

## Research Article

Ceren Hangul, Esin Guvenir Celik, Hacer Kaya, Onur Eroglu, Hilmi Uysal and Sibel Berker Karauzum\*



# Estradiol differentially regulates DUX4, $\beta$ -catenin and PAX3/PAX7 in primary myoblasts of facioscapulohumeral muscular dystrophy patients

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## Abstract

**Objectives:** There is a clinical variability and heterogeneity among Facioscapulohumeral Muscular Dystrophy (FSHD) patients. Escalation after menopause in women, early onset in men suggests that estrogen might be a protective factor on the course of FSHD. In spite of few molecular studies supporting the protective role of estrogen in FSHD *in vitro*, there is no study revealing the effect of estradiol on the protein levels of DUX4,  $\beta$ -catenin and PAX3/PAX7. In present study, we investigated the effect of estradiol treatment on the expressions of DUX4,  $\beta$ -catenin and PAX3/PAX7 protein levels.

**Materials and Methods:** Primary myoblasts of 63 and 71 years old (63yM/71yM) males; 47 years old (47yF) female FSHD patients were used. Cells were processed under these conditions; (i) untreated, (ii) 10 nM-30 min estradiol and (iii) 10 nM-4 h estradiol treated. The expression of DUX4, PAX3/PAX7 and  $\beta$ -catenin were examined by western-blotting.

**Results:** Expression of DUX4 significantly downregulated after 4 h treatment of estradiol while PAX3/PAX7 56 kDa

variant expression upregulated in 71yM cells.  $\beta$ -catenin and PAX3 expression was variable among the samples.

**Conclusion:** Our results suggest that estrogen might be a palliative treatment option via downregulation of DUX4 protein in DUX4 expressing FSHD patients.

**Keywords:**  $\beta$ -catenin; DUX4; estradiol; facioscapulohumeral muscular dystrophy; FSHD; PAX3; PAX7; skeletal muscle.

## ÖZ

**Amaç:** Fasioskapulohumeral Musküler Distrofi (FSHD) hastaları arasında klinik değişkenlik ve heterojenite gözlenmektedir. Erkeklerde hastalığın daha erken başlaması, kadınlarda ise menopoz ile bulguların şiddetlenmesi, östrojenin FSHD'nin seyri üzerinde koruyucu bir faktör olabileceğini düşündürmüştür. FSHD'deki koruyucu rolü üzerine birkaç *in vitro* çalışma mevcut olmakla beraber, östradiolün FSHD' de DUX4,  $\beta$ -catenin ve PAX3/PAX7 proteinlerinin düzeyleri üzerine olan etkisini ortaya koyan bir çalışma bulunmamaktadır. Mevcut çalışmamızda, östradiol muamelesinin DUX4,  $\beta$ -catenin ve PAX3/PAX7 protein düzeylerine etkisi araştırılmıştır.

**Gereç ve Yöntem:** 63 ve 71 yaşında erkek(63yE/71yE) ve 47 yaşında kadın (47yK) FSHD hastalarına ait primer miyoblast hücreleri; (i) kontrol (hiçbir muamele yapılmamış), (ii) 10 nM-30 dakika estradiol ve (iii) 10 nM-4-saat östradiol muamelesi sonrasında; DUX4,  $\beta$ -catenin ve PAX3/PAX7 ekspresyonunu belirlemek üzere western blot metodu ile incelenmiştir.

**Bulgular:** Östradiol ile 4 saat muamele sonucunda 71yE hastasına ait miyoblast hücrelerinde DUX4 ekspresyonunun down-regülasyonu, PAX3/PAX7 56 kDa varyantının up-regülasyonu gözlenmiştir.  $\beta$ -catenin ve PAX3 proteinleri, miyoblast örnekleri arasında değişken ekspresyon yanıtları sergilemiştir.

\*Corresponding author: Sibel Berker Karauzum, PhD, Department of Medical Biology and Genetics, Faculty of Medicine, Akdeniz University, Antalya, 07058, Turkey, Phone: +0090 532 727 89 41, E-mail: sibelberkerkarauzum@gmail.com

Ceren Hangul, Institute of Health Sciences, Akdeniz University, Antalya, Turkey. <https://orcid.org/0000-0001-6869-8802>

Esin Guvenir Celik and Hacer Kaya, Department of Molecular Biology and Genetics, Bilecik Şeyh Edebali University, Bilecik, Turkey

Onur Eroglu, Biotechnology Research and Application Center, Bilecik Şeyh Edebali University, Bilecik, Turkey

Hilmi Uysal, Department of Neurology, Faculty of Medicine, Akdeniz University, Antalya, Turkey

**Sonuç:** Bu çalışmanın sonuçları, DUX4 eksprese eden FSHD hastalarında östradiolün DUX4 down-regülasyonu yaparak palyatif bir tedavi seçeneği sunabileceğine işaret etmektedir.

**Anahtar Kelimeler:**  $\beta$ -katenin; DUX4; Fasioskapulohumeral Musküler Distrofi; FSHD; iskelet kası; östradiol; PAX3; PAX7.

## Introduction

Facioscapulohumeral Muscular Dystrophy (FSHD) (OMIM#158900), is an autosomal dominant genetic disease. Deletion in D4Z4 macrosatellite repeat located at chromosome 4q35 region is responsible from the disease. In normal population, D4Z4 contains 11 to 100 repeats and each unit consists of 3,300 bases involving DUX4 gene. In FSHD1 (95% of the patients) this repeat number is between 1 and 10 resulting from a deletion in this area [1]. This deletion causes chromatin relaxation ending up with the activation of DUX4 which is normally inactive in adult tissues except testis in men. If there is qA allele at the end of this repeat, this activation leads to the stabilization of DUX4 mRNA and the change of transcriptional network in FSHD muscle [2].

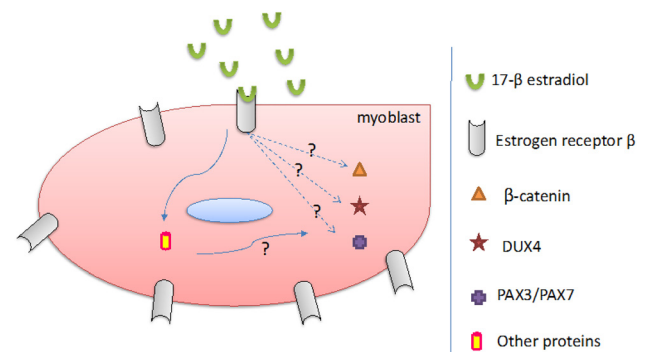
DUX4 is a double homeobox protein, especially expressed in embryological development [3, 4], its structure is similar to PAX protein family with their homeodomains. In one study, it was revealed that PAX3 and PAX7 are the only members that can compete with DUX4, while other homeodomain proteins as PAX6, Pitx2c, OTX1, Rax, Hesx1, MIXL1 and Tbx1 do not have this property [5]. Supporting this, overexpression of PAX3 and PAX7 in FSHD cell lines resulted in a decrease of DUX4 revealing a converse relation between DUX4 and PAX3/PAX7 [6]. DUX4 gene activation is a hallmark of FSHD. In addition to this, PAX7 target gene repression had been revealed as a biomarker in FSHD [7] just recently. PAX3 and PAX7 have splice variants; PAX3 have eight splice variants as 44.8/45.2/22.7/53.5/24.1/53/56/53.3 kDa and PAX7 have three splice variants as 56.9/55.1/56.7 kDa. Because it has not been clearly identified which variant or variants take a role in the pathology of FSHD, common antibody had been used.

DUX4 is a transcription factor and has many target proteins [8]. It was revealed that DUX4 overexpression resulted in either upregulation or downregulation of wide range of genes [9]. Until now, it has been difficult to attribute one or few responsible gene(s) for explaining the pathophysiology. In 2015, Banerji et al. used a new network

approach and suggested  $\beta$ -catenin as a central protein of this network [10] supporting previous studies that indicated impaired Wnt/ $\beta$ -catenin pathway in FSHD [11, 12].

FSHD is a progressive disease with an onset around 20–30s [13]. As named, Facioscapulohumeral Muscular Dystrophy affects primarily facial, scapular, humeral muscles and tibialis anterior muscles. There are clear variations in onset and clinical severity even in same family members carrying same repeat unit number. This indicates to the existence of additional modifier factors affecting severity. In men, onset is earlier and phenotype is more severe compared to women [14]. In women, it is observed that after childbirth [15, 16] and especially after menopause, there is clear aggravation of symptoms arousing a suspicion for the effect of estrogen on FSHD severity. Mul et al. identified no relation of clinical severity with lifetime estrogen exposure [17]. However, in our previous clinical study ratio of estradiol and progesterone to testosterone had been related to clinical severity independent of D4Z4 repeat unit [18]. Supportively, in one clinical study it is observed that anti-estradiol cancer therapy caused an aggravation of FSHD symptoms in contrast to other chemotherapeutic agents [19]. Later on, Teveroni et al. revealed the protective molecular effect of estradiol on FSHD cell lines by showing hypermethylation of DUX4's binding sites on DNA. Although direct regulation of DUX4 couldn't be shown, they revealed the downregulation of DUX4 target genes via epigenetic modulation after estrogen treatment [20]. Independent from DUX4, estrogen had also been shown to contribute satellite cell maintenance and muscle regeneration in females [21].

Based on the clinical difference between men and women in FSHD and in the light of aforementioned studies in literature, the purpose of this study was to reveal whether estrogen had an effect on the pathophysiology of FSHD at a molecular level. For this purpose, four different transcription factors which were indicated as key factors in literature had been investigated (Figure 1). These key



**Figure 1:** Representative schematic of the study.

transcription factors were; (i) **DUX4 protein** which locates in each D4Z4 repeat at chromosome 4q35 region; (ii)  **$\beta$ -catenin** which had been demonstrated as a central protein in the network of the FSHD, (iii) **PAX3 and PAX7** which are known to compete with DUX4 for DNA binding and also essential in embryologic development of skeletal muscles. Since the best reflecting *in vitro* study would be to evaluate the effect of estradiol on FSHD primary myoblasts, patient derived myoblasts had been used for investigation.

## Materials and methods

This study had been approved by Akdeniz University Faculty of Medicine Clinical Research Ethical Committee; Decision Number: 284, Date: 28.10.2015.

### Human myoblast cell culture

FSHD myoblast cell lines were obtained from Massachusetts University in the United States, from Professor Rabi Tawil and Don Henderson team. FSHD cell lines belonging to 63 and 71 years old males; 47 years old female who were diagnosed with FSHD1 had been used in the experiments. These cell lines are DUX4 positive cell lines in the level of DUX4 mRNA (Table 1).

Selected cell lines reflect; differently sized contracted repeat units, variable clinical severity, both male and female genders and different muscles where muscle biopsy had been sampled (Table 1). Instead of performing all procedures in one cell line, three cell lines had been used, to be able to reflect the clinical variability in FSHD patients [13], FSHD myoblast cells were grown F10 Nutrient Medium (Gibco11550-043) containing 20% heat-inactivated fetal bovine serum (Gibco26140-079), 1% penicillin/streptomycin (Gibco15070-063 10 ng/mL), Fibroblast Growth Factor (bFGF) (PromegaG5071), 1  $\mu$ M Dexamethazone (Sigma water soluble D2915), at 37 °C containing 5% CO<sub>2</sub> incubator.

FSHD myoblast cells were plated in 75 cm<sup>2</sup> flasks. Cells were allowed to attach to flasks overnight. Media was replenished for a period of 2–3 days after adhesion. As the cell number of the flasks reached 60–80% confluency, medium was removed for cell passaging; the cells were washed with 2 mL of Phosphate Buffered

Saline (PBS), incubated for 3 min with 3 mL of trypsin-EDTA (Ethylene-Diamine Tetraacetic Acid) (0.1%). At the end of this period, 3 mL of F10 medium was added to the flasks to stop trypsin activity. Cells were collected and centrifuged at 1,800 rpm for 7 min, then the supernatant was discarded, and 1 mL of fresh medium was added onto cell pellet. Homogenized cell suspension was counted in the hemocytometer.  $1.5 \times 10^6$  cells were plated to 75 cm<sup>2</sup> flasks. After two or three passages, when reached 60–80% confluency, adhesive cells in different plates were treated with 10 nM estradiol (E2-Sigma Aldrich-E2758) for 30 min, 4 h and non-treated (control). All cells were incubated at 37 °C in 5% CO<sub>2</sub>.

### Treatments

Estradiol had been dissolved in ethanol, diluted in medium to reach 10 nM concentration. 10 nM concentration had been identified from estradiol-FSHD cell study by Teveroni et al. 2017 and other two studies with myoblasts cultures in literature [20, 24, 25]. Estradiol treatment time had been identified as 72 h from Teveroni et al. 2017 [20]. However, after observing morphological destruction and high amounts of cell death in first experiments, literature re-scanning had been performed and time period was identified as two stages: 30 min and 4 h [26] to reveal time dependent expression of investigated proteins.

### Western blotting

Cells were collected by 1XPBS and centrifuged for 2 min at 13,200 rpm. The supernatant was discarded and radioimmuno-precipitation assay (RIPA) solution was added as an elution buffer on the cells. Phenyl-methyl-sulfonyl-fluoride (PMSF) was added to prevent degradation of proteins with protease activity just before addition to the RIPA solution. The samples were incubated at room temperature for 20 min on a shaker and centrifuged at +4 °C for 20 min at 13,200 rpm. Bradford method was used to determine the amount of protein [27]. Lysates were separated by SDS-PAGE gel electrophoresis using 12% polyacrylamide gels and then transferred to nitrocellulose membranes with semi-dry transfer system (iBlot2). Protein transfer was controlled by Ponceau Red staining.

Nitrocellulose membranes were blocked for 1 h with 5% milk powder without fat in Tris Buffered Saline (TBS) solution. After blocking, membranes were incubated with primary antibodies at 4 °C overnight in the same conditions. Primary antibodies used in 1:1,000 dilution as follows: anti DUX4 rabbit mab (ABCAM-E5-5), anti PAX3/PAX7 mouse mab (Santa Cruz-365843), anti  $\beta$ -catenin mab (Cell Signaling 9582S),  $\beta$ -actin (Cell Signaling) and GAPDH (Cell Signaling-2118).

After primary antibody labeling, membranes were washed with Tris Buffered Saline with Tween 20 (TBS-T) for 5 min of three times and then incubated with secondary antibodies attached with Horse Radish Peroxidase (HRP). For secondary antibody incubation 1:5,000 dilution was used for 1 h at 4 °C. Proteins were screened using ECL (Thermo-Fisher Scientific) in Syngene G:Box Chemi XRQ system without film.

For the proteins which have similar molecular weight and band pattern, stripping and re-staining had been performed. 0.5 M buffer with 6.8 pH (containing 20 mL SDS 10%, 12.5 mL Tris HCl, 0.8 mL  $\beta$ -mercaptoethanol, 67.5 mL distilled water) (abcam) had been used for harsh stripping protocol. Buffer solution was pre-heated to 50 °C; nitrocellulose membranes had been covered by buffer and incubated

**Table 1:** Cell lines and their detailed information.

Cell line code	Age at Bx*	Gender	Onset	CSS**	Repeat length & allele	Bx muscle
71yM	71	M	40	8	31 kb A	Deltoid
63yM	63	M	15	6	23 kb A	Vastus lateralis
47yF	47	F	47	1	19 kb A	Vastus lateralis

\*Bx, Biopsy; \*\*CSS, Clinic Severity Score. A scoring system for severity of the disease and could be reached on <https://www.urmc.rochester.edu/MediaLibraries/URMCMedia/fields-center/documents/ClinicalSeverityScoring.pdf>. Reference: [22, 23].

in 50 °C for 30–45 min with some agitation. Stripping solution was disposed, and membrane rinsed with water for 2 min. Then membrane was washed with TBS-T for 5 min. After this step, membrane was blocked with 5% milk powder and aforementioned steps were repeated for antibody labeling.

### Statistical analysis

“ImageJ application” program is used for the density measurement of the monitored bands. Each protein band had been measured three times and mean value of three measurement had been used. For the calculation of relative expression levels; each value had been divided to values of  $\beta$ -actin. Graphs with error bars including p values (determined by one-way Anova test) had been prepared in GraphPad Prism.

## Results

Three cell lines belonging to 71yM, 63yM, 47yF were investigated as following: (i) control (no estradiol treatment), (ii) 30 min 10 nM 17- $\beta$  estradiol (E2) treatment, (iii) 4 h 10 nM 17- $\beta$  estradiol (E2) treatment.

### The effect of E2 on DUX4 expression

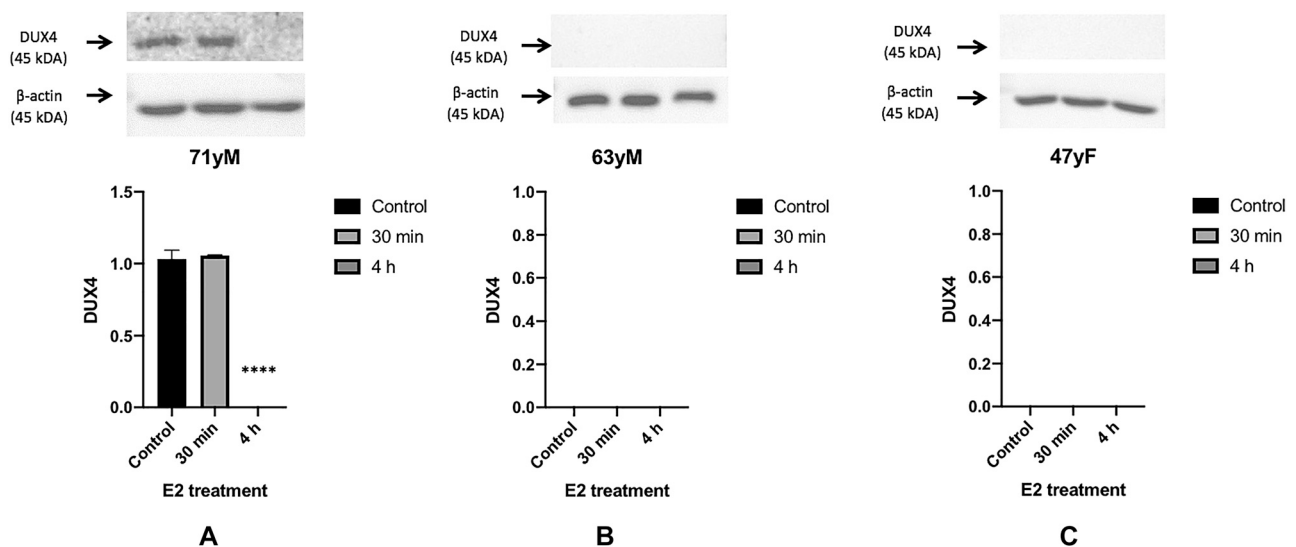
DUX4 expression decreased to zero at fourth hour after E2 treatment in 71yM ( $p < 0.0001$ ) (Figure 2A). In the myoblasts of 63yM and 47yF there were no DUX4 protein expression neither in control myoblasts nor E2 treated myoblasts (Figure 2B, C).

### The effect of E2 on $\beta$ -catenin expression

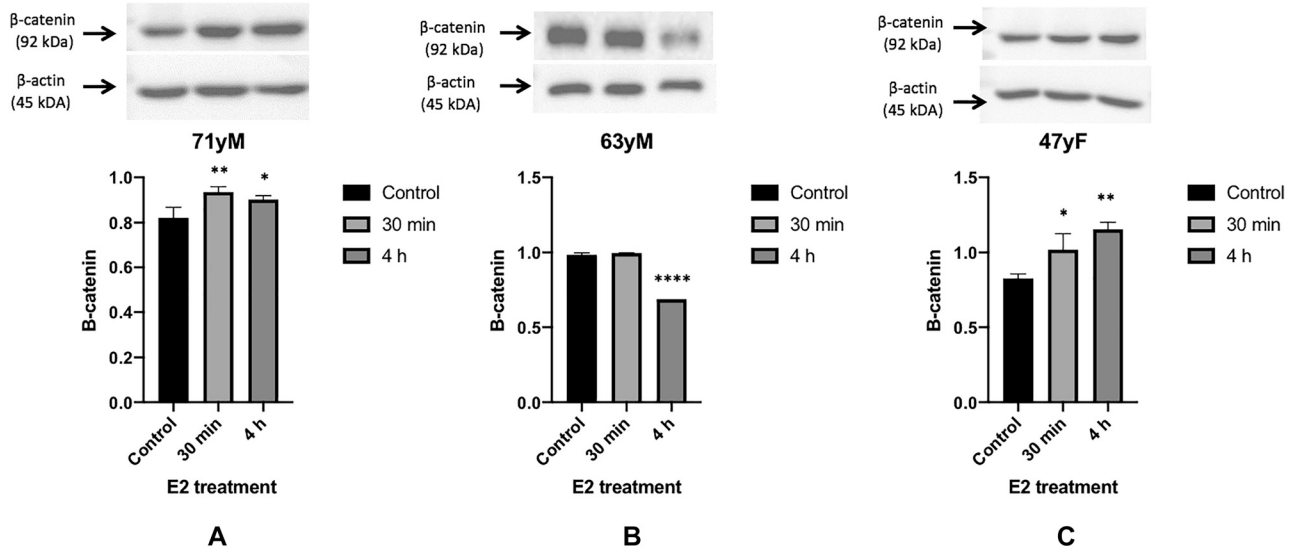
In 71yM,  $\beta$ -catenin exhibited significant increase at 30 min ( $p = 0.0084$ ) and 4 h ( $p = 0.0376$ ) after estradiol treatment compared to control myoblasts (Figure 3A). Similar results were observed in 47yF ( $\beta$ -catenin increased at 30 min [ $p = 0.0269$ ] and 4 h treatment [ $p = 0.0022$ ] compared to control myoblasts [Figure 3C]) while in 63yM, after 4 h treatment,  $\beta$ -catenin expression was found to be lowest level ( $p < 0.0001$ ) compared to control myoblasts (Figure 3B). These results revealed divergent  $\beta$ -catenin responses to E2 treatment in FSHD myoblasts.

### The effect of E2 on PAX3/PAX7 expression

PAX3/PAX7 labeled variant which expressed in all myoblasts belonging to three FSHD patients was 45 kDa. The expression of this variant was also present in control myoblasts where no E2 treatment, and the level of this protein exhibited divergent responses to E2 treatment similar to  $\beta$ -catenin while these responses were just in an opposite direction that  $\beta$ -catenin exhibited. In 71yM its expression gradually decreased in time dependent manner ( $p < 0.0001$ ), after 4 h of E2 treatment there were no expression (Figure 4A). Similar to that, in 47yM expression was found to be the lowest level at 4 h after E2 ( $p < 0.0001$ ) (Figure 4B). In 63yM, in contrast, its expression reached the highest level at 4 h after E2 ( $p < 0.0001$ ) (Figure 4C).



**Figure 2:** Demonstration of time-dependent DUX4 expression by western blotting in the presence of E2. (A) in 71yM, \*\*\*\*  $p < 0.0001$ , (B) in 63yM, (C) in 47yF. Control (untreated); 30 min (exposure to 10 nM E2 for 30 min); 4 h (exposure to 10 nM E2 for 4 h). In western blotting images; above DUX4, below  $\beta$ -actin had been demonstrated.

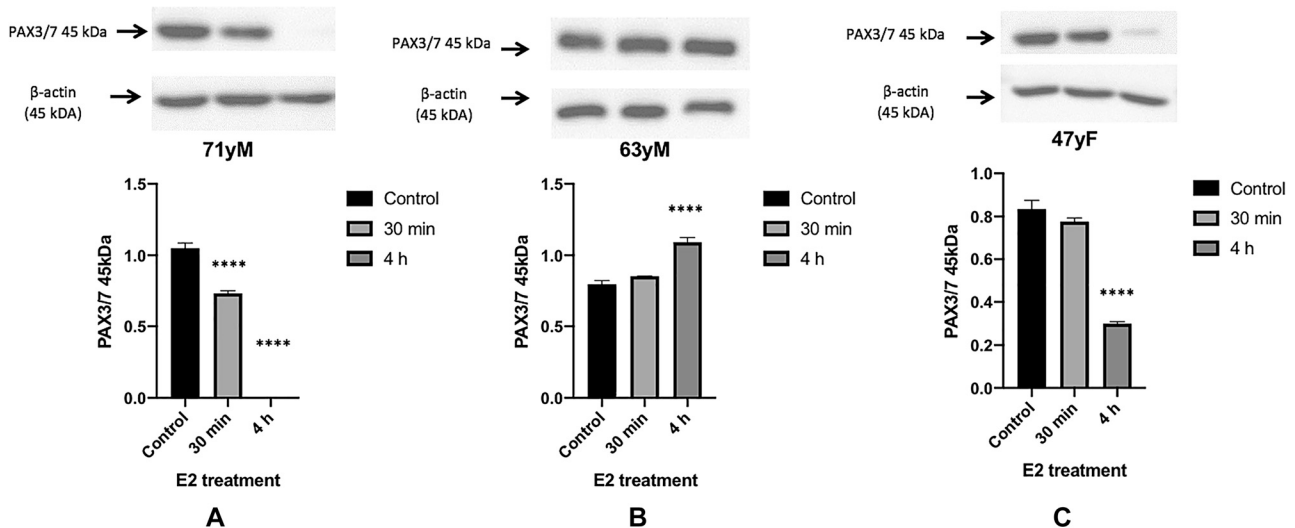


**Figure 3:** Demonstration of time-dependent  $\beta$ -catenin expression by western blotting in the presence of E2. (A) in 71yM, \*\*  $p=0.0084$ , \*  $p=0.0376$ , (B) in 63yM \*\*\*\*  $p<0.0001$ , (C) in 47yF \*  $p=0.0269$ , \*\*  $p=0.0022$ . Control (Untreated); 30 min (exposure to 10 nM E2 for 30 min); 4 h (exposure to 10 nM E2 for 4 h). In western blotting images; above  $\beta$ -catenin, below  $\beta$ -actin had been demonstrated.

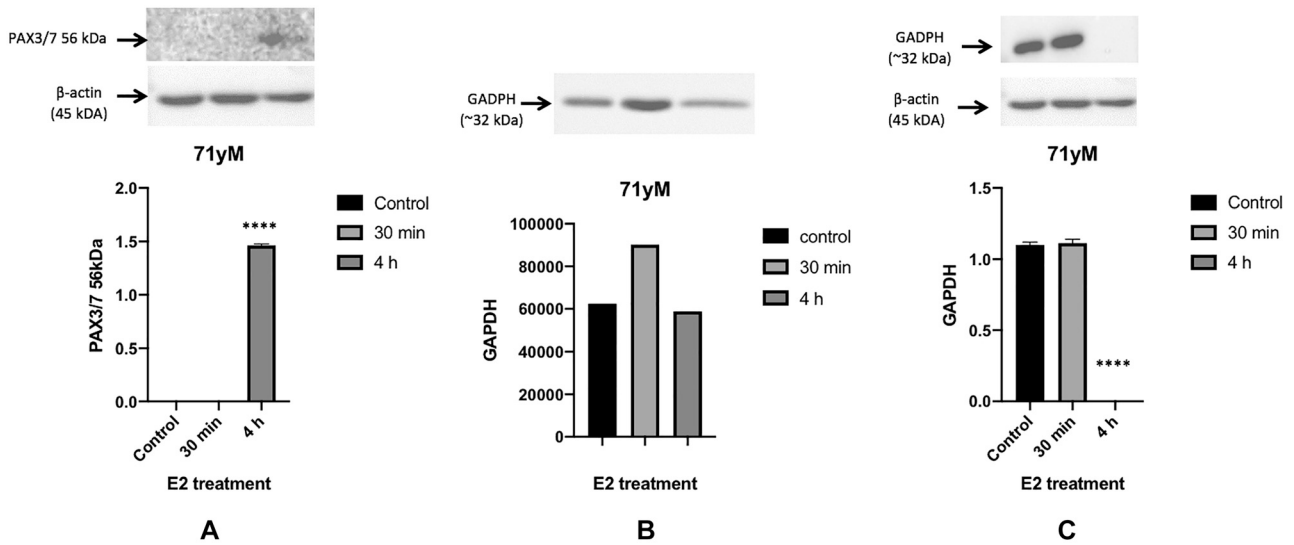
Additionally, in 71yM, expression of another PAX3/PAX7 variant which was 56 kDa had been observed after 4 h of E2 ( $p<0.0001$ ), while there were no expression of this protein neither in non-E2 treated myoblasts nor 30 min E2 treated myoblasts (Figure 5A). 56 kDa variant was observed to be expressed only in 71yM myoblasts similar to DUX4 of whose expression zeroized at same time point.

### The effect of E2 on GAPDH

GAPDH was used as housekeeping protein at first experiments. However, GAPDH protein bands were unexpectedly observed to be dependent to E2 treatment (Figure 5B). Repetition of GAPDH experiment via using  $\beta$ -actin as housekeeping protein, revealed that GAPDH expression



**Figure 4:** Demonstration of time-dependent 45 kDa PAX3 expression by western blotting in the presence of E2. (A) 71yM \*\*\*\*  $p<0.0001$ , (B) 45 kDa PAX3 variant in 63yM \*\*\*\*  $p<0.0001$ , (C) 45 kDa PAX3 variant in 47yF \*\*\*\*  $p<0.0001$ . Control (Untreated); 30 min (exposure to 10 nM E2 for 30 min); 4 h (exposure to 10 nM E2 for 4 h). In western blotting images; above PAX3, below  $\beta$ -actin had been demonstrated. Because PAX3 and  $\beta$ -actin have similar weight, stripping protocol had been applied.



**Figure 5:** (A) Demonstration of time-dependent 56 kDa PAX3/PAX7 expression by western blotting in the presence of E2 in 71yM \*\*\*\*  $p < 0.0001$ , (B) Demonstration of time-dependent GAPDH expression by western blotting in the presence of E2 without housekeeping protein, (C) Demonstration of time-dependent GAPDH expression \*\*\*\*  $p < 0.0001$ .

had clearly been affected from E2. After 4 h treatment GAPDH expression dramatically decreased in time dependent manner compared to control ( $p < 0.0001$ ) (Figure 5C). Therefore,  $\beta$ -actin had been replaced as housekeeping protein in continuing experiments.

## Discussion

In present study, the effect of E2 on FSHD myoblast had been researched through DUX4,  $\beta$ -catenin and PAX3/PAX7 protein levels. Teveroni et al. (2017) observed no change of DUX4 mRNA levels. On the other hand, after E2 treatment, downregulation of DUX4 target proteins (such as TRIM43 and ZSCAN4) had been revealed in differentiating FSHD myoblasts [20]. In our study, however, DUX4 protein level had shown to be affected by E2 treatment. After 4 h of E2, the expression of DUX4 was zeroised in myoblasts of 71yM. Inconsistency in the results of our and the previous study might arise from (i) using different cell types as myoblast cells vs. differentiating cells (ii) having different estrogen exposure times as 4 vs. 72 h and (iii) identifying different DUX4 structures as mRNA vs. protein. Despite divergent results, both of these studies underpin the protective role of estrogen in FSHD via DUX4.

In muscles of FSHD patients; DUX4 is expressed only in 1 of every 1,000 nuclei [28, 29] and usually not detectable in every FSHD patient cell line due to its low levels of expression [5]. In our study, DUX4 protein had been detected in untreated primary myoblasts of solely 71yM but

not in myoblasts of 63yM or 47yF. The reason why DUX4 expression is seen only in this patient may be due to the following differences: 71yM's Clinical Severity Score (CSS) was eight with highest score in between cell lines, on the other hand 63yM's score was 6 and 47yF's score was one, at the time of the biopsy. Also, variance in estrogen response between women and men might be conceivable additional factor effecting DUX4 expression in myoblasts. In 47yF, due to gender factor, relative higher estrogen response may suppress DUX4 expression. Another critical point to discriminate is the muscle group where the biopsy had been sampled. Muscle involvement of FSHD usually starts from the face and upper extremity; then proceeds to the lower extremity [30]. In 71yM, biopsy was taken from the deltoid muscle, while in 63yM and 47yF biopsies were taken from vastus lateralis muscle which is later involved at the progression of FSHD (Table 1). Because deltoid muscle is expected to be more severely affected than vastus lateralis, 71yM is expected to have higher DUX4 levels in which sampling was from deltoid muscle.

Temporal specific action of Wnt/ $\beta$ -catenin signaling has multiple functions in myogenesis including myoblast differentiation, muscle regeneration and satellite cell proliferation [31]. Role of  $\beta$ -catenin in FSHD pathophysiology is not completely clear yet, however, there are studies that investigated its function. In one study, opposite interplay had been suggested for DUX4 and  $\beta$ -catenin. It was concluded that DUX4 transcription was suppressed when Wnt/ $\beta$ -catenin signaling was activated in FSHD1 and FSHD2 primary human myoblasts [11]. Supporting this

result, Banerji et al. revealed that DUX4 disrupted Wnt/ $\beta$ -catenin signaling pathway in C2C12 mice myoblasts [10]. In a study with human peripheral blood mononuclear cells of FSHD patients, a significant decrease in both cytosolic and nuclear fraction of  $\beta$ -catenin was observed compared to the control group [12]. In present study we observed a decrease in DUX4 and an increase in  $\beta$ -catenin expression in 71yM via E2 treatment. This reverse relation of DUX4 and  $\beta$ -catenin is compatible with previous researches in literature and this response in 71yM with E2 treatment supported the protective role of estrogen. However, non-DUX4 expressing myoblasts (63yM, 47yF) exhibited divergent responses to E2. These divergent responses put  $\beta$ -catenin into a more complex position in FSHD pathophysiology.

There is not any expression of neither PAX3 nor PAX7 protein from the advanced stages of skeletal muscle differentiation. Expression of PAX3 and PAX7 is limited to undifferentiated muscle cells [32], that's why in our study, both PAX3 and PAX7 proteins were expected to be expressed in myoblasts. We observed two differently sized protein bands for PAX3 and PAX7 in western blotting as; 56 and 45 kDa in 71yM.

Since both PAX3 and PAX7 have 56 kDa splice forms, this band might belong to any of these forms. Strikingly, 56 kDa variant of PAX3/PAX7 expression had been stimulated 4 h after estradiol treatment, while DUX4 expression had been downregulated with estradiol treatment and there was no expression at the time of 4 h. 56 kDa PAX3/PAX7 response support the competition with DUX4 that was revealed by Bosnakovski et al. [5]. They also revealed when PAX3/PAX7 upregulated DUX4 is no longer toxic. Altogether, previously published studies together with our results support the protective role of estrogen by upregulating 56 kDa variant of PAX3/PAX7 expression and downregulating DUX4 expression.

Since PAX7 does not have a 45 kDa form, 45 kDa protein band corresponds to 44.8 kDa variant or 45.2 kDa variant of PAX3. This 45 kDa variant of PAX3 observed in all myoblasts (71yM, 63yM, 47yF) and exhibited divergent responses to estradiol treatment, interestingly just in an opposite direction to  $\beta$ -catenin responses. The divergent responses of PAX3 to estradiol might be explained with different variant expression which was not possible show technically. Not only this, but also there are differences both in the formation and development of facial and body muscles embryonically [33]. Therefore, different muscle groups may show different gene expression patterns. Good proof for that is a study with rats that investigated PAX3 and PAX7 levels in between extensor digitorum longus and in soleus muscles. As a result, PAX3 mRNA

levels were found to be similar to each other in different muscle groups, while PAX7 mRNA levels were not similar to each other [32]. Because of that, independent from the severity, sampling from different muscle origin from 71yM and 63yM/47yF might also explain the variance in the expression of DUX4 protein levels. However,  $\beta$ -catenin exhibited also divergent responses among cell lines. An additional possibility is the variations in the estrogen response element (ERE) sequence that affect the binding and the transcriptional activation [34] of PAX3 and  $\beta$ -catenin leading to divergent responses. Because each myoblast belongs to one individual's unique genome, genetic or epigenetic polymorphisms located in ERE sequences might potentially affect the responses.

GAPDH have six different spliced variants; 36.1 kDa (three variants), 31.5 kDa (two variants), 27.9 kDa (one variant). In our study, we observed a band profile at 32 kDa level. This result indicates that 31.5 kDa isoform(s) is expressed in FSHD myoblasts. Expression of 31.5 kDa variant of GAPDH protein isoform in FSHD myoblasts is a new knowledge for the literature. Additionally, this variant shows to be affected by estradiol treatment. Joe et al. found that GAPDH gene had the target region for 17- $\beta$  estradiol binding on rat brain due to the rapid and significant increases in GAPDH activity by addition of 10 nM estradiol [35]. This study might explain the response in FSHD myoblast cells. To reveal the effect clearly, by using  $\beta$ -actin and GAPDH together, we showed that in FSHD myoblasts, estradiol treatment clearly changed GAPDH protein levels. Because of this, following experiments had been performed by using  $\beta$ -actin as internal control instead of GAPDH. For future FSHD studies concerning estradiol treatment, we recommend not to use GAPDH as house-keeping protein.

## Limitations of the study and conclusion

With this novel study, it was revealed that estradiol could protect myoblasts via regulating DUX4, and estradiol can significantly modify  $\beta$ -catenin and PAX3/PAX7 levels in FSHD derived primary myoblasts. There were some limitations of present study: (i) primary myoblast cells are quite difficult to handle, especially when affection of a muscle disease would be considered. Additionally, there are limited period of passages to perform experiments before the differentiation of myoblasts. (ii) the levels of DUX4 protein is too low to detect especially in protein structure; (iii) neither  $\beta$ -catenin protein nor PAX3/PAX7 protein variants have not been studied with estradiol treatment in FSHD myoblasts, and there were not any

**Table 2:** Summary of the affection (increase:  $\blacktriangle$ /decrease:  $\blacktriangledown$ /no change: (-)/no expression: 0) of selected proteins in all cell lines after estradiol treatment.

	DUX4 (45 kDa)			$\beta$ -catenin (92 kDa)			PAX3/PAX7 (56 kDa)			PAX3 (45 kDa)			GAPDH (31.5 kDa)
	71yM	63yM	47yF	71yM	63yM	47yF	71yM	63yM	47yF	71yM	63yM	47yF	71yM
C-30 min	–	0	0	$\blacktriangle$	–	$\blacktriangle$	–	0	0	$\blacktriangledown$	–	–	–
C-4 h	$\blacktriangledown$	0	0	$\blacktriangle$	$\blacktriangledown$	$\blacktriangle$	$\blacktriangle$	0	0	$\blacktriangledown$	$\blacktriangle$	$\blacktriangledown$	$\blacktriangledown$

c, control; 30 min, 30 minutes; 4 h, 4 hours.

literature finding to compare our results during experimental period.

Even though an uphill struggle, we thought the best way to reflect our hypothesis would be using FSHD myoblasts and identified key transcription factors. We observed different responses at 30 min and 4 h with estradiol, because of this we concluded that the temporal timing is very important to design future studies including multiple time sections rather than only one temporally design. Otherwise, it would be impossible to capture zero level of DUX4 and stimulation of 56 kDa PAX3/PAX7 expression.

Level changes of all these transcription factors from different patients' cell lines at 30 min and 4 h after estradiol had been summarized in Table 2. In this table, each patient's myoblast exhibited completely divergent DUX4,  $\beta$ -catenin and PAX3/PAX7 responses with estradiol treatment. Knowing that each of these transcription factors have multiple targets, marks of distinct molecular signatures among FSHD patients will be highlighted better. These divergent molecular responses bear considerable resemblance to clinical variability of FSHD patients and that indicates a unique molecular underlying structure for each patient. Supporting this, in literature, studies on FSHD revealed that methylation status of D4Z4 is shown to be different among patients [36] in addition to D4Z4 contraction. There are also multiple other pathways are corrupted as mitochondrial dysfunction, oxidative stress [37] which can be driven by multiple other genetic and epigenetic factors. Conclusion here is that, it is necessary to evaluate FSHD clinical features and pathophysiology in combination of genetic, individual and environmental factors. As information is growing up, FSHD shifts to multifactorial disease rather than being a sole genetic disease.

Our major purpose in investigating for the role of estrogen in female and male FSHD patients was to contribute to the FSHD treatment for future generations. Estrogen is a candidate treatment option in FSHD. However, known potential risks as cancer and thromboembolism make estrogen treatment questionable. In our previous clinical

study on FSHD, we revealed approximately 12.5% of our FSHD patients had high fibrinogen levels [38]. That is why, it would be more logical to use local therapies or softer estrogen analogs. Supporting this Banerji et al. recently revealed that phytoestrogens as genistein rescued the impaired muscle phenotype in FSHD [39], which could be one of the potential therapeutic options for alleviating FSHD soon.

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**Competing interests:** The authors declare no conflict of interest.

**Informed consent:** Human biopsy samples were obtained from Rochester University Medical Center; informed consent for biopsy samples was obtained from all individual participants.

**Ethical approval:** All procedures performed in this study were in accordance with the ethical standards of Akdeniz University Institutional Ethical Committee (Decision Number: 284, Date: 28.10.2015) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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