Studying non-mammalian models? Not a fool's ERRand!

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Abstract

Through studies in mammalian model systems, the orphan nuclear receptor Estrogen-Receptor Related (ERR) α has been shown to interfere with estrogen signaling and may therefore be an interesting pharmaceutical target in estrogen-related pathologies. ERRα is also involved in energy storage and consumption and its modulation may be of relevance in the treatment of obesity and diabetes. Recent data have also been published studying the effects of this receptor, as well as other members of the ERR family, in non-mammalian animal model systems. Besides indications concerning their mechanisms of action, this analysis demonstrated a role for ERRα in controlling cellular movements and suggested that ERR receptors may be implicated in a more subtle range of processes than originally envisioned.

Introduction

Recent data have attracted attention to the ERRα orphan nuclear receptor in at least two scientific domains. First, ERRα has been shown to interact with estrogen signaling [1], a key factor in the promotion of breast cancer, itself the major cause of death by cancer among females. This has led various laboratories to analyze the expression of ERRα in human tumors (including those of mammary origin) in correlation to diverse anatomo-pathological criteria [2-5]. The long-term goal is to determine the roles played by ERRα in various aspects of cancer and, depending on these roles, to propose the receptor as a target for the design of new anticancer therapies. Second, ERRα has recently been implicated in the regulation of energy metabolism. Indeed, ERRα-defective mice are resistant to diet-induced obesity [6] and the receptor seems to participate in the regulation of mitochondrial biogenesis [7] and lipid storage and consumption [8-9]. As obesity and its associated disorders (including diabetes and cardiovascular diseases) have reached epidemic proportions in Western societies, one easily understands why any gene whose product may be involved at any level in the metabolic syndrome attracts attention as a potential target for pharmaceutical design.

The recent focus of studies into two main areas of ERRα biology (i.e. estrogen signaling/cancer and metabolism), however, does not diminish interest in investigating new aspects

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of regulatory function for ERRs. It is important to determine these roles for at least three reasons. First, modulating the expression/activity of ERRα as a therapy demands that we are able to predict the consequences of these modulations on other, non-intentional target tissues in order to avoid undesirable side-effects. Second, uncovering other functions of the receptor could lead to its implication in other diseases. Last, basic knowledge of the roles of a gene product *per se* cannot be neglected.

In addition, ERRα has two closely related paralogs in mammals (ERRβ and ERRγ), the roles of which are still poorly understood. When studying gene functions, non-mammalian models can offer a variety of advantages. These include genetic simplicity in invertebrates or experimental accessibility in fish early development. Given the frequent conservation of functions along evolution, the results gathered using these animals could lead us to envision new aspects of ERR activities in mammals. The purpose of this review is to summarize the data obtained with such non-mammalian model systems.

The ERR subfamily: an evolutionary view

The family of nuclear receptors (NRs) comprises 48 transcription factors in human (21 in *Drosophila melanogaster*; [10-11]) which all share a similar organization in terms of protein domains. Among NRs one finds proteins whose activity is regulated by the presence of a hydrophobic ligand and others, referred to as "orphan" receptors, for which no natural ligand has yet been described [12]. Based on phylogenetical analysis, six NR subfamilies have been described each of which have been proposed to have diversified by duplications of an orphan receptor [13-14]. Subfamily 3 is composed of three subgroups: NR3A (Estrogen Receptors [ERs]), NR3B (Estrogen-Receptor-Related receptors [ERRs]) and NR3C (Steroid Receptor [SRs]: Androgen- [AR], Glucocorticoid- [GR], Progesterone- [PR] and Mineralocorticoid- [MR] receptors).

The phylogenetic relationship of ERRs to the two other subgroups is unclear. ERRs have been originally described as most closely related to the ERs (hence their name [14-15]). However this view has been challenged by recent analysis suggesting that ERRs are equally related to ERs

and SRs [16], although this result is controversial [17]. Although the order of ER-ERR-SR divergence is still unresolved, it is clear that the ancestor of all bilaterian metazoans possessed one member of each subgroup (*i.e.* one ER, one ERR and one SR). Given the uncertainty about the phylogeny and the paucity of data concerning very early metazoa (*e.g.* Cnidarians and sponges), it is, to date, impossible to ascertain which gene was at the origin of the whole NR3 group. Gene loss has played an important role during the subsequent evolution of the subfamily. Indeed, if an ER gene has been found in the mollusk *Aplysia californica* [16], it is not present in *D. melanogaster* nor in *Caenorhabditis elegans* [10, 18], suggesting a loss in all ecdysozoans (comprising nematodes and arthropods). An ERR gene has been found in *D. melanogaster* and in the mosquito *Anopheles gambiae* but not in *C. elegans* [10, 17-18], suggesting a loss in the nematode genome which has been highly dynamic for nuclear receptors [19]. No ERR has yet been described in lophotrochozoa (*e.g.* Mollusks) although its presence is clearly implied by phylogenetical analysis (Figure 1).

From a single ancestral ERR, as still found in insects but also in non-vertebrate chordates (such as ascidians and amphioxus; [20-22]) to the three vertebrate ERRs [23], two waves of gene duplications were necessary. The first took place before the emergence of lamprey and resulted in the separation of ERRα from an ancestor of both ERRβ and ERRγ. Indeed, in mammals these two genes are more closely related to each other than to ERRα, and furthermore, a cDNA fragment equally resembling both ERRβ and ERRγ could be isolated from lamprey (P-L Bardet and J-M Vanacker, unpublished). Before the emergence of fish, another round of gene duplication took place, separating ERRβ from ERRγ, and also ERRα from an ERRδ, the latter being lost in mammals but maintained in some fish (such as fugu and zebrafish; [17, 24]). Additional gene duplications and losses took place specifically in various fish lines [17]. In mammals, ERRs have been cloned in bovine, mouse, rat and human [15, 25-27].

Molecular properties of ERRs

Apart from mammals, only ERRs originating from *D. melanogaster*, amphioxus and zebrafish have been studied for their molecular properties (Table 1). All ERRs studied to date bind

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to and activate transcription in a seemingly constitutive manner through the estrogen-response element (ERE) and the ERR-response element (ERRE), at least in transient transfection experiments [23].

Mouse ERRβ and ERRγ, but not ERRα, can be deactivated by high doses of 4-OH-tamoxifene (OHT) a mixed anti-estrogen [28]. This differential behavior is conserved in zebrafish where ERRγ and ERRδ, but again not ERRα, are sensitive to OHT [24]. *D. melanogaster* ERR requires the mutation of three amino acids in its ligand-binding domain, changing them to mammalian ERRβ/γ type, to react to OHT [29]. Transactivation by amphioxus ERR, in which these three positions are identical to mouse ERRβ/γ (but not to mouse ERRα) can also be inhibited by OHT (P-L Bardet and J-M Vanacker, unpublished).

Both *D. melanogaster* and amphioxus ERR are expressed as two isoforms differing by the presence or absence of an in-frame alternative exon at the beginning of the ligand-binding domain [18, 30]. Functional studies in the amphioxus have revealed that the resulting long ERR isoform behaves as a dominant negative transcription factor over the short isoform, but only through the ERRE [30]. On EREs, both the long and short isoforms activate transcription. Whether this is also true for *D. melanogaster* ERR has not been determined. However the fact that the size and insertion point of the alternative exon are roughly similar between amphioxus and *D. melanogaster* suggests functional conservation, despite obvious sequence divergence. In both animals the isoforms are differentially expressed during development, with the ratio of long/short mRNA progressively rising as development proceeds [30-31].

The dimeric *vs.* monomeric nature of the DNA binding by mammalian ERR proteins has been a subject of debate. The long amphioxus ERR isoform binds as a monomer on and does not activate transcription through ERRE sequences whereas it homodimerizes on and activates transcription through ERE sequences [30]. This observation suggests that dimerization is required for transactivation, a hypothesis that has recently been fueled [32]. Indeed Protein Kinase C (PKC) δ-driven phosphorylation shifts the binding of human ERRα on an ERRE site from a monomeric to a dimeric state. Correlatively, PKCδ also enhances transactivation exerted by ERRα on the single

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ERRE of the pS2 gene promoter. In contrast the ERRα gene promoter itself, which comprises multiple ERREs (altogether forming an ERE-like DNA stretch) is constitutively activated by ERRα (*i.e.* in a phosphorylation-independent manner).

Parallels can therefore be drawn between amphioxus and human ERR (at least for ERRα). In both experimental systems, evidence was obtained proposing that dimerization is necessary for transactivation. Regulation of dimerization (*vs.* monomer state) and consequently of transactivation is conspicuous on the ERRE but not on the ERE. The modalities of this regulation seem to differ, alternative splicing for amphioxus, phosphorylation in the human. These two events thus offer the possibility, with a single orphan ERR nuclear receptor, to finely regulate the transcription of certain target genes as a function of developmental and/or physiological state in the absence of a natural ligand.

Early developmental effects of ERR genes

Expression of the unique ERR gene in *D. melanogaster* is detectable very early after egg laying which indicates maternal transmission of its RNA [31] (Table 2). This transcript is rapidly degraded and reappears later under various alternatively spliced isoforms. Maternal transmission of ERR RNA was also observed in the zebrafish for ERRα but not for the other ERRs, which are expressed much later [24]. As no information is available concerning the mouse ERRs before blastocyst stage, it is not known whether they are maternally transmitted. However expression of ERRα and ERRβ in embryonic stem (ES) cells indicates an early requirement [25, 33]. In contrast, ERRγ is only detectable by Polymerase Chain Reaction (PCR) experiments at eleven days post-coitum (E11) but not at E7 [34]. In the ascidian *Herdmania curvata* the unique ERR is among the earliest NRs detected by PCR experiments, at the 64-cell stage (well before gastrulation) although not at the 32-cell stage [21], rendering maternal transmission unlikely. In the amphioxus, ERR expression is first detected at a later (neurula) stage [22].

In the mouse, expression of ERRβ in the extra-embryonic ectoderm is necessary to the formation of the placenta [35]. However, the functions of ERR genes in embryonic tissues are

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poorly characterized. Early developmental functions of ERRα have been studied in the zebrafish [24]. Inhibiting the activities of ERRα from very early stages (1- to 4-cell stages), using a morpholino (an antisense oligonucleotide that specifically inhibits the translation of the targeted transcript [36]) or a dominant-negative strategy results in a dramatic delay in the cellular movements (occurring 6 to 9 hours later) that precede and are necessary to gastrulation [37]. In particular, the convergence of lateral cells toward the dorsal part of the zebrafish embryo is strongly reduced upon ERRα knockdown; as a consequence the antero-posterior axis appears broader in mutant animals. Conversely, overexpressing ERRα has no effect on the above-mentioned cell convergence, but results in the perturbation of the extension of the antero-posterior axis, which consequently appears shorter. Interestingly, this phenomenon occurs in the absence of any effect on tissue determination: specific gene expressions that are characteristic of a presumptive tissue remain unaltered. In mice the requirement of ERRα for normal embryonic development does not appear as crucial. Indeed mice knocked-out for ERRα are born without any gross alteration [6]. Whereas this could suggest that the functions of the receptor during early development are different between fish and mouse, this could also indicate that the loss of ERRα in knock-out mice is compensated for by some unknown mechanisms. However the above-mentioned results point to roles for ERRα in regulating cellular movements, a broadly observed phenomenon not only during development but also in the course of various physio-pathological phenomena, such as inflammation, atherosclerosis, wound healing or cancer metastasis. The mechanism through which zebrafish ERRα exerts its effects on cellular movements is presently unknown. However osteopontin, a transcriptional target of ERRα [38], could be a possible connective link. This protein acts as a chemotaxis factor in the recruitment of inflammatory cells during atherosclerosis or rheumatoid arthritis [39-40 and ref therein] and is likely involved in metastatic behavior [41-42]. If confirmed in mammals, this movement-regulating role of ERRα could open a brand new field of investigations.

ERRs in the brain

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In zebrafish, ERR genes were found co-expressed in groups of neurons in the rhombomeres (which are repeated embryonic structures) of the hindbrain during development, in a temporal sequence of appearance: first ERRγ, then β and last α [22]. It is tempting to hypothesize a cross-regulation of the expression of these receptors as has been demonstrated in human cells where ERRγ up-regulates the expression of ERRα [43]. The unique amphioxus ERR was also found expressed in the homolog of the posterior brain during development [22]. Strikingly this expression also appeared in an iterated manner labeling regularly spaced motoneuron pairs along the antero-posterior axis, although no morphological segmentation is obvious in cephalochordates. ERR thus constitutes a conserved marker of cellular/molecular segmentation even when anatomical segmentation has not yet appeared in evolution. Expression of ERRs in mammalian rhombomeres has not been investigated.

In mouse embryonic brain, ERRβ has been detected by PCR experiments [44], but its detailed expression pattern has not been documented. In contrast ERRα and ERRγ display a complex, partly overlapping expression pattern in this organ [33, 45]. ERRα is detected in the neural tube 10.5 post-coitum and its expression is enhanced when cells leave the proliferative (ventricular) zone for the intermediate zone where they differentiate into neurons. Though the roles of ERRα in the brain are unknown, one could hypothesize that ERRα is required for the cellular movements necessary to this process and/or in the control of proliferation/differentiation. ERRγ expression is also enhanced in differentiating motoneurons [45]. Altogether, ERR receptors are found in the brain in a broad range of chordate species. This should be a strong signal to investigate thoroughly their roles in this organ, which remain undetermined.

ERRs in muscles.

Expression of ERRs in muscles has been demonstrated in various species. Indeed, amphioxus ERR is expressed in the dorsal parts of the somites, a population of cells that give rise to slow-twitch muscles [22]. Zebrafish ERRα is also present in the adaxial cells [24], which are also progenitors of slow-twitch muscles. In mouse, ERRα (and also ERRγ) is much more expressed in

the soleus (slow-twitch) than in the vastus lateralis (fast-twitch) muscle [8]. There is thus a strong evolutionary conservation of the predominant expression of ERRα in slow *vs* fast muscles.

Use of lipids as a fuel and a high number of mitochondria are distinctive features of slow muscle fibers (for a review, see [46]). Mouse ERRα (acting together with its interacting partner, the coactivator PPARgammaCoactivator-1α [PGC-1α]) has been proposed to regulate lipid storage and/or consumption, as well as promoting mitochondrial biogenesis [7-9], functions that are likely to be conserved through evolution. The elevated ERRα expression in differentiating cells or their immediate precursors (adaxial cells in the zebrafish, dorsal somitic cells in the amphioxus) suggests an additional role for the receptor in differentiation, more specifically in the slow muscle lineage. This hypothesis is consistent with the demonstrated functions of PGC-1α in muscles [47]. Indeed, when overexpressed in transgenic mice, this coactivator converts fast muscles into slow ones, a phenomenon accompanied by an elevated number of mitochondria and by a corresponding switch in energy source. In addition to this, expression of amphioxus ERR in neurons appears in the same cells that innervate the slow fibers in which the receptor is also present [22]. It could thus be that amphioxus ERR not only influences the independent physiologies of slow muscles and motoneurons, but also the connections between these cell components, a hypothesis that remains to be tested.

Conclusions and future prospects

Although the molecular mechanisms of action of ERRs have recently gained understanding, the physio-pathological functions exerted by these receptors in mammals remain largely unidentified. Studies in non-mammals that include comparative analysis of expression patterns as well as determination of functions may provide clues as to which mammalian tissues/cells are targeted by the receptors. Such an “evolution-based” approach assumes that the functions of the receptor are conserved throughout the animal reign. It is tempting to propose that the near-exclusive expression of ERRα in slow- *vs* fast-twitch muscles in all chordates studied (amphioxus, zebrafish,

mammals) provides a proof of principle to this assumption although the function of non-mammals ERR in energy homeostasis (a key function of mouse ERRα) has not been determined.

Strong (positive and negative) correlations have been observed between the expression of ERR receptors and cell proliferation and/or differentiation. In mouse, this not only includes neurons and muscle cells where expression of ERRα (and also of ERRγ in motoneurons) is elevated upon differentiation, but also ES cells in which ERRβ expression decreases upon differentiation-inducing retinoic acid treatment [25]. One or several ERR receptors acting either together or in an opposing manner may thus contribute to the regulation of proliferation and/or differentiation in different tissues. Another path to be explored originates from the demonstration, in the zebrafish, of an effect of ERRα on cellular movement. This general phenomenon is important in many physio-pathological processes that ERR receptors may thereby regulate. Whether this is also true for mammalian ERRα and/or extends to the other ERR paralogs remains to be determined, as well as the effector targets activated by these transcription factors.

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Legend to figure 1

Evolution of the ERR subfamily.

Ancestral ERR is predicted from phylogeny to have existed in the ancestor of bilaterian metazoans and may still be present in modern lophotrochozoans (such as mollusks). ERR (as well as other NR3 receptors) has been lost in worm phylum but maintained in insects. Position of global gene duplication events is indicated. Number and subtype of resulting ERR receptors (see text for details) are indicated. Insects include *D. melanogaster* (fruit fly) and *Anopheles gambiae* (malaria mosquito); cyclostomes include lampreys (where a β/γ fragment has been identified in *Petromyzon marinus*) and myxines; early chordates include *Brachiostoma floridae* (amphioxus), *Ciona intestinalis* and *Herdmania curvata* (two ascidians); teleost fish include *D. rerio* (zebrafish), *Takifugu rubripes* (fugu) and *Tetraodon nigroviridis* (tetraodon). Specific fish duplications are not represented.

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