages of transfected clones and thereafter the transgene expression was lost as cells were passaged suggests an inhibitory effect of 8*S*-LOX expression on the MT1/2 cell proliferation as well. In fact, loss of transgene expression was also observed in other papilloma cells that were transfected with K1/K10 (Kartasova et al. 1992), or loricrin genes (Hohl et al. 1991). It therefore appears that expression of differentiation-related genes in papilloma cells is not compatible with mitotic activity of those cells. In the case of 15*S*-LOX-2 (a human homolog of 8*S*-LOX that is involved in differentiation of prostate tissue (Brash et al. 1997; Shappell et al. 1999)), stable clones of prostate carcinoma cells were initially established, however the transgene expression was selectively lost starting with passage 3 (Bhatia et al. 2003). From this point of view we can speculate on the reason why 8*S*-LOX is not expressed or induced, in general, in established murine

keratinocyte cell lines, unlike in primary keratinocytes. It is likely that complete repression of 8*S*-LOX would benefit the growth of continuously proliferating cells.

On the other hand, forced expression of 8*S*-LOX was sustained much longer in CH72 carcinoma cells compared to that in MT1/2 or 308 papilloma cells. This may be partly because carcinoma cells do not respond to differentiating signals, as others have reported. For example, in the case of K1 or K10 stable transfectants, K1 or K10 expression was compatible with mitotic activity of SLC-1 carcinoma cells, although it was not with that of 308 papilloma cells (Kartasova et al. 1992). In fact, we could not observe any indication of keratinocyte differentiation such as K1 expression or differentiated cell morphology in CH72-8*S*-LOX stable clones. Forced expression of 8*S*-LOX, however, may have inhibited cell proliferation through mechanisms other than induction of differentiation. Although we do not know the exact mechanism yet, considering data on the effect of exogenous 8*S*-HETE on cell proliferation, it is tempting to speculate that constitutive production of 8*S*-HETE in CH72-8*S*-LOX stable clones arrested a subpopulation of the cells at G1 phase. It is also possible that 8*S*-HETE induced contact inhibition, since we found fewer cells at confluence with the 8*S*-LOX clones compared to the neo clones. Interestingly, the inhibitory effect of 8*S*-LOX expression on CH72 cell proliferation was much more pronounced in xenografts. The fact that 8*S*-LOX expression was not

detected in the tumors isolated from the 8*S*-LOX clone xenografts suggests a possibility of selection against cells expressing 8*S*-LOX in tumors. In the case of K10 stable transfectants of carcinoma cells, they proliferated in culture while expressing K10, however, the expression was lost in xenografted tumors (Kartasova et al. 1992). Of note is that the tumor suppressing effect of 8*S*-LOX clones apparently depends on enzyme activity, because one of the 8*S*-LOX stable clones that expressed 8*S*-LOX protein but did not have enzyme activity did not show any significant difference in xenograft tumor growth compared to that of neo clones (data not shown).

Observations that the level of 8*S*-LOX expression as well as the increased 8*S*-HETE levels in TPA-induced hyperplastic skin and in reversible papillomas, but not in carcinomas (Nair et al. 2000), have been presented as evidence that 8*S*-HETE is involved in tumor promotion as well as in malignant progression. However, our clear demonstration on the selective expression of 8*S*-LOX in differentiated regions of normal skin and of tumors strongly suggests the level of 8*S*-LOX expression is closely correlated with the extent of differentiation. Interestingly, this pattern of 8*S*-LOX expression is very similar to that of K1/K10 expression during mouse skin carcinogenesis. That is, K1 is also strongly expressed in TPA-induced hyperplastic skin and papillomas (Huitfeldt et al. 1991; Nelson and Slaga 1982; Toftgard et al. 1985); however the expression is reduced during progression of papillomas toward malignancy (Nischt et al. 1988) and is

absent in squamous cell carcinomas (Yuspa 1985). Of importance is that not only does 8*S*-LOX expression accompany keratinocyte differentiation but also 8*S*-LOX expression actively participates in inducing keratinocyte differentiation and in inhibiting skin tumorigenesis. Previously reported strong induction of K1 by exogenous 8*S*-HETE treatment of primary keratinocytes supports this active participation of 8*S*-LOX in keratinocyte differentiation (Muga et al. 2000). Conversely, knocking out protein kinase Ceta (PKCeta), which has been shown to be involved in keratinocyte differentiation, resulted in higher sensitivity to skin carcinogenesis than wild type (Kashiwagi et al. 2002). In addition, several *in vitro* data have also shown that suppression of malignancy in tumor- and normal-cell hybrids resulted from restoration of the terminal differentiation program in tumor cells that was imposed by the normal cell partner (Stanbridge et al. 1982). From this point of view, although we can not completely exclude a possibility of other physiological changes resulting from 8*S*-LOX overexpression in C57BL/6J mouse skin or in tumor cells, most likely, increased differentiation conferred by 8*S*-HETE mediates the anticarcinogenic effects of forced 8*S*-LOX expression.

In summary, our results demonstrate that the expression of 8*S*-LOX is closely associated with keratinocyte differentiation and the forced expression inhibits mouse skin tumorigenesis possibly through its ability to induce keratinocyte differentiation. This finding provides not only documentation on the anticarcinogenic role of 8*S*-HETE in tumor development but also the possibility

of the application of 8*S*-HETE to other human diseases. For example, it could be applied to human skin diseases such as psoriasis or hyperkeratosis that result from an imbalance between proliferation and differentiation of keratinocytes. Therefore, more profound mechanistic studies in the future on the molecular function of 8*S*-HETE in inducing keratinocyte differentiation as well as inhibiting tumorigenesis would be expected to provide significant rationale for targeting 8*S*-LOX/8*S*-HETE to treat or to prevent certain human diseases including cancer.

Table 3. 1. Production of 8*S*-HETE and 9*S*-HODE in wild type and 8*S*-LOX transgenic mouse skin.

Pulverized skin from wild type and 8*S*-LOX/C57BL/6J transgenic mice was homogenized and thereafter subjected to organic extraction. The metabolites were analyzed by LC/MS/MS as described in Materials and Methods. The level of eicosanoids was normalized per mg of protein. Data were obtained from two separate experiments using 2 mice in each group and the values are mean ± S.D.

Mouse	8*S*-HETE *(μg/mg protein)*	9*S*-HODE *(μg/mg protein)*
WT	2.66 ± 0.26	114.23 ± 4.47
8*S*-LOX/C57BL/6J	8.40 ± 0.75[a]	101.23 ± 16.32

[a] $p < 0.01$ (Student t-test) vs. WT

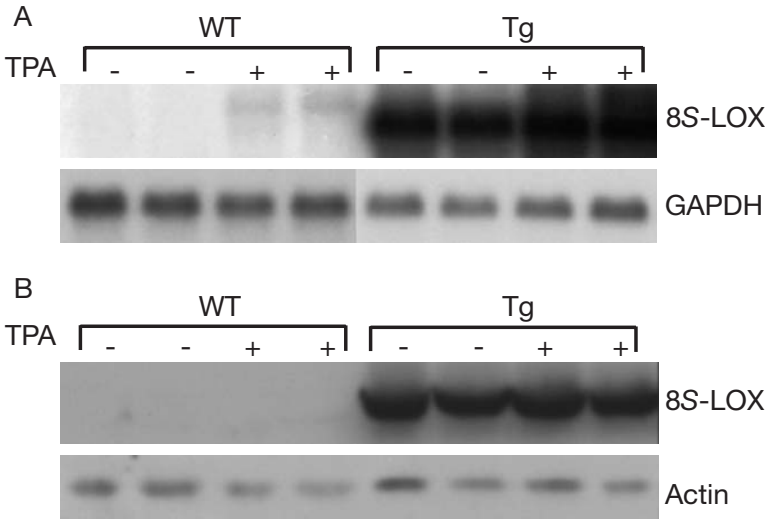


Fig. 3. 1. Transgene expression in 8*S*-LOX/C57BL/6J transgenic mice.

(A) Northern blot of total RNA (10 μg) isolated from wild type and 8*S*-LOX transgenic mouse skin treated for 9 h with acetone (-) or TPA (4 μg; +). The blot was hybridized with a radiolabeled 8*S*-LOX cDNA probe, stripped, and re-hybridized with a radiolabeled GAPDH cDNA probe to control for loading. Each lane represents RNA from individual mice. (B) Western blot of total protein (30 μg) isolated from wild type and 8*S*-LOX transgenic mouse skin treated for 9 h with acetone or TPA (4 μg). The blot was hybridized with antisera against 8*S*-LOX, stripped, and re-hybridized with a β-actin antibody to control for loading.

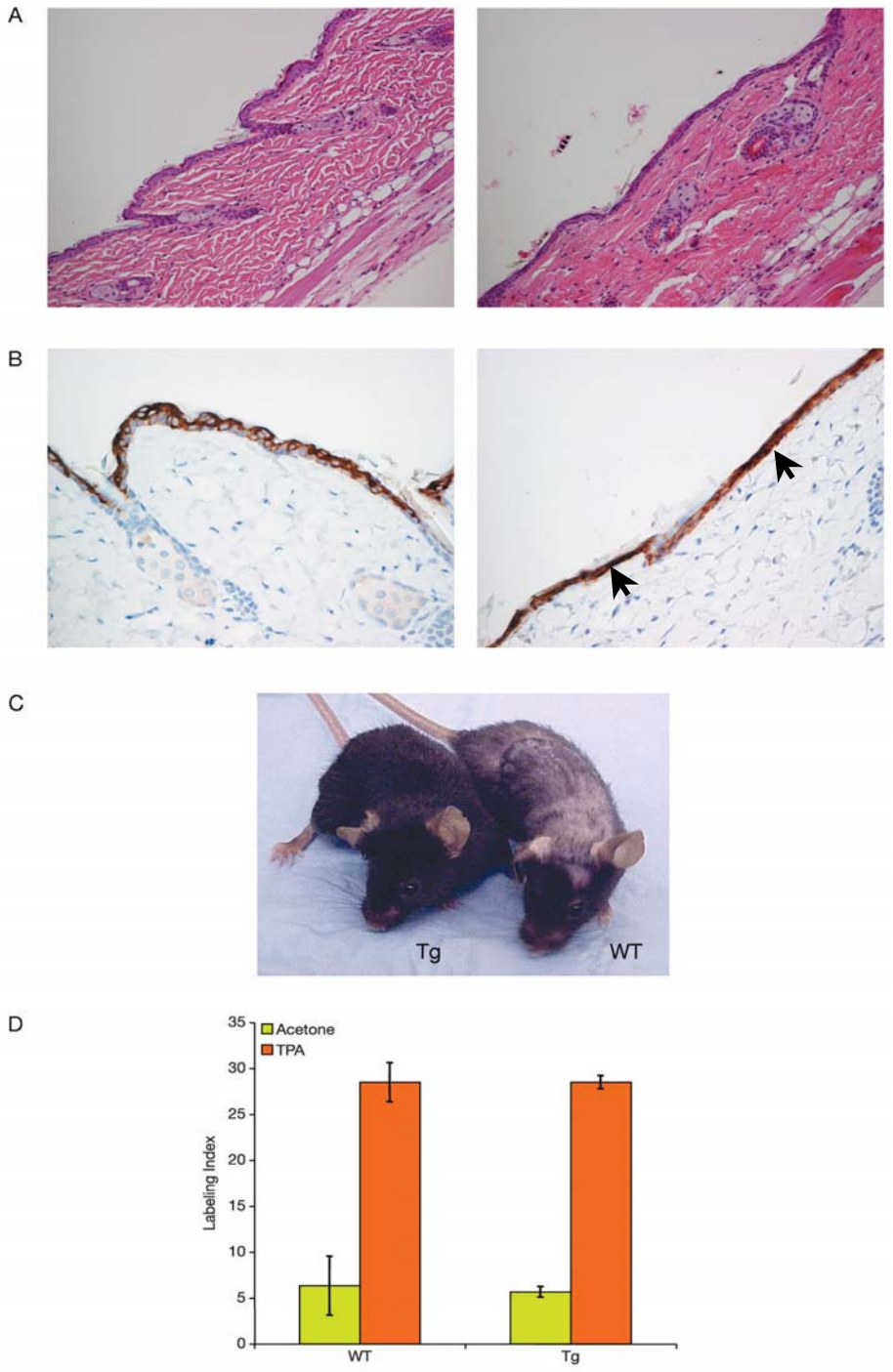


Fig. 3. 2.

Fig. 3. 2. Characterization of 8*S*-LOX/C57BL/6J transgenic mice.

(A) Histological analysis of wild type and 8*S*-LOX transgenic mouse skin. Dorsal skin of wild type (left) and 8*S*-LOX transgenic mice (right) was removed and fixed in formalin. Tissue sections were stained with H&E and photographed at 200X. (B) Serial sections were immunostained with K1 and photographed at 400 X. Note stronger K1 staining in suprabasal cells of wild type mice and aberrant K1 staining in basal cells (arrow head) of transgenic mice. (C) Photograph of wild type and 8*S*-LOX transgenic mouse in the middle of two-stage skin carcinogenesis protocol. The dorsal skins of the mice were shaved and subjected to DMBA initiation and TPA promotion as described in Materials and Methods. The picture was taken after 8 weeks of twice a week TPA (4 μg) treatment. Note the hair re-growth in the 8*S*-LOX transgenic (Tg) mice, but not on wild type (WT) mice. (D) Labeling index of wild type and 8*S*-LOX transgenic mice. The dorsal skins of the mice were shaved and treated with either acetone or TPA (4 μg) for 24 h. BrdU was injected i.p. 1 h before sacrifice and dorsal skin was removed and fixed in formalin. Tissue sections were immunostained with an antibody against BrdU and BrdU positive cells were counted. The labeling index represents the percentage of BrdU positive cells relative to the total number of basal cells in the interfollicular epidermis. Each value is the mean ± S.D. of labeling indices from at least 3 mice/group.

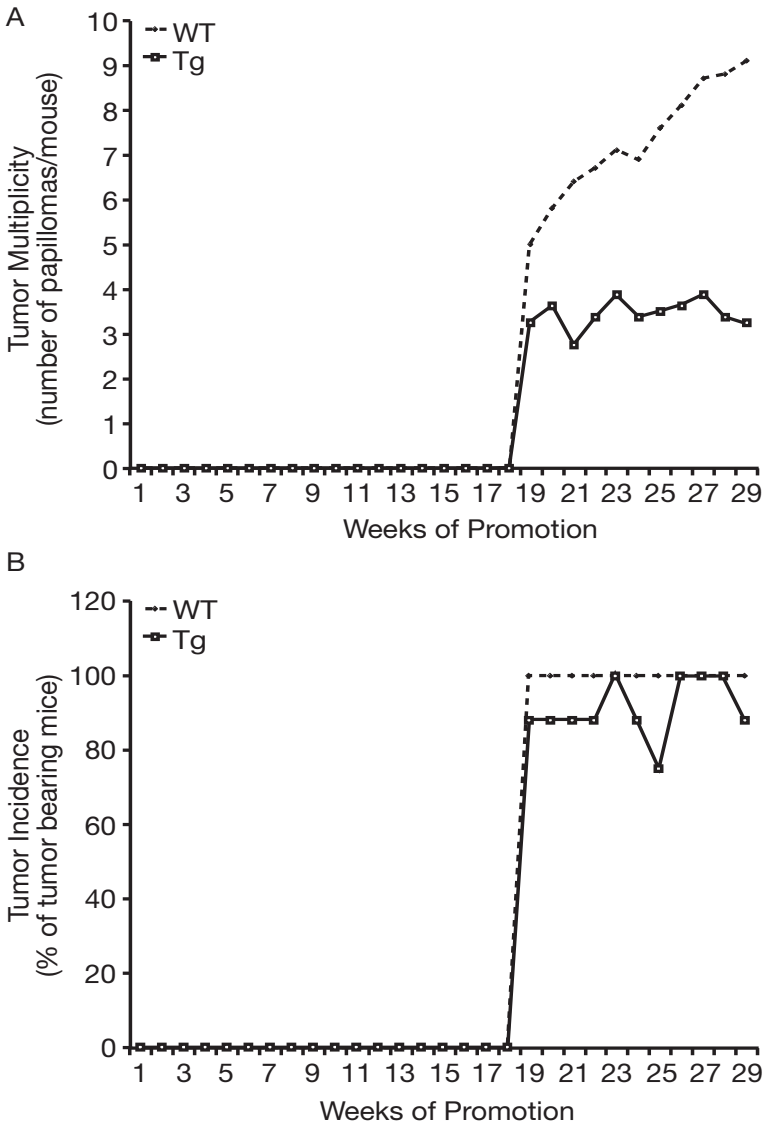


Fig. 3. 3. Two-stage skin carcinogenesis study.

Twenty female mice each of wild type and 8*S*-LOX/C57BL/6J transgenic were initiated with 200 nmol (50 μg) DMBA and promoted with 6.5 nmol (4 μg) of TPA in 200 μl of acetone twice a week for 15 weeks. The mice were then treated with same dose of TPA five times a week for another 14 weeks. (A) Tumor multiplicity (average number of papillomas/mice) and (B) incidence of tumors (percentage of mice bearing tumors) were recorded weekly.

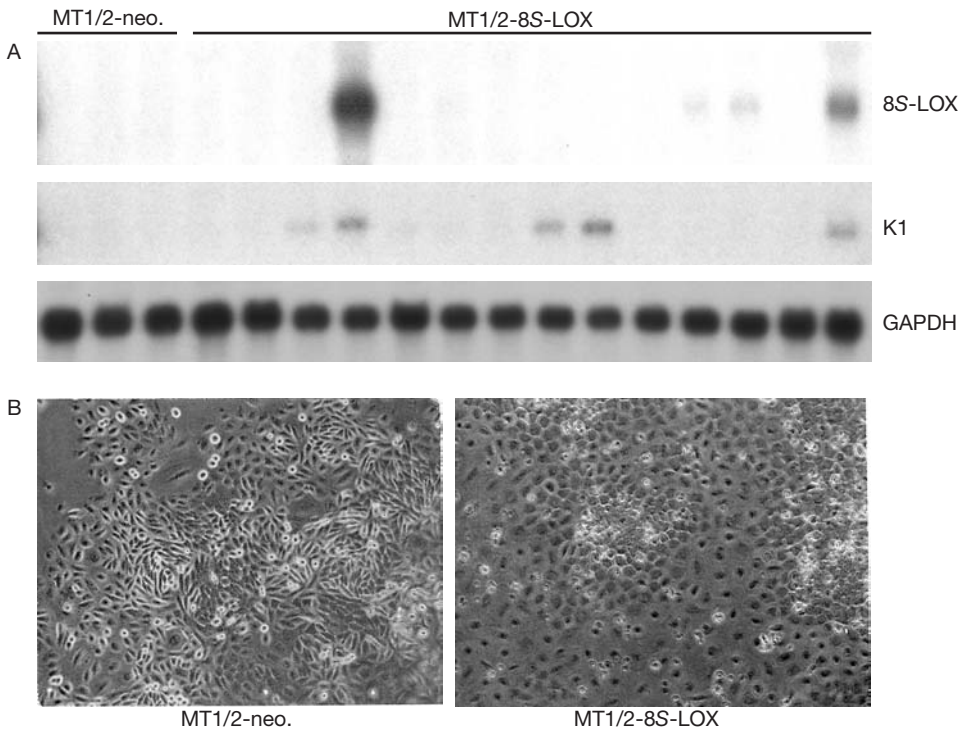


Fig. 3. 4. Generation and characterization of MT1/2-8*S*-LOX stable clones.

(A) Northern blot of total RNA (10 μg) isolated from G418-resistant clones of MT1/2 cells transfected with pCIneo or pCIneo-8*S*-LOX expression vector. The blot was hybridized with a radiolabeled 8*S*-LOX cDNA probe. After stripping, the same blot was hybridized with a radiolabeled K1 cDNA probe and thereafter re-hybridized with a radiolabeled GAPDH cDNA probe to control for loading. (B) Phase contrast photographs of representitive MT1/2-pCIneo or MT1/2-8*S*-LOX stable clones in culture. Note the more flattened cell morphology in MT1/2-8*S*-LOX clones.

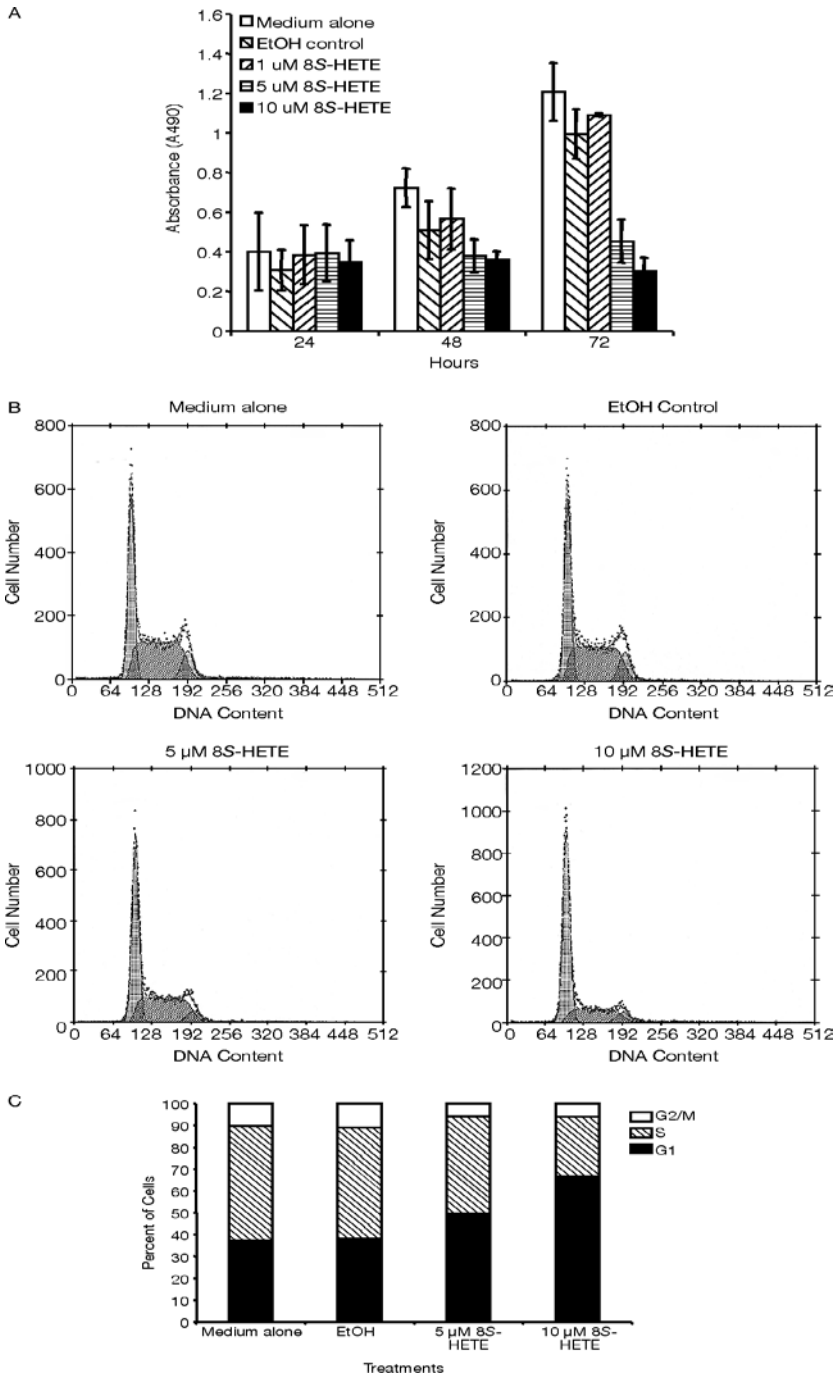

A
Medium alone
EtOH control
1 uM 8S-HETE
5 uM 8S-HETE
10 uM 8S-HETE
Absorbance (A490)
Hours
B
Medium alone
EtOH Control
5 µM 8S-HETE
10 µM 8S-HETE
Cell Number
DNA Content
C
G2/M
S
G1
Percent of Cells
Medium alone
EtOH
5 µM 8S-HETE
10 µM 8S-HETE
Treatments

Fig. 3. 5.

Fig. 3. 5. Effects of exogenous 8*S*-HETE on the proliferation of CH72 carcinoma cells.

(A) Cell proliferation assay. CH72 cells plated in 96-well plates (8×10^3 cells/well) were fed with fresh media containing 0.3% ethanol (vehicle), 1, 5, or 10 μM of 8*S*-HETE 24 h after plating. The cell number of viable cells was determined every 24 h as described in Materials and Methods. The data are the mean ± S.D. of at least three independent experiments each done in triplicate. (B) Cell cycle analysis. CH72 cells were plated in 60 mm dishes (7×10^5 cells/dish) and fed with fresh media containing 0.1% ethanol (vehicle), 5, or 10 μM of 8*S*-HETE at approximately 80% cell confluency. After 24 h of treatment, cells were trypsinized, fixed in ethanol, stained with propidium iodide, and subjected to flow cytometry. The data are representative of three independent experiments with similar results. (C) Quantification of the cell cycle analysis shown in (B).

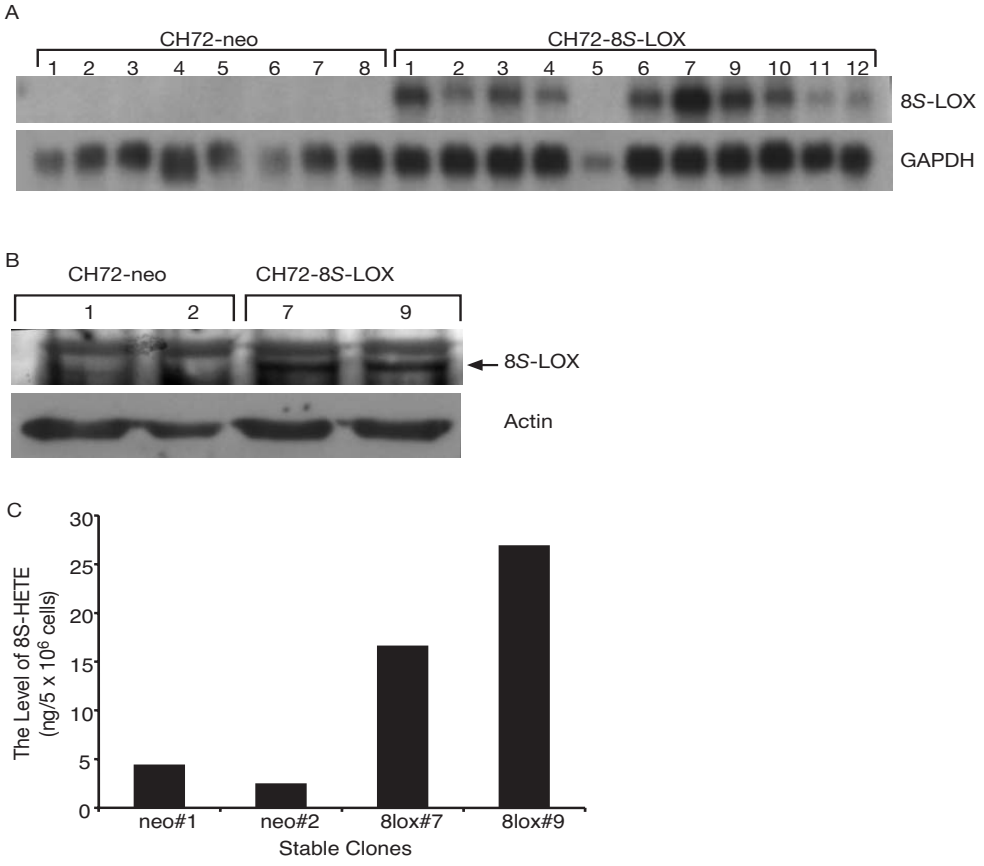


Fig. 3. 6. 8*S*-LOX transgene expression and activity in CH72-8*S*-LOX stable clones.

(A) Northern blot of total RNA (10 μg) isolated from pooled clones of CH72 cells stably transfected with pCIneo or pCIneo-8*S*-LOX expression vector. The blot was hybridized with radiolabeled 8*S*-LOX cDNA probe, stripped, and re-hybridized with radiolabeled GAPDH cDNA probe to control for loading. (B) Western blot of total protein (30 μg) isolated from CH72-pCIneo (#1 and #2) or CH72-8*S*-LOX (#7 and #9) clones (lanes 1, 2, 3, and 4, respectively). The blot was hybridized with antisera against 8*S*-LOX, stripped, and re-hybridized with a β-actin antibody to control for loading. (C) Production of 8*S*-HETE in stably transfected CH72 cells. Cells in log phase were treated with 50 μM of AA for 30 min and the media was collected. The metabolites were analyzed by LC/MS/MS as described in Materials and Methods. The level of eicosanoids was normalized to total cell number. The data are representative of three independent experiments with similar results.

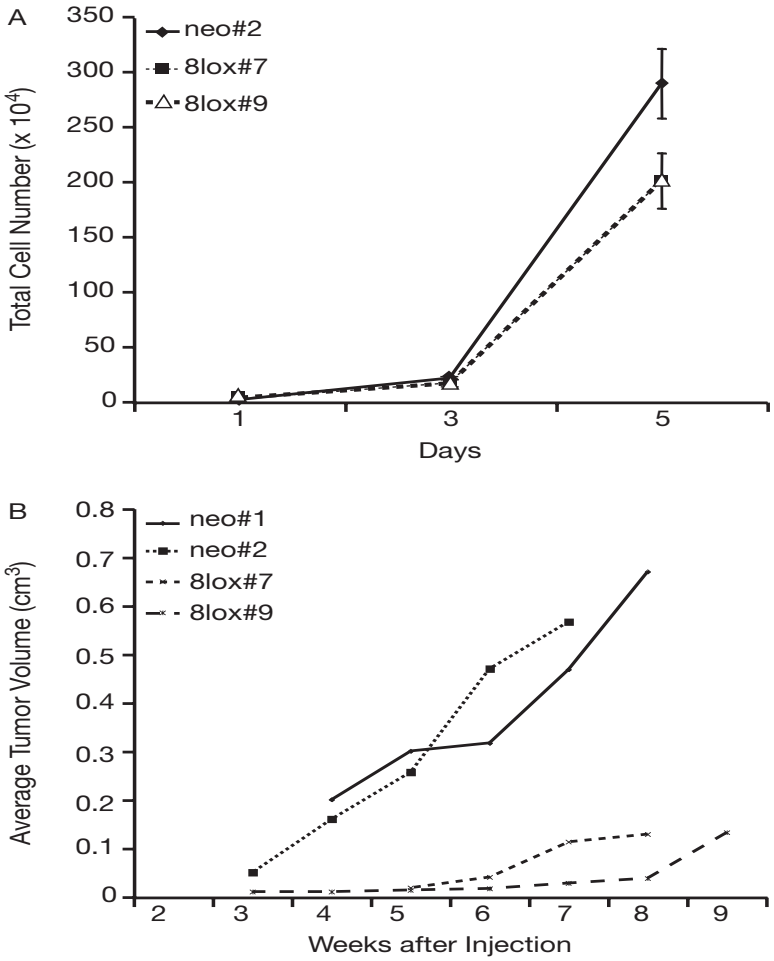


Fig. 3. 7. Proliferation of CH72-8S-LOX stable clones *in vitro* and *in vivo*.

(A) *In vitro* proliferation of CH72-neo and CH72-8S-LOX stable clones. Cells were plated at 5×10^4 cells/60 mm dish and media changed every two days. At day 1, 3, and 5 after plating, the cells were trypsinized and the total cell number was calculated by hemacytometer. The data are the mean ± S.D. of at least three independent experiments each done in triplicate. (B) *In vivo* growth of xenograft tumors. Cultured CH72-8*S*-LOX (#7 and #9) and CH72-neo (#1 and #2) stable clones (5×10^6 cells/site) were subcutaneously injected into four different sites (two shoulders and two flanks) of 2 female athymic nude mice each. Tumor diameters (length and width) were measured once a week and tumor volume was estimated as 1/2 (length × width^2). The data are representative of two independent experiments.

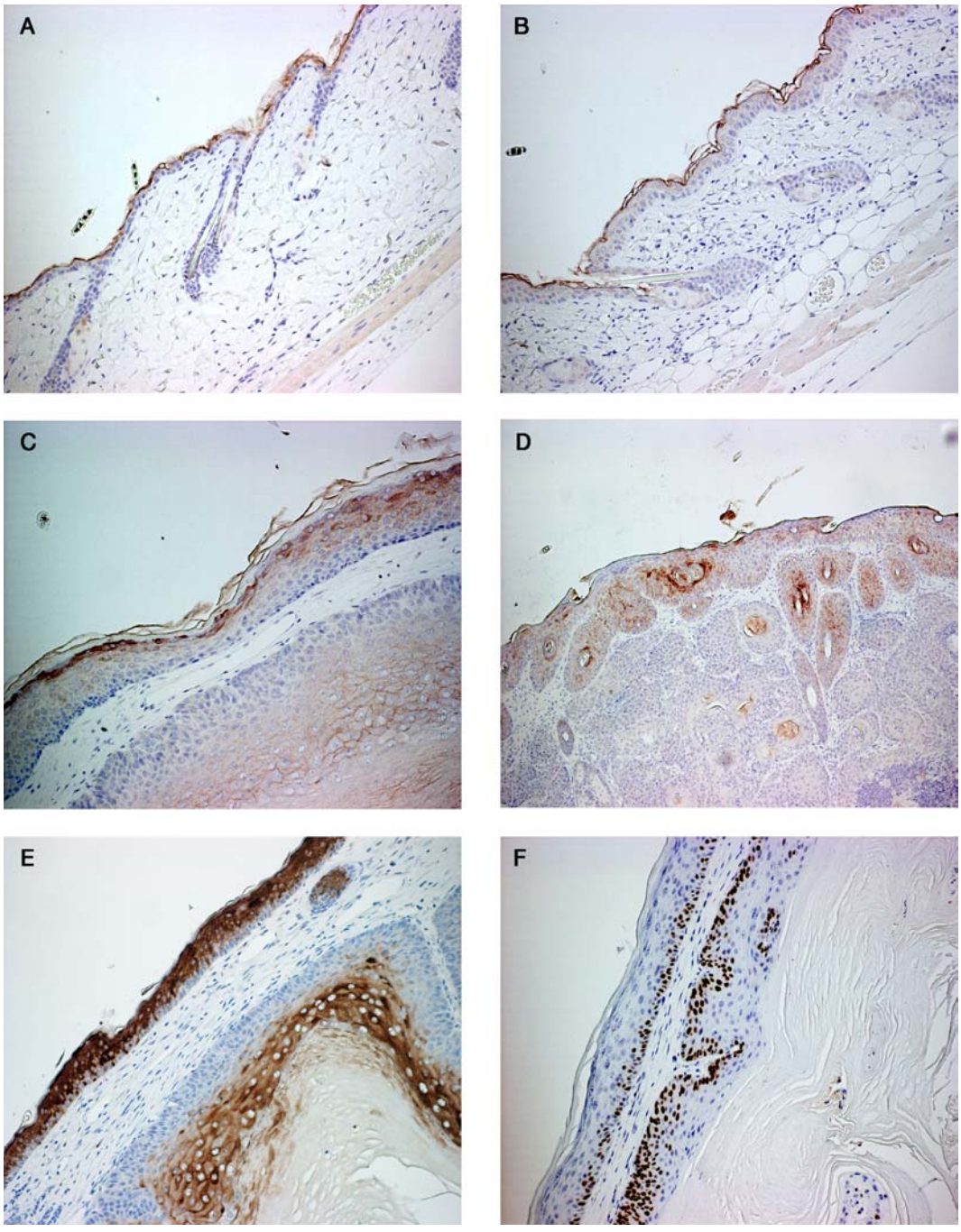


Fig. 3. 8.

Fig. 3. 8. 8*S*-LOX expression in mouse skin and in skin tumors.

Tissue sections of (A) normal skin, (B) TPA-treated skin (4 μg of TPA for 24 h treatment) and (C) a papilloma from wild type C57BL/6J mouse and (D) a carcinoma from FVB mouse were immunostained with antisera against 8*S*-LOX. Since none of the C57BL/6J mice developed carcinomas in our two-stage carcinogenesis protocol, carcinomas from similar skin carcinogenesis protocol in FVB mice were used for this experiment. Serial sections from the same papilloma as in (C) were also immunostained with antibodies against (E) K1 (a marker for differentiation) and (F) Ki67 (a marker for proliferation). Sections were photographed at X 200. Data are representative of at least three independent tissues.

CHAPTER IV

SUMMARY, DISCUSSION AND FUTURE DIRECTIONS

4.1. Summary

Mouse skin has served as an excellent model to study the multistage nature of the development of various human cancers. As has been seen in a number of human cancers, excessive arachidonic acid (AA) metabolism has also been observed during mouse skin carcinogenesis and moreover, skin tumor development can be effectively inhibited by inhibitors of various parts of the AA cascade (Fischer et al. 1987; Fischer et al. 1982; Kato et al. 1983; Nakadate et al. 1982).

8*S*-lipoxygenase (8*S*-LOX) is a recently cloned AA metabolizing enzyme, which is specifically highly expressed during mouse skin carcinogenesis (Bürger et al. 1999; Fürstenberger et al. 1991; Gschwendt et al. 1986; Jisaka et al. 1997). That is, it is not expressed at a detectable level in normal skin, however, it is highly induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment of the skin and is constitutively expressed in early stage papillomas. This is a very unique feature of expression compared to other LOXs present in mouse skin.

Several studies have suggested possible roles of 8*S*-hydroxyeicosatetraenoic acid (8*S*-HETE), an AA metabolite of 8*S*-LOX, in mouse skin tumor development. These include the ability of 8*S*-HETE to induce chromosomal damage (Bürger et al. 1999) and/or to induce keratinocyte differentiation (Muga et al. 2000). Despite these interesting observations, however, questions concerning how 8*S*-LOX expression is regulated and what the functional role of 8*S*-LOX/8*S*-HETE is during mouse skin carcinogenesis had not been addressed.

In the first study, we studied a molecular mechanism by which TPA induced 8*S*-LOX expression in SSIN primary keratinocytes. The level of 8*S*-LOX mRNA was highly increased as soon as 3 h after TPA treatment of these cells and the TPA-induced mRNA expression was inhibited by a transcription inhibitor, actinomycin D. We therefore further studied the transcriptional regulation of 8*S*-LOX expression by cloning its promoter. Approximately 2 kb of the cloned promoter does not have a TATA box or a CCAAT box and a transcription initiation site was mapped to -27 bp from the ATG translation start site. The promoter was highly responsive to TPA in TPA promotion-sensitive SSIN but not in promotion-resistant C57BL/6J primary keratinocytes. A TPA responsive element (TRE) of the promoter was identified as a Sp1 binding site, located -77 to -68 from the ATG, and Sp1, Sp2, and Sp3 proteins were identified as the proteins that bind to the TRE. Since the binding of these proteins to the TRE was significantly increased by TPA treatment and inhibition of the binding decreased

TPA-induced promoter activity as well as 8*S*-LOX mRNA expression, we concluded that increased binding of Sp1, Sp2, and Sp3 to the TRE of the 8*S*-LOX promoter is a mechanism through which 8*S*-LOX expression is regulated by TPA in keratinocytes.

In the second study, we investigated the functional role of 8*S*-LOX/8*S*-HETE in mouse skin carcinogenesis using a series of gain-of-function studies. Targeted C57BL/6J transgenic mice overexpressing the 8*S*-LOX gene under control of the loricrin promoter showed a more differentiated epidermal phenotype as well as reduced tumor development compared to wild type mice in a two-stage skin carcinogenesis protocol. Forced expression of the 8*S*-LOX gene in MT1/2, a murine papilloma cell line, caused a more differentiated cell morphology as well as K1 expression. Overexpression of 8*S*-LOX in CH72, a murine carcinoma cell line, did not affect cell differentiation, but, inhibited cell proliferation by 30% *in vitro* and by 86% in *in vivo* xenografts. Moreover, exogenous addition of 5 μM 8*S*-HETE to CH72 cells also significantly inhibited cell proliferation and caused cell cycle arrest at G1 phase. From immuno-histochemical analyses, we also found 8*S*-LOX expression was strictly limited to the differentiated compartment of skin epidermis even in the process of skin tumor development. Collectively these data suggest that 8*S*-LOX is closely associated with keratinocyte differentiation and that enforced expression of 8*S*-

LOX can inhibit mouse skin tumorigenesis possibly through its ability to induce keratinocyte differentiation.

4.2. Discussion and Future Directions

Since the entire human genome sequence has been unveiled, it is likely that many genes found in non-human species are receiving less attention. 8*S*-LOX is not an exception. Although the synthesis of 8-HETE was identified in a few human tissues and cancers, it appears that 8*S*-LOX is a murine specific gene, which discourages active research on it. Nevertheless, several strong reasons for persuing additional studies are described below:

The first is the regulation of gene expression. There are several pieces of evidence showing that diverse LOX family members originated from the same ancestral gene, including similar chromosomal localization, gene organization, and regulation (Fürstenberger et al. 2002; Hoshiko et al. 1990; Liu et al. 1997). For this reason, understanding a particular LOX would be expected to greatly enhance our understanding of other LOX family members as well. In the present study, we demonstrated that the basal and TPA-induced 8*S*-LOX gene expression in keratinocytes was transcriptionally regulated by binding of the Sp1 transcription factor family to a Sp1 binding site in its promoter. In fact, Sp1 and Sp1 binding sites have been reported as critical trans- and cis-acting factors for

the regulation of other LOX genes as well (Chen and Chang 2000; Hoshiko et al. 1990; Liu et al. 1997; Silverman et al. 2002). Beyond this point, however, the identity of the signal pathway responsible for the alteration of DNA binding activity, protein synthesis, or transactivation activity of Sp1 in the regulation of LOX gene expression has not been studied in depth. In the case of 8*S*-LOX, increased Sp1, Sp2, and Sp3 binding to a Sp1 binding site in the 8*S*-LOX promoter is proposed as a mechanism through which 8*S*-LOX gene expression is regulated by TPA. Therefore, our future studies will be directed toward identifying the upstream signal transduction pathways that lead to increased binding of those factors to the TRE of the 8*S*-LOX promoter. One of the first things that we can test is the phosphorylation status of Sp1 factors. Recent reports have shown that PKC-mediated Sp1 phosphorylation increases Sp1 binding to a Sp1 binding site (Pal et al. 1998; Pal et al. 2001; Zheng et al. 2000). Since PKC is a cellular receptor for TPA, it is possible that phosphorylation of Sp1, Sp2, and Sp3 following the activation of PKC may enhance their DNA binding activity to the 8*S*-LOX promoter. On the other hand, we may also need to consider a possibility of PKC-independent post-translational activation of Sp1. According to the reports of Torgeman et al., stimulation of Sp1 binding was mediated by formation of a Sp1-p53 protein complex following TPA treatment (Torgeman et al. 2001) in a PKC-independent way (Torgeman et al. 1999). This observation suggests TPA may modulate DNA binding activity of Sp1 by regulating its

interaction with other transcription factors or co-factors. The other interesting area that we want to further investigate in the future is to elucidate a mechanistic basis for the very low level of 8*S*-LOX gene transcription in C57BL/6J primary keratinocytes. C57BL/6J mice are known as a TPA tumor promotion-resistant strain and they do not develop many tumors in a classical two-stage skin carcinogenesis protocol (Fischer et al. 1989). Many researchers have extensively studied C57BL/6J mice from genetic, molecular biologic, and biochemical points of view to find critical genetic or cellular components that are responsible for their resistance to TPA tumor promotion (DiGiovanni et al. 1993; Fischer et al. 1988a; Fischer et al. 1989; Naito et al. 1988; Yamamoto et al. 1988). Despite these efforts, however, the critical components have not yet been identified. In the current and previous studies, we have observed that 8*S*-LOX is highly induced by TPA in TPA tumor promotion-sensitive strains of mice, but not in C57BL/6J. This is one of the most unique phenomena observed between promotion-sensitive and -resistant strains of mice. Therefore, to identify cis-, trans-acting factors, or upstream signal transduction pathways that are responsible for the TPA-induced 8*S*-LOX expression in C57BL/6J primary keratinocytes and to compare them with those of SSIN primary keratinocytes may provide a clue in the search for those critical components. Collectively, we expect that pursuit of these studies in the future may well provide significant insight into how TPA promotes tumorigenesis on a global level as well.

The homology of 8*S*-LOX with human 15*S*-LOX-2 is the second reason for continued studies on 8*S*-LOX. Although 8*S*-LOX is not encoded by the human genome, it shares 78% identity in amino acid composition with human 15*S*-LOX-2 (Jisaka et al. 1997). Moreover, both of them use arachidonic acid as a preferred substrate and produce 8*S*-HPETE and 15*S*-HPETE, respectively. Despite these structural and catalytic similarities, however, their tissue and cellular localization are quite distinctive from each other. That is, the expression of 15*S*-LOX-2 was identified in human prostate, lung, cornea, skin, and brain, whereas the expression of 8*S*-LOX has been reported only in mouse skin and brain (Brash et al. 1997; Jisaka et al. 1997). Moreover, in skin, 8*S*-LOX was detected in differentiated keratinocytes and in hair follicles, whereas 15*S*-LOX-2 was detected in the basal layer of the epidermis, sebaceous glands, and other adnexa (Shappell et al. 2001b). Based on these observations, it had been thought that they are not functional homologs but just structural homologs of each other. However, recently reported studies suggest that they may share functional roles as well. Although 15*S*-LOX-2 is not generally expressed in differentiated keratinocytes of epidermis, strong immunostaining for 15*S*-LOX-2 was observed in differentiated secretory sebocytes (Shappell et al. 2001b). Considering that 15*S*-HETE is a ligand of PPARγ, which is involved in adipocyte differentiation (Huang et al. 1999; Shappell et al. 2001a), both the 8*S*-LOX and 15*S*-LOX-2 gene products appear to be involved in tissue differentiation through PPARs. That is,

8*S*-HETE causes keratinocyte differentiation through PPARα and 15*S*-HETE contributes to sebocyte differentiation through PPARγ. In prostate, 15*S*-LOX-2 was specifically expressed in the glandular prostate epithelial cells *in vivo* but not in basal cells or other cell types including stromal cells (Bhatia et al. 2003). More interestingly, the protein level and enzymatic activity of 15*S*-LOX-2 have been shown to be downregulated in prostate cancers compared with normal and benign prostate tumors (Jack et al. 2000; Shappell et al. 1999). Moreover, exogenous 15*S*-HETE treatment or restoration of 15*S*-LOX-2 expression in prostate cancer cells inhibits cell proliferation *in vitro* and *in vivo* (Bhatia et al. 2003; Tang et al. 2002). These observations are very similar to what we observed with 8*S*-LOX in mouse skin and skin cancer cells in our second study. Again, although the tissues in which of 8*S*-LOX and 15*S*-LOX-2 are expressed are different, both genes appear to function as a suppressor of specific tumor development. Considering this suggested functional similarity, further investigation on the functional role of 8*S*-LOX in keratinocyte differentiation as well as in skin tumor inhibition may increase our understanding on the functional role of human 15*S*-LOX-2. Such investigations will include: 1) Generation of 8*S*-LOX knockout mice in promotion-sensitive mice to clarify the role of 8*S*-LOX in mouse skin; 2) Generation of a bitransgenic C57BL/6J mice in which 8*S*-LOX is targeted to the skin of PPARα null animals to determine whether the observed effects of 8*S*-LOX overexpression in C57BL/6J mice were PPARα mediated ones; 3) Identify

downstream targets of 8*S*-HETE-mediated cell cycle inhibition in carcinoma cells, and 4) Generation of targeted 8*S*-LOX transgenic mice under the control of K6 promoter, which is known to be constitutively upregulated in mouse skin tumors to confirm prodifferentiating, anticarcinogenic property of 8*S*-LOX.

In conclusion, here we studied the regulation and the function of 8S-LOX in mouse skin carcinogenesis. Considering its promising contributions to understanding other LOX family members, the mechanisms of tumorigenesis, as well as differentiation, we expect that a more through understanding of the regulation and function of 8*S*-LOX in the future could lead to novel approaches to the prevention or treatment of many human diseases including cancers.

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