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University of Nevada, Reno

**Activation of the Akt/mTOR Pathway and the Effects of Increased Myometrial mTOR signaling During Pregnancy**

Senior Thesis in the Biochemistry and Molecular Biology Major

By

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Tamara Valentine, Ph.D, Director, **Honors Program**

## Abstract

Preterm labor is defined as labor before 37 weeks of gestation and it is one of the leading causes for prenatal fatality and morbidity (Hirota et al 2011). Due to babies being delivered prior to full development, preterm labor can cause many problems and can also endanger the mother. Prior research has shown that the P13K/Akt/mTOR signaling pathway is related to birth timing in mouse models (Hirota et al 2011). It has also been shown that rapamycin, an inhibitor of mTOR, can rescue preterm labor in these mouse models (Hirota et al 2011). In this study, western blots were used on human myometrial samples to determine if total mTOR protein is increased in preterm and term laboring vs. preterm and term non-laboring myometrial tissue to determine if abnormal activation of the Akt/mTOR pathway might be related to the onset of preterm labor.

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## **Introduction**

Preterm labor is a major health concern and can endanger the mother and the child. (Lockwood and Kuczynski, 2001). Preterm birth can occur after the spontaneous onset of labor and can be caused by multiple pathologic processes, such as infection, vascular disorders, decidual senescence, uterine overdistention, decline in progesterone action, cervical disease, breakdown of maternal-fetal tolerance, stress and other unknown factors (Romero et al 2014). In a prior study by Hirota et al (2011), mTOR signaling activity was increased in uterine endometrial cells in mouse models of preterm birth and the mTOR inhibitor, rapamycin, rescued the preterm phenotype in this model (Hirota et al, 2011). The Akt/mTOR pathway is a prototypic survival pathway that is activated in many types of cancers and could be related to the timing of birth (LoPiccolo et al 2007). Akt is a serine/threonine protein kinase, which is expressed in most tissues while mTOR is the mammalian target of rapamycin, which is a master regulator of protein translation (LoPiccolo et al 2007). Akt is the upstream positive regulator of mTOR (Hahn-Windgassen et al 2005) and regulates mTOR by a number of growth factors. To determine if activation of Akt/mTOR signaling in the myometrium might contribute to preterm birth, experiments were performed to determine if increased myometrial mTOR phosphorylation or protein expression was present in patient myometrial samples (non-laboring vs. preterm labor) as well as increased Akt phosphorylation, another indicator of mTOR pathway activation.

## **Methods**

### **Western Blots of Samples with mTOR and phospho-mTOR**

Human uterine biopsies were obtained from women undergoing Caesarian section with informed consent and IRB approval. Myometrial protein was extracted in MAPK (mitogen-

activated protein kinase) buffer. Two mL of MAPK buffer was used for each sample and used on a dissociator with the setting: RNA (ribonucleic acid) 2. Samples were then centrifuged for 10 minutes and the supernatant was collected. Protein concentration was determined by EZQ Assay (protein quantification kit) and samples were quantified in triplicate. Samples (TNL=term non laboring, TL=term laboring, 01,2=samples from a telomerized/immortalized pregnant human myometrium cell line) were put on ice and diluted 3:1 with 4X SDS (Sodium dodecyl sulfate) PAGE (polyacrylamide gel electrophoresis) sample buffer with 25% 2-mercaptoethanol and double distilled water. The samples were boiled for 5 minutes and then put on ice. Electrophoresis buffer was prepared using 100mL of 10X electrophoresis buffer (40mM Tris, 20mM acetic acid, 1mM EDTA[edetic acid]) and 900mL of cold double distilled water. Two 4-20% precast acrylamide gels were obtained (Biorad). A western blot apparatus was set up and samples were loaded (10-40ug of protein per lane were loaded). The gels were run at 200V for 1 hour. The gels were transferred to a nitrocellulose membrane using the Bio-Rad Turbo Transfer Blot (The high MW setting was used). The membranes were blocked in TBS (tris buffered saline)+ 5% BSA (bovine albumin serum) at room temperature for 1 hour. The primary antibody (mTOR 7C10 Rabbit mAB from Cell Signaling Technology) was prepared (mTOR/phospho-mTOR 1:1000 in TBS-T +5%BSA). Ten mL antibody solution was prepared for each membrane. Membranes were incubated with the primary antibody overnight at 4°C. After incubation, primary antibody was recovered and membranes were washed with TBS-T (Tris buffered saline with 0.1% Tween) three times for 5 minutes at room temperature. The secondary antibody (Alexa Fluor 680 Goat Anti-Rabbit IgG from Life Technologies) (Anti-rabbit Alexa fluor 680 1:20000 in TBS-T + 5% BSA) was prepared.

Ten ml antibody was prepared for each of the membranes. The membranes were incubated with the secondary antibody for 1 hour at room temperature. The secondary antibody was recovered and the membrane was washed three times with TBS-T + 5% BSA for 5 minutes at room temperature. The membrane was washed once with TBS for 5 minutes at room temperature. The membrane was then scanned using the Odyssey Licor machine. Primary antibody for GAPDH (glyceraldehyde 3-phosphate dehydrogenase) from Cell Signaling Technology (1:1000 in TBS-T + 5% BSA) was prepared. GAPDH was used to normalize the data. Membranes were incubated with GAPDH for 1 hour at room temperature. The antibody was recovered and the membrane was washed three times with TBS-T for 5 minutes at room temperature. The secondary antibody was prepared (Anti-mouse IRDye 800 1:20000 in TBS-T + 5% BSA) and the membrane was incubated with the secondary for 1 hour at room temperature. The antibody was recovered and the membrane was washed three times with TBS-T for 5 minutes at room temperature and washed once with TBS for 5 minutes at room temperature. The membrane was scanned using the Odyssey Licor machine.

*Western Blots of Samples with Total Akt (SKB1) and phospho-Akt (Ser 473)*

Protein was isolated from human uterine myometrial samples and quantified using EZQ Assay protocol as described above. Nine samples were put on ice (variations of TNL, TL, PTNL, PTL, and NP; TNL=terminal non-labor, TL=term labor, PTNL=preterm non-labor, PTL=preterm term labor, NP=non-pregnant) and prepared in duplicate. Samples were prepared with 4X SDS (sodium dodecyl sulfate) with 25% 2-mercaptoethanol. Samples were prepared with a total volume of 36uL and protein concentration of 44ug and samples were treated with the 4X SDS with 25% 2-mercaptoethanol and double distilled water. The

samples were then boiled for 5 minutes. Samples were put on ice immediately after boiling. Electrophoresis buffer was prepared (100mL of 10X Electrophoresis buffer and 900mL of cold double distilled water). 4-20% acrylamide gel was obtained and prepared with the electrophoresis apparatus (cassette). Electrophoresis apparatus was set up and 30uL and 40ug of protein was loaded. The gel was run at 200V for 1 hr (or until blue dye passed black line of gel). Gel was transferred to nitrocellulose membrane using BioRad Turbo Transfer Blot (Mixed MW setting was used). Blocking membrane was prepared (Total Akt=PBS (phosphate buffered saline) and 2% milk, 10mL) (Phospho-Akt=TBS (tris buffered saline) and 2% milk, 10mL). The membranes were blocked for 1 hr. Primary antibody was prepared (Total Akt: Total Akt (SKB1 clone) from Millipore in PBS-T (phosphate buffered saline and 0.1% Tween) and 2% milk 1:500, 10mL) (Phospho-Akt: Phospho-Akt (Ser 473 clone) from Millipore in TBS-T (tris buffered saline and 0.1% Tween) and 2% milk 1:250, 10mL). Membranes were incubated with respective primary antibody overnight in -20C room. Secondary antibody was prepared (Anti-mouse IRDye 800 1:20000 in TBS-T + 2% milk, 10mL) (Anti-mouse IRDye 800 1:20000 in PBS-T + 2% milk, 10mL). Primary antibody was recovered and membranes were washed with either PBS-T (Total Akt) or TBS-T (Phospho Akt) three times for 5 minutes at room temperature. Membranes were incubated with secondary antibody for 1 hr. Secondary antibody was recovered and membranes were washed with either PBS-T (Total Akt) or TBS-T (Phospho-Akt) three times for 5 minutes at room temperature. Membranes were washed with PBS (Total Akt) or TBS (Phospho-Akt) once for 5 minutes at room temperature. Membranes were scanned using Odyssey Licor. Primary antibody, GAPDH from Cell Signaling, was prepared (GAPDH in TBS-T and 2% milk 1:1000, 10mL) (GAPDH in PBS-T and 2% milk

1:1000, 10mL). Membranes were incubated with GAPDH for 1 hr at room temperature. Secondary antibody was prepared (Anti-mouse IRDye 800 1:20000 in TBS-T + 2% milk, 10mL) (Anti-mouse IRDye 800 1:20000 in PBS-T + 2% milk, 10mL). The membranes were washed three times with either TBS-T or PBS-T for 5 minutes at room temperature. Membranes were incubated with secondary antibody for 1 hr at room temperature. The membranes were washed three times with either TBS-T or PBS-T for 5 minutes at room temperature. Membranes were washed once with TBS or PBS for 5 minutes at room temperature. Odyssey Licor software was used to quantify band intensities. Data was normalized by taking Akt/GAPDH, phospho-Akt/GAPDH, and normalized phospho/normalized total Akt (**Figures 11, 12, 13**). Statistical analyses were performed using ANOVA to compare TNL, TL, PTNL, PTL with NP.

## **Results**

### *Western Blots of Samples with mTOR and phospho-mTOR*

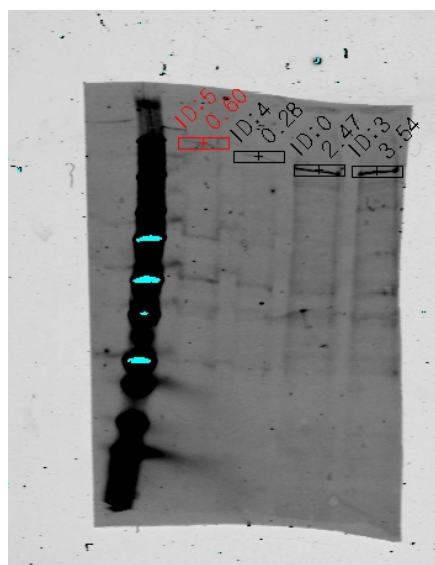
Western blots were done to observe if total mTOR expression was increased in human myometrial samples. Early western blots of samples (TNL, TL and HTRT cells only) detected faint bands at the expected size for mTOR and phospho-mTOR with 20ug protein per lane (**Figure 1**). Western blots with 40ug protein were more successful (**Figure 2**). However, the correct band was undetectable in subsequent western blots and I was unable to obtain quantifiable data with these antibodies.

### *Western Blots of Samples with Total Akt (SKB1) and phospho-Akt (Ser 473)*

To determine if Akt/mTOR pathway activation was increased in human myometrial samples, western blots were performed. Initial western blots were performed to optimize the antibody specificity. For Total Akt (SKB1 clone), PBS with 2% milk yielded the best

results. For phospho-Akt (Ser 473), TBS with 2% milk, the best results were obtained. The predicted 60 kD bands were observed when using Total Akt (**Figure 3 and 5**) and phospho-Akt (**Figure 7 and 9**). With GAPDH, the expected 37 kD bands were observed (**Figure 4 and 6, Figure 8 and 10**). For phospho Akt, the P value was 0.1996. For Total Akt, the P value was 0.0796.

250kd-  
150kd-  
100kd-  
75kd-  
50kd-  
37kd-  
25kd-  
20kd-  
  
15kd-  
10kd-



**Figure 1. Western Blot of mTOR with 10ug/20ug**

Figure displays a western blot of mTOR. Bands at 297kd is observed. 4-20% acrylamide gel.

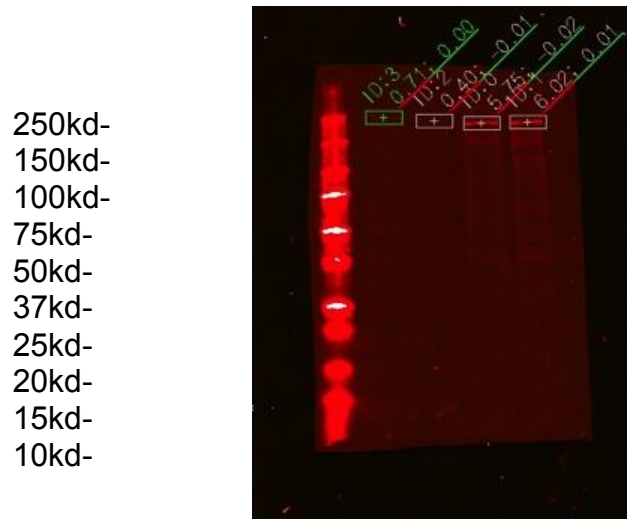
Lane 1: MW

Lane 2: TNL 551 (10ug)

Lane 3: TNL 534 (10ug)

Lane 4: 01 (20ug)

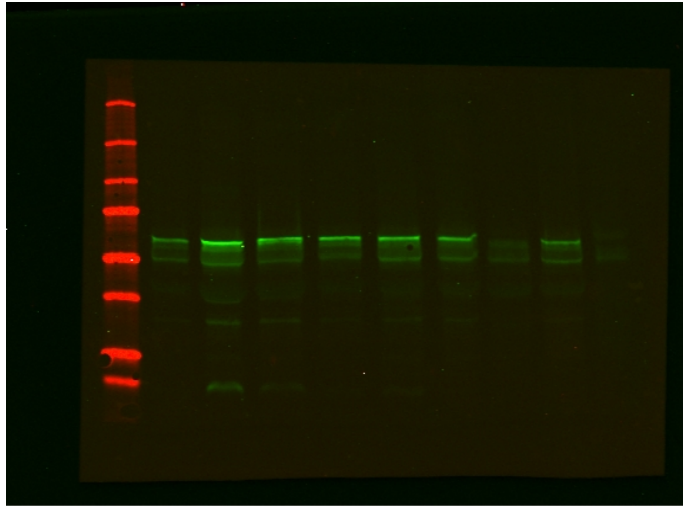
Lane 5: 02 (20ug)



**Figure 2. Western Blot of Phospho-mTOR with 10ug/20ug**

Lane 1: MW  
Lane 2: TNL 551 (10ug)  
Lane 3: TNL 534 (10ug)  
Lane 4: 01 (20ug)  
Lane 5: 02 (20ug)

250kd-  
150kd-  
100kd-  
75kd-  
50kd-  
37kd-  
25kd-  
20kd-  
15kd-  
10kd-



### Figure 3. Western Blot of Total Akt

The figure displays a western blot of Total Akt with 9 samples. Band at 60 kD on a 4-20% acrylamide gel.

Lane 1: MW

Lane 4: TL 571

Lane 7: PTL 303

Lane 10: NP 454

Lane 2: TNL 539

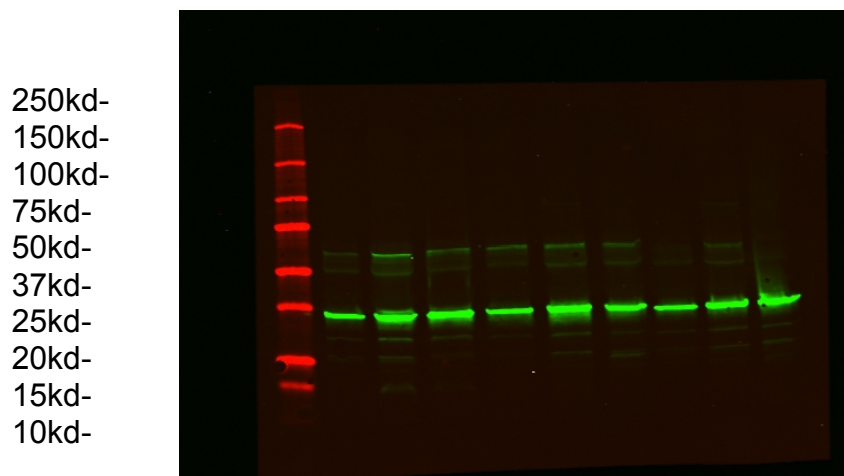
Lane 5: TL 301

Lane 8: PTL 369

Lane 3: TNL 540

Lane 6: PTNL 107

Lane 9: NP 452



**Figure 4. Western Blot of Total Akt & GAPDH**

The figure displays a western blot with Total Akt and GAPDH on 4-20% acrylamide gel. A band at 37 kD can be observed.

Lane 1: MW

Lane 2: TNL 539

Lane 3: TNL 540

Lane 4: TL 571

Lane 5: TL 301

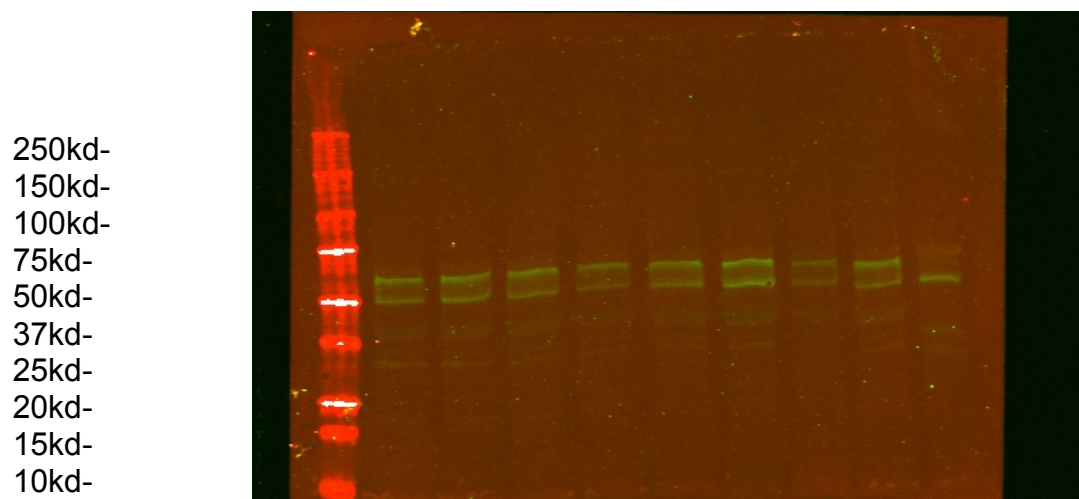
Lane 6: PTNL 107

Lane 7: PTL 303

Lane 8: PTL 369

Lane 9: NP 452

Lane 10: NP 454



**Figure 5. Western Blot of Total Akt**

Figure displays western blot of 9 samples with Total Akt on 4-20% acrylamide gel. A band at 60kD is observed.

Lane 1: MW

Lane 2: TNL 542

Lane 3: TNL 545

Lane 4: TL 315

Lane 5: TL 322

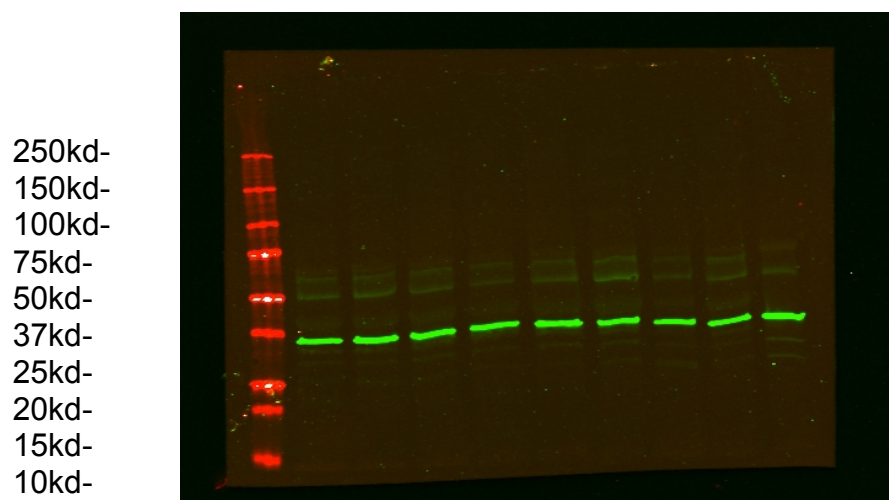
Lane 6: PTNL 343

Lane 7: PTNL 345

Lane 8: PTL 415

Lane 9: NP 526

Lane 10: NP 530



**Figure 6. Western Blot of Total Akt & GAPDH.**

Figure displays western blot image on 4-20% acrylamide gel with Total Akt & GAPDH. Band at 37kD can be observed. Lane 8 is blank due to not having enough of PTL 415 to load onto gel.

Lane 1: MW

Lane 2: TNL 542

Lane 3: TNL 545

Lane 4: TL 315

Lane 5: TL 322

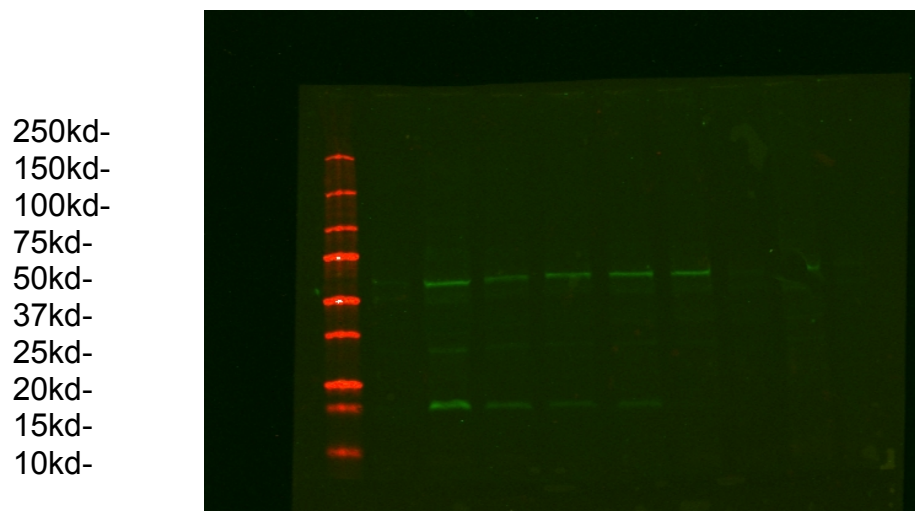
Lane 6: PTNL 343

Lane 7: PTNL 345

Lane 8: PTL 415

Lane 9: NP 526

Lane 10: NP 530



### Figure 7. Western Blot of Phospho-Akt

Figure displays a western blot on a 4-20% acrylamide gel with 9 samples. A 60kD band can be observed.

Lane 1: MW

Lane 2:TNL 542

Lane 3: TNL 545

Lane 4:TL 315

Lane 5: TL 322

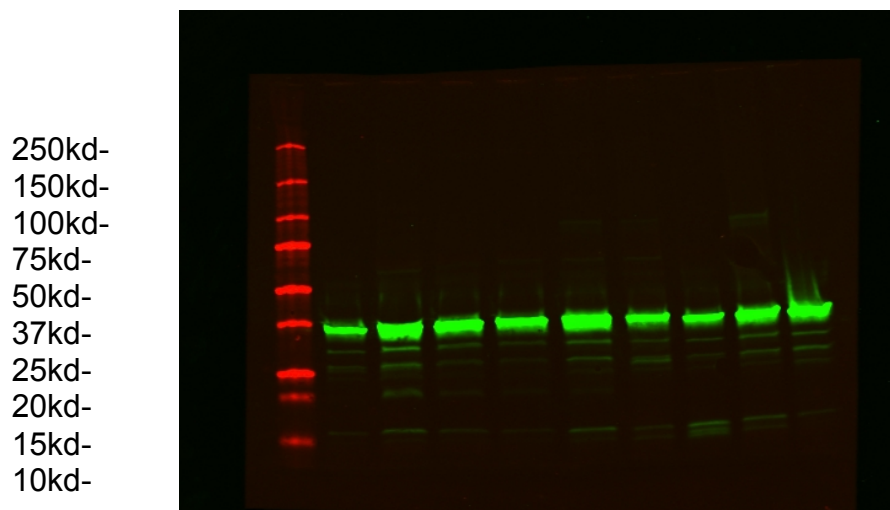
Lane 6: PTNL 343

Lane 7: PTNL 345

Lane 8: PTL 415

Lane 9: NP 526

Lane 10: NP 530



**Figure 8: Western Blot of Phospho-Akt & GAPDH**

Figure displays a western blot on a 4-20% acrylamide gel with 9 samples. A 37kD can be observed.

Lane 1: MW

Lane 3: TNL 545

Lane 5: TL 322

Lane 7: PTNL 345

Lane 9: NP 526

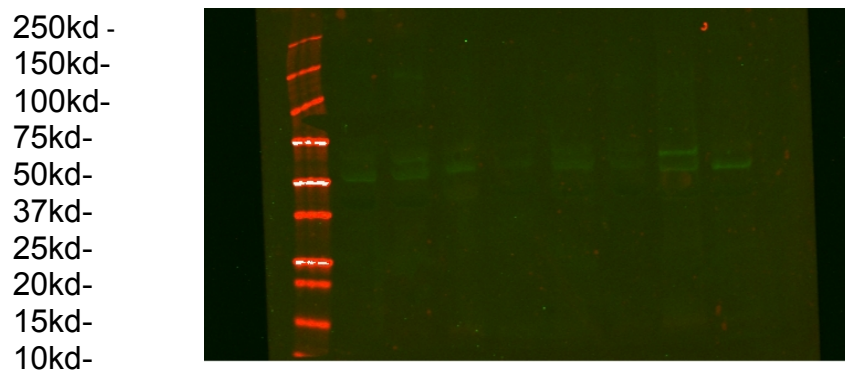
Lane 2:TNL 542

Lane 4:TL 315

Lane 6: PTNL 343

Lane 8: PTL 415

Lane 10: NP 530



**Figure 9: Western Blot of Phospho-Akt**

Figure displays a western blot on a 4-20% acrylamide gel with 8 samples. A 60 kD band can be observed.

Lane 1: MW

Lane 3: TNL 552

Lane 5: TL 544

Lane 7: PTNL 123

Lane 9: PTL 522

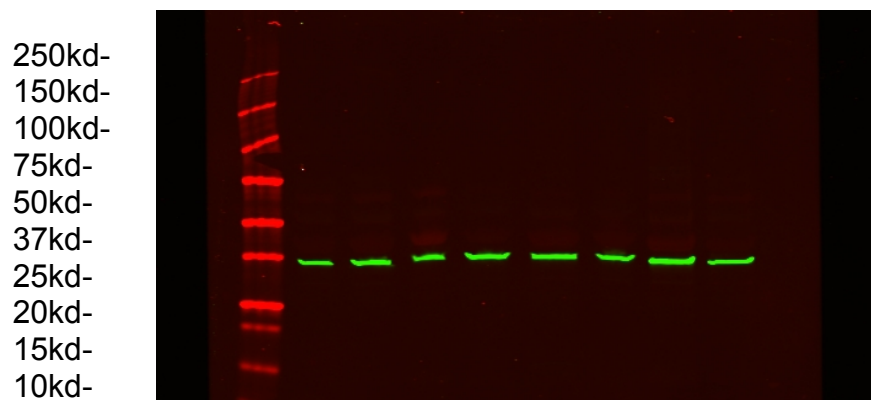
Lane 2:TNL 551

Lane 4:TL 541

Lane 6: PTNL 121

Lane 8: PTL 118

Lane 10: N/A



**Figure 10: Western Blot of Phospho-Akt & GAPDH**

Figure displays a western blot on a 4-20% acrylamide gel with 8 samples. A 37kd band can be observed.

Lane 1: MW

Lane 2:TNL 551

Lane 3: TNL 552

Lane 4:TL 541

Lane 5: TL 544

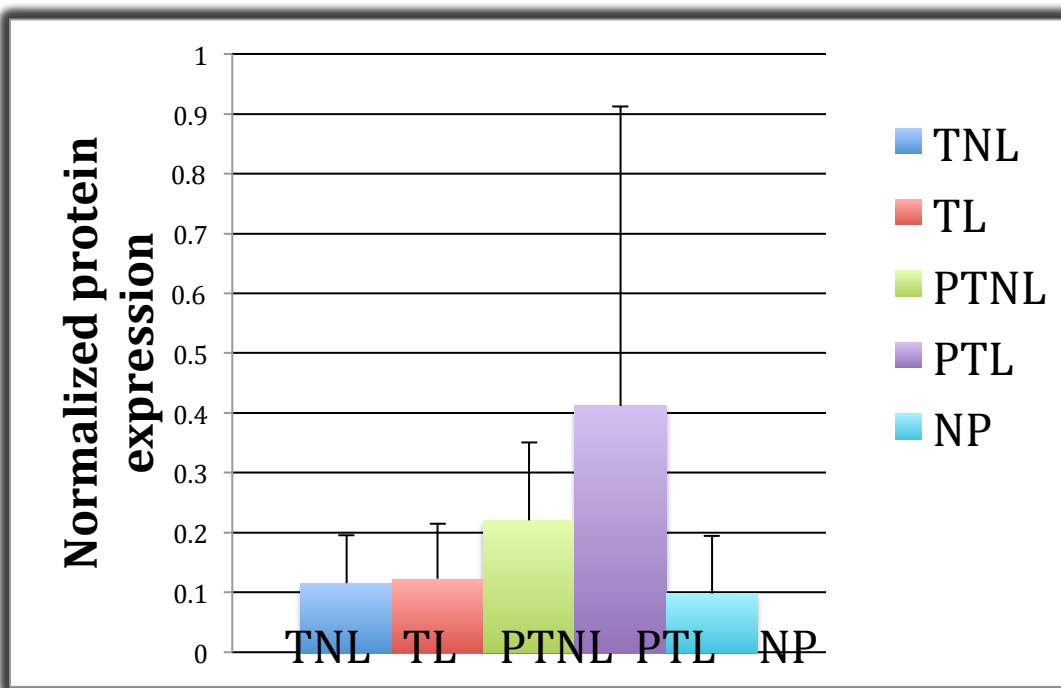
Lane 6: PTNL 121

Lane 7: PTNL 123

Lane 8: PTL 118

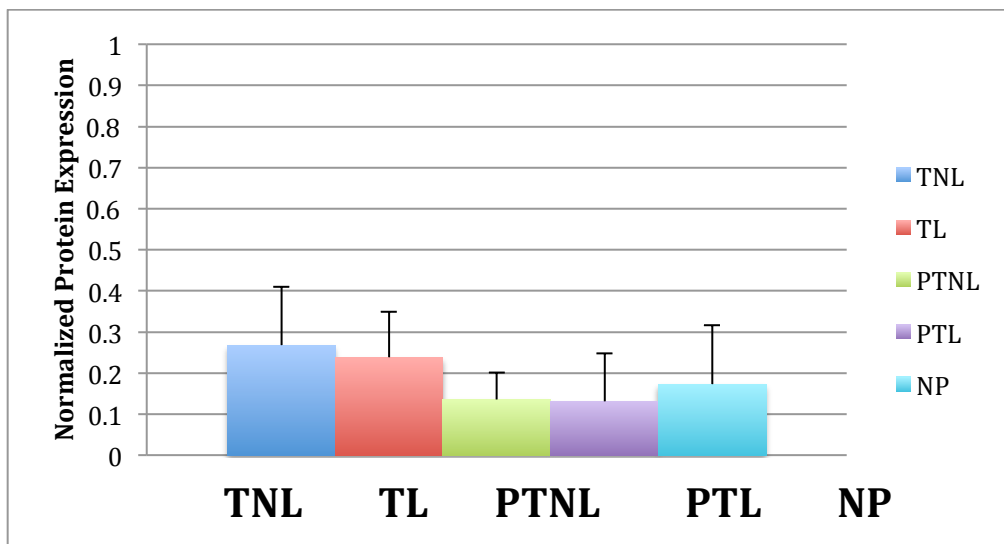
Lane 9: PTL 522

Lane 10: N/A



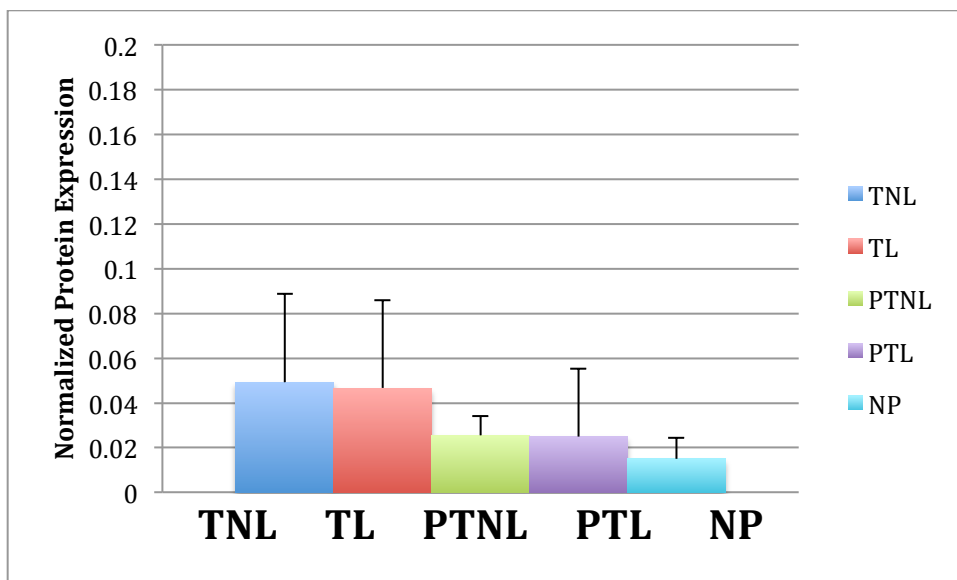
**Figure 11: Phospho-Akt:Total Akt**

Figure displays quantification of phospho-Akt in human myometrial samples. There were no statistical differences between the groups. Mean  $\pm$  standard deviation with n=8-10 per group.



**Figure 12. Total Akt Protein Expression**

Figure displays quantification of Total Akt in human myometrial samples. There were no statistical differences between the groups. Data represents mean  $\pm$  standard deviation, n=7-10 per group.



**Figure 13. Phospho-Akt Protein Expression**

Figure displays quantification of Phospho-Akt in human myometrial samples. There were no statistical differences between the groups. Data represents mean  $\pm$  standard deviation, n=5-10 per group.

## Discussion

The goals of these experiments were to determine whether mTOR expression and/or Akt/mTOR pathway activation is increased in preterm and term laboring myometria versus non-laboring myometria. Initial experiments were performed to detect mTOR and phospho-mTOR in human myometrial tissue. Faint bands were observed in the western blots of mTOR and phospho-mTOR (**Figures 1 and 2**). This could be due to low expression of mTOR in the samples and these results could not be quantitated. Next, human uterine myometrial protein extracts were analyzed for Akt phosphorylation, an indicator of Akt/mTOR pathway activation. Data was normalized taking densitometric values from total Akt/GAPDH (**Figure 3,4,5 and 6**) and phospho-Akt/GAPDH (**Figures 7,8,9 and 10**), followed by normalized total Akt/normalized phospho-Akt (**Figure 11**). These values were input to ANOVA and though they passed the normality test, the P values were greater than 0.05 indicating no significance differences between groups. I observed a large range of expression and activation levels, which could be due to the patient samples having a lot of variation in ethnicity, age, weight, contractile state, stage of labor or pregnancy, gestational parity, or other factors.

Future experiments could be performed to determine how uterine distention or other factors could affect the Akt/mTOR pathway and affect birth timing. If future experiments do find abnormal upregulation of the myometrial Akt/mTOR pathway in some cases of preterm labor, one might be able to identify the specific effectors as potential biomarkers or targets to treat preterm labor. Being able to compare total mTOR expression and Akt/mTOR activation leads to better understanding of the PI3K/Akt/mTOR pathway in pregnant human myometrium.

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