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The mitochondrial fatty acid synthesis (mtFASII) pathway is capable of mediating nuclear-mitochondrial cross talk through the PPAR system of transcriptional activation

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ABSTRACT

Mammalian cells contain two fatty acid synthesis pathways, the cytosolic FASI pathway, and the mitochondrial FASII pathway. The selection behind the conservation of the mitochondrial pathway is not completely understood, given the presence of the cytosolic FAS pathway. In this study, we show through heterologous gene reporter systems and PCR-based arrays that overexpression of MECR, the last step in the mtFASII pathway, causes modulation of gene expression through the PPAR pathway. Electromobility shift assays (EMSAs) demonstrate that overexpression of MECR causes increased binding of PPARs to DNA, while cell fractionation and imaging studies show that MECR remains localized to the mitochondria. Interestingly, knock down of the mtFASII pathway lessens the effect of MECR on this transcriptional modulation. Our data are most consistent with MECR-mediated transcriptional activation through products of the mtFASII pathway, although we cannot rule out MECR acting as a coactivator. Further investigation into the physiological relevance of this communication will be necessary to better understand some of the phenotypic consequences of deficits in this pathway observed in animal models and human disease.

1. Introduction

Mammalian cells contain at least two fatty acid synthesis (FAS) pathways, the cytosolic FASI pathway, and the mitochondrial FASII pathway. The cytosolic pathway is catalyzed by one multifunctional protein, fatty acid synthase, that carries out all of the steps necessary for fatty acid synthesis [1]. The mitochondrial fatty acid synthesis pathway (mtFASII) is reminiscent of prokaryotic FAS systems in that it consists of a separate protein performing each catalytic step [2,3]. The selection behind the conservation of the mitochondrial pathway is not completely understood, given the presence of the cytosolic FAS pathway. While the main function of the ancestral type II system in bacteria is the synthesis of glycerophospholipids, the pathway also creates intermediates that are diverted to synthesize other molecules, such as lipopolysaccharides, vitamins such as the protein-bound coenzymes biotin and lipoic acid, and the acylated homoserine lactones involved in density-dependent signaling [2,3].

In eukaryotic cells, the fate of mtFASII pathway products is less certain. The mtFASII pathways of *Neurospora* [4,5], plants [6,7], and mammals [8] have all been demonstrated to synthesize saturated fatty acids from malonate. Because most of the lipids in mitochondria are imported from the cytoplasm where they were synthesized by the FASI pathway [9,10], *de novo* synthesis of phospholipids from these fatty acids is unlikely. Experiments in fungi, however, suggest that these mtFASII products may be important in phospholipid side chain remodeling. Deletion of *acp-1*, the gene for the mtFASII acyl carrier protein in *Neurospora*, results in a 4-fold increase in mitochondrial lysophospholipids [11]. Deletion of mtFASII pathway genes in *Saccharomyces cerevisiae* results in a more than 50% reduction in cardiolipin levels [12,13], and loss of 90–95% of the normal level of lipoic acid [12].

Some understanding of the role of mammalian mtFASII has come from knocking down expression of pathway components in cultured cells and mice. ACP is a small (8 kD) mitochondrial protein essential to mtFASII, acting as a carrier for the fatty acids as they are elongated. ACP has also been identified as a component of respiratory chain complex I in *Neurospora crassa* and mammals [14–16]. siRNA-medicated knock down of the gene for ACP (*NDUFAB1*) in HeLa cells led to decrease in lipoylated proteins, slower cell growth, reduction in the specific activity of complex I of the electron transport chain, and loss of mitochondrial membrane

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potential [17]. Knock down of *Mcat*, the gene for malonyl CoA-acyl carrier protein transacylase that converts malonyl-CoA to malonyl-ACP in the mtFASII pathway, results in a dramatic phenotype in mouse, including shortened life span, baldness, weight loss despite food intake, loss of white fat, and hypothermia [18] emphasizing the important physiological role of this pathway in mammalian systems.

Upregulation of another mtFASII pathway enzyme, mitochondrial trans-2-enoyl-CoA reductase (MECR), in transgenic mouse heart resulted in cardiac dysfunction and dysmorphic mitochondria [19]. MECR, which catalyzes the last step in the mtFASII pathway [20], was originally identified as interacting with peroxisome proliferator-activated receptor α (PPARα) in the two-hybrid system [21]. The transcriptional activity of the PPARs is modulated by ligands such as the hypolipidemic fibrate drugs that cause peroxisome proliferation, and naturally occurring ligands such as fatty acids, eicosanoids, and phospholipids. These ligands affect the binding of proteins (coactivators and corepressors) that modulate the transcriptional activity of the PPARs [22].

Although MECR also interacts with several other nuclear hormone receptors, evidence of its ability to affect transcription has not been demonstrated. In this study, we show that upregulation and downregulation of members of the mtFASII pathway cause modulation of PPARdriven expression.

2. Materials and methods

2.1. Plasmid construction

To create the MECR overexpression plasmid, the entire open reading frame of *Mecr* was amplified from mouse cDNA using primers 5′-CCAGATCTGCCGCCACCATGGTGGTCAGCCAGCGAGTG-3′ and 5-TGGAGAGATCTCATGGTGAGAATCTGCTTCG-3. The resulting PCR product was cloned into the pCR2.1 vector using the TA cloning kit (Invitrogen) to create pMecr-TA. A FLAG epitope tag was created at the C terminus of *Mecr* by annealing complementary oligonucleotides 5′-GATCCACCATGGATTACAAGGATGACGTACGATAAGA-3′ and 5′-GATCTCTTATCGTCGTCATCCTTGTAATCCATGGTG-3′ and ligating the resulting product into the pMecr-TA vector that had been digested with BglII, creating pMecr-flagTA. The region encoding MECR-flag was then removed by digestion with EcoRI and BglII, and cloned into pSG5 (Agilent Technologies, Inc.), creating a plasmid expressing *Mecr* under the control of the SV40 promoter.

To create the reporter plasmid containing the *Mecr* promoter controlling luciferase expression, the region 700 bp upstream of the start codon of *Mecr* was amplified from mouse genomic DNA and cloned into the pCR2.1 vector using the TA cloning system (Invitrogen). The *Mecr* promoter region was then excised from pCR2.1 using EcoRI, and ligated into EcoRI site of pGL2-basic (Promega) upstream of the luciferase gene.

Mitochondrially targeted dsRED (m-dsRED) was a gift from J. Nunnari at University of California, Davis [23].

MECR–GFP was constructed by amplifying the entire open reading frame of *Mecr* from mouse cDNA using primers listed above, and ligation of the PCR product cut with BglII into the BglII site of pEGFP-N3 (gift from the D. Piston laboratory, Vanderbilt University).

2.2. Co-activation assays

To test if MECR has an effect on PPAR-driven transcription, a luciferase reporter-based transcriptional activation assay was used. The promoter of the carnitine palmitoyltransferase Ib gene (*Cpt1B*) [24], or acyl coA oxidase (*Aco1*) gene [25], both of which contain PPAR response elements (PPREs), were independently used to drive luciferase reporter gene expression. The reporter plasmid (CPT-luc or ACO-luc) was transiently co-transfected into HeLa cells using FuGene HD (Roche) with plasmids expressing peroxisome proliferator-activated receptor alpha (Pparα) [26] or gamma (Pparγ) and retinoid X receptor alpha (Rxrα) [26] transcription factors. The contribution of MECR was assessed by cotransfection of a plasmid driving overexpression of mouse *Mecr* under control of the SV40 promoter.

2.3. Cell fractionation/western blot

HeLa cells were transfected with either MECR or control (pSG5) plasmid and PPARα and RXRα plasmids. Cells were harvested and fractionated using the standard cell fractionation kit from Mitosciences. Antibodies specific to each cell fraction where used for Western immunoblots: GAPDH (Santa Cruz Biotechnology sc-20357) for cytosol, Lamin A/C (Cell Signaling Technology 4C11) for nuclear, and PDH (Abcam/Mitosciences MSp06) for mitochondrial. Polyclonal MECR antibodies were created by immunizing rabbits with mouse MECR-specific synthetic peptide (CSEVPLQGYQQALEASMKPF) conjugated to keyhole limpet hemocyanin (KLH) (Proteintech). MCAT antibody (sc-390858) is from Santa Cruz Biotechnology.

2.4. Confocal fluorescent microscopy

Confocal microscopy of transfected HeLa cells was conducted on a Zeiss LSM 510 with a 65X oil-immersion objective. M-dsRED and MECR–GFP transfected cells were imaged sequentially following excitation by lasers emitting at 543 and 488 nm, respectively.

2.5. Electrophoretic mobility shift assays

Evaluation of binding of transcription factors to DNA was performed using the PPAR EMSA kit from Signosis according to manufacturer instructions. Nuclear extracts were isolated using the nuclear extraction kit from Cayman Chemicals. Labeled probe was incubated with nuclear extracts from HeLa cells transfected with MECR, PPARα, RXRα, or control expression vectors. Flag M2 monoclonal antibodies for supershift experiments were obtained from Sigma. PPAR antibodies for supershift experiments were obtained from Santa Cruz Biotechnology.

2.6. siRNA medicated RNA knock down

Knock down of the mtFASII pathway was achieved using Qiagen Flexitube siRNAs specific for the gene for ACP (*NDUFAB1*) or MCAT (*MCAT*). HeLa cells were transfected with siRNA using HiPerfect Transfection Reagent (Qiagen). Knock down efficiency was measured after 96 h using real time quantitative RT-PCR or by Western immunoblot for MCAT.

2.7. Real time quantitative RT-PCR

Total RNA was isolated using TRIzol Reagent (Life Technologies) according to manufacturer's protocols. First-strand cDNA was created from total RNA using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Life Technologies). Quantitative RT-PCR was performed using TaqMan Expression Assays (Life Technologies) on the ABI 7900 platform according to manufacturer's protocols.

3. Results

3.1. Overexpression of MECR increases PPAR-dependent transcriptional activation

In order to determine the effect of MECR on transcriptional activation, an *in vitro* heterologous transcriptional activation system was used. The reporter consisted of a plasmid bearing the promoter of CPT1B, which contains a PPRE, driving transcription of the reporter gene for luciferase (CPT-luc). This reporter plasmid was transiently cotransfected into HeLa cells with expression plasmids for PPARα and RXRα. Upon overexpression of MECR, PPARα-dependent luciferase activity increased approximately 2-fold (Fig. 1A). A similar result was obtained when a promoter containing PPREs from the acyl-CoA oxidase upstream region fused to the luciferase reporter (ACO-luc) [25] was used (Fig. 1B). The effect of MECR overexpression on transcription also extended to PPARγ-driven expression, as shown in Fig. 1C. In none of these cases did expression of MECR without coexpression of a PPAR cause a change in expression of reporter plasmid (data not shown). These results indicate that increases in MECR are capable of increasing transcription via the PPARs.

3.2. MECR regulates its own expression via PPARs

The modulation of nuclear gene expression by MECR, a mitochondrial protein, is reminiscent of transcriptional regulation by HMG CoA synthase, another mitochondrial protein. HMG CoA synthase regulates its own gene expression by interaction with PPARα [27]. In order to determine if MECR acts to regulate its own transcription, a plasmid was constructed in which the promoter of *Mecr* was placed upstream of the luciferase gene. When cotransfected with RXRα and PPARα or PPARγ expressing plasmids, luciferase-associated luminescence was increased approximately 50% (Fig. 1D). This indicates that MECR is acting to increase its own transcription through the PPARs.

3.3. Overexpression of MECR changes transcriptional regulation of several genes regulated by PPARs

We next examined the effect of overexpression of MECR on other genes regulated by PPARs using a RT^2 Profiler™ PCR Array (SABiosciences). This array profiles the expression of 84 key genes involved in PPAR activation and response. Cells overexpressing MECR had significant increase of *FABP4* expression (3.3-fold upreg-

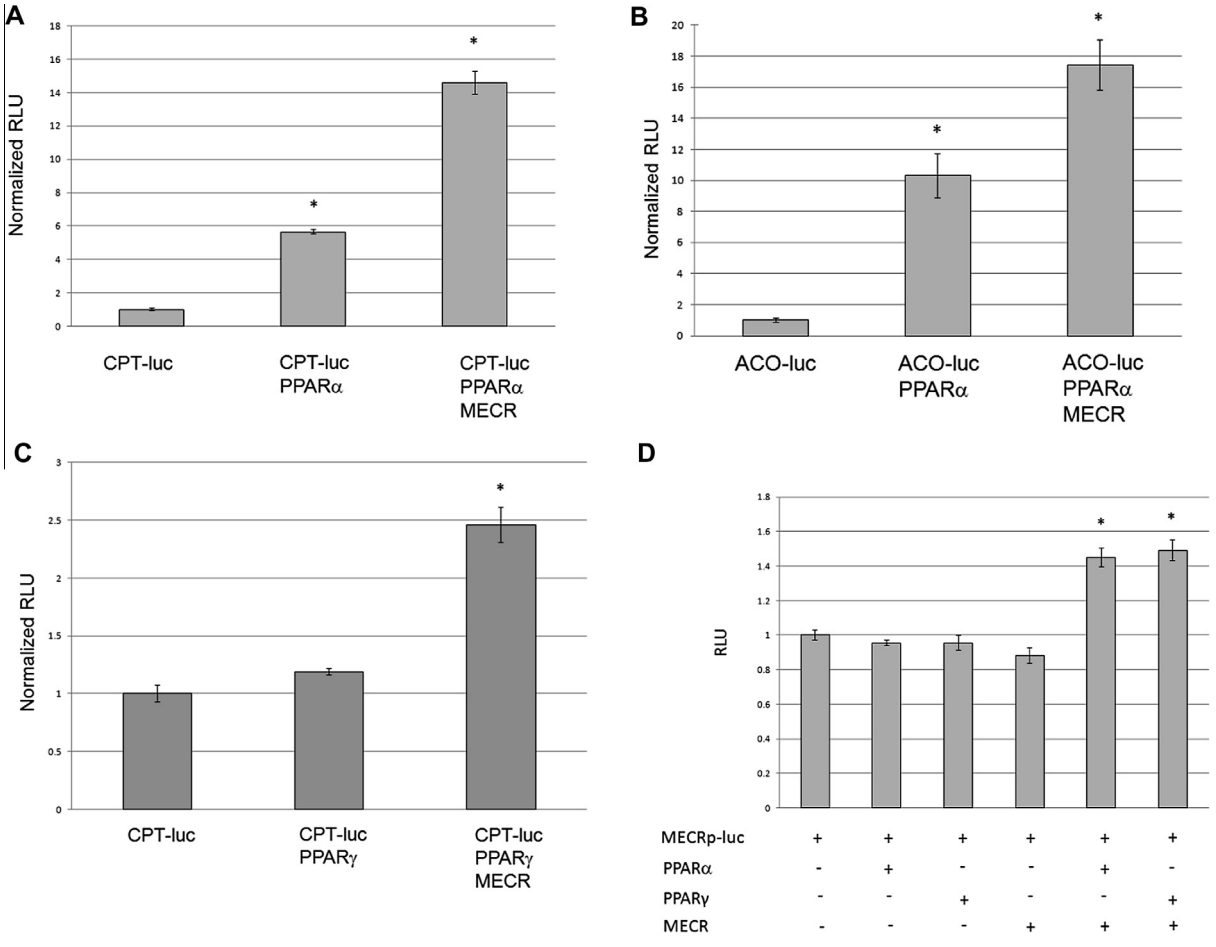

Fig. 1. Overexpression of MECR causes increased transcriptional activation through PPARα and PPARγ. The contribution of MECR to PPAR-based transcriptional activation was measured using a heterologous reporter system. The reporter constructs CPT-luc and ACO-luc were cotransfected with PPARα (A) and (B) or PPARγ (C), in the absence or presence of a pSG5-MECR expression vector as indicated. A renilla expression vector was cotransfected in all conditions to control for transfection efficiency. Bars represent mean ± (S.E.) renilla-corrected RLU, normalized = (1.0) to the activity of the reporter (CPT-luc) cotransfected with pSG5. Data represent at least three independent trials performed in triplicate. The asterisks indicate that the mean values are significantly different ($p < 0.05$). (A) Overexpression of MECR enhances PPARα-mediated transactivation of the *Cpt1b* gene promoter in HeLa cells. (B) Overexpression of MECR enhances PPARα-mediated transactivation the *Aco1* gene promoter in HeLa cells. (C) Overexpression of MECR enhances PPARγ-mediated transactivation of the *Cpt1b* gene promoter in HeLa cells. (D) To determine if MECR has an effect on its own transcription, the region 700 base pairs upstream of mouse *Mecr* gene was cloned upstream of luciferase in a reporter plasmid (Mecr-luc). The reporter construct was cotransfected with either PPARα or PPARγ, in the absence or presence of pSG5-Mecr expression vector as indicated.

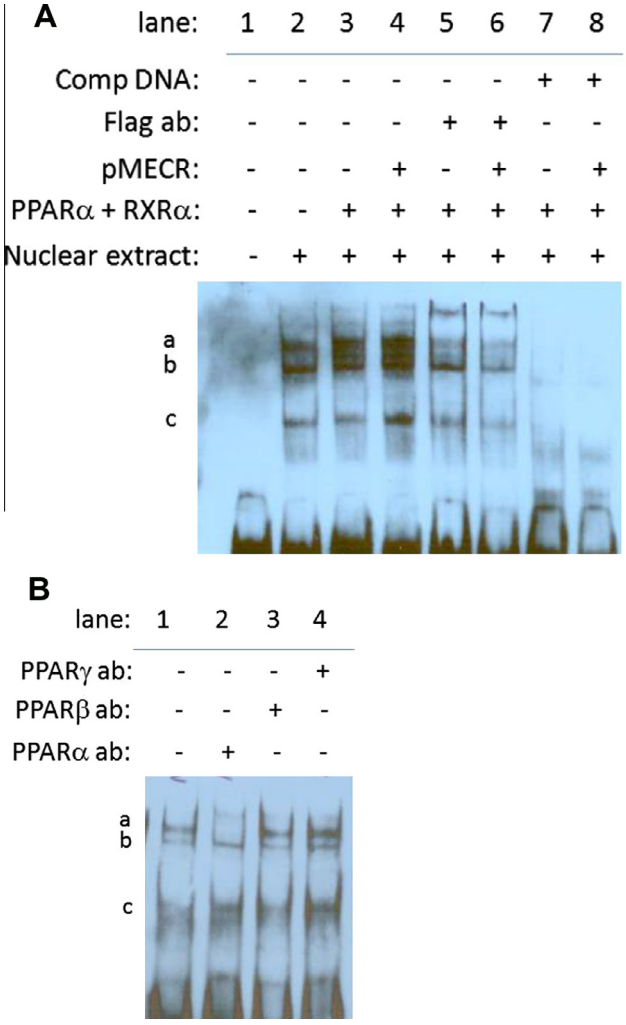

Fig. 2. Overexpression of MECR causes an increase in protein binding to PPRE as assessed by EMSA. EMSAs were used to evaluate the effect of MECR overexpression on the binding of PPARs to PPREs in DNA. (A) Lane 1 was loaded with biotin-labeled PPRE DNA probe only. Untransfected HeLa cell nuclear extract was added to the sample in lane 2. Sample in lanes 3–8 contained nuclear extract from cells transfected with PPARα and RXRα Lanes 4, 6, and 8 contained nuclear extract made from cells transfected with PPARα, RXRα, and MECR-expressing plasmids. Lanes 7–8 are the same as lanes 3–6 except that competitor (unlabeled) DNA was added. (B) Lanes 1–4 contain probe and nuclear extracts from cells transfected with PPARα and RXRα. Antibodies to PPARα, PPARβ, and PPARγ were added to lanes 2, 3, and 4 respectively to test for supershifted complexes.

ulation) and significant decrease of *ELN* expression (1.8-fold downregulation). Affected gene *FABP4* encodes a fatty acid binding protein that has a role in transport of fats within the cell, fat deposition in animals, and metabolic syndrome [28]. FABP4 has been reported to carry long chain fatty acids and retinoic acid to nuclear hormone receptors such as the PPARs and RXRs [29]. *ELN*, the gene for elastin, has previously shown to be downregulated by PPAR pathways [30].

3.4. Overexpression of MECR causes increased binding of PPARs to DNA

Electrophoretic mobility shift assays (EMSAs) were performed to further investigate the effect of overexpression of MECR on DNA binding by PPARs. When HeLa nuclear extracts were added to a PPRE-containing probe, several bands were shifted upward, suggesting several transcription factors (PPARs) bound to this PPRE (Fig. 2A, lane 2). When nuclear extracts were isolated from HeLa cells transfected with plasmids overexpressing PPARα and RXRα, the upper DNA/protein complex band (a) becomes more pronounced (Fig. 2A, lane 3). Band a is also supershifted when PPARα antibodies are added to the reaction (Fig 2B, lane 2), suggesting that band a represents DNA complexed with PPARα. When MECR is overexpressed in the nuclear extracts, complex bands a and c increase in intensity, suggesting increased PPAR binding to DNA (Fig. 2A, lane 4). All of the binding is specific to the probe, since it can be competed away by unlabeled probe (Fig. 2A, lanes 7–8). FLAG epitope-specific antibody was added to the DNA binding reaction to identify whether MECR was present in the binding complexes. While the FLAG antibody should recognize only the plasmid-expressed MECR, the change in the intensity of the bands was the same in the lane without MECR–FLAG in the reaction (Fig. 2A, lane 5) as it was in the lane with MECR–flag (Fig. 2A, lane 6), suggesting any change in response to the FLAG antibody is not specific to MECR.

3.5. Cellular localization of MECR

There are at least two models, which are not mutually exclusive, for how MECR might be affecting transcription through the PPARs. In the first model, MECR directly interacts with PPARs and acts as a coactivator of transcription. In the second model, MECR overexpression leads to increased flux through the mtFASII pathway, resulting in products that activate PPARs in the role of agonist/antagonists. The first model would rely on MECR translocating to the nucleus with PPAR to activate transcription. To test the localization of MECR, we performed subcellular fractionation of HeLa cells transfected with PPARα-expressing plasmid, and tested the presence of MECR in these fractions. Cells transfected with control vector (Fig. 3A, lanes 1–3), pMECR (lanes 4–6) or pMECR, pPPARα, and pRXRα (lanes 7–9) were isolated 24 h post-transfection by trypsinization and centrifugation. Mitochondrial, nuclear, and cytosolic fractions were isolated by detergent-based extraction, and samples were subjected to polyacrylamide gel electrophoresis and immunoblotting. While a very small fraction of the MECR was seen in the cytosolic and nuclear fraction, MECR was predominantly in the mitochondrial fraction. The amount of MECR in the nucleus is even less when coexpressed with PPARα, which is opposite the result expected if PPARα pulls MECR into the nucleus (Fig. 2A).

Additionally, fluorescent confocal microscopy was performed to investigate the location of MECR when coexpressed with PPARα. Cells were cotransfected with m-dsRED plasmid to mark mitochondria, MECR–GFP plasmid to visualize MECR, and PPARα expressing plasmid. MECR–GFP (Fig. 3 middle panel) colocalized with mitochondrially targeted dsRED (Fig. 3 left panel) whether PPARα was coexpressed or not.

3.6. MECR causes PPAR-dependent increases in transcription through its role in the mtFASII pathway

In the second model of how MECR may affect transcription through the PPARs, overexpression of MECR acts to upregulate the mtFASII pathway so that it produces a fatty acid or fatty acid derivative that acts as an agonist/antagonist to PPARs. If the second model is true, knock down of the mtFASII pathway would interfere with the transcriptional activation caused by overexpression of MECR. In order to test this hypothesis, *NDUFAB1*, which encodes the 8 kD ACP to which malonate is attached and lengthened to fatty acids by the rest of the mtFASII pathway, and *MCAT*, which encodes the malonyl CoA-acyl carrier protein transacylase that converts malonyl-CoA to malonyl-ACP in the mtFASII pathway were separately knocked down with siRNA. After 72 h, expression of the *ACP1* transcript was reduced 90% (Fig. 4A), resulting in a 50%

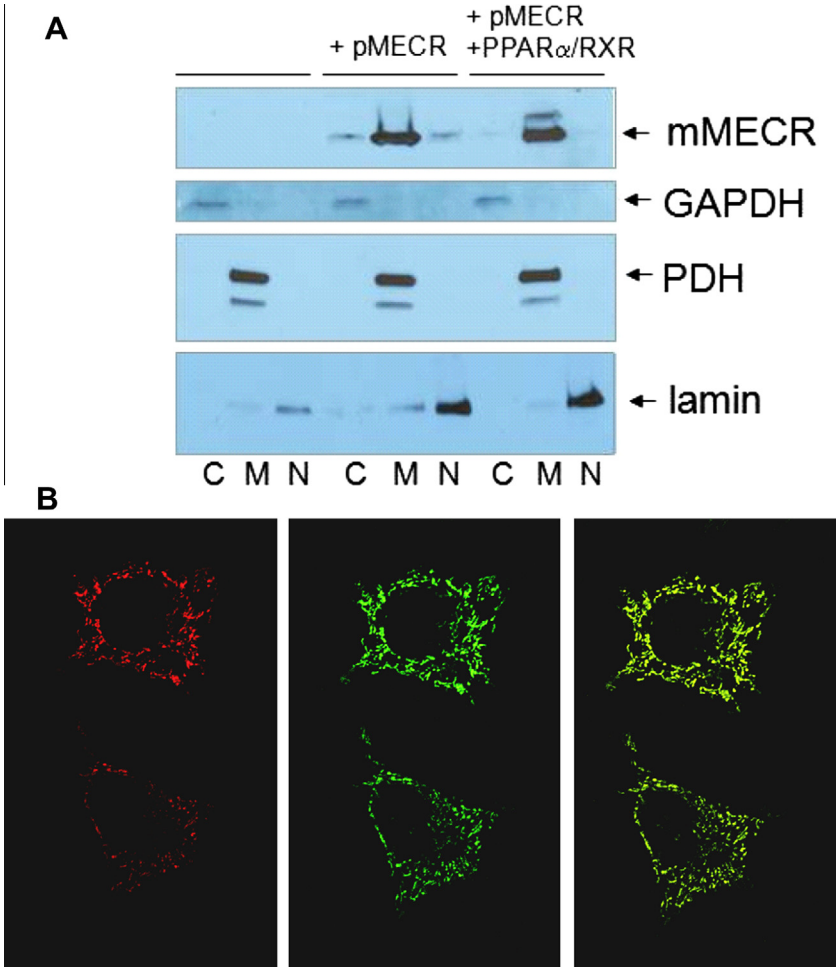

Fig. 3. MECR is localized to the mitochondria. (A) HeLa cells transfected with either empty vector (lanes 1–3), pMECR (lanes 4–6), or pMECR, pPPARα and pRXRα (lanes 7–9) were fractionated into nuclear (N), cytoplasmic (C), and mitochondrial (M) fractions. Samples from each fraction were subjected to polyacrylamide gel electrophoresis (PAGE) and Western immunoblotting. GAPDH was used as a cytosolic marker, pyruvate dehydrogenase (PDH) was used as a mitochondrial marker, and lamin was used as a nuclear marker. (B) In cells transfected with pPPARα (all panels), MECR–GFP (center panel) colocalizes with mitochondria (m-dsRED) (left panel) as seen as yellow in overlay (right panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reduction in transcriptional activation by overexpression of MECR (Fig. 4B). After 72 h of *MCAT* siRNA treatment, the MCAT protein was reduced approximately 2-fold (Fig. 4C), and a 30% reduction in transcriptional activation by overexpression of MECR was apparent (Fig. 4C). The effect of knock down of the mtFASII pathway on the transcriptional activation by MECR suggests that MECR-dependent activation works through products of the mtFASII pathway.

4. Discussion

At least one other example of a mitochondrial enzyme changing nuclear gene transcription through the PPAR pathway has been reported. HMG CoA synthase, a mitochondrial enzyme involved in cholesterol synthesis and ketogenesis, also upregulates its own expression through the PPAR pathway. HMG CoA synthase interacts with PPARα and translocates to the nucleus to upregulate its own expression [27]. The interaction between HMG CoA synthase and PPAR is enhanced by palmitoylation of HMG CoA synthase, increasing transcriptional activation [31]. Vila-Brau et al. report that HMG CoA synthase regulates fatty acid oxidation and *FGF21* expression, but only when it is catalytically active [32]. They suggest that HMG CoA synthase acts not as a transcriptional coactivator interacting with PPARs, but as a regulator of metabolism and expression via its role in ketogenesis and through the pathway product acetoacetate [32].

While we have shown that upregulation of MECR causes modulation of transcription through the PPAR pathway, questions remain about the mechanism of this regulation. In one possible model, MECR acts as a transcriptional coactivator of PPAR to promote its binding to DNA. This model requires direct interaction of MECR and PPAR, and the nuclear localization of the complex. We did not find evidence of a relocation of MECR to the nucleus, or of an interaction of MECR and PPAR by supershifted complexes in EMSAs. It is possible that the interaction of PPAR and MECR is dependent on the palmitoylation of MECR, similar to the mechanism reported for HMG-CoA synthase. Upregulation of MECR would cause an increase in the mtFASII pathway product palmitate, increasing palmitoylation of MECR, and subsequent interaction between MECR and PPAR. Alternatively, MECR could be acting exclusively through its role in the mtFASII pathway, and it is products of the pathway that act as agonists/antagonists of PPARs. It is reasonable that products of the mtFASII pathway might be acting as agonists for the PPARs, since endogenous fatty acids and their derivatives, including the FASI product 16:0/18:1 glycerophosphocholine (GPC), are already known to activate PPAR transcription [33,34]. Our data are most consistent with MECR-mediated transcriptional activation through products of

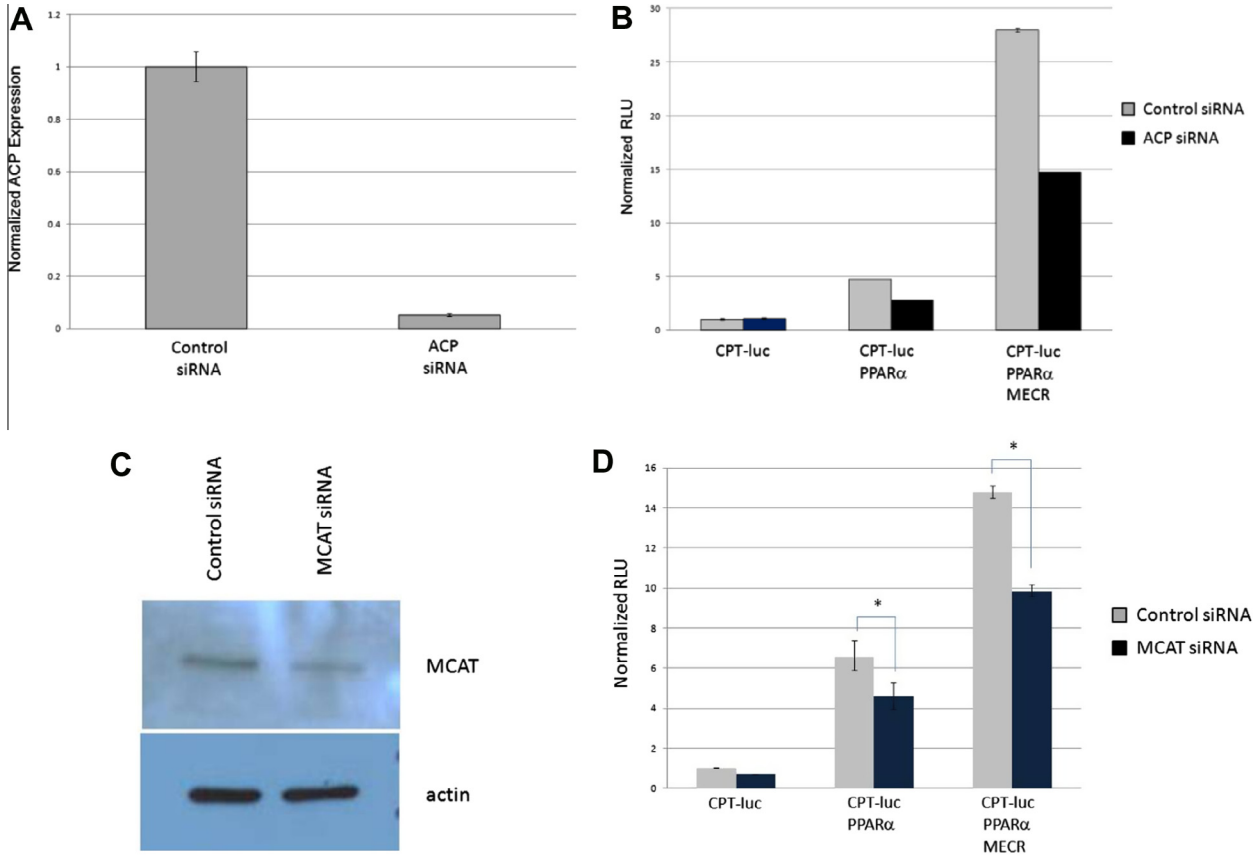

Fig. 4. Knock down of the mtFASII pathway lessens the transcriptional activation by MECR. The genes encoding ACP *(NDUFAB1)* and MCAT were knocked down using Qiagen Flexitube siRNAs. (A) Expression of *NDUFAB1* was decreased by over 90% using siRNA. (B) Knock down of *NDUFAB1* reduced the transcriptional activation caused by overexpression of MECR by approximately 50%. (C) Cells knocked down for MCAT had 50% less MCAT protein. (D) Knock down of MCAT reduced the transcriptional activation caused by overexpression of MECR by approximately 30%.

the mtFASII pathway as we do see a decrease in MECR-stimulated transcription when the mtFASII pathway is downregulated, suggesting that products of the mtFASII pathway are involved in MECR-driven transcriptional activation.

Acknowledgment

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