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Inducing Major Urinary Protein (MUP) expression in AML12 hepatocytes

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Abstract

Many social behaviors in mice, such as aggression, mating and territory marking, are mediated by the major urinary proteins (MUPs) present in their urine. While the mouse genome codes for 21 MUPs, any given male mouse only expresses a subset of these proteins at a defined concentration. Mice are able to detect the identity and concentration of the MUPs they encounter, and as such, these proteins appear to act as an "individuality signal." However, how a unique subset of MUPs is chosen for expression remains largely unknown. This study focuses on the control of gene expression of the 21 MUP genes, consisting of the highly similar "central" MUPs and the variable, divergent "peripheral" MUPs. In an effort to understand the mechanisms controlling MUP choice and expression, this study explored the role that testosterone, dihydrotestosterone, and growth hormone treatments play in mediating MUP expression in the model system of AML12 male hepatocytes. Utilizing RNA isolation, cDNA synthesis, and Polymerase Chain Reaction (PCR), it was found that treatment with these hormones was insufficient to induce MUP expression. Analysis of mouse growth hormone receptor (mGHR) expression in hormone treated cells indicate that mGHR is actively being expressed in the cell line. In addition, analysis of mouse androgen receptor (mAR) expression in the AML12 cell line indicate that mAR is not being actively expressed. Methylation inhibition treatments were also performed using 5-aza-2'-deoxycytidine (DAC). The DAC treatments suggest that solely inhibiting methylation is not sufficient to induce MUP expression. Because of their complex expression patterns, the MUP gene family serves as a good model system for the study of long standing molecular biology questions regarding mechanisms that control gene expression.

1. Introduction

Pheromones can be defined as chemical cues that are emitted and detected by members of the same species, and elicit social behaviors that play a key role in intra-species specific interactions[13]. While they are thought to be present in most terrestrial vertebrates, their functionality is still largely unknown[13]. Despite the many unanswered questions regarding the nature of pheromones, their ability to innately influence behavioral responses, such as aggression and female attraction, is apparent[3].

Mice are social animals that heavily rely on pheromones in the regulation of social behaviors. There are a variety of major urinary proteins (MUPs) present in mouse urine that act as genetically-encoded pheromones in species-specific interactions[8, 9]. MUPs are a group of lipocalin proteins that are synthesized in the liver and excreted in urine, allowing for these social interactions to take place[4, 5, 8, 9]. They are predominantly found in male urine, and these proteins play

a large role in important social behaviors such as mating, aggression, and territory marking[4, 5, 10, 2, 6].

Many mammalian species express MUPs, but the MUP gene family is specifically expanded in the mouse genome[8]. The mouse genome has at least 21 distinct MUP genes, and a subset of 4 to 12 of the MUP genes are expressed by an individual male mouse[11]. Which proteins are expressed and the relative ratio of expression remains stable throughout an adult male mouse's life[11]. These proteins are important because the identity and concentration of MUPs excreted by a male mouse is what allows him to define his particular scent as "self" – ultimately allowing the distinction between "self" and "non-self"[6].

Previous studies have shown that MUPs can be divided into two separate groups, the "central" MUPs and "peripheral" MUPs, which correspond to the locations of the MUP genes along chromosome 4[12]. It has been observed that the central MUPs are near-identical, their sequences differing by as little as one amino acid, and could likely be the primary basis of individual variation in expressed MUPs[8]. However, the peripheral MUPs have much more sequence variability than the central MUPs, which is likely because the MUPs in this region diverged earlier. In addition, peripheral MUPs have been identified as more specialized than the central MUPs[9]. These peripheral MUPs are of particular significance because of their individual ability to act like traditional pheromones; some peripheral MUPs have been shown to be able to independently trigger intra-species specific behaviors[9, 13, 2]. For instance, MUP20 is consistently chosen for expression, and has been found to have importance in mating by stimulating female memory of male scent, and it also plays a role in eliciting aggression[12, 2]. Because almost all males choose specific peripheral MUPs for expression, how they are being individually chosen as part of a MUP signature is of particular interest.

While it is known that these proteins play a large role in social behaviors, the mechanisms controlling MUP choice and expression remain to be fully understood. In addition, because of the apparent complexity of MUP expression patterns, MUPs serve as a good model system to study factors controlling gene expression. To date, studies involving MUP expression have exclusively been conducted *in vivo*. This study focuses on identifying ways in which MUP expression can be induced in a cell culture. A cell culture model system allows for advantages that are not so easily attained when working in live animals; a cell culture would allow for precise manipulation of the variables involved in MUP expression and a greater ease in studying the effects of these manipulations.

It has been shown in previous studies that hormones such as testosterone, growth hormone, and thyroxine play an important role in MUP expression, and the administration of these hormones *in vivo* can produce measurable changes in the expression as well[4, 5, 8, 7]. Some findings even indicate that the administration of a combination of hormones, such as growth hormone and thyroxine, is capable of increasing MUP mRNA levels up to 1000-fold[7]. On a cellular level, testosterone diffuses across the cell membrane into the cytoplasm where it can either bind to an androgen receptor or be reduced to 5α-dihydrotestosterone (DHT). It has been found that in human cells, DHT is capable of binding to an androgen receptor nearly five times more strongly than testosterone[1]. This indicates that DHT is a strong competitor and a promising option for hormone treatments in mouse cells due to the high genetic similarity between humans and mice.

This study begins to formulate a treatment plan using testosterone, DHT, and mouse growth hormone (mGH) that would allow for induction of MUP expression in the AML12 liver cell line. Current results indicate that the administration of these hormones is not sufficient to induce MUP expression in cell culture. These results were further analyzed by examining the presence of mouse growth hormone receptor (mGHR) and mouse androgen receptor (mAR) expression in hormone treated cells. Analysis of mGHR and mAR expression suggest that mGHR is being actively expressed in the cell line, but mAR is not being actively expressed. In order to determine if methylation has a role in inhibiting MUP expression in culture, 5-aza-2'-deoxycytidine (DAC) treatments were also performed. However, results from this treatment indicate that DAC treatment alone is not sufficient in turning on MUP expression and may require additional variables to produce a measurable change.

2. Methods

2.1. Cell Culture

The male hepatocyte AML12 cell line was obtained from ATCC. The cells were cultured in 1:1 F-12/DMEM supplemented with fetal bovine serum (FBS), dexamethasone, and ITS according to ATCC guidelines. Prior to hormone treatments, cells were cultured in 12-well plates ($2x10^5$ cells/well) with serum-free DMEM supplemented with charcoal-stripped FBS and L-Glutamine for 24 hours. Cells were incubated at 5% CO_2, 37°C, and 95% humidity.

2.2. Hormone Treatments

2.2.1. testosterone

Testosterone concentrations of 100, 500, and 750 nM were tested. An equivalent volume of the vehicle (acetonitrile) was used as a control for maximum and minimum testosterone concentrations. Treatment periods of 48 hours and 96 hours were used, and over the treatment period, existing media was removed from each well and replaced with new media and designated treatment every 24 hours.

2.2.2. DHT

Treatment of cells with DHT was performed at concentrations of 100, 250, and 750 nM. An equivalent concentration of the vehicle (MeOH) was used as a control for maximum and minimum DHT concentrations. Treatment periods of 48 hours and 96 hours were used, and over the treatment period, existing media was removed from each well and replaced with new media and designated treatment every 24 hours.

2.2.3. growth hormone

Treatment of the AML12 cells was also completed with mGH combined with varying concentrations of DHT. A constant mGH concentration of 100 μg/mL was maintained for each sample in combination with DHT concentrations of 1, 10, 100, 500, and 1000 nM. A treatment period of 96 hours was tested, and over the treatment period, existing media was removed from each well and replaced with new media and designated treatment every 24 hours.

2.3. RNA Isolation and cDNA Synthesis

Following the treatment period, the cells for each treatment were harvested by washing with PBS and performing trypsinization. RNA isolation was then performed using a QIAGEN RNeasy Mini Kit. RNA concentrations and purity were recorded using a NanoDrop. The isolated RNA for each sample was then used to synthesize a corresponding cDNA library by reverse transcription.

2.4. MUP Expression

PCR was performed in which the DNA from each sample was tested with primers designed to detect expression of central MUPs collectively (forward 5'-ATGAAGATGCTGCTGCTG-3'; reverse 5'-TCATTCTCGGGCCTGGAG-3') and peripheral MUPs collectively (forward 5'-ATGAAGCTGCTGCTGCCG-3'; reverse 5'-TCATTCTCGGGCCTCGAG-3'). A control reaction that tested for β-actin expression was also completed for each sample. Expression was visualized using gel electrophoresis.

2.5. mGHR and mAR Expression

Detection of mGHR expression in AML12 cells was performed using primers (forward 5'-GCAGCCATGGGAAGAGGAG-3'; reverse 5'-CTCCACGAATCCCGGTCAAA-3') designed to target the mGHR sequence in the synthesized cDNA. Primers (forward 5'TCCGGACCTTATGGGGACAT-3'; reverse 5'-ACTCCTGGCTCAATGGCTTC-3') were also designed for detection of mAR expression in the cells. The primers for both receptors were tested against C57 liver cDNA and against samples that had been treated with mGH. Expression was visualized using gel electrophoresis. To confirm that the designed primers were capable of detecting mAR, primers were tested against cDNA from the vomeronasal organ (VNO) and the C57 liver sample.

2.6. Methylation Inhibition

Treatment of cells using 5-aza-2'-deoxycytidine was performed at concentrations of 0.5, 1.0, 2.0, 5.0, and 10.0 μM. A treatment period of 72 hours was tested, and over the treatment period, existing media was removed from each well and replaced with new media and designated treatment every 24 hours.

3. Results

3.1. AML12 MUP Expression

Prior to beginning hormone treatments, an initial PCR amplification of the cDNA from cultured AML12 cells displayed β-actin expression but a lack of central or peripheral MUP expression.

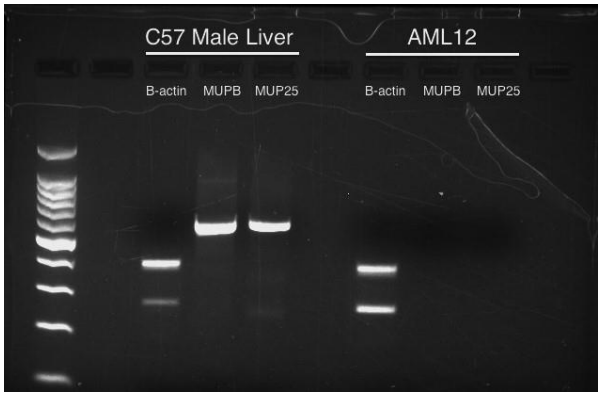

Figure 1. MUP Expression in AML12 cell line

Figure 1. The control sample, consisting of cDNA from the liver of an adult male C57 mouse, displays desired central (MUPB) and peripheral (MUP25) MUP bands near the 600bp mark. cDNA obtained from the AML12 cell line displays active expression of β-actin but does not display central or peripheral expression.

3.2. Hormone Treatments

3.2.1. testosterone

Treatment with testosterone at concentrations of 100 and 500 nM over a 48-hour treatment period did not induce MUP expression. Similarly, treatments at concentrations of 100, 500, and 750 nM over a 96-hour treatment did not result in MUP expression being induced.

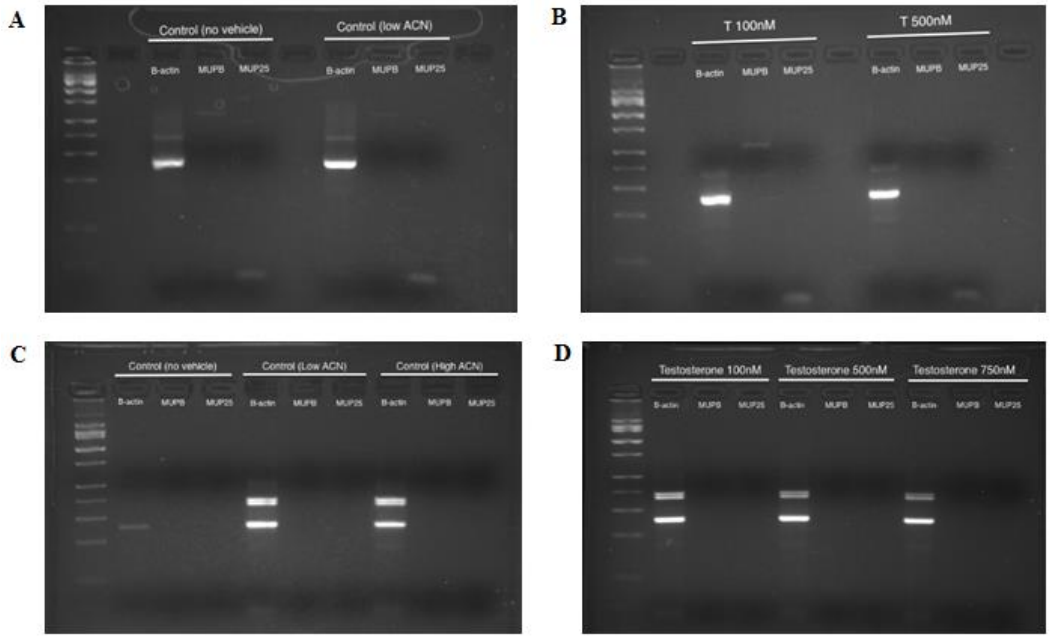

Figure 2. MUP expression following testosterone treatment

Figure 2. Testosterone treatments over a 48-hour treatment period display only β-actin expression in cells (*A*, *B*). The 96-hour treatment period produced identical results, with only β-actin expression detected (*C*, *D*). At all concentrations and in both treatment periods, there was an absence of both central and peripheral expression.

3.2.2. DHT

Treatment with DHT at concentrations of 100 and 500 nM over a 48-hour treatment period did not induce MUP expression. Following treatments at concentrations of 100, 500, and 750 nM over a 96-hour treatment did not result in MUP expression being induced.

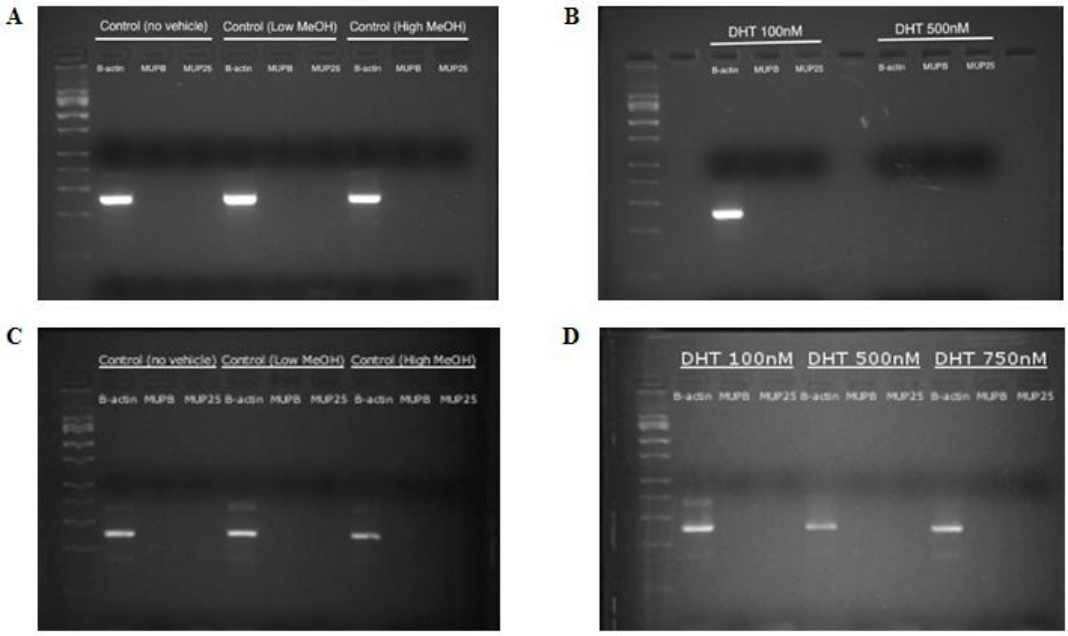

Figure 3. MUP expression following DHT treatment

Figure 3. DHT treatments over a 48-hour treatment period display only β-actin expression in cells, excluding the 500 nM treatment (*A, B*). The 96-hour treatment period produced identical results, with only β-actin expression detected (*C, D*). At all concentrations and in both treatment periods, there was an absence of both central and peripheral expression.

3.2.3. growth hormone

Treatment with growth hormone and at concentrations of 1, 10, 100, 500, and 1000 nM DHT over a 96-hour treatment period was not sufficient in inducing MUP expression.

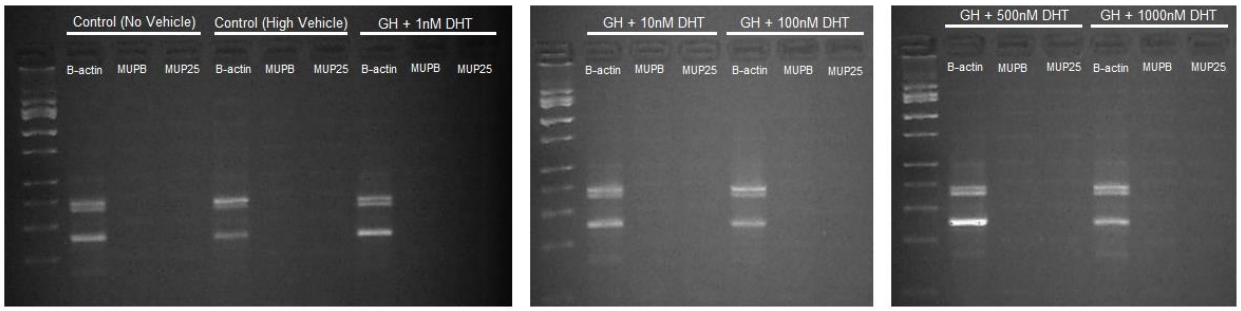

Figure 4. MUP expression following growth hormone and DHT treatment

Figure 4. Growth hormone and DHT treatments over a 96-hour treatment period display only control β-actin amplification in cells. At all concentrations, there was an absence of both central and peripheral MUP expression.

3.3. mGHR and mAR Expression

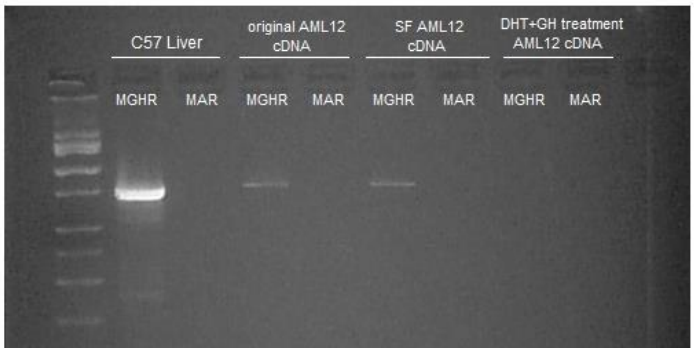

Figure 5. mGHR and mAR expression in various AML12 samples

Figure 5. cDNA taken from the liver of a C57 mouse was used as a control to test for mGHR and mAR expression. Different AML12 cDNA samples were also tested with mGHR and mAR primers. Original AML12 cDNA from cells cultured in 1:1 F-12/DMEM displayed mGHR expression only. AML12 cDNA from cells cultured in the supplemented serum-free DMEM also displayed only mGHR expression. The AML12 cDNA from cells treated with growth hormone and 500nM DHT did not display expression of either mGHR or mAR.

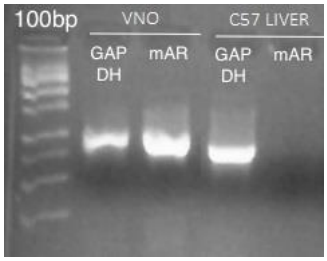

Figure 6. mAR expression in VNO and C57 Liver samples

Figure 6. The cDNA obtained from the VNO tissue displayed expression of the GAPDH control as well as mAR. As seen before, the cDNA from the C57 liver did not display active mAR expression, and only expression of the GAPDH control was observed.

3.4. DAC Treatment

Treatment of cells with DAC at concentrations of 0.5, 1.0, 2.0, 5.0, and 10.0 μM over a 72-hour treatment period did not result in MUP expression being induced. The DAC treatment also did not appear to have a noticeable impact on the expression of mGHR or mAR in cells.

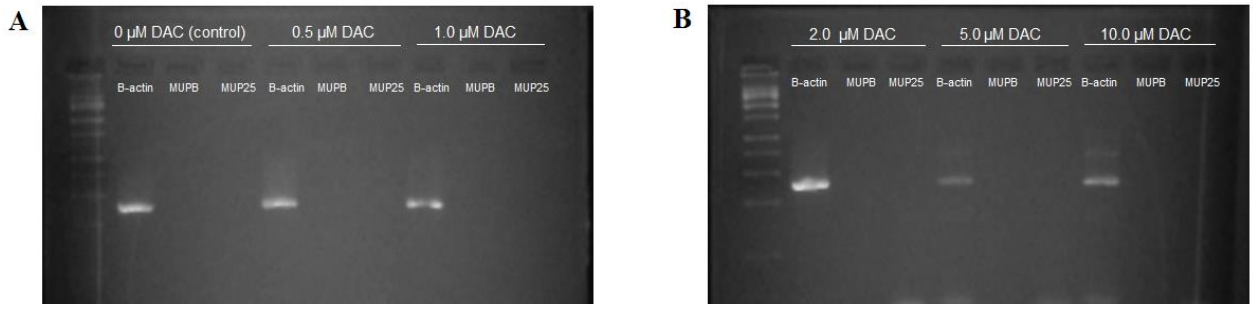

Figure 7. MUP expression following treatment with DAC

Figure 7. Treatment with DAC over a 72-hour treatment period produced β-actin expression in all treatment samples. There was no expression of central or peripheral MUPs detected at any concentration of DAC.

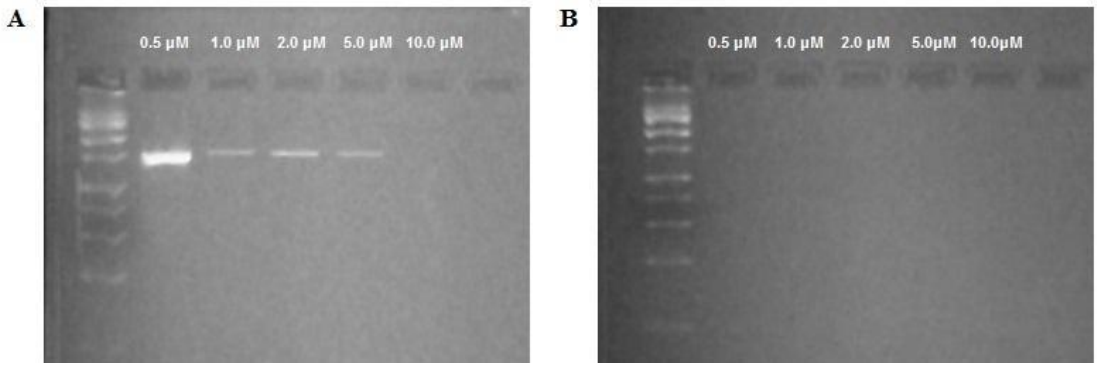

Figure 8. mGHR and mAR expression in DAC treated cells

Figure 8. Expression of mGHR was found in all concentrations of DAC treatment except for in the 10.0 μM sample (*A*). Expression of mAR was not present in any of the treated samples (*B*).

4. Discussion

The results of each hormone treatment suggest that, at the chosen concentrations and treatment periods, the administering of testosterone, DHT, or growth hormone in culture is not sufficient to induce MUP expression. While it has been shown that testosterone and growth hormone can elicit changes in MUP expression in live mice, there are many variables that are different or absent in cell culture that have an apparent effect on the expression of MUPs. Despite the ease of seeing results from *in vivo* treatments, developing a protocol that can produce similar results *in vitro* will provide a greater understanding of the complexity of MUP expression and a way to manipulate the involved signaling pathways more easily.

In all of the treated AML12 samples analyzed, expression of mAR was not detected. The absence of mAR expression in the AML12 cells suggests that another variable may be influencing MUP expression aside from the sole presence of the testosterone or DHT. The absence of mAR could also suggest that the hormones administered did not have a receptor to bind to, which likely would have prevented or hindered the typical cellular processes of testosterone and DHT. In contrast, mGHR appears to be actively expressed in the AML12 cell line. This indicates that the use of growth hormone in treatments has an increased chance of eliciting a cellular response within the cells and will play an integral role in future treatments.

In an effort to determine if DNA methylation was inhibiting the induction of MUP expression in this cell line, treatment with DAC, a methylation inhibitor, was performed. The results of this treatment provided similar results to those of the previous hormone treatments, with no expression of either central or peripheral MUPs and only β-actin being actively expressed. In addition to this, treatment with DAC also did not appear to have any effect on the expression of mGHR or mAR. These results suggest that DAC alone is not sufficient to induce MUP expression and may require the presence of other variables in order to produce a change in the current lack of expression.

Further studies would explore the use of DAC treatments in combination with other variables, such as histone deacetylase (HDAC) inhibitors and hormones. For example, a treatment utilizing DAC and Trichostatin A (TSA), an HDAC inhibitor, would allow for DNA to be more accessible to transcription factors, increasing gene expression in the cells. Another option would be utilizing both DAC and TSA in combination with a hormone. Should either of the inhibitors increase gene expression, there may be an increased ability for testosterone, DHT, or growth hormone to have an impact on influencing MUP expression.

Future plans for this project would also involve experimenting with combinatorial treatments, using several hormones at the same time in an attempt to mimic compositions found *in vivo*. As studies have suggested, combinations of certain hormones, such as growth hormone and thyroxine, have the ability to greatly increase the levels of MUP expression in mice[7]. Since mGHR is actively being expressed by the AML12 cells, providing growth hormone a receptor to bind to, there is a greater chance that utilizing a treatment with growth hormone could produce induction of MUP expression. Therefore, combining growth hormone in an experiment with thyroxine may have the capability to elicit expression of MUPs in culture.

Although a working protocol to induce MUP expression in cell culture has yet to be established, the findings of this study provide the groundwork necessary to build a model system. These data highlight the challenge of producing a working treatment system and speak to the complexity of MUP expression, reinforcing the validity of using the MUP gene family as a model system to study mechanisms controlling gene expression.

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