Novel Targets for the Transcription Factors MEF2 in MA-10 Leydig Cells¹

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ABSTRACT

Testosterone production by Leydig cells is a tightly regulated process requiring synchronized expression of several steroidogenic genes by numerous transcription factors. Myocyte enhancer factor 2 (MEF2) are transcription factors recently identified in somatic cells of the male gonad. In other tissues, MEF2 factors are essential regulators of organogenesis and cell differentiation. So far in the testis, MEF2 factors were found to regulate Leydig cell steroidogenesis by controlling *Nr4a1* and *Star* gene expression. To expand our understanding of the role of MEF2 in Leydig cells, we performed microarray analyses of MEF2-depleted MA-10 Leydig cells, and the results were analyzed using Partek and Ingenuity Pathway Analysis software. Several genes were differentially expressed in MEF2-depleted Leydig cells, and 16 were validated by quantitative RT-PCR. A large number of these genes are known to be involved in fertility, gonad morphology, and steroidogenesis. These include *Ahr*, *Bmal1*, *Cyp11b1*, *Hsd3b1*, *Hsd17b7*, *Map2k1*, *Nr0b2*, *Pde8a*, *Por*, *Smad4*, *Star*, and *Tsc22d3*, which were all downregulated in the absence of MEF2. In silico analyses revealed the presence of MEF2-binding sites within the first 2 kb upstream of the transcription start site of the *Por*, *Bmal1*, and *Nr0b2* promoters, suggesting direct regulation by MEF2. Using transient transfections in MA-10 Leydig cells, small interfering RNA knockdown, and a MEF2-Engrailed dominant negative, we found that MEF2 activates the *Por*, *Bmal1*, and *Nr0b2* promoters and that this requires an intact MEF2 element. Our results identify novel target genes for MEF2 and define MEF2 as an important regulator of Leydig cell function and male reproduction.

fertility, gonad morphology, Leydig cells, microarray, myocyte enhancer factor 2, steroidogenesis

INTRODUCTION

The myocyte enhancer factor 2 (MEF2) family of transcription factors consists of four members (MEF2A, 2B, 2C, and 2D) encoded by distinct genes. The MEF2 proteins have been well studied in muscles and in the brain (reviewed in [1]). These transcription factors play critical roles in the transmission of extracellular signals to the genome and in the activation of genetic programs that control cell differentiation, proliferation, morphogenesis, survival and apoptosis of a wide range of cell types (reviewed in [1]). All members contain two highly conserved domains in the N-terminal region, the MADS-box (MCM1, agamous, deficiens, SRF) and the MEF2 domain, both involved in DNA binding and protein dimerization while the C-terminal region contains the trans-activation domain ([2, 3] and reviewed in [4]). The mRNA of all MEF2 factors is alternatively spliced leading to several MEF2 isoforms [5]. MEF2 factors mediate their effects on gene expression by binding to the palindromic AT-rich sequence (C/T)TA (A/T)₄TA (G/A) present in the promoter region of target genes. MEF2 factors are also known to cooperate with transcription factors, including members of the GATA family [6, 7].

Recently we reported the presence of MEF2 factors in the mammalian gonad, specifically in the testis and not in the ovary [8]. Within the testis, MEF2A and 2D were found in the nucleus of both somatic cell populations, Sertoli and Leydig cells, throughout development and into adulthood [8]. In addition to MEF2, CAMK1 is also present in Leydig cells [9], and MEF2 was found to cooperate with CAMK1 to regulate the expression of the nuclear receptor *Nr4a1/Nur77* [8]. In these cells, NR4A1 regulates the expression of several steroidogenic genes such as *Star* [9], *Cyp17a1* [10], and *HSD3B2* [11]. The position of MEF2 upstream of a panregulator of steroidogenic genes, NR4A1, in a regulatory cascade suggests a broad role for MEF2 on several steroidogenic genes in Leydig cells [8]. In agreement with this, we recently reported that the *Star* gene encoding the STAR protein, an essential component of the cholesterol transport machinery, is a direct target of MEF2 [7]. Consistent with this, in MEF2-deficient Leydig cells, steroidogenesis was significantly decreased [7], indicating that MEF2 is an important contributor to steroidogenesis in these cells.

Despite this, there is still very limited information regarding the role(s) and target(s) of MEF2 in Leydig cells. In the present study, we used a microarray approach to compare the transcriptome of wild-type and MEF2-depleted MA-10 Leydig cells. We found that several genes involved in key processes, including steroidogenesis, fertility and gonad morphology, were deregulated in the absence of MEF2.

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TABLE 1. Oligonucleotides used in this study.

Application ^a	Description ^b	Sequence ^c
qPCR	<i>Ahr</i>	Forward: 5'-GACACAGAGACCGGCTGAAC-3' Reverse: 5'-CTACACTGGTCTGGCCTC-3'
	<i>Bmal1</i>	Forward: 5'-GGCTGCAGTGAATGCTTTTGG-3' Reverse: 5'-CGATTGCAGTCCACACCAC-3'
	<i>Celf6</i>	Forward: 5'-GAAGGACCACGACGCCATC-3' Reverse: 5'-CATGCAGTGCACCTGCGG-3'
	<i>Cyp1b1</i>	Forward: 5'-GGACATCTTCGGAGCCAG-3' Reverse: 5'-GACCGTATTCTTGGGGATGTAG-3'
	<i>Cyp17a1</i>	Forward: 5'-GGATAGTAGTAGCTCCTGGCAG-3' Reverse: 5'-GAGCGTAGACAGATCTCGGG-3'
	<i>Hsd3b1</i>	Forward: 5'-AGCTGCCCCGTGATCTTTTCAGCC-3' Reverse: 5'-CGCATGCCTGCTTCGTGACCA-3'
	<i>Hsd17b7</i>	Forward: 5'-CTCATCCACTGCAACATTAC-3' Reverse: 5'-GAAGCTGAGTACAGGCTGTA-3'
	<i>Lhcr</i>	Forward: 5'-CAGTGGCACCTTCAGGGGG-3' Reverse: 5'-GGGTAGGTCAGCGTGGCAACC-3'
	<i>Map2k1</i>	Forward: 5'-GGGAACGAAGGATGATGAC-3' Reverse: 5'-CCATGCAGATGCTGATCTC-3'
	<i>Nr0b2</i>	Forward: 5'-TACCCAGGGTGCCAGCCATC-3' Reverse: 5'-TGCAGGTGTGCGATGTGGCAG-3'
	<i>Nr4a1</i>	Forward: 5'-GGCTTCTTCAAGCGCACAGT-3' Reverse: 5'-GCTGCTTGGGTTTTGAAGGTAG-3'
	<i>Pde8a</i>	Forward: 5'-GTGCAATTTGGCCCGATGAG-3' Reverse: 5'-GATGTCATGGAGTTTGTCTCTGG-3'
	<i>Por</i>	Forward: 5'-CTGAGGCAGTGGCTGAAGAA-3' Reverse: 5'-CTTTTCCACGAAGCTGCTCTC-3'
	<i>Smad4</i>	Forward: 5'-GTGTCATAGACAAGGTGGGAAAG-3' Reverse: 5'-GTGTAGATCAGGCCACCTCC-3'
	<i>Star</i>	Forward: 5'-TTGGGCATACTCAACAACCA-3' Reverse: 5'-CCTTGACATTTGGGTTCAC-3'
	<i>Tsc22d3</i>	Forward: 5'-CTTCGTGAGCAACTTTCGGC-3' Reverse: 5'-CAATCTTGTGTCTAGGGCCACC-3'
PCR	<i>Bmal1 FL</i>	Forward: 5'-CGCGGATCCGGTTCAAAAAGGACGAAGTGGCC-3' Reverse: 5'-GGGGTACCCCTACTTTCCGACCAATCCGCTC-3'
	<i>Bmal1 min</i>	Forward: 5'-CGCGGATCCGGCTAACGGGAAGAGGCAGGTATC-3' Reverse: 5'-GGGGTACCCCTACTTTCCGACCAATCCGCTC-3'
	<i>Nr0b2 FL</i>	Forward: 5'-ATGTAAGCTTGGATCCTCCTGTGCCAGTTCTCAGGACAAG-3' Reverse: 5'-CGGGGTACCCCTTGTTCCTGTTCTTCCAGCTAT-3'
	<i>Nr0b2 min</i>	Forward: 5'-CGCGGATCCGTTTGGCCAGTCCCTTC-3' Reverse: 5'-CGGGGTACCCCTTGTTCCTGTTCTTCCAGCTAT-3'
	<i>Nr0b2 min mut</i>	Forward: 5'-CGCGGATCCGTTTGGCCAGTCCCTTCCGCCCATCAAGG ATATAAac gGCACTCACAG-3' Reverse: 5'-CGGGGTACCCCTTGTTCCTGTTCTTCCAGCTAT-3'
	<i>Por FL</i>	Forward: 5'-CGCGGATCCGCATACATGCACACGCACAC-3' Reverse: 5'-GGGGTACCCACACAGACAGGTCCTGG-3'
	<i>Por min</i>	Forward: 5'-CGCGGATCCCTCCTTACACCACACCAC-3' Reverse: 5'-GGGGTACCCACACAGACAGGTCCTGG-3'
siRNA	<i>Mef2a</i>	Forward: 5'-CAGGUGGUGGCAGUCUUGGAAUGAA-3' Reverse: 5'-UUCAUCCAAGACUGCCACCACCUG-3'
	<i>Mef2d</i>	Forward: 5'-ACUUCCAGGGAGGCAAAGGUUAA-3' Reverse: 5'-UUAACCCUUGCCUCCUGGGAAGU-3'
EMSA	<i>Nr4a1 WT</i>	Forward: 5'-GAAAA CTATTATAG ATCAA-3' Reverse: 5'-TTGAT CTATAA TAGTTTTC-3'
	<i>Nr4a1 mut</i>	Forward: 5'-GAAAA CgATTATc GATCAA-3' Reverse: 5'-TTGAT CgATAA TcGTTTTC-3'

^a EMSA, electrophoretic mobility shift assay; qPCR, quantitative RT-PCR; siRNA, small interfering RNA.^b FL, full length; min, minimal; mut, mutated.^c Cloning sites are underlined, MEF2 site is in bold, and mutations are in lowercase.

MATERIALS AND METHODS

Protein Purification and Western Blot Analysis

Cells were rinsed twice with ice-cold PBS, and nuclear proteins were prepared as described previously [9]. Nuclear proteins (15 µg) were fractionated by SDS-PAGE and transferred onto polyvinylidene fluoride membrane (Millipore Canada). Immunodetection was performed using an ECL approach according to the manufacturer's protocol (GE Healthcare Life Sciences). Detection of MEF2 and LAMIN B was performed using an anti-MEF2 antiserum (C-21, 1:500 dilution; Santa Cruz Biotechnology) and an anti-LAMIN B antiserum (C-20, 1:500 dilution; Santa Cruz Biotechnology), respectively.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSAs) were performed using nuclear extracts from MA-10 Leydig cells treated with control (scrambled) small interfering RNA (siRNA) or siRNA directed against MEF2A/2D as described previously [8]. Double-stranded oligonucleotides used as probes or in competition experiments are listed in Table 1. Three distinct anti-MEF2 antisera (3 µg) were used: anti-MEF2 (SC-313; Santa Cruz Biotechnology), anti-MEF2A (9736; Cell Signaling), and anti-MEF2D (SC-13921; Santa Cruz Biotechnology).

FIG. 1. MEF2 protein levels and DNA-binding activity in MEF2-depleted MA-10 Leydig cells. Control (scrambled) siRNA and siRNA against MEF2A/2D were transfected in MA-10 Leydig cells. Forty-eight hours after transfection, total RNA and nuclear proteins were isolated. **A**) MEF2 protein levels were determined by Western blot analysis. Representative blots of three independent experiments are shown. The bands were quantified using the Java-based ImageJ processing software (lower panel) (** $P < 0.01$). **B**) MEF2 DNA-binding activity was determined by EMSA using a double-stranded ^{32}P -labeled oligonucleotides corresponding to the MEF2 element at -284 bp of the *Nr4a1* promoter [8]. Binding was challenged with increasing doses of unlabeled oligonucleotides corresponding to the wild-type (WT) or mutated (Mut) MEF2 element (black triangles; molar excesses of 2 $\times$ and 5 $\times$). MEF2 binding was confirmed by supershift using a MEF2 antiserum for all MEF2 family members, a MEF2 antiserum specific for MEF2A, and a MEF2 antiserum specific for MEF2D. A rabbit immunoglobulin G was used as the negative control.

RNA Isolation

Total RNA was extracted using Trizol reagent (Life Technologies) according to the manufacturer's protocol. RNA quantity, purity, and integrity were controlled by absorbance at 260/280 nm and by agarose gel electrophoresis. For the microarrays, a second RNA purification step was performed using an RNeasy mini column kit (Qiagen), and RNA integrity was verified using an Agilent Bioanalyzer (Agilent Technologies Canada).

Sample Labeling and Microarray Hybridization

DNA microarray analyses were performed using Affymetrix Mouse Gene 1.0 ST arrays. Chips were processed according to the Affymetrix standard protocol as explained in [12]. Microarray hybridization was carried out by the Microarray Facility of the Centre Hospitalier Universitaire de Québec Research Centre. Three independent RNA samples treated with scrambled siRNA (negative control) or siRNA against MEF2A/2D were analyzed by microarray. The sequences of the siRNA oligonucleotides are provided in Table 1.

Microarray Analyses

Microarrays were performed as described in [12], and data were analyzed using Partek software version 6.6 update 6.13.0621 (Partek Inc.). Robust multiarray analysis, which is a normalization approach, was applied to the microarray data. The threshold value has been set at 1.5-fold change. Robust multiarray analysis corrects for the background and transforms microarray data into logarithmic data. Differentially expressed genes were analyzed using two-tailed *t*-test, two-sample equal variance comparison between the siRNA control group and the siRNA against MEF2A/2D group. The threshold value has been fixed at 1.2-fold change, P -value < 0.05 , and a false discovery rate (FDR) < 0.25 for Ingenuity Pathway Analysis (IPA; Ingenuity Systems).

Accession Number

Microarray data have been deposited in the Gene Expression Omnibus database and can be assessed using the GSE64128 dataset accession number.

RNA Isolation and Traditional and Quantitative PCRs

Synthesis of cDNA was performed as described in [9]. Quantitative RT-PCRs (qPCRs) were performed using a LightCycler 1.5 instrument and the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) according to the manufacturer's protocol using the following conditions: 10 min at 95°C followed by 40 cycles of denaturation (5 sec at 95°C), annealing (5 sec at 62°C), and extension (20 sec at 72°C) for *Celf6*, *Cyp17a1*, *Hsd17b7*, *Lhcgr*, *Pde8a*, *Por*, *Smad4*, and *Tsc22d3*. For *Ahr*, *Bmal1*, *Map2k1*, and *Nr0b2*, annealing conditions were 5 sec at 64°C. For *Hsd3b1*, *Star*, and *Nr4a1*, annealing conditions were 5 sec at 60°C. For *Cyp11b1* the following conditions were used: 5% dimethyl sulfoxide was added and annealing was performed at 64°C for 5 sec. At the end of each extension step, there was a single acquisition of fluorescence. *Rpl19* was used as the internal control [9, 13]. All the oligonucleotides used in qPCRs are described in Table 1. Specificity of the PCR products was confirmed by analyzing the melting curve and by sequencing (Centre de génomique de Québec, Québec, Canada). Quantification of gene expression was performed using the Livak method [14] and is expressed as a ratio of target to *Rpl19* levels. Each amplification was performed in duplicate using four different preparations (same three used in microarrays and an additional one) of first-strand cDNAs from different RNA extractions.

Plasmids

The mouse *Por* (-1116 to $+37$ bp and -67 to $+37$ bp), *Bmal1* (-1241 to $+44$ bp and -91 to $+44$ bp), and *Nr0b2* (-1757 to $+26$ bp and -74 to $+26$ bp) promoter fragments were obtained by PCR using the HiFi polymerase

FIG. 2. Microarray analysis of MEF2-depleted Leydig cells. Microarray experiments were performed using Affymetrix Mouse Gene 1.0 ST arrays. The results were analyzed with the Partek software. **A)** PCA mapping with the two populations: scrambled siRNA (Ctl) and siRNA against MEF2A/2D. **B)** Heatmap showing normalized gene expression data with a fold change ± 1.5 and a $P < 0.05$. Relative intensity scale is indicated below the heatmap.

(Clontech Laboratories) as recommended by the manufacturer using genomic DNA from MA-10 Leydig cells as template and the pairs of primers described in Table 1. The PCR conditions were as follow: 40 cycles of denaturation (10 sec at 98°C), annealing (15 sec at 55°C), and extension (120 sec at 72°C) for *Nr0b2* (−1757 to +26 bp), *Por* (−1116 to +37 bp), and *Bmal1* (−1241 to +44 bp). For *Nr0b2* (−74 to +26 bp), *Por* (−67 to +37 bp), and *Bmal1* (−91 to +44 bp), the annealing conditions were 60 sec at 58°C. The PCR products were cloned in a modified pXP1 firefly luciferase reporter vector [15] and confirmed by sequencing (Centre de génomique de Québec, Québec, Canada). The −74 to +26 bp *Nr0b2* reporter harboring a mutation of the MEF2-binding site was obtained by PCR using a forward primer containing the mutation (Table 1). The MEF2D and MEF2-Eng expression vectors were previously described [8]. The *Nr4a1* and *Ins13* reporters were previously described [16, 17]. The *Amhr2* reporter [18] was kindly provided by Dr. Jose Teixeira (Michigan State University).

Cell Culture and Transfections

Mouse MA-10 Leydig cells [19], provided by Dr. Mario Ascoli (University of Iowa), were grown [20] and transfected [9] as described previously. In knockdown experiments, MA-10 Leydig cells were transiently transfected with 200 nM of scrambled siRNA or siRNA directed against MEF2A/2D (Life Technologies) using 21.5 μ l jetPRIME (Polyplus-transfection). The sequences of the siRNA oligonucleotides are listed in Table 1. Forty-eight hours posttransfection, RNA and proteins were isolated from the same transfected cells. Proteins were used in Western blots to validate the efficiency of MEF2 knockdown. For gene reporter assays, MA-10 Leydig cells were transfected using jetPRIME as recommended by the manufacturer along with 250 ng of the pcDNA3 empty expression vector or expression vectors for MEF2D or MEF2-Eng along with 250 ng of the firefly luciferase promoter reporter in 24-well plates. Each experiment was performed four times, in triplicate.

Statistical Analyses

Statistical analyses were done using the paired Student *t*-test. For all statistical analyses, $P < 0.05$ was considered significant.

RESULTS

Microarray Analysis

To begin deciphering the role(s) of MEF2 factors in Leydig cells, MA-10 Leydig cells were depleted of MEF2A/2D and their transcriptome compared to that of MA-10 cells treated with scrambled siRNA. As shown in Figure 1A, siRNA against MEF2A/2D significantly reduced MEF2 protein levels in MA-10 Leydig cells as revealed by Western blot analysis. EMSA/supershifts also confirmed a significant decrease in MEF2 DNA-binding activity (Fig. 1B).

Total RNA was also isolated from these siRNA-transfected MA-10 Leydig cells (scrambled and directed against MEF2A/2D) for use in microarray experiments. Microarray analysis, principal component analysis (PCA) mapping, and heatmap design were performed using the Partek software with a threshold value fixed at 1.5-fold change. The PCA mapping diagram (Fig. 2A) shows the result distribution and confirms the reproducibility of the microarray data as the three scrambled siRNA samples are grouped on the left side while the three siRNA samples against MEF2A/2D are grouped on the right side. Figure 2B shows the heatmap of 64 affected genes of which seven were upregulated and 57 downregulated (−1.5- to +1.5-fold change) in MEF2-depleted MA-10 Leydig cells, and Table 2 provides the name, fold change, and P -value for each gene. The fact that some genes were not affected indicates that changes in gene expression were not due to alteration of vital cellular functions in the MEF2 knockdown cells.

Novel Cellular Functions Regulated by MEF2

The microarray data were next analyzed using the IPA software with a threshold value fixed at 1.2-fold change, P -value < 0.05 , and FDR < 0.25 . This software interconnects different genes involved in the same pathways or cellular functions. Using Z-scores, which indicate the mean distribution of results, this analysis revealed that several cellular functions were affected by the absence of MEF2 factors. Table 3 lists the 10 most affected cellular functions. Of interest, genes affected by MEF2 knockdown were found to be involved in cell death and survival, cellular development, reproductive system development and functions, and fertility (Table 3). Molecular network analysis with the IPA software revealed that various genes, up- or downregulated, are involved in fertility or/and gonad morphology (Fig. 3A). For example, *Smad4* and *Ahr* were downregulated and are associated with both functions. *Tsc22d3* was also downregulated but is only associated with

TABLE 2. The 64 most affected genes in MEF2-depleted MA-10 Leydig cells.

Gene symbol	P-value	Fold change	FDR ^a	Gene symbol	P-value	Fold change	FDR
<i>Atp1a3</i>	4.37E-04	1.567	0.045	<i>Gsta3</i>	1.39E-04	-2.388	0.037
<i>Ilitm7</i>	4.67E-04	2.080	0.047	<i>Id1</i>	4.25E-04	-1.607	0.045
<i>Maged1</i>	1.02E-04	1.685	0.037	<i>Id2</i>	1.46E-04	-1.717	0.037
<i>Man2a1</i>	9.21E-05	1.833	0.036	<i>Insig1</i>	9.00E-05	-1.592	0.036
<i>Mlc1</i>	1.60E-05	1.522	0.036	<i>Lin7c</i>	2.43E-04	-1.639	0.041
<i>Prkci</i>	2.33E-05	1.608	0.036	<i>Lin9</i>	1.45E-04	-1.596	0.037
<i>Pttg1ip</i>	3.74E-04	1.759	0.045	<i>Lonrf3</i>	1.86E-04	-1.677	0.040
<i>10339015</i>	2.63E-04	-1.794	0.042	<i>Mastl</i>	1.25E-04	-1.501	0.037
<i>10340676</i>	8.79E-05	-1.563	0.036	<i>Mybl1</i>	1.22E-05	-1.524	0.036
<i>10434556</i>	5.34E-05	-1.537	0.036	<i>Nek7</i>	5.13E-04	-1.517	0.047
<i>1700025G04Rik</i>	2.83E-04	-1.579	0.043	<i>Net1</i>	1.44E-05	-1.707	0.036
<i>1700106N22Rik</i>	2.70E-04	-1.572	0.041	<i>Nqo1</i>	4.67E-04	-2.413	0.047
<i>4930486L24Rik</i>	2.77E-04	-1.645	0.042	<i>Osgin2</i>	1.12E-05	-1.572	0.036
<i>Adamts9</i>	5.09E-05	-1.775	0.036	<i>Palmd</i>	5.58E-04	-1.601	0.048
<i>Akr1c14</i>	1.20E-04	-1.849	0.037	<i>Pgap1</i>	3.52E-05	-1.628	0.036
<i>Anln</i>	4.85E-05	-1.511	0.036	<i>Ptprj</i>	5.86E-04	-1.819	0.048
<i>Arhgap19</i>	1.21E-05	-1.981	0.036	<i>Purb</i>	8.04E-06	-1.647	0.036
<i>Atpaf1</i>	4.48E-05	-1.742	0.036	<i>Rb1</i>	1.95E-04	-1.663	0.040
<i>Cd63</i>	3.00E-04	-1.509	0.043	<i>Rnf19b</i>	3.77E-04	-1.621	0.045
<i>Cd109</i>	3.37E-04	-2.570	0.044	<i>Slbp</i>	3.92E-05	-1.647	0.037
<i>Cdkn1a</i>	2.21E-04	-2.636	0.041	<i>Slc25a24</i>	4.56E-04	-2.237	0.046
<i>Chmp5</i>	1.91E-04	-1.586	0.040	<i>Slc40a1</i>	3.76E-04	-2.405	0.045
<i>Coch</i>	4.66E-05	-1.758	0.036	<i>Serp1</i>	1.76E-05	-1.944	0.036
<i>Cpd</i>	1.45E-04	-1.840	0.037	<i>Spag5</i>	4.55E-05	-1.522	0.036
<i>E2f2</i>	1.59E-04	-1.720	0.038	<i>Spred2</i>	3.10E-04	-1.588	0.043
<i>Elmo1</i>	1.66E-04	-1.561	0.038	<i>Stk17b</i>	7.19E-05	-1.662	0.036
<i>Fam40b</i>	9.70E-05	-2.131	0.036	<i>Tlcd2</i>	1.12E-04	-1.727	0.037
<i>Fam81a</i>	1.83E-05	-1.723	0.036	<i>Tmem38b</i>	1.87E-04	-1.890	0.040
<i>Foxm1</i>	6.15E-04	-1.517	0.050	<i>Tor3a</i>	3.16E-04	-1.639	0.043
<i>Fuca2</i>	1.28E-04	-1.614	0.037	<i>Txnrd1</i>	1.45E-04	-1.702	0.037
<i>Gas2l3</i>	2.76E-04	-1.641	0.042	<i>Usp46</i>	2.18E-04	-1.922	0.041
<i>Gpsm2</i>	2.63E-04	-1.556	0.042	<i>Zdhhc2</i>	7.55E-05	-1.779	0.036

^a FDR, false discovery rate.

gonad morphology whereas the downregulated *Map2k1* gene only associates with fertility. *Lhcgr* was upregulated, and this gene is associated with fertility, gonad morphology, and synthesis of steroids. *Pde8a* was downregulated and is only associated with synthesis of steroid (Fig. 3B). The fact that these genes are known to be involved in fertility, gonad morphology, and synthesis of steroid supports the interconnection between these networks.

Although transcription factors can regulate gene expression from distantly located elements with respect to the transcription start site, proximal promoter regions usually contain key regulatory elements. We therefore performed in silico analyses of the proximal promoters (2000 bp upstream of the transcription start site) of genes deregulated in MEF2-depleted MA-10 Leydig cells to identify prospective MEF2-binding sites. This analysis revealed two categories of genes (Table 4). The first corresponds to genes containing MEF2-binding site(s) in their proximal promoters that could be direct MEF2 targets.

The second group consists of genes without MEF2-binding sites in the first 2 kb upstream of the transcription start site. As such, they are either indirect MEF2 targets or the MEF2 elements are located outside of the first 2 kb. In these two groups of genes, we selected key genes involved in fertility, gonad morphology, and synthesis of steroid for further analysis.

Novel MEF2 Target Genes

Two genes, *Star* and *Nr4a1*, previously described as targets for MEF2 in Leydig cells [7, 8] were present in the microarray data and served as internal controls. As expected, *Star* mRNA levels were reduced in MEF2-deficient cells while *Nr4a1* was not affected (Fig. 4, K and O). This is consistent with the fact that MEF2 regulates basal *Star* expression [7] while it is involved in hormone-induced, but not basal, *Nr4a1* expression [8]. Thirteen additional genes known to be involved in

TABLE 3. Cellular functions affected in MEF2-depleted Leydig cells.

Category	Functions	P-value	Z-score	Genes
Embryonic development	Size of embryo	7.52E-05	-5.919	58
Cell death and survival	Cell survival	9.21E-08	-5.769	219
Cell death and survival	Cell viability	5.37E-07	-5.045	201
Cellular growth and proliferation	Cell proliferation	2.83E-10	-4.593	520
Cell death and survival	Cell viability of tumor cell lines	5.41E-09	-4.506	133
Reproductive system development and function	Fertility	7.71E-08	-3.803	72
Cellular assembly and organization	Microtubule dynamics	2.79E-06	-3.649	166
Cellular function and maintenance	Microtubule dynamics	2.79E-06	-3.649	166
Cancer	Growth of tumor	6.63E-06	-3.469	74
Cellular assembly and organization	Organization of cytoskeleton	1.73E-07	-3.338	197

FIG. 3. MEF2 knockdown in Leydig cells affects genes involved in testicular functions. Affymetrix microarray results were analyzed with the IPA software. MEF2 knockdown affected numerous genes involved in fertility and gonad morphology (A), and steroidogenesis (B). Downregulated genes are in gray while upregulated genes are in black. Each geometric figure represents a class of protein: a trapeze for a transporter, a horizontal rectangle for a ligand-dependent nuclear receptor, a triangle for a kinase, a diamond for an enzyme, a vertical rectangle for a G protein-coupled receptor, a square for a growth factor, a vertical oval for a transmembrane receptor, and a horizontal oval for a transcription factor.

reproductive system development and function as well as in steroidogenesis were chosen for further analysis: *Ahr*, *Bmal1*, *Cyp11b1*, *Hsd3b1*, *Hsd17b7*, *Map2k1*, *Nr0b2*, *Pde8a*, *Por*, *Smad4*, *Tsc22d3*, *Cyp17a1*, and *Lhcgr* (Fig. 4). The fold

change and *P*-values for the selected genes from the microarray data are summarized in Table 4. The effects of MEF2 knockdown on these genes were validated by qPCR and the fold changes were similar to those of the microarray data

TABLE 4. Novel target genes for MEF2 in MA-10 Leydig cells.

Process	Gene symbol	Gene name	Fold change ^a	P-value	FDR ^b	MEF2 site
Fertility	<i>Map2k1</i>	Mitogen-activated protein kinase kinase 1	↓1.24	0.0057	0.09	Yes
	<i>Por</i>	P450 oxidoreductase	↓1.21	0.0168	0.13	Yes
Morphology of gonad	<i>Bmal1</i>	Aryl hydrocarbon receptor nuclear translocator-like	↓1.44	0.0191	0.13	Yes
	<i>Nr0b2</i>	Nuclear receptor subfamily 0, group B, member 2	↓2.10	0.0042	0.08	Yes
	<i>Tsc22d3</i>	TSC22 domain family protein 3	↓1.21	0.0472	0.20	Yes
Fertility, morphology of gonad	<i>Ahr</i>	Aryl hydrocarbon receptor	↓1.33	0.0128	0.12	Yes
	<i>Lhcgr</i>	Luteinizing hormone/choriogonadotropin receptor	↑1.35	0.0194	0.13	No
	<i>Smad4</i>	SMAD family member 4	↓1.21	0.0025	0.07	Yes
Steroidogenesis, synthesis of steroid	<i>Ahr</i>	Aryl hydrocarbon receptor	↓1.33	0.0128	0.12	Yes
	<i>Cyp1b1</i>	Cytochrome P450, family 1, subfamily B, polypeptide 1	↓1.42	0.0264	0.15	Yes
	<i>Cyp17a1</i>	Cytochrome P450, family 17, subfamily A, polypeptide 1	↑1.32	0.0359	0.17	Yes
	<i>Hsd17b7</i>	17Beta-hydroxysteroid dehydrogenase type 7	↓1.33	0.0124	0.11	Yes
	<i>Hsd3b1</i>	3Beta-hydroxysteroid dehydrogenase type 1	↓1.35	0.0848	0.26	Yes
	<i>Lhcgr</i>	Luteinizing hormone/choriogonadotropin receptor	↑1.35	0.0194	0.13	No
	<i>Nr4a1</i>	Nuclear receptor subfamily 4, group A, member 1	↑1.39	0.0178	0.13	Yes
	<i>Pde8a</i>	Phosphodiesterase 8A	↓1.22	0.0006	0.05	Yes
	<i>Por</i>	P450 oxidoreductase	↓1.21	0.0168	0.13	Yes
	<i>Star</i>	Steroidogenic acute regulatory protein	↓1.82	0.0052	0.09	Yes
	<i>Celf6</i>	CUGBP, Elav-like family member 6	↔1.00	0.9999	0.99	No

^aUpward arrow (↑) denotes an increase; downward arrow (↓) denotes a decrease; left-right arrow indicates no change.

^bFDR, false discovery rate.

(Table 5). As shown in Figure 4, mRNA levels for *Ahr*, *Bmal1*, *Cyp1b1*, *Hsd3b1*, *Hsd17b7*, *Map2k1*, *Nr0b2*, *Pde8a*, *Por*, *Smad4*, and *Tsc22d3* were decreased by 38%, 48%, 35%, 23%, 21%, 29%, 47%, 20%, 40%, 42%, and 43%, respectively, in MEF2-depleted MA-10 Leydig cells. On the other hand, mRNA levels for *Cyp17a1* and *Lhcgr* were increased by ~2-fold in the absence of MEF2 (Fig. 4, M and N). As a control, mRNA levels for the splicing gene *Celf6* [21] were also validated by qPCR and no significant change was observed (Fig. 4P). In agreement with this, the *Celf6* proximal promoter does not contain an MEF2-binding site (Table 4). This also indicates that MEF2 knockdown did not globally affect gene transcription. Thus, MEF2 represents a novel regulator of several genes essential for Leydig cell function.

MEF2 Activates the *Por*, *Bmal1*, and *Nr0b2* Promoters

Three genes important for Leydig cell functions and containing MEF2-binding sites in their proximal promoters were selected for functional promoter assays. The first gene codes for the electron donor POR (CYPOR) essential for steroidogenesis [22]. To test whether MEF2 directly regulates *Por* expression, a -1116 to +37 bp fragment of the mouse *Por* promoter was isolated, fused to the luciferase reporter gene, and used in transient transfections of MA-10 Leydig cells. As shown in Figure 5A, the *Por* promoter was activated about 2-fold by MEF2D. We next used siRNA against MEF2A/2D to deplete MA-10 Leydig cells of MEF2 factors and this led to a 35% decrease in *Por* promoter activity (Fig. 5A). Similar results were obtained when a MEF2-Eng dominant negative protein was used to compete with endogenous MEF2 factors (Fig. 5A). These results indicate that endogenous MEF2 factors are involved in *Por* transcription. The activating effect of MEF2D and repressive effect of knocking down MEF2 expression (siRNA) or activity (MEF2-Eng) were essentially lost when a minimal *Por* promoter construct (-67 to +37 bp) that lacks the two MEF2 elements was used (Fig. 5A).

The second gene selected for promoter studies encodes the BMAL1 (ARNTL, MOP3) protein. BMAL1 is a bHLH-PAS protein involved in circadian rhythmicity. BMAL1 is rhythmically expressed in Leydig cells and regulates *Star* promoter activity [23]. Homozygous *Bmal1* knockout male mice are infertile due to insufficient testosterone production [23]. To test a direct role of MEF2 in *Bmal1* transcription, a -1241 to +44 bp *Bmal1* promoter fragment was isolated and transfected in MA-10 Leydig cells. This promoter contains two potential MEF2 elements. Overexpression of MEF2D led to a 2-fold activation of the *Bmal1* promoter whereas knocking down MEF2 protein levels (siRNA) or activity (MEF2-Eng) resulted in decreased promoter activity by 70% for siRNA and 25% for MEF2-Eng (Fig. 5B). These effects were lost when a shorter -91 to +44 bp promoter fragment that no longer contains the MEF2 elements was used (Fig. 5B).

The nuclear receptor NR0B2 (SHP), a gatekeeper of steroidogenesis, which acts as a negative regulator of the expression of several key steroidogenic genes in Leydig cells [24], was chosen for functional studies because it was downregulated in MEF2-deficient Leydig cells (Fig. 4G) and its proximal promoter contains a potential MEF2 element (Table 4). As shown in Figure 5C, the *Nr0b2* promoter was activated 2.7-fold by MEF2D. Conversely, *Nr0b2* promoter activity was significantly reduced when MEF2 protein levels were reduced (siRNA) or MEF2 activity was inhibited (MEF2-Eng). Similar effects were observed with a -74 bp *Nr0b2* reporter construct that retains the MEF2 element (Fig. 5C). However, mutation of the MEF2 element in the context of the -74 bp reporter abrogated MEF2 responsiveness (Fig. 5C).

To further validate the specificity of MEF2-dependent activation of *Por*, *Bmal1*, and *Nr0b2*, three additional proximal promoters were used as negative control: 1) the *Nr4a1* promoter, which contains MEF2-binding sites (*Nr4a1* expression was unchanged in the microarray data); 2) the *Ins13* promoter devoid of MEF2 element (*Ins13* expression was unaffected in the microarrays); and 3) the *Amhr2* promoter, which lacks MEF2 site (*Amhr2* expression was reduced by 1.3-

FIG. 4. MEF2 regulates expression of genes implicated in fertility, gonad morphology, and steroidogenesis. MA-10 Leydig cells were transfected with scrambled siRNA (control, white bars) or with siRNA against MEF2A/D (black bars), and total RNA was isolated and used in qPCR. The genes validated by qPCR are involved in steroidogenesis, gonad morphology, and steroidogenesis: (A) *Ahr*, (B) *Bmal1*, (C) *Cyp1b1*, (D) *Hsd3b1*, (E) *Hsd17b7*, (F) *Map2k1*, (G) *Nr0b2*, (H) *Pde8a*, (I) *Por*, (J) *Smad4*, (K) *Star*, (L) *Tsc22d3*, (M) *Cyp17a1*, (N) *Lhcgr*, and (O) *Nr4a1*, as well as in mRNA splicing (P) *Celf6*. Levels of mRNA were normalized to *Rpl19* ($\pm$ SEM) (*** $P \leq 0.001$, ** $P < 0.01$, * $P < 0.05$).

TABLE 5. Fold change comparison between microarray and qPCR data.

Gene symbol	Fold change ^a	
	Microarray	qPCR ^b
<i>Ahr</i>	↓1.33	↓1.7
<i>Bmal1</i>	↓1.44	↓2.0
<i>Cyp11b1</i>	↓1.42	↓1.5
<i>Hsd3b1</i>	↓1.35	↓1.3
<i>Hsd17b7</i>	↓1.33	↓1.3
<i>Map2k1</i>	↓1.24	↓1.4
<i>Nr0b2</i>	↓2.10	↓2.0
<i>Pde8a</i>	↓1.22	↓1.2
<i>Por</i>	↓1.21	↓1.7
<i>Smad4</i>	↓1.21	↓1.7
<i>Star</i>	↓1.82	↓1.9
<i>Tsc22d3</i>	↓1.21	↓1.7
<i>Cyp17a1</i>	↑1.32	↑2.0
<i>Lhcgr</i>	↑1.35	↑2.0
<i>Nr4a1</i>	↑1.39	↑1.6
<i>Celf6</i>	↔1.00	↓1.1

^aUpward arrow (↑) denotes an increase; downward arrow (↓) denotes a decrease; left-right arrow indicates no change.

^bqPCR, quantitative RT-PCR.

fold, $P = 0.01$ in the microarray data). As expected, none of these promoters were responsive to MEF2D (Fig. 5D).

DISCUSSION

Recently, MEF2A/2D were identified in Leydig cells [8]. In these cells, MEF2 factors were found to regulate the expression of *Nr4a1* [8], a regulator of several steroidogenic genes, and of *Star* [7], a protein essential for steroidogenesis. In addition, steroid hormone production is significantly reduced in MA-10 Leydig cells depleted in MEF2, further supporting a role for these factors in steroidogenesis [7]. Despite these recent findings, limited information is available regarding the role(s) of this family of transcription factors in Leydig cells. Furthermore, mice knockout models do not provide any information on the role(s) of MEF2 in the testes because *Mef2*-deficient mice either die (neonatally or embryonically) or have no overt defects because of redundancy mechanisms [25–27]. To gain new insights on the role(s) of MEF2 factors in Leydig cells, we performed siRNA-mediated knockdown of MEF2A/2D in the MA-10 Leydig cell line and found that several genes involved in key processes were deregulated in the absence of these factors.

Global Roles of MEF2 in Regulating Genes Involved in Gonad Morphology, Fertility, and Steroidogenesis

In the mammalian gonads, MEF2 factors are expressed only in testes throughout development and into adulthood [8], thus suggesting important roles for these factors in gonadal differentiation and male reproductive function. Microarray data analyzed using the IPA software revealed three main biological functions that were affected in MEF2-depleted cells: fertility, gonad morphology, and steroid synthesis. This is in line with the well-established roles for MEF2 factors in organogenesis and development of various cell types and tissues (reviewed in [1]). The expression of several genes involved in these processes was significantly reduced in MEF2-deficient cells.

One of these genes is *Tsc22d3*. TSC22D3 (GILZ, glucocorticoid-induced leucine zipper) is a member of the TSC22-domain leucine zipper family. Despite being present in several cell types and tissues, including the kidney, brain, lung, heart, thymus, liver, and testis, the role of TSC22D3 remains to

be fully defined [28]. Interestingly, male knockout mice for *Tsc22d3* are infertile due to lower size and weight of the testes, absence of germ cells, and increased apoptosis in the seminiferous tubules [28, 29]. In these mice, testosterone levels are high as are FSH (follicle-stimulating hormone) levels whereas LH levels are normal, suggesting that the feedback loop is deficient [28]. Moreover, in *Tsc22d3*-deficient mice Leydig cells are hyperplastic [28]. Thus, TSC22D3 is important for male fertility and appears to be downstream of MEF2.

The gene encoding MAP2K1 (mitogen-activated protein 2 kinase 1) or MEK1 (MAP/ERK kinase 1) was also significantly downregulated in MEF2-depleted MA-10 Leydig cells. The MAPK pathway is an important regulator of Leydig cell proliferation, survival, and steroidogenesis [30–32]. Conditional knockout of *Map2k1* and *Map2k2* leads to decreased expression of Leydig cell-specific markers such as STAR, CYP11A1, and CYP17A1, and the number of Leydig cells per testis is also significantly reduced although testicular weight is not affected [33]. In addition, *Map2k1*^{−/−}/*Map2k2*^{−/−} mice exhibit hypoandrogenemia consistent with deficient Leydig cell function. The fertility of 3-mo-old *Map2k1*^{−/−}/*Map2k2*^{−/−} mice declines, and this reduction in fertility was not associated with spermatogenic defects [33]. Our current data revealed that MEF2 is required for proper expression of the *Map2k1* gene in Leydig cells, a gene coding for a kinase essential for steroidogenesis and male fertility.

In MEF2-depleted MA-10 Leydig cells, *Smad4* gene expression was reduced by 50%. SMAD4 (mothers against decapentaplegic homolog 4) belongs to the SMAD family of transcription factors that are induced by members of the TGFβ superfamily of growth factors, several of which are essential for testis development (reviewed in [34]). *Smad4* knockout in Sertoli and Leydig cells leads to abnormal formation of testis cords, seminiferous tubules, and vasculature during embryogenesis [35]. In aging Sertoli/Leydig-specific *Smad4* knockout mice, the testes hemorrhage, develop tumors, including Leydig cell adenoma, and spermatogenesis is arrested [35]. Overexpression of SMAD4 also leads to testis defects and fertility issues, and these mice exhibit Leydig cell hyperplasia [36]. Thus, SMAD4 plays critical roles in testis development, Leydig cell differentiation and function, as well as in male fertility, and our current data position MEF2 upstream of SMAD4 in Leydig cells.

NR0B2 (SHP, small heterodimer partner) is an atypical nuclear receptor that lacks a DNA-binding domain [37]. During postnatal development, NR0B2 is expressed in interstitial cells and in the seminiferous tubules whereas in the adult testis it is only present in Leydig cells [24]. Mice deficient in NR0B2 have higher testosterone levels and increased *Star* and *Cyp11a1* gene expression [24]. NR0B2 is a critical regulator of steroid production in Leydig cells, and in our present study we found that *Nr0b2* mRNA levels were decreased in MEF2-depleted MA-10 Leydig cells. Functional studies also revealed that MEF2 directly regulates *Nr0b2* gene expression by acting directly on the *Nr0b2* promoter. Thus, MEF2 is upstream of NR0B2 in a molecular cascade of steroidogenic regulators.

Expression of several genes involved in detoxification was significantly reduced in MEF2-depleted Leydig cells including *Ahr* and *Cyp1b1*. Upon activation, the AHR (aryl hydrocarbon receptor) receptor is translocated to the nucleus where it dimerizes with ARNT (aryl hydrocarbon receptor nuclear translocator protein) [38], binds to DNA, and activates expression of target genes [39]. AHR ligands include polycyclic aromatic hydrocarbons and dioxins, which are environmental pollutants known to lower Leydig cell number,

FIG. 5. MEF2 activates the *Por*, *Bmal1*, and *Nr0b2* promoters in MA-10 Leydig cells. MA-10 Leydig cells were transfected with controls as indicated (open bars; empty expression vector, scrambled siRNA, or Eng expression vector), expression vector for MEF2D (hatched bars), expression vector for MEF2-Eng (grey bars), or siRNA against MEF2A/2D (black bars) along with reporter constructs for the (A) *Por* promoter (–1116 to +37 bp and –67 to +37 bp), (B) the *Bmal1* promoter (–1241 to +44 bp and –91 to +44 bp), or (C) the *Nr0b2* promoter (–1757 to +26 bp, –74 to +26 bp wild type, –74 to +26 bp mutated in the MEF2 element as depicted by the large X). (D) As controls, MA-10 Leydig cells were transfected with the *Nr4a1* promoter (–1013 to +52 bp) that contains MEF2-binding sites and was not affected in microarray data, the *Ins13* promoter (–1086 to +5 bp) that does not contain MEF2-binding site and was not affected in microarray data, and the *Amhr2* promoter (–1600 to +50 bp) that lacks MEF2-binding site and was reduced in the microarray data. Results are shown as fold activation over the respective control. An asterisk indicates a statistically significant difference from the respective control ($P < 0.05$).

impair cholesterol mobilization, inhibit steroidogenic enzyme activity, and perturb androgen production and action. This leads to defects in the development of the male reproductive tract and external genitalia (reviewed in [40], see also [41–43]) and fertility disorders in humans [44]. One of the downstream targets of AHR is *Cyp1b1* [39], which encodes an enzyme involved in drug metabolism. CYP1B1 is expressed in the testis, ovary, and adrenal gland, and its expression is stimulated by adrenocorticotropin [45], cAMP [46], and AHR ligands [47]. *Cyp1b1*-null mice are phenotypically normal probably because of redundancy and compensation by other CYP1 members [48]. Triple knockout (*Cyp1a1/Cyp1a2/Cyp1b1*) mice exhibit pseudohermaphroditism (retention of Müllerian and Wolffian duct derivatives) and bilateral cystic ovaries [49]. Thus, CYP1 members are important for sexual development and masculinization. Whether MEF2 regulates *Cyp1b1* expression directly or indirectly by acting on *Ahr* expression remains to be established.

The circadian cycle is essential for reproductive function homeostasis [50]. The circadian protein BMAL1 (brain and muscle Arnt-like protein-1) is a master regulator of other clock genes. Homozygous *Bmal1* knockout male mice are infertile owing to low sperm count and low testosterone levels [23]. In Leydig cells of *Bmal1*-deficient mice, *Star* mRNA and protein levels are reduced, and this is due to the fact that BMAL1 directly regulates *Star* transcription [23]. Our current data indicate that MEF2 is essential to maintain normal *Bmal1* expression in Leydig cells and that MEF2 directly activates the *Bmal1* promoter.

POR or CYPOR (P450 oxidoreductase or cytochrome P450 oxidoreductase) is an electron donor essential for steroidogenesis and masculinization of the male fetus. Humans with mutations in the *POR* gene suffer from congenital adrenal hyperplasia and exhibit disorders of sexual development such as undervirilization [51, 52]. Our present data indicate that proper expression of the *Por* gene requires normal MEF2 levels in Leydig cells.

In Leydig cells, steroidogenesis is regulated mainly by LH-activated cAMP-dependent pathways. Poststimulation, cAMP is degraded via the action of phosphodiesterases (PDE), mainly PDE8A [53, 54]. Consistent with sustained high cAMP levels, *Pde8a*^{-/-} male mice have increased testosterone levels [53]. Interestingly, *Pde8a* expression was decreased in MEF2-depleted MA-10 Leydig cells.

Leydig cells express several hydroxysteroid dehydrogenases essential for cholesterol synthesis and steroidogenesis. In MEF2-depleted MA-10 Leydig cells, expression of *Hsd17b7*, which encodes an enzyme essential for cholesterol synthesis [55, 56], was reduced. This would suggest a role for MEF2 in cholesterol synthesis in addition to steroidogenesis in Leydig cells.

All the genes discussed so far were all downregulated in MEF2-depleted Leydig cells. However, two genes *Cyp17a1* and *Lhcgr* were upregulated. CYP17A1 is a steroidogenic enzyme essential for testosterone production while LHCGR (LH receptor) is essential for Leydig cell differentiation and hormonal stimulation. MEF2 factors are predominantly transcriptional activators, but they can also repress gene expression. This is dictated by the nature of their associated cofactor (reviewed in [2]). Therefore, it is possible that MEF2 could be involved in the repression of *Cyp17a1* and *Lhcgr* gene expression, either via direct repression or indirectly by activating the expression of repressors such as NR0B2. As such, MEF2 would be involved in the stimulatory phase of steroid hormone production by activating steroidogenic genes ([7, 8] and this study) as well as in the subsequent inhibitory phase in order to maintain adequate steroid hormone levels. Another interesting possibility is that MEF2-deficient Leydig cells compensate for the decreased steroid production caused by reduced expression of several genes involved in steroidogenesis. Such a compensatory mechanism has been described previously in *Star*-deficient mice that result in lipid congenital adrenal hyperplasia. In the absence of STAR, steroidogenesis is severely decreased, and cholesterol transport in the mitochondria is blunted. To compensate for the lack of steroid hormone production, there is an increase in the expression of genes involved in cholesterol production, which results in lipid droplet accumulation in the cytoplasm of the cell [57].

In conclusion, we have identified and characterized novel MEF2 target genes that are known to be involved in fertility, gonad morphology, and steroidogenesis using the MA-10 Leydig cell line as the model. However, our study using microarrays revealed a large number of genes affected by the absence of MEF2 in Leydig cells. For several of these genes, no information is available regarding their potential role in reproductive function, which should not be interpreted as having no role in this process. Additional work will be required to determine whether these MEF2-regulated genes are important regulators of Leydig cell function and male reproduction.

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