6-16-2021

A Novel in vitro Method for Pelvic Floor Dysfunction in PCOS Women

Ruben Vila
Portland State University

Spring 6-16-2021

A Novel in vitro Method for Pelvic Floor Dysfunction in PCOS Women

Ruben Vila

A Novel in vitro Method for Pelvic Floor Dysfunction in PCOS Women

By
Ruben Vila

An undergraduate honors thesis submitted in partial fulfillment of the requirements for the degree of
Bachelor of Science

In

University Honors

And

Biology

Thesis Advisor
Ov Slayden

Portland State University
2021

Abstract:

Background:

Poly Cystic Ovary Syndrome and Pelvic Floor Dysfunction are reproductive diseases with comorbidities whose novel relationship deserves further investigation.

Methods:

Levator Ani Muscle/Paravaginal Attachment was dissected from female Rhesus Macaque and subjected to tissue dissociation. Cells were treated with serum level hormones E2, T, and DHT. PCR, ICC, and IHC were used, followed by statistical analysis.

Results:

RNA analysis show differences in relative expression for endpoints ER, PR, AR, Ki-67, Col1A, and Vimentin. IHC along with ICC showed expression and localization *in vivo.*

Conclusions:

A novel *in vitro* model was created from macaque Levator Ani Muscle/Paravaginal Attachment for Pelvic Floor Dysfunction in Poly Cystic Ovary Syndrome women. Treatment of cells with androgens revealed a potential therapeutic target and possible etiology of PFD in PCOS women.

Introduction:

Poly Cystic Ovary Syndrome (PCOS) is clinically diagnosed using the Rotterdam criteria with the presence of hyperandrogenemia, poly cystic ovary/ovaries, and anovulation (1). Hyperandrogenism is defined as elevated serum levels of free testosterone (T) leading to overexpression of androgen receptor (AR) (2-4). These elevated levels of T have widespread effects such as deepening of voice, hirsutism, and acne (5). Androgens have long been known to be anabolic drivers of muscle growth and strength (6). It stands to reason that overexpression of androgen receptor would cause strengthening of muscles around the body such as those in the pelvic floor. Yet, the incidence rate for women with PCOS having Pelvic Floor Dysfunction (PFD) symptoms, does not align with this reasoning (1, 7). Even with increased levels of T, estrogen (E2) levels are usually found to be within normal ranges, with some cases having increased levels of E2 (8). The increased levels are mostly commonly attributed to the presence of hyperandrogenemia, due to the aromatization of T (9). The pelvic floor has been shown to be responsive to steroid hormones (10). The effects of Estrogen Receptor (ER) have been the subject of scrutiny, with the discourse community divided between if it upregulates collagen content or has no significant affect (10-15). Much of the debate has limited the role of AR in the regulation of collagen in the pelvic floor. The weakening of the muscles and ligaments that constitute the pelvic floor are crucial in development of Pelvic Floor Dysfunction (PFD) (16).

PFD and Pelvic Organ Prolapse (POP) is caused when the pelvic floor muscles and ligaments are weakened(17-19). This weakening is not attributed to atrophy, and although menopause is a risk factor, it does not account for all cases of PFD (14, 19-26). The role of the Levator Ani Muscle (LAM) and Paravaginal Attachment (PA) are of most relevance (27-29). These tissues have been attributed to having the most important role in pelvic floor support and, make up most of the pelvic floor(27, 28). They are also responsive to steroid hormones (10). Studies have been limited in the manner by which they can study this novel relationship between PFD and PCOS. They have been required to use imaging techniques or been confined to small biopsy samples of tissues that are not vital to pelvic floor support, such as the vaginal wall (14, 18, 27-32). This is why establishing a relevant *in vitro* model is a necessary advancement for the field. Stromal cells are the best target cell because of their role in maintenance of pelvic floor support, they express receptors for the various steroid hormones, and their role in tissue remodeling during wound healing (31-38). To our knowledge, fibroblasts from human pelvic floor tissue have never been isolated from these tissues because of the difficulty associated with dissecting a large enough tissue sample from the LAM/PA. These studies were carried out at the Oregon National Primate Research Center (ONPRC). Dr. Cecily Bishop has characterized a macaque model of PCOS that allows access to these unique tissues (3). The rhesus macaque provide the best animal model for PFD (39), because like women they have mostly upright posture, they give birth to an infant with a relatively large head resulting in damage to the pelvic floor, and naturally display PFD with age.

The aim was to characterize expression and localization of these steroid receptors in these LAM and PA stromal cells. It included observing the effects of androgens on collagen production and characterization of collagen deposition. In order to do this, this novel *in vitro* method was first developed.

Materials and Methods:

Rhesus Macaque Samples:

A study done through the National Centers for Translational Research in Reproduction and Infertility (NCTRI), subjected juvenile female macaque to testosterone (T) (3). The other treatment groups were out of the scope of this current study. Juvenile macaques that had not undergone menarche were sedated using ketamine, and a small 1cm incision was done on the left subscapular region of the of the upper back. A testosterone implant was placed subcutaneously, and the incision was stitched closed. All procedures were done in a sterile field. Similar procedures were taken for replacing the implants. Animal samples were also taken from miscellaneous necropsies in which, macaques that were otherwise unassociated with any study were euthanized. Euthanization was done in a case by case basis and was most often attributed to GI issues. Animal care was done by the department of comparative medicine (DCM). Animal use was reviewed and approved by the ONPRC Institutional Animal Care and Use Committee.

Cell Culture:

Tissue obtained was done during scheduled necropsies for the mentioned NCTRI research grant headed by Cecily Bishop. The reproductive tract was isolated and transported to the lab workstation in a Hanks Balanced Salt Solution (Hanks) with HEBES added. LAM and PA were then dissected at the bench workstation and placed in a DEMEM/F12 + 1% antibiotic/antimycotic solution. Tissue could be left in 4C in this solution for up to 24hrs and stromal cells could still be isolated and viable. Tissue was then placed in a petri dish and was rinsed with Hanks in order to wash away as much of the blood as possible. Tissue was then minced in the DEMEM/F12 + 1% solution until pieces were very fine, ~1-2mm in diameter. Tissue was then placed in digestion solution in order to break fibroblasts free from their ECM matrix. Three digestion solutions were made; ~10mL of DEMEM/F12 + 1% was placed in a sterile filter along with collagenase type I at a concentration of about 2mg/ml. Dispase was added at about 80uL for every 10mL and was at a concentration of 5U/mL. Tissue was vacuumed in an automatic pipettor and was placed in the first collagenase wash. The digestion solution was then placed in water bath at 37C and was swirled every 5 minutes for 20min. The supernatant was then placed in a separate tube and diluted 1:1 with growth medium containing 10% FBS, this is important as it stops collagenase type I activity and prevents it from digesting the desired stromal cells. Cells were strained twice through a 40um strainer; each digestion solution was treated the same and was collected in conical tube. The collection of supernatants was then spun down and cells were resuspended in growth medium and plated to seed. Cells were kept in incubator at 37C with 5% CO2. Cells were then treated with 10nM concentrations of steroid hormones E2, E2+T, and E2+DHT at 60% confluence and with growth medium + 2% FBS.

RNA Analysis:

RNA was extracted at 100% confluence with Monarch RNA extraction kit from New England Biolabs. Real-Time PCR was done on 6 target endpoints where mRNA levels were measured from *in vitro* cell lines. Each target was subject to 4 treatment groups; control, E2, E2+T, and E2+DHT. The DHT group is important because it does not allow for aromatization. This allows for the ER component to be eliminated. These groups were selected in order to mimic the *in vivo* conditions present in PCOS women. Each treatment group had a total of 8 individual cell groups taken and grown from 3 different monkeys. This was done in order to provide relevant statistical power. True statistical power was beyond the resource means and would require subjecting a large amount of rhesus macaques to a single study, which is not possible. A calculation done in order to calculate the needed sample size was a total of 116 with 58 in each group. When comparing 4 independent study groups this number compounded and was completely unreasonable. Yet, this study still provides clinical insight in which trends can be seen. 3 different rhesus macaques were taken in order to provide a broader insight, if this was not done a single cell line divided into multiple lines could be biased. mRNA was targeted for ERalpha (ERa), Progesterone Receptor (PGR), Androgen Receptor (AR), Ki-67, Collagen type I alpha (Col1A),

and Vimentin (VIM). mRNA was measured by cycle threshold values (Ct) in reference to S10 the ribosomal protein. Target values were then plotted on their own standard curve and the results were calculated against S10 levels. S10 has been a standard to use in creating standard curves because of its uniformity in cells and low reactivity. mRNA levels were also corrected for Vimentin. Vimentin being a non-hormonally regulated structural protein, it is a marker of proliferation and migration in fibroblasts. It is present in all mesenchymal derived cells and serves as an intermediate filament protein. Providing another needed reference point.

Statistics:

A one-way ANOVA was run on each treatment group. Each treatment group for each individual target was subjected to a Shapiro-Wilk test for normality and the each produced a normal distribution (P>.05). Each target group was subjected to Levene's test for equality of variance and was normalized to fit all assumptions needed to have statistically relevant data. Results were subjected to Grubb's test for outliers. Sample group means, standard deviation, and standard error were all calculated.

Immunohistochemistry:

Immunohistochemistry (IHC) was done on paraffin embedded tissue blocks and OCT microwave embedded frozen tissue blocks using a standard method (40). Paraffin samples were able to be processed in at room temperature while frozen OCT blocks were done at 4C on the first day; then room temperature on the second day. Sections were cut at 5 um and were mounted on charged slides. Antibodies for AR, Col1A, and Ki-67 were diluted to 1:40, 1: 400, and 1:400 respectively. Frozen sections were kept at -80 C for 48hrs to allow full adherence to the slide. They were then subjected to microwave antigen retrieval and immediately fixed in paraformaldehyde 4%. Slides were subjected to rinsing washes and blocked using 2% normal horse serum in 1% BSA. Avidin and biotin were blocked for independently using Vector Labs avidin/biotin blocking kit. Antibodies were diluted in 1% BSA and were kept on slides overnight (~16hrs) at 4C for binding. Signal enhancement was done using the ABC/DAB method with biotinylated secondary antibodies diluted 1:200. Ready to use ABC complex peroxidase solution was placed on sections and allowed to bind for at least 1hr.

Paraffin slides were signal enhanced using the mentioned method above. Antigen retrieval was done using a citrate buffer solution at pH 6 in the pressure cooker at 15 psi for 7 minutes. Slides were then blocked for using 2% normal goat serum and primary antibodies were allowed to bind overnight at 4C.

Pictures were taken using Zeiss microscope at 20X magnification.

Immunocytochemistry:

Cells that were being resuspended for passaging were subjected to chamber slides. An aliquot of resuspension ~100uL was placed in each individual chamber of the chamber slide. Growth medium with 10% FBS ~1 mL was placed in each chamber. Slides were then placed in incubator at 37C with 5% CO2. Chamber slides were then fixed with 4% paraformaldehyde for 10 min at (37C). Cells were rinsed with packed PBS (.01M) and incubated with Triton X 100 at 37C for 30minutes. Triton is a permeabilization reagent which creates small holes in the membrane and allows for entrance of antibodies. Cells were rinsed with packed PBS and blocked with 2% normal serum and incubated for 1hr. Primary antibody was diluted and chamber slides were covered in parafilm and kept overnight at 4C to allow time for binding. Cells were then rinsed with packet PBS and secondary antibody was added diluted 1:200 and incubated for 30min at room temp. ABC ready to use solution was added and incubated for 30 min at room temp. DAB was mixed in DI water at pH was 7.6. 30% Hydrogen peroxide ~27 uL was added to activate DAB and cells were incubated at room temp for 5min. Chambers were removed and mounted with aqueous mounting medium and allowed to dry for ~2 hrs before cover slipping.

Results:

mRNA Transcripts:

PGR was significantly elevated in all three treatment groups (p<.05). Expression of PGR was lower in the presence of androgens (p=.08) (figure 1B). Ki-67 also seemed to be downregulated in the E2 group with slightly higher expression when androgens were present, which were not significant (p>.05) (figure 1D). Vimentin also has similar

expression across all groups, with differences that are not significant (figure 1F). Col1a, ERa, and AR when corrected for vimentin, yielded significant differences. Col1a was downregulated in the presence of androgens (p=.05) (Figure 2A). ERa and AR yielded significance at the 90% CI, in which the presence of androgens also downregulated their relative expression (p=.1) (figure 2 B&C). It seems that when androgens are present a trend towards downregulation occurs. The DHT group saw similar trends at the T group, even without the aromatization component of androgens, the effects are similar. Leading to the conclusion that the effects are most likely due to expression of the AR, not through ER.

Immunostaining:

Trichrome staining previously done on the LAM and PA reveals the connection between the two tissues. The collagen matrix extends deep into the LAM and surrounds various groups of muscle cells/bundles. Fibroblasts are also imbedded in the collagen matrix between the LAM muscle bundles. Staining for COL1A reveals that majority of the collagen in the LAM is COL1A and majority if not all of it in the PA. Staining against AR shows that fibroblasts in the LAM and PA are also positive for this receptor, and that a majority of these cells are positive. Also, an interesting feature is the nature of ERa and ERb localization in the LAM and PA. ERa seems to be localized to the stromal cells themselves, while ERb is only localized to the muscle cells. Staining done for Ki-67 shows the quiescent nature of these fibroblasts with only a few positive cells in the pictured regions of interest. Showed for comparison is a picture of basal vaginal epithelium on the same section that is also stained for Ki-67.

Immunocytochemistry:

Steroid receptor targets staining demonstrated expression and localization of the receptors in fibroblasts from the LAM and PA. Fibroblasts were seen to express ER, AR, PR, and Ki-67 cells were also seen to stain positive. These receptors are localized to the nucleus and were present in majority of the cells.

Discussion:

Fibroblasts are an adaptive cell type with a crucial role in the development of PFD in PCOS women. Thanks to the resources at the ONPRC, an *in vitro* model was able to be developed from the LAM and PA. A useful model in studying the novel relationship between PCOS and PFD. As was shown the response to androgens from these fibroblasts is dynamic and can change cell nature. When exposed to androgens in culture fibroblasts tend to reduce their production of Col1a. Col1a is an extremely important protein that provides flexibility and strength in the pelvic floor. The Col1a deposition seems to be controlled by the expression of steroid receptors. In the presence of androgens T and DHT, collagen production was downregulated. The similar findings for both the T and DHT group supports the evidence that this is most likely an AR dependent function. The vimentin ratios provided a guide for what function the cell is prioritizing. Fibroblast production of vimentin increases during proliferation and migration. The cells prioritize proliferation and migration when androgens are present. The constant elevated levels of T in PCOS women are likely causing the reduction in collagen deposition and compromised support. The role of proliferation also seems to point to the likelihood of some fibrosarcoma. The role of androgens in cancers has been confirmed in other carcinomas (41). This relationship in the pelvic floor deserves further study.

The inhibition of ERa points to some negative feedback from AR in response to elevated levels of androgens. This coincides with the literature, that AR has a downregulatory effect on ER expression. When the vimentin ration of these two receptors were done, they were also downregulated in the presence of androgens. ERa is a proposed up-regulator of collagen deposition and its expression leads to inhibition of enzymatic degradation of collagens (13, 15). Its downregulation in combination with collagen suppression, supports the case even further for androgen's negative effects. This prioritization of cellular proliferation leads to less expression of ERa and therefore less collagen. There are also the effects of AR being downregulated by the presence of its own ligands. E2 upregulated AR and when in combination with T and DHT, its expression compared to vimentin production was also downregulated. Again, the cell is prioritizing production of intermediate filaments and structural cell proteins over extracellular components such as steroid receptors.

PGR was upregulated in each of the treatment groups, and this is most attributed to the E2 present in each group. PGR is known to be a E2 sensitive which is supported by the results; but it also has an inhibitory affect towards ER (42). When T and DHT were present PGR seemed to be downregulated, although these were not significant (p>.05). The presence of T and DHT does not cause a significant downregulation, this allows for higher expression of PGR. Expression of PGR leads to suppression of ER and therefore less collagen production.

The immunostaining provided greater insight into the specific type of collagen being produced. The over 10 forms of collagen make it difficult to predict what exactly is being deposited by these fibroblasts. The staining for Col1a demonstrated that majority of the PA and LAM are made up Col1a. Staining for Ki-67 was not extremely proliferative in the LAM and PA. The fibroblasts may be prioritizing intracellular components, but not DNA synthesis. Previous literature indicates that elevated levels of androgens cause cell quiescence in fibroblasts (43). While this may seem contradictory to the current findings, they instead help narrow the possibilities of what is taking place. The cell may very well be prioritizing proliferation but failing to divide. These relationships merit further exploration.

Conclusion:

This study provided a needed *in vitro* model for studying the relationship between PFD in PCOS women. The isolation of fibroblasts helped confirm their presence in these tissues, and immunostaining helped elucidate their localization. Treatment of cells with E2, T, and DHT showed that AR reduces collagen production and causes prioritization of intracellular function over ECM deposition in fibroblasts.

Figures:

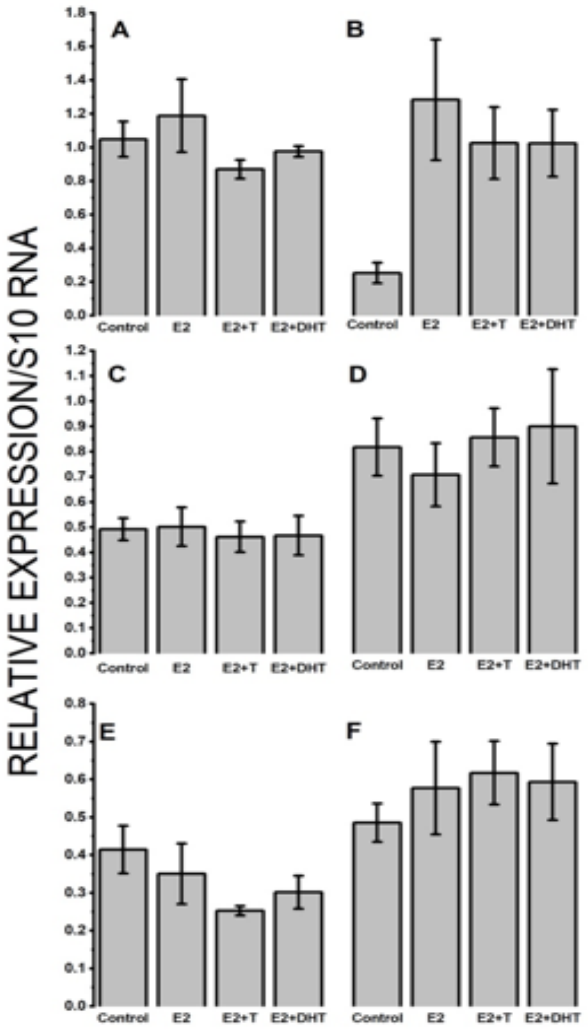
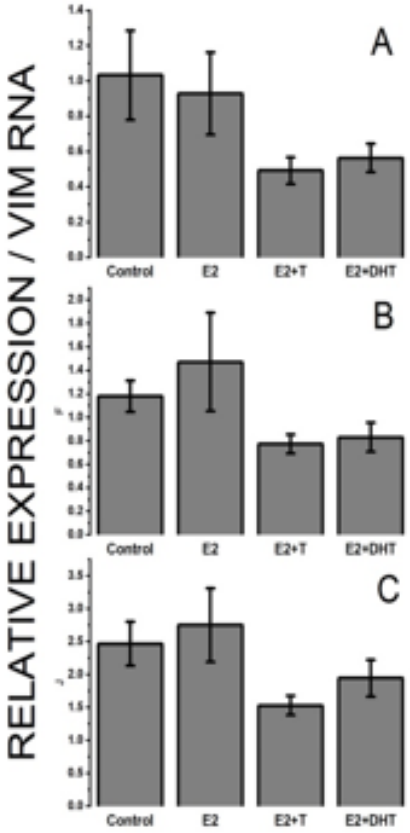
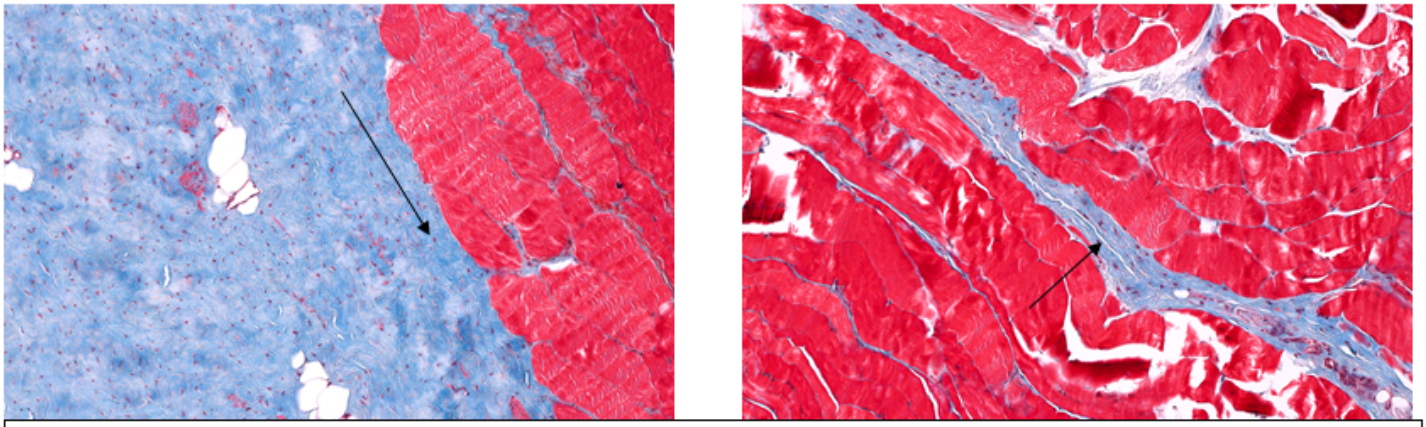


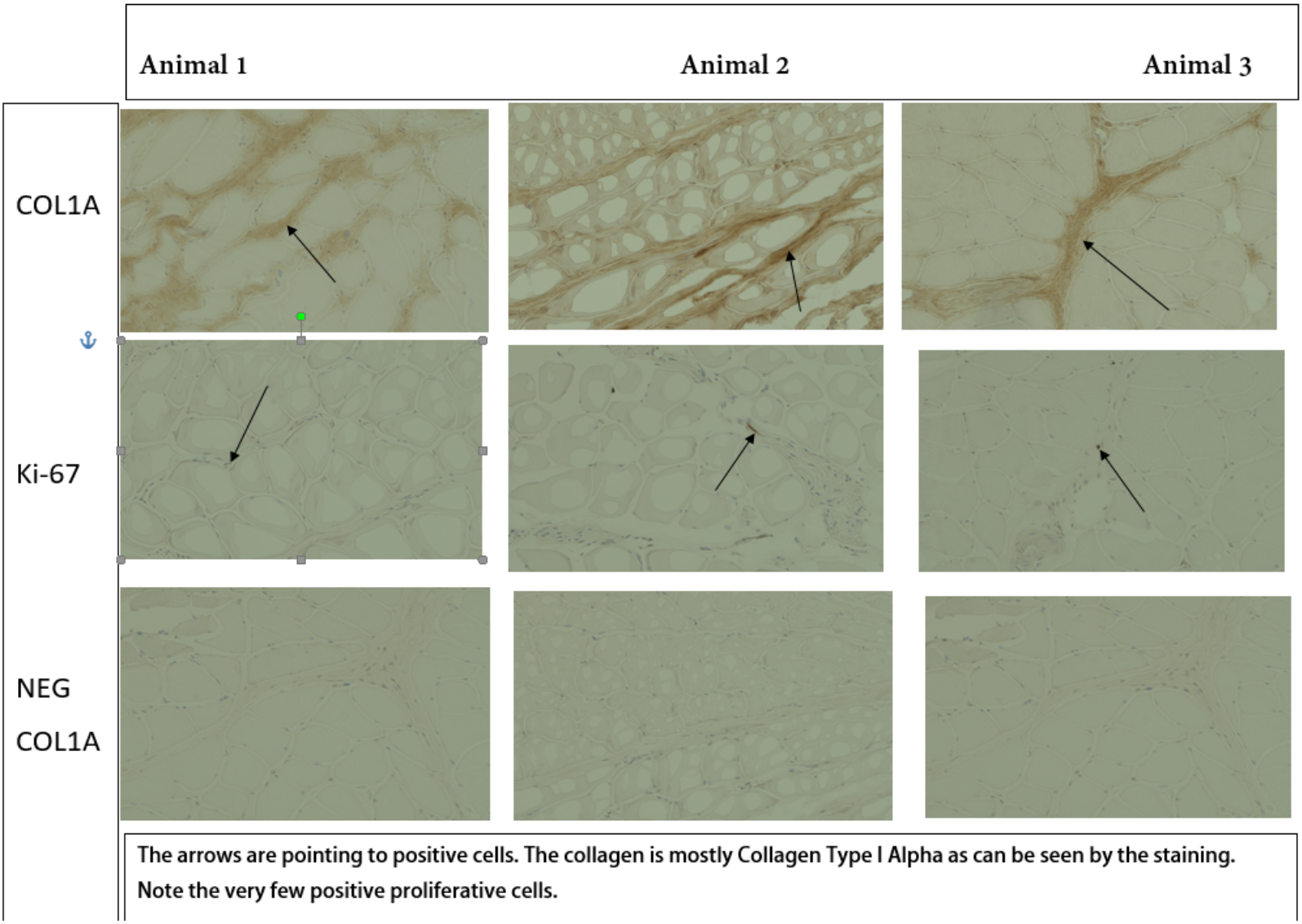
Figure 1: The histograms for RNA transcripts and their relative expression corrected against S10 RNA. Graphs that are adjacent are scaled equally. (A) ERa. (B) PGR; all groups had a significant difference compared to the control ($p<.05$). (C) Expression of AR. (D) Expression of Ki-67 a cell cycle marker. (E) the expression of Col1a. (F) Expression of Vimentin.



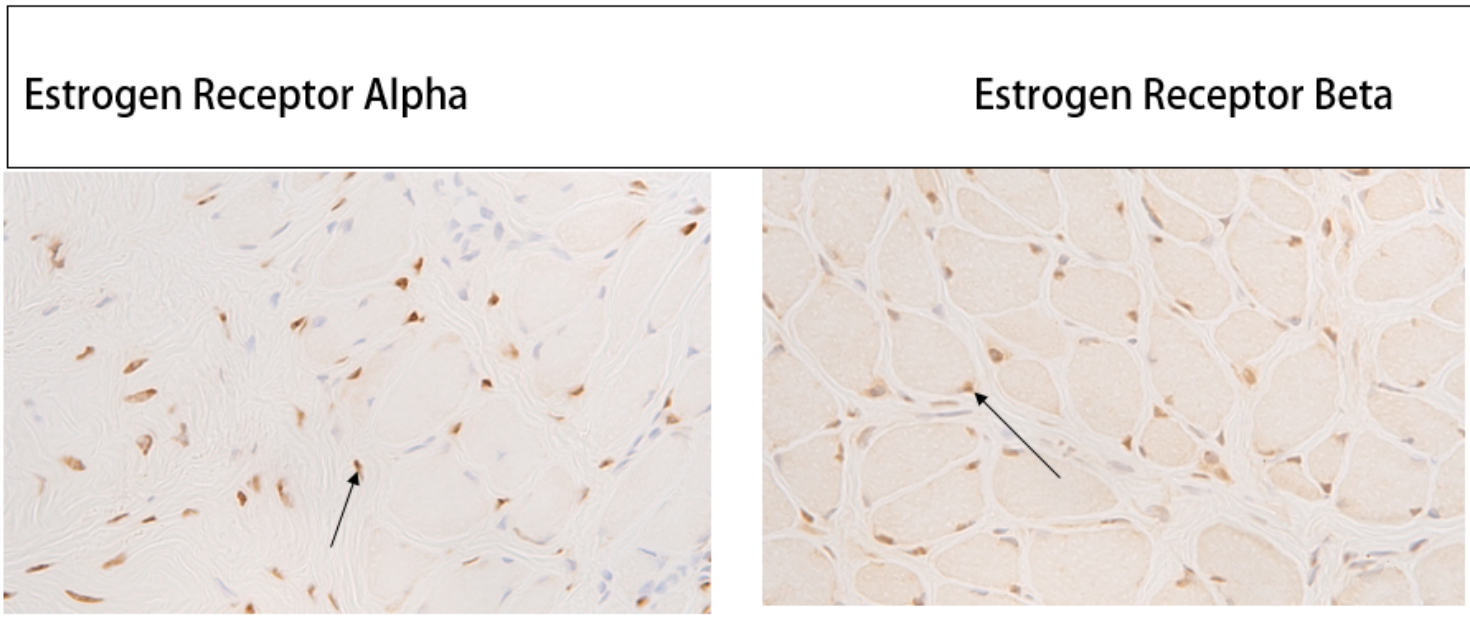
mRNA ratios corrected against VIM ratios of mRNA. (A) In Col1A both the E2+T/DHT downregulated collagen production significantly from the control ($p<.05$). (B) Similarly, T also downregulated AR expression ($p=.01$) and the DHT group had similar effects, although not significant ($P=.1$). (C) The trend continues as androgens downregulated ERa expression. The E2+T group was significant against both the control and E2 group ($p<.05$).



Trichrome stain of the Levator Ani Muscle and Paravaginal Attachment (Left 10X) and the Levator Ani Muscle longitudinal (Right 10X). The Arrows point to the dense collagen matrix (Left) and the fibroblast nuclei embedded in the Levator Ani Muscle (Right).



The arrows are pointing to positive cells. The collagen is mostly Collagen Type I Alpha as can be seen by the staining. Note the very few positive proliferative cells.



Localization of the two subtypes of estrogen receptor can be seen to be distinct. ER-a is localized to the stromal cells and ER-b is localized to the muscle cells.

References:

1. Saei Ghare Naz M, Ramezani Tehrani F, Behroozi-Lak T, Mohammadzadeh F, Kholosi Badr F, Ozgoli G. Polycystic Ovary Syndrome and Pelvic Floor Dysfunction: A Narrative Review. Res Rep Urol. 2020;12:179-85. Epub 2020/05/23. doi: 10.2147/RRU.S249611. PubMed PMID: 32440514; PMCID: PMC7213900.
2. Bishop CV, Mishler EC, Takahashi DL, Reiter TE, Bond KR, True CA, Slayden OD, Stouffer RL. Chronic hyperandrogenemia in the presence and absence of a western-style diet impairs ovarian and uterine structure/function in young adult rhesus monkeys. Hum Reprod. 2018;33(1):128-39. Epub 2017/12/01. doi: 10.1093/humrep/dex338. PubMed PMID: 29190387; PMCID: PMC5850861.
3. Bishop CV, Stouffer RL, Takahashi DL, Mishler EC, Wilcox MC, Slayden OD, True CA. Chronic hyperandrogenemia and western-style diet beginning at puberty reduces fertility and increases metabolic dysfunction during pregnancy in young adult, female macaques. Hum Reprod. 2018;33(4):694-705. Epub 2018/02/06. doi: 10.1093/humrep/dey013. PubMed PMID: 29401269; PMCID: PMC6454458.
4. Iwaszkiewicz-Pawlowska A, Zoch-Zwierz W, Biernacka A. [Primary hyperlipoproteinemia type V]. Pol Tyg Lek. 1992;47(46-48):1067-8. Epub 1992/11/16. PubMed PMID: 1305725.
5. Rocha A, Oliveira F, Azevedo R, Silva V, Peres T, Candido A, Gomes K, Reis F. Recent advances in the understanding and management of polycystic ovary syndrome [version 1; peer review: 3 approved]. F1000Research. 2019;8(565). doi: 10.12688/f1000research.15318.1.
6. Davey RA, Grossmann M. Androgen Receptor Structure, Function and Biology: From Bench to Bedside. Clin Biochem Rev. 2016;37(1):3-15. Epub 2016/04/09. PubMed PMID: 27057074; PMCID: PMC4810760.
7. Taghavi SA, Bazarganipour F, Allan H, Khashavi Z, Reisi N, Dosha N, Aghili F, Keramati M, Zahedi S, Aji-Ramkani A. Pelvic floor dysfunction and polycystic ovary syndrome. Hum Fertil (Camb). 2017;20(4):262-7. Epub 2017/06/22. doi: 10.1080/14647273.2017.1292003. PubMed PMID: 28635410.
8. Laven JS, Imani B, Eijkemans MJ, Fauser BC. New approach to polycystic ovary syndrome and other forms of anovulatory infertility. Obstet Gynecol Surv. 2002;57(11):755-67. Epub 2002/11/26. doi: 10.1097/00006254-200211000-00022. PubMed PMID: 12447098.
9. Walters KA. Androgens in polycystic ovary syndrome: lessons from experimental models. Curr Opin Endocrinol Diabetes Obes. 2016;23(3):257-63. Epub 2016/02/13. doi: 10.1097/med.0000000000000245. PubMed PMID: 26866639.
10. Soderberg MW, Johansson B, Masironi B, Bystrom B, Falconer C, Sahlin L, Ordeberg GE. Pelvic floor sex steroid hormone receptors, distribution and expression in pre- and postmenopausal stress urinary incontinent women. Acta Obstet Gynecol Scand. 2007;86(11):1377-84. Epub 2007/10/27. doi: 10.1080/00016340701625446. PubMed PMID: 17963065.
11. Campbell L, Emmerson E, Davies F, Gilliver SC, Krust A, Chambon P, Ashcroft GS, Hardman MJ. Estrogen promotes cutaneous wound healing via estrogen receptor beta independent of its antiinflammatory activities. J Exp Med. 2010;207(9):1825-33. Epub 2010/08/25. doi: 10.1084/jem.20100500. PubMed PMID: 20733032; PMCID: PMC2931162.
12. He K, Niu G, Gao J, Liu JX, Qu H. MicroRNA-92 expression may be associated with reduced estrogen receptor beta1 mRNA levels in cervical portion of uterosacral ligaments in women with pelvic organ prolapse. Eur J Obstet Gynecol Reprod Biol. 2016;198:94-9. Epub 2016/01/25. doi: 10.1016/j.ejogrb.2016.01.007. PubMed PMID: 26803387.
13. Leblanc DR, Schneider M, Angele P, Vollmer G, Docheva D. The effect of estrogen on tendon and ligament metabolism and function. J Steroid Biochem Mol Biol. 2017;172:106-16. Epub 2017/06/21. doi: 10.1016/j.jsbmb.2017.06.008. PubMed PMID: 28629994.
14. Borazjani A, Kow N, Harris S, Ridgeway B, Damaser MS. Transcriptional Regulation of Connective Tissue Metabolism Genes in Women With Pelvic Organ Prolapse. Female Pelvic Med Reconstr Surg. 2017;23(1):44-52. Epub 2016/09/17. doi: 10.1097/SPV.0000000000000337. PubMed PMID: 27636223; PMCID: PMC5161600.

15. Nallasamy S, Yoshida K, Akins M, Myers K, Iozzo R, Mahendroo M. Steroid Hormones Are Key Modulators of Tissue Mechanical Function via Regulation of Collagen and Elastic Fibers. Endocrinology. 2017;158(4):950-62. Epub 2017/02/17. doi: 10.1210/en.2016-1930. PubMed PMID: 28204185; PMCID: PMC5460796.
16. Hallock JL, Handa VL. The Epidemiology of Pelvic Floor Disorders and Childbirth: An Update. Obstet Gynecol Clin North Am. 2016;43(1):1-13. Epub 2016/02/18. doi: 10.1016/j.ogc.2015.10.008. PubMed PMID: 26880504; PMCID: PMC4757815.
17. Chung SH, Kim WB. Various Approaches and Treatments for Pelvic Organ Prolapse in Women. J Menopausal Med. 2018;24(3):155-62. Epub 2019/01/24. doi: 10.6118/jmm.2018.24.3.155. PubMed PMID: 30671407; PMCID: PMC6336571.
18. Friedman T, Eslick GD, Dietz HP. Risk factors for prolapse recurrence: systematic review and meta-analysis. Int Urogynecol J. 2018;29(1):13-21. Epub 2017/09/19. doi: 10.1007/s00192-017-3475-4. PubMed PMID: 28921033.
19. Giarenis I, Robinson D. Prevention and management of pelvic organ prolapse. F1000Prime Rep. 2014;6:77. Epub 2014/10/25. doi: 10.12703/P6-77. PubMed PMID: 25343034; PMCID: PMC4166938.
20. <Expression of estrogen receptors in the pevic floor of pre and post menopausal wmen presenting pelvic organ prolapse - 2011.pdf>.
21. Bezerra LO, de Oliveira MCE, da Silva Filho EM, Vicente da Silva HK, Menezes de Oliveira GF, da Silveira Goncalves AK, Pegado R, Micussi M. Impact of Pelvic Floor Muscles Training Isolated and Associated with Game Therapy on Mixed Urinary Incontinence: A Randomized Controlled Trial. Games Health J. 2020. Epub 2020/07/28. doi: 10.1089/g4h.2019.0207. PubMed PMID: 32716652.
22. Gong R, Xia Z. Collagen changes in pelvic support tissues in women with pelvic organ prolapse. Eur J Obstet Gynecol Reprod Biol. 2019;234:185-9. Epub 2019/02/03. doi: 10.1016/j.ejogrb.2019.01.012. PubMed PMID: 30710765.
23. Islam RM, Oldroyd J, Rana J, Romero L, Karim MN. Prevalence of symptomatic pelvic floor disorders in community-dwelling women in low and middle-income countries: a systematic review and meta-analysis. Int Urogynecol J. 2019;30(12):2001-11. Epub 2019/06/06. doi: 10.1007/s00192-019-03992-z. PubMed PMID: 31165221.
24. Lim VF, Khoo JK, Wong V, Moore KH. Recent studies of genetic dysfunction in pelvic organ prolapse: the role of collagen defects. Aust N Z J Obstet Gynaecol. 2014;54(3):198-205. Epub 2014/03/01. doi: 10.1111/ajo.12169. PubMed PMID: 24575973.
25. Liu X, Zhao Y, Pawlyk B, Damaser M, Li T. Failure of elastic fiber homeostasis leads to pelvic floor disorders. Am J Pathol. 2006;168(2):519-28. Epub 2006/01/27. doi: 10.2353/ajpath.2006.050399. PubMed PMID: 16436666; PMCID: PMC1606509.
26. Maldonado PA, Wai CY. Pelvic Organ Prolapse: New Concepts in Pelvic Floor Anatomy. Obstet Gynecol Clin North Am. 2016;43(1):15-26. Epub 2016/02/18. doi: 10.1016/j.ogc.2015.10.001. PubMed PMID: 26880505.
27. Nyangoh Timoh K, Moszkowicz D, Zaitouna M, Lebacle C, Martinovic J, Diallo D, Creze M, Lavoue V, Darai E, Benoit G, Bessede T. Detailed muscular structure and neural control anatomy of the levator ani muscle: a study based on female human fetuses. Am J Obstet Gynecol. 2018;218(1):121 e1- e12. Epub 2017/10/11. doi: 10.1016/j.ajog.2017.09.021. PubMed PMID: 28988909.
28. Rostaminia G, White D, Hegde A, Quiroz LH, Davila GW, Shobeiri SA. Levator ani deficiency and pelvic organ prolapse severity. Obstet Gynecol. 2013;121(5):1017-24. Epub 2013/05/03. doi: 10.1097/AOG.0b013e31828ce97d. PubMed PMID: 23635738.
29. Trutnovsky G, Kamisan Atan I, Ulrich D, Martin A, Dietz HP. Levator ani trauma and pelvic organ prolapse - a comparison of three translabial ultrasound scoring systems. Acta Obstet Gynecol Scand. 2016;95(12):1411-7. Epub 2016/09/14. doi: 10.1111/aogs.13018. PubMed PMID: 27622984.

30. Raizada V, Mittal RK. Pelvic floor anatomy and applied physiology. Gastroenterol Clin North Am. 2008;37(3):493-509, vii. Epub 2008/09/17. doi: 10.1016/j.gtc.2008.06.003. PubMed PMID: 18793993; PMCID: PMC2617789.
31. Ruiz-Zapata AM, Kerkhof MH, Zandieh-Doulabi B, Brolmann HA, Smit TH, Helder MN. Fibroblasts from women with pelvic organ prolapse show differential mechanoresponses depending on surface substrates. Int Urogynecol J. 2013;24(9):1567-75. Epub 2013/04/13. doi: 10.1007/s00192-013-2069-z. PubMed PMID: 23579290; PMCID: PMC3745620.
32. Wen Y, Wani P, Zhou L, Baer T, Phadnis SM, Reijo Pera RA, Chen B. Reprogramming of fibroblasts from older women with pelvic floor disorders alters cellular behavior associated with donor age. Stem Cells Transl Med. 2013;2(2):118-28. Epub 2013/01/24. doi: 10.5966/sctm.2012-0092. PubMed PMID: 23341439; PMCID: PMC3659753.
33. Li Y, Hong L, Liu C, Min J, Hong S, Hu M, Zhao Y, Yang Q, Tang J, He S. Effect of puerarin on collagen metabolism of fibroblasts in pelvic tissue of women with pelvic organ prolapse. Mol Med Rep. 2018;17(2):2705-11. Epub 2017/12/06. doi: 10.3892/mmr.2017.8112. PubMed PMID: 29207080.
34. Liu C, Yang Q, Fang G, Li BS, Wu DB, Guo WJ, Hong SS, Hong L. Collagen metabolic disorder induced by oxidative stress in human uterosacral ligamentderived fibroblasts: A possible pathophysiological mechanism in pelvic organ prolapse. Mol Med Rep. 2016;13(4):2999-3008. Epub 2016/03/05. doi: 10.3892/mmr.2016.4919. PubMed PMID: 26936098; PMCID: PMC4805094.
35. Mackey AL, Magnan M, Chazaud B, Kjaer M. Human skeletal muscle fibroblasts stimulate in vitro myogenesis and in vivo muscle regeneration. J Physiol. 2017;595(15):5115-27. Epub 2017/04/04. doi: 10.1113/JP273997. PubMed PMID: 28369879; PMCID: PMC5538230.
36. Ruiz-Zapata AM, Kerkhof MH, Ghazanfari S, Zandieh-Doulabi B, Stoop R, Smit TH, Helder MN. Vaginal Fibroblastic Cells from Women with Pelvic Organ Prolapse Produce Matrices with Increased Stiffness and Collagen Content. Sci Rep. 2016;6:22971. Epub 2016/03/12. doi: 10.1038/srep22971. PubMed PMID: 26965792; PMCID: PMC4786799.
37. Wakao J, Kishida T, Fumino S, Kimura K, Yamamoto K, Kotani SI, Mizushima K, Naito Y, Yoshikawa T, Tajiri T, Mazda O. Efficient direct conversion of human fibroblasts into myogenic lineage induced by co-transduction with MYCL and MYOD1. Biochem Biophys Res Commun. 2017;488(2):368-73. Epub 2017/05/16. doi: 10.1016/j.bbrc.2017.05.059. PubMed PMID: 28501623.
38. Zong W, Zyczynski HM, Meyn LA, Gordy SC, Moalli PA. Regulation of MMP-1 by sex steroid hormones in fibroblasts derived from the female pelvic floor. Am J Obstet Gynecol. 2007;196(4):349 e1-11. Epub 2007/04/04. doi: 10.1016/j.ajog.2006.12.019. PubMed PMID: 17403418.
39. Stewart AM, Cook MS, Esparza MC, Slayden OD, Alperin M. Architectural assessment of rhesus macaque pelvic floor muscles: comparison for use as a human model. Int Urogynecol J. 2017;28(10):1527-35. Epub 2017/03/13. doi: 10.1007/s00192-017-3303-x. PubMed PMID: 28285397; PMCID: PMC5593758.
40. Almeida-Francia CC, Keator CS, Mah K, Holden L, Hergert C, Slayden OD. Localization and hormonal regulation of endometrial matrix metalloproteinase-26 in the rhesus macaque. Hum Reprod. 2012;27(6):1723-34. Epub 2012/03/22. doi: 10.1093/humrep/des086. PubMed PMID: 22434853; PMCID: PMC3357194.
41. Augello MA, Den RB, Knudsen KE. AR function in promoting metastatic prostate cancer. Cancer Metastasis Rev. 2014;33(2-3):399-411. Epub 2014/01/16. doi: 10.1007/s10555-013-9471-3. PubMed PMID: 24425228; PMCID: PMC4452946.
42. Slayden OD. Translational In Vivo Models for Women's Health: The Nonhuman Primate Endometrium--A Predictive Model for Assessing Steroid Receptor Modulators. Handb Exp Pharmacol. 2016;232:191-202. Epub 2016/01/02. doi: 10.1007/164_2015_22. PubMed PMID: 26721679.
43. Castoria G, Giovannelli P, Di Donato M, Ciociola A, Hayashi R, Bernal F, Appella E, Auricchio F, Migliaccio A. Role of non-genomic androgen signalling in suppressing proliferation of fibroblasts and fibrosarcoma cells. Cell Death Dis. 2014;5:e1548. Epub 2014/12/06. doi: 10.1038/cddis.2014.497. PubMed PMID: 25476896; PMCID: PMC4649827.