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A Nuclear Receptor Atlas: 3T3-L1 Adipogenesis

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The differentiation of a preadipocyte into a mature adipocyte represents a fundamental process in biology that requires a scripted program of transcriptional events leading to changes in gene expression. As part of our contribution to the Nuclear Receptor Signaling Atlas (NURSA), we used quantitative real-time PCR to profile the temporal expression of all 49 members of the mouse nuclear receptor superfamily at selected time points during differentiation of 3T3-L1 cells into mature, lipid-bearing adipocytes using two differentiation inducers [DMI (a cocktail of dexamethasone, 3-isobutyl-1-methylxanthine, and insulin) and rosiglitazone]. We also included a comparative analysis of nuclear receptor expression in mouse primary preadipocytes and mature adipocytes. In addition to confirming the expression of receptors known to be required for adipogenesis, this analysis revealed the existence of a tightly regulated transcriptional cascade that appeared in three distinct temporal phases. The first phase began within 4 h of adipogenic initiation with the transient, sequential expression of four previously uncharacterized receptors, followed by biphasic expression of a second subset, and ended with the sequential increase in a third receptor subset over a period of 2 wk after initiation. The discovery that these receptors may serve as adipogenic biomarkers and as potential therapeutic targets in adipose-related diseases highlights the utility of quantitative expression profiling as a method for directing mechanism-based approaches to study complex regulatory pathways. (*Molecular Endocrinology* 19: 2437–2450, 2005)

OBESITY REPRESENTS a tremendous international health problem and serves as a significant risk factor for many diseases such as diabetes, cancer, heart disease, and hypertension (1, 2). Obesity is characterized by increased adipose tissue mass that results from both increased fat cell number and increased fat cell size. The number of adipocytes present in an organism is determined to a large degree by the adipocyte differentiation process (3). One of the best characterized and widely used *in vitro* models to study adipocyte differentiation has been the 3T3-L1 preadipocyte cell line. This cell line was isolated from nonclonal Swiss 3T3 cells and is already committed to the adipocytic lineage (4). When treated with an empirically derived, prodifferentiation regimen that includes cAMP, insulin, and glucocorticoids, these cells undergo differentiation to mature fat cells over a 4- to 6-d period. Within 24–36 h of hormonal induction, cells re-enter the cell cycle, undergo one to two rounds of mitosis or clonal expansion, after which they permanently withdraw from the cell cycle, begin to accumulate lipid, and undergo terminal differentiation into mature adipocytes (5).

Many of the genes and their respective proteins associated with adipogenesis have been identified through research using this established adipogenic cell line. Among the most important adipogenic regulators discovered to date are members of the CCAAT/enhancer binding protein (C/EBP) and peroxisome proliferator-activated receptor (PPAR) families of transcription factors (6). For example, C/EBPβ and C/EBPδ are expressed transiently and relatively early (within 24 h) after stimulation of adipocyte differentiation, whereas C/EBPα and PPARγ are expressed relatively late (36–48 h) into the differentiation process (7, 8). Despite identification of these and other important proteins involved in adipogenesis, an understanding of the complete mechanism by which precursor cells become adipocytes, particularly during the early stages of differentiation, is far from complete. A crucial

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Abbreviations: aP2, Adipocyte protein 2; AR, androgen receptor; C/EBP, CCAAT/enhancer binding protein; COUP-TFII, chicken ovalbumin upstream promoter-transcription factor II; DMI, a cocktail of dexamethasone, 3-isobutyl-1-methylxanthine, and insulin; ER, estrogen receptor; ERRα, estrogen-related receptor α; GCNF, germ cell nuclear factor; GR, glucocorticoid receptor; LXR, liver X receptor α; MR, mineralocorticoid receptor; NGFI-B, nerve growth factor-induced gene B; NOR1, neuron-derived orphan receptor 1; NR, nuclear receptor; NURR1, nuclear receptor-related factor 1; NURSA, Nuclear Receptor Signaling Atlas; PPAR, peroxisome proliferator-activated receptor; QPCR, quantitative, real-time PCR; RAR, retinoic acid receptor; REV, reverse; RORα, retinoic acid-related orphan receptor α; RXRα, retinoid X receptor α; SDEV, standard deviation; TRα, thyroid hormone receptor α; VDR, vitamin D receptor.

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step to understanding this process requires the quantitative and temporal characterization of the transcriptional regulators involved.

Nuclear receptors represent ideal transcriptional regulatory candidates because of their well-known roles in governing gene expression in essential biological systems, including cell growth and differentiation, development, reproduction, and metabolism (9). The nuclear receptor superfamily includes the classic endocrine receptors that mediate the actions of steroid hormones, thyroid hormones, and the fat-soluble vitamins A and D, as well as a large number of so-called orphan receptors, whose ligands, target genes, and physiological functions were initially unknown (10). In addition to PPARγ, several other nuclear receptors such as the glucocorticoid, estrogen, and retinoic acid receptors (GR, ER, and RARs) have also been reported to regulate adipogenesis (11–13). However, the role of the majority of the rest of the nuclear receptors, and in particular the orphan members of the family, remains largely unknown.

As contributors to the Nuclear Receptor Signaling Atlas (NURSA), one of our main objectives is to provide to the scientific community a comprehensive analysis of nuclear receptor function during fundamental biological processes, such as adipogenesis. Toward that goal, and as a first step to exploring the role of nuclear receptors in adipocyte differentiation, we characterized the gene expression profiles of nuclear receptors during differentiation of murine 3T3-L1 cells. RNA samples were collected at different time points of differentiation, and their expression patterns were obtained by using a high-throughput quantitative, real-time PCR (QPCR) method. The resulting nuclear receptor expression matrix revealed the existence of a complex, temporally regulated transcriptional network involving numerous, previously unsuspected receptors during adipogenesis. As well as providing a springboard for further studies directed toward understanding the adipogenic process, this work establishes a convincing rationale for using a gene family-selective approach to studying a complex biological process.

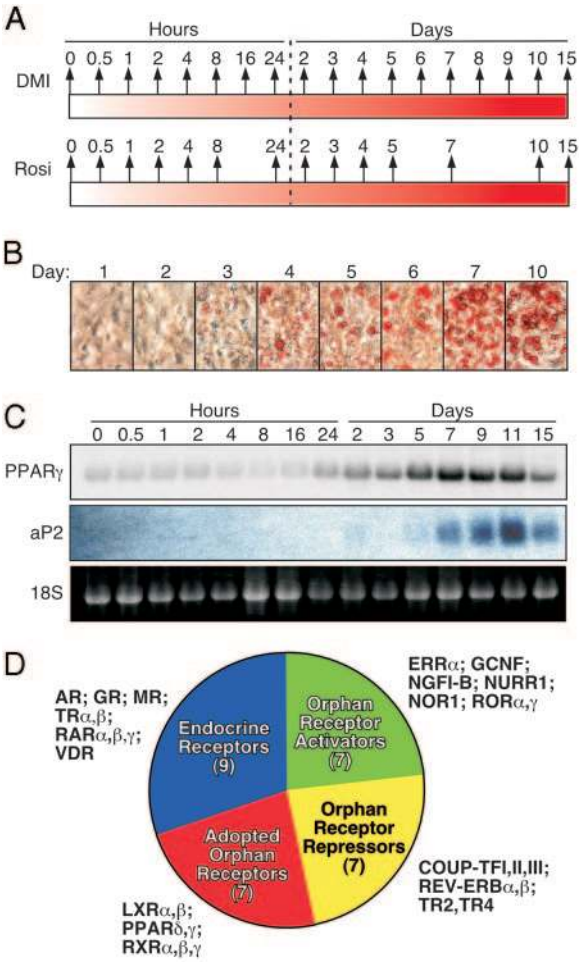

Fig. 1. Experimental Design for Expression Profiling during 3T3-L1 Adipocyte Differentiation

A, Postconfluent 3T3-L1 preadipocytes were induced to differentiate by DMI or rosiglitazone (Rosi) and processed for QPCR analysis at the indicated times over a period of 15 d. B, DMI-induced adipocyte differentiation was monitored by Oil Red O staining at the indicated time points (magnification, ×200). C, DMI-induced expression of known adipogenic markers was monitored by Northern blot analysis using PPARγ and aP2 cDNA probes. Ethidium bromide staining of 18S RNA served as a loading control. D, Composition of nuclear receptors expressed during 3T3-L1 cell differentiation. Thirty of the 49 known nuclear receptors were expressed, including nine endocrine receptors; seven adopted orphan receptors that bind to low-affinity dietary lipids; and 14 orphan receptors, consisting of seven constitutive activators and seven constitutive repressors.

RESULTS

Expression of Nuclear Receptors during 3T3-L1 Cell Differentiation into Adipocytes

Mouse 3T3-L1 cells were induced to differentiate using two different protocols: DMI (a cocktail of dexamethasone, 3-isobutyl-1-methylxanthine, and insulin) or rosiglitazone alone (see *Materials and Methods*). RNA was prepared from cells at different time points after adipogenic induction as indicated in Fig. 1A. In addition to the 3T3-L1 cells, we also harvested RNA from primary preadipocytes and mature adipocytes taken from the epididymal fat pads of C57Bl6 mice (see *Materials and Methods*). The mRNA samples from the primary cells and each time point of the differentiation experiments were then processed by performing TaqMan-based QPCR analysis as described (14), using validated primers against all 49 members of the mouse nuclear receptor superfamily. The names and abbreviations of the 49 mouse receptors are listed in Supplemental Table 1, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org. In a par-

allel experiment, histological staining for cytoplasmic lipid accumulation using Oil Red O was used to monitor the adipogenic process after inducing 3T3-L1 cells with DMI, confirming that the cells had fully differentiated (Fig. 1B). A similar analysis was used to assay rosiglitazone-induced differentiation (data not shown), although we note that the onset of differentiation induced by rosiglitazone alone was delayed by several days compared with that induced with DMI. As a further confirmation of adipocyte differentiation and to provide an alternate validation of the QPCR approach, we also performed Northern blotting against a selected set of nuclear receptor mRNAs (data not shown), as well as to adipocyte protein 2 (aP2) and PPARγ (Fig. 1C), two well-known markers of adipogenesis. As expected, the Northern blot analysis completely validated the QPCR data.

Of the 49 mouse nuclear receptors, 30 were expressed at some point during 3T3-L1 adipogenesis (Table 1 and Fig. 1D). Evaluation of the expressed receptors showed that 17 were expressed in a temporal-specific fashion that delineated a clear set of transcriptionally regulated boundaries (see below). A complete listing of the entire QPCR raw data set can be found at www.NURSA.org.

Table 1. NR Gene Expression during 3T3-L1 Differentiation

Induced or Variable Expression	Constitutive Expression	Low or Undetectable Expression (Ct $\geq$ 33)
AR	COUP-TFI	CAR
COUP-TFII	COUP-TFIII	DAX1
LXRα	ERRα	ERα
MR	GCNF	ERβ
NGFI-B	GR	ERRβ
NOR1	LXRβ	ERRγ
NURR1	PPARδ	FXRα
PPARγ	RARα	FXRβ
RORα	RARβ	HNF4α
RORγ	RARγ	HNF4γ
REV-ERBα	RXRβ	LRH1
REV-ERBβ	TR2	PNR
RXRα	TR4	PPARα
RXRγ		PR
TRα		PXR
TRβ		RORβ
VDR		SF1
		SHP
		TLX

CAR, Constitutive androstane receptor; DAX1, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X-chromosome, gene 1; FXR, farnesoid X receptor; HNF, hepatocyte nuclear factor; LRH1, liver receptor homolog 1; PNR, photoreceptor cell-specific nuclear receptor; PR, progesterone receptor; PXR, pregnane X receptor; SF1, steroidogenic factor 1; SHP, small heterodimer partner; TLX, tailless homolog.

Sequential Expression of the Nuclear Receptor (NR) 4A and Vitamin D Receptor (VDR) Nuclear Receptor Subfamilies Marks the Earliest Stages of Adipocyte Differentiation

A comparison of the temporal expression of the nuclear receptors revealed that four receptors, including the NR4A subfamily of receptors [nerve growth factor-induced gene B (NGFI-B), nuclear receptor-related factor 1 (NURR1), neuron-derived orphan receptor 1 (NOR1)], and the VDR, were expressed in a tightly regulated, sequential, and transient manner (Fig. 2). These receptors exhibited very low to undetectable expression in preadipocyte 3T3-L1 cells, but were induced to relatively high levels within the first 4 h of DMI stimulation. NGFI-B was the earliest induced gene with expression beginning at 0.5 h and peaking at 1 h, followed by NURR1 and NOR1 peaking at 1–2 h, and VDR peaking at 4 h. Remarkably, the expression of these receptors decreased back to baseline levels within 16–24 h after adipogenic induction. Thus, the NR4A gene family may represent the earliest known markers of adipogenic induction in these cells and the transient, stenotic nature of their expression may be one reason they have not been characterized previously during adipogenesis.

Interestingly, NGFI-B, NOR1, and VDR showed similar expression patterns in both DMI- and rosiglitazone-induced 3T3-L1 differentiation. However, NURR1 was not inducible by rosiglitazone, but instead its expression was decreased significantly below its already low level during rosiglitazone-induced 3T3-L1 differentiation. Not surprisingly, primary preadipocytes and mature adipocytes showed patterns of expression that closely mirrored 3T3-L1 cells before and after differentiation.

Phasic Expression of GR, Mineralocorticoid Receptor (MR), Retinoic Acid-Related Orphan Receptor α (RORα), Reverse (REV)-ERBα, REV-ERBβ, Retinoid X Receptor α (RXRα), Chicken Ovalbumin Upstream Promoter-Transcription Factor II (COUP-TFII), and Thyroid Hormone Receptor α (TRα) Distinguishes Early, Middle, and Late Transcriptional Boundaries during Adipocyte Differentiation

Five nuclear receptors (GR, MR, RORα, REV-ERBα, and REV-ERBβ) were expressed in a biphasic manner (Fig. 3), with higher levels of expression during the first several hours of adipogenesis, followed by a prolonged period of lower level expression that gradually increased back to preadipocyte levels after several days. This pattern was complemented by sequential, mutually exclusive peaks of expression of COUP-TFII expression, TRα and RXRα at 24 h (for COUP-TFII) and 48 h (for RXRα and TRα) (Fig. 3). The expression patterns for these receptors was similar in response to both DMI- and rosiglitazone-induced differentiation. In primary cells, the expression of most of these receptors was substantially higher in mature adipocytes relative to preadipocytes. Together, the tempo-

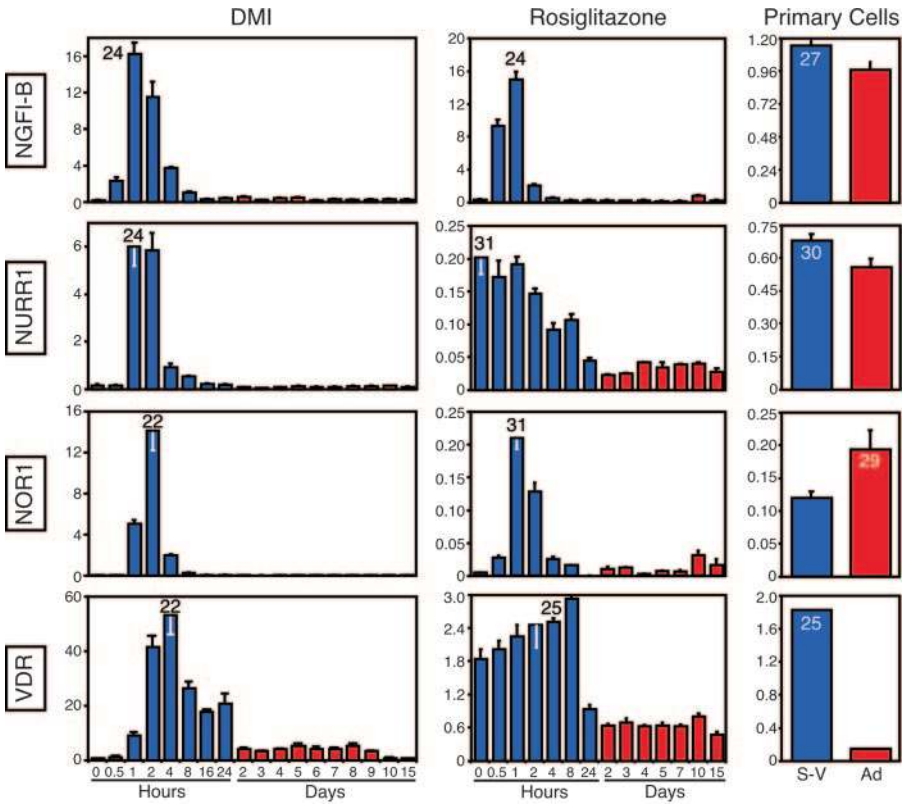

Fig. 2. Nuclear Receptors Expressed Early in 3T3-L1 Differentiation

The rapid, sequential induction and decay of four nuclear receptor mRNAs (NGFI-B, NURR1, NOR1, and VDR) mark the earliest stage of adipocyte differentiation. Postconfluent 3T3-L1 preadipocytes were induced to differentiate by DMI or rosiglitazone. Primary cells were isolated from pooled white adipose mass of 2-month-old mice (n = 10). *Blue* and *red bars* represent early (in hours) or late (in days) time points, respectively. Relative mRNA values (± SDEV) are shown on the y-axis and were obtained and normalized as described in *Materials and Methods*. Note the differences in scale of the y-axes. The cycle time for the highest mRNA expression value is shown for each experiment. S-V, Stromal-vascular fraction; Ad, mature adipocytes.

ral expression pattern of these receptors appeared to mark distinct, transcriptionally regulated boundaries appearing at early, middle, and late stages of adipocyte differentiation.

PPARγ, Liver X Receptor α (LXRα), RORγ, Androgen Receptor (AR), TRβ, and RXRγ Mark the Latest Phase of Adipocyte Differentiation

The latest stages of adipogenesis (starting 24 h after hormonal induction) were characterized by the gradual, but substantial increase in the expression of five nuclear receptors, beginning with PPARγ, LXRα, and RORγ (Fig. 4). As previously reported, PPARγ and LXRα are key markers of the differentiation program and of mature adipocytes (15, 16). As expected, their pattern of expression correlated well with the expression of other known markers of adipogenesis, including lipoprotein lipase, sterol regulatory element binding protein-1c, adipoQ, aP2, fatty acid synthase, phosphoenolpyruvate carboxykinase (PEPCK), resistin, and adipsin (Supplemental Fig. 1). Again, the expression of these receptors was similar in both DMI- and rosiglitazone-treated cells, although expression after rosiglitazone induction was delayed. This finding was consistent with the observation that rosiglitazone-treated 3T3-L1 cells took longer to differentiate.

The latest time-dependent boundary of nuclear receptor expression during 3T3-L1 differentiation was marked by the appearance of AR and RXRγ, the last two receptors to be induced by DMI (Fig. 4). Interestingly, RXRγ was the only nuclear receptor mRNA that was undetectable up to 3 d postadipogenic induction and then increased its expression dramatically, peaking by d 15. Rosiglitazone induced RXRγ in a similar fashion to DMI but failed to induce AR. In contrast, rosiglitazone induced thyroid hormone receptor β (TRβ), whereas DMI did not. These differences may reflect the relatively low level of expression for these receptors that is achieved by these inducers (Ct = 31 and 33 for AR and TRβ, respectively) because we note that mature primary adipocytes showed significant levels of both receptor mRNAs.

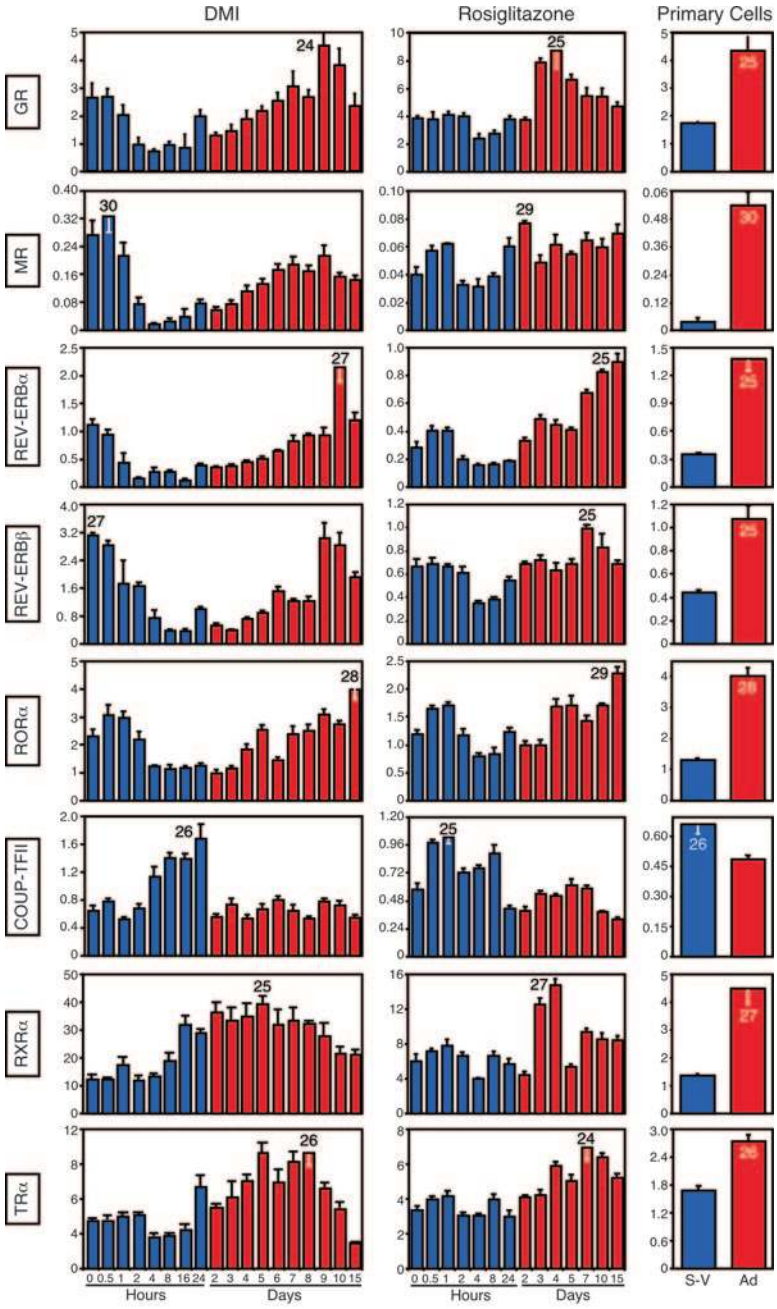

Fig. 3. Nuclear Receptors Expressed in Multiple Phases throughout 3T3-L1 Differentiation

Five nuclear receptors (GR, MR, REV-ERBα, and β, RORα) exhibited biphasic expression at early and late time points, whereas three receptors (COUP-TFII, RXRα, TRα) were sequentially expressed during the middle to late periods of adipogenesis. *Blue* and *red bars* represent early (in hours) or late (in days) time points, respectively. Relative mRNA values ($\pm$ SDEV) are shown on the y-axis and were obtained and normalized as described in *Materials and Methods* and Fig. 2. Note the differences in scale of the y-axes. The cycle time for the highest mRNA expression value is shown for each experiment. S-V, Stromal-vascular fraction; Ad, mature adipocytes.

Receptors Expressed throughout Adipogenesis

As shown in Fig. 5, 12 receptors were expressed throughout the adipogenic program at relatively constant levels. Included in this group were COUP-TFI, COUP-TFIII, estrogen-related receptor α (ERRα), germ cell nuclear factor (GCNF), GR, LXRβ, PPARδ, RARα, RARβ, RARγ, RXRβ, and testis orphan receptors 2 and 4 (TR2, TR4). One of these receptors, RARβ, was barely within the level of detection in the 3T3-L1 cell differentiation experiments, and in primary cells it was expressed but only in preadiopocytes. In contrast, 21 members of the nuclear receptor superfamily were expressed at negligible (Ct $>$ 33) to undetectable levels in 3T3-L1 cells throughout the differentiation program, regardless of the inducing medium (Table 1).

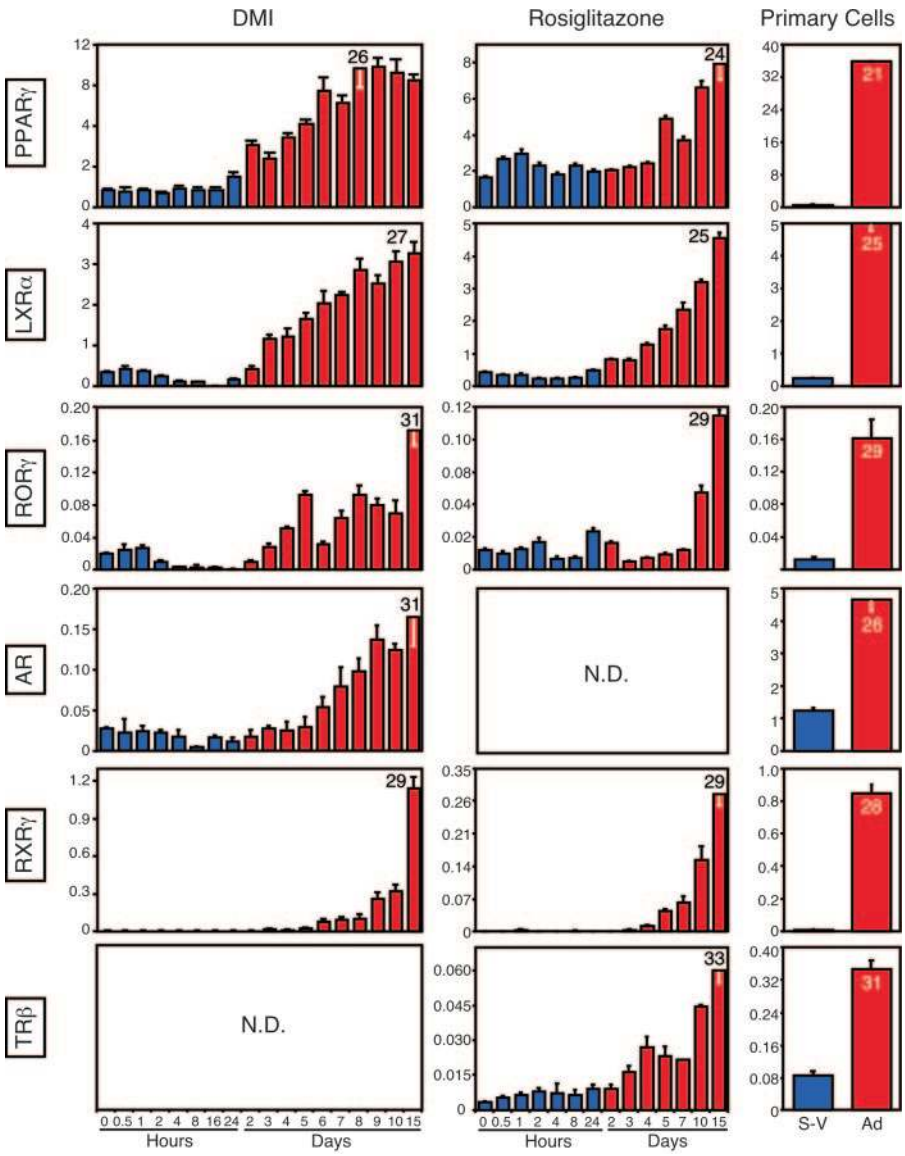

Fig. 4. Nuclear Receptors Expressed Late in 3T3-L1 Differentiation

The sequential increase in expression of PPARγ, LXRα, RORγ, AR, TRβ, and RXRγ represent the last nuclear receptors to be expressed during adipogenesis and, thus, characterize fully differentiated adipocytes. *Blue* and *red bars* represent early (in hours) or late (in days) time points, respectively. Relative mRNA values (± SDEV) are shown on the y-axis and were obtained and normalized as described in *Materials and Methods* and Fig. 2. Note the differences in scale of the y-axes. The cycle time for the highest mRNA expression value is shown for each experiment. S-V, Stromal-vascular fraction; Ad, mature adipocytes; ND, not detected.

DISCUSSION

The Nuclear Receptor Expression Matrix during Adipogenesis

One approach to the study of complex biological processes such as adipogenesis is to characterize the regulatory genes involved in the process. Completion of the sequencing and annotation of human and mouse genomes has permitted such characterizations by using high-throughput expression profiling techniques, such as QPCR. The definition of the nuclear receptor gene superfamily has allowed us to take this approach one step further by studying the temporal expression of a single class of transcriptional regulators during an important biological process. In this study, we focused on 3T3-L1 cell differentiation into adipocytes, the most commonly used model to study adipogenesis. We analyzed the expression of nuclear receptors during adipogenesis after induction by two different, but well-known inducing protocols, DMI and rosiglitazone (a PPARγ agonist). In general, the expression pattern resulting from both protocols was remarkably similar, indicating a common pathway is

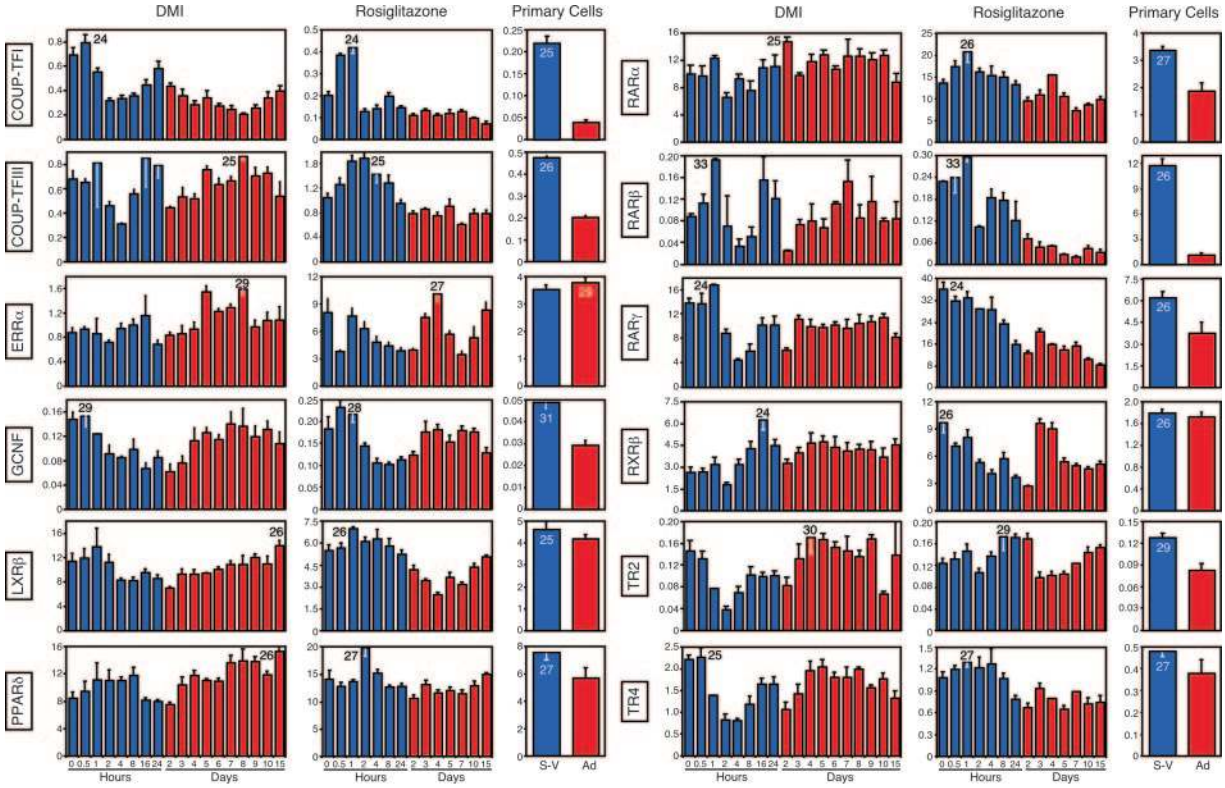

Fig. 5. Nuclear Receptors Constitutively Expressed during 3T3-L1 Differentiation

Twelve nuclear receptors were expressed throughout adipogenesis, including RARα, β, and γ, and RXRβ, whose ligands are known for their potent effects on fat metabolism. The adipocyte-related roles of the other orphan receptors in this group remain unknown. *Blue* and *red bars* represent early (in hours) or late (in days) time points, respectively. Relative mRNA values ($\pm$ SDEV) are shown on the y-axis and were obtained and normalized as described in *Materials and Methods* and Fig. 2. Note the differences in scale of the y-axes. The cycle time for the highest mRNA expression value is shown for each experiment. S-V, Stromal-vascular fraction; Ad, mature adipocytes.

used by both stimulants, even in the early stages of differentiation (see the following paragraph). We also included the expression profiling for primary preadipocytes and mature adipocytes isolated from white adipose tissue. Taken together, our data revealed a surprisingly dynamic variation in nuclear receptor expression, both in terms of the kinds of receptors and their periods of expression. The resultant atlas of nuclear receptor expression can be divided into one of three categories: 1) receptors expressed in a transient or periodic pattern during adipocyte differentiation; 2) receptors expressed at relatively similar levels throughout the differentiation process; or 3) receptors expressed at undetectable levels during the entire course of differentiation. Below, we discuss these categories and how the analysis of these data might be used to reveal cross-regulatory interactions between nuclear receptors and the transcriptional cascades that govern adipogenesis.

Early Receptors

One unexpected finding was the ephemeral and sequential nature of the expression signatures for numerous receptors that revealed four distinct, temporally regulated cascades (summarized in Fig. 6). For example, the NR4A subfamily (NGFI-B, NURR1, NOR1) and VDR were expressed sequentially at high, but short-lived levels beginning at 30 min and lasting for only a few hours after adipogenic activation. Because the earliest events governing adipogenesis are largely unknown, these four genes may represent a novel transcriptional cascade controlling the earliest stage of inducible 3T3L1 differentiation. The rapid induction of the NR4A receptor mRNAs may be due in part to the initial response of the differentiation medium, which includes serum and DMI in one case, and only a PPARγ agonist in the other case. Indeed, the NR4A subfamily is known for its early response to a number of factors, including serum (17). The induction of VDR early in the adipogenic program is also noteworthy. This receptor was transiently expressed only during the first 24 h and at one of the highest levels recorded for a nuclear receptor mRNA. This finding is in agreement with reports from others showing high levels of transiently expressed VDR early in adipocyte differentiation (18). The role of vitamin D in fat metabolism has been implicated by population studies re-

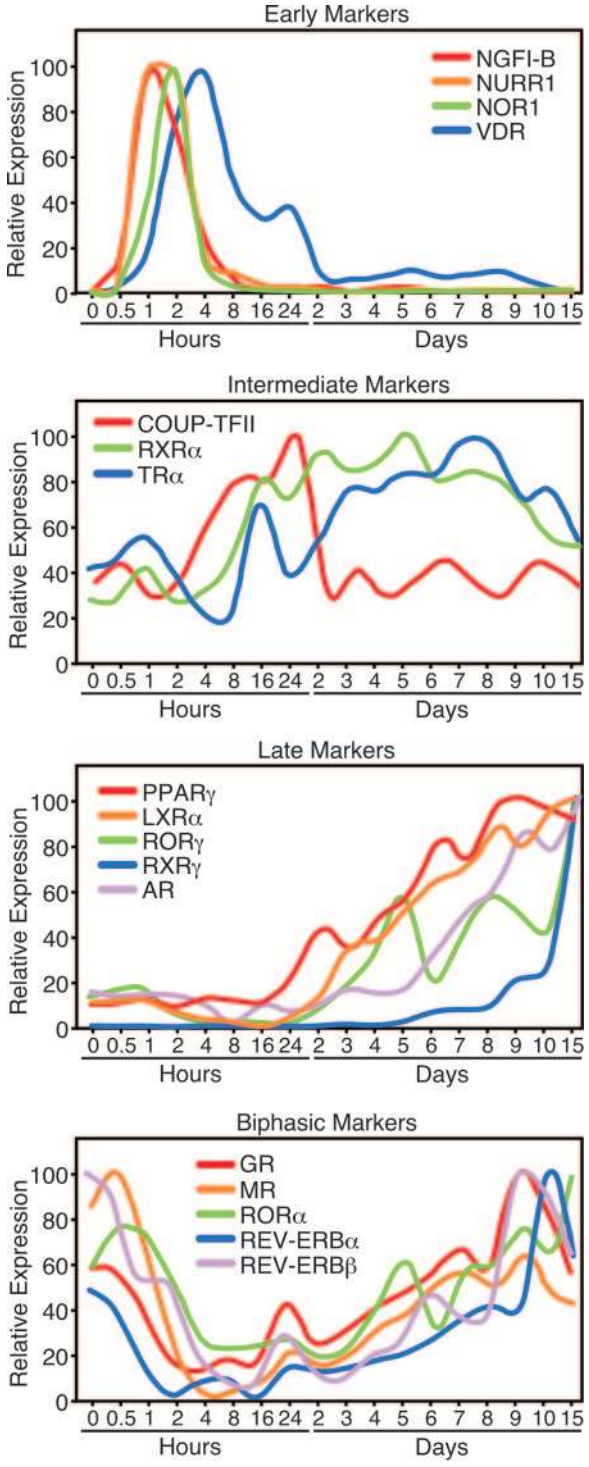

Fig. 6. Nuclear Receptor Expression Cascades during 3T3-L1 Differentiation

Analysis of the temporal regulation of nuclear receptor expression during adipogenesis revealed four expression cascades: early (NGFI-B, NURR1, NOR1, and VDR), intermediate (COUP-TFII, RXRα, and TRα), late (PPARγ, LXRα, RORγ, AR, and RXRγ), and biphasic (GR, MR, RORα, and REV-ERBα and β). Together, these cascades define distinct temporal boundaries that may represent specific transcriptional events (see text for details). Data from Figs. 2–4 were replotted in linear fashion to visualize the cascades. The maximum expression detected for each receptor over the time course of the experiment was set at 100%, and relative expression at all other time points is depicted.

porting that VDR gene polymorphisms are associated with obesity in type 2 diabetes with early age of onset (19). Moreover, serum 1,25-dihydroxy vitamin D concentrations are negatively associated with obesity (20, 21). In addition, at least one *in vitro* study suggested that VDR activation might inhibit adipogenesis (22).

Another intriguing possibility is that this group of receptors governs the cell cycle arrest in the early stage of adipogenesis that is believed to be important for promoting terminal adipocyte differentiation (23). NGFI-B, NURR1, and VDR promote cell cycle arrest by up-regulating cell cycle inhibitors such as p21, p27 etc. in other cell types (24–26). Thus, the rapid and transient expression of NGFI-B, NURR1, and VDR may be involved in the regulation of cell cycle exit and thereby permissive for terminal adipocyte differentiation.

With respect to a potential transcriptional cascade, it is tempting to speculate that the downstream targets of the NR4A orphan receptors and VDR might also be other nuclear receptors or transcription factors. C/EBPβ is one example of a factor whose sequence of expression follows the NR4A subfamily (Supplemental Fig. 1). In addition, we note that the sequential pattern of NR4A receptor expression is remarkably similar to that observed during macrophage activation [see companion paper by Barish et al. (27)]. Given the known roles of the NR4A subfamily in lymphocyte survival and neuron differentiation (28, 29), these studies suggest a common role for these orphan receptors in early cellular processes. Whether the dramatic changes in expression of these receptors are required for adipogenesis and macrophage activation awaits further study.

Biphasic Receptors

Several receptors showed biphasic expression. In particular, GR, MR, REV-ERBα and β, and RORα were expressed during the first few hours of differentiation, followed by a period of decreased expression lasting several days, and increased expression again late in the differentiation process. A potential role for MR in adipogenesis is intriguing considering MR, which is activated by mineralocorticoids in specific tissues like kidney, is also a high-affinity receptor for glucocorticoids in other tissues. The fact that glucocorticoids are potent regulators of adipose, and are included in the adipocyte differentiation medium, suggests that in addition to GR, MR may play an important role in mediating the adipogenic effects of glucocorticoids early on. GR has already been implicated in mediating the effects of glucocorticoids in the early steps of preadipocyte differentiation and promoting obesity in Cush-

ing's syndrome and during prolonged steroid therapy (11). Comparing the individual contributions of MR and GR will be of future interest. Although roles for REV-ERBα and β, and RORα in adipogenesis have not yet been elucidated, we note that the early temporal pattern of expression for these receptors is reminiscent of the circadian pattern that is seen in other tissues (30, 31).

Late-Stage Receptors

The later stages of 3T3-L1 differentiation were delineated by the expression of receptors that likely define the fully mature adipocyte. These include PPARγ and LXRα, which are known to play key roles in differentiation and maintenance of mature fat cells. Convincing evidence exists to support the contention that PPARγ is a crucial regulator of the adipogenic program (32, 33). In contrast, LXRα is not required for adipogenesis (34) but instead is likely an important mediator of lipogenesis (16, 34, 35). This conclusion is consistent with the delayed onset of LXRα expression compared with that of PPARγ, which may directly regulate LXRα expression. TRα represents another receptor that was also expressed relatively late during differentiation, peaking at 3 d after adipogenic induction. This finding is consistent with a recent study in 3T3-L1 adipocytes suggesting that TRα, like LXRα, might play an important role in lipogenesis (36).

In contrast to the proadipogenic effects of PPARγ, LXRα, and TRα, activation of AR has been reported to inhibit adipogenesis and AR-deficient male mice have been shown to develop late onset obesity (37, 38). These studies suggest that AR may serve as a negative regulator of adipose development. In the present study, we found that AR was significantly increased at 6 d after induction, by which time 3T3-L1 cells were fully differentiated into adipocytes. How AR expression affects adipogenesis is not known, but the finding of this receptor's unique expression profile warrants further study. One possibility is that AR promotes or inhibits release of factors from mature adipocytes that in turn regulate preadipocyte differentiation in a paracrine manner. The expression of RORγ also appeared relatively late in differentiation, and likewise, its role remains unknown.

It is interesting to note that several members of the same receptor subfamily were expressed in dramatically different patterns. Examples include the PPARs (γ is inducible; δ is constitutively expressed), LXRs (α is induced; β is constitutive), COUP-TFs (II is induced; I and III are constitutive), and the RXRs (α and γ are induced; β is constitutive). The meaning of these relationships is not clear, but it suggests that these receptors are not functionally redundant within their own subfamily. In the case of the RXRs (each of which is expressed in a different temporal pattern) their expression profile may reflect the need of a particular receptor partner to heterodimerize with a specific RXR subtype and thereby transactivate select sets of target genes at a given time point. Consistent with this hypothesis, we observed a reciprocal pattern of expression for RXRα and RXRγ.

Constitutively Expressed Receptors

The second category of receptors includes those that were expressed throughout the differentiation program (Fig. 5). Among these are several well-studied, ligand-dependent receptors (LXRβ, PPARδ, RARα, RARβ, RARγ, RXRβ), as well as a number of orphan receptors whose functions during adipogenesis are not known (COUP-TFI, COUP-TFIII, ERRα, GCNF, TR2, TR4). Although their expression levels did not appear to be temporally regulated, investigating the roles of receptors in this group that have not been characterized previously in adipogenesis may be revealing. For example, COUP-TFI and III were highly expressed throughout the differentiation process. Although a role for these orphans in adipogenesis is not known, mutation of Svp, the fly homolog of COUP-TFs, is known to impair fat body development in *Drosophila* (39), suggesting that a similar role may be found in mammals. ERRα and GCNF were also expressed during adipocyte differentiation and are known for their important roles during development and differentiation (40–42). In particular, ERRα-deficient mice are resistant to diet-induced obesity, suggesting a role for this receptor in fat metabolism (43).

Receptors that Are Not Expressed

In addition to the nuclear receptors that are expressed, it is worth mentioning that expression of many members (19 receptors) in this gene family was very low or undetectable during 3T3-L1 differentiation (Table 1). Although this result implies that many of these receptors play little or no role in adipogenesis, it is important to note that 3T3-L1 cells represent a working model for studying adipogenesis *in vitro*. Differentiation of 3T3-L1 cells may not completely recapitulate the adipogenic process that occurs *in vivo* because they are aneuploid and exist out of the context of their normal extracellular matrix and supporting structure. Thus, whereas some nuclear receptors are not expressed in 3T3-L1 cells, they may still be important in adipose tissue. For example, both ERα and ERβ are expressed and have important roles in white adipose tissue (for example, see Ref. 44), but neither is expressed in 3T3-L1 cells.

Conclusions

In summary, we have determined the temporal and quantitative expression of the nuclear receptor gene family during 3T3-L1 adipogenesis. The results reveal the expression of a number of receptors not previously known to be involved in adipocyte differentiation. The unexpected finding that several receptors were expressed only during the first several

Table 2. NR TaqMan Primer and Probe List

Receptor	Formal Name	mRNA Accession No.	QPCR Primers (5′–3′)	TaqMan Probe
			accgcagctaggaataatgga	
18S		X00686	gcctcagttccgaaaacca	VIC-ACCGCGGTTCTATTT
			tgtcaactccaggatgctctact	
AR	NR3C4	NM_013476	tggctgtacatccgagacttg	6FAM-TTCAATGAGTACCGCATGC
			gctgcaagggcttcttcag	
CAR	NR1I3	NM_009803	aacggacagatgggaccaa	6FAM-CAGTCAGCAAAACC
			tgctattcacgtcagatgcttgt	
COUP-TFI	NR2F1	NM_010151	cagggcacactgtgatttctc	6FAM-CCACATCGAAAGCC
			gcatgagacgggaagctgtac	
COUP-TFII	NR2F2	NM_009697	cgttggtcagggcaaactg	6FAM-AGGCATCCTGCCTCT
			aagggaccgtgctctttaacc	
COUP-TFIII	NR2F6	NM_010150	gagggctgcaagagtttcttc	6FAM-CCGGTCCAACCGTGAC
			tccggtggtgctgatcaa	
DAX	NR0B1	NM_007430	tctccactgaagaccctcaatgt	6FAM-TTTCACGCACTGCAG
			gcagatagggagctggttca	
ERα	NR3A1	NM_007956	tggagattcaagtccccaaa	6FAM-CACTCTCTTTGCCCAG
			gccaacctcctgatgcttct	
ERβ	NR3A2	NM_010157	tcgtacaccgggaccacat	6FAM-TGTCAGGCACATCAGTAA
			agcaagccccgatgga	
ERRa	NR3B1	NM_007953	gagaagcctgggatgctctt	6FAM-TGGTGACCACTATCTCTCGAT
			cagatcgggagcttgtgttc	
ERRβ	NR3B2	NM_011934	tggtccccaagtgtcagact	6FAM-CCAAGCACATCCCAGG
			acttggctgaccgagagttg	
ERRγ	NR3B3	NM_011935	gccagggacagtgtggagaa	6FAM-ATGGGCAAAACATATTC
			tccggacattcaaccatcac	
FXRα	NR1H4	NM_009108	tcactgcacatcccagatctc	6FAM-TCACAAGTTCACCCCGC
			catacaagggctaatgaagtttacca	
FXRβ	NR1H5	NM_198658	ttttgacgccttctgtaatgc	6FAM-AATTTAACCACTGAGGATCAG
			cctccctcacagtgtacagcaa	
GCNF	NR6A1	NM_010264	tgtgatataggtagatgagtcgttcaa	6FAM-AAGAACTCCACAGATTTAGT
			gcaagtggaaacctgctatgc	
GR	NR3C1	NM_008173	catacatgcagggtagagtcattctt	6FAM-TTGCTCCTGATCTGATTAT
			accaagaggtccatggtgttt	
HNF4α	NR2A1	NM_008261	gtgccgagggacgatgtag	6FAM-ACGTGCTGCTCCTAG
			atgaccaggtggccctctt	
HNF4γ	NR2A2	NM_013920	tgtagctccaagcagcagatg	6FAM-CTCCAGCATGGGCTCT
			tgggaaggaagggacaatctt	
LRH-1	NR5A2	NM_030676	cgagactcaggaggttgttgaa	6FAM-ACTCCACCATCATCTCA
			aggagtgtcgacttcgcaaa	
LXRα	NR1H3	NM_013839	ctcttcttgccgcttcagttt	6FAM-CAGAAGAACAGATCCGCTT
			aagcaggtgccagggttct	
LXRβ	NR1H2	NM_009473	tgcattctgtctcgtggttgt	6FAM-CTGAAGGCGTCCACCAT
			agcaggcctttgaggtcatt	
MR	NR3C2	XM_356093	aaggccccaccattcatg	6FAM-CATGCCTGACTCTGG
			acggtccctgcacagctt	
NGFIB	NR4A1	NM_010444	atgcgattctgcagctcttc	6FAM-ACCCGACGAGGGTCCT
			agtgtcgggatggttaaggaa	
NOR1	NR4A3	NM_015743	acgacctctcctccctttca	6FAM-ACTGTCTGTACGCACAA
			gcacttcggcggagttg	
NURR1	NR4A2	NM_013613	ggaatccagcccgtcaga	6FAM-TGTCCGCTCTCTTCATTCA
			aggtgatgctaagccagcatag	
PNR	NR2E3	NM_013708	gaggagcaatttcccaaacc	6FAM-CTCACCACCCCAGCCA
			acaaggcctcagggtacca	
PPARα	NR1C1	NM_011144	gccgaaagaagcccttacag	6FAM-ACATGCGTGAACTCCGT
			gcctcgggcttccactac	
PPARδ	NR1C2	NM_011145	agatccgatcgcacttctca	6FAM-AAGGGCTTCTTCCGCC
			caagaataccaaagtgcgatcaa	
PPARγ	NR1C3	NM_011146	gagctgggtcttttcagaataataag	6FAM-AGAACCTGCATCTCC
			gcttgcatgatcttgtgaaaca	
PR	NR3C3	NM_008829	tgtccgggattggatgaat	6FAM-CACCTGTACTGCTTGAATA
			caaggccaatggctacca	
PXR	NR1I2	NM_010936	cgggtgatctcgcaggtt	6FAM-ATCCTTCACACGTCAT
			ccagcttccagtcagtggtta	

Table 2. *Continued*

Receptor	Formal Name	mRNA Accession No.	QPCR Primers (5′–3′)	TaqMan Probe
RARα	NR1B1	NM_009024	tgctctgggtctcgatggt acagatctccgcagcatcag	6FAM-AGCACACCGTCCCCA
RARβ	NR1B2	NM_011243	gcattgatccaggaatttcca ccatgctttgtatgcaatgaca	6FAM-TACACGTTCGGCACCTTT
RARγ	NR1B3	NM_011244	ttctgaatgctgcgtctgaag gggcacaagcaacattacca	6FAM-CCTTGCAGCCTTCACAG
REV-ERBα	NR1D1	NM_145434	cacgtccccacacaccttac tgggacttttgaggttttaatgg	6FAM-CAGTAGCACCATGCCA
REV-ERBβ	NR1D2	NM_011584	gtgacagtccgttcctttgc accgtgtccatggcagaac	6FAM-ACGATTTGCTTCATTATT
RORα	NR1F1	NM_013646	tttccaggtgggatttggat ggcagacccacacctacga	6FAM-AGAACACCTTGCCCAGAA
RORβ	NR1F2	NM_146095	cagagcctccctggacttg tctacacggccctggttct	6FAM-TTTGATACGCCTTGATTT
RORγ	NR1F3	NM_011281	atgttccactctcctcttctcttg cggaacagcgctcacagt	6FAM-TGCCAACCGTCCTGG
RXRα	NR2B1	NM_011305	agctccgtcttgtccatctg caaacggctctgtgcaatct	6FAM-AGCACCCTGTCAAAGATG
RXRβ	NR2B2	NM_011306	agccctcgcagctgtaaac gccaccctggaggcctata	6FAM-CCATAGTGCTTGCCTG
RXRγ	NR2B3	NM_009107	agcagaagcttggcaaacct cccttatccggctgagaatt	6FAM-AGTATCCGGAACAGCC
SF-1	NR5A1	NM_139051	ccaggtcctcgtcgtacga cgatcctcttcaacccagatg	6FAM-CTTCCGTTCAGCGGAC
SHP	NR0B2	NM_011850	agggctccaagacttcacaca agcccgccggatcaa	6FAM-CCAGGCCTCCGTGCC
TLX	NR2E1	NM_152229	caagcgtagaccccgtagtg cgatcatggcgaccatagaa	6FAM-CCGCATTTTAGATATCCCTT
TR2	NR2C1	NM_011629	atgaactgcttgccctgtgt gtcatgagtctctccaccatcct	6FAM-TCTGCTGCTCTGTAACAATCT
TR4	NR2C2	XM_132700	gctttatccggtcaccagaaa ggatggaattgaagtgaatggaa	6FAM-CATTGTCAACCACCTACAGA
TRα	NR1A1	NM_178060	ccgttctttctttttcgctttc ctcttctcacggttctcctc	6FAM-AGTGCCAGGTCACC
TRβ	NR1A2	NM_009380	aaccagtgccaggaatgt ggcttccacttcaacgctatg	6FAM-CTTTAAGAAATGCATCTATG
VDR	NR1I1	NM_009504	atgctccgcctgaagaaac	6FAM-TTGCAGCCTTCACAGG

CAR, Constitutive androstane receptor; DAX, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X-chromosome; 6FAM, 6-carboxyfluorescein; FXR, farnesoid X receptor; HNF, hepatocyte nuclear factor; LRH-1, liver receptor homolog-1; PNR, photoreceptor cell-specific nuclear receptor; PR, progesterone receptor; PXR, pregnane X receptor; SF-1, steroidogenic factor-1; SHP, small heterodimer partner; TLX, tailless homolog.

hours after stimulation hints at a highly scripted program of early transcriptional events followed by rapid mRNA turnover. Whether these changes have meaningful biological consequences for the adipogenic program awaits future study. At minimum, these studies provide a feasible rationale for further investigating the role of these receptors during adipogenesis, and how their expression is so tightly regulated. Finally, the use of this high-throughput QPCR approach to provide an atlas of nuclear receptor expression should be amenable to other biological processes. Given the therapeutic potential of nuclear receptors as ligand-dependent transcription factors, such an approach should have promising translational value.

MATERIALS AND METHODS

Cell Culture and Differentiation

3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin in a 5% CO_2 humidified atmosphere and allowed to reach confluence. Differentiation of 2-d, postconfluent preadipocytes was induced by two protocols using either DMI or the thiazolidinedione, rosiglitazone. For DMI, postconfluent preadipocytes were incubated with a cocktail of 5 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine in DMEM supplemented with 10% fetal bovine serum for 48 h, then the culture medium was replaced every 48 h with DMEM supplemented with 10% fetal bovine serum and 5 μg/ml insulin. For rosiglitazone, postconfluent preadipocytes were incubated with 1 μmol/liter rosiglitazone in DMEM supplemented with 10% fetal

Table 3. Adipogenesis Markers SYBR Green Primer List

Receptor	mRNA Accession No.	QPCR Primers (5′–3′)
		accgcagctaggaataatgga
18S	X00686	gcctcagttccgaaaacca
		cagtggatctgacgacaccaa
AdipoQ	NM_009605	gaacaggagagcttgcaacagt
		aggacgacctcattctttttaagc
Adipsin	NM_013459	acttctttgtcctcgtattgcaa
		agtgaaaacttcgatgattacatgaa
aP2	NM_024406	gcctgccactttccttgtg
		gacatcagcgcctacatcga
C/EBPα	NM_007678	tcggctgtgctggaagag
		atttctatgagaaaagaggcgtatgt
C/EBPβ	NM_009883	aaatgtcttcactttaatgctcgaa
		ttccaacccctttccctgat
C/EBPδ	NM_007679	ctggagggtttgtgttttctgt
		gctgcggaaacttcaggaaat
FAS	NM_007988	agagacgtgtcactcctggactt
		ggccagattcatcaactggat
LPL	NM_008509	gctccaaggctgtaccctaag
		caccatcacctcctggaaga
PEPCK	NM_011044	gggtgcagaatctcgagttg
		acaagaagatcaaacaagacttcaact
Resistin	NM_022984	cagcaatttaagccaatgttcttt
		ggagccatggattgcacatt
SREBP1c	NM_011480	ggcccgggaagtcactgt

SREBP1c, Sterol regulatory element binding protein-1c; FAS, fatty acid synthase; LPL, lipoprotein lipase; PEPCK, phosphoenolpyruvate carboxykinase.

bovine serum, and the same culture medium was replaced every 48 h.

Isolation of Primary Adipocytes

Two-month-old male C57BL/6J mice were used to obtain primary adipose cells essentially as described previously (45). Briefly, the epididymal fat pads were removed, minced, and digested using collagenase (3 mg of collagenase/1 g of fat tissue) at 37 C for 0.5 h. After 10 min centrifugation at 1200 rpm, the mature adipocytes were located on the top white layer and the stromal-vascular fraction containing preadipocytes was in the pellet.

Oil Red O staining

Oil Red O staining was performed as previously described with minor modifications (46). In brief, cells were washed twice with PBS and fixed with 10% formalin in PBS for 15 min. After two washes in PBS, cells were stained for at least 1 h in freshly diluted Oil Red O solution (six parts Oil Red O stock solution and four parts H_2O; Oil Red O stock solution is 0.5% Oil Red O in isopropanol). The stain was then removed and the cells were washed twice with water, with or without counterstain (0.25% giemsa for 15 min), and then photographed.

RNA Isolation and QPCR

Total cellular RNA was isolated from 3T3-L1 cells at various times after adipogenic induction and from primary adipocytes using RNA Stat-60 (Tel-Test, Friendswood, TX) as previously described (14). The mRNA levels in each sample were measured using the TaqMan-based standard curve assay with an ABI (Foster City, CA) 7900HT Sequence Detection System as described previously (14) (Tables 2 and 3). PCR efficiencies were calculated from the slope of the resulting standard curves using the formula

$$E = 10^{\frac{-1}{slope}}$$

where E is efficiency. The calculated efficiency was used to convert cycle times (Ct) from log to linear scale using E^{-ct}. Normalized mRNA levels were obtained by dividing the averaged, efficiency-corrected nuclear receptor values by that of 18S for each sample

$$\left(\frac{(E_{NHR})^{-Ct_{NHR}}}{(E_{18S})^{-Ct_{18S}}}\right).$$

The resulting values were multiplied by 10^6 and plotted $\pm$ SD [standard deviation (SDEV)] from triplicate sample wells. The primer/probe sets for the 49 mouse nuclear receptors and other adipogenic markers were validated as described and are available in Tables 2 and 3 and on our web site at www.NURSA.org.

Northern Analysis

Northern analysis was performed according to standard techniques. Total RNA (10 μg) was fractionated on formaldehyde 1% agarose gels. RNA was transferred to nylon membranes and cross-linked by UV irradiation. Blots were hybridized with ^{32}P-labeled aP2 and PPARγ cDNA probes. After washing, blots were exposed to x-ray film at −80 C overnight and then developed.

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