Evidence for Epigenetic Changes in the Estrogen Receptor Alpha Promoter in Lymphangioleiomyomatosis (LAM)

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Abstract: Lymphangioleiomyomatosis (LAM) is a rare but often fatal disease, characterized by the abnormal proliferation of smooth muscle cells of the lung. LAM occurs almost exclusively in women and its pathology correlates with mutations in the tuberous sclerosis complex 2 (TSC2) gene and expression of the hormone estrogen. One of the hallmarks of LAM lesions is the anomalous expression of the intracellular receptor for estrogens, ERα, and the distinct gender specificity of LAM would support the hypothesis that this anomalous expression of ERα plays an essential role in the pathology of the disease. Our previous studies have defined a direct link between the TSC2 gene product tuberin and estrogen signaling through ERα. The objectives of this study were to investigate epigenetic changes in the ERα promoter as a mechanism for upregulation of ERα expression in LAM disease. The results of this study provide evidence for: a) use of multiple ERα promoters in human airway smooth muscle cells and LAM- associated cell lines, b) epigenetic changes in the promoter of the ERα gene in LAM-associated cell lines and LAM lesions, and c) differential binding of histone deacetylase 1 and methyl-CpG binding proteins in human airway smooth muscle and LAM cells. These studies cumulatively suggest the upregulation of ERα expression associated with LAM disease may in part be a consequence of demethylation at the ERα promoter.

Keywords: Tuberous sclerosis, tuberin, methylation, TSC2.

INTRODUCTION

Lymphangioleiomyomatosis (LAM) is a rare disease occurring almost exclusively in women and characterized by proliferation of smooth muscle cells of the lung [1-3]. The most common presenting symptoms are dyspnea and pneumothorax. Although LAM lesions are composed of multiple cell populations, the predominant proliferating cell found in LAM lesions appears to be the myofibroblast-like spindle-shaped cells that express immunoreactivity to a variety of smooth muscle cell markers [4, 5]. Uncharacteristic of smooth muscle cells of the lung, these cells also express the cell surface protein gp100 (HMB-45 positive) and intracellular receptors for the hormones estrogen (ERα) and progesterone (PR) [6, 7]. It is these latter 2 that have been hypothesized to play an integral role in the gender specificity of LAM.

LAM pathogenesis has been shown to correlate with both expression of the hormone estrogen [8-10] and mutations in the TSC2 gene [11, 12]. Although exceptions have been identified [13-18], the vast majority of LAM patients fall into the category of post-pubescent but pre-menopausal women. This observation would strongly suggest estrogen hormone signaling plays a role in LAM pathogenesis, and is further supported by published observations that pregnancy in women with LAM seems to exacerbate the disease while anti-estrogen therapies including oophorectomy [19], ovarian irradiation [20] and progesterone administration [9, 21] appear to lessen the severity of the disease.

LAM occurs either as an isolated disorder (sporadic LAM) or in association with tuberous sclerosis complex TSC (TSC-LAM). TSC is an autosomal dominant neurocutaneous disease that leads to the development of hamartomatous lesions in a variety of tissue types including brain, kidney, heart, skin, eye and lung [22]. TSC has been genetically mapped in humans to two distinct loci, TSC1 and TSC2 [23], and both sporadic LAM and TSC-LAM have been linked to mutations in the TSC genes [12]. The TSC1 gene encodes hamartin, a 130 kilodalton protein containing 1164 amino acids [24]. The TSC2 gene encodes a 198 kilodalton protein called tuberin, containing 1807 amino acids [25]. Together these proteins have been identified in complexes regulating cell growth, cell adhesion, cell migration and a variety of intracellular signaling pathways (reviewed in: [26, 27]). Tuberin and hamartin also appear to maintain distinct cellular locations [28, 29] and distinct cellular functions that include regulation of nuclear receptor gene activities [30], cell motility [31-33], cytokinesis [34] and cell growth [35-38].

Previous studies from our laboratory and that of others have demonstrated that tuberin can directly impact ERα-mediated genomic and nongenomic signaling [39-42]. Cumulatively these data support the hypothesis that, if ERα is present in a cell, disruption of tuberin/hamartin-associated signaling events can directly impact signaling pathways mediated by ERα. Consistent with this hypothesis is the corollary that LAM gender specificity and pathogenesis is linked to the up-regulation of ERα expression in tuberin/

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hamartin disrupted lung smooth muscle cells. The objective of this study was to investigate a potential mechanism that could serve as either a cause or exacerbation of ERα anomalous expression in lung smooth muscle cells. Evidence is presented for epigenetic changes, in the form of CpG demethylation, occurring at the promoter region of the ERα gene in LAM-associated cell lines and LAM lesions. These changes are further shown to correlate with reduced association of this promoter with the transcription repressor histone deacetylase 1 (HDAC1) and a methyl-CpG binding protein. Cumulatively these data support the hypothesis that the ERα promoter in LAM lesions is subject to epigenetic changes that facilitate an upregulation of ERα gene expression.

MATERIALS AND METHODOLOGY

Cell Lines and Lung Tissues

The LAM cell line and control human airway smooth muscle (HASM) cells used in these studies were as previously described [43] and a generous gift from Drs. Vera Krymskaya and Ray Panettieri (University of Pennsylvania, Philadelphia, PA). The LAM cell line was established from a LAM lung lesion and previously characterized as having mutations in both TSC2 alleles and expressing ERα. These cells were used in low passage (between 5 through 10). The second LAM-associated TSC2 defective cell line employed in these studies (AML-101) was a generous gift from Rachel Squillace at the Rothberg Institute (Guilford, CT). The AML-101 cell line is a transformed version of the LAM-associated AML-621 estrogen responsive human angiomyolipoma-derived cell line described by Yu *et al*. [44]. The human embryonic kidney-293 (HEK-293) and human breast cancer-derived MCF-7 cell lines were purchased from ATCC (Manassas, VA).

The 5 LAM lung tissue specimen used in these studies were obtained from the NIH repository of LAM patient diseased lung tissues and/or DNA preparations. These tissues were obtained under an IRB protocol (#04-0715-P3G) approved by the University of Kentucky Institutional Review Board. The tissues in the NIH LAM repository are derived exclusively from women (between 21 and 65 years) and of all ethnic groups. These women suffer from end-stage LAM disease and as a result undergo lung transplantation. The tissues were snap frozen lung specimen that were defined by pathology as being derived from LAM lesions or normal lung. The tissue samples were provided with number identifiers only and those identifiers have been redesignated as L1-L5 for these studies.

The 2 female human normal lung samples used in these studies were specimen obtained under IRB approval (#00-0556-P3R) from the Pathology Department at the University of Kentucky. These samples were provided as snap frozen tissues with number identifiers. The identifiers have been simplified to the N1 and N2 designations used here. The rarity of this disease and the limited availability of tissues did not permit age matching of tissues for the LAM specimen and normal controls.

ERα Promoter-Driven Luciferase Constructs

Using overlapping oligonucleotides, the 70 bp ERα 5'-UTR sequence shared by all ERα promoters [45, 46] was initially subcloned upstream of a firefly luciferase gene in the pGL3-Basic vector (Promega, Madison, WI, USA). This construct served as the mother vector for subcloning the ERα-A, B, C, E and F promoters. These promoter fragments were isolated from human genomic DNA using oligonucleotides (see Table **1**) that permitted fusion of the respective promoters into a unique *Mlu I* restriction site upstream of the common 70 bp ERα 5'-UTR sequence. The sequence integrity of all constructs was validated by DNA sequencing.

ERα Promoter-Specific RT-PCR Analyses

RNA was isolated from cells and tissues using Trizol reagent (Sigma, St. Louis, MO, USA) according to manufacturer's protocols. To characterize ERα promoter usage in LAM and MCF-7 cells, standard RT-PCR analyses utilizing ERα-A, B, C, D, E and F specific nested PCR primers, along with a common ERα exon 2 primer were used as previously described [46]. Briefly, tandem pairs of forward primers specific for each ERα promoter (A-F) along with a common tandem pair of reverse primers for ERα exon 2 were used in sequential nested PCR reactions. Primers and reaction conditions were exactly as previously described [46]. Parallel GAPDH RT-PCR analyses (see table for specific primers) were performed for normalizing data. PCR products were separated on 2% agarose gels and identified by ethidium bromide staining.

Western Blot Analyses

Protein from tissues and cell lines was isolated and analyzed for ERα expression in Western blot analyses as we have previously described [47]. The antibody (AB-15) used for ERα detection was purchased from Lab Vision Corporation (Fremont, CA, USA).

Methylation Analyses

Sodium bisulfite modifications of DNA were performed using a commercially available kit (EZ DNA Methylation Kit, Zymo Research, Orange, CA, USA) according to manufacturers instructions. Briefly, 1 ug of DNA was incubated at 37^{o}C for 15 min in 1x M-dilution buffer. To this was added 2-fold excess CT Conversion Reagent. This was incubated 12 hr at 50^{o}C. Following the 12 hr incubation samples were mixed with an equal volume of M-Binding Buffer and loaded onto a Zymo-Spin I Column. Columns were centrifuged briefly at 12,000 g, washed with 200 ul of M-Wash Buffer, 200 ul of M-Desulphonation Buffer, 200 ul of M-Wash Buffer and eluted with 10 ul of M-Elution Buffer.

For direct characterization of methylated DNA sequences, PCR primers were designed (see Table **1**) to non-CpG containing sequences that surround the ERα-A promoter sequence. These were used in cycle sequencing reactions with bisulfite modified DNAs from the various cell lines as described in the relevant figure legends. A commercially available cycle sequencing kit (Amersham Biosciences, Pittsburg, PA, USA) was used according to the manufacturers detailed instructions. For analyzing the relative demethylation at the various GpG in our sequencing autoradiographs, a Kodak Imaging 3.5 System and its associated 1D Image Analysis Software was used. Bands were normalized to background and an average pixel density

Table 1. Various Oligonucleotide Primers Used in Plasmid Constructions, RT-PCR Analyses and CpG Methylation Sequencing

pGL3-Basic ERα Promoter Cloning Primers		
Primer	**Sequence**	**Gene Location**
Promoter-A Forward	5'-AAGTAAAGTTCAGGGAAGCTG-3'	-170a
Promoter-A Reverse	5'-AGGGTGCAGACCGTGTCC-3'	+216
Promoter-B Forward	5'-CAGCTGCTAAATATAGCTGTC-3'	-518
Promoter-B Reverse	5'-CAACTTTAAGTACTGGTCTCC-3'	-20
Promoter-C Forward	5'-GAAAGCATAGGGTACTTTC-3'	-2,248
Promoter-C Reverse	5'-CATGGAGAACAGCAATCCTCA-3'	-1,749
Promoter-D Forward	5'-TTATGGGCAGAGAGGAAGC-3'	-4,175
Promoter-D Reverse	5'-CTGGCTCTCTCAGGTGAAG-3'	-3,655
Promoter-E Forward	5'-ATAGACTTTTAAGAGCTCTTTG-3'	-151,414b
Promoter-E Reverse	5'-CCTTCTTTGGTAGCTACAGA-3'	-150,915
Promoter-F Forward	5'-TTTAGTTTAGCTGTAGCTAAC-3'	-117,522
Promoter-F Reverse	5'-TTGAAGAGAAGATTATCACTC-3'	-117,015
RT-PCR and ChIP Primers		
Primer	**Sequence**	**Gene Location**
ERα Forward	5'-ACAGGCCAAATTCAGATAATCGACG-3'	811c
ERα Reverse	5'-CAGGCTGTTCTTCTTAGAGCGTTTGAT-3'	1270
GAPDH Forward	5'-TCATCCATGACAACTTTGGTATCGTG-3'	590d
GAPDH Reverse	5'-TCATATTTGGCAGGTTTTTCTAGACGG-3'	869
Primers Used for CpG Methylation Sequencing		
Primer	**Sequence**	**Gene Location**
Forward	5'-AACCACCTAAAAAAAAAACAC-3	+149
Reverse	5'-CTTTAATATAAAAAATCATAATC-3'	+236

[a]Source of sequence information for promoters A, B, C, D and the methylation sequencing primers are from GenBank Accession # AL356311.
[b]Source of sequence information for promoters E and F are derived from GenBank Accession # AB090237.
[c]Source of sequence information for ERα PCR primers is from GenBank Accession #NM000125.
[d]Source of sequence information for GAPDH PCR primers is from GenBank Accession #NM002046.

value for a set region of interest (band) was established. Bands with pixel density values within 40% of this value were categorized as (+), bands with pixel density values 10% or less of this value were categorized as (-), bands with pixel density values 11-60% below this average were classified weak and bands with pixel density values greater than 140% of this average were classified as strong.

ChIP Assays

Modified chromatin immunoprecipitation (ChIP) assays were performed essentially as previously described [48]. Briefly, LAM and HASM cells, treated and untreated with 2uM 5-azadeoxycytidine for 96 h, were incubated 10 min in 1% formaldehyde, brought to 0.125 M glycine and incubated another 5 min. The cells were lysed by Dounce homogenization in lysis buffer (5mM PIPES, 85mM KCl, 0.5% Nonidet P-40 and protease inhibitors) and nuclei were isolated by centrifugation. Nuclei were lysed in nuclei lysis buffer (50mM Tris, 10mM EDTA, 1% SDS and protease inhibitors) and chromatin was sheered by sonication. Sonicated lysates were diluted with 2 volumes of IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, 167 mM NaCl and protease inhibitors) and incubated overnight with a rabbit polyclonal anti-MeCpG2 (Imgenex, San Diego, CA, USA: IMG-297) antibody or a rabbit polyclonal HDAC1 (Imgenex, San Diego, CA, USA: IMG-337) antibody. *Staphylococcus aureus* (Staph A: Pansorbin, Calbiochem, San Diego, CA, USA) cells were added, incubated 15 min, centrifuged and the pellet was washed with IP dilution buffer. Protein complexes were eluted by the addition of elution buffer (50mM NaHCO3, 1% SDS). Elutions were brought to 0.3M NaCl and incubated at 65^{o}C for 5 hr to reverse the crosslinking. DNA was purified by standard phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. This DNA sample was analyzed by PCR analyses for the presence of ERα promoter-A DNA (see Table **1** for primers used).

Demethylation Analyses

The impact demethylation has on ERα mRNA expression was analyzed as previously described [49]. Briefly, LAM and HASM cells were plated at 50% confluency in 10cm dishes. Cells were treated with 2uM 5-azadeoxycytidine (5-azadC: Sigma, St. Louis, MO) for 96 hours, changing the media every 24 hours. Twenty-four hours prior to harvesting, the cells were treated with 5mM 4-phenylbutyrate (4-PBA: Calbiochem, San Diego, CA, USA). RNA was isolated from cells and cDNA was synthesized from 5ug of total RNA from treated and untreated LAM and HASM cells using a commercially available First Strand cDNA Synthesis Kit (Fermentas, Amherst, NY). This cDNA was analyzed for ERα mRNA expression using a SYBR Green Real-Time PCR Kit (Stratagene, San Diego, CA, USA) and an Mx4000 Real-Time PCR machine (Stratagene). PCR primers for these studies can be found in Table **1**.

Statistical Analyses

Statistical differences between experimental groups were determined by ANOVA and Tukey's multiple comparison analysis. Data are expressed as means ± SEM for 3 independent experiments. P values equal to or less than 0.05 were considered significant.

RESULTS

LAM-Associated Cell Lines Express ERα mRNA *via* Multiple ERα Promoters

The 5' regulatory region for the ERα gene is a complex structure consisting of up to 7 different promoters, designated A to F and T (reviewed in: [45]), that span >150 kb of DNA. The predominant promoters utilized for expression of the ERα gene in most cells and tissues are the A and B promoters, although there have been some reported novel cancer and tissue-specific switching of promoter utilization. To identify which of the ERα promoters are used in LAM, total RNA was isolated from a LAM cell line and an AML immortalized cell line [43]. A set of nested primers that distinguish the various ERα promoters, together with a common ERα exon 2 primer, were designed as previously described [50] and used in RT-PCR analyses of the respective RNAs (Fig. **1**). As positive and negative controls

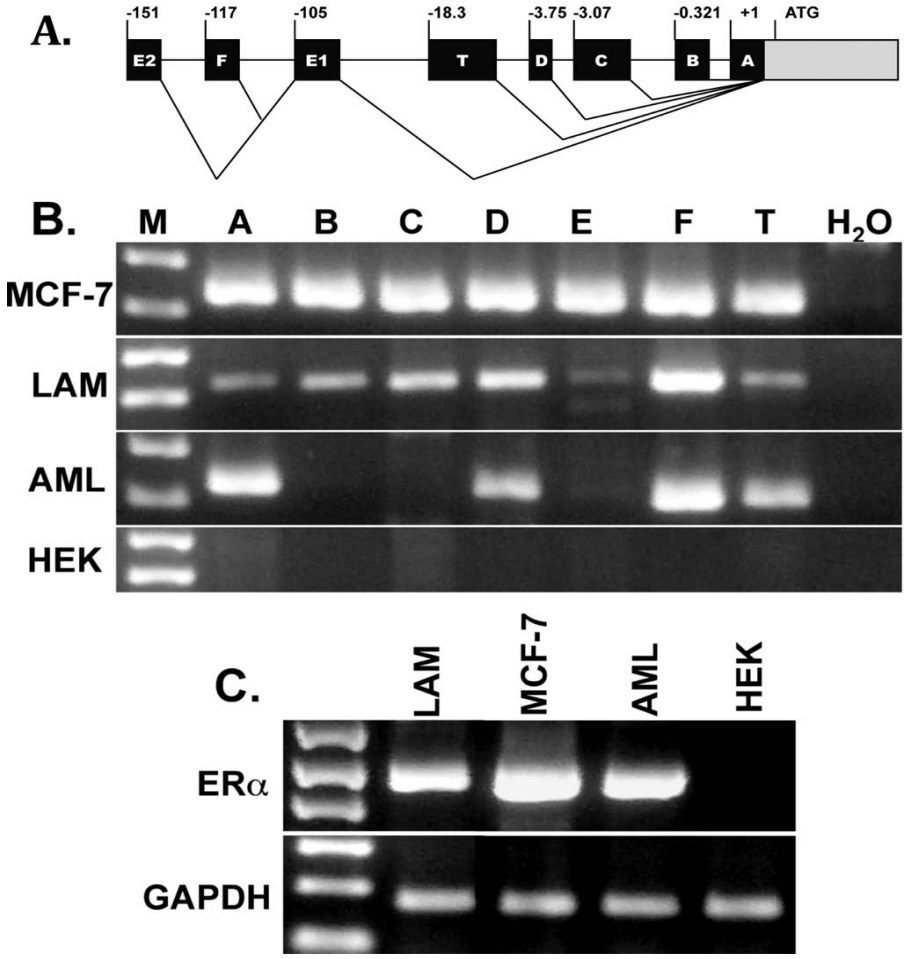


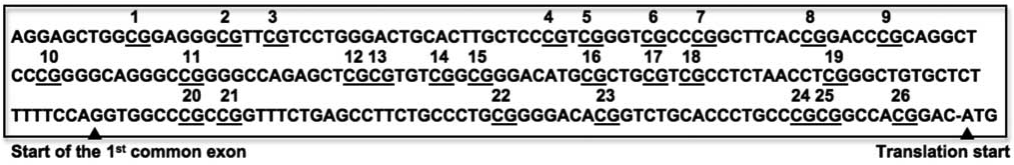
Fig. (1). Cells derived from a LAM lesion utilize promoters A, B, C and F of the ERα gene to express ERα mRNA. (**A**) A schematic diagram of the ERα gene and the different promoters identified to regulate it as previously described [45]. (**B**) A series of nested PCR primers as described in the Methods section were used to qualitatively evaluate the promoter usage in total RNA samples derived from a MCF-7 cell line, a LAM cell line, an AML cell line and a HEK cell line. PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. The marker (M) bands for each gel corresponds to 200 and 300 base pair DNA fragments from a standard 1kb ladder sample. (**C**) As controls for these studies, the RNA samples used in B were also analyzed by PCR technologies for total ERα mRNA expression and GAPDH mRNA expression.

for these studies, parallel analyses were performed on MCF-7 and HEK-293 cell lines respectively. As seen here, primers specific for the ERα -A, B, C, D, E, F and T were amplified in the LAM cell line, while the analysis of the AML cell line suggest ERα expression is restricted to the usage of promoters A, D, F and T.

LAM Cell Lines and LAM Tissues Appear Demethylated in their Proximal ERα-A Promoter

Global demethylation has been reported for a variety of cancers and this event has been shown to activate previously silenced genes [51, 52]. To investigate this possibility for the ERα promoter in LAM disease, a human airway smooth muscle (HASM) cell line, a LAM cell line, an LAM-associated angiomyolipoma (AML) cell line, tissue specimen derived from 5 LAM lesions (accessed through the NIH LAM repository of archived tissues) and 2 tissue specimen derived from normal female lung tissue, were examined with respect to the methylation status of their ERα -A promoter (Fig. **2**). This promoter region spans 233 bp upstream of the ATG start site and encompasses 70 bp of the first common exon (a region shared by all ERα promoters). As seen in Fig. (**2A**), the full-length promoter contains 26 CpGs, a number of which have previously been shown to be susceptible to methylation [53, 54]. The proximal 70 bp sequence of this promoter incorporates 7 of these 26 CpGs. Genomic DNA was isolated from cells and tissues, and unmethylated cytidine residues were converted to uradine residues (thymidine in the PCR amplified DNA) using a commercially available sodium bisulfite kit. The treated DNA was subsequently sequenced using standard PCR-based cycle sequencing technologies and analyzed by autoradiography for CpG conversion to TpG, or resistance to this conversion due to methylation. Fig. (**2B**) maps the results of these analyses for the entire promoter A region for HASM and LAM cells, and the proximal 70 bp region of promoter A for the AML cell line, the 5 LAM tissue specimen and the 2 normal lung tissue specimens. Clearly distinguishable differences in methylation status were identifiable when comparing LAM, HASM and AML cell lines. Unfortunately LAM tissue specimen are by nature heterogenous and were therefore much more subtle with

A.



B.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
HASM	S	+	w	-	w	w	+	w	w	w	+	w	w	S	+	+	+	+	-	-	w	S	w	S	S	w
LAM	S	+	w	-	+	S	+	w	w	w	+	-	w	S	-	w	-	-	-	-	-	-	-	-	-	-
AML																				-	-	-	-	-	-	-
L1																				-	w	w	w	-	w	-
L2																				-	-	w	w	-	w	-
L3																				-	-	-	w	-	w	-
L4																				-	w	S	w	-	-	-
L5																				-	-	w	-	-	w	-
N1																				w	w	S	w	S	S	w
N2																				w	S	S	w	S	S	w

\+ = Methylation
\- = No Methylation
W = Weak Methylation
S = Strong Methylation

C.

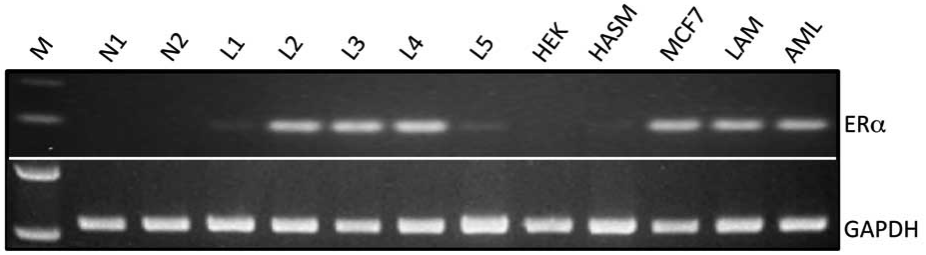


Fig. (2). The ERα-A promoter is selectively demethylated in cells derived from LAM lesions. (**A**) The ERα promoter sequence (+1 to +233: [45]) analyzed in this study is presented, and the 26 methylatable CpG structures are underlined. The first arrowhead identifies the AG residue corresponding to the 1st exon splice site at which all ERα promoter sequences are spliced, and the second arrow identifies the ATG translation initiation site. (**B**) A table of the results from sequence analysis comparing CpG methylation in LAM cells and HASM cells for the 26 CpG motifs found in ERα promoter-A, and a similar analysis comparing methylation of the ERα promoter-A distal 7 CpG motifs in a LAM-associated angiomyolipoma cell line (AML), 5 LAM tissue samples (L1-L5) and 2 normal lung specimen (N1-N2). See the Methods section for further elaboration of the +/-, weak and strong designations. (**C**) Agarose gel profile of RT-PCR characterization of ERα and GAPDH mRNA expression in the various cell lines and tissues examined in these studies. (M = DNA ladder markers).

regard to their global differences in methylation status at the seven CpG sites analyzed. In spite of this limitation it was clear that at least for the limited number of LAM tissues analyzed, methylation differences were present, and that LAM cells and tissue specimens are or become hypomethylated in the distal region of their ERα-A promoter. To provide support for the transcriptional upregulation of ERα in these various tissues and cell lines, we isolated total RNA and performed RT-PCR analyses on them (Fig. **2C**). As seen here, all lung tissue samples, to varying degrees, expressed levels of ERα mRNA greater than that observed for the normal lung tissues.

Trans-Regulation at ERα Promoters is Altered in LAM Cells

It is logical to assume that *cis* modifications to a promoter result in altered *trans*-acting factors associating with that promoter. Whether the *trans* factors are causing the *cis* modifications or vice versa is unestablished, but *trans* factors ultimately define the amount and rate of transcription. In an initial look at *trans*-regulation of the ERα promoter in LAM cells, we analyzed the ERα promoter A region for the binding of two proteins, methyl-CpG binding protein-2 (MeCP2) and histone deacetylase-1 (HDAC1), previously observed to bind to and transcriptionally repress ERα promoters [55-57]. Utilizing chromatin immunoprecipitation technology, formaldehyde-crosslinked chromatin from LAM and HASM cells was selectively immunoprecipitated using MeCP2 (Fig. **3A**) and HDAC-1 (Fig. **3B**) antisera. Crosslinking was reversed, DNA was isolated and ERα promoter-A DNA was identified by PCR. As seen here, noticeably more MeCP2 and HDAC-1 were bound to promoter-A in HASM cells than in LAM cells, and binding of MeCp2 in HASM cells could be inhibited if these cells were preincubated with the methylation inhibitor 5-aza-deoxycytidine.

In an extension of the above studies we investigated the impact chemical alteration of global DNA methylation and deacetylation had on ERα mRNA expression in LAM and HASM cells. LAM and HASM cells were incubated for 96

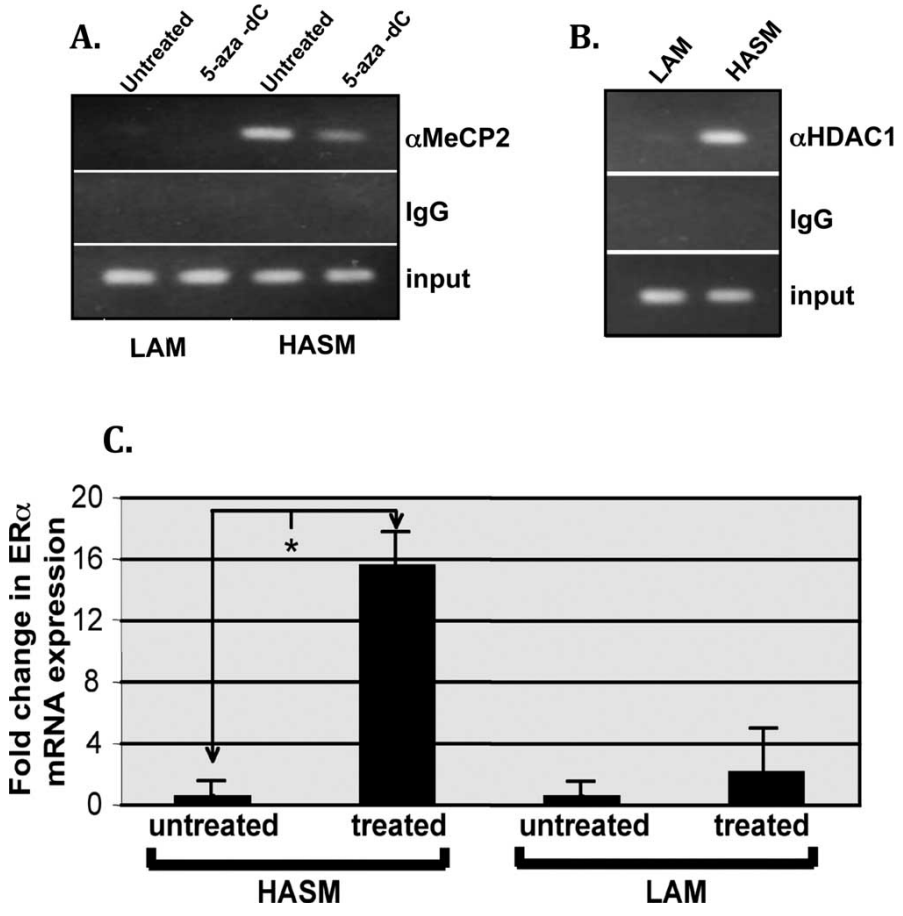


Fig. (3). Alterations in *trans*-acting factor binding at the ERα-A promoter in LAM cells correlate with ERα mRNA expression. (**A**) Chromatin Immunoprecipitation analyses identify selective binding of methyl-CpG-2 binding protein and HDAC-1 to the ERα promoter-A region in HASM cells. DNA-protein complexes from formaldehyde-fixed LAM and HASM cell lysates were immunoprecipitated using antisera to either methyl-CpG binding protein-2 (MeCP2) or HDAC-1. DNA was extracted from these immunoprecipitates and PCR analyses were performed using ERα-promoter-A specific primers. PCR products were analyzed on 1.5% agarose gels *via* ethidium bromide staining. These results are representative of this experiment performed minimally 3 times. (**B**) LAM and HASM cells were incubated for 96 hours in the presence of the demethylating agent 2uM 5-aza and the final 24 hours in the presence of the HDAC inhibitor 5mM 4-PBA. (**C**) RNA was isolated and analyzed for the expression of ERα mRNA using SYBR green Real-Time PCR technologies. Results are presented as the fold change observed for RNA analyzed from untreated cells. Statistical bars represent ±SEM for 3 independent experiments. Asterisk indicates statistically significant differences ($p \leq 0.05$).

hours in the presence of the methylation inhibitor 5-azadC and the final 24 hours in the presence of the HDAC inhibitor 4-PBA. RNA was isolated and analyzed for the presence of ERα mRNA using SYBR green Real-Time PCR technologies (Fig. **3C**). As seen here, inhibition of DNA methylation and histone acetylation results in a substantial increase in ERα mRNA levels in HASM cells, but only a minor increase in LAM cells.

DISCUSSION

The gender specificity of LAM and direct correlations with mutations in the TSC2 gene, support the hypothesis that disruption of normal TSC2 function can sensitize lung smooth muscle and kidney cells to the mitogenic activity of circulating estrogens. Consistent with this hypothesis is the observation that LAM lesions also display an anomalous expression of ERα, suggesting a correlative hypothesis that mutations in the TSC2 gene upregulates the expression of ERα. The mechanism for this upregulation is unknown but could be through upregulation of transcription of the ERα gene, upregulation of translation of the ERα mRNA, tissue-specific inhibition of ERα protein degradation or any combination of these. In this study we investigate the potential for upregulation of ERα expression through epigenetic changes at the ERα gene promoter that might facilitate upregulation of the expression of ERα mRNA in LAM. Unfortunately, the human ERα promoter is not a simple promoter to work with in that it has been shown to extend greater than 150 kb upstream of the 1st coding exon [45]. As many as 7 different ERα promoters have been identified and in several instances selective promoter usage has been correlated with carcinogenesis [46, 58]. In the LAM-associated cell lines examined in these studies it was observed that transcription expression of the ERα gene was actively being initiated from multiple ERα promoters. Although these studies were limited to looking at steady-state levels of ERα mRNA and therefore do not directly address transcription rates, our previously published studies demonstrating tuberin's ability to localize to the nucleus [40, 42] and tuberin's ability to directly impact transcription regulation [30, 41] would support a direct impact on ERα mRNA transcription as opposed to ERα mRNA degradation.

The most obvious mechanisms for altered transcription of the ERα gene in LAM would be through some alteration in cis-acting elements at the ERα promoter, alteration in *trans*-factor binding at the ERα promoter or a combination of both. Epigenetic changes at the human ERα gene promoter have been reported for a variety of cancers [53, 54]. In this study we looked at altered methylation of the CpG residues in the proximal (A) promoter of the ERα gene. A substantial amount of demethylation was observed to occur in both a LAM lesion-derived cell line and LAM tissue specimen. It was further observed that the alteration in methylation in the LAM cell line was accompanied by a reduced binding of the transcriptional repressors MeCpG2 and HDAC1 at the ERα promoter. These observations have been made for ERα [59] as well as other nuclear receptor gene promoters in which epigenetic changes have been linked to alterations in expression of those genes [60, 61], and would suggest that in LAM lesions there is a demethylation at the ERα gene promoter resulting in upregulation of transcription of the ERα mRNA. Further studies are required to conclusively establish whether this is directly caused by mutations in the TSC2 gene and disruption of tuberin/hamartin signaling, or a downstream consequence of tumor formation.

Although the above data support a potential role for epigenetic changes at the ERα promoter in the pathology of LAM disease, a variety of previously published data suggest the mechanism is not as simple as this. These include studies from our laboratory and others that demonstrate: a) tuberin can impact ERα non-genomic signaling through its ability to modulate PDGF signaling [39, 62], p90RSK/ERK signaling and RHEB/mTOR signaling [42, 63]; b) tuberin can inhibit ERα genomic signaling through its ability to directly bind ERα, directly bind ERα's coregulatory protein calmodulin and to block ERα DNA binding [41, 42]; c) a study that demonstrates estrogen can promote the survival and pulmonary metastasis of tuberin-null cells [64]; d) a study that demonstrates nongenomic estrogen activities can regulate tuberin stability [65]; and e) a study that provides evidence for upregulation of ERα protein expression in LAM lesions through a post-transcriptional-protein degradation mechanism [66]. Although some of these findings might appear to be somewhat contradictory, they most probably reflect the dedifferentiated state and complexities of LAM lesions. Whether any of these, or some alternative mechanism (e.g. regulation of ERα mRNA translation), is the driving force of (rather than a consequence of) LAM pathology will require further studies.

CONCLUSION

In summary, data in this manuscript, for the first time, identify changes in methylation at the ERα promoter associated with the pathogenesis of LAM. These epigenetic changes were further observed to correlate with the expression of ERα in LAM cells and tissues, and correlate with the binding of transcription regulators at the ERα promoter.

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