NOTE TO USERS

Isolation and characterization of RA-target genes from keratinocytes potentially involved in epithelial tumorigenesis

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ABSTRACT

Retinoic acid (RA), the principal biologically active retinoid, is essential for normal differentiation of a wide variety of cell types. Vitamin A deficiency predisposes to certain cancers and it has since been discovered that retinoids display antitumorigenic activity in many models. Keratinocytes with a null mutation in the RARγ and RARαγ genes are predisposed to tumorigenesis *in vivo*. This outcome is reversed by the re-introduction of a functional RAR, suggesting that some retinoid-target gene(s) are implicated in tumour formation in this model. Suppressive subtractive hybridization techniques have been used to isolate RA-responsive genes no longer regulated in RAR-null keratinocytes. This work led to the cloning of *gas3*, a member of the growth arrest-specific gene family. Our studies have demonstrated that *gas3* expression is regulated by RA and is greatly reduced following tumor promotion, indicating that this gene could potentially inhibit epidermal tumorigenesis elicited by retinoids.

SUMMARY

L'acide rétinoïque (AR), la principale forme biologiquement active de la vitamine A, est essentielle à la différentiation de plusieurs types cellulaires. La déficience à la vitamine A cause certains cancers et depuis, il a été découvert que les rétinoides ont un effet antitumorigénique dans plusieurs modèles. Ils ont aussi le potentiel de guérir quelques cancers épithéliaux, incluant le carcinome de la peau. L'injection de keratinocytes homozygote pour une mutation nulle pour les récepteurs RARγ et RARαγ dans des souris nudes augmente l'incidence de la tumorigénège *in vivo*. Cet effet est inversé par la ré-introduction d'un RAR fonctionnel, ce qui suggère que certains gènes cibles sont impliqués dans la formation de tumeurs dans ce modèle. Des techniques de soustraction par hybridation, en utilisant des kératinocytes déficients en RARαγ qui ne répondent plus à l'AR, ont été employées afin d'identifier des gènes cibles de l'AR. Ces études ont menées au clonage de *gas3*, codant pour une protéine de la famille des gènes impliqués dans l'arrêt de la croissance cellulaire. Nos études ont démontrés que *gas3* est régulé par l'AR et que son expression est fortement réduite suite à la promotion de tumeurs, indiquant que ce gène aurait un rôle potentiel dans l'inhibition de la tumorigénège élicitée par les rétinoides dans l'épiderme.

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ABBREVIATIONS

AD:	Activation domain
ADH:	Alcohol dehydrogenase
AF-1:	Activation function-1
AF-2:	Activation function-2
ALDH:	Aldehyde dehydrogenase
AK:	Actinic keratoses
AP-1:	Activating protein-1
BCC:	Basal cell carcinoma
BM:	Basement membrane
CBP:	CREB-binding protein
Cdk:	Cyclin dependent kinase
CEL:	Cholesteryl ester hydrolase
CRABP:	Cellular retinoic-acid binding protein
CRBP:	Cellular retinoid binding protein
CHX:	Cycloheximide
DBD:	DNA binding domain
DEJ:	Dermal-epidermal junction
DMBA:	7,12-dimethylbenz[a]anthracene
DMSO:	Dimethyl sulfoxide
dnRAR:	dominant negative RAR
DR:	Direct repeat
DRIP:	vitamin D receptor-interacting protein
ECM:	Extracellular matrix
EGFR:	Epidermal growth factor receptor
ER:	Estrogen receptor
Gas3:	Growth arrest-specific gene 3
GR:	Glucocorticoid receptor
HAT:	Histone acetyltransferase
HDAC:	Histone deacetyltransferase
4-HPR:	N-(4-hydroxyphenyl) retinamide
HRE:	Hormone response element
ID:	Interacting domain
IKK:	I kappa kinase
IRS:	Inner root sheat
LBD:	Ligand-binding domain
MAPK:	Mitogen activated protein kinase
MRGX:	MORF-related factor X
N-CoR:	Nuclear receptor corepressor
NF-κB:	Nuclear factor-κB
NR:	Nuclear receptor
ODC:	Orthinine decarboxylase
ORS:	Outer root sheat
PKA:	Protein kinase A
PKC:	Protein kinase C

PMP22:	Peripheral myelin protein 22
PPAR:	Peroxisome proliferator-activated receptor
RA:	Retinoic acid
RAL:	Retinaldehyde
RALDH:	Retinaldehyde dehydrogenase
RAR:	Retinoic acid receptor
RARE:	Retinoic acid response element
RBP:	Retinol binding protein
RD:	Repression domain
REH:	Retinyl ester hydrolase
RID:	Receptor interacting domain
RoDH:	Retinol dehydrogenase
ROL:	Retinol
RXR:	Retinoid X receptor
SCC:	Squamous cell carcinoma
SDR:	Short-chain alcohol dehydrogenase/reductase
SMRT:	Silencing mediator for retinoic acid and thyroid hormone receptors
SRC:	Steroid receptor coactivator
SRRP:	Small proline-rich region protein
Tgase:	Transglutaminase
TGFβ:	Transforming growth factor β
TR:	Thyroid hormone receptor
TRAP:	Thyroid hormone receptor-associated protein
TRE:	Thyroid receptor response element
TTR:	Transthyretin
VAD:	Vitamin A deficiency
VDR:	Vitamin D_3 receptor

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Chapter 1

Literature Review

1.1. Vitamin A metabolism

1.1.1. Vitamin A uptake, storage and metabolism

All vitamin A in the body is acquired from the diet either as preformed vitamin A (retinyl esters) or provitamin A carotenoids (β-carotene) (Blaner and Olson, 1994; Napoli, 1996; Vogel *et al.*, 1999). Animal fats and fish liver oils are major sources of retinyl esters whereas carotenoids are primarily found in yellow and green vegetables. Dietary retinoids undergo a series of metabolic conversions within the lumen or the mucosa of the small intestine, resulting in the production of all-*trans*-retinol (vitamin A), which represents the most abundant retinoid in blood (Figure 1-1; Vogel *et al.*, 1999). However, processing of dietary retinoids depends on their source of origin. Retinyl esters are hydrolyzed to ROL whereas provitamin A carotenoids are oxidized to retinal and subsequently reduced to ROL (Gottesman *et al.*, 2001; Napoli *et al.*, 1999b). It has long been thought that triglyceride lipase and cholesteryl ester hydrolase (CEL) are able to hydrolyze retinyl esters (Blaner and Olson, 1994; Van Bennekum *et al.*, 2000). However, other evidence demonstrates that a hydrolase activity intrinsic to the brush border membrane of the small intestine cleaves retinyl palmitate (Rigtrup and Ong, 1992; Rigtrup *et al.*, 1994). This notion is further supported by the fact that the rate and amount of vitamin A taken up by the small intestine from bolus doses of ROL, retinyl acetate and retinyl palmitate, is equivalent between CEL-deficient and wild-type mice (Vogel *et al.*, 1999).

The synthesized ROL is then reesterified by lecithin:retinol acetyltransferase (LRAT) within the enterocyte and is packaged, along with other dietary lipids, into nascent chylomicrons (Blaner and Olson, 1994; Vogel *et al.*, 1999). Of note, LRAT uses ROL bound to cellular retinol-binding protein II (CRBPII, discussed in section 1.1.5) as a substrate for esterification (Ong, 1994). Following secretion of these lipoproteins into the lymphatic system, the majority is taken and stored by the liver and other organs, with the liver serving as the main site of storage (Blaner and Olson, 1994). For biological activity, ROL must be first oxidized to all-*trans* retinaldehyde (RAL) and

subsequently to all-*trans* RA by the action of several enzymes (discussed in section 1.1.2 and 1.1.3).

ROL travels in the general circulation complexed to its specific plasma transport protein, retinol-binding protein (RBP, Gottesman *et al.*, 2001; Quadro *et al.*, 2003; Vogel *et al.*, 1999). Moreover, retinol-*Rbp* circulates in the blood in a 1:1 molar complex with transthyretin (TTR), which prevents glomerular filtration of RBP (Blaner and Olson, 1994; Vogel *et al.*, 1999). It has long been thought that the sole function of RBP was to deliver ROL to tissues (Blaner and Olson, 1994). In particular, targeted disruption of *Rbp* in mice has provided interesting insights into the role of this protein in vitamin A metabolism. *Rbp*-null mice are unable to mobilize ROL from hepatic stores and have impaired vision during the first several months of life (Quadro *et al.*, 1999, 2003; Vogel *et al.*, 2002). However, when these mice are maintained on a standard chow diet, they accumulate sufficient RAL levels to achieve normal vision by 4-5 months of age (Quadro *et al.*, 1999). Therefore, $Rbp^{-/-}$ mice are able to use RBP-independent pathways to mobilize retinoids. These mechanisms include circulation of albumin bound RA, retinyl esters in chylomicrons and chylomicron remnants, and provitamin A carotenoids that can be converted to RA within tissues (Blaner and Olson, 1994; Gottesman *et al.*, 2001; Vogel *et al.*, 1999). However, RBP knockout mice accumulate significantly higher concentrations of hepatic ROL than wild-type animals by 5 months of age (Quadro *et al.*, 2003). Their hepatic levels of total ROL do not fall after exposure to a vitamin A-deficient diet, clearly indicating that they cannot mobilize hepatic retinoid stores. These studies suggest that the major role of RBP is to ensure that ROL is available for maintaining normal cellular functions in times of inadequate vitamin A intake.

1.1.2. Biosynthesis of retinaldehyde

The reversible oxidation of ROL to RAL is catalyzed by two different classes of ROL dehydrogenases, referred as medium-chain alcohol dehydrogenase (ADH) and short-chain alcohol dehydrogenase/reductase (SDR; Figure 1-1; Duester, 2000, 2001; Duester *et al.*, 2003; Jörnvall *et al.*, 1995). All members of the ADH and SDR families require NAD+ to catalyze the oxidation of substrates. Therefore, in the

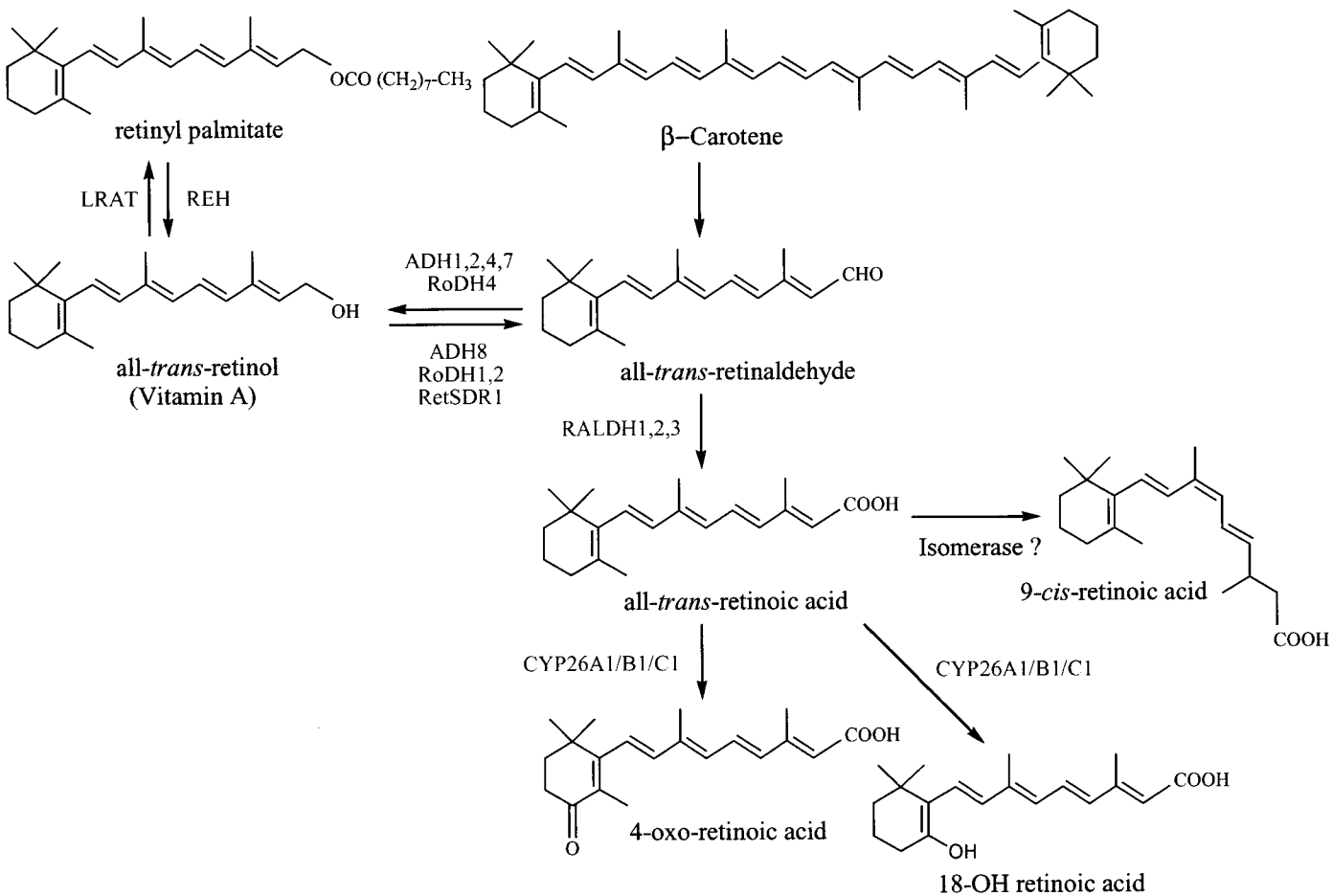


Figure 1-1. Schematic representation of vitamin A metabolism.
ADH, alcohol dehydrogenase; LRAT, lecithin:retinol acetyl transferase; RALDH, retinaldehyde dehydrogenase; REH, retinyl ester hydrolase; RetSDR,; RoDH, retinol dehydrogenase. See text for details.

presence of NAD, ROL will be oxidized to RAL whereas the reverse reaction will take place in the presence of NADH.

Medium-chain alcohol dehydrogenases (ADHs). Orthologs of the ADH family have been identified in mouse, rat, human, fish and frog (Duester, 2000). The medium-chain alcohol dehydrogenases are dimeric zinc metalloenzymes that are divided into eight classes in vertebrates (ADH1-8; Duester *et al.*, 1999; Duester, 2000, 2001). ADH1-7 are NAD-dependent enzymes whereas ADH8 is NADP(H)-dependent. These enzymes have different affinities for all-*trans* ROL, 9-*cis* and 13-*cis* retinoid isomers as well as ethanol (Boleda *et al.*, 1993; Duester *et al.*, 1999; Yang *et al.*, 1994). In support of this, ADH8 has very high activity for reduction of all-*trans* ROL (kcat/km = 33.8 $\mu M^{-1}Xmin^{-1}$) and also has moderate affinity for 9-*cis* RAL (kcat/Km = 7.8 $\mu M^{-1}Xmin^{-1}$; Duester, 2000). However, only six ADHs (ADH1, 2, 3, 4, 7, 8) are able to use ROL as substrate. Of these, ADH1, ADH3 and ADH4 have been found to play an important role in retinoid metabolism (Ang *et al.*, 1996a, 1996b; Duester *et al.*, 2003; Yang *et al.*, 1994).

ADH1 is conserved across many species including frogs, bony fish, birds, rodents and humans (Duester *et al.*, 1999). Both ADH1 and ADH4 are expressed in many retinoid target tissues (Ang *et al.*, 1996a, 1996b; Bilanchone *et al.*, 1986; Deltour *et al.*, 1997; Haselbeck *et al.*, 1997a; Hoffmann *et al.*, 1998; Vonesch *et al.*, 1994). ADH1 is also found in the liver where it represents about 1% of cytosolic protein (Vallée *et al.*, 1983). In addition, ADH4 is more efficient at metabolizing ROL compared with other members of the family (Yang *et al.*, 1994). *Adh1* and *Adh4*-null mice exhibit no obvious embryonic defects when maintained on a sufficient vitamin A diet (Deltour *et al.*, 1999a, 1999b; Duester *et al.*, 2003). However, $Adh1^{-/-}$ mice show a severe reduction in ROL metabolism when subjected to large doses of ROL, leading to excessive vitamin A toxicity (Molotkov *et al.*, 2002a). On the same line, $Adh4^{-/-}$ newborn mice, but not *Adh1*-null mutants, exhibit reduced growth and die by day 15 when deprived of vitamin A (Deltour *et al.*, 1999b; Molotkov *et al.*, 2002a). Interestingly, $Adh1{:}Adh4^{-/-}$ double mutant mice do not have a more severe vitamin A toxicity than *Adh1*-null mutants, and exhibit postnatal lethality by day 24 during VAD,

similar to *Adh4*-null mice (Molotkov *et al.*, 2002a). Overall, these studies indicate that ADH1 and ADH4 have non-overlapping roles in RA synthesis.

ADH3 is a ubiquitous enzyme that was first described as a glutathione-dependent formaldehyde dehydrogenase (Fernandez *et al.*, 1995). However, studies of *Adh3*-null mice revealed that it is associated with ROL metabolism (Duester *et al.*, 2003; Molotkov *et al*, 2002b). In contrast to both $Adh1^{-/-}$ and $Adh4^{-/-}$ mice, *Adh3*-null mutants exhibit reduced viability and growth when maintained on a retinoid sufficient diet. Moreover, $Adh3^{-/-}$ mice display high vitamin A toxicity when given large doses of ROL, have a postnatal lethal phenotype evoked by VAD and exhibit reduced RA-generation *in vivo* (Molotkov *et al.*, 2002a, 2002b). Overall, these results suggest that ADH3 performs an essential role in retinoid signaling with additional contributions from ADH1 and ADH4 (Ang *et al.*, 1996a, 1996b; Molotkov *et al.*, 2002a). Moreover, neither ADH1 nor ADH4 can fully compensate for ADH3 function when ROL becomes limiting (Molotkov *et al.*, 2002b).

Short-chain alcohol dehydrogenase/reductase (SDR). These enzymes were originally purified as microsomal ROL dehydrogenases in the liver, where they are highly expressed (Leo *et al.*, 1987; Napoli and Race, 1990). SDRs are evolutionarily related to the ADH family, but are smaller and do not require a catalytic metal ion for activity (Duester, 2000, 2003; Persson *et al.*, 1995). The vertebrate SDR family is composed of eight members, including RoDH1-4, CRAD1, CRAD2, RDH5 and retSDR1 (Chai *et al.*, 1997; Driessen *et al.*, 1998; Duester, 2000; Gamble *et al.*, 1999; Haeseleer *et al.*, 1998). Of these, RoDH1, RoDH3-4, CRAD1-2 and RDH5 are NAD- or NADP-dependent whereas RoDH2 and retSDR1 are NADPH-dependent (Haeseleer *et al.*, 1998). SDRs, like ADHs, have wide substrate specificities encompassing hydroxysteroids, retinoids as well as other alcohols and aldehydes of physiological importance (Duester, 2000). They also show different affinities towards retinoids, including all-*trans*, 9-*cis* and 11-*cis* ROL. With exception of RDH5, no genetic investigations or *in vivo* studies have been reported and thus, a role for these enzymes in retinoid signaling has not been clearly established (Driessen *et al.*, 2000; Duester *et al.*, 2003; Shang *et al.*, 2002).

1.1.3. Biosynthesis of retinoic acid

Various aldehyde or retinaldehyde dehydrogenases (ALDH or RALDH) have been proposed to catalyze the oxidation of RAL to RA (Figure 1-1; Duester, 1999, 2000; Duester *et al.*, 2003; Napoli, 1996). The vertebrate ALDH family consists of 16 distinct enzymes, with RALDH1 (ALDH1A1), RALDH2 (ALDH1A2) and RALDH3 (ALDH1A3) representing cytosolic forms (Vasiliou *et al.*, 1999; Yoshida *et al.*, 1998). RALDHs require NAD as a coenzyme to catalyze oxidation of substrates. Of the 16 members of the family, only RALDH1, RALDH2, RALDH3 and ALDH2 are able to use retinal as a substrate (Duester *et al.*, 2003). Unlike ROL oxidation, the oxidation of retinal to RA is irreversible.

Retinaldehyde dehydrogenase 1 (RALDH1). RALDH1 is expressed in a subset of RA-dependent embryonic tissues as well as adult organs including the eye, thymus, lung and the liver, among others (Ang and Duester., 1999a; Duester, 2001; Wagner *et al.*, 2000). The expression of RALDH1 in the embryonic eye has been linked to synthesis of RA and injection of mouse or frog RALDH1 mRNA into *Xenopus* embryos results in premature RA synthesis during embryogenesis (Ang and Duester, 1999b; McCaffery *et al.*, 1999). Thus, these studies provided the first insight that this protein could synthesize RA *in vivo*. However, *Raldh1*$^{-/-}$ mice are viable and exhibit normal retinal morphology even though there is complete absence of this protein in the dorsal neural retina, which is inconsistent with an essential role for RALDH1 in development of the eye (Fan *et al.*, 2003). This suggests that other RA-generating enzymes may compensate for proper retina development. In support of this, RALDH3 is expressed in the dorsal retinal pigment epithelium (RPE) and ventral neural retina and has a 10-fold higher activity than RALDH1 for oxidation of RAL (Grün *et al.*, 2000). Thus, it has been proposed that RALDH3 in the RPE is sufficient to generate RA observed in the dorsal eye of *Raldh1*$^{-/-}$ embryos (Fan *et al.*, 2003). However, it still remains unclear what role RALDH1 might play in retinoid signaling *in vivo*.

Retinaldehyde dehydrogenase 2 (RALDH2). RALDH2 is highly conserved across many species including mouse, human, chick and zebrafish (Begemann *et al.*, 2001; Swindell *et al.*, 1999; Zhao *et al.*, 1996). RALDH2 is first expressed in the embryonic mesoderm on each side of the primitive streak concomitant with RA

production at embryonic day (E) 7.5 (Niederreither *et al.*, 1997). Subsequently, RALDH2 is expressed in the posterior part of the embryo up to the base of the headfold, including undifferentiated somites, the optic vesicle, inner ear, flank mesoderm, posterior hindbrain mesenchyme, posterior heart mesoderm, trunk mesoderm, proximal limb bud and in various organs at foetal stages (Berggren *et al.*, 1999; Niederreither *et al.*, 1997). It is also expressed in several retinoid-target tissues in the adult, indicative of a role in retinoid signaling (Duester, 2001; Haselbeck *et al.*, 1999; Zhao *et al.*, 1996).

Overexpression of RALDH2 in *Xenopus* embryos, like RALDH1, results in increased RA levels, indicative of a role for this enzyme in retinoid metabolism (Haselbeck *et al.*, 1999). *Raldh2* null mutants suffer from embryonic lethality at E10.5 (Mic and Duester, 2003; Mic *et al.*, 2002; Niederreither *et al.*, 1999, 2001, 2003). Interestingly, *Raldh2*$^{-/-}$ embryos develop normally until E8.5 at which point they fail to undergo axial rotation and become shortened along their anteroposterior axis by E9.5. Other defects include arrested development of trunk mesoderm, lack of heart looping and chamber morphogenesis, impaired posterior hindbrain development, and lack of development of branchial arches (Duester, 2001, 2003; Niederreither *et al.*, 2000, 2001). In general, *Raldh2* null mutants lack RA everywhere except the eye. Furthermore, these malformations can be rescued by maternal RA administration from E7 to at least E8.5 (Mic *et al.*, 2002; Niederreither *et al.*, 2003). These studies therefore provide strong evidence that RALDH2 is indispensable for generation of RA during embryonic development.

Retinaldehyde dehydrogenase 3 (RALDH3). RALDH3 was recently isolated from mouse and chick (Grün *et al.*, 2000; Li *et al.*, 2000a; Mic *et al.*, 2000; Suzuki *et al.*, 2000). The human homolog is known as ALDH6 or ALDH1A3 (Hsu *et al.*, 1994). RALDH3 is highly restricted to the eye, more particularly the ventral retina, dorsal retina and pigment epithelium, olfactory pit, otic vesicle and at low levels in the forebrain and ureteric buds (Duester *et al.*, 2003; Grün *et al.*, 2000; Mic *et al.*, 2000; Suzuki *et al.*, 2000). Similar to RALDH1 and RALDH2, it is able to oxidize RAL *in vitro* (Grün *et al.*, 2000). Moreover, injection of ALDH inhibitors into the ectodermal site of RALDH3 expression adjacent to the forebrain in chick results in frontonasal developmental defects (Schneider *et al.*, 2001). *Raldh3*-null mutants suffer from severe

malformations restricted to ocular and nasal regions, including choanal atresia (a rare and heritable developmental defect), followed by death at birth (Dupé *et al.*, 2003). Interestingly, these defects are highly similar to those observed in vitamin A deficient embryos and RAR-null mutants (Dickman *et al.*, 1997; Lohnes *et al.*, 1994; Wendling *et al.*, 2000; White *et al.*, 2000b;). Furthermore, maternal administration of RA between E8.5 and E14.5 prevented choanal atresia and nasolacrimal groove persistence. Fan *et al.* hypothesized that RALDH3 could provide sufficient RA for retina development when RALDH1 is missing (Fan *et al.*, 2003). However, the ventral retina appears normal at E18.8 in *Raldh3* knockout mice, suggesting that RALDH1 could compensate for RALDH3 (Dupé *et al.*, 2003). Overall, these studies strongly indicate that RALDH3 is critical to provide RA for nasal development and to prevent choanal atresia, that may be due to mutations in the *Raldh3* locus.

1.1.4. Degradation of retinoic acid

Tight regulation of RA levels during embryogenesis is critical as either RA excess or deficiency have detrimental effects resulting in various malformations. The availability of RA during embryogenesis is achieved by retinoid synthesizing enzymes, including members of the ALDH and RALDH families. However, vertebrates have also evolved degradative mechanisms to protect tissues from inappropriate exposure to RA. RA degradation involves several pathways including oxidation, formation of glucuronides and taurine conjugates, with oxidation being the major route of degradation (Genchi *et al.*, 1996; Kojima *et al.*, 1994; Shirley *et al.*, 1996).

The cytochrome *P*-450 family has known to be involved in RA metabolism for some time (van Wauve *et al.*, 1990; Williams and Napoli, 1987). Recently, a new member, referred as P450RAI or Cyp26A1, was identified in zebrafish, mouse, human, chick and *Xenopus* (Blentic *et al.*, 2003; de Roos *et al.*, 1999; Fuji *et al.*, 1997; Haque *et al.*, 1998; Swindell *et al.*, 1999; White *et al.*, 1996). Cyp26A1 is believed to be essential in inactivation of all-*trans* RA. In support of this, overexpression of Cyp26A1 in several cell lines results in higher production of polar metabolites including 4-hydroxy, 4-oxo and 18-hydroxy RA from all-*trans* RA (Fuji *et al.*, 1997; White *et al.*, 1996). Exogenous RA can strongly induce the expression of *Cyp26A1*, which is

required for induction of reporter gene expression in response to RA (Abu-Abed *et al.*, 1998; Loudig *et al.*, 2000; Ray *et al.*, 1997; Sonneveld *et al.*, 1998). This is believed to create a negative feedback loop whereby RA induces *Cyp26A1* expression resulting in RA degradation (Abu-Abed *et al.*, 1998; Fuji *et al.*, 1997; Ray *et al.*, 1997; White *et al.*, 1996).

Studies in mouse and *Xenopus* have shown that *Cyp26A1* exhibits a complex spatiotemporal pattern of expression during development (de Roos *et al.*, 1999; Holleman *et al.*, 1998; McCaffery *et al.*, 1999). Of particular interest, *Cyp26A1* transcripts are expressed in the primitive streak and the mesoderm of the early gastrulating mouse embryo (E6.5-E7.5) whereas expression shifts more posteriorly, becoming restricted to the neuropore, the hindgut and the tail bud, by late gastrulation (E8.5-E10; de Roos *et al.*, 1999; Fujii *et al.*, 1997). From E9.5 onwards, *Cyp26A1* transcripts can also be detected in various regions including the hindbrain, pharynx, tailbud, neural retina, stomach, cartilage condensations of the digits and epithelium of the limbs among others (de Roos *et al.*, 1999; Fujii *et al.*, 1997).

Targeted inactivation of *Cyp26A1* results in embryonic lethality by mid-late gestation and results in severe morphogenetic defect, which are similar to RA administration, suggesting that Cyp26A1 is essential in controlling RA levels during development. (Abu-Abed *et al.*, 2001; Padmanabhan, 1998). These defects include spina bifida, agenesis of caudal portions of the digestive and urogenital tracts, vertebral transformations and abnormal hindbrain patterning (Abu-Abed *et al.*, 2001; Sakai *et al.*, 2001). Furthermore, the *Cyp26A1*$^{-/-}$ phenotype seems to result from the deleterious effects of excess embryonic RA rather than a lack of signaling by polar RA metabolites like 4-oxo-RA (Niederreither *et al.*, 2002). To support this notion, injection of *Cyp26A1* into *Xenopus* embryos is able to rescue the developmental defects induced by excess RA administration (Hollemann *et al.*, 1998). Niederreither *et al.* hypothesized that if Cyp26A1 functions to protect the embryo from excess RA, the phenotype of *Cyp26A1*$^{-/-}$ mice should be attenuated in a *Raldh2*$^{+/-}$ background whereas it should be exaggerated if Cyp26A1-generated metabolites are essential. Indeed, the early lethal phenotype of Cyp26A1-null mice is largely rescued in a RALDH2$^{+/-}$ background (Niederreither *et al.*, 2002). This genetic evidence suggests that these polar derivatives

are biologically inactive and thus, are not involved in retinoid signaling but rather are likely catabolites (Niederreither et al., 2002). These studies also suggest that Cyp26A1 and RALDH2 generate local embryonic RA concentrations that must be delicately regulated to allow proper development.

The limbs are largely normal in *Cyp26A1*$^{-/-}$ mutants, indicating that other RA-degrading enzymes can compensate for the lack of Cyp26A1. Indeed, a second P450RAI enzyme, termed Cyp26B1, has been cloned from mouse, zebrafish, chick and human (Abu-Abed *et al.*, 2002; MacLean *et al.*, 2001; Nelson, 1999; White *et al.*, 2000a). Its enzymatic activity is indistinguishable from that of Cyp26A1 (White *et al.*, 2000a). *Cyp26B1* is expressed in the distal region of the developing limb bud, indicating that it could compensate for lack of Cyp26A1 during limb development (MacLean *et al.*, 2001; Yashiro *et al.*, 2004). Indeed, targeted deletion of Cyp26B1 results in severe limb malformations (meromelia) including impaired proximal-distal patterning, increased apoptotic cell death of the proximal region and delayed chondrocyte maturation (Yashiro *et al.*, 2004). Interestingly, excess RA evokes exactly the same limb defects as seen in Cyp26B1-null mutants. Thus, Yashiro *et al.* propose that mesenchymal cells in the developing limb establish their proximal-distal (P-D) identity according to a concentration of RA determined by RA synthesis in the trunk (where RALDH2 is expressed) and RA degradation at the distal end of the limb (where Cyp26B1 is located). Their results provide evidence for an RA gradient along the P-D axis of the developing limb.

Cyp26C1, the third member of the CYP26 family of cytochrome P450s, was cloned recently from human and mouse (Tahayato *et al.*, 2003; Taimi *et al.*, 2004). Cyp26C1 is expressed in the hindbrain and branchial arches at early developmental stages and becomes strongly induced in the tooth buds and inner ear at late stages of embryogenesis (Tahayato *et al.*, 2003). Similar to Cyp26A1 and Cyp26B1, Cyp26C1 is able to convert RA to more polar metabolites and this activity is indistinguishable from the other two members of the family (Taimi *et al.*, 2004). Although Cyp26C1 shares significant sequence similarity with Cyp26A1 and Cyp26B1, it exhibits a unique catalytic activity as it can metabolize 9-*cis* RA to 4-*hydroxy*-9-*cis* RA and 4-*oxo*-9-*cis* RA (Chithalen *et al.*, 2002; Montplaisir *et al.*, 2002; Taimi *et al.*, 2004). However, the

physiological role of 9-*cis* RA remains unclear. To summarize, the distinct pattern of expression of the three Cyp26 enzymes support unique roles for each during embryogenesis.

1.1.5. Intracellular retinoid binding proteins

Due to their hydrophobic nature, retinoids must associate with proteins in an aqueous environment. To date, several evolutionary conserved intracellular retinoid binding proteins have been characterized in vertebrates: type I and II cellular ROL-binding proteins (CRBPI and CRBPII) and type I and II cellular RA-binding proteins (CRABPI and CRABPII; Li *et al.*, 1996; Ong, 1987, 1994). CRBPs recognize both ROL and retinal while CRABPs specifically bind to RA and some of its metabolites.

Cellular retinol binding proteins (CRBPs). Although highly conserved between rat, human, mouse, pig and chick orthologs, CRBPI and CRBPII exhibit remarkably different tissue distribution (Li *et al.*, 1996). Of note, CRBPI expression domains include multiple tissues during embryogenesis and also in the adult (Dolle *et al.*, 1990; Gustafson *et al.*, 1993; de Leeuw *et al.*, 1990; Wardlaw *et al.*, 1997; Zetterstrom *et al.*, 1994). In contrast, CRBPII is highly restricted to the intestine in the adult and in the yolk sac and foetal liver (Crow *et al.*, 1985; Li *et al.*, 1986; Noy, 2000; Schaefer *et al.*, 1989).

Both holo-CRBPI and holo-CRBPII deliver ROL to LRAT for esterification (Blaner and Olson, 1994; Noy, 2000; Ong *et al.*, 1988; Yost *et al.*, 1988). CRBPs also prevent spontaneous nonenzymatic isomerization and oxidation of ROL, which occurs in their absence (Noy, 2000; Ong *et al.*, 1988; Yost *et al.*, 1988). Furthermore, apo-CRBPI acts as an inhibitor of LRAT, thereby stimulating hydrolysis of retinyl esters and subsequent release of ROL to the circulation (Herr and Ong, 1992; Noy, 2000). However, *Crbpl* null mutant mice do not display any signs of congenital abnormalities related to RA deficiency, indicating that it is largely dispensable for RA synthesis (Ghyselinck *et al.*, 1999). Nevertheless, it does play an important role in vitamin A storage and mobilization as *Crbpl*$^{-/-}$ mice have decreased capacity to store ROL and to maintain retinyl ester stores in the liver due to impaired transfer of dietary ROL from hepatocytes to stellate cells.

The exclusive expression of CRBPII in absorptive cells, where it represents about 0.4-1% of total cytosolic protein, suggests that it plays a role in intestinal absorption (Noy, 2000; Ong *et al.*, 1994). In support of this, overexpression of CRBPII in Caco-2 cells leads to increased uptake and esterification of ROL (Levin, 1993). Similar to CRBPI, CRBPII binds both all-*trans* retinol and all-*trans* retinal and determines the specificity of enzymes that will gain access to these substrates (Napoli, 2000; Napoli *et al.*, 1991; Noy, 2000). *Crbp*II$^{-/-}$ mice appear healthy and fertile but have reduced hepatic stores (a 40% reduction) of ROL when maintained on a vitamin A-enriched diet (Ghyselinck *et al.*, 1999; E *et al.*, 2002). However, increased neonatal lethality is achieved when vitamin A is reduced during mid-late gestation (E *et al.*, 2002). This phenotype is greatly reduced when dietary vitamin A is increased to sufficient levels, suggesting that neonatal mortality was due to inadequate delivery of vitamin A to the developing fetus. Because CRBPII is highly expressed in the small intestine, E *et al.* had hypothesized that its primary function was to facilitate intestinal absorption and metabolism of ROL (E *et al.*, 2002). However, the increased neonatal mortality observed in *CrbpII*-null mice may be primarily due to the loss of extra intestinal CRBPII. Overall, these studies indicate that foetal CRBPII is required to ensure adequate delivery of RA to the developing fetus when maternal vitamin A is limited.

Cellular retinoic acid-binding proteins (CRABPs). Both CRABPI and CRABPII are expressed during embryonic development and in adult tissues although they do not co-exist in the same cell (Maden, 1994; Noy, 2000; Ruberte *et al.*, 1992; Zheng and Ong, 1998). CRABPII is found in almost every tissue while CRABPI expression is restricted to the skin, uterus, ovary and choroid plexus.

CRABPI and CRABPII have been suggested to solubilize and protect RA in the cytosol and to transport retinoids between different cellular compartments (Noy, 2000; Takase *et al.*, 1986). To support this notion, both CRABP types can be detected in both the cytosol and nuclei of cells (Takase *et al.*, 1986). CRABPI also appears to transfer RA to CYP26-isoenzymes while CRABPII facilitates transport of RA to the nucleus (Delva *et al.*, 1999; Donovan *et al.*, 1995; Noy, 2000). Thus, CRABPI stimulates the conversion of RA to more polar metabolites such as 4-hydroxy-, 4-oxo-, and 18-hydroxy-retinoic acids by serving as a substrate for the Cyp26 family (Fuji *et al.*, 1997;

White *et al.*, 1996). However, both of these proteins seem to be dispensable for retinoid metabolism and function under normal physiological conditions as *CrabpI*, *CrabpII* or *CrabpI:CrabpII* double null mice are fertile, healthy and develop normally with the exception of a minor limb defect in *CrabpII*$^{-/-}$ and *CrabpI:CrabpII*$^{-/-}$ mice (Fawcett *et al.*, 1995; Lampron *et al.*, 1995).

1.2. The retinoic acid receptors

1.2.1. The nuclear hormone receptor superfamily.

The cloning of the RA receptors (RARs) in the late 1980's marked the beginning of an era leading to a better understanding of the retinoid signaling pathway (Giguère *et al.*, 1987; Giguère and Evans, 1990; Mangelsdorf *et al.*, 1990; Petkovich *et al.*, 1987). The RARs belong to the nuclear receptor (NR) family, which includes receptors for progestins (PR), estrogens (ER), androgens (AR), glucocorticoids (GR), thyroid hormone (TR), vitamin D_3 (VDR) as well as orphan receptors (Aranda and Pascual, 2001; Evans, 1988; Mangelsdorf *et al.*, 1995; Ribeiro *et al.*, 1995). These receptors all share a common modular structure comprised of a variable N-terminal domain, a well conserved DNA-binding domain (DBD), a hinge region, a ligand binding domain (LBD), and both a ligand-dependent and independent activation function (AF) (Figure 1-2; Aranda and Pascual, 2001; Kumar and Thompson, 1999; Tenbaum and Baniahmad, 1997).

Multiple receptor isoforms can be generated for several NRs to achieve additional complexity as this is the case for the TRs and RARs (Aranda and Pascual, 2001). For RARα and RARγ, two different isoforms (RARα1, RARα2, RARγ1 and RARγ2) are created by alternate promoter usage while the combination of differential splicing, isoform-specific start codons and alternate promoter usage produces four isoforms for RARβ in mice (RARβ1 to RARβ4; Kastner *et al.*, 1990; Leroy *et al.*, 1991; Nagpal *et al.*, 1992; Zelent *et al.*, 1991).

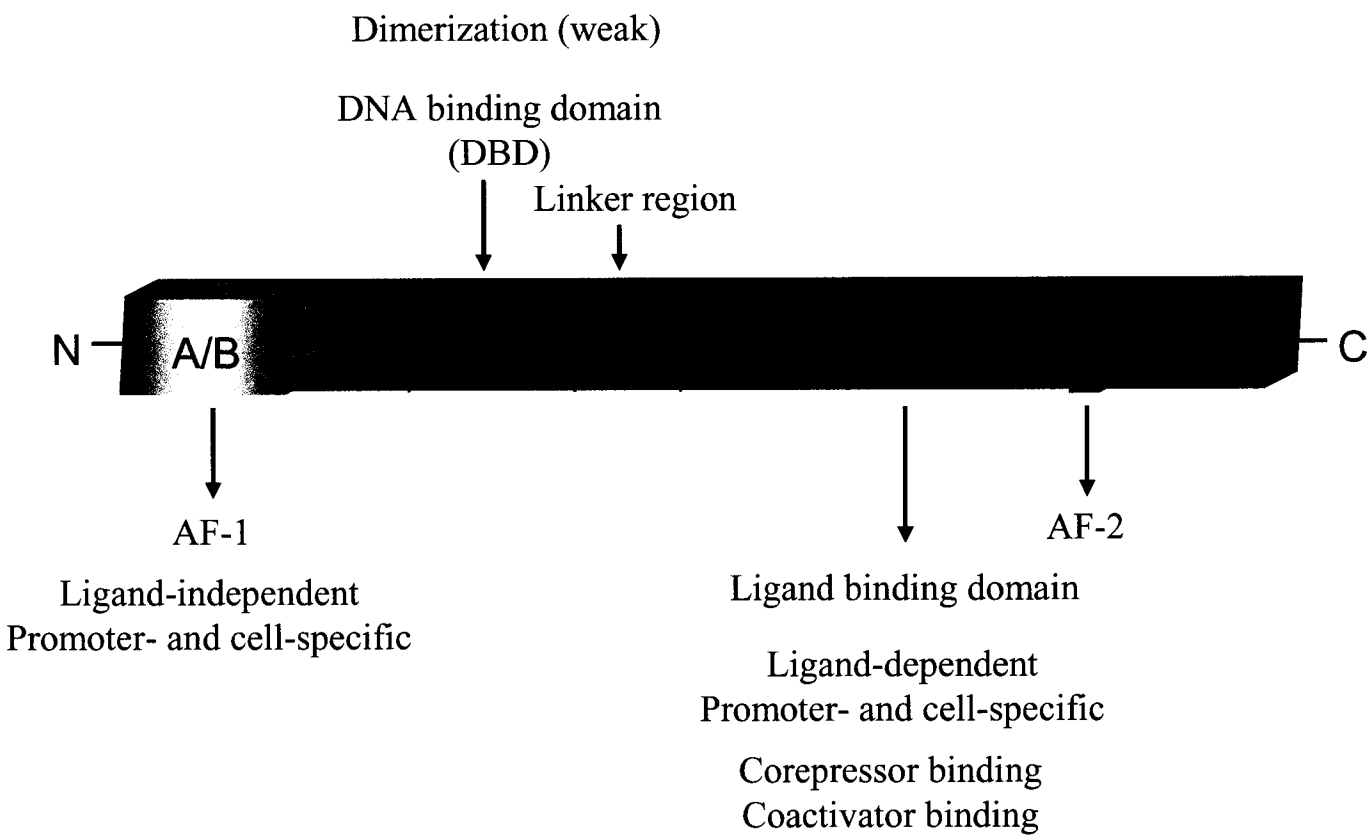


Figure 1-2. Structure of nuclear hormone receptors.
Nuclear receptors are composed of six modular domains; a DNA binding domain, a hinge region, a ligand binding domain and both a ligand-dependent and independent activation functions. AF: activation function; DBD: DNA binding domain;

The amino-terminal region (A/B) is highly variable both in size and sequence. The N-terminal region of the RARs contains a transcriptional activation function (AF-1) that can act autonomously and ligand-independently (Nagpal *et al.*, 1993). An interesting feature of the AF-1 domain of RARα and RARγ is that it contains consensus phosphorylation sites for cyclin-dependent kinases (Rochette-Egly, 2003). As the serine residues targeted by cdk7 lie within surfaces that interact with transcription factors, it is believed that their phosphorylation would help the recruitment of coactivators and thus increase the efficiency of chromatin derepression (Bastien *et al.*, 2000; Rochette-Egly *et al.*, 1997). The AF-1 domain of RARγ can also be phosphorylated by p38MAPK, positively regulating its transcriptional properties through the recruitment of the ubiquitylation machinery (Gianni *et al.*, 2002a, 2002b, 2003; Kopf *et al.*, 2000). However, phosphorylation of Serine 66 on RARγ2 is also a signal for degradation by the 26S proteasome (Gianni *et al.*, 2003).

The RARs, like all other nuclear receptors, contain a sequence-specific DNA binding domain (C) that has the most conserved amino acid sequence (66 residues) within the family. It contains two zinc-finger motifs, which may be considered as an invariant pattern of eight cysteines, arranged in two groups of four, so as to coordinate the binding of two zinc atoms (Kumar and Thompson, 1999). These zinc fingers form two α-helices, the first of which is involved in site-specific recognition and discrimination of binding (Lee *et al.*, 1993). This α-helix, termed the recognition helix, makes specific contacts with the bases of the conserved half-site of the hormone response element (HRE) in the major groove of DNA (Aranda and Pascual, 2001; Kumar and Thompson, 1999). The second α-helix, which encompasses a carboxy-terminal extension, forms a right angle with the recognition helix and is involved in DNA binding and dimerization with the retinoid X receptors (RXRs; Mangelsdorf and Evans, 1995; Rastinejad, 2001).

The LBD (E) not only serves the function of providing a specific ligand binding site, but is also involved in dimerization, coregulator binding and ligand-dependent transcriptional activation (Aranda and Pascual, 2001; Kumar and Thompson, 2003; Nagpal *et al.*, 1993; Warnmark *et al.*, 2003). The LBD is composed of 12 α-helices

(H1-H12) and one β-turn (between H5 and H6) arranged as a three-layered antiparallel α-helical sandwich harboring an internal ligand binding core (Gronemeyer and Miturski, 2001; Kumar and Thompson, 1999). The ligand binding cavity is very hydrophobic and can only accommodate small lipophilic molecules. All ligand-activated receptors possess a carboxy-terminal activation function (AF-2), which is central to the recruitment of coactivators and co-repressors (Aranda and Pascual, 2001; Mangelsdorf and Evans, 1995; Wurtz *et al.*, 1996). As the AF-2 domain forms part of the ligand-binding pocket, its conformation would predict whether RARs are bound by co-repressors or co-activators. The AF-2 domain also seems to be a target for phosphorylation, which acts to enhance the transcriptional activities of nuclear receptors (Rochette-Egly, 2003). For example, phosphorylation of RARα by PKA on serine residues between H9 and H10 within the LBD seems to enhance its transcriptional activity by helping heterodimerization and coregulator binding (Rochette-Egly *et al.*, 1995).

In the absence of ligand, the activation function (AF-2) forms an amphipathic α-helix (corresponds to the C-terminal helix H12), that extends at a 45° angle from the body of the core structure. The presence of ligand alters this structure in such a way that the LBD becomes more compact. Ligand binding appears to shift the AF-2 helix over the ligand binding pocket, thus releasing co-repressors and creating a surface for the binding of coactivators (Glass and Rosenfeld, 2000; Gronemeyer and Miturski, 2001). In this model, referred as the mouse trap model, ligand binding repositions H11 in the continuity of H10 so as to relocate H12 on top of the ligand binding pocket (Glass and Rosenfeld, 2000; Kumar and Thompson, 1999; Moras and Gronemeyer, 1998). In this position, H12 seals the ligand binding cavity and further stabilizes ligand binding.

1.2.2. RXR, a common DNA binding partner

All NRs bind to specific DNA sequences known as hormone response elements (HREs) which consist of a minimal core hexad consensus sequence (5'-PuGGTCA-3'). Response-element recognition occurs subsequently with the formation of dimers between the receptor DBDs. Steroid receptors bind to their cognate response elements

as homodimers while type II NRs function as heterodimers (Aranda and Pascual, 2001; Forman and Evans, 1995; Mangelsdorf and Evans, 1995).

It was shortly discovered after the identification of the RARs, that their effect on gene transcription was potentiated by another group of nuclear receptors, the retinoid X receptors (RXRs). Thereafter, it was soon realized that the RXRs were a heterodimeric partner for various hormone and orphan receptors (Bugge *et al.*, 1992; Kliewer *et al.*, 1992a, 1992b; Mangelsdorf and Evans, 1995). The RXRs are also members of the nuclear hormone receptor superfamily and thus, they share the same modular structure (Figure 1-2). However, RXRs can only associate with 9-*cis* RA whereas RARs can bind to both all-*trans* RA and 9-*cis* RA (Allenby *et al.*, 1993; Heyman *et al.*, 1992; Levin *et al.*, 1992; Minucci *et al.*, 1997). The ligand-induced transcriptional activity of RXR is abolished when complexed with RAR, TR and VDR (Forman and Evans, 1995; Kurokawa *et al.*, 1994; Mangelsdorf and Evans, 1995). The formation of the RXR/RAR heterodimer prevents RXR from binding to 9-*cis* RA, which indicates that RXR is a silent partner.

Similar to RARs, multiple isoforms can also be generated for RXRs (RXRα1, RXRα2, RXRβ1, RXRβ2, RXRγ1 and RXRγ2; Liu *et al.*, 1993; Nagata *et al.*, 1994; Seleiro *et al.*, 1994). RXRγ gives rise to two functional mRNA species (RXRγ1 and RXRγ2) that differ at their 5'-ends (Seleiro *et al.*, 1994). Of note, RXRγ1 seems to be predominant in the liver, brain and muscle whereas RXRγ2 is expressed in the eye, dorsal root ganglia, cardiac and skeletal muscle. The RXRγ1 and RXRγ2 proteins are predicted to differ in their N-terminal domain, in a region though to modulate transcriptional activation by this receptor. Similarly, transcription from different promoters followed by alternative splicing give rise to two isoforms of RXRβ (Nagata *et al.*, 1994). Thus, the existence of multiple RXR isoforms may increase the range of heterodimers formed between RXR and the RARs, TRs, VDRs and PPARs.

1.2.3. Retinoic acid response elements

The formation of dimers between the receptor DBDs is influenced by specific repertoires of high-affinity response elements. The nuclear hormone receptor superfamily can be divided into two, with the steroid receptors binding to response

elements containing palindromic repeats whereas RXR homo- and heterodimers bind to direct repeat (DR) sequences (Aranda and Pascual, 2001; Glass, 1994; Khorasanizadeh and Rastinejad, 2001; Koenig *et al.*, 1990; Mangelsdorf and Evans, 1995).

The RARs, like other steroid hormone receptor members, recognize the same hexameric DNA core motif with the consensus half-site 5'-PuGGTCA-3' (purine being A or G, Aranda and Pascual, 2001; Gronemeyer and Miturski, 2001; Mangelsdorf and Evans, 1995). Direct repeats with one to five base pairs of spacing (DR1-DR5) are binding sites for various RXR-NR heterodimers, with target selectivity dictated by the 1-to-5 rule (Aranda and Pascual, 2001; Mangelsdorf and Evans, 1995; Rastinejad, 2001; Rastinejad *et al.*, 1995). According to this rule, the major element contributing to receptor binding specificity is the half-site spacing, which likely causes geometric constraints. DNA binding specificity and polarity to DRs seems to be mediated by the dimerization interface of the DBDs of the two partners (Mader *et al.*, 1993; Kurokawa *et al.*, 1994; Perlmann *et al.*, 1993; Zechel *et al.*, 1994a, 1994b). In addition to these parameters, differences in the sequence of the half-site and in the flanking nucleotides 5' upstream of the response element can have tremendous effects on binding efficiency and transcriptional activities of NRs (Mader *et al.*, 1993; Rastinejad, 2001).

The type of response element to which the RARs bind can directly affect whether they function as activators or repressors of transcription. For example, the DBDs of RXR and RAR can associate with significant cooperativity on DR2 and DR5 elements (Predki *et al.*, 1994; Rastinejad *et al.*, 2000). Several studies have established that RXR occupies the 5' half-site on the response element while the partner occupies the 3' half-site (Kurokawa *et al.*, 1993; Perlmann *et al.*, 1993; Predki *et al.*, 1994; Zechel *et al.*, 1994). On DR1 elements, RXR can also bind as both a homo- and a heterodimer with RAR. However, the polarity of the RXR/RAR heterodimer is reversed on a DR1 such that RAR now occupies the 5' half-site, thus converting an activator of transcription to a potent repressor (Kurokawa *et al.*, 1994, 1995; Rastinejad *et al.*, 2001). Moreover, RARs do not respond to its ligand on this later element.

1.3. Transcription of retinoid target genes

1.3.1. RARs and transcriptional corepressors

The ability of nuclear receptors to switch from an inactive to an active state by simple addition of ligand has provided some insights with respect to their mechanism of action. The knowledge that the TRs and RARs actively repress transcription in the absence of hormone led to the search for corepressors. Two large proteins, referred as N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator for RAR and TR), have been isolated as RAR and TR interacting proteins (Chen and Evans, 1995; Horlein *et al.*, 1995; Sande and Privalski, 1996; Seol *et al.*, 1996). Unliganded TR and RAR interact strongly with N-CoR and SMRT *in vitro* and N-CoR and SMRT mediate repression of TR and RAR reporter genes in a ligand-independent manner. Moreover, the addition of ligand induces dissociation of N-CoR/SMRT from both RAR and TR with a concomitant induction in gene expression (Chen and Evans, 1995; Horlein *et al.*, 1995).

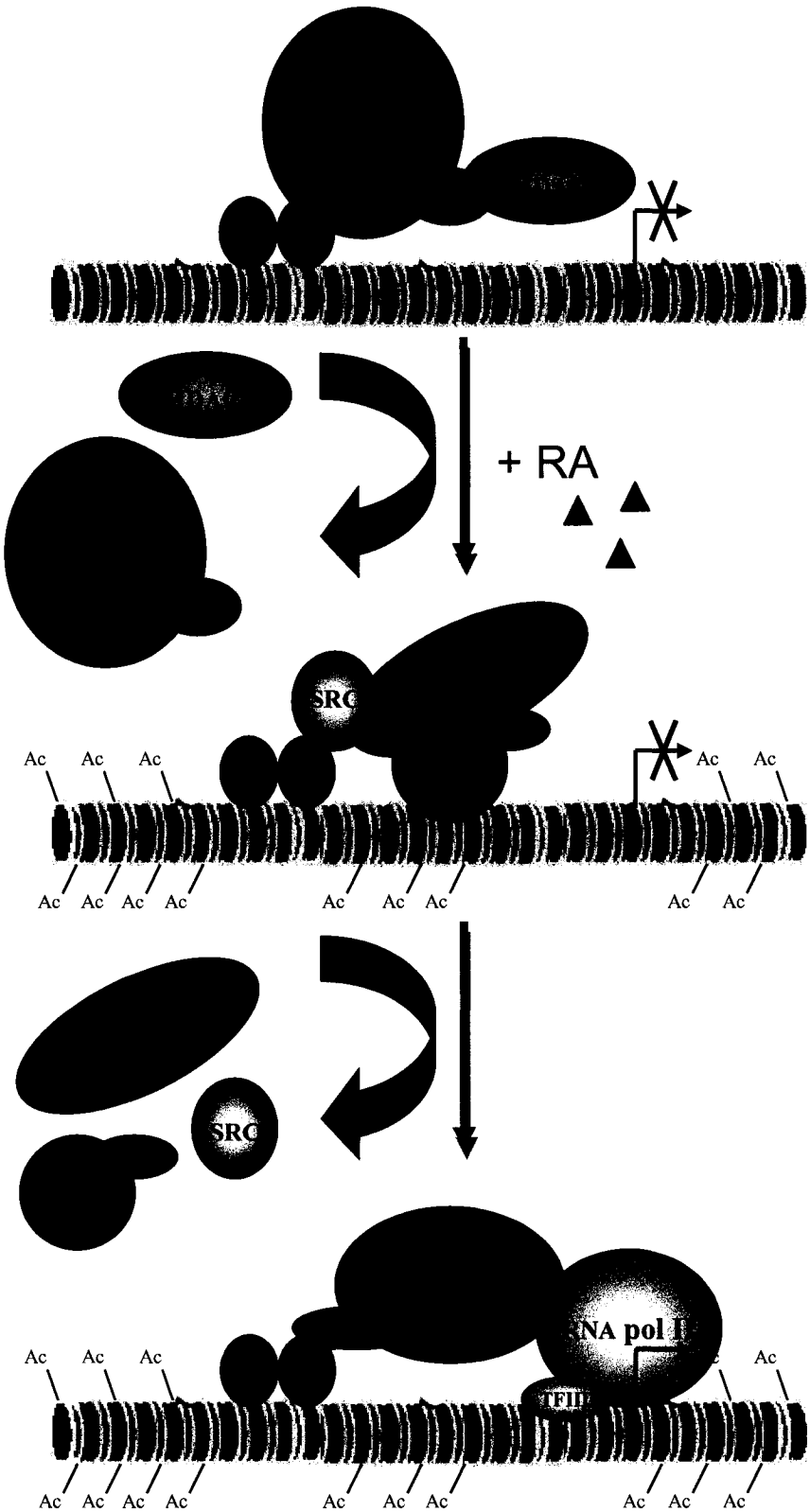
N-CoR and SMRT contain multiple domains indispensable for gene silencing, including three repression domains (RD), a histone interacting domain and two receptor interacting domains (ID), which have been mapped to their C-terminal referred as ID1 and ID2, also known as LXXI/HIXXXI/L helices (discussed below, Cohen *et al.*, 1998; Horlein *et al.*, 1995; Hu and Lazar, 1999; Seol *et al.*, 1996; Zamir *et al.*, 1996). Horlein *et al.* demonstrated that loss of the CoRNR box attenuates repression by unliganded TR, indicating that this motif is required for efficient TR and RAR-mediated transcriptional repression. Moreover, NRs have distinct preferences and affinities for both ID boxes. For example, RAR binds to ID1 while RXR preferentially interacts with ID2 (Hu and Lazar, 2000).

Crucial insights into the potential mechanisms of transcriptional silencing by nuclear receptors were provided by the cloning of yeast Sin3 (mammalian homologs being Sin3A and Sin3B) and the discovery that these proteins interact with N-CoR and SMRT (Alland *et al.*, 1997; Ayer *et al.*, 1995; Heinzel *et al.*, 1997; Nagy *et al.*, 1997). The Sin3 scaffolding proteins are components of very large protein complexes that

contain histone deacetylases, termed RPD3 in yeast and HDAC1/ HDAC2 (class I deacetylases) in mammals (Hassig *et al.*, 1997; Laherty *et al.*, 1997; Taunton *et al.*, 1996; Zhang *et al.*, 1997). mSin3 and HDAC are required for repression by unliganded receptors, suggesting that corepressors function by recruiting the mSin3-HDAC complex (Heinzel *et al.* 1997; Nagy *et al.*, 1997). Interestingly, Heinzel *et al.* demonstrated that HDAC inhibitors decrease repression by TR or N-CoR fused to a heterologous DNA binding domain, clearly demonstrating that HDACs contribute to repression by NRs. N-CoR/SMRT also associates with class II deacetylases HDAC4, HDAC5 and HDAC7 in a Sin3-independent manner (Huang *et al.*, 2000). Moreover, both N-CoR and SMRT exist in large protein complexes with HDAC3 and this seems to be one mechanism leading to repression of the TR (Ishizuka and Lazar, 2003). HDAC3 is also activated by the deacetylase activating domain (DAD) of SMRT and mutations in the DAD abolishes interactions between HDAC3 and SMRT, suggesting that SMRT does not only act as a platform for HDAC recruitment, but also functions as an important component of an active HDAC3 enzyme (Guenther *et al.*, 2001).

The N-terminal domain of N-CoR and SMRT contains three transferable repression domains (RD1, RD2 and RD3; Hu and Lazar, 2000; Li *et al.*, 1997b) that are crucial for association with mSin3 and HDACs. N-CoR/SMRT recruits mSin3 through RD1 and interacts with HDACs through RD2 and RD3 (Alland *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997). Thus, in the absence of ligand, RARs associate with N-CoR/SMRT, which recruit histone deacetylases principally by associating with mSin3 (Figure 1-3). This hypothesis is reinforced by the fact that the ternary complex formed between RAR or TR, N-CoR/SMRT and HDACs is sufficient to grant active repression on RARE or TRE reporters *in vitro* (Aranda and Pascual, 2001; Nagy *et al.*, 1997). The role of HDACs is to maintain a repressive nucleosomal structure. This is accomplished, at least in part, by deacetylation of lysine residues within the N-terminal domain of core histones. Histone tails become more basic upon deacetylation and, subsequently, they interact more tightly with the DNA backbone, which is acidic in nature. As a consequence, the DNA becomes more compact and less accessible to the basal transcription machinery.

Figure 1-3. Transcriptional regulation by the retinoic acid receptors. CBP, CREB-binding protein; DRIP, vitamin D receptor-interacting proteins; HDAC, histone deacetyltransferase; NCOR, nuclear receptor corepressor; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; SMRT, silencing mediator for retinoid and thyroid hormone receptor; SRC, steroid receptor-coactivator; TRAP, TR-associated proteins. See text for details.


+ RA
SRC
Ac
SRC
NA pol II
TFII

1.3.2. RARs, transcriptional coactivators and histone acetylation

In general, coactivators may play one or more of the following roles in NR transcriptional activity: 1) they function as bridging factors to recruit other coactivators to DNA-bound nuclear receptors, 2) they acetylate histones at target gene promoters and 3) they function as bridging factors between the DNA-bound receptors and the basal transcription machinery (Leo and Chen, 2000; Malik and Roeder, 2000; McKenna *et al.*, 1999).

SRC/p160 coactivators. Among the first coactivators identified were members of the p160/SRC protein family (Aranda and Pascual, 2001; Cavailles *et al.*, 1994; Freedman, 1999). There are currently three related members of the p160 coactivator family referred as SRC-1/NcoA-1 (steroid receptor co-activator-1/nuclear receptor co-activator-1), TIF2/GRIP-1/NcoA-2 (transcriptional intermediary factor 2/glucocorticoid receptor interacting protein-1/nuclear receptor co-activator-2) and p/CIP/ACTR/TRAM-1/AIB-1 (p300/CBP interacting protein/activator of the thyroid and RA receptor/thyroid receptor activated molecule-1/amplified in breast cancer-1) (Anzick *et al.*, 1997; Chen *et al.*, 1997; Li *et al.*, 1997a; Onate *et al.*, 1995; Takeshita *et al.*, 1997; Torchia *et al.*, 1997). Although p160 cofactors were originally identified as steroid receptor coactivators (SRC), and interact with many nuclear receptors (PR, ER, TR, RXR, GR, PPAR, RAR), they also interact with diverse transcription factors (NFκB, SMAD3, AP-1), supporting a role for these coactivators in multiple signaling pathways (Lee *et al.*, 1998; Na *et al.*, 1998; Yanagisawa *et al.*, 1999).

The SRC family possesses distinct domains that are required for coactivation, including a highly conserved amino-terminal basic helix-loop-helix (bHLH), three receptor interaction domains (RIDs), an activation domain (AD) as well as histone acetyltransferase (HAT) activity. The RID of SRC coactivators is required to mediate ligand-dependent association with NRs (Ding *et al.*, 1998; Leo and Chen, 2000; Onate *et al.*, 1998; Voegel *et al.*, 1998). Detailed analysis of the RID revealed that it is composed of an LXXLL motif, termed the NR box (discussed below; Heery *et al.*, 1997). Evidence for the requirement of this motif in mediating association between SRC members and NRs has been provided by both site-directed mutagenesis and peptide competition experiments (Ding *et al.*, 1998; Heery *et al.*, 1997; Torchia *et al.*,

1997). The AD of SRC factors also contains three LXXLL motifs that have been linked to interactions with CBP/p300 (McInerney *et al.*, 1998; Voegel *et al.*, 1998). Moreover, anti-CBP antibodies abolish the binding of SRC-1 to RAR and eliminate the ability of SRC-1 to coactivate RAR, suggesting that CBP/p300 is required for the coactivation and NR binding function of SRC-1.

The C-terminal domain of both SRC-1 and SRC-3 contains HAT activity, suggesting that they might modify the chromatin structure (Xu and Li, 2003). However, the HAT activity of these two proteins is much weaker than those of CBP, p300 or p/CAF. Inactivation of SRC-1 HAT activity does not affect its coactivation function on chromatinized templates, indicating that it is not essential for initiation of transcription. Rather, the p160 coactivators may play major roles through the direct and indirect recruitment of other coactivators.

CBP/p300. The general transcriptional activator CBP was originally identified on the basis of its interaction with the *trans*-activating protein CREB (cAMP-response element binding protein) in response to cAMP (Kwok *et al.*, 1994). In addition, a highly related protein, termed p300, was isolated through its association with the viral E1A protein (Eckner *et al.*, 1994). CBP/p300 is able to potentiate the transcription of a wide range of nuclear receptors (RXR, TR, RAR and ER among others) as well as a number of other transcription factors (AP-1, Jun, Fos, NF-κB and STATs among others; Aranda and Pascual, 20001).

CBP/p300 contains discrete domains that are required for diverse functions, including a RID at its amino-terminus, a bromodomain, a HAT domain, a CH/3 domain and a SRC-1 interaction domain located at its carboxy-terminus (Kamei *et al.*, 1996). As for p160 coactivators, the RID of CBP/p300 contains several NR boxes indispensable for ligand-dependent binding to nuclear receptors (Heery *et al.*, 1997; McKenna *et al.*, 1999). In addition, microinjection of anti-CBP antibodies abolishes transactivation by GR, RAR and RXR whereas overexpression of CBP/p300 potentiates ligand-dependent transcriptional activity of these receptors (Chakravarti *et al.*, 1996; Yao *et al.*; 1998).

CBP/p300 also interacts with the p160 coactivators through an SRC interacting domain (Kamei *et al.*, 1996; Torchia *et al.*, 1997; Voegel *et al.*, 1998). This interaction

has been identified both *in vivo* and *in vitro* and provides receptors with two different ways to interact with CBP/p300. One mechanism involves direct interaction of the NR with the N-terminal RID of CBP and the other through interaction with the p160 coactivators, the latter seems to be the prevalent model. In support of this, CBP is unable to restore transcriptional activity from RARE-linked reporter genes after immunodepletion of SRC-1 (McKenna *et al.*, 1999; Torchia *et al.*, 1997). As different regions of CBP/p300 are involved in interactions with NRs and SCR coactivators, they may form a functional ternary complex at target gene promoters. CBP/p300 also possess potent HAT activity (Aranda and Pascual, 2001; Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996). Therefore, the recruitment of HAT-containing transcription factors by NRs may lead to the opening of the condensed chromatin structure, leading to the assembly of the pre-initiation complex and to transcriptional activation.

TRAP/DRIP mediators. The search for additional receptor-interacting cofactors led to the discovery of the TRAP/DRIP complex. These coactivators were originally purified as a thyroid hormone receptor-associated protein complex (TRAP) and a vitamin D_3 receptor-interacting protein complex (DRIP; Fondell *et al.*, 1996; Ito and Roeder, 2001; McKenna et al., 1999; Rachez *et al.*, 1998). These studies have shown that the TRAP/DRIP cofactors enhance ligand-dependent transactivation of TR and VDR on chromatinized templates, raising the possibility that they activate a broader range of NRs. Indeed, the TRAP/DRIP complex also acts as a coactivator for several NRs (PPAR, RAR, RXR, ER, GR), through a single subunit, referred as TRAP220/DRIP205 (Hittelman *et al.*, 1999; Rachez *et al.*, 2000; Yuan *et al.*, 1998).

Consistent with these findings, expression of a truncated form of TRAP220 represses TR, VDR and PPAR activation whereas overexpression of full-length TRAP220 enhances their function (Hittelman *et al.*, 1999; Ito and Roeder, 2001). In contrast to p160 coactivators and CBP/p300, the TRAP/DRIP complex is devoid of HAT activity. Rather, many TRAP/DRIP components are highly similar to mediator proteins, raising the possibility that they function by bridging the nuclear receptor-coactivator complex to the basal transcription machinery (Gu *et al.*, 1999; Ito *et al.*, 1999; Näär *et al.*, 1999). In support of this, numerous mediator proteins have been

found to associate with RNA polymerase II, raising the possibility that they are an integral component of the RNA polymerase II holoenzyme (Myer and Young, 1998; Malik and Roeder, 2000).

1.3.3. Determinants for coactivator and corepressor binding

A common feature for the p160/SRC and CBP/p300 coactivator families is the presence of a leucine-rich motif of the consensus sequence LXXLL, which is present in the RID of these coactivators (Glass and Rosenfeld, 2000; Heery *et al.*, 1997). This motif, also termed the NR box, forms a short α-helix that directly interacts with helix 12 of the AF-2 domain (Darimont *et al.*, 1998). Corepressors also possess a conserved sequence, similar to the LXXLL motif, within their two RIDs (Hu and Lazar, 1999; Nagy *et al.*, 1999; Perissi *et al.*, 1999). This motif, referred as the CoRNR box or as a LXXI/HIXXXI/L helix, represents an extended α-helix that is both necessary and sufficient for binding to unliganded TR and RAR (Perissi *et al.*, 1999). Molecular modeling of coregulator binding has revealed that the NR and CoRNR boxes dock into the hydrophobic pocket of the LBD formed by helices 3-6 and the AF-2 helix (Glass and Rosenfeld, 2000; Perissi *et al.*, 1999).

Discrimination between the association of coactivators or corepressors occurs upon ligand binding. Hormone binding results in a displacement of helix 12 such that it now covers the hydrophobic ligand binding pocket (Figure 1-3). This results in the formation of a charge clamp that associates strongly with the LXXLL helix of coactivators and further positions this helix to allow the leucine side chains to pack tightly into the hydrophobic groove of the LBD (Glass and Rosenfeld, 2000). In the absence of ligand, the CoRNR box extends into the same hydrophobic pocket but does not depend on the charge clamp formed by the AF-2 helix and helix 3. Rather, the conformation of the AF-2 helix acts to enhance the binding of corepressors (Glass and Rosenfeld, 2000). The ligand-activated charge clamp is very specific for the length of the LXXLL helix of coactivators and it is believed that ligand binding inhibits the association of corepressors with nuclear receptors. Therefore, it has been suggested that the AF-2 helix has evolved to discriminate between the LXXLL motif and the CoRNR box, allowing a ligand-dependent switch of nuclear receptor activity.

1.4. Skin development

1.4.1. Structure of the skin

The skin is a large organ that protects the body against a wide range of environmental threats including ultraviolet (UV) radiation, chemical carcinogens, viruses and other pathogens and prevents dehydration. Mammalian skin can be divided into three layers; the epidermis, the dermis and the hypodermis (Figure 1-4; Haake *et al.*, 2001; Oadland, 1991).

Epidermis. Located at the surface of the skin, the epidermis forms a protective barrier. The epidermis is a thin and continuous layer of stratified keratinizing epithelium (Figure 1-4). It is composed of multiple cell types including keratinocytes, melanocytes, Langerhans cells and Merkel cells (Haake *et al.*, 2001; Oadland, 1991). Keratinocytes, which contain keratin intermediate filaments, represent the major epidermal cell type (90-95%). Keratins filaments are the major structural proteins of the epidermis, provide integrity to the epidermis and participate in the formation of the stratum corneum (Candi *et al.*, 1998; Fuchs, 1995; Smack *et al.*, 1994). Keratins are organized into two families, the acidic type I (K10-K20) and basic type II (K1-K9) which form pairs (Fuchs, 1995; Haake *et al.*, 2001). Melanocytes are derived from the neural crest and are responsible for pigment production in the epidermis (Erickson, 1993). Langerhans cells are important composants of the immune response in the epidermis and are the primary cells responsible for the uptake, processing and presentation of soluble antigens to T lymphocytes (Haake *et al.*, 2001; Romani *et al.*, 1989). Merkel cells are the neuroendocrine cells of the skin. They function as mechanoreceptors and make synaptic contacts with nerve endings to form the Merkel cell-neurite complex (Narisawa *et al.*, 1996; Tachibana, 1995).

The epidermis is organized into four layers: 1) basal cell layer (BL), 2) spinous cell layer (SL), 3) granular cell layer (GL), and 4) stratum corneum (SC; Haake *et al.*, 2001; Oadland, 1991). The basal layer is directly connected to a basement membrane via hemidesmosomes (Burgeson and Christiano, 1997; Green and Jones, 1996; Jones *et al.*, 1998). These connections not only contribute to epidermal integrity but also provide signals for keratinocyte survival, differentiation, migration and proliferation.

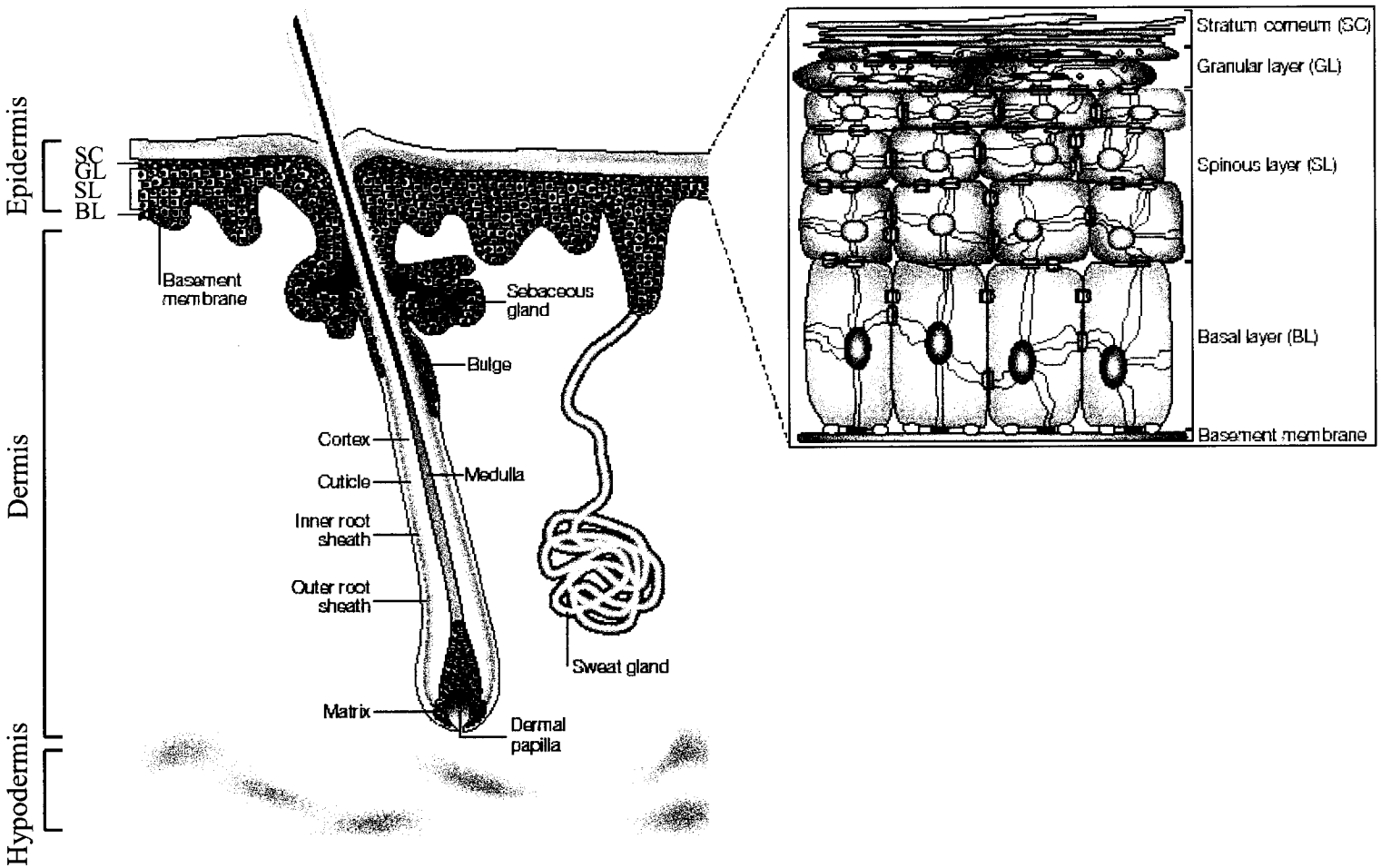


Figure 1-4. Structure of the skin.

The skin is divided into three layers: the dermis, epidermis and hypodermis. The epidermis is further subdivided into the basal layer (BL), the spinous layer (SL), the granular layer (GL) and the stratum corneum (SC). The epidermis and the dermis are separated by the basement membrane. See text for details.

Taken from Fuchs E and Raghavan S, *Nature Genetics Reviews*, 2002, 3: 199-209.

The basal layer is composed of stem cells (10%), post-mitotic differentiated cells (5%) and rapidly proliferating cells, referred as the transit amplifying cells (Jones and Watt, 1993; Haake *et al.*, 2001). The transit amplifying cells have low self-renewal capacity and a high probability of terminal differentiation. When they commit to terminal differentiation, they withdraw from the cell cycle, downregulate integrins, lose contact with the basement membrane and start their ascension towards the surface of the skin (Barrandon and Green, 1987; Fuchs and Raghavan, 2002; Watt, 1998). The terminal differentiation program of the epidermis, called keratinization, involves several changes including the loss of ability to proliferate, dehydration, increase in cell size and flattening, altered synthesis of proteins and lipids and receptors and formation of new organelles (Byrne and Hardman, 2002). The end point of keratinization is the formation of dead, enucleated and flattened cells that provide a protective barrier to the skin. These cells are shed and continuously replaced by inner cells that move upwards. Thus, the epidermis is in a constant state of dynamic equilibrium and self-renewal. The entire differentiation program from the basal layer to the surface of the skin takes about 10-14 days.

The spinous layer (stratum spinosum or prickle cell layer) lies just above of the basal layer (Haake *et al.*, 2001; Oadland, 1991). It is primarily composed of keratinocytes intertwined with desmosomes (focal junctions) that results in the formation of so-called ''spines''. Keratinocytes of the suprabasal spinous layer are more polyhedral, whereas those of the upper layer adopt a more flattened, larger shape with additional organelles (lamellar granules; Haake *et al.*, 2001). Keratin 1 and 10 (K1/K10) are upregulated in the suprabasal layer, concomitant with the onset of differentiation whereas expression of K5 and K14 is restricted to the basal layer (Byrne *et al.*, 1994).

The granular layer, or the stratum granulosum, is composed of two to three layers of differentiated keratinocytes that can be recognized by their cytoplasmic basophilic keratohyalin granules (Haake *et al.*, 2001; Holbrook, 1989). Keratohyalin granules form a meshwork composed of profilagrin and keratin filaments (Fukuyama *et al.*, 1980). Loricrin, a protein of the cell envelope, and profilaggrin (a histidine-rich

protein) are found within keratohyalin granules. Proteolysis of profilaggrin occurs from transition of a granular to a cornified cell. The breakdown product, filaggrin, acts as a scaffold to promote aggregation of keratin filaments (Haake *et al.*, 2001). Lamellar granules, regarded as membrane-bound organelles, are first detected in the upper spinous layer but are only active at the granular-cornified layer interface. They act to deliver lipid precursors of the stratum corneum to the intercellular space and process them to neutral lamellae that will coat the surface of cornified cells (Madison, 2003).

The stratum corneum, which is the outermost layer, is in direct contact with the environment. It is composed of multiple layers of dead, enucleated, flattened and differentiated keratinocytes (Byrne and Hardman, 2002; Haake *et al.*, 2001; Odland, 1991). These cells, referred to as corneocytes, are filled with an aggregated keratin matrix and are surrounded by a very dense impermeable cornified envelope cross-linked to external lipids (Kalinin *et al.*, 2002; Swartzendruber *et al.*, 1987). The cornified cell envelope is composed of about 20 proteins including involucrin, loricrin, cystatin A, S100 family proteins, small proline-rich region proteins (SRRPs) and some desmosomal proteins (Kalinin *et al.*, 2002; Madison, 2003; Steinert and Marekov, 1999; Steinert, 2000). The first sign of cornified envelope assembly is the association of envoplakin, periplakin and involucrin with keratin intermediate filaments and desmosomal proteins at the plasma membrane. These proteins are cross-linked together and with other lipid components by transglutaminase 1 (TG1; Nemes *et al.*, 1999). Shortly thereafter, SPRR proteins in the cytoplasm are cross-linked into oligomers by transglutaminase 3 (TG3; Candi *et al.*, 1999; Steinert *et al.*, 1999; Tarcsa *et al.*, 1998). SPRR proteins act to cross-bridge the envelope constituents in order to fortify the envelope structure (Steinert *et al.*, 1998). Loricrin, which constitutes the major protein of the cornified envelope, becomes attached to the complex to complete barrier formation. As the isopeptide bond formed by TGase enzymes cannot be cleaved, this provides a convenient mechanism for the formation of an insoluble, stable and mechanically resistant barrier (Kalinin *et al.*, 2002). The protein envelope is then covalently attached to ceramides, the major constituents of the lipid envelope (Kalinin *et al.*, 2002). The lipid envelope is formed by very long chain ω-hydroxyacids amide-linked to glycosylated sphingosine bases.

Dermis. The dermis is a connective tissue located beneath the epidermis (Figure 1-4). It protects the body against mechanical injury and supplies the epidermis with nutrients. Mature dermis is primarily composed of fibroblasts, collagen, elastic fibers, extracellular matrix (ECM) and interfibrillar glycosaminoglycans (GAG)/proteoglycan gel (Byrne and Hardman, 2002; Haake *et al.*, 2001; Odland, 1991). Collagen provides the skin with tensile strength and elasticity, contributes 75% of the skin's dry weight and aggregates to form bundles (Vuorio and Crombrugghe, 1990). These bundles are made up of three chains, all having a helical domain consisting of (Gly-X-Y) repeats, where X is a proline and Y a hydroxyproline (Haake *et al.*, 2001; Prockop and Kivirikko, 1995). Each chain is glycosylated and assembled into soluble procollagen molecules prior to secretion from the endoplasmic reticulum. They are then cleaved by proteases and cross-linked to form collagen fibrils, filaments and networks (Vuorio and Crombrugghe, 1990). About 80-90% of the collagen in the dermis is type I collagen and 8-12% is type III collagen, with small contributions of type V and VI (Haake *et al.*, 2001). Elastic fibers are composed of a fibrillar component, termed fibrilin, which is embedded in the matrix component, elastin. Its sole function is to return the skin to its normal shape following mechanical stress (Haake *et al.*, 2001; Odland, 1991).

The dermis is subdivided into two anatomical regions, the papillary dermis and the reticular dermis (Haake *et al.*, 2001; Oadland, 1991). The papillary dermis is the thinner of the two layers and is closest to the epidermis. It is characterized by the presence of small bundles of collagen fibrils and elastic fibers, which are loosely distributed. In contrast, the reticular dermis is composed of dense collagenous and elastic connective tissue that forms large interwoven fiber bundles. Thus, the reticular dermis contributes to the strong mechanical properties of the skin.

The major dermal cell type is the fibroblast, which is responsible for synthesis of matrix proteins such as collagen and elastin (Haake *et al.*, 2001). Other cell types include monocytes, macrophages and dermal dendrocytes, which constitute the phagocytic system of the skin.

Basement membrane. The basement membrane (BM) physically separates the dermal and epidermal compartments (Burgeson and Christiano, 1997; Kalluri, 2003). It is highly cross-linked and insoluble, providing mechanical stability. The BM interacts

with epidermal cells via integrins while it is anchored to the dermis via fibrils (Bruckner-Tuderman, 1999; Byrne and Hardman, 2002). About 50 proteins are known to make up the dermal-epidermal junction with collagens comprising 50% of all BM proteins (Kalluri, 2003). The most important components of the basement membrane include type IV collagen, laminin, heparan-sulfate proteoglycans and nidogen/entactin. Minor components include agrin, osteopontin, fibulins, type XV collagen and type XVIII collagen (Kalluri, 2003; Schittny and Yurchenco, 1989; Yurchenco and Schittny, 1990).

The first sign of DEJ assembly is the synthesis of functional components by basal keratinocytes and dermal fibroblasts. Following secretion, laminin, anchored to the cells via integrins and dystroglycans, initiates BM scaffold formation by self-polymerization (Kalluri, 2003). Deposition of laminin polymers leads to association and bridging with type IV collagen network at the cell surface, a process facilitated by nidogen/entactin (Aumailley *et al.*, 1989, 1993; Kalluri, 2003). The other components of the BM will then interact with this scaffold to generate a functional DEJ.

1.4.2. Growth and differentiation of epidermal keratinocytes

The morphology and function of the epidermis depends on a balance between terminal differentiation and proliferation. If this balance is perturbed, diseases such as psiorasis and epidermal cancers may result. Numerous studies have highlighted the importance of diverse signaling pathways in the epidermis (Fuchs and Raghavan, 2002).

As described above, most basal keratinocytes have a limited proliferative potential and undergo terminal differentiation, starting in the spinous layer, when they exit the cell cycle. Extracellular calcium (Ca^{2+}) has profound effects on keratinocytes *in vitro*. Keratinocytes die below an extracellular concentration of 0.01 m*M* Ca^{2+} but proliferate rapidly between calcium concentrations of 0.01 and 0.09 m*M* (Hennings *et al.*, 1980a, 1980b; Kulesz-Martin *et al.*, 1984). Above 1.2 m*M*, keratinocytes vertically stratify, express terminal differentiation products and differentiate (Pillai *et al.*, 1990; Yuspa *et al.*, 1989). Thus, lower Ca^{2+} concentrations promote continued proliferation of keratinocytes *in vitro* whereas higher calcium concentrations leads to differentiation. As keratinocytes undergo terminal differentiation, the Ca^{2+} gradient peaks in the granular

layer in adult epidermis, indicative of a role in epidermal keratinization (Dlugosz and Yuspa, 1993). The discovery of calcium sensing receptors (CaR) in suprabasal keratinocytes further strengthens this hypothesis (Bikle *et al.*, 1996; Oda *et al.*, 1998, 2000). Komuves *et al.* demonstrated that $CaR^{-/-}$ mice display an increased number of proliferating cells and show alterations in terminal differentiation (Komuves *et al.*, 2002). Thus, Ca^{2+} is a critical regulator of epidermal differentiation.

Transduction of the Ca^{2+} signal is associated with activation of protein kinase C (PKC), which is essential for keratinocyte maturation. Four PKC isoforms (α, δ, ε and η) are present throughout the mouse epidermis. Of note, PKCε is found in the proliferative basal layer, PKCα is located to the spinous and granular layers, PKCη is expressed in the granular layer and PKCδ is moderately expressed throughout the epidermis with slightly higher levels in the basal/lower spinous layer (Denning, 2004). The different expression patterns of these PKC isoforms likely reflects different roles in the epidermis. PKCε drives keratinocyte proliferation, PKCα triggers irreversible growth arrest in suprabasal cells whereas PKCη and PKCδ downregulate keratin 1 and 10 and induce expression of loricrin, involucrin, filaggrin and transglutaminase during the spinous to granular cell transition (Denning, 2004; Deucher *et al.*, 2002; Kashiwagi *et al.*, 2002; Tibudan *et al.*, 2002). Moreover, an RARE was found in the promoter of PKCα, suggesting that the RARs increase PKCα gene expression (Desai *et al.*, 1999). Both PKCα and PKCγ are able to phosphorylate RARα, resulting in a decrease in transcriptional activity (Delmotte *et al.*, 1999). Thus, these two PKC isoforms may modulate the activity of RARα *in vivo*.

Another important player in dictating terminal differentiation is the I kappa α kinase (IKKα), a member of the NF-κB pathway. IKKα, together with its regulatory subunit IKKγ, phosphorylates cytoplasmic IκB, leading to its degradation (Ghosh and Karin, 2002; Karin and Ben-Neriah, 2000). Degradation of IκB results in nuclear translocation and activation of the transcription factor NF-κB. In adult epidermis, NF-κB is expressed in the cytoplasm of basal keratinocytes and in the nucleus of suprabasal cells (Li *et al.*, 1999). Targeted inactivation of IKKα leads to several defects, including developmental arrested after epidermal stratification but before the onset of terminal

differentiation and barrier formation (Hu *et al.*, 1999; Li *et al.*, 1999). These defects are not dependent on NF-κB as the activity of NF-κB was unaffected in epidermal keratinocytes. Rather, these studies suggest that IKKα seems to be responsible for the production of a secreted factor that controls terminal differentiation of keratinocytes.

The epidermal growth factor receptor (EGFR) is a phosphoglycoprotein that posses tyrosine kinase activity (Cohen *et al.*, 1980). In adult epidermis, EGFR is expressed in the basal layer and, to a lesser extent, in the first suprabasal layer (Jost *et al.*, 2000; Nanney *et al.*, 1990). EGFR promotes epidermal thickness and stimulates epidermal keratinocyte proliferation (Cohen, 1965; Cohen and Elliott, 1963). It has been demonstrated that the EGFR is a critical regulator of DNA synthesis and cell cycle progression (G1 to S phase) in cultured keratinocytes (Coffey *et al.*, 1988; Jost *et al.*, 2000; Kobayashi *et al.*, 1998). Moreover, EGF treatment inhibits expression of early and late terminal differentiation markers including K1, K10, transglutaminase 1 and cornified envelope precursors (Monzon *et al.*, 1996; Peus *et al.*, 1997). Consistent with these findings, $EGFR^{-/-}$ mice display a thinner epidermis due to hypoproliferation as well as disorganization of hair follicles (Hansen *et al.*, 1997; Miettinen *et al.*, 1995; Threadgill *et al.*, 1995).

The transcription factor activating protein-1 (AP-1) is composed of homo- and heterodimers of Jun (cJun, Jun B and Jun D), Fos (cFos, Fos B, Fra-1 and Fra-2) and ATF (ATF2, ATF3 and B-ATF; Angel and Karin, 1991; Shaulian and Karin, 2002). Jun proteins can form homodimers as well as heterodimers with the various Fos and ATF proteins. Fos members are unable to form stable homodimers whereas ATF proteins are able to homodimerize. AP-1 regulates transcription of target genes from TPA-response elements (5'-TGAG/CTCA-3'; Angel *et al.*, 1987; Bowden *et al.*, 1994).

Several Jun and Fos proteins are expressed in the epidermis. In newborn mice, c-Jun, Jun D, c-Fos, Fra-1 and Fra-2 can be detected in basal cells while Jun B and Fra-2 are expressed in the granular layer (Carrasco and Bravo, 1995; Rutberg *et al.*, 1996). In addition, c-Jun, Jun D and Fra-1 are also expressed in the spinous layer. The distinct pattern of expression of the Jun and Fos proteins suggests that they might play a role in the epidermal proliferation and differentiation. In support of this, an increasing number of genes expressed in the epidermis harbour AP-1 binding sites in their promoters.

These include transglutaminases and several members of the keratin gene family like K1, K5, K6, K8, K14, K18 and K19 (Angel *et al.*, 2001; Eckert and Walter, 1996). However, the phenotype of c-Fos, Fos B or JunD-null mutants does not provide evidence for a critical role of these proteins in the skin (Jochum *et al.*, 2001).

1.5. Pathogenesis of squamous cell carcinoma

1.5.1. Cutaneous squamous cell carcinoma

Nonmelanoma skin cancer is the most prevalent cancer in North America. About 80% of nonmelanoma skin cancers are basal cell carcinoma (BCC) and 20% are squamous cell carcinomas (SCCs; Alam and Ratner, 2001). BCCs are rarely fatal but they can, in some cases, be invasive and destructive (Alam and Ratner, 2001; Miller, 1991). SCCs, on the other hand, are invasive and more than 10% will metastasize (Kwa *et al.*, 1992; Moller *et al.*, 1979). Actinic keratoses (AK) have been identified as a precursor of SCC (Alam and Ratner, 2001). Also, AKs are far more common than SCCs, which makes them a good target for chemoprevention. Numerous risk factors are involved in SCC development with chronic exposure to UV radiation being the single most critical factor. UV radiation produces mutations in DNA, usually thymidine dimers, which, if not correctly repaired, may lead to tumor formation (Grossman and Leffel, 1997).

1.5.2. The multistage skin carcinogenesis model

Mouse SCC can be induced by sequential application of chemical carcinogens and tumor promoters. Multistage carcinogenesis involves a complex set of genetic and epigenetic changes, which occur in a predictable sequence in experimental models (Figure 1-5; Yuspa, 1998). These studies have provided great insight into the biological processes that are associated with SCC.

The most common chemical carcinogenesis protocol is a two-stage protocol, which involves the administration of a single dose of the polycyclic aromatic hydrocarbon 7,12-dimethyl-benz[*a*]anthracene (DMBA) followed by multiple applications of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Slaga,

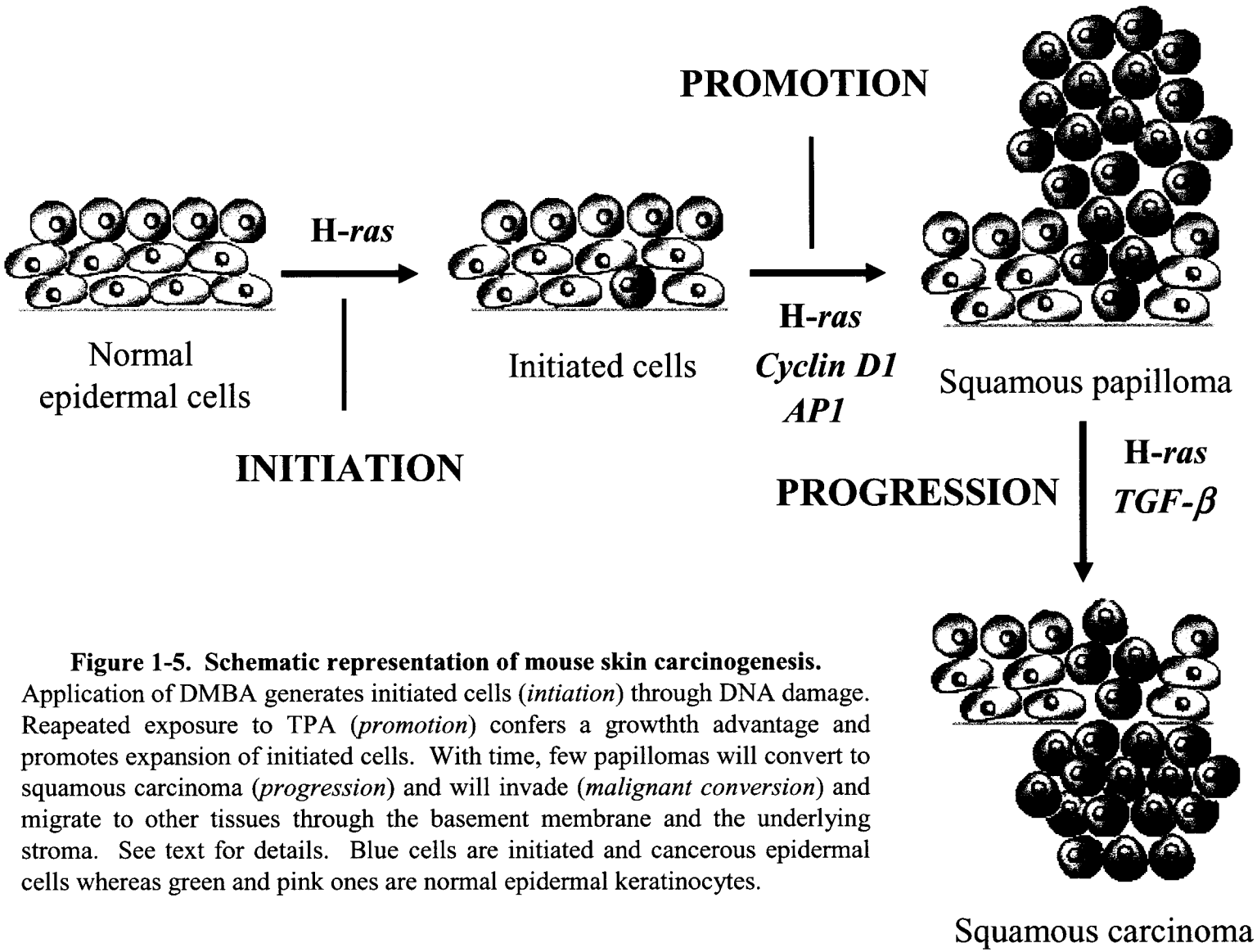


Figure 1-5. Schematic representation of mouse skin carcinogenesis. Application of DMBA generates initiated cells (*intiation*) through DNA damage. Reapeated exposure to TPA (*promotion*) confers a growthth advantage and promotes expansion of initiated cells. With time, few papillomas will convert to squamous carcinoma (*progression*) and will invade (*malignant conversion*) and migrate to other tissues through the basement membrane and the underlying stroma. See text for details. Blue cells are initiated and cancerous epidermal cells whereas green and pink ones are normal epidermal keratinocytes.

1984; Yupsa, 1994). Initiation is mutagenic in nature, resulting in DNA damage. DMBA intercalates into the major groove of DNA to cause simple mutations like frameshifts, transversions and point mutations and is an irreversible event. DMBA produces a subtle change in keratinocyte phenotype, which is unrecognizable at this point (Yupsa, 1994). Cells that have mutations (in the ras gene for example) that favor clonal expansion are the most likely to be subsequently expanded by tumor promoters (Boutwell, 1974; Iannaccone *et al.*, 1987). Tumour promoters, such as phorbol esters, activate protein kinase C (PKC) and accelerate terminal differentiation of normal keratinocytes as well as DMBA mutagenized cells (Yuspa *et al.*, 1980, 1982). In contrast to initiation, promotion is reversible and involves epigenetic changes.

The consequence of initiation and promotion is the formation of squamous papillomas, each representing an expanded clone of initiated cells. Papillomas are characterized by a high rate of proliferation, altered expression of keratinocyte markers, blocked or delayed terminal differentiation and hyperplasia (Roop *et al.*, 1988; Yupsa, 1998). Tumors may have a greater risk of progression and are termed ''high risk'' papillomas while those that will regress with time are called ''low risk'' squamous papillomas (Yupsa, 1998). Premalignant progression is a generally spontaneous process that does not require further exposure to tumor promoters and is the major time-dependent phase of cancer development. However, most papillomas will not become malignant. Major genetic abnormalities associated with premalignant progression of papillomas include aneuploidy and chromosomal aberrations, in particular trisomies of chromosme 6 and 7 (Aldaz *et al.*, 1987, 1989; Conti *et al.*, 1986). Malignant conversion is characterized by invasion through the basement membrane and migration into the underlying stroma and involves changes in cell-cell and cell-matrix interactions (Yupsa, 1998). Squamous carcinomas may differ in their degree of differentiation; some remain histologically similar to papillomas whereas other are very poorly differentiated.

1.5.3. Signaling pathways involved in mouse two-stage skin carcinogenesis

Initiation and promotion. Activating mutations in the harvey *Ras* (*Ha-ras*) oncogene have been implicated in tumor initiation (Balmain and Pragnell, 1983;

Zoumpourlis *et al.*, 2003). Consistent with this, targeted deletion of the *Ha-ras* gene correlates with reduced tumor formation of DMBA-treated mouse epidermis (Brown *et al.*, 1986; Ise *et al.*, 2000). Furthermore, primary mouse keratinocytes expressing the *Ha-ras* oncogene produce squamous papillomas when grafted onto nude mice. The Ras proteins are small G-proteins that transduce mitogenic signals and are constitutively activated by point mutations of codons 12, 13 or 61 (Zoumpourlis *et al.*, 2003). In virtually all DMBA-initiated tumours, the *Ha-ras* gene is activated by a mutation at codon 61, leading to accelerated cell growth and inhibition of apoptosis (Balmain and Pragnell, 1983; Brown *et al.*, 1990; Quintanilla *et al.*, 1986).

Several studies have demonstrated that the *cyclin D1* gene, a cell cycle associated nuclear protein active in the G1 phase, is overexpressed in squamous carcinomas but not in skin papillomas (Bianchi *et al.*, 1993; Yamamoto *et al.*, 2002). When grafted onto nude mice, cyclin D1-knockout keratinocytes form squamous papillomas with significantly reduced size compared to wild-type and heterozygous keratinocytes (Yamamoto *et al.*, 2002). Cyclin D1 deficiency also leads to an 80% reduction in squamous tumour development in the mouse two-step carcinogenesis protocol (Robles *et al.*, 1998; Rodriguez-Puebla *et al.*, 1999). Robles *et al.* thus suggested that Ras-mediated tumorigenesis was dependent on signaling pathways that act through cyclin D1 (Robles *et al.*, 1998). Cyclin D1 forms a complex with cyclin-dependent kinase (cdk) 4 and cdk6, which are also induced in keratinocytes in response to oncogenic ras (Zoumpourlis *et al.*, 2003). Taken together, these results indicate that there is a strong correlation between activated ras and cyclin D1 expression.

In the mouse multistage carcinogenesis model, repeated topical application of TPA results in induction of AP-1 target genes (Bowden *et al.*, 1994). Moreover, several malignant SCC cells lines express constitutive AP-1 DNA binding and transactivation (Strickland *et al.*, 1988). High levels of phosphorylated, and therefore activated, c-Jun, Fra-1, Fra-2 and ATF-2 proteins correlate with increased malignant frequency (Zoumpourlis *et al.*, 2000). These studies bring strong evidence that AP-1 plays a critical role in tumor promotion, progression and/or metastasis (Barthelman *et al.*, 1998; Bowden *et al.*, 1994; Dong *et al.*, 1994). Consistent with this, a dominant negative form of c-Jun lacking the transcriptional activation domain attenuates SCC formation,

indicating that AP-1 does play a role in malignant conversion (Alani *et al.*, 1991; Brown *et al.*, 1993).

Numerous studies have shown that sustained activation of NF-κB is linked to human cancers (Fuchs and Raghavan, 2002; Karin *et al.*, 2002). However, recent evidence shows that colon cancer cells have decreased NF-κB activity through an interaction with the β-catenin protein (Deng *et al.*, 2002). In mice overexpressing a stabilized IκB in the epidermis, NF-κB is permanently inhibited and the skin becomes sensitized to developing SCCs (Seitz *et al.*, 1998; van Hogerlinden *et al.*, 1999). Taken together, these results diverge from the protumorigenic function of NF-κB seen in other tumours, thereby suggesting that NF-κB may play different roles in epithelial cells. In addition, co-expression of *Ras* and *IκBα* generates skin tumours resembling SCCs when grafted onto nude mice, indicating that cooperativity between Ras signaling and NF-κB function is enough to drive the formation of SCCs (Dajee *et al.*, 2003). Thus, it seems that Ras prevents the susceptibility to apoptosis by allowing cells to progress through the cell cycle. However, the exact mechanism underlying the interplay between Ras, NF-κB inhibition and SCC development requires further studies.

Conversion and progression. The secreted factor transforming growth factor β1 (TGFβ1) plays very important roles in epidermal homeostasis and alterations leading to the enhancement or repression of the TGFβ pathway have been linked to carcinogenesis (Wang, 2001). However, its role in tumor promotion and malignant progression has been controversial. TGFβ1 is induced in the epidermis in response to TPA and its expression is often elevated in tumor cell *in vivo* (Akhurst *et al.*, 1988; Fowlis *et al.*, 1992; Krieg *et al.*, 1991). Also, loss of the mitogenic response to TGFβ1 occurs in some tumors and has been proposed to contribute to tumor outgrowth (Manning *et al.*, 1991). Moreover, intracutaneous injections of TGFβ1 suggest that it acts as a tumor promoter. TGFβ1-null keratinocytes, transduced with mutant v-ras^{Ha}, and grafted on nude mice produce tumors with enhanced malignant conversion rates compared to wild-type keratinocytes (Glick *et al.*, 1994). However, immunohistochemical analysis of endogenous TGFβ1 expression in tumors *in vivo* has suggested that TGFβ1 attenuates malignant conversion. In an attempt to resolve these controversial results, Cui *et al.*

generated transgenic mice with keratinocyte-specific expression of TGFβ1 (Cui *et al.*, 1995). Using the murine multistage carcinogenesis model, they have found that such transgenic mice exhibit reduced benign tumor growth at an early stage whereas TGFβ1 enhances malignant progression rates at later stages.

The α6β4 integrin is a receptor for the laminin family of BM components and provides a link to the intermediate filament cytoskeleton and thus, it is important for epidermal integrity (Mercurio, 1995; Mercurio and Rabinovitz, 2001). Enhanced expression of α6β4 integrin has been linked to malignant progression in mouse skin and many other cancers (Rabinovitz and Mercurio, 1996; Tennenbaum *et al.*, 1993). Loss of polarization of α6β4 integrin and extended expression in the suprabasal layers correlates with an increased invasive phenotype of carcinomas (Rabinovitz and Mercurio, 1996; Tennenbaum et al., 1993; Witkowski et al., 2000). This likely relates to the finding that α6β4 integrin is able to mediate migration of cells through its ability to associate with the cytoskeleton and promote the formation and stabilization of fillopodia (Rabinovitz and Mercurio, 1997). This integrin can also stimulate the activity of phosphoinositide 3-OH phosphate (PI3-K) in invasive carcinoma cells, suggesting further mechanisms of action in invasive carcinoma (Mercurio and Rabinovitz, 2001; Shaw *et al.*, 1997). Indeed, the lipid products of PI3-K, phosphatidylinositol 3,4,5-trisphosphate, could play a direct role in the formation of actin-containing motility structures as they are able to bind a number of proteins that regulate actin assembly (Hartwig *et al.*, 1996; Lu *et al.*, 1996).

1.6. Role of RA and RARs in the epidermis

1.6.1. RA, RARs and epidermal development

The idea that retinoids play critical roles in epithelial differentiation came from studies of vitamin A deficiency (VAD), which exhibits squamous metaplasia of diverse epithelia (Frazier and Hu, 1931; Moon, 1994; Wolbach and Howe, 1925). The effects of retinoids on cell growth have been studied extensively both *in vivo* and *in vitro*. When applied topically on human skin, retinoids increase the proliferation rate of basal keratinocytes leading to an accelerated turnover of epidermal cells and thickening of the

suprabasal layers (Connor *et al.*, 1986; Fisher and Voorhees, 1996; Thacher *et al.*, 1997). Vitamin A deprivation from the culture medium *in vitro* induces keratinocyte terminal differentiation whereas addition of vitamin A inhibits this process (Fuchs and Green, 1981).

RXRα, RXRβ, RARα and RARγ are expressed in the epidermis with RXRα and RARγ being the predominant receptors (Fisher and Voorhees, 1996). It should also be noted that RARγ represents 90% of RARs in skin with the remaining 10% being RARα (Fisher *et al.*, 1994). A role for these receptors in epidermal differentiation has been inferred from studies of transgenic mice expressing a dominant negative (dn) form of RARα in the basal epidermis (Saitou *et al.*, 1995). The transgenic newborn mice were characterized by very thin, shiny, wrinkleless and dry skin (Saitou *et al.*, 1995). The suprabasal layers of these mice was severely reduced, indicating that there is a block of differentiation. However, the complete absence of both RARα and RARγ in adult epidermis does not markedly disturb the proliferation or differentiation of the epidermis, indicating that they are largely dispensable under normal conditions (Chapellier *et al.*, 2002; Ghyselinck *et al.*, 2002). In support of this, the skin of RARα-null mutants appear normal while the skin of RARγ-null mutants only shows minor defects in granular keratinocyte differentiation (Fisher *et al.*, 1994; Ghyselinck *et al.*, 1997; Lohnes *et al.*, 1993; Lufkin *et al.*, 1993). However, Chapellier *et al.* showed that topical treatment of skin with retinoids results in a marked increase in keratinocyte proliferation mediated principally by RARγ/RXR heterodimer (Chapellier *et al.*, 2002).

Recent studies have shown that ablation of RXRα in basal keratinocytes results in hair follicle degeneration and hyperproliferation of interfolicullar keratinocytes (Li *et al.*, 2000b, 2001). As RXR acts as an heterodimeric partner for various NRs, these results probably reflect a role for either RXR/PPAR, RXR/TR or RXR/VDR signaling in hair follicle cycling as the RXR/RAR heterodimer is not critically involved in keratinocyte proliferation under physiological conditions (Chapellier *et al.*, 2002). Indeed, VDR and RXR knockouts give similar epidermal phenotypes (Li *et al*, 2000b, 2001; Yoshizawa *et al.*, 1997).

1.6.2. RA, RARs and epithelial tumorigenesis

The connection between vitamin A status and cancer dates back to 1925 when Wolbach and Howe reported that VAD rats exhibit premalignant tumors, which can be reversed by vitamin A repletion (Wolbach and Howe, 1925). VAD has also been linked to a higher incidence of cancer in both experimental animals and humans (Moon *et al.*, 1994). Thus, these studies have led to the hypothesis that retinoids protect the organism against the development of benign and malignant tumours.

Retinoids have been found to be effective in inhibiting the induction of cancer in several models. Several lines of evidence suggest that RA inhibits carcinogenesis by interfering with the promotion stage in the murine multistage carcinogenesis model (Verma *et al.*, 1979, 1982; Verma, 2003). In support of this, a single application of RA one hour before TPA treatment resulted in a 60% reduction in tumour multiplicity (Verma, 2003). Conversely, when RA application was delayed as long as 24 hours post-TPA treatment, RA did not inhibit skin tumour formation. Thus, in order to inhibit the induction of skin tumors, RA treatments had to be scheduled close to TPA application. In addition, an 82% reduction in the number of skin tumours was obtained when RA was applied in conjunction with TPA during the entire promotion period (30 weeks), demonstrating that inhibition of skin tumour promotion is dependent on the duration of RA treatment (Verma *et al.*, 1979). Furthermore, retinoids have been shown to inhibit the growth of transformed cells and the appearance of precancerous lesions in various organs (Lotan *et al.*, 1990; Sun and Lotan, 2002; Verma *et al.*, 1979, 1982, 1986). They are also able to inhibit carcinoma development from chemically-induced squamous papillomas, further providing evidence that retinoids inhibit both the promotion and progression stage during tumorigenesis (Chen *et al.*, 1994, 1995; De Luca *et al.*, 1993, 1996).

The inhibitory action of RA on skin carcinogenesis appears to be mediated at the level of RARs. Several studies have indicated that neoplastic cells progressively lose RARs during tumour promotion and malignant progression (Darwiche *et al.*, 1995; Xu *et al.*, 2001). For example, transformation of mouse epidermal keratinocytes with oncogenic Ha-*ras* also results in concomitant reduction in RAR transcript levels (Darwiche *et al.*, 1996). RARα transcripts are abundant in papillomas and

differentiated carcinomas but are lost in undifferentiated carcinoma and more malignant spindle cell carcinoma (Darwiche *et al.*, 1995; Xu *et al.*, 2001). RARγ, on the other hand, is expressed in the suprabasal layers of the epidermis and papillomas but not in differentiated or undifferentiated carcinomas nor in spindle cell carcinoma (Darwiche *et al.*, 1995; Xu *et al.*, 2001). Moreover, we have shown that RARγ is the predominant receptor that mediates RA-induced cell cycle arrest in wild-type keratinocytes (Goyette *et al.*, 2000). Also of note, RARγ-null or RARαγ-null keratinocytes are predisposed to Ras-induced tumorigenesis, indicating that loss of RARγ predisposes to tumor formation (Chen *et al.*, 2004). Although expressed in the epidermis, RXR transcripts remain at high levels in both normal epidermis and tumors, suggesting that RARs mediate the anti-tumor retinoid activity in skin (Darwiche *et al.*, 1995).

Although RARβ is not expressed in the epidermis, several lines of evidence suggest that loss of RARβ is associated with tumor progression (Altucci and Gronemeyer, 2001). For example, downregulation of RARβ is observed in a diverse range of solid tumours including head and neck SCC, non-small cell lung cancer, colon, breast, oesophageal and cervical cancers (Altucci and Gronemeyer, 2001; Sun and Lotan, 2002). Furthermore, the growth inhibitory effects of RA in these solid tumours correlate with increased expression of RARβ and/or induced RARβ2 promoter activity, suggesting that RARβ acts as a tumour suppressor. In support of this, transgenic mice expressing anti-sense RARβ2 develop solid tumours of the lung whereas constitutive overexpression of RARβ2 decreases the tumoriginicity of lung carcinoma cells (Berard *et al.*, 1996; Houle *et al.*, 1993). This is in contrast to RARβ-null mice, which do not develop tumors (Ghyselinck *et al.*, 1997). There is also accumulating evidence that the RARβ2 promoter may be hypermethylated and thus silenced in several epithelial cancers (Hayashi *et al.*, 2001a; Ivanova *et al.*, 2002; Kwong *et al.*, 2002; Lamy *et al.*, 2001). Consistent with this, treatment with 5-aza-2'-deoxycytidine restores the inducibility of the RARβ gene by RA in breast cancer carinoma cells (Bovenzi and Momparler, 2001; Widschwendter *et al.*, 2000).

Aberrant expression of RARα has been associated with acute myeloid leukemia (APL). APL is characterized by a block in differentiation of hematopoietic cells at the

promeylocytic stage (Lin *et al.*, 2001). In almost all cases, APL is associated with a t(15;17) chromosomal translocation that fuses the RARα gene and the promeylocytic leukemia gene (PML; Piazza *et al.*, 2001). In rare cases, the RARα gene can be fused to the promeylocytic leukemia zinc finger (PLZF) gene, the nucleophosmin (NPM) gene, the nuclear mitotic apparatus (NUMA) or the signal transducer and activator of transcription 5B (STAT5B; Pandolfi, 2001; Zelent *et al.*, 2001).

The PML-RARα fusion protein acts as a potent transcriptional repressor by virtue of a higher affinity for corepressors (Grignani *et al.*, 1998; He *et al.*, 1998; Lin *et al.*, 1998). Thus, signaling is constituvely repressed by corepressor-associated HDAC activity under physiological RA levels, leading to transcriptional silencing and a maturation block. Consistent with this, HDAC inhibitors, in combination with RA, can overcome the repressor activity of PML-RARα and trigger terminal differentiation of APL cells (Lin *et al.*, 1998, 2001). Lin *et al.* proposed a model to explain APL pathogenesis mediated by oncogenic activation of RARα. In this model, transcriptional repression and promeylocytic differentiation blockage occurs under physiological RA levels (10^{-8}-10^{-9} M RA) as a result of increased association with corepressor complexes whereas pharmacological RA concentrations leads to the association of coactivator complexes, activation of gene expression, leukemia cell differentiation and clinical remission (Lin *et al.*, 2001).

A number of studies have implicated RA in the regulation of myeloid differentiation. In support of this, exogenous RA can induce granulocyte differentiation of the myeloid leukemia cell line HL-60 (Breitman *et al.*, 1980). Moreover, the PML-RARα fusion protein in APL has been shown to negatively inhibit RA signalling (Lin *et al.*, 1999). Also, several RA-target genes such as *p21WAF*, *C/EBPβ* and *HoxA1* are known to play important roles in regulating myeloid cell proliferation and differentiation and, it is conceivable that repression by PML-RARα leads to differentiation blockage (Lin *et al.*, 2001). Activation of these target genes by pharmacological concentrations of RA may induce a cascade of gene expression that triggers leukemia cell differentiation and clinical remission (Lin *et al.*, 1999). Also worthy to mention, the PML-RARα fusion protein can also inhibit gene expression through non-DNA binding mechanisms. Of note, the PML-RARα fusion protein is able

to abolish DNA binding and transactivation of the neutrophilic differentiation factor C/EBPα, leading to a blockage of myeloid differentiation (Pabst *et al.*, 2001).

Topical application of TPA to mouse skin causes a decrease in the expression of both RARα and RARγ transcripts (Kumar *et al.*, 1994). As TPA increases protein kinase C (PKC) signaling, this result suggests that PKC-dependent mechanisms may be involved in RAR silencing. Consistent with this, Darwiche *et al.* showed that inactivation of PKC activity can restore RAR levels in Ras-transformed keratinocytes (Darwiche *et al.*, 1996).

Activated AP-1 plays important roles in epithelial tumorigenesis, especially in the promotion stage (section 1.5.3; Bowden *et al.*, 1994; Jochum *et al.*, 2001). Thus, there has been considerable effort to identify compounds that could downregulate AP-1 activity and thereby oppose the unregulated cell growth leading to the formation of papillomas and squamous cell carcinomas (SCCs). RARs, like several other NRs, have been reported to inhibit AP-1 activity and several mechanisms have been proposed to underlie this *trans*-repression (Karin *et al.*, 1997; Pfahl, 1993; Schule *et al.*, 1991). These include competitive titration between RAR and AP-1 for common coregulators such as CBP/p300, downregulation of c-Jun N-terminal kinase (JNK) activity and inhibition of AP-1 DNA binding (Benkoussa *et al.*, 2002; Caelles *et al.*, 1997; Kamei *et al.*, 1996; Schule *et al.*, 1991).

1.6.3. Retinoids and chemoprevention of cancer

Retinoids have been found to be effective in suppressing tumor development in several carcinogenesis models including skin, breast, bladder, pancreas, prostate, lung and oral cavity (Lotan, 1996; Moon *et al.*, 1994). Retinoids have been tested in a number of clinical trials and are able to suppress precancerous lesions, such as oral leukoplakia, cervical dysplasia and xeroderma pigmentosum (Sun and Lotan, 2002). Isotretinoin (13-*cis*-RA) reduces second aerodigestive tract tumors in patients with head and neck cancers (Lotan, 1996; Niles, 2002). RA has also been used topically for the treatment of AK, a pre-malignant precursor of SCC (Lotan, 1996).

Nearly all cases that are diagnosed with APL undergo clinical remission with retinoid monotherapy although many patients will relapse (Freemantle *et al.*, 2003).

The basis for the success of the RA-based differentiation therapy is that RARα agonists will bind to the PML-RARα LBD, resulting in dissociation of the HDAC-corepressor complex (Pandolfi, 2001). Subsequent, activation of gene expression results in the induction of terminal differentiation and clinical remission. Given that HDAC activity underlies APL, research has focussed on the development of HDAC inhibitors (HDACi) as a new therapeutic avenue. Treatment with the HDACi sodium phenylbutyrate, in combination with RA, results in clinical remission for 7 months before the patient developed resistance against this treatment (Warrell *et al.*, 1998). HDACi plus RA has also been shown to induce PLZF-RARα APL cell differentiation, indicating that these compounds could be used to treat primary RA-resistant APL cases (Lin *et al.*, 2001). Reversal of DNA hypermethylation by the demethylating agent 5-aza-2'-deoxycitidine has also been shown to restore RA-mediated differentiation of several leukemia and solid tumor cells *in vitro* (Altucci and Gronemeyer, 2001).

Even though retinoids offer a potential avenue for therapy, 10-15 % of patients suffer from the RA syndrome, the main side effect of retinoid treatment (Altucci and Gronemeyer, 2001; Miller, 1998). Typical signs include fever, respiratory distress, weight gain, oedema of the lower extremities, pleural or pericardial effusions, and, occasionally, renal failure. Furthermore, chronic administration of high doses of RA to APL patients can lead to hypervitaminosis A, characterized by anorexia, weight loss, bone and joint pain, apolecia, fever, cracked and bleeding lips and skin and mucous membrane changes, among others (Miller, 1998). Except for bone toxicity, most of these side effects are reversible upon discontinuation of treatment. Another limitation frequently encountered in clinical use of RA is resistance, which is often associated with relapse of APL. Resistance is thought to be partly due to enhanced expression of P450 and lipoxygenase enzyme activities by RA (Fontana and Rishi, 2002; Muindi and Young, 1993). Induced CRABPII expression is another mechanism by which cells may acquire resistance to RA.

To circumvent these problems, novel retinoids have been developed. One such candidate, N-(4-hydroxyphenyl) retinamide (4-HPR), is being intensively investigated. The modification of the carboxyl group in RA with *N*-4 hydroxyphenyl creates a molecule with reduced toxicity as a chemopreventing agent (Fontana and Rishi, 2002;

Wu *et al.*, 2001). 4-HPR mediates its effects by binding to, and activating, RARγ and to a lesser extent RARβ (Fanjul *et al.*, 1996; Fontana and Rishi, 2002). However, RAR-independent pathways have also been suggested to mediate the apoptotic effects of 4-HPR, as this compound is active in retinoid-resistant cells (Delia *et al.*, 1993; Goyette *et al.*, 2000; Wu *et al.*, 2001). Several lines of evidence indicate that 4-HPR can prevent chemically induced tumours of the breast, prostate, bladder and skin (Pollard *et al.*, 1991; Slawin *et al.*, 1993; Ulukaya and Wood, 1999). Moreover, 4-HPR is highly effective against head and neck squamous carcinoma, ovarian adenocarcinoma and breast adenocarcinoma (Wu *et al.*, 2001). Also, 4-HPR seems to reduce the occurrence of oral carcinoma in patients with leukoplakia, again indicating that it might be highly effective against various cancers (Chiesa *et al.*, 1992). As for other retinoids, 4-HPR has also been shown to reduce secondary tumor formation in patients with breast or head and neck cancers (Wu *et al.*, 2001).

1.7. Hypothesis

As discussed, many studies have shown that RARs are effective in suppressing tumor development in diverse model systems (Lotan, 1996). Evidence also indicates that the anti-tumor activity of retinoids is mediated through inhibition of tumor promotion (Verma, 2003). Overall, this indicates that retinoids are chemopreventive agents and that physiological levels of retinoids protect the organism against the development of pre-cancerous lesions, as well as conversion to malignancy.

RARγ is the predominant receptor in the epidermis, and mediates RA-induced growth arrest in transformed keratinocytes (Goyette *et al.*, 2000). RARγ-null and RARαγ-null Ha-ras transformed keratinocytes develop tumours when injected in nude mice, indicating that RARγ may also act as a tumour suppressor (Chen *et al.*, 2004). Since the RARαγ-null keratinocyte cell line behaves exactly as the RARγ-null keratinocyte cell line, it became a valuable model to study the means by which RARs suppress tumor formation in the epidermis. However, it remains unknown which RA-target gene mediates tumour suppression in the epidermis. I reasoned that the genes that were induced by RA in wild-type, but not in RARαγ-null, keratinocytes were likely candidates to play an important role in epithelial tumorigenesis. To investigate this, I used a suppression subtractive library to isolate genes that were induced by RA in wild-type keratinocytes relative to the RARαγ-null keratinocyte cell line. Second, given that tumor formation is associated with loss of RAR transcripts, I hypothesized that one or more of the genes would be profoundly reduced in papillomas and/or squamous cell carcinomas (Darwiche *et al.*, 1996). To this end, I have employed the well-defined murine two-stage carcinogenesis model to generate papillomas and demonstrated that expression of one putative RA-target gene is indeed severely reduced in papillomas.

Chapter II

Materials and Methods

2. Materials and Methods

2.1. Suppressive subtractive hybridization

Poly A^+ RNA was purified from wild type and RARαγ-null keratinocyte cultures using the QuickPrep™ Micro mRNA purification kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. First-strand cDNA synthesis was carried out with 2 μg of poly A^+ RNA using the SMART™ PCR cDNA synthesis kit (Clontech) according to the manufacturer's instructions. The cDNA was used as starting material for the subtraction using the Clontech PCR-Select™ cDNA subtraction kit according to the manufacturer's instructions. Subtracted cDNA fragments were cloned into the pGEM®-T cloning vector (Promega) according to the manufacturer's instructions. *E.coli* was transformed with the ligated DNA by electroporation, and plasmid DNA was isolated using the alkali lysis technique. Clones of interest (identified by reverse northern blots) were end-sequenced with T7 by automated sequencing.

2.2. Reverse Northern analysis

Thirty microliters of plasmid DNA containing sub cloned PCR fragments from the subtractive library was digested with Sst I/Sst II and run on a 1.5% agarose gel. The DNA was denatured by two washes of 45 minutes in 0.5 M NaOH and then transferred to a hybond N+ membrane according to the manufacturer's instructions. The membranes were hybridized in a formamide-based buffer [40% deionized formamide, 0.9 M NaCl, 50 mM sodium phosphate buffer (pH 6.5), 2 mM EDTA, 4x Denhardt's, and 1% SDS] at 42°C overnight with $1.0X10^6$ cpm/ml cDNA probe generated from poly A+ RNA from RA (10^{-6} M) treated wild-type or RARαγ$^{-/-}$ keratinocyte cultures as follows: 1/4th of the poly A+ RNA was precipitated at 4°C and resuspended in 10 μl of DEPC H_2O. Half of the mRNA was reverse transcribed with 300 units of M-MLV reverse transcriptase (Invitrogen) in a mixture of 1X first-strand buffer, 10 mM DTT and 40 units RnaseOUT™ recombinant ribonuclease inhibitor at 37°C for 1 hour. The reaction was labelled with 50 μCi of ^{32}P-α-dCTP with the rediprime^{II} labelling kit (Amersham Pharmacia Biotech) for 2 hours at 37°C. Membranes were washed at 65°C

twice for 20 minutes in 2X SCC, 0.1% SDS (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and once for 15 minutes in 1X SCC, 0.1% SDS. Signal was revealed by autoradiography using X-Omat film (Kodak). Signal intensities from the membrane hybridized with the probe derived from wild-type cells were compared to the membrane hybridized with the probe from RAR$\alpha\gamma^{-/-}$ cells in order to identify potential RA-regulated clones.

2.3. Generation of immortalized cell lines and treatment of cells

2.3.a. Primary keratinocyte culture and immortalization

Derivation of the wild-type and RAR$\alpha\gamma^{-/-}$ keratinocyte cell lines have already been described (Goyette *et al.*, 2000).

2.3.b. Treatment of immortalized keratinocyte cell lines

For northern blot experiments, cells were seeded in 100 mm plates (approximately $3x10^5$ cells/plate) treated the following day with RA (1μM) or 4-HPR (1μM) in SMEM supplemented with 10% Fetal Calf Serum, adenine (24μg/ml) and $MgCl_2$ (1.5 mM). Control cultures were treated with DMSO only. RA was dissolved in DMSO at a concentration of 10^{-2} M and 4-HPR was dissolved in ethanol at a concentration of 10^{-2} M. For northen blot analysis, cells were harvested 2-48 hours post-treatment, snap frozen, and stored at -80°C prior to RNA extraction.

To assess the requirement for *de novo* protein synthesis, cells were treated for 1 hour with 15 mg/ml or 30 mg/ml of cycloheximide (Sigma) prior to RA treatment. Otherwise, cells were treated with carrier (DMSO), RA (1 μM) or 15 μg/ml or 30 μg/ml cycloheximide. Cells were harvested 6 hours post-treatment, snap frozen, and stored as described above prior to Northern blot analysis.

In some experiments, cells were treated with RA (1 μM) and/or TPA (100 ng/ml). TPA was dissolved at a concentration of 1 mg/ml. For Northern blot analysis, cells were harvested 6-24 hours post-treatment, snap frozen, and stored at -80°C prior to RNA purification.

2.4. RNA isolation

2.4.a Total RNA

Total RNA was extracted from frozen skin samples, tumour samples or cell pellets using Trizol® reagent (Life Technologies) according to the manufacturer's directions. Skin and tumour samples were homogenized using a tissue tearor (Biospec Products) in 1-2 ml of Trizol®. The RNA pellet was resuspended in an appropriate volume of deionized formamide and stored at –80°C.

2.4.b Poly A+ RNA

Poly A+ RNA was isolated from RA (10^{-6} M) treated wild-type and RAR$\alpha\gamma^{-/-}$ keratinocyte cultures using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Poly A+ RNA was stored at -80°C.

2.5. Northern blot analysis

Fifteen microgram of total RNA was size fractionated on a 1% agarose-formaldehyde gel in MOPS buffer and transferred to a MAGNA nylon membrane (Amersham Pharmacia Biotech) in 10X SSC overnight as described by the manufacturer's instructions. RNA was fixed to the membrane by crosslinking under UV light for 5 minutes. Hybridizations were performed overnight at 65°C in a SSC-based buffer (6X SSC, 4X Denhardt's, 0.4% SDS, 160 µg/ml yeast RNA) supplemented with 0.1 mg/ml of denatured salmon sperm DNA with approximately 10^6 CPM/ml of denatured probe prepared by random labelling with the rediprime^{II} labelling kit (Amersham Pharmacia Biotech). Blots were washed in 2X SSC, 0.1% SDS two times at 65°C and signal was revealed by autoradiography using X-Omat film (Kodak).

2.6. Animals

Female FVB/N mice, 4 weeks of age and CD-1 mice, 6-8 weeks of age, were obtained from the Jackson laboratory. Mice were housed for at least two weeks prior to

use. Mice were fed rodent chow (Purina 5001 Rodent Chow; Purina Mills, Richmond, IN) and water *ad libitum*.

2.7. Treatment of animals

2.7.a. Skin

Female CD-1 mice, 2-3 months of age, were treated with 100 mg/Kg RA by oral gavage. Animals were shaved on their dorsal surface 24 hours post-treatment and animals were sacrificed by cervical dislocation. Dorsal skin sections were fixed in Bouin's fixative for 3-4 days and washed in 50% and 70% ethanol for two days.

2.7.b. Tumour studies

Tumour studies have been described previously (Owens *et al.*, 1999). Briefly, female FVB/N mice, 6 weeks of age, were shaved on their dorsal surface. After two weeks, mice that were in the resting phase of their hair cycle received one topical application of 200 nmol of DMBA in 200 µl acetone. One week later, mice were treated topically twice a week for a period of 6 weeks with 5 nmol TPA in 200 µl acetone. TPA treatments were stopped for one week, after which mice were treated topically with 200 nmol DMBA in 200 µl acetone (second initiation step). One week later, treatment with 5 nmol TPA was resumed for 40 more weeks. Papillomas were scored once weekly. FVB/N mice were sacrificed by cervical dislocation, skin tumours were harvested and small portions were snap frozen and stored at -80°C prior to northern blot analysis.

2.8. Immunohistochemistry

Dorsal skin samples from 2-3 month old CD-1 mice were fixed in Bouin's solution, embedded in paraffin and sectioned at 7 µm. Immunohistochemistry was performed as described previously (Attar *et al.*, 1997; Imakado *et al.*, 1995). Briefly, after deparaffination and rehydration, sections were blocked with 10% serum (Sigma) in PBS/0.2% Tween-20 at room temperature for 1 hour and subsequently incubated with primary antibodies (K5 (Covance), 1:500 dilution; gas3 (from Suter U), 1:500 dilution) at 4°C overnight. Slides were then washed three times with PBS/0.2% Tween-20 re-

blocked with 10% serum and incubated with biotinylated secondary antibodies (1:150 dilution, Vector Laboratories) for one hour. Samples were then washed and incubated with streptavidin-horseradish peroxidase (1:1000, Perkin Elmer) and reactivity revealed by incubation with diamino benzidine (DAB, Sigma) solution in TBS (DAB, 0.05%; imidazole, 0.01 M; $NiCl_2$, 0.064%; H_2O_2, 0.009%) for 5-10 minutes. Slides were counterstained with methyl green (Sigma) before mounting.

Chapter III

Results

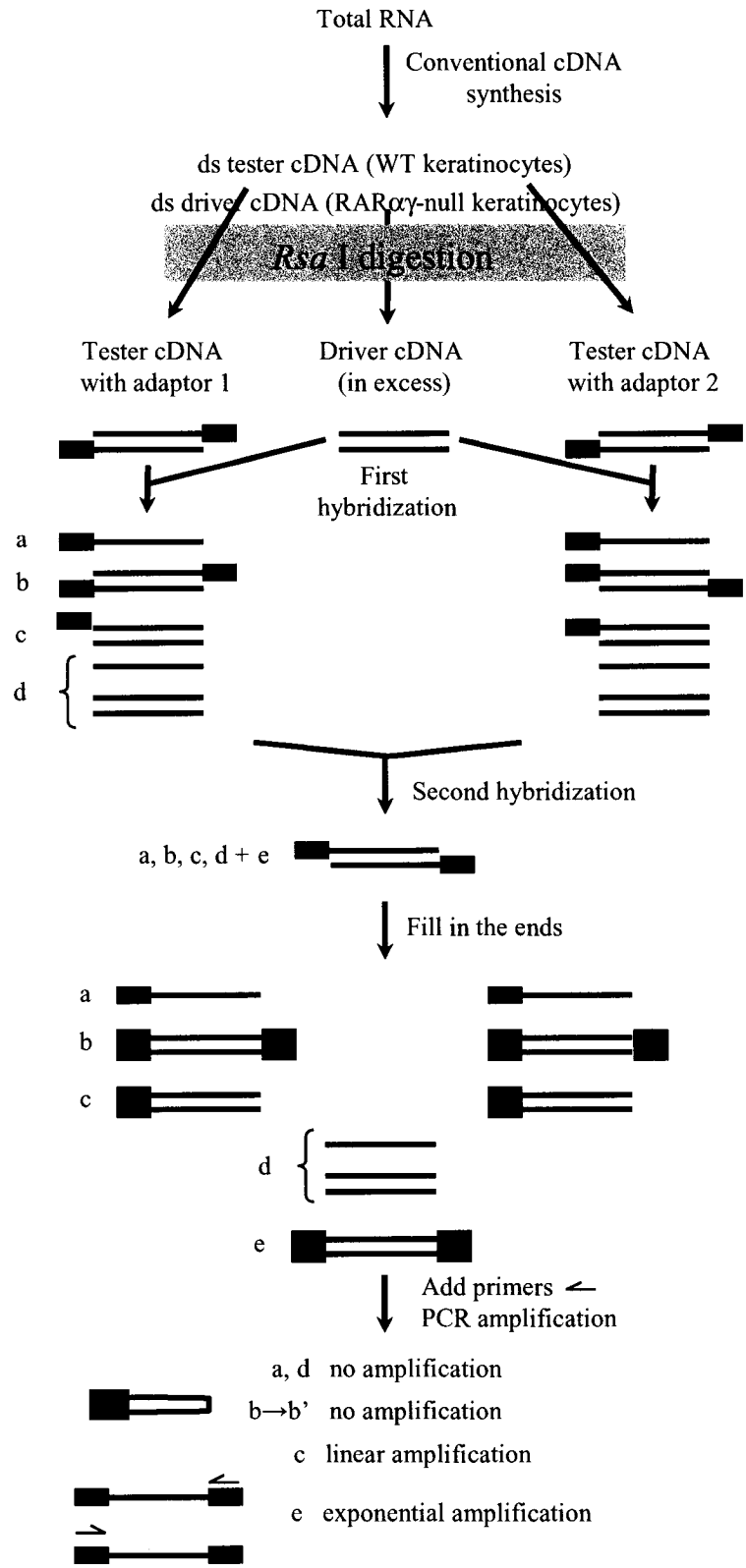
3. Results

3.1. Suppression Subtractive Hybridization (SSH)

Unlike other subtraction methods, very small amounts of RNA (500ng-2μg poly A^+ RNA) are required for SSH. This amount can be easily derived from little material. Moreover, SSH can overcome many of the limitations of other subtraction methods and also offers greater than 1000-fold enrichment for differentially expressed genes.

The forward subtraction, used here, was designed to enrich for genes that are expressed in RA-treated wild-type keratinocytes (tester population) but reduced or absent in treated RARαγ-null keratinocytes (driver population). To generate the subtracted cDNA library, several steps were required including mRNA preparation, cDNA synthesis, adaptor ligation, hybridization of the tester and driver populations and PCR amplification of the subtracted cDNA fragments (Figure 3-1). RNA was isolated from wild-type and RARαγ-null keratinocytes treated for 6 hours with 1 μM RA. The restriction enzyme *Rsa*I was used to digest cDNA into smaller fragments because it generated the largest average size of fragments (about 600 bp; Figure 3-1). Furthermore, cutting cDNAs into small fragments allowed a better representation of individual genes and prevented the formation of complex networks arising from long DNA fragments that may inhibit the formation of appropriate hybrids. In the first hybridization step, an excess driver cDNA was added to each tester cDNA, and the samples were heat denatured and allowed to anneal. The remaining single-stranded cDNAs were normalized, meaning that low and high abundance cDNA became roughly equal. Furthermore, tester single-stranded cDNAs were enriched for differentially expressed sequences as non-target cDNAs present in the tester and driver cDNA form hybrids. In the second hybridization step, the samples from the first hybridization were mixed together and fresh denatured driver cDNA was added to further eliminate common sequences between the two populations. New hybrid molecules (b, c and e; Figure 3-1) were formed which consisted of differentially expressed cDNAs with different adaptors on each end. The last step remaining was PCR amplification of the differentially expressed cDNAs. In the first amplification, only double-stranded cDNAs with different adaptor sequences on each end were amplified. In the second

Figure 3-1. Suppression Subtractive Hybridization (SSH)
The SSH technique was designed to enrich for genes that are expressed in wild-type keratinocytes but reduced or absent in RARαγ-null keratinocytes. Briefly, this technique relies on several steps including cDNA synthesis, adaptor ligation, hybridization of the tester and driver populations and PCR amplification of the subtracted cDNA fragments. See text for details.


Total RNA
Conventional cDNA synthesis
ds tester cDNA (WT keratinocytes)
ds driver cDNA (RARαγ-null keratinocytes)
Rsa I digestion
Tester cDNA with adaptor 1
Driver cDNA (in excess)
Tester cDNA with adaptor 2
First hybridization
a
b
c
d
Second hybridization
a, b, c, d + e
Fill in the ends
a
b
c
d
e
Add primers
PCR amplification
a, d no amplification
b→b' no amplification
c linear amplification
e exponential amplification

amplification, nested PCR was used to further reduce background and to enrich for differentially expressed fragments. The cDNA fragments were then cloned into an A-T cloning vector to generate the subtracted cDNA library.

3.2. Identification of differentially expressed transcripts from SSH

The subtracted cDNA samples were compared by reverse Northern analysis as described in Section 2.2 using DNA isolated from single colonies. Briefly, inserts were excised by digestion with Sst I/Sst II, obtained from SSH and run on duplicate agarose gels and transferred to nylon membranes. The blots were hybridized with equivalent c.p.m. of ^{32}P-labeled cDNA. Briefly, poly A+ RNA was purified from wild-type and RARαγ-null keratinocytes treated for 6 hours with 1 μM RA and reverse transcribed with M-MLV reverse transcriptase. The cDNA reaction was then labelled with ^{32}P-α-dCTP. *Tgase II* or *claudin 6* cDNAs were included in all blots as positive controls. *Tgase II* is a classic RA-target gene in the skin and, as expected, was recovered several times from our screen. We showed that claudin 6 was modestly induced in wild-type and undetectable in RARαγ-null keratinocytes and thus, it was also a good control for differential expression. The fact that both *claudin 6* and *Tgase II* were more abundant in wild-type keratinocytes versus RARαγ-null keratinocytes indicated that this pool was enriched for RA-induced genes (Figure 3-2).

One thousand two hundred-and-forty-one clones were screened by reverse Northern and 725 cDNA clones generated a signal after exposure of one to three days (Table 3-1). cDNA fragments that represent low abundance messages could likely not be detected by this method. Of the 725 clones, 219 (17.6%) were more abundant in the RA-treated wild-type cDNA population relative to RARαγ-null. However, some cDNAs gave a very strong signal in wild-type keratinocytes. These were homologous to retrotransposons or as ribosomal RNAs (green dots on Figure 3-2). Retrotransposons and ribosomal RNAs are known RA-target genes but they were not interesting for our studies (Islam *et al.*, 1993). In order to avoid picking these candidate genes, we decided to choose clones whose expression in wild-type keratinocytes was less than that typical of retrotransposons and ribosomal RNAs.

Figure 3-2. Differential expression of RA-candidate genes revealed by reverse northern analysis.

Equal amounts of digested cDNA clones were run on two agarose gels and transferred to nylon membranes. The blots were hybridized with equivalent c.p.m. of ^{32}P-labeled cDNA generated from RNA extracted from wild-type and RARαγ-null keratinocyte cell lines treated for 6 hours with 1μM RA. Comparison of the blots reveals that some genes are induced in wild-type compared to RARαγ-null cultures. *Claudin 6* or *Tgase II* (depending on the membranes) were used as positive controls whereas β-actin served as a control for RNA integrity. Red dots represent candidate clones that were chosen whereas the green dot corresponds to rRNA.

Wild-type RAR population

RARαγ-null population

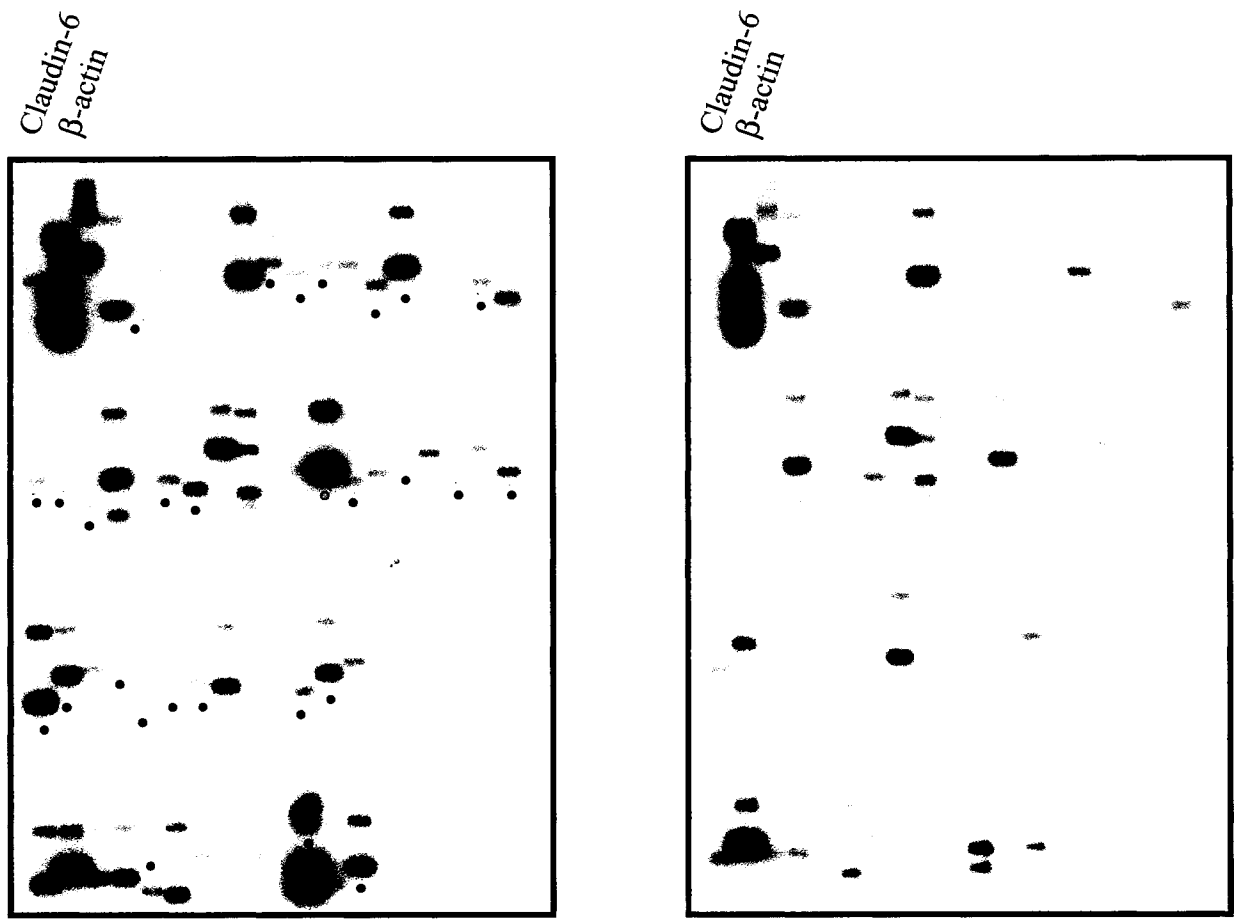


Table 3.1. Suppression Subtractive Hybridization Results

Reverse Northern Analysis	
Total number of clones screened	1241
Total number of clones giving a signal	725
Total number of clones that appeared more abundant in wild-type keratinocytes	219
Percentage of visible clones induced by RA	30.2 %

Northern Analysis	
Total number of clones screened	75
Total number of clones giving a signal	68
Induced by RA in wild-type keratinocytes, but not in RARαγ$^{-/-}$ cells	32
Not induced by RA in wild-type keratinocytes, absent/lower in RARαγ$^{-/-}$ cells	7
Not induced by RA in wild-type or RAR-null keratinocytes	29
Percentage of visible clones induced by RA	47.0 %

3.3. Identification of RA-regulated genes

To validate regulation and to eliminate any remaining false positives, Northern blot analysis was performed using RNA from wild-type or RARαγ-null keratinocytes treated for 6 hours with 1 μM RA (data not shown). As shown in Table 3-1, 68 of the 75 clones tested gave a signal by Northern analysis. Of these, 32 (42.3%) clones appeared to be induced by RA while the remaining 36 (53.3%) clones exhibited no difference between untreated and RA-treated wild-type keratinocytes but reduced in RARαγ$^{-/-}$ keratinocytes. Three classes of expression were observed, 32 clones were induced in wild-type keratinocytes 6 hours post-treatment with RA and were expressed, but not induced, in RARαγ-null cells. The second category was composed of 29 clones that were not induced by RA in either wild-type or RARαγ-null keratinocytes. The remaining 7 clones were also not induced in wild-type cells 6 hours post-treatment but their expression was undetectable in RARαγ-null keratinocytes. Only the clones that were induced by RA in wild-type keratinocytes and those that were not up regulated but largely reduced in RAR-null keratinocytes on Northerns were end-sequenced. A summary of sequence homologies and regulation is presented in Table 3-2.

For time course analysis, Northern blots were performed using RNA from wild-type and RARαγ-null keratinocytes. The wild-type cell line was treated with 1 μM RA and harvested at different time points ranging from 2 hours to 48 hours. RARαγ-null keratinocytes were treated with either 1 μM RA or 1 μM 4-HPR and harvested at 24 hours. As described in section 1.6.3, 4-HPR binds and activates RARγ but can also mediate its effects through RA-independent mechanisms. Thus, it was a valuable tool to determine if RA-regulated genes could also be induced independently of RARs in keratinocytes.

Clones #1 and #2 are homologous to the *Tgase I* and *II* genes. Transglutaminases are enzymes that catalyze various post-translational modifications, including transamidation, esterification and hydrolysis of proteins, and are induced by RA in skin (Lorand and Graham, 2003; Rubin and Rice, 1986; Yuspa *et al.*, 1982). As expected, we recovered Tgase II several times from the screen (Table 3-2), suggesting that the screen worked efficiently and that *Tgase II* was a good control for time course analysis.

Table 3.2. RA-regulated candidate genes

Clone Number	*Sequence homology*	*Expression pattern*
1	Transglutaminase I	induced in WT, not induced in DM
2	Transglutaminase II	induced in WT, not induced in DM
3	Transglutaminase III	induced in WT, not induced in DM
4	Gas 3	induced in WT, not induced in DM
5	NFAT5	induced in WT
6	Mpf	induced in WT
7	Not sequenced	not induced in WT, not induced in DM
8	Not sequenced	not induced in WT, not induced in DM
9	VL-30	induced in WT, not induced in DM
10	KIAA0227	induced in WT, not induced in DM
11	Not sequenced	not induced in WT, not induced in DM
12	MEA6/MGEA11	induced in WT, not induced in DM
13	No stong homologies	induced in WT, not induced in DM
14	Not sequenced	induced in WT, not induced in DM
15	VL-30	induced in WT, not induced in DM
16	KIAA0852	induced in WT, not induced in DM
17	VL-30	induced in WT, not induced in DM
18	Not sequenced	induced in WT, not induced in DM
19	TM protein ER/Golgi	induced in WT, not induced in DM
20	Claudin 6	induced in WT, not induced in DM
21	No stong homologies	induced in WT, not induced in DM
22	MRGX	not induced in WT, not induced in DM
23	Claudin 6	not induced in WT, absent in DM
24	MRGX	not induced in WT, not induced in DM
25	CTLA-2 α/β	induced in WT, not induced in DM
26	clone 1958033	not induced in WT, absent in DM
27	No stong homologies	induced in WT, not induced in DM
28	No stong homologies	not induced in WT, absent in DM
29	A330094D24	induced in WT, not induced in DM
30	MRGX	induced in WT, not induced in DM
31	Retrovirus	not induced in WT, absent in DM
32	MRGX	not induced in WT, not induced in DM
33	Not sequenced	not induced in WT, not induced in DM
34	Claudin 6	not induced in WT, absent in DM
35	Not sequenced	induced in WT, not induced in DM
36	Not sequenced	Not visible
37	Not sequenced	not induced in WT, not induced in DM
38	Not sequenced	not induced in WT, not induced in DM

Table 3.2. Cont'd RA-regulated candidate genes

Clone Number	*Sequence homology*	*Expression pattern*
39	Not sequenced	not induced in WT, not induced in DM
40	Claudin 6	not induced in WT, absent in DM
41	Not sequenced	Not visible
42	VL-30	not induced in WT, not induced in DM
43	Not sequenced	induced in WT, not induced in DM
44	Not sequenced	not induced in WT, not induced in DM
45	Not sequenced	Not visible
46	Not sequenced	Not visible
47	Not sequenced	not induced in WT, not induced in DM
48	Claudin 6	induced in WT, absent in DM
49	MRGX	not induced in WT, not induced in DM
50	Not sequenced	induced in WT, not induced in DM
51	Not sequenced	not induced in WT, not induced in DM
52	Not sequenced	not induced in WT, not induced in DM
53	Tm7sf1	induced in WT, absent in DM
54	Claudin 6	not induced in WT, absent in DM
55	Cystatin B	induced in WT, not induced in DM
56	VL-30	not induced in WT, not induced in DM
57	MRGX	not induced in WT, not induced in DM
58	VL-30	not induced in WT, not induced in DM
59	Transglutaminase II	induced in WT, not induced in DM
60	Not sequenced	not induced in WT, not induced in DM
61	Not sequenced	induced in WT, not induced in DM
62	Not sequenced	not induced in WT, not induced in DM
63	Gag	not induced in WT, not induced in DM
64	VL-30	not induced in WT, not induced in DM
65	Not sequenced	induced in WT, not induced in DM
66	Transglutaminase II	induced in WT, not induced in DM
67	Not sequenced	not induced in WT, not induced in DM
68	Not sequenced	Not visible
69	Not sequenced	Not visible
70	Not sequenced	not induced in WT, not induced in DM
71	Not sequenced	not induced in WT, not induced in DM
72	Not sequenced	not induced in WT, not induced in DM
73	Not sequenced	Not visible
74	Not sequenced	not induced in WT, not induced in DM
75	Not sequenced	not induced in WT, not induced in DM

DM, double mutant or RARαγ-null keratinocytes; WT, wild-type keratinocytes

Expression of *Tgase II* in wild-type keratinocytes was induced as early as 8 hours, with maximal levels attained at 48 hours following RA treatment while *Tgase I* was up-regulated after 2 hours but its expression only peaked at 24 hours (Figure 3-3). Tgase I and Tgase II were both expressed in RARαγ-null keratinocytes, but at lower levels, and were not induced 24 hours post-treatment with RA or 4-HPR. The basal expression also suggests that other pathways regulate these genes in keratinocytes.

Clone #4 is homologous to a gene called growth arrest-specific gene 3 (*gas3*), also known as peripheral myelin protein 22 (*Pmp22*) in humans. Gas3 is an important component of the peripheral nervous system (PNS) and point mutations in gas3 cause severe neuropathies including the Charcot-Marie-Tooth disease type 1, Dejerine- Sottas syndrome and hereditary neuropathy with liability to pressure palsies, among others (Jetten and Sutter, 2001). Although well characterized in the PNS, its function has never been studied in skin. The expression of *gas3* in wild-type keratinocytes was induced as early as 2 hours in response to RA with maximal levels attained 48 hours post-treatment (Figure 3-3). *Gas3* was not induced following RA nor 4-HPR treatments in RARαγ-null keratinocytes. However, *gas3* is still expressed at low levels in these cells, suggesting that it is regulated, in part, by retinoid-independent mechanisms in keratinocytes.

Clone #55 is homologous to cystatin B, a cystein protease inhibitor. The expression of cystatin B in wild-type keratinocytes was slightly induced after 8 hours with maximal levels attained 16 hours post-treatment (Figure 3-3). Cystatin B transcripts were also highly expressed in RARαγ-null keratinocytes. Several lines of evidence have indicated that cystatin B is expressed in head and neck squamous cell carcinoma, especially during metastasis and inflammation (Budihna *et al.*, 1996).

A second category of candidate genes that were isolated was those that were not induced by RA on time courses but that were largely reduced or undetectable in RARαγ-null keratinocytes. Clone #20 is homologous to claudin 6, which encodes a tight junction protein of the epidermis. This protein had previously been shown to be induced by RA in F9 cells (Kubota *et al.*, 2001). In our time course studies, expression of *claudin 6* appeared to be very slightly up-regulated by RA after 2 hours, with

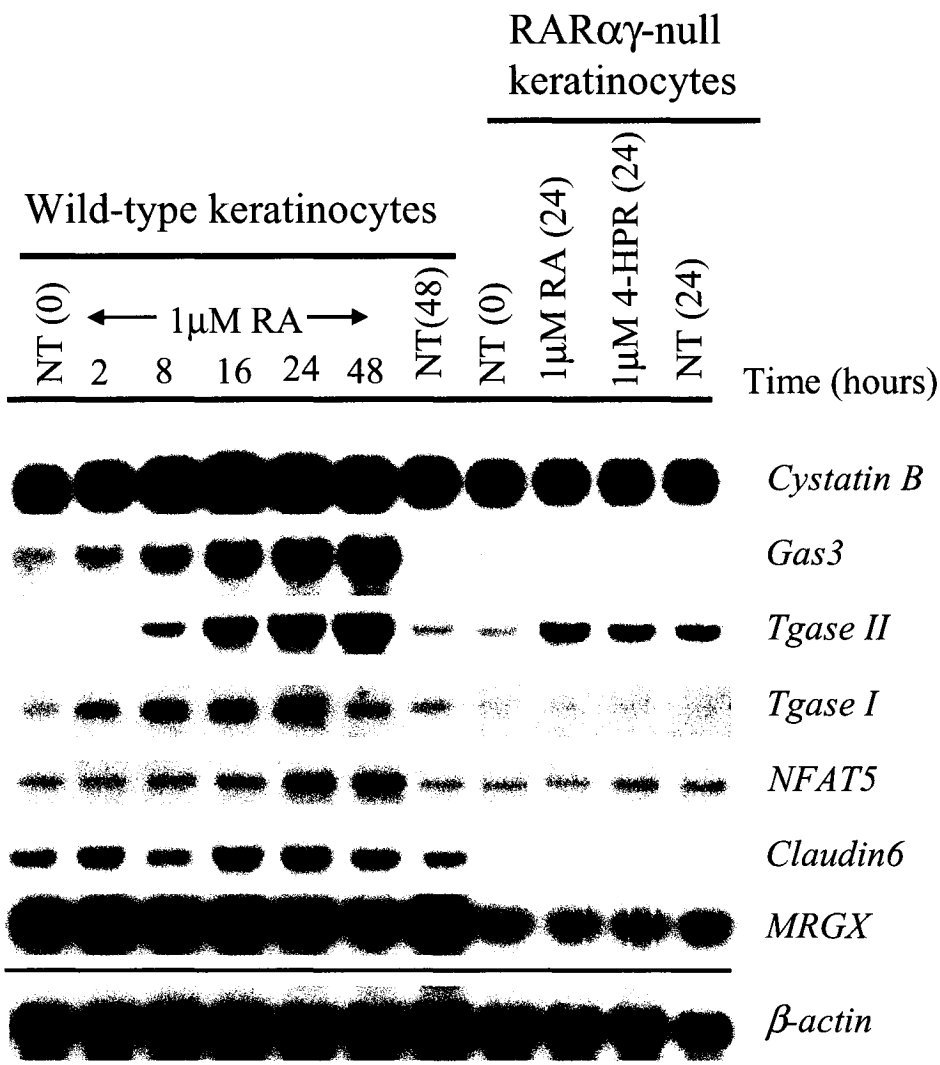


Figure 3-3. Temporal induction of retinoic acid-responsive genes
Northern blot analysis of total RNA (15μg) from wild-type and RARαγ-null immortalized keratinocytes treated for various times with all-*trans* RA (1μM) or 4-HPR (1μM). The blot was probed with cDNAs encoding *Cystatin B*, *gas3*, *Tgase II*, *Tgase I*, *NFAT5*, *Claudin 6* or *MRGX* as indicated. Hybridization to β-*actin* was used as a loading control.

maximum levels attained at 24 hours post-treatment (Figure 3-3). Moreover, *claudin 6* mRNA was undetectable in RARαγ-null keratinocytes. We isolated claudin 6 six times from the subtracted library (clone #20, #23, #34, #40, #48, #54; Table 3-2). However, induction was reproducible (Table 3-2). This may reflect differences in cell densities at the time the cells were treated or other variables.

Clone #22 is homologous to a gene called MORF-related growth factor X (MRGX), a member of the MORF-related transcription factor gene family located on chromosome X (Bertram *et al.*, 1999). These transcription factors are involved in cell senescence in mice and humans. MRGX was not affected by RA treatment in wild-type keratinocytes but its expression was reduced in the RARαγ-null cell line (Figure 3-3).

Primary and secondary screens as well as time course studies were done with immortalized keratinocytes. In order to assess if *gas3* was induced by RA in a more physiological context, we tested its pattern of expression in primary keratinocytes. Northern blot analysis on RNA from primary keratinocytes treated with 1 μM RA and harvested at time points ranging from 2 hours to 48 hours was performed. As with immortalized wild-type keratinocytes, expression of *gas3* was slightly induced by RA after 2 hours with maximal levels attained 48 hours post-treatment (Figure 3-4). Figure 3-4 also shows two different *gas3* expression levels over a period of 48 hours (lane 1 and 7). This is due to trace amounts of RA in the fetal calf serum as it is not treated to remove RA. The pattern of expression of *gas3* was similar to that of Tgase II, a known RA-target gene.

3.4. Gas3 expression in skin in vivo

In order to assess if *gas3* is expressed in skin *in vivo*, we performed immunohistochemichal analysis.

Transverse dorsal skin sections were incubated with primary antibodies against K5 or Gas3. K5, a marker of basal keratinocytes, was restricted to basal keratinocytes in the epidermis and in hair follicles (Figure 3-5B, C). Gas3 was expressed throughout the epidermis (Figure 3-5D). Moreover, positive staining was also detected in hair follicles (Figure 3-5E) and in sebaceous glands (Figure 3-5F). We are currently investigating the epidermal expression of Gas3 in response to RA.

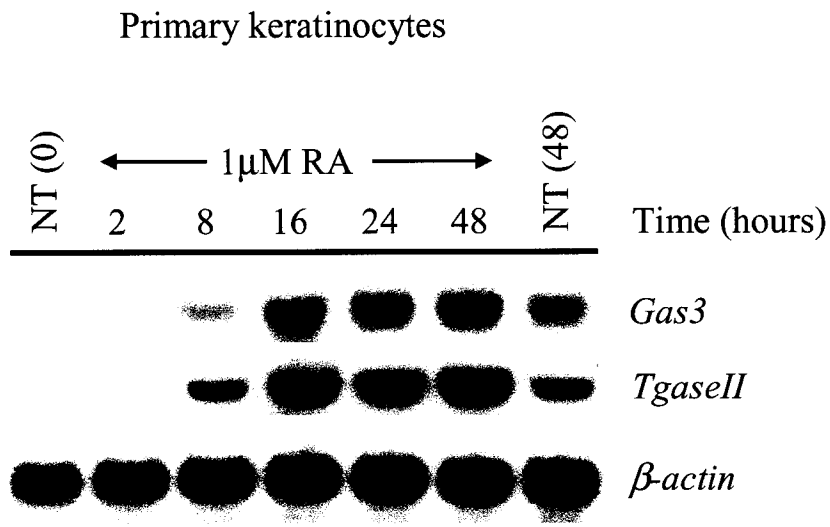


Figure 3-4. *Gas3* is induced by RA in primary keratinocytes.
Northern blot analysis of total RNA (15μg) from primary keratinocytes treated for different times with 1 μM RA. The blot was probed with cDNAs encoding *Gas3* or *Tgase II* as denoted on the right. β-actin was used as loading control.

3.5. Gas3 induction by RA is independent of de novo protein synthesis

Retinoic acid induces the transcription of a number of genes in the epidermis. Some genes may be direct targets while others are indirectly regulated. As an example, *Tgase II* is expressed in the epidermis, is induced very early in our time course studies and has been shown to be a direct RA-target gene (Nagy *et al.*, 1997). It was very tempting to speculate that *gas3* is also a direct RA-target gene as it was induced very early in response to RA in keratinocytes, similar to *Tgase II.* To begin to test this hypothesis, cycloheximide (CHX) poisoning experiments were performed.

Northern blot analysis using RNA from wild-type keratinocytes treated with carrier (DMSO), 10^{-6} M RA and/or 15μg/ml or 30μg/ml CHX and harvested 6 hours post-treatment were performed. Cycloheximide has been shown to block protein synthesis in a number of other systems at these concentrations. Cells were pre-treated for one hour with CHX before addition of RA to the culture medium. As expected, *gas3* mRNA levels were induced 6 hours following RA treatment (Figure 3-6). An increase in message abundance was also observed upon CHX treatment. This suggests an increase in stability of gas3 message, a general phenomenon observed for many genes. *Gas3* mRNA levels were also highly induced upon treatment with RA and CHX. These results strongly indicate that induction of *gas3* does not depend on *de novo* protein synthesis, consistent with it being a direct RA-target gene.

3.6. Expression of gas3 is reduced in papillomas

RAR transcripts are frequently lost following epidermal tumor promotion and malignant progression (Darwiche *et al.*, 1995). Recent studies from our lab have indicated that RARs, in particular RARγ, suppress Ha-*Ras* induced tumorigenesis and functions as a tumor suppressor gene in the epidermis (Chen *et al.*, 2004). To further investigate the relationship between *gas3* and tumorigenesis, we have assessed the expression of *gas3* in chemically induced papillomas.

Papillomas were generated by the mouse multistage carcinogenesis protocol, as described in section *2.7.b.* Northern blot analysis revealed that *gas3* expression was profoundly reduced in all papillomas tested (Figure 3-7). High expression of K10,

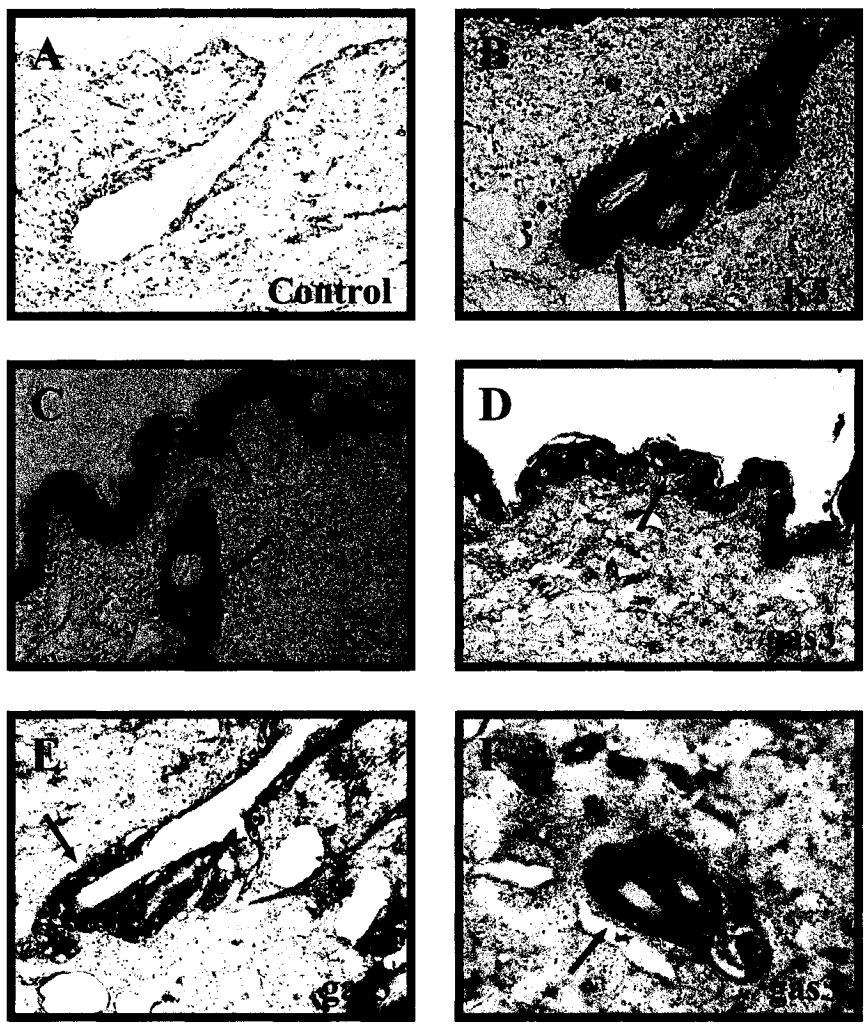


Figure 3-5. Gas3 expression in murine skin.
Immunohistochemical analysis of transverse dorsal skin sections from female CD-1 mice stained with K5 (B, C) or gas3 (D, E, F) primary antibodies. (A) No primary antibody control. K5 expression is restricted to hair follicles (B) and the basal layer in the epidermis (C). Expression of gas3 was located to the epidermis (D), hair follicles (E) and sebaceous glands (F). Arrow indicates positive staining. 400X.

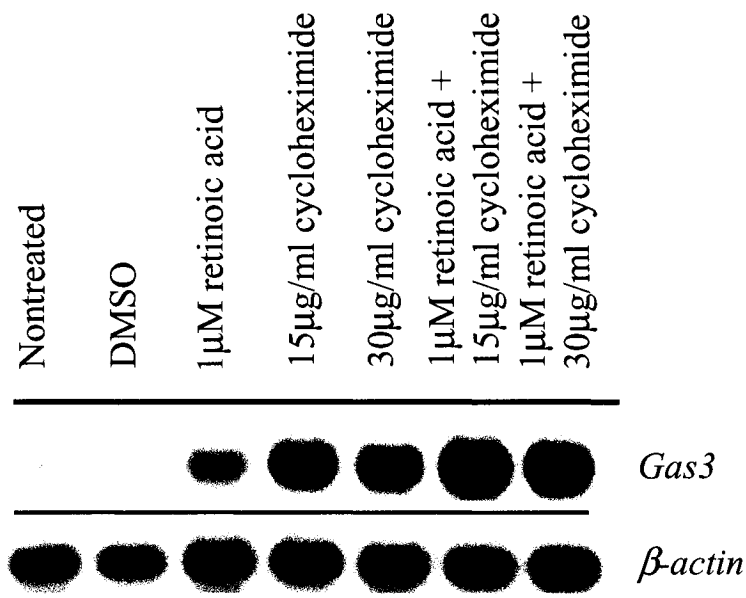


Figure 3-6. *Gas3* induction by RA is independent of *de novo* protein synthesis.

Northern blot analysis of total RNA (15μg) from wild-type immortalized keratinocytes treated for 6 hours with carrier (DMSO), 10^{-6} M RA and/or 15mg/ml or 30mg/ml cycloheximide. The blot was probed with *gas3* cDNA as noted on the right. Hybridization with *β-actin* was used as a loading control.

indicative of tumour progression, was also observed in these samples (Nelson and Slaga, 1982). Analysis of *gas3* in squamous carcinomas has not been carried out yet. These studies suggest that suppression of *gas3* is an early event in carcinogenesis.

3.7. Antagonistic effects of RA and TPA

TPA is widely used in the murine two-stage carcinogenesis model to promote skin tumour formation. RA has been shown to inhibit papilloma formation by interfering with this promotion step. To investigate the relationship between *gas3*, RA and/or TPA, we have assessed expression of *gas3* in response to RA and/or TPA.

Wild-type keratinocytes treated with DMSO, 10^{-6} M RA and/or 100 ng/ml TPA and harvested for northern blot analysis at different time points ranging from 6 to 24 hours. We used 100 ng/ml TPA because it is the concentration used to induce the formation of tumors in diverse models. As expected from our time course studies (Figure 3-3), *gas3* was induced in response to RA treatment. Of note, we found that TPA had the opposite effect on expression of *gas3* at all time points tested (Figure 3-8). Furthermore, repression of *gas3* was also observed upon combined treatment with RA and TPA, with RA having a slight effect on *gas3* expression. Indeed, inhibition of *gas3* by TPA may correlate with the increase in the number of papillomas observed on skin. In order to address that *gas3* behaves as a tumor suppressor, we need to perform dose-response experiments with TPA and find if TPA has an effect on *gas3* promoter.

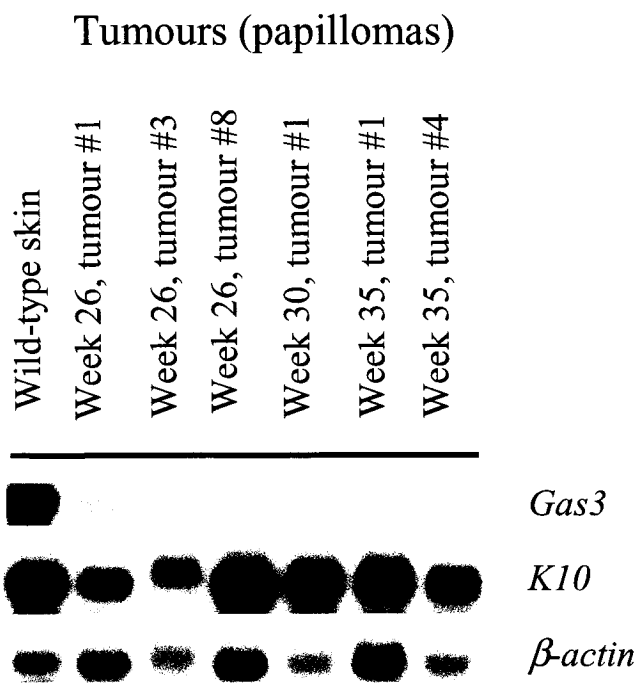


Figure 3-7. *Gas3* expression is reduced in papillomas.
Northern blot analysis of total RNA (15μg) from wild-type skin or papillomas harvested between weeks 26 and 35 following tumor promotion. Papillomas were generated by the mouse two-stage skin carcinogenesis protocol. The blot was probed with cDNA encoding *Gas3* or *K10* (positive control) as denoted on the right. Hybridization to β-*actin* was used as loading control.

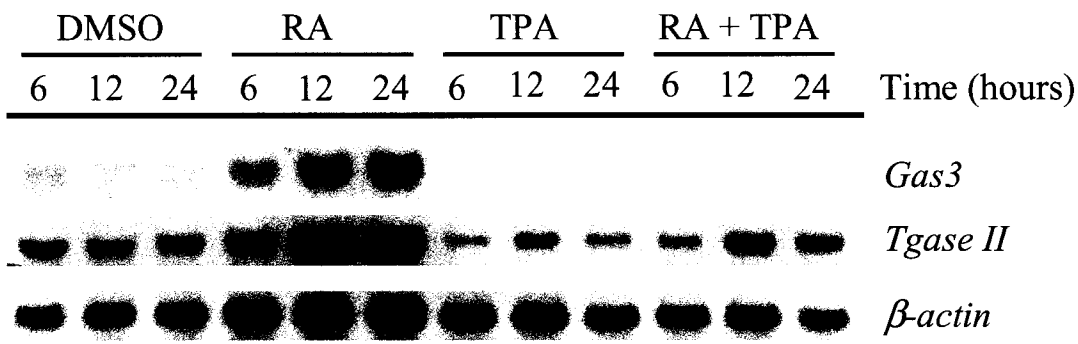


Figure 3-8. TPA antagonizes the induction of *gas3* by RA.
Northern blot analysis of total RNA (15μg) from wild-type keratinocytes treated for various times with carrier (DMSO), 1 μM RA and/or 100ng/ml TPA. The blot was probed with cDNA encoding *Gas3* or *Tgase II* as denoted on the right.

Chapter IV
General Discussion

4.1. Isolation of RA-regulated genes from keratinocytes

The epidermis expresses RARα and RARγ, with RARγ being the predominant receptor subtype (Fisher and Voorhees, 1996). Studies of transgenic mice expressing a dominant negative form of RARα in the epidermis provided evidence that the retinoid receptors were involved in keratinocyte differentiation (Saitou *et al.*, 1995). In contrast, the complete absence of RARs in the epidermis does not alter the proliferation potential of basal keratinocytes nor does it cause epidermal hyperproliferation, suggesting that they are not involved in the control of homeostatic epidermal proliferation under resting conditions (Chapellier *et al.*, 2002). On the other hand, these authors proposed that RA-induced epidermal hyperplasia is mediated by RXRα/RARγ heterodimers, suggesting that retinoid signaling plays important roles under pharmacological conditions.

Treatment of skin with tumor promoters or exposure to ultraviolet radiation leads to a decrease in expression of the RARs in the epidermis (Kumar *et al.*, 1994; Wang *et al.*, 1999). Furthermore, tumor promotion and malignant conversion are associated with the progressive loss of RARs in the skin (Darwiche *et al.*, 1995). Together, these studies strongly indicate that RARs are involved in epithelial tumorigenesis. Moreover, retinoids have been shown to interfere with tumorigenesis by attenuating the effects of tumor promoters.

The RARαγ-null keratinocyte cell line, developed by our group, is devoid of all RARs and is resistant to growth arrest in response to RA (Goyette *et al.*, 2000). Furthermore, *ras*-transduced RARαγ$^{-/-}$ keratinocytes form tumors *in vivo* and this outcome is reversed by re-introduction of a functional RAR, suggesting that the RARs act as tumor suppressors (Chen *et al.*, 2004). This cell line was therefore a valuable model to determine the means by which RARs suppress epidermal tumor formation. As there is still a gap in our knowledge of the genes that function downstream of the RARs involved in epithelial tumorigenesis, I have used suppression subtractive hybridization (SSH) to isolate genes that were expressed in wild-type keratinocytes in response to RA treatment versus RARαγ-null keratinocytes. Using reverse northern analysis, the mRNA levels of 219 clones isolated by SSH appeared more abundant in RA-treated wild-type relative to RAR-null keratinocytes. Several of these clones were assessed for

RA-regulation by Northern blot analysis and 42.3% were up-regulated in response to RA treatment.

Tgase I and Tgase II belong to a family of enzymes that carry out various biological functions, including cell envelope formation, to maintain skin integrity (Lorand and Graham, 2003). Transglutaminases have long been known to be induced by RA in the skin (Rubin and Rice, 1986; Yuspa *et al.*, 1982). As *Tgase II* was recovered several times in the screen, this indicates that the wild-type pool was enriched for RA-induced genes. *Tgase* I and *Tgase II* mRNA levels were induced early in response to RA with maximum levels attained 24 hours and 48 hours post-treatment but were not up-regulated by RA or 4-HPR in RARαγ-null keratinocytes.

Expression of *cystatin B* peaked early in response to RA with maximum levels attained at 16 hours. Moreover, *cystatin B* was not up-regulated by RA or 4-HPR in RAR-null keratinocytes. Many studies have associated cystatins with tumour invasion and metastasis in SCC of the head and neck (Budhina *et al.*, 1996; Strojan *et al.*, 2000, 2001). Furthermore, Ebert *et al.* observed that the activity of cystatin B in lung tumours correlated with survival probability, suggesting that cystatin B could act as a prognostic factor (Ebert *et al.*, 1997). This is likely a paradox as the same protein cannot behave differently in other tumors. Overall, these results suggest that cystatin B might play a role in epithelial tumorigenesis.

Even though some RA-target genes were not induced in wild-type keratinocytes, they may still be involved in epithelial differentiation. In support of this, we observed that the expression of several candidate genes was strongly reduced in RARαγ-null keratinocytes but not induced in wild-type keratinocytes. One of these genes was *claudin 6*, a tight junction (TJ) protein that belongs to a gene family with at least 20 members (Tsukita and Furuse, 2000). Claudins are integral components of TJs, which serve a major role in cell-cell adhesion of endothelial and epithelial cells. Transgenic mice overexpressing claudin 6 suffer from barrier dysfunction and die within two days, suggesting that this gene is involved in epidermal barrier formation (Turksen and Troy, 2002). Furthermore, several studies have reported reduced or undetectable *claudin 6* mRNA in mammary cancer cell lines, indicating that it may function as a tumour marker (Quan and Lu, 2003). In agreement with this, we have found that *claudin 6*

mRNA levels were undetectable in RAR-null keratinocytes. Also of note, Kubota *et al.* demonstrated that RA induces the expression of *claudin 6* in F9 cells (Kubota *et al.*, 2001). Similarly, we found that expression of claudin 6 was slightly induced 2 hours post-treatment in wild-type keratinocytes with maximum levels attained after 24 hours.

4.2. Regulation of gas3 expression by RA

The *pmp22* gene, also known as *gas3*, codes for a glycoprotein that belongs to the extended tetraspan membrane protein family (Taylor *et al.*, 1995). *Gas3* is expressed in schwann cells of the peripheral nervous system (PNS), where it localizes to the compact myelin, and in various non-neuronal tissues during embryogenesis and in the adult (Baechner *et al.*, 1995). The highest levels of *gas3* mRNA outside the PNS is found in epithelial cells of the lungs and intestines. However, the function of Gas3 in these tissues has not been resolved. Although the function of Gas3 in the PNS is also largely undefined, it is well established that mutations, deletions and duplications are responsible for several heritable demyelinating peripheral neuropathies, including Charcot-Marie-Tooth disease type 1 (CMT1; Jetten and Suter, 2001).

Using our immortalized cell lines, we found that *gas3* was induced by RA in wild-type keratinocytes. This induction could be detected very early in response to RA treatment, with maximal levels attained 48 hours post-treatment. Consistent with this, we also showed that *gas3* was up-regulated in primary keratinocytes as early as 2 hours in response to RA. Immunohistochemistry indicated that Gas3 is located in the epidermis, hair follicles and sebaceous glands. This pattern of expression in skin correlates with that of RARα and RARγ, consistent with Gas3 playing a role in RARα and RARγ-dependent epidermal functions.

4-HPR, a synthetic retinoid, can inhibit growth and induce apoptosis in various model systems (Webber *et al.*, 1999). Although it can act directly via RARγ, and to a lesser extent RARβ, its effects are also mediated by RA-independent mechanisms (Clifford *et al.*, 1999; Delia *et al.*, 1993; Formelli *et al.*, 1996; Goyette *et al.*, 2000). Interestingly, 4-HPR is able to induce apoptosis of RARαγ-null keratinocytes, clearly demonstrating an RA-independent mechanism (Chen *et al.*, 2004). Thus, this provided a good model to determine whether some RA-target genes could be induced

independently of the RARs in keratinocytes. My work demonstrated that *gas3* was neither induced in response to RA nor 4-HPR treatment in RAR-null keratinocytes, indicating that RA-induction of *gas3* is mediated solely by the RARs. As RAR transcripts are reduced in SCC, and *gas3* mRNA levels are strongly reduced in RARαγ-null keratinocytes, it is tempting to speculate that RARs mediate their tumour suppressive function, at least in part, through activation of *gas3* in the epidermis.

4.3. RA regulation of gas3 gene expression

As members of the *Tgase* family are direct RA-target genes, we speculated that *gas3* was directly regulated by retinoids based on its similar pattern of expression in keratinocytes. Cycloheximide (CHX) is a glutarimide antibiotic that specifically inhibits the translation of mRNA on the large 80S ribosomal subunit in eukaryotes (Bennett *et al.*, 1965; Obrig *et al.*, 1971). In response to CHX treatment, some messenger RNAs accumulate in the cell since they cannot be translated. This suggests that a subset mRNAs, which would normally be degraded early, are more stable (Tobey *et al.*, 1966). Consistent with this, *gas3* mRNA levels were strongly induced in response to CHX treatment. A further increase in message abundance was observed upon treatment with RA and CHX. Thus, this induction suggests both an increase in transcription and stability of gas3 message, a general phenomenon for immediate-early target genes (Ichikawa *et al.*, 2003; Magun et Rodland, 1995).

Additional studies are required to confirm if *gas3* is a direct RA-target gene. The *gas3* gene is regulated by two promoters that give rise to two different mRNAs that differ only in their 5' non-coding region (Saberan-Djoneidi *et al.*, 2000; Suter *et al.*, 1994). These two promoters are located immediately upstream of two alternative 5'-noncoding exons (exon 1A and 1B), where transcripts containing exon 1A (CD25 mRNA) are highly expressed in schwann cells whereas transcripts containing exon 1B (S13 mRNA) are predominant in non-neuronal tissues. RT-PCR studies have demonstrated that CD25 mRNA can be detected in the heart, lung, testis, tongue and skeletal muscle, in addition to its well-defined expression in peripheral nerves (van de Wetering *et al.*, 1999). Even though these tissues are strongly innervated organs that can contain small amounts of myelinated peripheral nerves, this strongly suggests that

promoter 1A is active in other tissues. We believe that one of these promoters may contain an RARE. To test this hypothesis, wild-type keratinocytes were transiently transfected with a luciferase reporter plasmid driven by promoter 1A. Although promoter 1A did not seem to respond to RA, an RARE may be located in promoter 1B or a region outside the two promoters.

4.4. Is gas3 involved in epithelial tumorigenesis?

Tumorigenesis is a complex multistage process that involves both genetic and epigenetic changes that occur in a predictable sequence (Yuspa, 1994, 1998). These changes affect cellular processes that control cell proliferation, differentiation, DNA repair and apoptosis. Understanding the molecular basis of tumorigenesis is essential for the development of therapeutic targets for cancer prevention and treatment. The murine model of chemical induced carcinogenesis is one of the best-defined experimental *in vivo* models of epithelial tumorigenesis (Yuspa, 1994). The development of squamous cell carcinoma in multistage carcinogenesis involves three stages including initiation, promotion and progression (Marks and Furstenberger, 1990). Exogenous retinoids are able to attenuate the effects of tumour promoters in this model (Chen *et al.*, 1995; De Luca *et al.*, 1996; Sun and Lotan, 2002). This observation prompted much research into retinoids as chemopreventive agents. Since then, retinoids have been found to display antitumorigenic effects in diverse model systems.

We were tempted to speculate that this gene plays a role in epithelial tumorigenesis as *gas3* mRNA levels were barely detectable in RARαγ-null keratinocytes. Our key finding was that suppression of *gas3* occurs early during multistep mouse skin carcinogenesis. Indeed, expression of *gas3* mRNA was detected in normal skin but was profoundly reduced in squamous papillomas. Hence, this suggest that downregulation of *gas3* is a common event in the development of SCC and may act as a tumour suppressor gene in the epidermis.

4.5. TPA represses expression of gas3

Promotion causes selective clonal outgrowth of initiated cells to produce benign squamous cell papillomas. This selective growth advantage results from the growth

stimulatory effects of exogenous tumor promoters. The most potent tumor promoters are the phorbol esters, like TPA, which activate PKC and give a growth advantage to DMBA-initiated cells. Retinoids, on the other hand, are able to attenuate the effects of tumour promoters in this model.

To understand the relationship of *gas3* expression in epithelial tumorigenesis, we investigated the effects of TPA on *gas3* in wild-type keratinocytes. Our major finding was that TPA repressed *gas3* mRNA levels as early as 6 hours post-treatment. This is a very rapid response, indicating that *gas3* is lost early during tumor promotion. *Gas3* expression was also repressed in wild-type keratinocytes treated with both RA and TPA. However, this block in induction by RA may be due, in part, by the concentration of TPA used for these studies. Even though it is the concentration most frequently used in cell culture, it might be too high for RA to overcome the block when cells are treated with both RA and TPA. Another possibility is that TPA would be dominant over RA for *gas3* expression. Therefore, this would mean that *gas3* is not a key player in TPA-mediated tumor promotion. To test this possibility, one could perform dose-response experiments with TPA, by keeping RA concentration constant, and look at *gas3* mRNA levels. If RA is able to counteract the effects of TPA at lower doses of TPA, then gas3 is an important player in TPA-mediated tumor promotion.

Within cells, TPA mimics the effects of the second messenger diacylglycerol (DAG), activating several PKC isoforms and influencing cellular signalling and gene regulation (Mellor and Parker, 1998). TPA also induces the expression of many genes associated with signal transduction, cell cycle, epithelial differentiation, cell metabolism and cell structure. These include *Vl30*, *Jun B*, *cathepsin*, *serine proteinase*, *metallothionein 1* and *2*, s100 protein family and *orthinine decarboxylase* (*ODC*), among others (Schlingemann *et al.*, 2003). One class of transcription factors that mediates gene regulation in response to phorbol esters is the AP-1 complex. Increased expression of AP-1 target genes is observed both after treatment of murine skin with TPA and in different stages of skin tumor development (Tuckermann *et al.*, 1999). It is therefore possible that downregulation of gas3 may be mediated by TPA-induced expression of AP-1.

The efficacy of retinoids in chemoprevention of cancer can be explained by several mechanisms. It has been suggested that the growth inhibitory effects of RA are mediated by *trans*-repression of the AP-1 complex (Goyette *et al.*, 2000; Jochum *et al.*, 2001; Karin *et al.*, 1997). The potential mechanisms that underlie *trans*-repression include competitive titration between RAR and AP-1 for common co-regulators such as CBP/p300, downregulation of c-Jun N-terminal kinase activity and inhibition of AP-1 DNA binding (Benkoussa *et al.*, 2002; Caelles *et al.*, 1997; Kamei *et al.*, 1996; Schule *et al.*, 1991). Recently, it has been suggested that RA does not seem to deplete AP-1 coactivators or AP-1 monomers but rather targets JunB and/or Fra-1 dimers (Suzukawa and Colburn, 2002). In support of this, TPA treatment increases the transcriptional activities of full-length JunB and Fra-1, but not the transactivation domain fusions, whereas RA suppresses their activity. It is possible that gas3 is an AP-1 target. To test this possibility, transient transfections with a luciferase reporter plasmid driven by the gas3 promoter or a region located outside of the promoter could be performed. Therefore, induction of gas3 expression in response to RA may result from the *trans*-repression of AP-1.

4.6. Future Perspectives

Our model suggests that RA activates the transcription of various target genes in the epidermis, including *gas3* and we hypothesize that Gas3 may contribute to the antitumorigenic effects of retinoids.

RARs are able to induce growth arrest of wild-type transformed keratinocytes. Cell cycle arrest is associated with the induction of $p21^{cip1}$ and $p27^{kip1}$ and loss of *cdk1* expression (Gartel *et al.*, 1996; Sgambato *et al.*, 2000). RARs have been found to impact on the expression of these proteins, correlating with growth arrest (Dimberg *et al.*, 2002; Langenfeld *et al.*, 1997; Liu *et al.*, 1996). However, the primary targets mediating RA-induced growth arrest are presently unknown. Several groups have reported that overexpression of *gas3* induces apoptosis and growth arrest of schwann cells (Fabbretti *et al.*, 1995; Zoidl *et al.*, 1995). However, growth appeared to be restricted to the induction of cell cycle entry of resting cells. In order to test if Gas3 carries out the same function in keratinocytes, we could generate wild-type

keratinocytes stably overexpressing *gas3* using a conditional system such as tet-on/off that allows induction/repression of gas3 expression by the simple addition/removal of tetracycline.

We have demonstrated that *gas3* mRNA levels are profoundly reduced in skin papillomas, suggesting that Gas3 may act as a tumor suppressor gene. To further confirm this hypothesis, one could study gas3 expression in several skin SCC cell lines. We now have *Gas3*$^{-/-}$ mice in our possession (generously provided by Dr. Ueli Suter). In order to test if Gas3 is truly involved in tumorigenesis, one could use the murine multistage carcinogenesis protocol, and compare Gas3$^{-/-}$ to wild-type mice. If Gas3 behaves as a tumor suppressor, we would expect to see an increased number of papillomas on gas3-null mice, tumors that appear more rapidly or a higher frequency of papillomas that progress to SCCs. Another way to test our theory would be to inject *ras*-transformed RARαγ-null keratinocytes stably overexpressing *gas3* into nude mice. Similar studies, from our lab, have clearly indicated that loss of RARγ or RARαγ leads to tumor formation (Chen *et al.*, 2004). Therefore, we anticipate that overexpression of *gas3* will inhibit tumor development in these mice. This studies will bring stronger evidence that gas3 is downstream of the RARs and inhibits tumor promotion.

Some of my work suggest that downregulation of *gas3* occurs early upon TPA treatment. To confirm that this outcome also occurs *in vivo*, we could repeat the same experiment but using mice instead of transformed keratinocytes. Thus, immunohistochemistry analysis of murine skin treated for various times with either acetone, RA and/or TPA could be performed.

4.7. Summary

The genes that function downstream of the RARs, which are involved in epithelial tumorigenesis are largely unknown. My work led to the cloning of *gas3* as a gene induced very early by RA in immortalized and primary keratinocytes. Moreover, *gas3* is expressed in the epidermis and epidermal appendages, indicating that it may play a role in RARα and RARγ epidermal functions. *Gas3* expression is also profoundly reduced in skin squamous cell papillomas and is repressed by TPA 6 hours post-treatment, consistent with a tumour suppressor function. Together, these studies

may lead to a better understanding of the role of gas3 in the epidermis and in tumorigenesis.

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Canadian Nuclear Safety Commission | Commission canadienne de sûreté nucléaire

03055-1-08.0

R SUBSTANCES AND ON DEVICES | PERMIS PORTANT SUR LES SUBSTANCES NUCLÉAIRES ET LES APPAREILS À RAYONNEMENT | Licence Number / Numéro de permis

AIRE DE PERMIS

Conformément à l'article 24 (2) de la Loi sur la sûreté et la réglementation nucléaires, le présent permis est délivré à:

Institut de recherches cliniques
de Montréal
110, avenue des Pins Ouest
Montréal (Québec)
H2W 1R7
Canada

Ci-après désigné sous le nom de «titulaire de permis»

)DE

Ce permis est valide du 1er février 2003 au 31 janvier 2008.

/ITÉS AUTORISÉES

Le présent permis autorise le titulaire à posséder, transférer, importer, exporter, utiliser et entreposer les substances nucléaires et les équipements autorisés qui sont énumérés dans la section IV) du présent permis.

Le présent permis est délivré pour le type d'utilisation: études de laboratoire - 10 laboratoires ou plus où des radio-isotopes sont utilisés ou manutentionnés (836)

STANCES NUCLÉAIRES ET ÉQUIPEMENT AUTORISÉ

ARTICLE	SUBSTANCE NUCLÉAIRE	SOURCE NON SCELLÉE QUANTITÉ MAXIMALE	ASSEMBLAGE DE LA SOURCE SCELLÉE QUANTITÉ MAXIMALE	ÉQUIPEMENT - FABRICANT ET MODÈLE
1	Calcium 45	100 MBq	s/o	s/o
2	Cobalt 57	40 MBq	s/o	s/o
3	Cobalt 59	40 MBq	s/o	s/o
4	Chrome 51	400 MBq	s/o	s/o
5	Fer 59	2 GBq	s/o	s/o
6	Hydrogène 3	3 GBq	s/o	s/o
7	Iode 123	500 MBq	s/o	s/o
8	Iode 125	3 GBq	s/o	s/o
9	Iode 131	500 MBq	s/o	s/o
10	Phosphore 32	30 GBq	s/o	s/o
11	Phosphore 33	100 MBq	s/o	s/o
12	Rubidium 86	40 MBq	s/o	s/o
13	Soufre 35	3 GBq	s/o	s/o
14	Zinc 65	40 MBq	s/o	s/o
15	Césium 137	s/o	400 kBq	s/o
16	Radium 226	s/o	400 kBq	s/o
17	Césium 137	s/o	1110 kBq	Beckman LS series
18	Radium 226	s/o	370 kBq	Wallac Série 1200

La quantité totale d'une substance nucléaire non scellée possédée ne doit pas excéder la quantité maximale qui est indiquée pour une source non scellée correspondante. La quantité de substance nucléaire par source scellée ne doit pas excéder la quantité maximale indiquée par source scellée correspondante. Les sources scellées doivent être utilisées seulement dans l'équipement indiqué corrrespondant.

ROIT(S) OÙ LES ACTIVITÉS AUTORISÉES PEUVENT ÊTRE EXERCÉES

utilisées ou entreposées à (aux)
endroit(s) suivant(s):

110, avenue des Pins Ouest

Montréal (Québec)

TIONS

. Interdiction visant l'utilisation chez les humains
Le permis n'autorise pas l'utilisation des substances nucléaires dans ou sur le corps d'une personne.
(2696-0)

. Classification des zones, pièces et enceintes
Le titulaire de permis désigne chaque zone, pièce ou enceinte où on utilise plus d'une quantité d'exemption d'une substance nucléaire non scellée à un moment donné selon la classification suivante :

(a) de niveau élementaire si la quantité ne dépasse pas 5 LAI,
(b) de niveau intermédiaire si la quantité utilisée ne dépasse pas 50 LAI,
(c) de niveau supérieur si la quantité ne dépasse pas 500 LAI,
(d) de confinement si la quantité dépasse 500 LAI;
(e) à vocation spéciale, avec l'autorisation écrite de la Commission ou d'une personne autorisée par celle-ci.

À l'exception du niveau élementaire, le titulaire de permis n'utilise pas de substances nucléaires non scellées dans ces zones, pièces ou enceintes sans l'autorisation écrite de la Commission ou d'une personne autorisée par celle-ci.
(2108-1)

. Liste des laboratoires
Le titulaire de permis tient à jour une liste de toutes les zones, salles et enceintes dans lesquelles plus d'une quantité d'exemption d'une substance nucléaire est utilisée ou stockée.
(2569-1)

. Procédures de laboratoire
Le titulaire affiche en tout temps et bien en évidence dans les zones, les salles ou les enceintes où des substances nucléaires sont manipulées une affiche sur la radioprotection qui a été approuvée par la Commission ou une personne autorisée par la Commission et qui correspond à la classification de la zone, de la salle ou de l'enceinte.
(2570-1)

. Surveillance thyroïdienne
La personne
a) qui utilise à un moment donné une quantité d'iode 125 ou d'iode 131 volatiles dépassant :
(i) 5 MBq dans une pièce ouverte,
(ii) 50 MBq dans une hotte,
(iii) 500 MBq dans une boîte à gants,
(iv) toute autre quantité dans une enceinte de confinement approuvée par écrit par la Commission ou une personne autorisée par celle-ci;
(b) qui est impliqué dans un déversement mettant en cause plus de 5 MBq d'iode 125 ou d'iode 131 volatiles;
(c) chez laquelle on détecte une contamination externe à l'iode 125 ou l'iode 131;
doit se prêter à un dépistage thyroïdien dans les cinq jours suivant l'exposition.
(2046-7)

. Dépistage thyroïdien
Le dépistage de l'iode 125 et de l'iode 131 internes se fait :
(a) par mesure directe à l'aide d'un instrument capable de détecter 1 kBq d'iode 125 ou d'iode 131;
(b) par essai biologique approuvé par la Commission ou une personne autorisée par celle-ci.
(2600-1)

. Essai biologique thyroïdien
Si la charge thyroïdienne dans une personne depasse 10 kBq d'iode 125 ou d'iode 131, le titulaire de permis doit présenter immédiatement un rapport préliminaire à la Commission ou à une personne autorisée par

celle-ci. Dans un délai de 24 heures, la personne en question doit subir des essaies biologiques par une personne autorisée par la Commission à offrir un service de dosimétrie interne.
(2601-4)

9. Dosimétrie des extrémités
Le titulaire de permis veille à ce que toute personne qui manipule un contenant renfermant plus de 50 MBq de phosphore 32, de strontium 89, d'yttrium 90, de samarium 153 ou de rhénium 186 porte une bague dosimètre. Le dosimètre est fourni et lu par un service de dosimétrie autorisé par la Commission.
(2578-0)

9. Critères de contamination
En ce qui a trait aux substances nucléaires figurant au tableau «Classification des radionucléides» du guide sur les demandes de permis, le titulaire de permis veille à ce que :
(a) la contamination non fixée dans toutes les zones, pièces ou enceintes où on utilise ou stocke des substances nucléaires non scellées ne dépasse pas :
(i) 3 Bq/cm2 pour tous les radionucléides de catégorie A,
(ii) 30 Bq/cm2 pour tous les radionucléides de catégorie B,
(iii) 300 Bq/cm2 pour tous les radionucléides de catégorie C,
selon une moyenne établie pour une surface ne dépassant pas 100 cm2;
(b) la contamination non fixée pour toutes les autres zones ne dépasse pas :
(i) 0,3 Bq/cm2 pour tous les radionucléides de catégorie A,
(ii) 3 Bq/cm2 pour tous les radionucléides de catégorie B,
(iii) 30 Bq/cm2 pour tous les radionucléides de catégorie C,
selon une moyenne établie pour une surface ne dépassant pas 100 cm2.
(2642-2)

10. Déclassement
Avant le déclassement d'une zone, d'une pièce ou d'une enceinte où s'est déroulée l'activité autorisée, le titulaire de permis veille à ce que :
(a) la contamination non fixée pour les substances nucléaires figurant au tableau «Classification des radionucléides» du guide sur les demandes de permis ne dépasse pas :
(i) 0,3 Bq/cm2 pour tous les radionucléides de catégorie A,
(ii) 3 Bq/cm2 pour tous les radionucléides de catégorie B,
(iii) 30 Bq/cm2 pour tous les radionucléides de catégorie C,
selon une moyenne établie pour une surface ne dépassant pas 100 cm2;
(b) la mise en disponibilité de toute zone, pièce ou enceinte contenant une contamination fixée soit approuvée par la Commission ou une personne autorisée par celle-ci;
(c) toutes les substances nucléaires et tous les appareils à rayonnement ont été transférés conformément aux conditions du permis;
(d) tous les panneaux de mise en garde contre les rayonnements ont été retirés ou ont été rendus illisible.
(2571-2)

11. Stockage
Le titulaire :
a) veille à ce que seules les personnes autorisées par lui aient accès aux substances nucléaires radioactives ou aux appareils à rayonnement stockés;
b) veille à ce qu'à tout endroit occupé à l'extérieur de la zone, de la salle ou de l'enceinte de stockage le débit de dose provenant des substances ou appareils stockés ne dépasse pas 2,5 microSv/h;
c) a des mesures en place pour assurer que les limites de dose indiquées dans le Règlement sur la radioprotection ne sont pas dépassées en raison du stockage de ces substances ou appareils.
(2575-0)

12. Évacuation (laboratoires)
Lorsqu'il évacue des substances nucléaires non scellées dans une décharge municipale ou un réseau d'égouts, le titulaire de permis veille à ce que les limites suivantes ne soient pas dépassées :

COLONNE 1	COLONNE 2(a)	COLONNE 3(b)
-	LIMITES	LIMITES
Substance	solides à la	liquides

nucléaire	décharge municipale (quantité au kg)	(hydrosolubles) l'égout municipal (quantité par an)
Carbone 14	3,7 MBq	10 000 MBq
Chrome 51	3,7 MBq	100 MBq
Cobalt 57	0,37 MBq	1000 MBq
Cobalt 58	0,37 MBq	100 MBq
Hydrogène 3	37 MBq	1 000 000 MBq
Iode 125	0,037 MBq	100 MBq
Iode 131	0,037 MBq	10 MBq
Phosphore 32	0,37 MBq	1 MBq
Phosphore 33	1 MBq	10 MBq
Soufre 35	0,37 MBq	1000 MBq
Technétium 99m	3,7 MBq	1000 MBq

(a) Les limites indiquées à la colonne 2 s'appliquent aux quantités de déchets solides de moins de trois tonnes par an. Les substances nucléaires évacuées dans la décharge municipale doivent être sous forme solide et distribuées uniformément dans les déchets; la concentration doit être inférieure aux limites indiquées à la colonne 2. Lorsqu'on évacue plus d'une substance nucléaire à la fois, le quotient obtenu en divisant la quantité de chaque substance par sa limite correspondante de la colonne 2 ne doit pas dépasser un.

(b) Les limites indiquées à la colonne 3 s'appliquent à la forme liquide (hydrosoluble) de chaque substance nucléaire qui peut être évacuée par an et par bâtiment.
(2161-3)

13. Exigences concernant les contaminamètres
Le titulaire de permis met en tout temps à la disposition des travailleurs sur les lieux de l'activité autorisée un contaminamètre portatif en bon état de fonctionnement.
(2572-1)

14. Rapport annuel de conformité
Deux mois avant l'anniversaire de la date d'expiration indiquée à la section II de son permis, le titulaire de permis soumet par écrit à la Commission, ou à une personne autorisée par celle-ci, un rapport annuel de conformité de l'activité autorisée qui contient des renseignements opérationnels suffisants et sous une forme que la Commission juge acceptable.
(2916-4)

Fonctionnaire désigné en vertu du paragraphe 37(2)(c) de la Loi sur la sûreté et la réglementation nucléaires

Copie de la commission