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Transforming growth factor β1 impairs the transcriptomic response to contraction in myotubes from women with polycystic ovary syndrome

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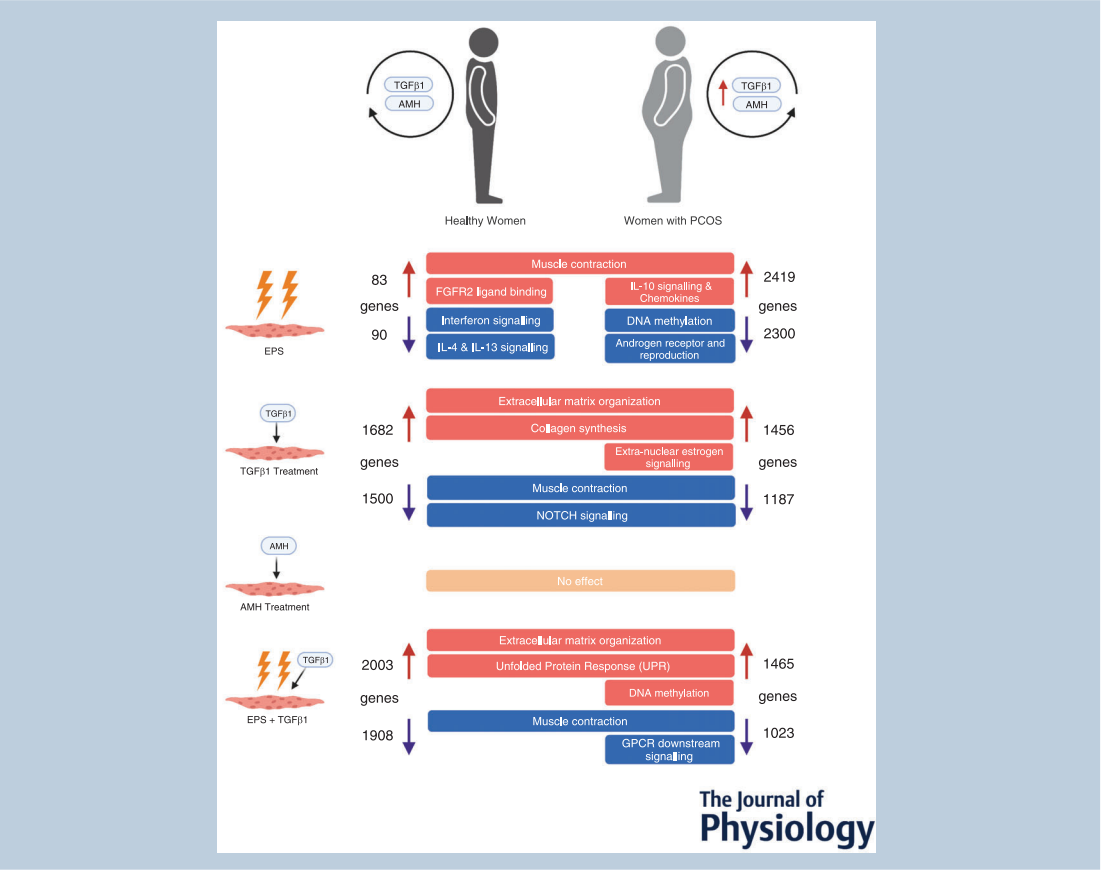

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Abstract Polycystic ovary syndrome (PCOS) is characterised by a hormonal imbalance affecting the reproductive and metabolic health of reproductive-aged women. Exercise is recommended as a first-line therapy for women with PCOS to improve their overall health; however, women with PCOS are resistant to the metabolic benefits of exercise training. Here, we aimed to gain insight into the mechanisms responsible for such resistance to exercise in PCOS. We employed an *in vitro* approach with electrical pulse stimulation (EPS) of cultured skeletal muscle cells to explore whether myotubes from women with PCOS have an altered gene expression signature in response to contraction. Following EPS, 4719 genes were differentially expressed (false discovery rate <0.05) in myotubes from women with PCOS compared to 173 in healthy women. Both groups included genes involved in skeletal muscle contraction. We also determined the effect of two transforming growth factor β (TGFβ) ligands that are elevated in plasma of women with PCOS, TGFβ1 and anti-Müllerian hormone (AMH), alone and on the EPS-induced response. While AMH (30 ng/ml) had no effect, TGFβ1 (5 ng/ml) induced the expression of extracellular matrix genes and impaired the exercise-like transcriptional signature in myotubes from women with and without PCOS in response to EPS by interfering with key processes related to muscle contraction, calcium transport and actin filament. Our findings suggest that while the fundamental gene expression responses of skeletal muscle to contraction is intact in PCOS, circulating factors like TGFβ1 may be responsible for the impaired adaptation to exercise in women with PCOS.

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Abstract figure legend Women with polycystic ovary syndrome (PCOS) have elevated levels of circulating transforming growth factor β (TGFβ) ligands, in particular TGFβ1 and anti-Müllerian hormone (AMH). Electrical pulse stimulation (EPS), a model of *in vitro* contraction, produced different transcriptomic responses in myotubes from healthy women and women with PCOS, as evidenced by changes in the number of genes and the associated Reactome pathways. EPS produced a pro-inflammatory response in myotubes from women with PCOS and supressed genes related to DNA methylation and reproduction. Treatment of myotubes with TGFβ1 resulted in increased expression of genes related to 'extracellular matrix organization' and 'collagen synthesis', and a downregulation of genes involved in 'muscle contraction' and 'NOTCH signalling' in both groups. Conversely, AMH had no effect. TGFβ1 treatment altered the response to EPS, resulting in the activation of genes related to 'extracellular matrix organization' and 'unfolded protein response' with the suppression of genes related to contractile function in both groups, while only altered 'DNA methylation' and 'GPCR signalling' pathways in myotubes from women with PCOS. Collectively, this highlights that dysregulated TGFβ1 signalling may influence skeletal muscle signalling in response to contraction and subsequent adaptations in women with PCOS.

Key points

- Gene expression responses to *in vitro* contraction (electrical pulse stimulation, EPS) are altered in myotubes from women with polycystic ovary syndrome (PCOS) compared to healthy controls, with an increased expression of genes related to pro-inflammatory pathways.
- Transforming growth factor β1 (TGFβ1) upregulates genes related to extracellular matrix remodelling and reduces the expression of contractile genes in myotubes, regardless of the donor's health status.
- TGFβ1 alters the gene expression response to EPS, providing a possible mechanism for the impaired exercise adaptations in women with PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is a complex endocrine condition that affects women of reproductive age. It is characterised by androgen excess (hyperandrogenism), ovulatory dysfunction and polycystic ovary morphology (Ehrmann, 2005). PCOS affects 8–13% of reproductive-aged women worldwide (Bozdag et al., 2016) and is associated with a number of morbidities including subfertility, insulin resistance (Cassar et al., 2016; Stepto et al., 2013), type 2 diabetes mellitus (T2DM) (Moran et al., 2010), psychological disorders (depression and anxiety) (Cooney et al., 2017), an increased risk of cardiovascular disease (Kakoly et al., 2019) and endometrial cancer (Dumesic & Lobo, 2013). While the aetiology of PCOS remains to be established, there are a number of proposed contributing factors such as genetic, environmental, circulating and *in utero* developmental programming factors (Franks et al., 2008; Sir-Petermann et al., 2002; Tata et al., 2018; Vázquez-Martínez et al., 2019).

Lifestyle intervention is a first-line therapy for women with PCOS to manage clinical features at the metabolic, reproductive and psychological level (Teede et al., 2018). Exercise is a key component of this lifestyle intervention (Stepto, Patten et al., 2019; Teede et al., 2018), inducing improvements in metabolic health. In particular, some of the exercise-induced molecular adaptations in skeletal muscle result in improvement in insulin sensitivity, increases in muscle mass, vascularisation, glucose transport and metabolism, and mitochondrial content (Egan & Zierath, 2013; Sylow & Richter, 2019). It has, however, been proposed that women with PCOS display an impaired response to exercise on metabolic health (Hansen et al., 2020; Harrison et al., 2012). Overweight women with PCOS display lesser improvements in insulin sensitivity compared to BMI-matched controls in response to 12 weeks of high intensity interval training (Harrison et al., 2012). Additionally, 14 weeks of mixed modality exercise training failed to improve glucose uptake, insulin signalling or peripheral blood flow in lean hyperandrogenic women with PCOS (Hansen et al., 2020). However, the precise mechanisms associated with the lack of such exercise-induced metabolic improvements remain elusive.

One of the numerous factors which could contribute to abnormal exercise responses in women with PCOS is the transforming growth factor β (TGFβ) signalling pathway. Dysregulated TGFβ signalling has been linked to the pathophysiology of PCOS, and in particular to the development of ovarian fibrosis and reproductive defects (Hatzirodos et al., 2011; Raja-Khan et al., 2014), and has been suggested to also affect peripheral tissues. Of interest, women with PCOS have elevated circulating levels of the TGFβ family ligands TGFβ1 and anti-Müllerian hormone (AMH) (Cassar et al., 2014; Irani et al., 2015; Raja-Khan et al., 2010; Sumbul et al., 2022; Tal et al., 2013). To the best of our knowledge, only one study to date conducted by our group has examined the effect of TGFβ signalling in skeletal muscle of women with PCOS (Stepto et al., 2020).

In individuals with T2DM, the mechanism behind reduced improvements in insulin sensitivity after exercise training has been attributed to TGFβ1 (Böhm et al., 2016). Low responders to exercise training have greater TGFβ1 activity, resulting in the suppression of AMP-activated protein kinase α2 (AMPKα2), ATP synthase subunit α, peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α), and mitochondrial transcription factor A (TFAM), key factors of the exercise-induced metabolic regulation (Böhm et al., 2016). A role of TGFβ signalling has also been proposed in women with PCOS following high-intensity exercise training, where exercise did not fully restore insulin sensitivity to levels comparable to overweight controls after the exercise training (Stepto et al., 2020). This modest effect of exercise was accompanied by an increased profibrotic gene expression profile associated with TGFβ signalling (Stepto et al., 2020), suggesting an effect of TGFβ signalling in exercise-induced adaptations.

Electrical pulse stimulation (EPS) represents an *in vitro* model of contraction in human primary and C2C12 myotubes (Nikolić et al., 2017). EPS increases the gene expression of myosin heavy chain 7 (*Myh7)* and 2 (*Myh2)*, and induces the expression of contraction-induced signal transduction proteins in primary myotubes similar to the *in vivo* exercise response (Son et al., 2019). Thus, EPS is considered an appropriate *in vitro* model to study the molecular responses to muscle contraction, and

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to explore how cell-autonomous factors influence these contraction-induced responses.

The present study sought to identify skeletal muscle pathways that may explain the aberrant responses to exercise observed in women with PCOS, and to determine whether TGFβ signalling plays a role in the altered response. We investigated the impact of two TGFβ ligands, TGFβ1 and AMH, on contraction-induced adaptations by applying *in vitro* EPS to myotubes from women with PCOS compared to healthy controls. We hypothesised that myotubes from women with PCOS would present with a different exercise-induced signature and that TGFβ signalling would play a major role in this adaptation.

Methods

Ethical approval

This study conformed to the standards set by the *Declaration of Helsinki*, except for registration in a database. Participants included in this study were part of the TGFβ-PCOS trial (ACTRN12618000155291) and the iHIT-PCOS trial (ACTRN12615000242527): https://www.anzctr.org.au/ (Australian New Zealand Clinical Trials Registry). Ethical approval was obtained from the Victoria University Human Research Ethics Committee (Reference HRE17-232), and all participants provided written informed consent prior to participation in the study.

Participants

Six overweight women with PCOS (body mass index (BMI) >25 kg/m^2) and six lean (BMI <25 kg/m^2) healthy women, all Caucasian, premenopausal and aged between 18 and 40 years, were included in this study (Supporting information Table S1). PCOS was diagnosed according to the Rotterdam Criteria (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004), and confirmed by an endocrinologist. The Rotterdam criteria required confirmation of two of the following: (i) oligo- or anovulation; (ii) clinical (hirsutism) and/or biochemical hyperandrogenism; and (iii) polycystic ovaries on ultrasound and exclusion of other causes of hyperandrogenism. The healthy control group consisted of women without any features of PCOS. The exclusion criteria included menopause or perimenopause, secondary causes of menstrual disturbance, pregnancy, smoking, type 1 or type 2 diabetes, uncontrolled hypertension ($>160/100$ mmHg), cardiovascular disease, established cardiovascular disease and use of medications that interfere with endpoints. All clinical measures and testing, including body composition assessment by dual energy X-ray absorptiometry (DXA) scan (iDXA GE Lunar Prodigy scanner; GE Healthcare, Milwaukee, WI, USA) and euglycaemic–hyperinsulinaemic clamps to assess insulin sensitivity were conducted after an overnight fast and in the early follicular phase of the menstrual cycle (days 1–7) for those participants with regular menstrual cycles, following the methods previously described (Moreno-Asso et al., 2022). Cardiorespiratory fitness level ($\dot{V}_{O_2peak}$) of all participants was assessed as previously described (Patten et al., 2022).

Muscle biopsies and primary myotube cultures

A muscle biopsy was obtained from the *vastus* lateralis using the modified Bergstrom technique (Bergström, 1975; Shanely et al., 2014) after an overnight fast. Following collection, approximately 40–50 mg of muscle was minced into small pieces ($<$1–2 mm^3), enzymatically dissociated with 0.05% Trypsin-EDTA (Thermo Fisher Scientific, Melbourne, Australia) and satellite cells selected using CD56$^+$ magnetic beads as described previously (Agley et al., 2013). Cells were cultured in growth medium (α-minimum essential medium (MEM) with 10% v/v fetal bovine serum, 0.5% v/v penicillin–streptomycin and 0.5% v/v amphotericin B) in coated flasks/plates with extracellular matrix gel (Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix, Thermo Fisher Scientific). Once cells reached 80% confluency, they were differentiated for 5 days in six-well plates using differentiation medium (α-MEM with 2% v/v horse serum, 0.5% v/v penicillin–streptomycin and 0.5% v/v amphotericin B) before starting the treatments.

TGFβ ligand treatments

To determine if the circulating factors TGFβ1 and AMH differently influence human primary myotube responses to *in vitro* EPS, the following six conditions were applied for 16 h in serum-free normal glucose media (α-MEM with 0.5% v/v penicillin–streptomycin and 0.5% v/v amphotericin B): (i) non-treated control (no treatment and no EPS); (ii) TGFβ1 (5 ng/ml); (iii) AMH (30 ng/ml); (iv) EPS; (v) EPS+TGFβ1 (5 ng/ml), and (vi) EPS+AMH (30 ng/ml) (Fig. 1).

Electrical pulse stimulation

Following the treatment, medium was removed and cells washed twice with phosphate-buffered saline and then placed in serum-free medium (α-MEM with 0.5% v/v penicillin–streptomycin and 0.5% v/v amphotericin B). Human primary myotubes were then stimulated with EPS using the C-Pace EP multichannel Culture Pacer (IonOptix, Milton, MA, USA) (Fig. 1), using a chronic low-frequency stimulation protocol of 11.5 V, 2 ms,

1 Hz for 6 h or remained unstimulated in serum-free medium for 6 h. This EPS protocol has been shown to activate exercise-like induced signal transduction improving glucose metabolism, promoting the secretion of myokines and causing structural changes in the myotubes (Nikolić et al., 2017).

RNA sequencing

Immediately following the 6 h treatment, total RNA was extracted using the Qiagen AllPrep DNA/RNA/miRNA universal kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quality of total RNA samples (500 ng) was assessed using the Agilent RNA 6000 Nano kit and Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA). Sequencing libraries were prepared according to Illumina TruSeq stranded total RNA with the Ribo-Zero Gold protocol (Illumina, San Diego, CA, USA) as previously described (Moreno-Asso et al., 2022). Qubit dsDNA HS assay kit (Thermo Fisher Scientific) was used for quantification of libraries, and quality control for base pair size and purity was examined using an Agilent high-sensitivity DNA chip and Bioanalyzer instrument (Agilent Technologies). Sequencing was performed on the NovaSeq 6000 (Illumina).

Bioinformatic analysis of RNA sequencing data

RNA-seq reads ($n \approx 36.8$ M) from FASTQ files were aligned using STAR (v2.7.2b) aligner (Dobin et al., 2013) with Ensembl (Cunningham et al., 2019) human annotation (GRCh38, release 98) resulting in 28 M reads on average (Supporting information, Fig. S1). One library (sample: P_c_EPS_A30_26) was excluded from downstream analysis due to low read number ($n = 137$). Gene coverages were counted using *featureCounts* (Liao et al., 2014) ($n \approx 23.7$) from the subread (v1.6.2) package. Genes with low expression were removed using the *filterByExpr* function from the edgeR package (Robinson et al., 2009) (v3.28.1), and thereby 19,231 genes were considered for differential expression analysis. A generalised linear model (GLM), *0 + group* (group is defined by merging experimental conditions 'Disease', 'EPS-treatment' and 'hormone-treatment'), was fitted by edgeR's (v3.32.1) *voomLmFit* function while blocking for participants in order to calculate differentially expressed genes with a false discovery rate (FDR) <0.05. Multidimensional scaling (MDS) plots were generated by using batch (participant ID) corrected logCPM values using the *removeBatchEffect* function from the edgeR package.

Pathway enrichment analysis

Gene set enrichment analysis (GSEA) was performed by using clusterProfiler (Yu et al., 2012) (v3.14.3) and Reactome database (ReactomePA R package (Yu & He, 2016), v1.30.10) with log 2-fold changes (logFC) calculated by the differential expression analysis. The REACTOME ontology terms with less than 10 and more than 500 genes were removed from the analysis. REACTOME ontology terms with q-value <0.05 were considered as enriched. The ontology terms with the genes were plotted as a network, CNET plots, with selected REACTOME ontology terms shown using the *cnetplot* function from the clusterProfiler package.

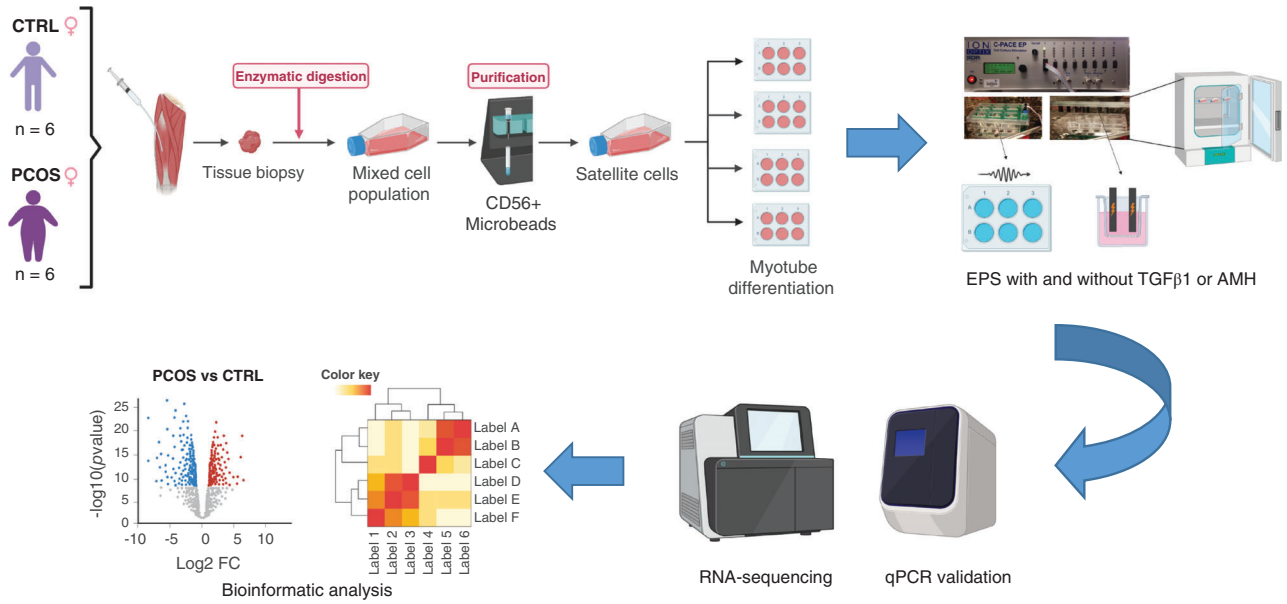

Figure 1. Schematic representation of the study design

Table 1. qRT-PCR primer sequences

Gene symbol	Primer sequence, 5′–3′	Accession no.
MYL2	F: GCTGAAGGCTGATTACGTTCG R: AGTCCAAGTTGCCAGTCACG	NM_000432.4
ACTA1	F: CACGATGTACCCTGGGATCG R: GCGGGGCGATGATCTTGA	NM_0 01100.4
TNNC2	F: CAGCAACCATGACGGACCA R: CCCAACTCCTTGACGCTGAT	NM_0 03279.3
IGF1	F: CCAAGACCCAGAAGGAAGTACA R: ACTCGTGCAGAGCAAAGGAT	NM_0 011 11284.2
COL7A1	F: AAAGGATGGAGTGCCTGGTATC R: TCCCCGTTCACCCTTGAG	NM_000094.4
COL10A1	F: CTTCTGCACTGCTCATCTG R: TATTCTCAGATGGATTCTGCGT	NM_000493.4
ACTB	F: GAGCACAGAGCCTCGCCTTT R: TCATCATCCATGGTGAGCTGGC	NM_0 01101.3
PPIA	F: GTCAACCCCACCGTGTTCTTC R: TTTCTGCTGTCTTTGGGACCTTG	NM_02 1130.4
B2M	F: TGCTGTCTCCATGTTTGATGTATCT R: TCTCTGCTCCCCACCTCTAAGT	NM_0 04048.2
GAPDH	F: AATCCCATCACCATCTTCCA R: TGGACTCCACGACGTACTCA	NM_0 012 89746.1
TBP	F: CAGTGACCCAGCAGCATCACT R: AGGCCAAGCCCTGAGCGTAA	NM_0 03194.4

F, forward primer; R, reverse primer.

Cluster analysis of expression patterns

To identify the similar gene expression patterns, the batch corrected logCPM values (see 'Bioinformatic analysis of RNA sequencing data') were *z*-score transformed across libraries, excluding AMH treated ones, and the average *z*-score was calculated by group. The *Clust* algorithm (v1.10.10) (Abu-Jamous & Kelly, 2018) was used to calculate the similar gene clusters, which resulted in 12 distinct clusters. The enriched gene ontology (GO) molecular function (MF) and biological process (BP) terms for the gene clusters 2 and 4, respectively, were identified by using over-representation analysis with a *q*-value cut-off below 0.05. Displayed GO terms were filtered using SimRel semantic similarity with a value of $C = 0.5$ to eliminate redundancy and prepared for visualisation using the REViGO tool (Supek et al., 2011; available online at http://revigo.irb.hr/).

Quantitative reverse transcription–PCR

Extracted mRNA was reverse transcribed using Bio-Rad iScript RT Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and a Thermocycler (Bio-Rad Laboratories). Quantitative reverse transcription–PCR (qRT-PCR) reactions were performed in duplicate using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories) and run in an Applied Biosystems QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific). Specific qRT-PCR forward and reverse primers were used for the amplification of selected genes (Table 1). Gene expression results were normalised using the geometric mean of the selected three most stable housekeeping genes (*PPIA* (cyclophilin), *B2M*, *GAPDH*) out of five analysed (*TBP*, *ACTB*, *PPIA*, *B2M*, *GAPDH*), using RefFinder (https://www.heartcure.com.au/reffinder/).

Statistical analyses

Statistical analysis for qRT-PCR gene expression was performed by two-way repeated measures of ANOVA with Bonferroni adjustment for multiple comparisons. Factors were *group* (CTRL, healthy controls; and PCOS, women with PCOS) and *treatment* (non-treated control, TGFβ1 treatment, AMH treatment, EPS treatment, and EPS+TGFβ1 treatment groups). This analysis was carried out using GraphPad Prism software version 8.2.1 (GraphPad Software Inc., La Jolla, CA, USA).

Results

EPS induces a greater gene expression response in myotubes from women with PCOS

Compared to the control group, women with PCOS showed profound insulin resistance and dysregulated

hormonal expression including hyperandrogenism (Supporting information Table S1). As these clinical features may influence skeletal muscle signalling in response to exercise, we aimed to assess changes in the myotube transcriptome in response to contraction in primary muscle cells from both women with PCOS and healthy controls using low-frequency EPS. An MDS plot of RNA-seq data displayed a clear separation by group (PCOS *vs*. healthy) and by EPS treatment (Fig. 2*A*), the latter being more apparent in myotubes from women with PCOS compared to myotubes from healthy women. We identified a total of 19 231 transcripts where 173 were differentially expressed following EPS in myotubes from healthy women (83 upregulated and 90 downregulated; FDR < 0.05) and 4719 differentially expressed (2419 upregulated and 2300 downregulated; FDR < 0.05) in myotubes from women with PCOS (Fig. 2*B* and Supporting information Dataset S1). GSEA using Reactome pathways database showed upregulation of genes involved in skeletal muscle contraction in both groups following EPS in first 20 enriched terms ranked by normalised enrichment score (NES) (Fig. 2*C* and Supporting information Dataset S2). Only in myotubes from women with PCOS, genes related to interleukin (IL)-10 signalling, extracellular ligands and chemokine binding, and cholesterol biosynthesis pathways were upregulated following EPS, while genes related to androgen receptor signalling, oxidative stress-induced senescence and epigenetic regulation of both DNA methylation and histone acetylation were downregulated (Fig. 2*D*). Relevant genes included in the IL-10 signalling and chemokines pathways are *CXCL10*, *TNF*, *IL1A*, *CXCL8*, *CCL20*, *PTGS2*, *CX3CL1*, *LIF*, *IL6* and *CSF3*, which were all highly induced following EPS (log 2-fold change greater than 1.6) (Fig. 2*D* and Supporting information Dataset S2). In contrast, myotubes from healthy women displayed a downregulation of genes related to several inflammatory pathways and immune responses after EPS treatment amongst the top 20 downregulated enriched terms (Fig. 2*C* and Supporting information Dataset S2). These results show that myotubes from women with PCOS display a greater responsiveness to EPS compared to myotubes from healthy women and support a pro-inflammatory response after muscle contraction in PCOS.

TGFβ1 upregulates genes related to extracellular matrix remodelling and reduces the expression of contractile genes in myotubes

To understand if elevated levels of TGFβ ligands could negatively affect skeletal muscle as observed in the reproductive tissues, we assessed changes in the transcriptomic profile in human primary myotubes following 16 h treatment with TGFβ1 or AMH. MDS plot of RNA-seq data showed a marked separation by TGFβ1 but no effect of AMH treatment on myotubes from both women with PCOS and healthy controls (Fig. 3*A*). We detected 1682 upregulated and 1500 downregulated genes (FDR < 0.05) in myotubes from healthy women following treatment with TGFβ1 (Fig. 3*B* and Supporting information Dataset S3). Similarly, in myotubes from women with PCOS there were 1456 upregulated and 1187 downregulated genes (FDR < 0.05) following TGFβ1 treatment (Fig. 3*B*). GSEA using the Reactome pathways showed upregulation of genes related to extracellular matrix remodelling, collagen synthesis and TGFβ signalling in both groups following TGFβ1 treatment (Fig. 3*C* and Supporting information, Dataset S4). In addition, TGFβ1 treatment resulted in the suppression of genes related to muscle contraction and NOTCH signalling in both groups (Fig. 3*C*). TGFβ1 treatment induced downregulation of genes associated to IL-6-type receptor ligand interactions and activation of matrix metalloproteinases and extra-nuclear oestrogen signalling only in myotubes derived from women with PCOS (Fig. 3*C*). Differential expression of a selection of genes (*ACTA1*, *MYL2*, *COL10A1* and *COL7A1*) from relevant enriched terms was validated by qRT-PCR (Fig. 3*D*). Altogether, these results suggest that TGFβ1 reprograms skeletal muscle cell cultures towards a less differentiated phenotype.

TGFβ1 inhibits the EPS-induced increase in genes related to myotube contractile functions

To test the hypothesis that TGFβ1 interferes with contraction-induced gene expression adaptations in muscle cells in PCOS, we examined the effects of TGFβ1 on the EPS treatment of primary myotubes from both women with PCOS and healthy controls. Differential gene expression detected with TGFβ1 and EPS compared with EPS alone in both groups was similar (healthy controls: 2003 upregulated and 1908 downregulated genes, FDR < 0.05; and PCOS: 1465 upregulated and 1023 downregulated, FDR < 0.05) (Fig. 4*A* and Supporting information Dataset S5). A selection of differentially expressed genes, *ACTA1*, *MYL2*, *TNNC2*, *IGF1*, *COL10A1* and *COL7A1*, was validated by qRT-PCR (Fig. 4*B*).

To get functional insight into the effect of TGFβ1 on the gene expression changes induced by *in vitro* contraction in human primary myotubes, we performed both GSEA and cluster analysis. GSEA showed an upregulation of genes involved in extracellular matrix remodelling and unfolded protein response/endoplasmic reticulum stress pathways and a downregulation of genes related to muscle contraction in both PCOS and healthy control groups (Fig. 4*C* and Supporting

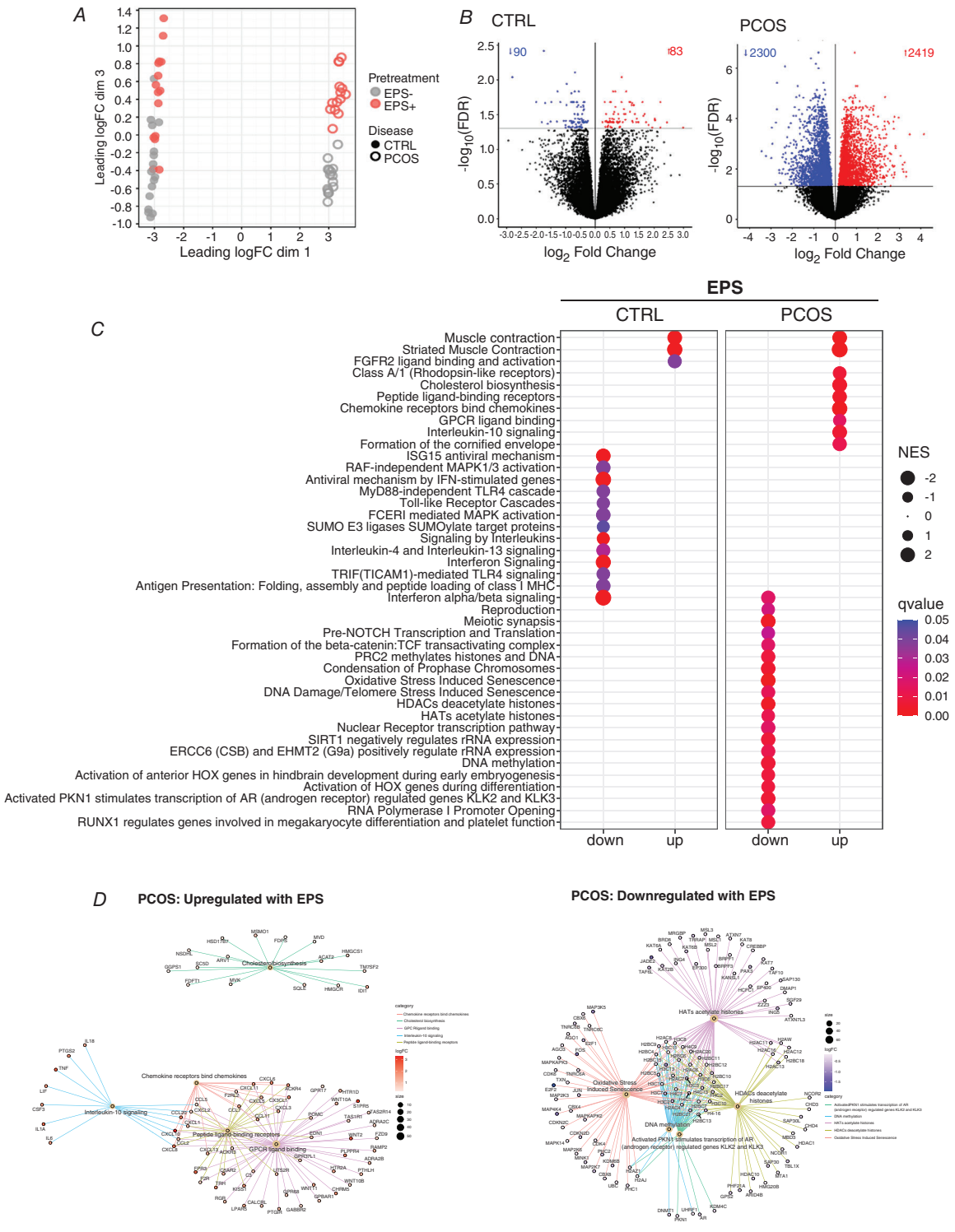

Figure 2. EPS-induced gene expression changes in primary myotubes
A, multidimensional scaling (MDS) plot of RNA-seq data from primary myotube cultures with (EPS+) and without (EPS−) EPS from women with PCOS (PCOS; n = 5 showing all data points for each condition) and healthy control women (CTRL; n = 5 showing all data points for each condition). *B*, volcano plot of all transcripts detected between myotubes with EPS and without in women with PCOS and healthy controls, with significantly upregulated genes (red) and downregulated genes (blue) highlighted (FDR <0.05). *C*, top 20 upregulated and downregulated Reactome pathways from GSEA in myotubes with EPS, in both women with PCOS and healthy controls (q-value <0.05). *D*, CNET plots showing selected significantly enriched Reactome ontology terms after EPS treatment within five selected upregulated and downregulated pathways, respectively, in myotubes from women with PCOS.

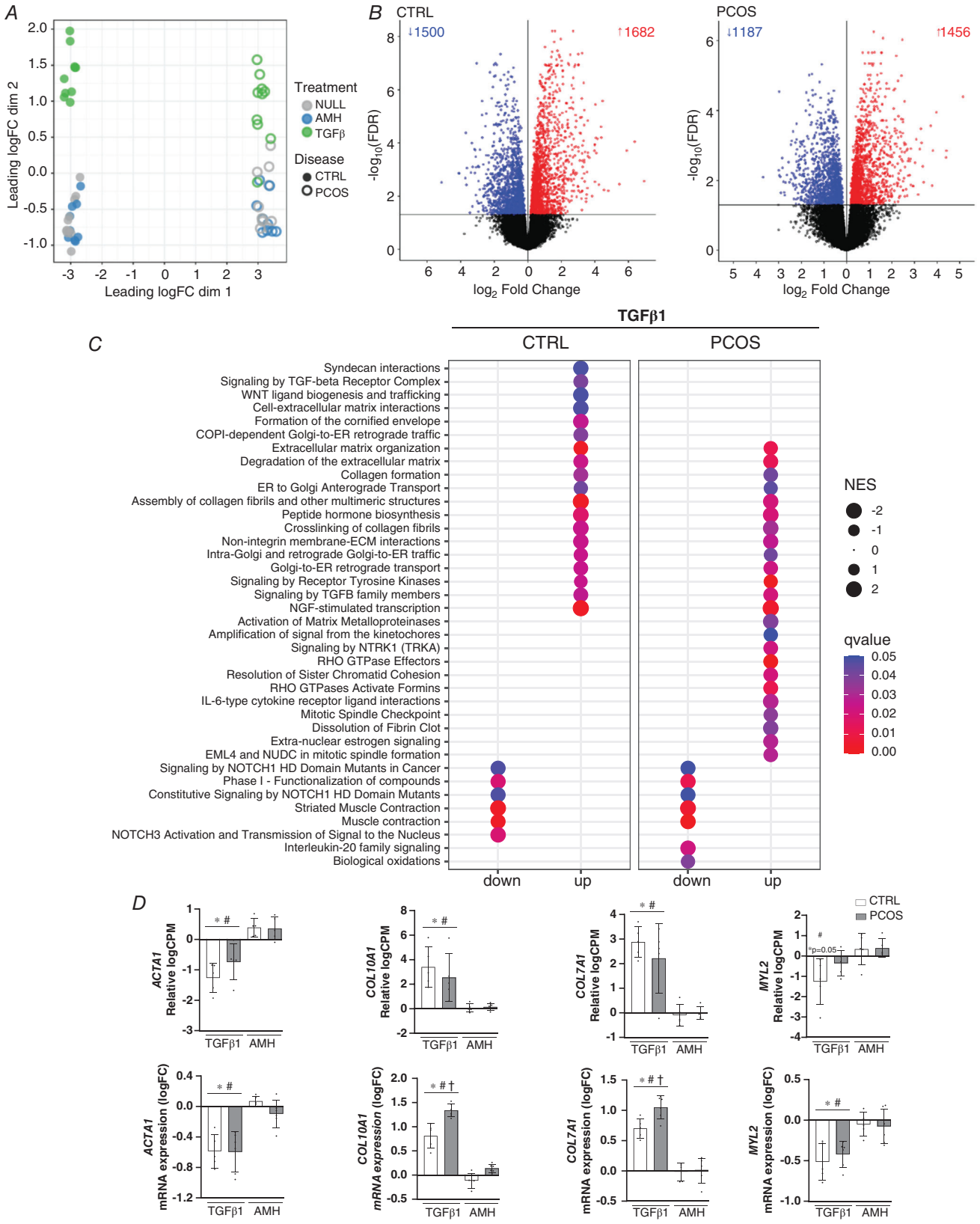

Figure 3. Effect of TGFβ1 and AMH treatment on primary myotubes
A, multidimensional scaling (MDS) plot of RNA-seq data from primary myotubes treated with TGFβ1, AMH or with no treatment (NULL) from women with PCOS (PCOS; n = 5 showing all data points for each treatment) and

healthy control women (CTRL; n = 5 showing all data points for each treatment). *B*, volcano plot of all transcripts detected between myotubes with TGFβ1 in women with PCOS and healthy controls, with significantly upregulated genes (red) and downregulated genes (blue) highlighted (FDR <0.05). *C*, top 20 upregulated and downregulated Reactome pathways from GSEA in myotubes treated with TGFβ1, in both women with PCOS and healthy controls (q-value <0.05). *D*, gene expression of selected differentially expressed genes with TGFβ1 and AMH. Relative logCPM indicates RNA-seq counts per million relative to non-treated samples levels from myotubes from PCOS women and healthy controls, respectively. Validation of mRNA expression levels by qRT-PCR is shown as logarithm of fold change (logFC) over non-treated sample. Values are represented as means ± SD (CTRL, n = 5; in white) and PCOS women (PCOS, n = 6; in grey). $*P_{adj} < 0.05$ TGFβ1 *vs*. no-treatment; $\#P_{adj} < 0.05$ TGFβ1 *vs*. AMH treatment within each group; $\dagger P_{adj} < 0.05$ TGFβ1 between groups.

information Dataset S6). In addition, myotubes derived from women with PCOS displayed an upregulation of genes related to DNA methylation and histone acetylation, and a downregulation of genes related to GPCR signalling and fatty acid metabolism, which was not observed in the healthy control group (Fig. 4*C*). Cluster analysis revealed 12 groups of genes with similar expression pattern (Fig. 5*A* and Supporting information Dataset S7). We identified three clusters of genes, labelled 2, 4 and 11, whose expression was activated by EPS but were downregulated when TGFβ1 treatment was added in both primary myotubes from women with PCOS and healthy controls (Fig. 5*A*). Conversely, expression of genes in clusters 3, 6 and 9 was higher when TGFβ1 was added to the EPS (Fig. 5*A*). Consistent with our GSEA findings of the RNA-seq data, over-representation analysis of these clusters using gene ontology, biological processes (GO:BP) or molecular function (GO:MF), respectively, revealed that genes in cluster 2 were involved in SMAD/activin signalling and phosphatidylinositol 3-kinase (PI3K) binding (Fig. 5*B* and Supporting information Dataset S8), while genes in cluster 4 were related to muscle contraction, muscle development and myotube differentiation, actin-filament based movement, and cellular calcium ion transport (Fig. 5*C* and Supporting information Dataset S8). No enriched pathways were found for genes in clusters 3, 6, 9 and 11. Collectively, these results show that the presence of TGFβ1 during muscle contraction affects the normal gene expression response such as pathways controlling intracellular signalling and constituents of the contraction apparatus.

Discussion

Here, we have used an EPS model of *in vitro* muscle contraction to map the transcriptomic response to contraction in primary myotubes from women with PCOS compared to that in healthy controls, and also examined the effect of TGFβ1 and AMH. We show that primary myotubes from women with PCOS display a greater responsiveness to EPS with a large pro-inflammatory response compared to healthy control women. Our results show that TGFβ1, but not AMH, alters the response to EPS by impairing contractile functions in primary myotubes from both groups of women and uniquely affects different pathways in each group.

Exercise activates inflammatory related pathways in human skeletal muscle, which promotes tissue repair and adaptation (Louis et al., 2007; Peake et al., 2017; Powers & Jackson, 2008). In our *in vitro* contraction model, we found that following EPS, the expression genes related to IL-10 signalling and chemokines was increased in myotubes from women with PCOS. In contrast, we did not see this response in myotubes from healthy women, despite EPS having previously been shown to induce the expression of genes related to interleukin and chemokine signalling in primary myotubes from healthy subjects (Raschke et al., 2013; Scheler et al., 2013). This signalling has been associated *in vivo* with increased secretion of myokines, such as CX3CL1 and CCL2, involved in exercise adaptive processes such as tissue repair and hypertrophy (Catoire et al., 2014; Hoffmann & Weigert, 2017; Raschke et al., 2013; Scheler et al., 2013). In response to EPS in myotubes from healthy women, we also observed a reduction in inflammatory related pathways including IL-4 and IL-13 signalling pathways. Genes associated with these pathways are linked with TNFα signalling, fibrosis and immune responses (Distler et al., 2019). In line with this, it has been previously shown that 8 h of EPS of myotubes from healthy subjects produces an anti-inflammatory effect by preventing TNFα-induced inflammatory signalling (Lambernd et al., 2012). Thus, the pro-inflammatory response to contraction of myotubes derived from women with PCOS may be a pathophysiological feature of PCOS aggravating a maladaptive response to exercise.

Comparing the gene expression response to contraction between PCOS and controls, we also observed a reduction in the expression of genes related to androgen signalling and epigenetic regulation by EPS in primary myotubes from women with PCOS. Consistent with the described hyperandrogenism in PCOS, an upregulation of androgen receptor signalling genes has been observed in the skeletal muscle of women with PCOS (Manti et al., 2020). Remarkably, following exercise training, women with PCOS exhibit a reduction in free androgen index and total testosterone levels (Patten et al., 2020). Our study, therefore, highlights a potential effect of muscle

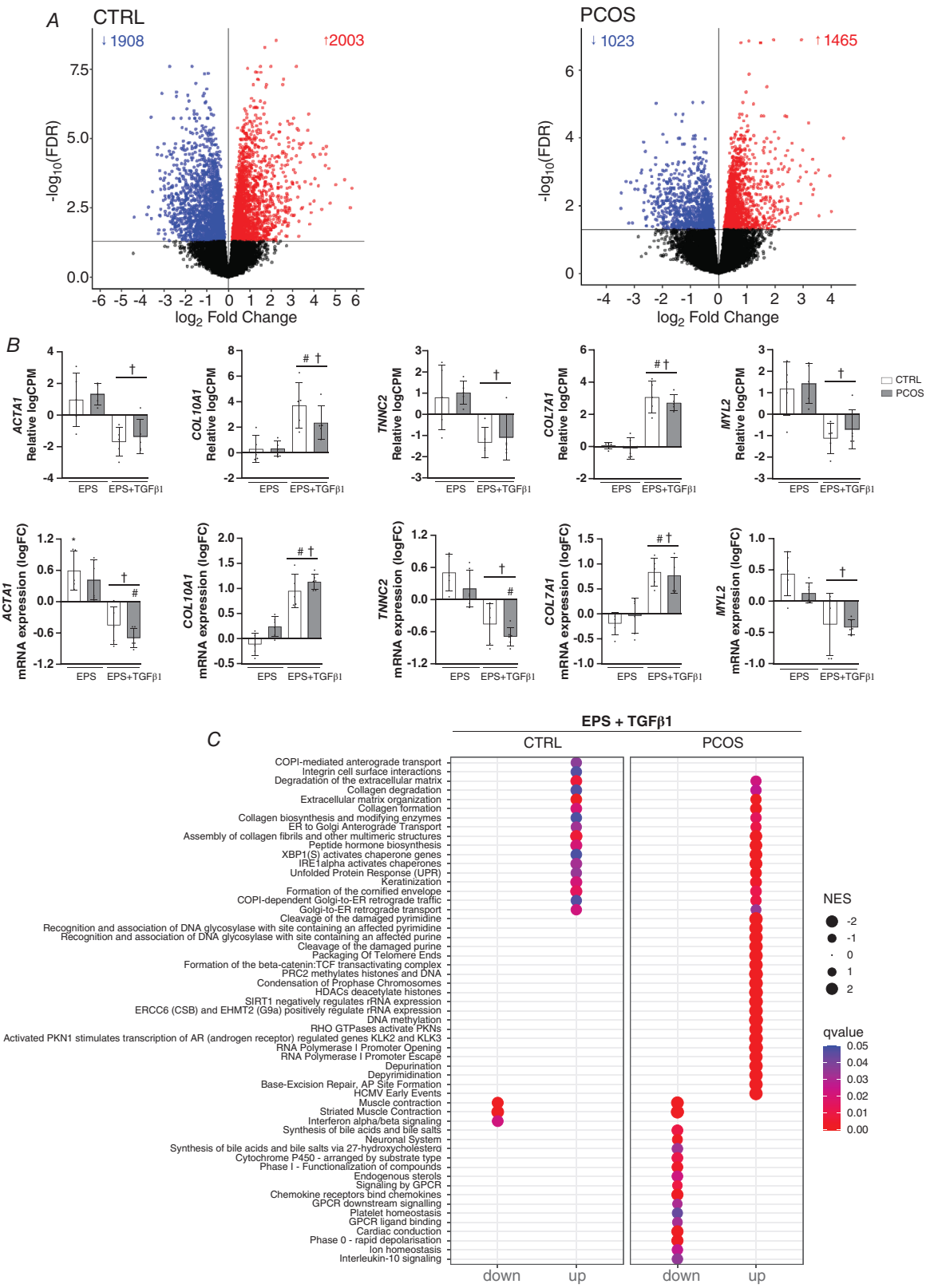

Figure 4. TGFβ1 inhibits the EPS-induced expression of contractile related genes, and increases expression of genes associated to DNA methylation in myotubes from women with PCOS
A, volcano plot of all transcripts detected between myotubes treated with EPS + TGFβ1 compared to EPS alone in women with PCOS (n = 5) and healthy controls (CTRL; n = 5), with significantly upregulated genes (red) and

downregulated genes (blue) highlighted (FDR < 0.05). *B*, gene expression of selected differentially expressed genes with EPS and EPS + TGFβ1. Relative logCPM indicates RNA-seq counts per million relative to non-treated samples levels from myotubes from PCOS women and healthy controls, respectively. Validation of mRNA expression levels by qRT-PCR is shown as logarithm of fold change (logFC) over non-treated sample. Values are represented as means ± SD (CTRL, n = 5; in white) and PCOS women (PCOS, n = 6; in grey). *C*, top 20 upregulated and downregulated Reactome pathways from GSEA in myotubes treated with TGFβ1 and EPS compared to EPS alone, in both women with PCOS and healthy controls (q-value <0.05). $*P_{adj} < 0.05$ EPS *vs*. no-treatment; $\#P_{adj} < 0.05$ EPS+TGFβ1 *vs*. no-treatment; $\dagger P_{adj} < 0.05$ EPS+TGFβ1 *vs*. EPS.

contraction by regulating androgen receptor signalling and thus supporting the role of exercise in improving hyperandrogenism. Of note, we found that the androgen receptor signalling pathway was downregulated in myotubes from women with PCOS following EPS. The associated ontology term included core histone genes (*H2A*, *H2B*, *H3B*, *H3C*, *H4* and *H4C*) which were shared across other downregulated terms associated with cellular senescence, DNA methylation and histone acetylation. In line with our findings, exercise-induced DNA hypomethylation has already been shown to occur immediately post-exercise in skeletal muscle (Barrès et al., 2012), with a similar response occurring in myotubes following EPS (Pattamaprapanont et al., 2016). Although our analysis was conducted in primary myotubes that were lysed immediately after EPS, we only observed this decrease in genes related to DNA methylation in myotubes derived from women with PCOS and not in those from healthy women. The downregulation of genes related to histone acetylation has also been reported to occur in response to metabolic stress in C2C12 myotubes (Jo et al., 2020), and has been suggested to promote expression of genes related to cell survival, whilst downregulating genes related to cellular functions that require large amounts of energy (Jo et al., 2020). Total histone 3 (H3) gene and protein expression are downregulated in skeletal muscle following acute resistance exercise, contributing to the dissembling of nucleosomes to allow transcriptional activation (Lim et al., 2020). Together with our data, evidence from the literature suggests that muscle contraction induces a greater metabolic stress and more disrupted oxidative homeostasis in PCOS compared to controls, resulting in the regulation of histones and epigenetic factors to favour cell adaptations through the regulation of transcription.

The differential gene expression profile in response to EPS between myotubes from women with PCOS and healthy controls in our study may not only be explained by the presence of intrinsic PCOS mechanisms but also associated, in part, to other retained characteristics from the *in vivo* phenotype, such as the fitness/training level of the donors (Bourlier et al., 2013). Indeed, in our study, women with PCOS had significantly lower cardiorespiratory fitness levels ($\dot{V}_{O_2peak}$) compared to healthy control women (Supporting information Table S1). Thus, a potential mechanism for the lack of gene expression plasticity may be through the existence of a skeletal muscle epigenetic memory from previous exercise training (Seaborne et al., 2018). However, we have previously shown that skeletal muscle-derived myotubes from women with PCOS do not retain *in vivo* impairments in energy metabolism or the altered expression of genes related to mitochondrial function, which are associated to exercise-induced adaptations (Moreno-Asso et al., 2022). Thus, a limitation of this study is the lack of an additional control group of myotubes from sedentary overweight women, which may have allowed for better identification of the differences that occur due to PCOS, excluding the influence of different fitness levels and obesity.

We also determined if elevated levels of TGFβ1 or AMH, similar to those observed *in vivo* in women with PCOS, would influence basal and EPS-induced gene expression. We previously proposed that AMH, which is associated with peripheral insulin resistance in women with PCOS, might be involved in tissue cross-talk to influence skeletal muscle metabolism (Nardo et al., 2009; Sahmay et al., 2018; Stepto, Moreno-Asso et al., 2019; Wiweko & Susanto, 2017). However, treatment with AMH did not affect gene expression in primary myotubes from either healthy women or women with PCOS in our study. Thus, our data indicate that AMH does not play a direct role in muscle metabolism or exercise-induced metabolic adaptations, at least in a cell autonomous system, despite its involvement in reproductive defects and proposed role in whole-body insulin resistance in women with PCOS (Wiweko et al., 2018).

In contrast to the lack of transcriptional alterations by AMH, TGFβ1 induced robust and similar responses in the gene expression profile of myotubes from both healthy women and women with PCOS despite intrinsic differences between donor groups, such as the presence of obesity. TGFβ1 increased gene expression of several extracellular matrix-related pathways, which can be linked to adverse tissue remodelling such as fibrosis and muscle pathologies (Ismaeel et al., 2019). Typically, TGFβ1 and tissue fibrosis are associated with increased collagen accumulation, in particular collagens 1 and 3 (Williams et al., 2015). However, our data show an increase after TGFβ1 treatment of gene expression of collagens 7 and 10 (*COL7A1* and *COL10A1*), which act as anchoring fibrils and network-forming collagens, respectively (Kaur & Reinhardt, 2015). These findings are consistent with our previous data showing no change in collagens 1 and 3 in primary myotubes treated with TGFβ1 (McIlvenna et al., 2021). This atypical response may be related to the 2D cell

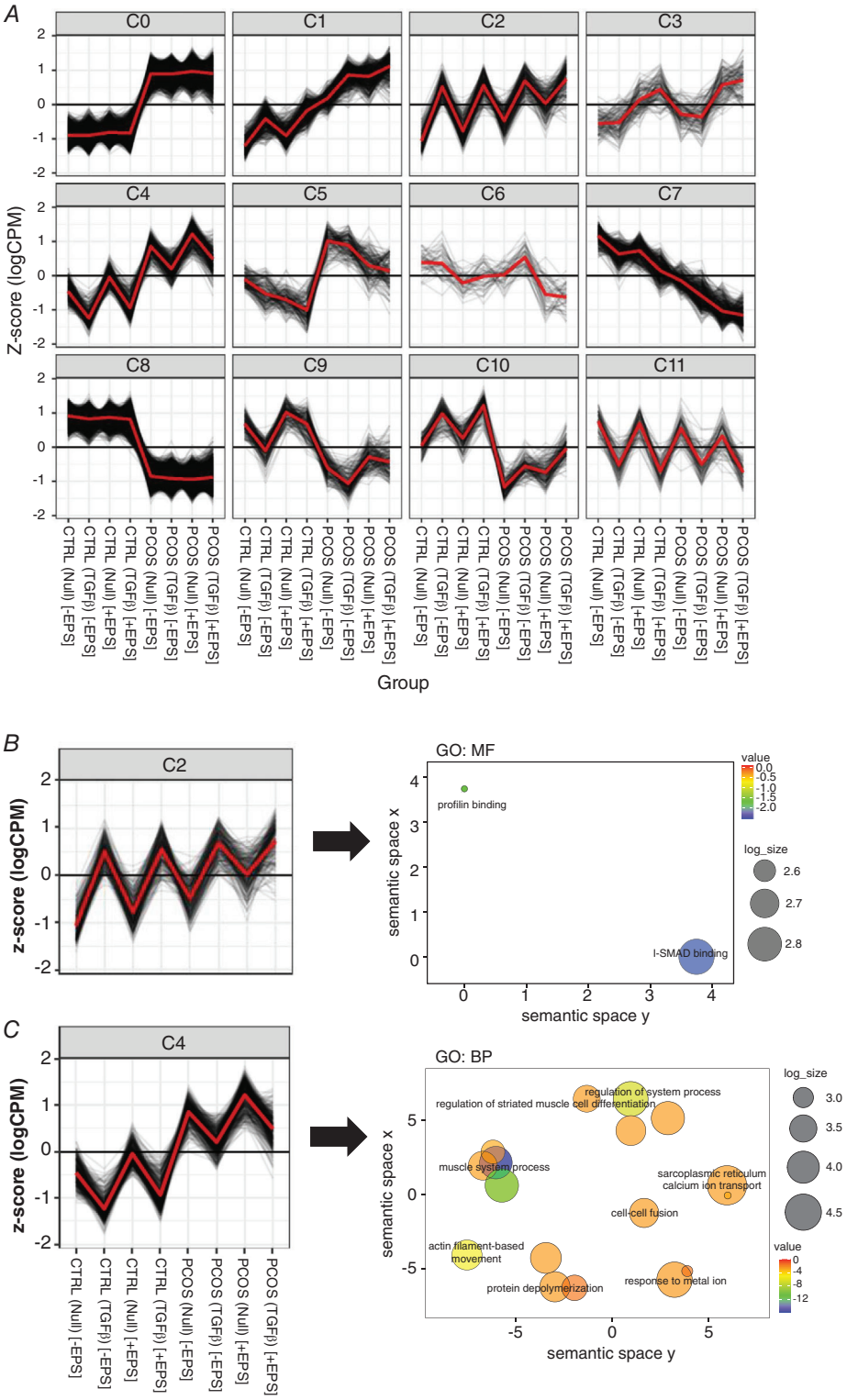

Figure 5. Cluster analysis of gene expression levels in myotubes with the different conditions
A, trend plots showing expression pattern of genes (FDR < 0.05) for the different conditions (no treatment ((Null)[−EPS]); TGFβ1 ((TGFβ)[−EPS]); EPS ((Null)[+EPS]); EPS + TGFβ1 ((TGFβ)[+EPS]) in both myotubes from women with PCOS (n = 5) and healthy controls (CTRL; n = 5). *B* and *C*, trend plots for cluster 2 and 4, respectively, and scatter plots representing enriched pathways obtained from over-representation analysis for each of the clusters using Gene Ontology – biological processes (GO:BP) or molecular function (GO:MF), respectively.

culture set-up, which does not fully capture the structural and dynamic complexities of the extracellular matrix *in vivo* (Li & Kilian, 2015; Nicolas et al., 2020). We and others have previously shown that skeletal muscle and cultured myotubes derived from women with PCOS have altered transcriptomic signatures, which include upregulation of genes related to extracellular matrix remodelling and collagens (Moreno-Asso et al., 2022; Nilsson et al., 2018; Stepto et al., 2020). Thus, taking into consideration that TGFβ1 levels are increased in women with PCOS (Irani et al., 2015; Raja-khan et al., 2010; Raja-khan et al., 2014; Tal et al., 2013), findings from our study support a role of TGFβ1 as a casual factor for the extracellular matrix dysregulation in women with PCOS.

In the present study, we also investigated whether TGFβ1 may be responsible for the aberrant metabolic adaptations to exercise observed in women with PCOS (Hansen et al., 2020; Stepto et al., 2020). We found that, regardless of the presence or absence of PCOS and intrinsic differences in the donor health status, TGFβ1 impairs the exercise-like gene expression signature in myotubes in response to EPS by interfering with key processes related to muscle contraction, calcium transport and actin filament, which all play a role in exercise-induced adaptations. This reduction in the expression of genes of the contractile apparatus was observed with TGFβ1 treatment alone and persisted after adding EPS. Our findings are in agreement with a recent study showing impaired muscle function induced by TGFβ1 injection in mice during muscle regeneration, and a disruption of genes regulating actin dynamics, affecting cell fusion/differentiation (Girardi et al., 2021). Similarly, excess TGFβ1 in mice leads to phosphorylation of SMAD3, increased NADPH oxidase 4 (NOX4) gene and protein expression and reactive oxygen species production in skeletal muscle, causing muscle weakness and reducing the intracellular calcium signalling needed for muscle contraction (Waning et al., 2015). In addition, excess SMAD signalling contributes to skeletal muscle and cardiac dysfunction in models of muscular dystrophy via calcium handling pathways (Goldstein et al., 2014). These studies support our findings showing a substantial increase in NOX4 gene expression and SMAD signalling in myotubes from both groups after EPS when treated with TGFβ1. Contrary to previous findings in myotubes from T2DM subjects showing a link between TGFβ1 and impairments in exercise-induced skeletal muscle mitochondrial metabolism (Böhm et al., 2016), we did not detect any reduced expression of mitochondrial genes with TGFβ1 in myotubes derived from either women with PCOS or healthy controls. This suggests a distinctive effect of TGFβ1 in skeletal muscle of women with PCOS compared to that in individuals with T2DM.

In conclusion, our study provides evidence that the gene expression response to contraction is altered in cultured primary myotubes from women with PCOS compared to healthy controls, with an increased expression of genes related to pro-inflammatory pathways in PCOS. We identified that TGFβ1 inhibits the gene expression response to *in vitro* contraction, providing further insight into the possible negative influence of TGFβ signalling on the gene expression response after exercise in PCOS. Elevated circulating levels of TGFβ1 may represent a mechanism by which the exercise response of skeletal muscle on gene expression plasticity is impaired in women with PCOS.

References

Abu-Jamous, B., & Kelly, S. (2018). Clust: Automatic extraction of optimal co-expressed gene clusters from gene expression data. *Genome Biology*, **19**(1), 172.

Agley, C. C., Rowlerson, A. M., Velloso, C. P., Lazarus, N. R., & Harridge, S. D. R. (2013). Human skeletal muscle fibroblasts, but not myogenic cells, readily undergo adipogenic differentiation. *Journal of Cell Science*, **126**, 5610–5625.

Barrès, R., Yan, J., Egan, B., Treebak, J. T., Rasmussen, M., Fritz, T., Caidahl, K., Krook, A., O'Gorman, D. J., & Zierath, J. R. (2012). Acute exercise remodels promoter methylation in human skeletal muscle. *Cell Metabolism*, **15**(3), 405–411.

Bergström, J. (1975). Percutaneous needle biopsy of skeletal muscle in physiological and clinical Research. *Scandinavian Journal of Clinical and Laboratory Investigation*, **35**(7), 609–616.

Böhm, A., Hoffmann, C., Irmler, M., Schneeweiss, P., Schnauder, G., Sailer, C., Schmid, V., Hudemann, J., Machann, J., Schick, F., Beckers, J., Hrabě de Angelis, M., Staiger, H., Fritsche, A., Stefan, N., Nieß, A. M., Häring, H.-U., & Weigert, C. (2016). TGF-β contributes to impaired exercise response by suppression of mitochondrial key regulators in skeletal muscle. *Diabetes*, **65**(10), 2849–2861.

Bourlier, V., Saint-Laurent, C., Louche, K., Badin, P. M., Thalamas, C., de Glisezinski, I., Langin, D., Sengenes, C., & Moro, C. (2013). Enhanced glucose metabolism is preserved in cultured primary myotubes from obese donors in response to exercise training. *Journal of Clinical Endocrinology and Metabolism*, **98**(9), 3739–3747.

Bozdag, G., Mumusoglu, S., Zengin, D., Karabulut, E., & Yildiz, B. O. (2016). The prevalence and phenotypic features of polycystic ovary syndrome: A systematic review and meta-Analysis. *Human Reproduction*, **31**(12), 2841–2855.

Cassar, S., Misso, M. L., Hopkins, W. G., Shaw, C. S., Teede, H. J., & Stepto, N. K. (2016). Insulin resistance in polycystic ovary syndrome: A systematic review and meta-analysis of euglycaemic–hyperinsulinaemic clamp studies. *Human Reproduction*, **31**(11), 2619–2631.

Cassar, S., Teede, H. J., Moran, L. J., Joham, A. E., Harrison, C. L., Strauss, B. J., & Stepto, N. K. (2014). Polycystic ovary syndrome and anti-Müllerian hormone: Role of insulin resistance, androgens, obesity and gonadotrophins. *Clinical Endocrinology*, **81**(6), 899–906.

Catoire, M., Mensink, M., Kalkhoven, E., Schrauwen, P., & Kersten, S. (2014). Identification of human exercise-induced myokines using secretome analysis. *Physiological Genomics*, **46**(7), 256–267.

Cooney, L. G., Lee, I., Sammel, M. D., & Dokras, A. (2017). High prevalence of moderate and severe depressive and anxiety symptoms in polycystic ovary syndrome: A systematic review and meta-analysis. *Human Reproduction*, **32**(5), 1075–1091.

Cunningham, F., Achuthan, P., Akanni, W., Allen, J., Amode, M. R., Armean, I. M., Bennett, R., Bhai, J., Billis, K., Boddu, S., Cummins, C., Davidson, C., Dodiya, K. J., Gall, A., Girón, C. G., Gil, L., Grego, T., Haggerty, L., Haskell, E., …, Flicek, P. (2019). Ensembl 2019. *Nucleic Acids Research*, **47**(D1), D745–D751.

Distler, J. H., Györfi, A. H., Ramanujam, M., Whitfield, M. L., Königshoff, M., & Lafyatis, R. (2019). Shared and distinct mechanisms of fibrosis. *Nature Reviews Rheumatology*, **15**(12), 705–730.

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics*, **29**(1), 15–21.

Dumesic, D. A., & Lobo, R. A. (2013). Cancer risk and PCOS. *Steroids*, **78**, 782–785.

Egan, B., & Zierath, J. R. (2013). Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metabolism*, **17**(2), 162–184.

Ehrmann, D. A. (2005). Polycystic ovary syndrome. *New England Journal of Medicine*, **352**(12), 1223–1236.

Franks, S., Webber, L. J., Goh, M., Valentine, A., White, D. M., Conway, G. S., Wiltshire, S., & McCarthy, M. I. (2008). Ovarian morphology is a marker of heritable biochemical traits in sisters with polycystic ovaries. *Journal of Clinical Endocrinology and Metabolism*, **93**(9), 3396–3402.

Girardi, F., Taleb, A., Ebrahimi, M., Datye, A., Gamage, D. G., Peccate, C., Giordani, L., Millay, D. P., Gilbert, P. M., Cadot, B., & Le Grand, F. (2021). TGFβ signaling curbs cell fusion and muscle regeneration. *Nature Communication*, **12**(1), 750.

Goldstein, J. A., Bogdanovich, S., Beiriger, A., Wren, L. M., Rossi, A. E., Gao, Q. Q., Gardner, B. B., Earley, J. U., Molkentin, J. D., & McNally, E. M. (2014). Excess SMAD signaling contributes to heart and muscle dysfunction in muscular dystrophy. *Human Molecular Genetics*, **23**(25), 6722–6731.

Hansen, S. L., Bojsen-Møller, K. N., Lundsgaard, A. M., Hendrich, F. L., Nilas, L., Sjøberg, K. A., Hingst, J. R., Serup, A. K., Olguín, C. H., Carl, C. S., Wernblad, L. F., Henneberg, M., Lustrup, K. M., Hansen, C., Jensen, T. E., Madsbad, S., Wojtaszewski, J. F. P., Richter, E. A., & Kiens, B. (2020). Mechanisms underlying absent training-induced improvement in insulin action in lean, hyperandrogenic women with polycystic ovary syndrome. *Diabetes*, **69**(11), 2267–2280.

Harrison, C. L., Stepto, N. K., Hutchison, S. K., & Teede, H. J. (2012). The impact of intensified exercise training on insulin resistance and fitness in overweight and obese women with and without polycystic ovary syndrome. *Clinical Endocrinology*, **76**(3), 351–357.

Hatzirodos, N., Bayne, R. A., Irving-Rodgers, H. F., Hummitzsch, K., Sabatier, L., Lee, S., Bonner, W., Gibson, M. A., Rainey, W. E., Carr, B. R., Mason, H. D., Reinhardt, D. P., Anderson, R. A., & Rodgers, R. J. (2011). Linkage of regulators of TGF-β activity in the fetal ovary to polycystic ovary syndrome. *FASEB Journal*, **25**(7), 2256–2265.

Hoffmann, C., & Weigert, C. (2017). Skeletal muscle as an endocrine organ: The role of myokines in exercise adaptations. *Cold Spring Harbor Perspectives in Medicine*, **7**(11), a029793.

Irani, M., Seifer, D. B., Grazi, R. V., Julka, N., Bhatt, D., Kalgi, B., Irani, S., Tal, O., Lambert-Messerlian, G., & Tal, R. (2015). Vitamin D supplementation decreases TGF-β1 bioavailability in PCOS: A randomized placebo-controlled trial. *Journal of Clinical Endocrinology and Metabolism*, **100**(11), 4307–4314.

Ismaeel, A., Kim, J. S., Kirk, J. S., Smith, R. S., Bohannon, W. T., & Koutakis, P. (2019). Role of transforming growth factor-β in skeletal muscle fibrosis: A review. *International Journal of Molecular Sciences*, **20**(10), 2446.

Jo, C., Park, S., Oh, S., Choi, J., Kim, E. K., Youn, H. D., & Cho, E. J. (2020). Histone acylation marks respond to metabolic perturbations and enable cellular adaptation. *Experimental & Molecular Medicine*, **52**, 2005–2019.

Kakoly, N. S., Moran, L. J., Teede, H. J., & Joham, A. E. (2019). Cardiometabolic risks in PCOS: A review of the current state of knowledge. *Expert Review of Endocrinology & Metabolism*, **14**, 23–33.

Kaur, J., & Reinhardt, D. P. (2015). Extracellular Matrix (ECM) Molecules. In *Stem cell biology and tissue engineering in dental sciences* (pp. 25–45). Academic Press.

Lambernd, S., Taube, A., Schober, A., Platzbecker, B., Görgens, S. W., Schlich, R., Jeruschke, K., Weiss, J., Eckardt, K., & Eckel, J. (2012). Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways. *Diabetologia*, **55**(4), 1128–1139.

Li, Y., & Kilian, K. A. (2015). Bridging the Gap: From 2D cell culture to 3D microengineered extracellular matrices. *Advanced Healthcare Materials*, **4**(18), 2780–2796.

Liao, Y., Smyth, G. K., & Shi, W. (2014). FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*, **30**(7), 923–930.

Lim, C., Shimizu, J., Kawano, F., Kim, H. J., & Kim, C. K. (2020). Adaptive responses of histone modifications to resistance exercise in human skeletal muscle. *PLoS One*, **15**(4), e0231321.

Louis, E., Raue, U., Yang, Y., Jemiolo, B., & Trappe, S. (2007). Time course of proteolytic, cytokine, and myostatin gene expression after acute exercise in human skeletal muscle. *Journal of Applied Physiology*, **103**(5), 1744–1751.

Manti, M., Stener-Victorin, E., & Benrick, A. (2020). Skeletal muscle immunometabolism in women with polycystic ovary syndrome: A meta-analysis. *Frontiers in Physiology*, **11**, 573505.

McIlvenna, L. C., Patten, R. K., McAinch, A. J., Rodgers, R. J., Stepto, N. K., & Moreno-Asso, A. (2021). Transforming growth factor beta 1 alters glucose uptake but not insulin signalling in human primary myotubes from women with and without polycystic ovary syndrome. *Frontiers in Endocrinology*, **12**, 732338.

Moran, L. J., Misso, M. L., Wild, R. A., & Norman, R. J. (2010). Impaired glucose tolerance, type 2 diabetes and metabolic syndrome in polycystic ovary syndrome: A systematic review and meta-analysis. *Human Reproduction Update*, **16**(4), 347–363.

Moreno-Asso, A., Altıntaş, A., McIlvenna, L. C., Patten, R. K., Botella, J., Mcainch, A. J., Rodgers, R. J., Barres, R., & Stepto, N. K. (2022). Non-cell autonomous mechanisms control mitochondrial gene dysregulation in polycystic ovary syndrome. *Journal of Molecular Endocrinology*, **68**(1), 63–76.

Nardo, L. G., Yates, A. P., Roberts, S. A., Pemberton, P., & Laing, I. (2009). The relationships between AMH, androgens, insulin resistance and basal ovarian follicular status in non-obese subfertile women with and without polycystic ovary syndrome. *Human Reproduction*, **24**(11), 2917–2923.

Nicolas, J., Magli, S., Rabbachin, L., Sampaolesi, S., Nicotra, F., & Russo, L. (2020). 3D extracellular matrix mimics: Fundamental concepts and role of materials chemistry to influence stem cell fate. *Biomacromolecules*, **21**(6), 1968–1994.

Nikolić, N., Görgens, S. W., Thoresen, G. H., Aas, V., Eckel, J., & Eckardt, K. (2017). Electrical pulse stimulation of cultured skeletal muscle cells as a model for in vitro exercise – possibilities and limitations. *Acta Physiologica*, **220**(3), 310–331.

Nilsson, E., Benrick, A., Kokosar, M., Krook, A., Lindgren, E., Källman, T., Martis, M. M., Højlund, K., Ling, C., & Stener-Victorin, E. (2018). Transcriptional and epigenetic changes influencing skeletal muscle metabolism in women with polycystic ovary syndrome. *Journal of Clinical Endocrinology and Metabolism*, **103**(12), 4465–4477.

Pattamaprapanont, P., Garde, C., Fabre, O., & Barrès, R. (2016). Muscle contraction Induces Acute hydroxymethylation of the exercise-responsive gene Nr4a3. *Frontiers in Endocrinology*, **7**, 165.

Patten, R. K., Boyle, R. A., Moholdt, T., Kiel, I., Hopkins, W. G., Harrison, C. L., & Stepto, N. K. (2020). Exercise interventions in polycystic ovary syndrome: A systematic review and meta-analysis. *Frontiers in Physiology*, **11**, 606.

Patten, R. K., McIlvenna, L. C., Levinger, I., Garnham, A. P., Shorakae, S., Parker, A. G., McAinch, A. J., Rodgers, R. J., Hiam, D., Moreno-Asso, A., & Stepto, N. K. (2022). High-intensity training elicits greater improvements in cardio-metabolic and reproductive outcomes than moderate-intensity training in women with polycystic ovary syndrome: a randomised clinical trial. *Human Reproduction* **37**(5), 1018–1029.

Peake, J. M., Neubauer, O., Della Gatta, P. A., & Nosaka, K. (2017). Muscle damage and inflammation during recovery from exercise. *Journal of Applied Physiology*, **122**(3), 559–570.

Powers, S. K., & Jackson, M. J. (2008). Exercise-induced oxidative stress: Cellular mechanisms and impact on muscle force production. *Physiological Reviews*, **88**(4), 1243–1276.

Raja-Khan, N., Kunselman, A. R., Demers, L. M., Ewens, K. G., Spielman, R. S., & Legro, R. S. (2010). A variant in the fibrillin-3 gene is associated with TGF-β and inhibin B levels in women with polycystic ovary syndrome. *Fertility and Sterility*, **94**(7), 2916–2919.

Raja-Khan, N., Urbanek, M., Rodgers, R. J., & Legro, R. S. (2014). The role of TGF-β in polycystic ovary syndrome. *Reproductive Sciences*, **21**(1), 20–31.

Raschke, S., Eckardt, K., Bjørklund Holven, K., Jensen, J., & Eckel, J. (2013). Identification and validation of novel contraction-regulated myokines released from primary human skeletal muscle cells. *PLoS One*, **8**(4), e62008.

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2009). edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, **26**(1), 139–140.

Sahmay, S., Aydogan Mathyk, B., Sofiyeva, N., Atakul, N., Azemi, A., & Erel, T. (2018). Serum AMH levels and insulin resistance in women with PCOS. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, **224**, 159–164.

Scheler, M., Irmler, M., Lehr, S., Hartwig, S., Staiger, H., Beckers, J., de Angelis, M. H., Häring, H.-U., & Weigert, C. (2013). Cytokine response of primary human myotubes in an in vitro exercise model. *American Journal of Physiology Cell Physiology*, **305**(8), C877–C886.

Seaborne, R. A., Strauss, J., Cocks, M., Shepherd, S., O'Brien, T. D., Van Someren, K. A., Bell, P. G., Murgatroyd, C., Morton, J. P., Stewart, C. E., & Sharples, A. P. (2018). Human skeletal muscle possesses an epigenetic memory of hypertrophy. *Science Reports*, **8**(1), 1898.

Shanely, R. A., Zwetsloot, K. A., Triplett, N. T., Meaney, M. P., Farris, G. E., & Nieman, D. C. (2014). Human skeletal muscle biopsy procedures using the modified bergström technique. *Journal of Visualized Experiments* (91), 51812.

Sir-Petermann, T., Maliqueo, M., Angel, B., Lara, H. E., Pérez-Bravo, F., & Recabarren, S. E. (2002). Maternal serum androgens in pregnant women with polycystic ovarian syndrome: Possible implications in prenatal androgenization. *Human Reproduction*, **17**(10), 2573–2579.

Son, Y. H., Lee, S. M., Lee, S. H., Yoon, J. H., Kang, J. S., Yang, Y. R., & Kwon, K. S. (2019). Comparative molecular analysis of endurance exercise in vivo with electrically stimulated in vitro myotube contraction. *Journal of Applied Physiology*, **127**(6), 1742–1753.

Stepto, N. K., Cassar, S., Joham, A. E., Hutchison, S. K., Harrison, C. L., Goldstein, R. F., & Teede, H. J. (2013). Women with polycystic ovary syndrome have intrinsic insulin resistance on euglycaemic-hyperinsulaemic clamp. *Human Reproduction*, **28**(3), 777–784.

Stepto, N. K., Hiam, D., Gibson-Helm, M., Cassar, S., Harrison, C. L., Hutchison, S. K., Joham, A. E., Canny, B. J., Moreno-Asso, A., Strauss, B. J., Hatzirodos, N., Rodgers, R. J., & Teede, H. J. (2020). Exercise and insulin resistance in PCOS: Muscle insulin signalling and fibrosis. *Endocrine Connections*, **9**(4), 346–359.

Stepto, N. K., Moreno-Asso, A., McIlvenna, L. C., Walters, K. A., & Rodgers, R. J. (2019). Molecular mechanisms of insulin resistance in polycystic ovary syndrome: unraveling the conundrum in skeletal muscle? *Journal of Clinical Endocrinology and Metabolism*, **104**(11), 5372–5381.

Stepto, N. K., Patten, R. K., Tassone, E. C., Misso, M. L., Brennan, L., Boyle, J., Boyle, R. A., Harrison, C. L., Hirschberg, A. L., Marsh, K., Moreno-Asso, A., Redman, L., Thondan, M., Wijeyaratne, C., Teede, H. J., & Moran, L. J. (2019). Exercise recommendations for women with polycystic ovary syndrome: Is the evidence enough? *Sports Medicine*, **49**(8), 1143–1157.

Sumbul, H. E., Avci, B. S., Bankir, M., Pekoz, B. C., Gulumsek, E., & Koc, A. S. (2022). Ovarian stiffness is significantly increased in polycystic ovary syndrome and related with anti-mullerian hormone: A point shear wave elastography study. *Ultrasound Quarterly*, **38**(1):83–88.

Supek, F., Bošnjak, M., Škunca, N., & Šmuc, T. (2011). REVIGO summarizes and visualizes long lists of gene ontology terms. *PLoS One*, **6**(7), e21800.

Sylow, L., & Richter, E. A. (2019). Current advances in our understanding of exercise as medicine in metabolic disease. *Current Opinion in Physiology*, **12**, 12–19.

Tal, R., Seifer, D. B., Shohat-Tal, A., Grazi, R. V., & Malter, H. E. (2013). Transforming growth factor-β1 and its receptor soluble endoglin are altered in polycystic ovary syndrome during controlled ovarian stimulation. *Fertility and Sterility*, **100**, 538–543.

Tata, B., Mimouni, N. E. H., Barbotin, A. L., Malone, S. A., Loyens, A., Pigny, P., Dewailly, D.i., Catteau-Jonard, S., Sundström-Poromaa, I., Piltonen, T. T., Dal Bello, F., Medana, C., Prevot, V., Clasadonte, J., & Giacobini, P. (2018). Elevated prenatal anti-Müllerian hormone reprograms the fetus and induces polycystic ovary syndrome in adulthood. *Nature Medicine*, **24**, 834–846.

Teede, H. J., Misso, M. L., Boyle, J. A., Garad, R. M., McAllister, V., Downes, L., Gibson-Helm, M., Hart, R. J., Rombauts, L., Moran, L., Dokras, A., Laven, J., Piltonen, T., Rodgers, R. J., Thondan, M., Costello, M. F., Norman, R. J., Baye, E., Brennan, L., … Woolcock, J. (2018). Translation and implementation of the Australian-led PCOS guideline: Clinical summary and translation resources from the International Evidence-based Guideline for the Assessment and Management of Polycystic Ovary Syndrome. *Medical Journal of Australia*, **209**, S3–S8.

Vázquez-Martínez, E. R., Gómez-Viais, Y. I., García-Gómez, E., Reyes-Mayoral, C., Reyes-Muñoz, E., Camacho-Arroyo, I., & Cerbón, M. (2019). DNA methylation in the pathogenesis of polycystic ovary syndrome. *Reproduction*, **158**, R27–R40.

Waning, D. L., Mohammad, K. S., Reiken, S., Xie, W., Andersson, D. C., John, S., Chiechi, A., Wright, L. E., Umanskaya, A., Niewolna, M., Trivedi, T., Charkhzarrin, S., Khatiwada, P., Wronska, A., Haynes, A., Benassi, M. S., Witzmann, F. A., Zhen, G., Wang, X., … Guise, T. A. (2015). Excess TGF-β mediates muscle weakness associated with bone metastases in mice. *Nature Medicine*, **21**, 1262–1271.

Williams, A. S., Kang, L., & Wasserman, D. H. (2015). The extracellular matrix and insulin resistance. *Trends in Endocrinology and Metabolism*, **26**, 357–366.

Wiweko, B., & Susanto, C. A. (2017). The effect of metformin and cinnamon on serum anti-mullerian hormone in women having PCOS: A double-blind, randomized, controlled trial. *Journal of Human Reproductive Sciences*, **10**, 31–36.

Wiweko, B., Indra, I., Susanto, C., Natadisastra, M., & Hestiantoro, A. (2018). The correlation between serum AMH and HOMA-IR among PCOS phenotypes. *BMC Research Notes*, **11**(1), 114.

Yu, G., & He, Q. Y. (2016). ReactomePA: An R/Bioconductor package for reactome pathway analysis and visualization. *Molecular Biosystems*, **12**, 477–479.

Yu, G., Wang, L. G., Han, Y., & He, Q. Y. (2012). ClusterProfiler: An R package for comparing biological themes among gene clusters. *OMICS*, **16**, 284–287.

Additional Information

Data availability statement

Sequencing data are archived (GSE199225) for public access at the Gene Expression Omnibus (GEO) Repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE199225). The rest of the data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Author contributions

L.C.M., N.K.S. and A.M-A. were involved in the conception and design of the present study. L.C.M., A.A., R.K.P., A.J.M., R.J.R., N.K.S., R.B. and A.M-A. were involved in the acquisition, analysis or interpretation of data. L.C.M., A.A., R.K.P., A.J.M., R.J.R., R.B. and A.M-A. were involved in the drafting of the manuscript and revising it critically for important intellectual content. All authors approved the final version of the manuscript submitted for publication. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Keywords

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Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

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