Commentary: The Year in Orphan Nuclear Receptors and Their Coregulators

David D. Moore

Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030

We are beginning to integrate the functions of individual orphan receptors into larger networks. It is particularly exciting that these basic studies are beginning to shed light on important diseases. We are now able to address not just physiological but also pathological functions of these proteins and their potential as targets for treating a surprisingly wide range of diseases. **(*Molecular Endocrinology* 25: 1983–1988, 2011)**

Orphan receptors come from the early days of gene cloning, when it became clear that there were more receptors than there were hormones. That was the discovery phase. Soon we were able to de-orphanize, that is, find ligands, especially natural physiological ligands, for these proteins. Once those connections were made, a series of experiments, typically gene knockouts, led to real insights into the basic functions of these proteins. Examples include the knockout of the *Lxrα* and *Lxrβ* by David Mangelsdorf, showing they are involved in cholesterol homeostasis, and the knockout of *Sf1* by the late Keith Parker, showing that it is very important in the development of the adrenals and other tissues.

Now we are in a new era and are beginning to integrate the functions of individual orphan receptors with each other in larger networks. It is particularly exciting that these basic studies are beginning to shed light on important diseases. We are now able to address not just physiological but also pathological functions of these proteins and their potential as targets for treating a surprisingly wide range of diseases. Based on this, my laboratory has adopted a new (and admittedly immodest) slogan: "No Disease Is Safe." By this we do not mean that diseases are bad but that no disease is safe from the therapeutic onslaught of nuclear receptors (NRs). The increasing range of therapeutic applications is evident in the top 10 papers that I reviewed at the ENDO 2011 Year In session. In chronological order:

"Anti-Diabetic Drugs Inhibit Obesity-Linked Phosphorylation of PPARγ by cdk5"

Choi *et al.* (1) observed that peroxisome proliferator-activated receptor-γ (PPARγ) is phosphorylated in response to a high-fat diet. This is associated with an increase in activity of a kinase called cyclin-dependent kinase 5 (cdk5) that responds to stress signals. They identified a set of PPARγ target genes that is regulated by cdk5, then identified the site of cdk5 phosphorylation on PPARγ and made a mutant in that site. Loss of cdk5 phosphorylation affects a subset of genes that includes adiponectin, CD36, and other important functional targets but not the more standard PPARγ targets that are associated with adipocyte differentiation specification. So, a specific subset of PPARγ targets are suppressed by cdk5 phosphorylation.

The researchers then showed that they could inhibit the phosphorylation of cdk5 not only by standard PPARγ agonists like rosiglitazone but also by, for example, an MRL24, a synthetic ligand compound that is a very poor agonist and activates very weakly. They showed that both

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in U.S.A.

doi: 10.1210/me.2011-1228 Received August 25, 2011. Accepted October 6, 2011.
First Published Online November 17, 2011

Abbreviations: BSEP, bile salt export pump; cdk5, cyclin-dependent kinase 5; dERR, *Drosophila* estrogen-related receptor; DLPC, dilauroyl phosphatidylcholine; ERR, estrogen-related receptor; FGF, fibroblast growth factor; FXR, farnesoid X receptor; GSK, glycogen synthase kinase; HDAC3, histone deacetylase 3; LRH-1, liver receptor homolog-1; LXR, liver X receptors; NCoR, NR corepressor; NR, nuclear receptor; PPARγ, peroxisome proliferator-activated receptor-γ; ROR, retinoid-related orphan receptor; SRC-2, steroid receptor coactivator 2; SREBP, sterol regulatory element-binding protein.

compounds are equally effective in decreasing cdk5 phosphorylation and in their antidiabetic effects. They also showed that ligand-binding actually stabilizes a particular region around the phosphorylation site that is far away from the standard activation function-2 (AF-2) surface that is conserved among NRs. A study of human diabetics supported the relevance of this site. In a panel of patients treated with rosiglitazone with a range of therapeutic efficacy, they observed a striking inverse correlation between the amount of phosphorylation of the cdk5 site and the degree of insulin sensitivity.

They concluded that cdk5 is a key modulator of PPARγ activity and that its effects can be blocked not only by agonist ligands but also by ligands that do not activate the receptor. This is exciting because it suggests that compounds that block the negative effect of cdk5 could have full therapeutic activity but none of the side effects associated with PPARγ activation. This could overcome the problems that have been associated with the PPARγ agonists that have been used to date and resurrect the PPARγ family as a real therapeutic target.

"Protein Evolution by Molecular Tinkering: Diversification of the Nuclear Receptor Superfamily from a Ligand-Dependent Ancestor"

In Bridgham *et al.* (2), the researchers delved far back into ancestral sequences of NRs to try to answer the question: Which came first, the receptor or the ligand?

The study compared NRs amino acid sequences across a very wide range of species and included new genome sequences, in particular, one from the sponge *Amphimedon queenslandica*. This represents the Porifera, the most anciently branched metazoan phylum. *A. queenslandica* has only two receptors, AqNR2 and AqNR1, and the authors were able to generate a phylogeny of all the current NRs that is rooted by these two receptors, which must be very close to the unique ancestral receptor. Interestingly, these two sponge receptors are most closely related to the mammalian hepatocyte nuclear factor 4, which binds fatty acids in the ligand-binding pocket.

The researchers then characterized the two sponge receptors and modeled them based on hepatocyte nuclear factor 4. They found that serum extracts, and also fatty acids, were able to very potently activate one of these receptors, AqNR1. This is fundamental evidence that the ancestral NRs was actually a ligand-binding receptor.

They have not yet been able to take the array of available sequences and reconstruct a unique ancestral NRs sequence, but they were able to infer conserved features. They propose that the ancestral receptor had the capacity to activate transcription in response to ligand binding. Thus, it appears that the ligand came first, and may well have been a fatty acid.

"Cellular Energy Depletion Resets Whole-Body Energy by Promoting Coactivator-Mediated Dietary Fuel Absorption"

The study by Chopra *et al.* (3) focuses more on the coregulators but starts with NRs. Bile acids are functional compounds that act as detergents in the diet to allow the absorption and digestion of lipids and lipid-soluble entities that otherwise would be difficult to get in. They are enzymatically produced in the liver, released from the gall bladder into the intestine, and are efficiently reabsorbed and recycled back to the liver. They are potentially toxic, and when their levels get too high in the liver, their production is repressed and their export is increased by pathways controlled by the nuclear bile acid receptor farnesoid X receptor (FXR).

Steroid receptor coactivator 2 (SRC-2), a coregulator, functions with FXR to activate expression of the bile salt export pump (BSEP), which is responsible for getting bile acids out of the hepatocyte and into the bile ducts. Thus, the authors showed that SRC-2 binds very nicely to the BSEP promoter, but this binding is lost in the FXR knockout liver. This is important because the SRC-2 knockouts have a defect in bile acid secretion into the lumen of the gut and therefore a defect in fatty acid absorption.

When mice are treated with radioactive palmitate, its appearance in the serum is markedly retarded in the SRC-2 knockout animals. From an opposite perspective, SRC-2 knockouts have higher triglycerides in the feces, but that phenotype can be reversed by dietary bile acid supplementation. Therefore, the SRC-2 knockout mice have a bile acid phenotype that affects the ability to absorb nutrients.

This story gets more interesting because AMP kinase, the well-known sensor of cellular energy balance, is a potent activator of SRC-2 function. The researchers screened for compounds that affected BSEP expression and found that the AMP kinase activator AICAR (aminoimidazole carboxamide ribonucleotide) is a very potent inducer of BSEP expression in hepatocytes. This response is dependent on SRC-2 and is markedly decreased in the SRC-2 knockouts. Unexpectedly, they found that AMP kinase is also recruited to the FXR/SRC-2 site in the BSEP promoter.

It therefore appears that AMP kinase actually promotes bile acid release from the liver. That is important

because the activation of AMP kinase when energy supplies are low in the liver is translated into a whole-body response that promotes energy absorption through triglycerides and lipids in the diet. So when hepatocyte energy is low, AMP kinase is activated, SRC-2 is activated, FXR is coactivated, BSEP is expressed, bile acids are secreted, and fat absorption and whole-body energy stores go back up.

"The *Drosophila* Estrogen-Related Receptor Directs a Metabolic Switch that Supports Developmental Growth"

Humans have three members of the estrogen-related receptor (ERR) or NR3B subgroup, ERRα, -β, and -γ, but flies have only one. Tennessen *et al.* (4) generated two different null alleles of the *Drosophila* version, *dERR*. The main phenotype of the mutant flies is that they are unable to maintain metabolic activity. Not too long before they die, the ATP levels are down significantly. This is also associated with decreased triglycerides and a marked increase in carbohydrates. An examination of gene arrays revealed that the transcripts for essentially all of the enzymes in the pathway of glycolysis are reduced. It appears that the dERR is a crucial integrator of glucose metabolism and glucose homeostasis and that it drives glycolysis.

In mammals, ERR have been associated primarily with increased mitochondrial biogenesis and oxidative phosphorylation. Although these specific functions are not completely consistent with the *Drosophila* results, there is certainly a general conservation of metabolic regulation. In addition, several more recent studies strongly associate mammalian ERR isoforms with regulation of glycolytic genes. It seems that metabolic regulation, and particularly control of carbohydrate metabolism, is a conserved ancestral function for this NRs subclass.

"Exercise and PGC-1α-Independent Synchronization of Type I Muscle Metabolism and Vasculature by ERRγ"

Narkar *et al.* (5) found that the ERRγ isoform is expressed in type I muscle fibers. Using a gain-of-function transgenic strategy to express ERRγ throughout skeletal muscle, they found that the type II glycolytic fibers were converted into the oxidative type I. Thus, this is yet another all-red-meat mouse, with ERRγ driving increased mitochondrial production in the type II fibers.

Consistent with this fiber type switch, the transgenic mice have more oxidative capacity and increased oxygen consumption as well as dramatically increased endurance. This is similar to a phenotype shown previously with PPARδ activation, which is associated with induction of PGC-1α as also observed in response to oxygen deprivation and exercise. That does not appear to be the case with the ERRγ transgenics, because PGC-1α levels were not changed.

Instead, the researchers observed activation of AMP kinase, which they had previously shown in the context of PPARδ activation can also promote the type I muscle fiber fate and better endurance. In this study, they show that ERRγ overexpression results in both AMP kinase activation and an increase in angiogenesis. In fact, ERRγ appears to drive the expression of angiogenic factors like vascular endothelial growth factor that promote the development of the increased blood supply that is needed to supply these endurance muscles. The prospect of substituting NRs activation for exercise is getting closer.

"A Circadian Rhythm Orchestrated by Histone Deacetylase 3 Controls Hepatic Lipid Metabolism"

This is another study (6) that focuses on both coregulators and orphan receptors. Heme is the rather unexpected ligand for Rev-erbα. Heme binding increases recruitment of the NR corepressor (NCoR) to the ligand-binding domain and decreases expression of a range of Rev-erbα targets, including those involved with circadian rhythm [because Rev-erbα and the retinoid-related orphan receptors (RORs) are part of the circadian core clock machinery), glucose and other metabolic pathways, and also production of heme itself.

In this paper, the researchers looked into the circadian connection. NCoR exerts its transcriptional repression function by bringing histone deacetylase 3 (HDAC3) to chromatin. They therefore examined the recruitment of Rev-erbα, NCoR, and HDAC3 over the 24-h cycle in the liver at the whole genome level. The HDAC3 result was particularly dramatic. Although HDAC3 protein levels do not change over the daily cycle, there were more than 10,000 HDAC3 binding sites 10 h after the lights came on, but only hundreds 12 h later. This could be explained by the observation that nearly all of the HDAC3 sites overlapped with Rev-erbα sites, and Rev-erbα protein levels and DNA binding are very strongly circadian. As expected, when HDAC3 recruitment was high, there was less RNA polymerase recruitment to nearby genes.

So what are these genes affected by HDAC3? It turns out that they are enriched for metabolic processes, and particularly lipogenesis. Consistent with this, acutely

knocking out HDAC3 in the liver caused a dramatic increase in hepatic triglycerides. The conclusion is that HDAC3 normally functions to shut off lipogenesis during the day. In its absence, lipogenesis goes unchecked, leading to an accumulation of fat in the liver, much more than would occur on a high-fat diet. The researchers observed similar but less robust results with a Rev-erbα knockout, which was consistent with their own results although not with an earlier paper (7). Although the basis for the difference is unclear, the overall picture that emerges is that Rev-erbα, NCoR, and HDAC3 coordinate circadian regulation of liver fat metabolism.

"FGF19 as a Postprandial, Insulin-Independent Activator of Hepatic Protein and Glycogen Synthesis"

The fibroblast growth factor (FGF) called FGF15 in mice and FGF19 in humans is induced in the distal small intestine by the bile acid receptor FXR (8). Because of the efficient intestinal reabsorption of bile acids along with nutrients, serum levels of FGF19 increase in response to feeding. This study shows that FGF19 exerts metabolic effects that overlap with, but are distinct from, those of the much better known feeding responses to insulin.

In the liver of mice treated with the more stable human FGF19, activation of the FGF receptor 4/βklotho complex induces the well-characterized ERK pathway. This in turn activates another kinase, MNK (MAPK iNteracting kinase), as well as p90RSK (p90 ribosomal S6 kinase), which is a well-recognized downstream target of insulin. This results in insulin-like effects that increase translation and protein synthesis.

Insulin also acts via the glycogen synthase kinases (GSK)-3α and GSK3β to induce glycogen synthesis. Thus, the loss of insulin in mice given streptozotocin reduces glycogen levels. If these mice are treated with FGF19, glycogen is rebalanced, returning to a more normal level. Like insulin, FGF19 treatment increases the phosphorylation of the inhibitory GSK. This inactivates them and increases glycogen synthase activity. However, the insulin and FGF19 pathways that target the GSK are distinct. Insulin activates Akt, which directly phosphorylates and suppresses the GSKs and also goes on to activate mTOR (mammalian Target Of Rapamycin) and S6K (S6 kinase), whereas the FGF19 effect depends on p90RSK.

Because FGF19 does not activate AKT or its downstream target mTOR, it does not share the lipogenic effects of insulin. FGF19 also affects bile acid synthesis in a pattern that is not shared by insulin. The authors suggest that both the important overlaps between the activities of FGF15 and -19, and insulin and their independent effects are consistent with the idea that both are produced in response to food. The insulin response is obviously very quick, whereas the FGF15/19 response is slower, late postprandial as opposed to immediate. They propose two hormones work together in temporally distinct phases to maintain metabolic processes such as glycogen and protein synthesis while also exerting distinct and specific effects on lipogenesis and bile acid homeostasis, for example.

"Digoxin and Its Derivatives Suppress T_H17 Cell Differentiation by Antagonizing RORγt Activity" and "Supression of T_H17 Differentiation and Autoimmunity by a Synthetic ROR Ligand"

These back-to-back papers (9, 10) report beneficial effects of ROR inverse agonists in mouse models of autoimmune disease. T0901317 was once considered to be a specific activator of LXR but has also been found to be an inverse agonist for RORα and RORγ. Solt *et al.* (10) identified a related compound, SR1001, which dials out the LXR effect but retains the inhibition of RORα and -γ. They showed by a variety of techniques that it binds both ROR isoforms directly, with the expected inhibitory effects on coactivator recruitment. Huh *et al.* (9) showed that the cardiac glycoside digoxin specifically inhibits RORγ transactivation and also identified related but less toxic compounds that retained this activity.

RORα and the RORγt isoform are key players in the development of T_H17 cells, a particular T helper subset that produces IL-17. T_H17 cells contribute to host response to pathogens but have also been associated with a variety of autoimmune diseases. Both SR1001 and digoxin inhibited differentiation of T_H17 cells and expression of IL-17 as well as the IL-23 receptor (IL-23R) and other markers. RORγt knockout mice are resistant to the development of experimental autoimmune encephalomyelitis, and both groups showed that their RORγt inverse agonists similarly delayed both onset and severity the disease. These results clearly raise the prospect of targeting RORγt to treat autoimmune disorders. However, RORα and -γ have roles outside of the immune system, and although Solt *et al.* (10) did not find any obvious toxic effects of SR1001 treatment, they did find that it affected both metabolic and circadian gene expression in the liver.

"Thiazolidinediones Enhance Sodium-Coupled Bicarbonate Absorption from Renal Proximal Tubules via PPARγ-Dependent Nongenomic Signaling"

This study (11) is a good example of an emerging theme in the nuclear receptors that is now being extended to the orphan receptors, that of nongenomic signaling. These researchers showed that PPARγ thiazolidinedione agonists very rapidly activate ERK phosphorylation and also increase the activity of the electrogenic Na^+-HCO_3^- cotransporter NBCe1 and the luminal Na^+/H^+ exchanger NHE3 in cultured kidney proximal tubules. Pioglitazone also rapidly activated ERK phosphorylation and activity of a different transporter, NHE1, in wild-type mouse embryonic fibroblasts, but not those from PPARγ knockouts. They strongly confirmed the nongenomic nature of this response by rescuing it in the mutant cells with a PPARγ construct completely lacking the DNA-binding domain. Finally, a 1-h acute treatment of rats with pioglitazone also activated ERK and significantly decreased urinary output and other functional parameters.

The conclusion is that PPARγ has both nongenomic and transcriptional effects in the kidney. The transcriptional effects are relatively well known, but the rapid effects depend on a new pathway that involves Src, the tyrosine kinase, not to be confused with the SRC coactivator, the epidermal growth factor receptor, and ERK. Both pathways seem to contribute to the edema that is a known side effect of PPARγ agonists.

"A Nuclear-Receptor-Dependent Phosphatidylcholine Pathway with Antidiabetic Effects"

Our recent paper in *Nature* (12) describes an unexpected antidiabetic effect of ligands for liver receptor homolog-1 (LRH-1), a somewhat obscure member of the NRs superfamily that has not been well characterized because the knockout is embryonic lethal. The liver-specific knockout does not show a strong phenotype, does have modestly decreased bile acid production, This was predicted from what was known about LRH-1 target genes.

Several years ago phospholipids were identified as potential ligands for LRH-1, based on several x-ray crystal structures, but the physiological relevance of this result was uncertain. Because the nucleus contains a large and dynamic pool of saturated phosphatidylcholine species, we screened such compounds for effects on LRH-1. We were surprised to identify dilauroyl phosphatidylcholine (DLPC), which has two unusually short 12-carbon saturated fatty acid side chains, as a highly specific LRH-1 agonist.

Acute experiments showed that DLPC could modestly increase bile acid levels, which we know in other contexts can have a beneficial impact on metabolic disorders. Experiments with the insulin-resistant db/db mouse showed that DLPC treatment dramatically improved insulin sensitivity. This was extended to high-fat-fed mice, where the DLPC-treated wild-type mice again showed improved glucose metabolism, whereas the LRH liver-specific knockouts did not. In both mouse models, the improved glucose homeostasis was associated with a dramatic decrease in fat accumulation in the liver. It is likely that this is due to the suppression of sterol regulatory element-binding protein (SREBP)-1c, the lipogenic transcription factor.

This antilipogenic effect of DLPC is the opposite of a pathway to insulin resistance that was described by the late Dennis McGarry in 1992 (13). Insulin levels increase in the early stages of insulin resistance. That results in induction of SREBP-1c, which increases fat accumulation in the liver. This increased steatosis, which is tightly correlated with insulin resistance, causes things to get worse, as insulin levels increase further and SREBP-1c follows along. This vicious cycle to insulin resistance is reversed by DLPC. Thus, the cycle runs backward when SREBP-1c expression is repressed, steatosis is decreased, insulin sensitivity is improved, and SREBP-1c expression is further decreased. We believe that this is a self-reinforcing pathway to improve insulin sensitivity. As we noted in our manuscript, a trial to test this prediction in humans is underway.

Acknowledgments

Address all correspondence and requests for reprints to: David Moore, Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030. E-mail: moore@bcm.edu.

Disclosure Summary: The author has nothing to disclose.

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