Chapter 1
History of Retinoic Acid Receptors

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Abstract The discovery of retinoic acid receptors arose from research into how vitamins are essential for life. Early studies indicated that Vitamin A was metabolized into an active factor, retinoic acid (RA), which regulates RNA and protein expression in cells. Each step forward in our understanding of retinoic acid in human health was accomplished by the development and application of new technologies. Development cDNA cloning techniques and discovery of nuclear receptors for steroid hormones provided the basis for identification of two classes of retinoic acid receptors, RARs and RXRs, each of which has three isoforms, α, β and ɣ. DNA manipulation and crystallographic studies revealed that the receptors contain discrete functional domains responsible for binding to DNA, ligands and cofactors. Ligand binding was shown to induce conformational changes in the receptors that cause release of corepressors and recruitment of coactivators to create functional complexes that are bound to consensus promoter DNA sequences called retinoic acid response elements (RAREs) and that cause

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opening of chromatin and transcription of adjacent genes. Homologous recombination technology allowed the development of mice lacking expression of retinoic acid receptors, individually or in various combinations, which demonstrated that the receptors exhibit vital, but redundant, functions in fetal development and in vision, reproduction, and other functions required for maintenance of adult life. More recent advancements in sequencing and proteomic technologies reveal the complexity of retinoic acid receptor involvement in cellular function through regulation of gene expression and kinase activity. Future directions will require systems biology approaches to decipher how these integrated networks affect human stem cells, health, and disease.

Abbreviations

ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation coupled with deep sequencing
cDNA	Complementary DNA
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinol binding protein
DNA	Deoxyribonucleic acid
DBD	DNA binding domain
LBD	Ligand binding domain
NMR	Nuclear magnetic resonance
RA	Retinoic acid
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RNA	Ribonucleic acid
RNA-seq	High throughput RNA sequencing
RXR	Retinoic X receptor
VAD	Vitamin A deficiency

Introduction: In Quest of a Mechanism of Action for Vitamin A

The idea that essential factors other than proteins, fat, starch, sugar, or minerals are present in food was a novel concept before the late 1880s. Ultimately this notion was verified by a series of human dietary supplementation studies and controlled experiments in animal models conducted between 1880 and 1920, which demonstrated that removal of these factors from the diet caused debilitating illnesses and death. The first discoveries in the field were made by Christiaan Eijkman and Frederick Gowland Hopkins who found that rice polishings contain substances preventing beriberi. These investigators received the Nobel prize for their work in 1929 [17]. In 1912, Casimer Funk identified the active fraction,

which was named water-soluble factor b, later described as thiamine [16]. Since this substance, which was vital for life belonged to a class of organic compounds called amines, he named it "vitamine" (vital amines) [98].

During this time, two groups, one led by Elmer McCollum and the other by Thomas Osborne and Lafayette Mendel, independently provided evidence for another essential substance that was named fat-soluble factor a [113]. By 1920, several other low abundance dietary factors were also being described, and Jack Drummond argued to the American Chemical Society that since there was no evidence for the presence of an amine in all of these "vitamines", it would be easiest for classification purposes to drop the *-e* on Funk's general reference to vital factors and refer to them as Vitamin A, Vitamin B, Vitamin C, etc. [29].

The structure of vitamin A, also known as retinol, was first reported by Paul Karrer and his collaborators in 1931 [55, 111], confirmed by the group of Heilbron the following year [44], and crystallized in 1937 [47]. The molecule is composed of 20 carbon atoms arranged as a beta-ionone ring with a conjugated isoprene tail that terminates with an alcohol functional group (Fig. 1.1).

Ongoing studies revealed that vitamin A serves as a precursor for active derivatives that impart two very different physiologic effects: (1) an aldehyde derivative (11-*cis* retinal), which is the active chromophore of vision [2, 7, 48, 128], and (2): an acid derivative (all-*trans* retinoic acid), which has the ability to reverse developmental defects in vitamin A deficient (VAD) animals [4, 5, 123] (Fig. 1.1). Further experiments by Arens and van Dorp suggested that retinoic acid (RA) could not be converted into vitamin A in vivo and thus, they concluded that RA was itself a hormone involved in cell growth and in development. Since that time, several other active vitamin A metabolites have been identified, and active compounds have been synthesized. In 1976, all of them were grouped as *retinoids* [117].

Clues as to how retinol and retinoic acid work inside cells came from studies performed with other fat soluble (lipophilic) hormones such as estrogens and glucocorticoids. In the 1960s, advancements in methods to synthesize radiolabelled hormones with sufficient specific activity for in vivo use and in techniques to count tritium in animal tissues [51] allowed the identification of binding proteins for these hormones [52], and also suggested that there was a link between their physiological action and transcription in the nucleus. This was the first indication that lipophilic hormones regulate gene transcription through nuclear receptors functioning as transcription factors.

More support for this concept came from Pierre Chambon's finding that administration of estradiol to immature chickens elicited an increase in liver *aggregate* polymerase that preceded an induction of protein synthesis [19, 130]. Subsequent studies found that estradiol induces translocation of an estrogen binding protein from the cytoplasm to the nucleus [53] and that steroid hormones induce transcription of specific subsets of genes [82, 96]. The generality of this phenomena in the animal kingdom was shown by studies in flies demonstrating that insect ecdysteroids induce alterations in chromatin dynamics, observed as *puffing* of chromosomes [6]. The final pieces of the puzzle came together in the 1980s with the discovery that glucocorticoid receptor proteins could be proteolytically cleaved into independently-functional, ligand-binding (LBD) and DNA-binding (DBD)

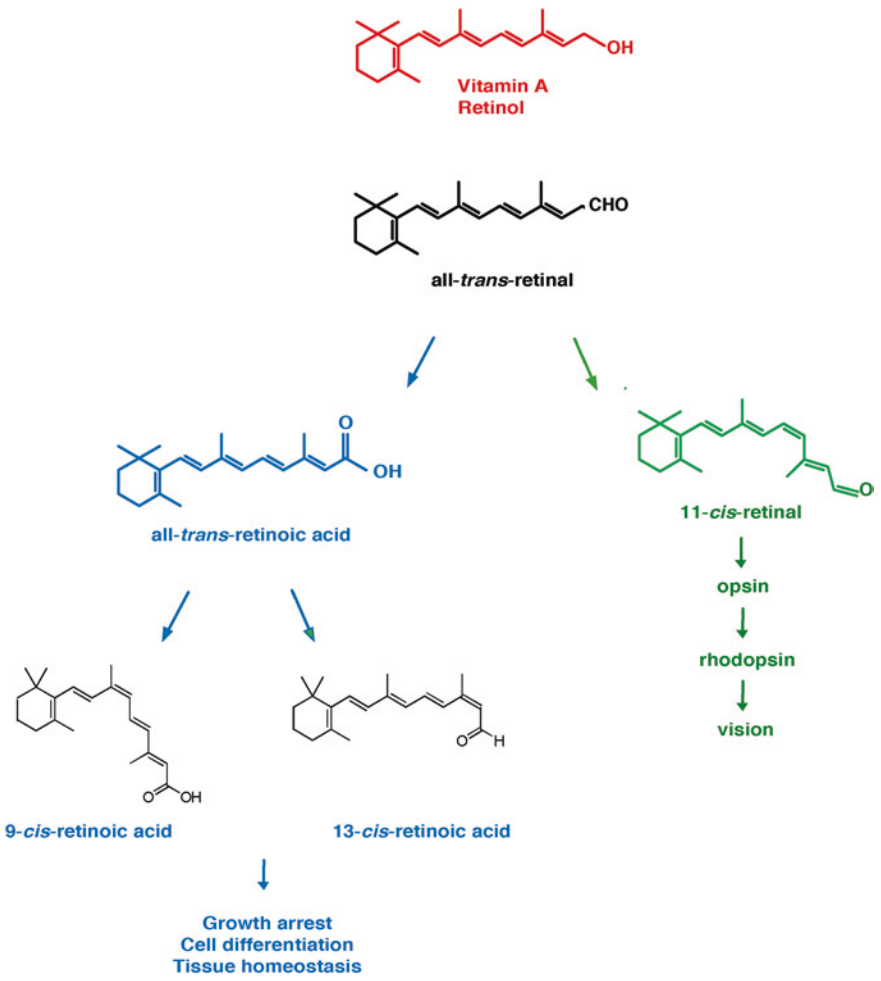

Fig. 1.1 Retinol and its main metabolites

domains [132], and that the DBD could bind to specific DNA sequence elements conferring glucocorticoid regulation of adjacent genes [20, 109].

At the same time, experimental evidence was accumulating that vitamin A and its derivatives also influence RNA and protein synthesis [24, 54, 134] and can bind intracellular proteins [8, 90]. In 1978, the group of Frank Chytil purified and characterized cytosolic proteins that bound retinol (CRBPI and CRBPII) or retinoic acid (CRABPI and CRABPII) [23, 91, 92]. However, subsequent studies performed by the same group revealed that CRBPs and CRABPs are present essentially in the cytosol and that these proteins merely serve as vehicles or *shuttles* transferring the ligand into the nucleus to specific binding sites on the chromatin [70, 71, 120, 121]. Also at that time, there appeared to be clear evidence that other retinoid receptors were present in the nucleus that could modulate the transcription of specific genes [43].

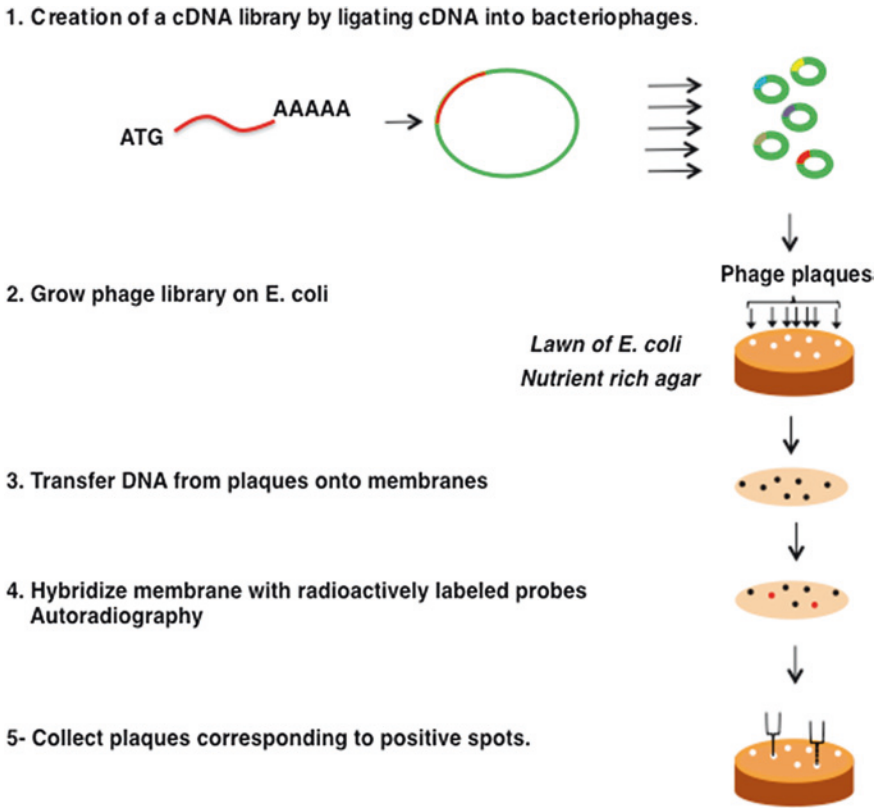

Fig. 1.2 Gene cloning using phage cDNA libraries

History: Cloning of the Nuclear Retinoic Acid Receptors

In the 1970s, progress in genetic technologies increased rapidly with promising possibilities for gene mapping. New procedures for DNA hybridization [27] and gene transfer [131] made possible gene localization to specific regions of chromosomes and gene cloning into DNA plasmids. A huge advance in molecular genetic studies was also provided by the purification and characterization of a microbial reverse transcriptase enzyme that could be engineered to generate DNA complementary (cDNA) to RNA in animals [116, 122]. This ability to generate DNA sequences representing processed RNA circumvented the problems of non-coding introns and exon splicing, and allowed mRNA isolated from cells to be copied into cDNA sequences that could then be individually cloned into plasmids [112] or bacteriophages to create libraries of all the DNA translation products expressed in a particular cell type.

In 1985, these technologies were used by the group of Pierre Chambon to clone the human estrogen [129] and glucocorticoid receptors [41] (Fig. 1.2). Phage

libraries were infected into bacteria growing on a petri dish at densities that allowed individual phage to be separately identified by the clear areas they produced in the bacterial lawn, called plaques. The cDNA in the plaques was transferred to a nitrocellulose membrane, which was then screened with a radioactively labeled query probe (for example, a nucleotide sequence with homology to putative hormone receptors). When exposed to X-RAY film, plaques hybridizing with the radioactive probe produced a spot that allowed their identification and isolation. Then, the isolated cDNA's could be used to produce large quantities of protein and could be genetically manipulated to mutate or excise coding regions for specific parts of the proteins, which allowed precise identification of functional areas.

Cloning of RARs

The first retinoic acid receptor, RARα, was independently identified in 1987 by the laboratories of Pierre Chambon and Ronald Evans. Chambon's group cloned RARα by using a consensus oligonucleotide probe corresponding to a highly conserved sequence in the DBD of several members of the nuclear receptor family (human and chicken estrogen receptor, human and rat glucocorticoid receptor, human progesterone receptor and viral oncogene erbA) [97]. This probe was radiolabeled and hybridized with a phage cDNA library created from the human breast cancer cell lines MCF-7 and T47D. Several phage plaques giving positive signals were obtained and rescreened with probes corresponding to the DBD of the human estrogen, progesterone and glucocorticoids receptors in order to eliminate clones of these receptors. The cDNA inserts of the remaining positive clones were subcloned into a plasmid vector and sequenced. One of the cDNA inserts contained an open reading frame encoding a 432 amino-acid protein with a predicted molecular mass of 47,682 Da. This sequence was referred to as hRAR.

Analysis of the cDNA-deduced hRAR protein sequence revealed that the regions corresponding to the DBD and the LBD possess a high degree of similarity with that of all other nuclear receptors. Several experiments were designed to define whether the cloned hRAR protein binds RA and whether, by analogy with the other members of the nuclear receptor family, it could act as a ligand-inducible transcription factor. A cDNA fragment encoding the LBD was subcloned into an expression vector and introduced in HeLa cells. Incubation of the extracts with radiolabelled ligands confirmed that the hRAR protein binds RA selectively and with high affinity. A chimeric receptor in which the DBD of hRAR is replaced by the DBD of the estrogen receptor was constructed and cotransfected into HeLa cells with a vit-tk-CAT reporter gene under the control of an estrogen response element. Addition of RA resulted in an increase in CAT reporter activity indicating that hRAR is a ligand-inducible trans-activator of transcription. Thus the first human nuclear retinoic acid receptor was cloned and is now named RARα.

A few month later, Evans' group published that they had independently cloned the same receptor using a different probe corresponding to a novel sequence with striking

similarity to the DBD of the steroid hormone receptors that they fortuitously identified in a human hepatocellular carcinoma [36]. Close on the heels of these groundbreaking publications, two independent groups led by Magnus Phfahl and Chambon used the same probe [9, 13, 25] and cloned a second human nuclear retinoic acid receptor, which depicted high homology with RARα. Both groups created chimeric proteins by swapping the DBD for that of the estrogen receptor and verified that RA could activate the chimeric protein to transactivate a reporter gene under the control of an estrogen responsive DNA sequence element. One group called the new receptor, RARε, based on its high expression in epithelial tissues [9], but the name proposed by the other group, RARβ, became the commonly known name for this second RAR [13]. Finally, efforts to clone the murine counterparts of the human RARα and RARβ receptors revealed the existence of a third RA receptor, RARγ [137]. DNA sequences of the mouse RARγ gene were then used to clone the human RARγ gene [58].

Subsequent research indicated that the three RARs are encoded by three distinct genes located in different chromosomes [50, 81]. Several isoforms of each RAR subtype were identified that differ in the N-terminal region as a result of alternative splicing or of the use of different promoters upstream of the gene. Two major isoforms were identified for RARα (RARα1 and RARα2) [67], four isoforms for RARβ (RARβ1, RARβ2, RARβ3, and RARβ4) [89, 138], and two isoforms for RARγ (RARγ1 and RARγ2) [37, 57]. Official classification of the nuclear receptors identified RARα as NR1B1, RARβ as NR1B2 and RARγ as NR1B3 [35].

Cloning of a Second Family of Nuclear Retinoid Receptors: The RXRs

In the late 1980s a novel strategy was used to isolate cDNA clones encoding DNA binding domains. The technique involves absorbing the proteins produced by a phage cDNA library onto nitrocellulose filters and probing the filters with radioactive, double-stranded DNA [114, 125]. In 1989, the Keiko Ozato group used this technique to isolate from mouse liver a cDNA clone encoding a protein capable of binding the conserved MHC class I regulatory element (CRE). Interestingly, this protein, H-2RIIBP (H-2 region II binding protein), had modular domains characteristic of the nuclear hormone receptors and could also bind estrogen response elements [42]. More research needed to be done however, to determine whether the H-2RIIBP protein was a nuclear hormone receptor. Then Mangelsdorf et al. performed a low stringency screen of human liver and kidney cDNA libraries using a probe corresponding to the DBD of RARα and isolated a novel nuclear receptor, which was substantially different from RARα and was referred to as hRXRα [79]. A few month later, the Michael Rosenfeld group screened a cDNA phage library from a thyroid tumor using a RAR response element and isolated an additional nuclear receptor, which exhibited remarkable homology to RXRα and which differed by only 2 amino acids from the H-2RIIBP protein. This protein was finally named RXRβ [133]. Subsequently, three murine RXRs (RXRα, RXRβ and RXRγ) encoded

by different genes were isolated [78]. Several studies attempted to clone the human RXRγ gene, but without results. Nevertheless, a human genomic locus encoding RXRγ has been mapped in chromosome 1 using florescence in situ hybridization (FISH) [3]. According to the official classification of nuclear receptors, RXRs are now identified as NR2B1 (RXRα), NR2B2 (RXRβ), and NR2B3 (RXRγ) [35].

The interesting feature of the RXR proteins is that they can activate transcription in response to RA, but are unable to bind all-*trans* RA due to the fact that they do not share significant homology with the LBD of RARs. Instead, *9-cis* retinoic acid was identified as a high affinity ligand for RXRs [45, 68]. However, today it is clear that *9-cis* RA cannot be detected in most tissues, and the existence of a physiological RXR ligand is still being investigated [34]. Nevertheless, several synthetic compounds that bind RXRs and not RARs, rexinoids, have been designed and have provided useful tools [94].

Establishment of the Basis of RARs and RXRs Mechanism of Action (1990–1995)

Once they were cloned, the sequences of the different RAR and RXR cDNAs were analyzed, aligned and compared to that of the other nuclear receptors [61]. This analysis revealed that all nuclear hormone receptors including RARs and RXRs exhibit a modular structure composed of 6 conserved regions designated A–F (Fig. 1.3a) with different degrees of conservation [65]. Region C, which corresponds to the DBD is the most conserved region with 94–97 % identity between RARs and with 60 % identity between RARs and RXRs. Region E encompasses the LBD and is also well conserved between RARs (84–90 % identity), but differs considerably between RARs and RXRs (27 % identity). Finally the A and F regions differ markedly among the 3 RARs, and the F region is lacking in RXRs.

During the same time, the promoter regions of endogenous genes that are controlled by RA were characterized, leading to the identification of specific *cis*-acting DNA elements that bind RARs (Fig. 1.3c). The first natural RA response element (RARE) identified was that in the RARβ2 promoter [26, 46, 119]. It consists of a direct repetition of 2 motifs (G/AGTTCA) separated by 5 base pairs and was called a DR5. During ensuing years, other RAREs that have different spacings between the direct repeats (DR1 and DR2) were discovered in the promoters of several other RA-responsive genes [31, 115].

RARs from crude cell extracts were able to bind RAREs, however several groups observed that high affinity binding required interactions with other nuclear factors [38, 66]. Chambon's group purified the nuclear accessory factor that enhanced the binding of RARs to RAREs in vitro and discovered that this protein was RXRβ [66]. This group also showed that RXRs form heterodimers with RARs and that interaction regions overlap with the LBDs and the DBDs of both partners [66]. Concomitantly, Rosenfeld's group also identified RXRβ as the factor that stimulates the binding of RARs to RAREs [133].

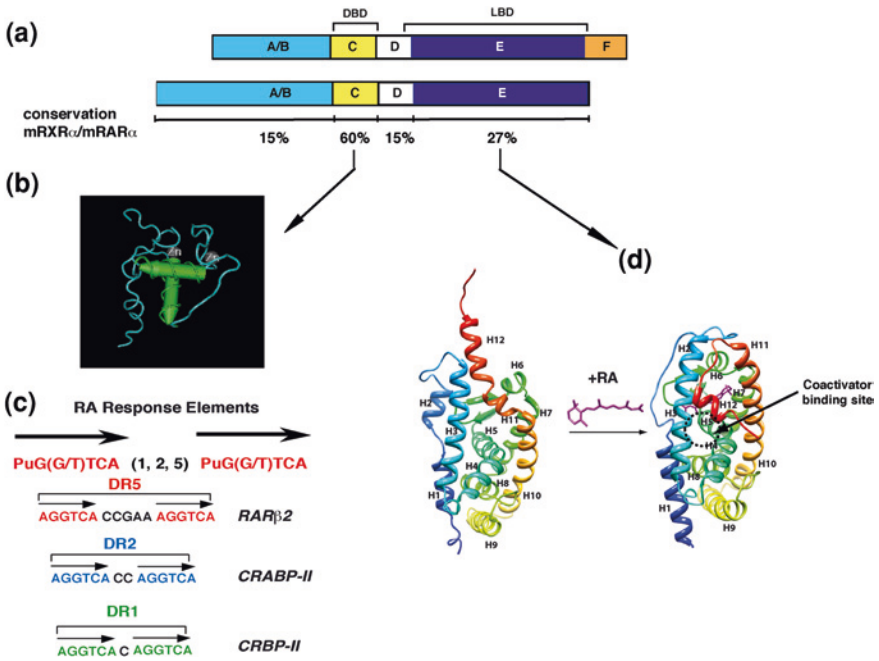

Fig. 1.3 Structure of RARs and of their DNA binding sites. **a** Schematic representation of the modular organization of RARs and RXRs with the functional domains. The conservation between RARα and RXRα is shown. **b** High resolution structure of the RXRα DNA binding domain NMR (mmdbId: 8588). **c** Description of the classical retinoic acid response elements (RAREs). **d** Structural changes induced upon RA binding. The crystal structures of the unliganded RXRα and liganded RARγ LBDs are shown with the binding domain for corepressors. Helices are represented as ribbons and labeled from H1 to H12. Adapted from Protein Data Bank 1lbd and 2lbd

In 1994–1995, knowledge of the mechanism(s) of action of RAR/RXR heterodimers and most nuclear receptors increased tremendously due to the discovery of co-activators that bind specific sequences of the LBD in response to RA [30, 62, 126, 127] and characterization of the three-dimensional structures of the DBD [64] (Fig. 1.3b) and the LBD [12, 100] (Fig. 1.3d). Most notably, the description of the crystallographic structures of the LBD in the absence and presence of RA revealed that the *on* switch for gene transcription by liganded RARs relies on structural rearrangements that create binding surfaces for co-regulators [18, 87] (Fig. 1.3d).

During this same time, peptides corresponding to amino acid sequences specific for each RAR and RXR subtypes were synthesized, allowing generation of antibodies recognizing not only the RAR and RXR proteins produced in vitro (recombinant proteins), but also endogenous RARs and RXRs [32, 105]. In addition to their utility in localizing endogenous receptor expression in tissues, the antibodies had an additional interesting benefit of revealing that endogenous RARs and RXRs migrated to different positions on polyacrylamide gels when compared with their recombinant counterparts. This later finding suggested the

native proteins had a higher relative molecular weight, possibly due to post translational modifications, such as phosphorylation, in vivo [33, 104, 106].

Genetic Evidence that RARs Transduce the Retinoid Signals in Vivo

Until the 1990s, the physiological functions of vitamin A and retinoids in vivo were mainly inferred from studies on vitamin A-deficient (VAD) animals. These studies demonstrated that vitamin A is required during pre- and post-natal development as well as in adult life. The VAD syndrome is associated with several congenital malformations during embryonic development and with defects in growth, vision, reproduction and maintenance of several tissues after birth. The cloning of RARs and RXRs raised the question of whether these nuclear receptors mediate the physiological effects of vitamin A and retinoids in vivo.

The development of the homologous recombination technology for targeted disruption of specific genes allowed the generation of mice lacking CRABPs, RARs or RXRs. Single or double knock out of CRABP I and II did not generate overt phenotypic abnormalities corroborating that these retinoic acid binding proteins are not required for the effects of RA during development [40, 60]. In contrast, knock out of RARα or RARγ exhibited congenital malformations and displayed some of the defects of the postnatal VAD syndrome with reduced postnatal viability [72, 74, 75]. Double mutants were also generated that recapitulated all of the fetal VAD syndrome malformations and that exhibited a dramatically reduced viability [73, 83]. All these results confirmed that the effects of vitamin A in development are indeed mediated by RARs. Subsequently, RXR knock out mice were also engineered, but the interpretation of the phenotypes was more complicated since RXR heterodimerize not only with RARs, but also with multiple other types of nuclear hormone receptors [56, 118]. Nevertheless, compound mutants in both RXR and RAR greatly exacerbated the VAD phenotypes [56] suggesting that the RAR/RXR heterodimers are the functional units that mediate vitamin A signaling in vivo.

Development of the Field: A Huge Explosion Has Occurred in the Field of RARs During the Last Two Decades

Between 1987 and 1995, RARs and RXRs were cloned and the basis for their mechanism of action was established (Fig. 1.4). During the next twenty years, up to now, knowledge in the field of RARs and RXRs has increased tremendously due to the development of novel genetic, biophysical and high throughput molecular technologies. Integration of these approaches has provided an in-depth view of the mechanism of action of RARs and of all nuclear receptors. These findings have been the subject of several recent comprehensive reviews, some of which are recapitulated in this book volume. In addition, several databases have been developed and are now publically

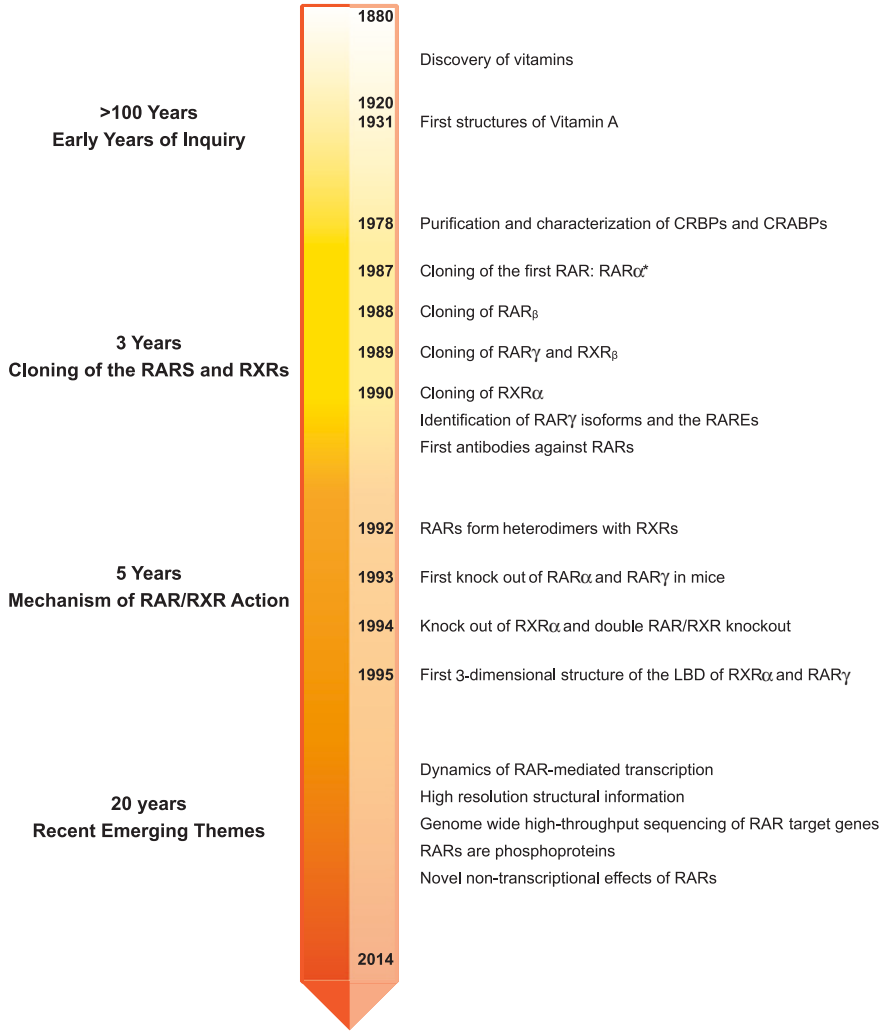

Fig. 1.4 Chronology of the main events in the field of vitamin A and RARs. *Petkovich et al. 1987; Giguere et al. 1987

available including [NURSA (http://www.nursa.org), Transcriptomine (http://www.nursa.org/transcriptomine) and IUPHAR (http://www.iuphar-db.org)].

Briefly, the novel findings discovered during the two last decades can be summarized as follows:

A large number of coactivator and corepressors have been identified and found to be components of multisubunit coregulator complexes exhibiting an ever-expanding diversity of enzymatic and epigenetic activities, exemplified by ATP-dependent nucleosome remodeling complexes, histone acetyl/deacetyl transferases (HATs and HDACs),

histone methyl transferases (HMTs) and histone demethylases [39]. The development of the chromatin immunoprecipitation (ChIP) technique revealed that transcription of RAR-target genes is a dynamic process and that RA binding switches RARs from an inactive state to an active state by promoting the exchange of corepressor complexes for coactivators [95, 102]. The consummate effects of these coregulators are the modification, remodeling and decompaction of chromatin to pave the way for the recruitment of the transcription machinery [28]. Today new proteins and complexes are still continuously being discovered increasing our knowledge of the complexity of RAR-mediated transcription. Moreover, the combination of different biophysical methods [X-ray crystallography, small angle X-ray (SAXs), fluorescence resonance energy transfer (FRET), nuclear magnetic resonance (NMR)] and the use of several synthetic agonists or antagonists for RARs and RXRs is providing a view of the dynamic structure of RAR-RXR heterodimers associated with coactivators on different response elements [101].

Subsequent to the sequencing of the human genome, the development of genome-wide profiling technologies such as RNA-seq (high throughput qPCR sequencing) and ChIP-seq (chromatin immunoprecipitation coupled with deep sequencing) has allowed the identification of novel RA response elements with different spacings [88] and novel RA-target genes [59, 77]. The genome-wide integrative analysis of RAR/RXR binding and transcriptional regulation has also provided a dynamic view of RA signaling [84]. Recently, RARs have been found to target the expression of microRNAs, introducing yet another level of complexity in the regulation of RAR-regulated gene transcription [93, 135, 136]. These recent approaches and findings are identifying novel mechanisms of action for RARs and are opening up promising new avenues for research and development.

During the last two decades, the concept arose that posttranslational modifications such as phosphorylation and ubiquitination are crucial for RARs activity [2]. Early studies using phosphopeptide analysis performed with radioactivity and large amounts of recombinant receptors resulted in the identification of a number of phosphorylation sites on RARs and RXRs [1, 63, 103, 107]. Subsequently, the emergence of new methods for enrichment of phosphopeptide samples and development of phosphospecific antibodies provided the ability to analyze phosphorylation of endogenous RARs in response to their cognate ligand or signaling pathways [14]. Now it is clear that RARs, as well as several other proteins, are rapidly phosphorylated in response to RA, subsequent to RA-induced activation of kinase cascades via a pool of RARs that are present in membrane lipid rafts [80, 99]. A concept that is gaining support is that phosphorylations induce subtle changes in the conformation of the receptors that modulate the association/dissociation of new coregulators [21, 108]. Another developing concept is that phosphorylation is a signal for the degradation of RARs by the ubiquitin proteasome system, a process that signals the end of transcription [11].

In situ analysis of endogenous RAR protein expression profiles became possible with the generation of purified highly-specific antibodies [15, 124]. Moreover, the development of novel conditional gene targeting strategies based on the use of the Cre recombinase allowed the generation of somatic mutations in individual genes in a specific cell type and at a given time in the life of a transgenic mouse [85, 86]. This novel strategy has yielded remarkable advances in understanding the roles played

by RARs and RXRs because it circumvents the limitations of previous transgenic approaches that led to early lethality and that were compromised by redundancies in receptor isoform expression in tissues under investigation [49, 69].

Future Directions

Today, 25 years after the cloning of RARs (Fig. 1.4), knowledge continues to evolve in the field of retinoid biology. The structures of RAR/RXR heterodimers bound to DNA have been solved, but they are still lacking the N-terminal domain which exhibits a quasi absence of defined secondary structures but confers considerable flexibility to RARs. The integration of data from several sources and from high-resolution biophysical approaches should provide the structure of the RAR/RXR heterodimers as full-length proteins bound to DNA with their coregulators intact.

A driving goal for future studies will be the discovery of the rules for cell fate specification integrated into a systems biology view of RAR/RXR actions and RA signaling. Application of computational models and programs to reconstruct differentiation-related gene networks obtained from different cell types should allow the prediction of RA-regulated gene network intricacies and the identification of key factors that direct cells towards a particular differentiation phenotype. Stem cells, which are pluripotent cells capable of generating all the differentiated cell types present in the body and which are responsive to RA, are currently, a most promising tool for such cell fate studies. Extrapolation of data generated from stem cell differentiation models should have applicability to a deeper understanding RAR-dysfunctional diseases that interfere with normal cell homeostasis and redirect normal cells to a more primitive, mitosis-driven state.

Recent findings are stretching the boundaries of our understanding of vitamin A action. These newer studies are indicating that the effects of vitamin A retinol and RA are not mediated only by RAR/RXR heterodimers and transcriptional processes, and this is opening up new avenues in the field. As an example, it has been found that RA can activate other nuclear receptors such as the peroxisome proliferator-activated receptor β/δ [110], providing a rationale for the long-noted, but poorly understood function of vitamin A in regulating energy balance. Moreover, recent findings are hinting that RA, as well as vitamin A itself, can have extranuclear, non-transcriptional effects and can activate kinase-signaling pathways [2, 10]. Consequently, one can speculate that, in addition to affecting the transcriptome, RA and retinol could also affect the phospho-proteome. Next generation, dual linear ion trap mass spectrometers coupled with Orbitrap technology should allow the identification of new panels of proteins that are phosphorylated in response to retinol or RA. The future objectives should be to integrate the RA-induced variations in the phospho-proteome with the transcriptome. Such an integrative study should pave the way to breakthroughs in disease-related research. The recent observation that RARs are present in the cytosol of specific cell types [22, 76] continues to open new areas in the mechanisms of action of vitamin A and RA.

Great progress has been made in deciphering how specific molecules and signaling pathways interact to mediate vitamin A/RA action. But much is left to be done to fully understand the complexities of their action at the cellular and sub-cellular levels and of their regulation in time and space throughout the life of an organism.

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