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Regulation of corepressor alternative mRNA splicing by hormonal and metabolic signaling

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Abstract

Alternative mRNA splicing diversifies the products encoded by the NCoR and SMRT corepressor loci. There is a programmed alteration in NCoR mRNA splicing during adipocyte differentiation from an NCoR δ isoform, which contains three nuclear receptor interaction domains, to an NCoR δ isoform that contains two nuclear receptor interaction domains. This alternative mRNA splicing of NCoR has profound effects on adiposity and on diabetes in mouse models. We report here that dexamethasone, a powerful regulator of metabolism and of adipocyte differentiation, confers this change in NCoR mRNA splicing in cultured adipocytes. We also demonstrate that changes in dietary components can consistently, if moderately, modulate the total transcript levels and the mRNA splicing of NCoR and SMRT in both cultured cells and intact mice. This ability of alternative corepressor mRNA splicing to respond to nutritional changes confirms its importance in regulating glucose and lipid metabolism, and its promise as a therapeutic candidate for metabolic disorders such as type 2 diabetes.

Keywords

Transcriptional regulation; Corepressors; Alternative mRNA splicing; SMRT; NCoR; Isoforms

1. Introduction

Two of the most extensively characterized transcriptional corepressors are the Silencing Mediator of Retinoic acid and Thyroid hormone receptor (SMRT) and the Nuclear Receptor Corepressor (NCoR), which are paralogs of one another (Perissi et al., 2010; Watson et al., 2012; Stanya and Kao, 2009; Mottis et al., 2013; Hu and Lazar, 2000; Astapova and Hollenberg, 2013). SMRT and NCoR help recruit a variety of chromatin-modifying enzymes, such as HDAC3, to various transcription factors, and are particularly important in mediating repression by the nuclear receptors (also known as nuclear hormone receptors) (Jepsen and Rosenfeld, 2002). SMRT and NCoR contain multiple domains both for tethering to their nuclear receptor partners (Receptor Interaction Domains, or RIDs), and for

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.mce.2015.06.036>.

tethering the chromatin modifiers that actually repress transcription (Silencing Domains, or SDs) (Fig. 1) (Hu and Lazar, 2000; Privalsky, 2004). Nuclear receptors comprise a large family of ligand-regulated transcription factors, including the thyroid hormone, peroxisome-proliferator activated, and glucocorticoid receptors. These nuclear receptors play key roles in the regulation of both glucose and lipid metabolism (Sonoda et al., 2008; Chawla et al., 2001; Spiegelman, 1998). Not surprisingly therefore, among their other functions, SMRT and/or NCoR control adipogenesis and regulate glucose sensitivity through their ability to alter expression of specific gene panels (Yu et al., 2005; Yamamoto et al., 2011; Nofsinger et al., 2008; Li et al., 2013, 2011; Guan et al., 2005; Ghisletti et al., 2009; Feige and Auwerx, 2007; Cohen, 2006). Changes in corepressor function are associated with human endocrine, metabolic, and oncogenic diseases (Astapova and Hollenberg, 2013; Yamamoto et al., 2011; Li et al., 2011; Rosen and Privalsky, 2011; Mengeling et al., 2011), and they are therefore plausible targets for therapeutic intervention (Perissi et al., 2010; Hsia et al., 2010).

SMRT and NCoR mRNAs are each alternatively spliced at several locations that retain or remove exons, thus yielding a host of unique protein isoforms (Fig. 1) (Short et al., 2005; Goodson et al., 2005a). Notably, these spliced isoforms can possess differing numbers of RIDs, distinct preferences for particular nuclear receptor partners (such as the thyroid hormone and peroxisome-proliferator activated receptors), and can vary in their responses to post-translational modifications (Malartre et al., 2004; Jonas et al., 2007; Goodson et al., 2005b; Faist et al., 2009). Different core-pressor isoforms can play drastically different physiological roles (Malartre et al., 2006). For example, one splice isoform, NCoR ω , arises from the incorporation of an exon (37b+) that encodes a 3rd RID and appears to inhibit adipose tissue formation. Conversely, NCoR δ incorporates a shorter version of this exon (37b-) that lacks this 3rd RID yet promotes adipose tissue formation (Goodson et al., 2011). Mice engineered to specifically ablate the NCoR ω isoform also (somewhat paradoxically) exhibit a greatly improved glucose sensitivity that is refractory to diet induced diabetes (Goodson et al., 2014). Given that the different corepressor isoforms play distinct roles in energy metabolism (Goodson et al., 2011, 2014), targeting specific corepressor isoforms and understanding their regulation may serve as a particularly promising means for the treatment of metabolic disorders.

We report here our identification of the hormonal and nutritive events that initiate these changes in alternative corepressor mRNA splicing, and thus can impact hormone regulation and metabolism. Our studies demonstrate that dexamethasone (DEX), a known powerful modulator of glucose metabolism (Qi et al., 2004), is the key driver of the previously reported changes in NCoR mRNA splicing during 3T3-L1 cell adipogenesis (Goodson et al., 2011). Moreover, rosiglitazone (ROSI), a distinct pharmacological inducer of adipogenesis and glucose utilization (Goodson et al., 2011; Fujiwara et al., 1988), also induces this shift in NCoR splicing. We further report that increasing the availability of glucose, but not fatty acids, shifts SMRT mRNA splicing in adipocyte and hepatocyte cell lines toward isoforms possessing a third RID domain. This glucose-induced shift in splicing also occurs in NCoR in hepatocytes at a comparable location to that in SMRT. Similar alterations in corepressor splicing also occur in both adipose and liver tissues in a whole mouse dietary study. We conclude that alternative core-pressor RNA splicing utilizes hormonal and nutritional cues to fine-tune the actions of their transcription factor partners, such as the nuclear receptors, and

thus the regulation of glucose and lipid metabolism in normal physiology. Corepressor splicing may therefore serve as a promising pharmacological target to treat type 2 diabetes and other endocrine disorders.

2. Materials and methods

2.1. Reagents

Insulin and rosiglitazone (ROSI) were purchased from Life Technologies and Cayman Chemical respectively. Dexamethasone (DEX), IBMX (3-isobutyl-1-methylxanthine), oleic acid and linoleic acid were obtained from Sigma–Aldrich.

2.2. Cell culture

3T3-L1 cells (ATCC, Manassas, VA) were propagated in Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM glucose, 1 mM sodium pyruvate (Life Technologies, Grand Island, NY) and 9% newborn calf serum (JR Scientific, Woodland, CA). Hepa1-6 cells (ATCC, Manassas, VA; a murine hepatoma cell line) and embryonic fibroblast cells derived from wild-type C57BL/6 mice (MEFs) were propagated in DMEM supplemented with 25 mM glucose and 9% fetal bovine serum (FBS; PAA Laboratories, Dartmouth, MA). All cells were propagated at 37 °C in a 5% CO₂ atmosphere.

2.3. 3T3-L1 differentiation

3T3-L1 preadipocytes were differentiated as previously described (Student et al., 1980). Adipogenesis was induced with single or multiple components of the cited differentiation cocktail on differentiation day 0, with omitted factor(s) replaced with vehicle only (either ethanol or DMSO); the cells were subsequently re-fed with medium containing insulin or 2.5 uM ROSI as indicated (Student et al., 1980). For acute treatments, 3T3-L1 cells were exposed for 48 h to ROSI, either as undifferentiated preadipocytes or as mature adipocytes previously differentiated by prior exposure to tripartite differentiation medium.

2.4. Oil Red O staining

On differentiation day 12, the 3T3-L1 adipocytes were stained for lipid accumulation with Oil Red O (Goodson et al., 2011; Kinkel et al., 2004) and counterstained with hematoxylin. Oil Red O staining was quantified through the analysis of six microscopic fields using the Color Threshold and Analyze Particle functions of NIH ImageJ 1.46r (Collins, 2007).

2.5. Nutritive manipulations of cultured cells

All cells were plated in six-well dishes containing the above media formulated with 25 mM glucose. For the glucose manipulation experiments, cells were switched 48 h before harvesting from this “propagation” media to the “treatment” media containing 1 mM sodium pyruvate, insulin (if 3T3-L1 adipocytes), 9% FBS, plus the levels of glucose (0, 5, 25, 35, or 50 mM) indicated. For the fatty acid manipulations, cells were switched 48 h before harvesting from the “propagation” media to “treatment” media containing 1 mM sodium pyruvate, insulin (if 3T3-L1 adipocytes), 25 mM glucose, 9% FBS, plus the levels of fatty

acid (0, 0.03, or 0.06 mM) indicated. Multiple biological replicates representing distinct experiments were performed on different days.

2.6. Diet manipulations in mice

Adult male wild-type C57BL/6 mice, 144–157 days old were fed ad libitum either a low sucrose, high fat diet (60% kcal fat, 20% kcal carbohydrate, 20% kcal protein; catalog D12492) or a high sucrose, low fat diet (10% kcal fat, 70% kcal carbohydrate, 20% kcal protein; catalog D12450B) purchased from Research Diets, Inc. (New Brunswick, NJ). Four to seven mice were used per diet (indicated). After 14 weeks on each diet, the mice were euthanized as approved by the AAALAC; tissues were harvested by dissection and were stored in RNAlater (Life Technologies, Grand Island, NY).

2.7. RT-PCR analysis of corepressor isoform expression

RNAs were isolated using QIAzol Lysis Reagent (Qiagen, Hilden, Germany) using the manufacturer's protocol. Complimentary DNAs (cDNAs) were synthesized using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany); a common reaction was used for all the cDNAs generated from each sample. The cDNAs derived from 25 ng RNA were amplified for 32 cycles using GoTaq DNA Polymerase (Promega, Madison, Wisconsin) and splice-specific primers (Supplemental Table 1). These splice-specific primer pairs flank each alternative splice-site and were used to yield multiple distinct-sized PCR products representing the individual corepressor isoforms generated from that particular alternative splicing location (Goodson et al., 2011, 2014). These PCR products were then loaded in individual lanes for each splice site, resolved by gel electrophoresis, detected by ethidium bromide staining, and quantified using an Alpha Innotech FluorChem 8900 and AlphaEase software (Version 3.1.2). The abundance of each alternatively spliced corepressor isoform was calculated as percentage of the sum of all the alternatively spliced isoforms produced at that location (i.e. each isoform was represented as a distinct sized PCR product migrating in a given gel lane). Our quantification was therefore internally controlled for primer/PCR reaction efficiency and was independent of any non-uniformity in loading, electrophoresis, staining, or destaining. The multiple alternatively spliced locations within a corepressor mRNA were analyzed using a panel of primer pairs, each specific for the given alternatively spliced location.

2.8. Quantitative real time PCR for endogenous gene expression

RNAs and cDNAs were analyzed by quantitative real-time PCR using SsoAdvanced SYBR Green Supermix (BioRad, Hercules, CA), a DNA Engine Opticon2 Real-Time Cyclor (BioRad, Hercules, CA) and gene-specific primers (Supplemental Table 1B). The expression levels of the total (i.e. pan-splice) transcripts for each corepressor were calculated using the Ct method (Livak and Schmittgen, 2001). Vehicle-only samples served as the reference Ct and the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene served as an endogenous normalization control.

3. Results

3.1. Dexamethasone (DEX) mediates the shift in alternative mRNA splicing of NCoR observed during 3T3-L1 differentiation in culture

The 3T3-L1 adipocyte model is used to mimic *in vivo* adipo-genesis by the treatment of undifferentiated 3T3-L1 cells, which display a fibroblast-like preadipocyte phenotype, with a “differentiation cocktail” that induces the cells to differentiate into a mature, lipid-accumulating, adipocyte-like phenotype (Rosen and Spiegelman, 2000). The classically-used differentiation cocktail consists of a tripartite mixture containing DEX, insulin, and IBMX (Rosen and Spiegelman, 2000). These three different cocktail components play distinct roles in mediating adipo-genesis and must be provided in a specific order to achieve a complete differentiation phenotype in the 3T3-L1 cells (Pantoja et al., 2008).

When stimulated to differentiate by the tripartite cocktail, there is a distinctive shift in NCoR splicing in the 3T3-L1 cells from mRNAs that include a 37b+ exon (producing NCoR ω) to mRNAs that include a 37b– exon (producing NCoR δ) (Goodson et al., 2011). There are no significant changes in total NCoR or SMRT mRNA expression, or alternative mRNA splicing at any other positions examined ((Goodson et al., 2011) and data not shown). Consistent with this observation, NCoR ω is anti-adipogenic and NCoR δ is proadipogenic when overexpressed in 3T3-L1 cells (Goodson et al., 2011). In agreement with these conclusions based on *ex vivo* over expression, a whole mouse knockout of NCoR ω increases adipose tissue size and weight gain, whereas the corresponding knockout of NCoR δ produces a lean phenotype (Goodson et al., 2014). The mouse knockout of NCoR ω also produces a substantial increase in glucose sensitivity that renders the mice more resistant to obesity-induced diabetes.

We emphasize that although the changes observed here in alternative corepressor splicing at exon 37 are relatively moderate in extent, they are highly statistically significant and highly reproducible. We also emphasize that artificial manipulation of the transcript ratios of NCoR ω to NCoR δ to a comparable extent cause profound effects on 3T3L1 adipogenesis, indicating that these modest alterations in NCoR splicing can play biologically relevant roles in the regulation of adipose differentiation (Goodson et al., 2011). Further, although NCoR ω represents a small percentage of the total NCoR in differentiated adipocytes *in vitro* and *in vivo*, ablation of even this modest residual amount by splice-specific knockout dramatically changes lipid and glucose metabolism in mice (Goodson et al., 2014).

We therefore next examined the roles of the individual differentiation cocktail components on corepressor splicing in the 3T3-L1 cells. DEX alone, or in combination with one or more other components, drove NCoR exon 37 splicing from NCoR ω (inclusion of the 37b+ exon) to NCoR δ (inclusion of the 37b– exon). This DEX-induced shift in NCoR gene expression was identical to that seen with the complete differentiation cocktail (Fig. 2A, right panel, compare bars 3, 5, 7 and 8). In contrast, DEX alone, or in combination with just one other factor, exhibited only an abortive differentiation phenotype (as assayed by lipid staining) on the 3T3-L1 cells compared to the complete trivalent cocktail (Fig. 2B). No changes were observed at the other NCoR and SMRT alternative splice sites examined (Fig. 2A, left and middle panel). We conclude that DEX is the necessary and sufficient component in the 3T3-

L1 differentiation cocktail that mediates the observed change in corepressor splicing, and that this change in splicing is not dependent on terminal adipogenesis. Further, DEX did not have an effect on the alternative splicing of NCoR mRNA when used on already mature, differentiated adipocytes (Supplemental Figure S1), thus linking the effects of DEX to the differentiation state of these cells.

3.2. Alternatively, a chronic exposure to ROSI also supports the shift in NCoR mRNA splicing

ROSI can be used as an alternative to insulin to support 3T3-L1 adipocyte differentiation after initiating this process with the tripartite cocktail (Goodson et al., 2011). ROSI is a synthetic agonist of peroxisome proliferator-activated receptor gamma (PPAR γ) and is thought to participate in adipocyte differentiation through a distinct mechanism from that of insulin (Chawla et al., 2001; Lehmann et al., 1995). We therefore compared three different courses of 3T3-L1 differentiation: the classical eight days of insulin treatment after initiation with trivalent cocktail ("Insulin"), four days of insulin treatment followed by four days of ROSI treatment after initiation ("Insulin + ROSI"), or eight days exclusively of ROSI treatment after initiation ("ROSI") (Fig. 3). Both of the ROSI courses helped maintain and enhance the change in splicing from the NCoR ω to the NCoR δ RNA isoform (i.e. exon 37b+ to exon 37b-), as well as inducing a more mature adipocyte phenotype in the Oil Red O assay ((Goodson et al., 2011) and data not shown). Therefore the shift in NCoR mRNA splicing is associated with, and perhaps required for, overt adipocyte differentiation induced by at least two different chemical agents. A moderate change in the mRNA splicing of SMRT at exon 40 and NCoR at exon 28 was also observed under these ROSI conditions (Fig. 3).

3.3. Acute exposure of 3T3-L1 cells to ROSI is sufficient to alter NCoR splicing

When 3T3-L1 preadipocytes were treated with ROSI for 48 h in the absence of an initiating exposure to the trivalent differentiation cocktail, we also observed a shift from incorporation of NCoR exon 37b+ to inclusion of exon 37b- (from the NCoR ω to the NCoR δ isoform) (Fig. 4A, bottom panel). Although this shift in NCoR mRNA splicing in undifferentiated 3T3-L1 cells was modest, it was still highly reproducible. This shift was more pronounced when the same 48-h ROSI treatment was applied to 3T3-L1 cells previously induced to form adipocytes by prior exposure to trivalent differentiation cocktail (Fig. 4B). No or modest changes were observed at the other NCoR and SMRT alternative splice sites examined.

3.4. Manipulations of glucose in the medium cause cell-type dependent changes in total corepressor mRNA expression levels

Increasing glucose in the media of mouse embryonic fibroblasts (MEFs) increased the levels of total SMRT (and likely NCoR) mRNA (using "pan" reactive, splice-independent, probes) (Fig. 5, top panel). Different results, however, were observed with different cell types: increasing glucose decreased total SMRT mRNAs in 3T3-L1 adipocytes (Fig. 5, middle panel), whereas it decreased both total SMRT and total NCoR mRNAs in Hepa1-6 liver cells (Fig. 5, bottom panel). Adding oleic acid had little or no effect on either NCoR or SMRT mRNA levels when these cells were maintained in standard 25 mM glucose (Fig. 5). We conclude that glucose treatment altered both total NCoR and SMRT transcript levels,

but that this effect is cell-type dependent and can be observed in the absence of a response to lipid.

3.5. Manipulations of glucose in the medium also altered mRNA splicing of both SMRT and NCoR mRNAs

In addition to examining the effect of nutrients on total core-pressor gene expression, their effects on alternative mRNA splicing of SMRT and NCoR were also tested. Elevating the glucose level from 0 to 50 mM in the medium of mature 3T3-L1 adipocytes caused a significant shift in mRNA splicing from the SMRT exon 40b-to the SMRT exon 40b+ isoform, e.g. SMRT δ to SMRT ω (Fig. 6A). The same glucose-induced change in SMRT mRNA splicing was also observed in Hepa1-6 hepatocytes and in MEFs (Fig. 6B, C). An analogous shift from NCoR δ to NCoR ω transcripts was observed in the Hepa1-6 cells (Fig. 6B), however this shift in NCoR either did not occur, or was slightly in the opposite direction in the 3T3-L1 adipocytes and in MEFs (Fig. 6A, C). A modest change was also observed in alternative mRNA splicing at exon 28 of SMRT in 3T3L1 cells exposed to increasing glucose (Fig. 6A). In contrast, changes in fatty acid concentration did not have any observable effect on corepressor mRNA splicing in any of the cell lines tested (Fig. 6).

To determine if the moderate alterations seen in SMRT splicing in response to glucose availability are able to mediate changes in biologically-relevant target gene expression, we artificially altered the transcript abundance ratios of SMRT isoforms in the Hepa1-6 cell line. This was achieved by modest overexpression of SMRT δ or SMRT ω in cells maintained in 25 mM glucose culture medium; the former thereby mimics the higher SMRT δ to ω mRNA ratios observed in these cells when deprived of glucose. Notably, this modest increase in SMRT δ to ω resulted in significant changes in expression of several glycolytic and metabolic genes, including AP2, HK2, and SLC2a1 (Suppl. Fig. S2). Conversely, the AP2 transcript levels changed reciprocally in response to overexpression of SMRT ω compared to SMRT δ , as might be expected, although the changes in expression of none of the 3 metabolic genes in response to SMRT ω was statistically significant versus a GFP control (perhaps suggesting that the effect of SMRT ω was already saturating before this manipulation). We conclude that even modest changes in corepressor mRNA splicing, comparable to those observed in response to our nutrient manipulations, can invoke significant differences in physiologically relevant gene expression.

3.6. Fructose also caused changes in corepressor mRNA splicing

A 48-h fructose treatment of the 3T3-L1 cells resulted in a modest shift from the SMRT δ to the SMRT ω isoform (Suppl. Fig. S3); this shift was smaller than, but paralleled, the effect of increasing glucose concentration. No effect of fructose was seen on NCoR splicing in these cells. The opposite was observed in Hepa1-6 hepatocytes, wherein fructose exposure induced a shift from the SMRT ω to the SMRT δ isoform and from the NCoR ω to the NCoR δ isoform at the mRNA level (at exons 40 and 37, respectively) (Suppl. Fig. S3).

3.7. Mice fed a high sucrose diet displayed shifts from SMRT δ to SMRT ω in adipose tissue and from NCoR δ to NCoR ω in liver tissue

Adult male C57BL/6N mice were fed either low sucrose (high fat) or high sucrose (low fat) diets (equivalent calories per gram feed) for 14 weeks. Mice on the high sucrose (low fat) diet gained less weight and exhibited better glucose and insulin sensitivity when challenged with glucose and insulin tolerance tests than mice on the low sucrose (high fat) diet (Goodson et al., 2014). No statistically significant changes in corepressor mRNA splicing were observed in subcutaneous white adipose tissue or in skeletal muscle on these two diets (Fig. 7B, D). However, similar to our cultured 3T3-L1 adipocytes, there was a shift in splicing from the SMRT exon 40b-transcript (SMRT δ) to the SMRT exon 40b+ transcript (SMRT ω) in the visceral white adipose tissue of mice fed the high sucrose diet (Fig. 7A). In common with our Hepa1-6 hepatocyte results, liver tissue harvested from mice on the high sucrose diet showed a reproducible shift from the NCoR exon 37b-transcript (NCoR δ) to NCoR exon 37b+ transcript (NCoR ω) (Fig. 7C). Of course, the changes we observe in the mice related to adiposity may be through a distinct mechanism from the changes observed as alterations in adipogenesis in the 3T3-L1 cell model.

4. Discussion

4.1. Corepressor mRNA splicing, which plays a key role in control of energy storage and glucose sensitivity, is responsive to relevant hormonal and pharmacological modulators of adipocyte differentiation

Alternative mRNA splicing generates NCoR and SMRT core-pressor isoforms that play different roles in development, in normal physiology, and in disease (Short et al., 2005; Goodson et al., 2005a, 2005b; Malartre et al., 2004; Jonas et al., 2007; Faist et al., 2009; Malartre et al., 2006; Goodson et al., 2011, 2014). Here we have determined how these alternative corepressor mRNA splicing events are regulated both physiologically and pharmacologically in relation to control of energy metabolism. The switch in NCoR exon 37 splicing during adipocyte differentiation, from a NCoR ω to a NCoR δ isoform transcript (Goodson et al., 2011), appears to be in response to the DEX component of the trivalent “cocktail” commonly utilized to induce adipogenesis in the 3T3-L1 cell model. DEX is a synthetic derivative of a natural hormone that regulates many key aspects of glucose and lipid metabolism (Qi et al., 2004; Wu et al., 1996), and DEX treatment alone appears to be both necessary and sufficient to alter NCoR mRNA splicing. These results are compatible with studies demonstrating that each of the three components of the differentiation cocktail play separate roles in inducing adipogenesis, and that the order of addition of these components is crucial to obtain full differentiation (Pantoja et al., 2008). DEX alone has been reported to induce a novel commitment state in 3T3-L1 cells termed the “DEX-primed preadipocyte” which displays a gene expression profile intermediate between that of a preadipocyte and a mature adipocyte (Pantoja et al., 2008). Our own findings suggest that changes in NCoR mRNA splicing may help define this novel DEX-induced commitment state. Furthermore, the fact that an acute 48-h DEX treatment does not further shift NCoR splicing in already mature adipocytes emphasizes the linkage of these changes in alternative splicing to events in the differentiation process itself. Notably, the effects of DEX on alternative NCoR mRNA splicing do not require complete adipogenesis and are also seen in

response to ROSI treatment. We therefore propose that these changes in NCoR mRNA splicing may favor or even promote adipogenesis, rather than simply being a secondary effect of adipocyte differentiation.

Although the changes in the ratios of the different corepressor isoforms as measured here are relatively moderate, we emphasize that our measurement techniques are internally controlled, thus avoiding potential problems such as differences in primer efficacy and loading variation, and that our results are highly reproducible in multiple biological replicates. We also emphasize that our current study focuses on the analysis of mRNA populations to study NCoR and SMRT alternative splicing in large part due to the technical limitations of studying these phenomena at the protein level. These corepressors are quite large (~250 kDa) and the modest changes in protein size due to alternative splicing can not be resolved by the electrophoresis/immunoblotting (using panspecific antisera) methods that we have attempted. The ability to raise isoform-specific antibody for use in immunoblotting is also limited, particularly for corepressor isoforms that are distinctive only due to their absence of a particular coding exon. To our knowledge, the only available splice-specific anti-NCoR or anti-SMRT antibody is limited to a single anti-NCoR ω reagent (which expresses an exon sequence not in NCoR δ), therefore preventing a comprehensive, appropriately quantitative analysis at the corepressor protein level. We also note, that in our own and other studies, SMRT and NCoR mRNAs levels have generally been found to be predictive of their corresponding protein levels in response to both nutritive manipulations (Yamamoto et al., 2011) and to artificial siRNA knockdowns (Yu et al., 2005).

4.2. Total corepressor gene expression levels and alternative mRNA splicing are modulated by glucose in a cell-type specific manner

We observed an increase in total NCoR transcripts when MEFs were treated with glucose that paralleled a similar observation by Yamamoto et al. (Yamamoto et al., 2011), although our own data did not achieve comparable statistical significance. Our own results also showed a statistically significant increase in total SMRT transcript levels in MEFs in response to higher glucose concentrations, which Yamamoto et al. did not, and a marked decrease of SMRT in mature 3T3-L1 adipocytes and of NCoR in Hepa1-6 liver cells under the same conditions. These results highlight the cell-specific nature of these phenomena, an important fact to consider when evaluating the roles of these changes in corepressor levels in the control of overall energy metabolism in the intact organism.

Increased glucose (or fructose) in the culture medium also caused changes in the alternative mRNA splicing of SMRT and NCoR. This too was cell-specific, in that glucose favored the splicing of both corepressors in hepatocytes to isoforms that contain three rather than two RIDs (e.g. ω versus δ), whereas in the 3T3-L1 adipocyte cell line this was observed only for the SMRT and not for the NCoR transcripts. We interpret these changes in corepressor mRNA levels as a response specific to the nutritive manipulation, rather than an artifact due to general cell distress. Our glucose manipulation experiments were carried out for only 48 h, during which time cells had access to alternate energy sources such as pyruvate and serum-derived nutrients. Microscope inspection of the cells revealed no observable morphological cytotoxic effects or detachment from the culture surface (a similar study during which

glucose was restricted for 72 h also showed normal 3T3-L1 morphology and physiology (Sabater et al., 2014)). Furthermore, trypan blue staining of the Hepa1-6 cells showed no significant cell death in our glucose manipulation experiments compared to cultures maintained with the standard 25 mM glucose (unpublished data).

Interestingly, the exon 40 and exon 37 splice sites that generate the ω and δ isoforms of SMRT and NCoR (respectively) are not truly homologous at the detailed sequence level, even though they have very similar effects on the overall RID composition of the corresponding proteins (Goodson et al., 2005a). This may be indicative of a convergent evolutionary process reflecting a shared selection for the synthesis of these larger NCoR and SMRT corepressor isoforms that exert a common metabolic function not conferred by the smaller NCoR and SMRT isoforms. Notably, experimental alteration of SMRT isoform mRNA levels to mimic the effects seen in response to glucose deprivation produced observable changes in expression of metabolically relevant target genes. This altered endogenous gene expression is therefore caused by the increased abundance specifically of the SMRT δ isoform, rather than due to an increase in total SMRT, since no significant changes in expression of these target genes were observed in the cell lines transfected instead by the SMRT ω isoform. This indicates that even modest changes in SMRT transcript isoform abundance can have observable biological actions.

4.3. Elevated dietary sucrose, an oligomer of glucose and fructose, also shifts corepressor mRNA splicing in vivo

We maintained wild-type C57BL/6N mice on diets that were low sucrose, high fat or high sucrose, low fat to examine if glucose and fructose influence corepressor mRNA splicing in an in vivo context. Presumably, the disaccharide sucrose is hydrolyzed in the gut of these animals to the corresponding monosaccharides. Mice fed the low sucrose, high fat diet gained more weight and became more intolerant to glucose and insulin than the mice on the high sucrose, low fat diet (Goodson et al., 2014). There was a shift in SMRT exon 40 mRNA splicing from the δ to the ω isoform in the visceral white adipose tissue of mice fed the high sucrose diet, similar to our observations in cultured 3T3-L1 adipocytes. Additionally, there was a shift from NCoR δ to NCoR ω transcripts in the livers of these mice, similar to our observations in the Hepa1-6 hepatocytes. At this point, we cannot establish if this effect on corepressor splicing in mice is a direct effect of dietary carbohydrate availability or is an indirect response to the more global and long term metabolic changes caused by the two different diets. Nonetheless, we do conclude that, directly or indirectly, the availability of specific monosaccharides influence corepressor mRNA splicing in both whole animals and cell lines in a tissue-type specific fashion.

Do these changes in corepressor splicing play a causal role in the development of type 2 diabetes? Elimination of the NCoR ω isoform from all tissues in a splice-specific knockout mouse model actually promotes glucose utilization and storage (Goodson et al., 2014); therefore the enhanced ratio of NCoR ω to NCoR δ observed in the liver of wild-type mice fed a high sucrose diet may conceivably induce the poor glucose utilization associated with type 2 diabetes. However, it is the wild-type mice on the low sucrose, high fat diet that become obese and develop type 2 diabetes. We also observed a shift to the SMRT ω

transcript in the visceral adipose tissue in response to these diets; unfortunately, we do not yet fully understand what these changes in SMRT corepressor mRNA splicing might mean. The SMRT and NCoR isoforms exert distinctive biological roles (Short et al., 2005; Goodson et al., 2005a; Malartre et al., 2004; Jonas et al., 2007; Goodson et al., 2005b; Faist et al., 2009; Malartre et al., 2006; Goodson et al., 2011, 2014) and the observed diet-induced changes in SMRT mRNA splicing may therefore have different impacts on glucose physiology than those seen in our splice-specific knockouts of NCoR. In addition, the reciprocal changes in the levels of fat versus sucrose in these diets (designed to achieve caloric balance) represents another factor that must be considered in interpreting our initial results.

The two diets change the levels of gene expression in the liver and visceral white adipose tissue of a variety of trans-acting splicing factors (unpublished data), some of which are associated with ZNF638, a factor which is known to regulate both NCoR splicing and adipogenesis (Du et al., 2014). Conceivably one or more of these splicing factors may be responsible for the observed alterations of splicing of NCoR and/or SMRT we observe in response to changes in nutrient availability; however this data is highly preliminary, and establishing a causal relationship and identifying any additional splicing factors involved in NCoR and SMRT alternative splicing, will require future studies. It should also be noted that the changes we observe in our *in vivo* mouse models relate to adiposity, and therefore may be through a distinct mechanism from the changes observed as alterations in adipogenesis in the 3T3-L1 cell model.

In conclusion, our findings support the concept that important dietary factors and hormonal regulators of energy metabolism modulate corepressor mRNA splicing. Since different corepressor splice variants play distinct key roles in glucose and lipid metabolism (Goodson et al., 2011, 2014), it is likely that their splicing is regulated in this way so as to maintain metabolic homeostasis. Furthermore, excessive exposure to these regulators, such as a high sugar diet or chronic glucocorticoid therapy, may lead to aberrant corepressor splicing and metabolic disease. We believe that pharmacologically targeting specific SMRT or NCoR splice isoforms may open up a novel and more precise therapeutic space for treating metabolic disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Schematics of the NCoR and SMRT corepressor proteins are represented. The effects of alternative mRNA splicing at NCoR exons 28 and 37, and at SMRT exon 40 are shown. Protein coding regions deleted or added by a splicing event are indicated, and vertical ovals represent nuclear receptor interaction domains.

Fig. 2.

DEX induces the change in NCoR mRNA splicing observed during 3T3-L1 cells differentiation. Cells were differentiated with various combinations of the individual factors within the tripartite differentiation cocktail. Means $\pm$ S.E.M. ($n = 3$ for SMRT Exon 40 and NCoR Exon 28, and $n = 5$ for NCoR Exon 37) are presented. $p < 0.05$ (*) and $p < 0.01$ (†) are indicated. (A) Effects of different combinations of the cocktail on alternative mRNA splicing of SMRT and NCoR. Corepressor transcript isoform abundance was determined as in Materials and Methods. (B) Effects of different combinations of the cocktail on lipid accumulation were quantified by Oil Red O staining as in Materials and Methods. Means $\pm$ S.E.M. ($n = 3$) are presented.

Fig. 3.

Chronic ROSI treatment replaces insulin in supporting the change in NCoR mRNA splicing observed during 3T3-L1 cell differentiation. Cells were treated with the tripartite differentiation cocktail on differentiation day 0, followed by: eight days of insulin treatment (“Insulin”), four days of insulin treatment and four days of ROSI treatment (“Insulin ROSI”), or eight days of ROSI treatment (“ROSI”). Corepressor transcript isoform abundance and statistics were determined as in Fig. 2. Mean $\pm$ S.E.M. ($n = 3$) are presented. $p < 0.01$ (†) and $p < 0.001$ (‡) are indicated.

Fig. 4.

Acute ROSI treatment also induces a change in corepressor mRNA splicing in 3T3-L1 cells. Preadipocytes (3T3-L1 cells never treated with the differentiation cocktail), or mature adipocytes (3T3-L1 cells at differentiation day 12 after exposure to the tripartite differentiation cocktail) were treated acutely for 48 h with ROSI. Corepressor transcript isoform abundance and statistics were determined as in Fig. 2. Mean $\pm$ S.E.M (n = 3) are presented. $p < 0.05$ (*) and $p < 0.01$ (†) are indicated.

Fig. 5.

The availability of glucose, but not fatty acids, alters the gene expression of total (pan-splice) NCoR and total (pan-splice) SMRT. The indicated cells were exposed for 48 h to glucose or oleic acid. Total levels (all splice isoforms) of NCoR and SMRT mRNAs were determined by q RT-PCR as in Materials and Methods. Means $\pm$ S.E.M. ($n = 4$ for MEFs, $n = 3$ for Hepa1-6 and 3T3-L1 cells) are presented. $p < 0.05$ (*), $p < 0.01$ (†) and $p < 0.001$ (‡) are indicated.

Fig. 6.

The availability of glucose, but not fatty acids, alters the alternative mRNA splicing of NCoR and SMRT. Cells were exposed for 48 h to the concentrations of glucose, oleic acid or linoleic acid indicated, using (A) differentiated 3T3-L1 adipocytes (day 12), (B) Hepa1-6 liver cells, or (C) MEFs. Relative transcript isoform abundance and statistics were determined as in Fig. 2. Means $\pm$ S.E.M. (n = 3 for all experiments, except n = 6 for NCoR Exon 28 at the 50 mM concentration) are shown. $p < 0.05$ (*), $p < 0.01$ (†), $p < 0.001$ (‡), and $p < 0.0001$ (§) are indicated.

Fig. 7.

A low sucrose versus high sucrose diet alters the mRNA splicing of NCoR and SMRT in intact mice. Adult male, wild-type C57BL/6N mice were fed a low sucrose, high fat diet (LSD) or a high sucrose, low fat diet (HSD) (equivalent calories per gram diet) for 14 weeks. At the end of the study, mRNAs from the indicated tissues were analyzed as in Materials and Methods. Corepressor transcript isoform abundance is displayed in (A) visceral white adipose tissue (vWAT), (B) subcutaneous white adipose tissue (sWAT), (C) liver, or (D) skeletal muscle. Means $\pm$ S.E.M. (n = 7 for LS/HFD vWAT, n = 3 for HS/LFD vWAT, n = 4 for sWAT, n = 7 for LS/HFD liver, n = 4 for HS/LFD liver, n = 4 for skeletal muscle) are presented.