Hindlimb Immobilization, But Not Castration, Induces Reduction of Undercarboxylated Osteocalcin Associated With Muscle Atrophy in Rats

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ABSTRACT

Undercarboxylated osteocalcin (ucOC) has been implicated in skeletal muscle insulin sensitivity and function. However, whether muscle mass and strength loss in atrophic conditions is related to a reduction in ucOC is not clear. We hypothesized that both immobilization and testosterone depletion would lead to reductions in ucOC, associated with not only the degree of muscle atrophy but also changes to atrophy signaling pathway(s) in male rats. We subjected 8-week-old male Fischer (F344) rats to 7 days of hindlimb immobilization 10 days after castration surgery. Hindlimb immobilization, but not castration, resulted in a significant reduction in ucOC (30%) and lower ucOC was correlated with the degree of muscle loss and muscle weakness. ucOC levels, the expression of ucOC-sensitive receptor G protein-coupled receptor, class C, group 6,

member A (GPRC6A), as well as the activity of extracellular signal-regulated kinase (ERK) and 5′ adenosine monophosphate–activated protein kinase (AMPK) were associated with the expression and activity of a number of proteins in the mammalian target of rapamycin complex 1 (mTORC1) and Forkhead Box O (FOXO) signaling pathways in a muscle type–specific manner. These data suggest that ucOC may have other effects on skeletal muscle in addition to its insulin sensitizing effect. © 2016 American Society for Bone and Mineral Research

KEY WORDS: UNDERCARBOXYLATED OSTEOCALCIN; MUSCLE ATROPHY; TESTOSTERONE; HINDLIMB IMMOBILIZATION; CASTRATION

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Introduction

Bone is an endocrine organ involved in energy metabolism and possibly male fertility.[1] These functions are achieved by the undercarboxylated form of osteocalcin (ucOC).[2,3] Osteocalcin may play a role in cell growth and muscle fiber strength in skeletal muscles.[4–6] Indeed, there is some evidence suggesting that osteocalcin-deficient mice are characterized by lower muscle mass and weaker muscle strength.[7] In humans, lower ucOC/OC is associated with lower hip flexor, hip abductor, and quadriceps muscle

strength.[8] Furthermore, ucOC treatment promoted increased extensor digitorum longus (EDL) muscle cross-sectional area and grip strength in mice and myotube formation in C2C12 myoblast cultures in vitro.[6]

Muscle atrophy is a consequence of homeostatic imbalance between protein synthesis and protein degradation.[9] It is possible therefore, that the loss of muscle mass and strength during atrophic conditions might be, at least in part, attributed to lower circulating ucOC. However, it is not clear whether atrophic conditions, as seen following limb immobilization, has an impact on serum ucOC levels.

Testosterone is an anabolic hormone produced by the Leydig cells of the testes and plays a major role in the regulation of muscle mass in males.[10,11] Loss of testosterone, as a consequence of aging or castration surgery, leads to muscle atrophy and muscle weakness.[12,13] Testosterone modulates muscle anabolism not only via signaling networks that are directly orchestrated by itself, but also indirectly via other hormones such as follicle-stimulating hormone, luteinizing hormone, and growth hormone[14,15] due to widely expressed androgen receptors in endocrine organs, including bone.[16–18] Indeed, there are suggestions for the existence of a reciprocal interaction between bone and gonads.[19,20] ucOC promotes testosterone secretion in testes.[21] In turn, testosterone is implicated in bone growth, maturation, and maintenance.[20] However, whether testosterone loss has an impact on circulating ucOC level is unknown. As such, it is not clear whether reductions in testosterone level indirectly influence skeletal muscle mass via decreases in ucOC level.

The beneficial effects of ucOC on skeletal muscle are likely mediated via its putative receptor G protein-coupled receptor, class C, group 6, member A (GPRC6A).[22] Within the ucOC-GPRC6A cascade, extracellular signal-regulated kinase (ERK) and 5' adenosine monophosphate–activated protein kinase (AMPK) are proposed downstream

kinases[7,23] that are also known to be involved in the proliferation, growth, and lifespan of muscle cells.[24–26]

Under atrophic conditions, there are several signaling pathways actively responsible for muscle loss. The mammalian target of rapamycin complex 1 (mTORC1) cascade plays a vital role in cell proliferation and growth.[27] Its dysfunction has been suggested as one of the main mechanisms underlying muscle atrophy.[28,29] The phosphorylation of mTOR as well as the phosphorylation of 70-kDa ribosomal protein S6 kinase (P70S6K), one of the major mTORC1 downstream targets, are indicative of mTORC1 activation.[30,31] Another important target of mTORC1 is unc-51-like autophagy activating kinase 1 (ULK1). Phosphorylation of ULK1 at ser757 by mTORC1 blocks its pro-autophagy functions.[32] Forkhead Box O (FOXO) family proteins are also critical in the process of muscle atrophy.[33] Phosphorylation of FOXO family members inhibit their translocation into the nucleus, thereby inhibiting the transcription of Muscle-specific RING Finger protein1 (MuRF1) and Muscle Atrophy F-box (MAFbx/Fbx32), the two important skeletal muscle-specific ubiquitin E3 ligases that tag proteins for proteolysis via the proteasome.[33] Furthermore, protein kinase B (AKT) is functions as a critical convergent point, regulating both mTORC1 and FOXO signaling pathways via phosphorylation.[28] The upstream signaling partner of AKT, insulin receptor substrate 1 (IRS-1), directly activated by the insulin receptor, plays a role in these pathways, and can be downregulated via negative feedback in atrophic muscles.[34] Nevertheless, it is not clear how these signaling proteins interact with the ucOC-GPRC6A cascade under conditions of muscle atrophy.

The aims of this study were to test the hypotheses that (1) hindlimb immobilization and castration surgery lead to reductions in ucOC levels and (2) the reductions in ucOC correlate with muscle loss and related muscle signaling proteins. We also hypothesized that the expression/activity of downstream proteins in ucOC signaling

cascade will be correlated with the muscle atrophy signaling network. Given that different types of muscles respond differently during immobilization-induced atrophy,[35,36] these correlations between signaling proteins would also be in a muscle type-specific manner.

Materials and Methods

Animals

Male Fischer (F344) rats (n = 31, body weight = 224.1 ± 3.1 g) were purchased ~8 weeks of age (Animal Resource Centre, Canning Vale, WA, Australia) and housed in pairs in a light- and climate-controlled room (12:12 hours of light and dark, 20°C to 22°C) with *ad libitum* access to water and standard animal chow (AIN93G; Specialty Feeds, Glenn Forrest, WA, Australia). All experiments and procedures were approved by the Animal Ethics Committee at Victoria University and in accordance with the Australian code of practice for the care and use of animals in scientific research.

Castration surgery and hindlimb immobilization

After 1 week of acclimatization, animals were randomly allocated into castration or sham groups. Testosterone levels were manipulated by performing a bilateral orchiectomy or sham surgery via a scrotal incision under sterile conditions. Pain relief was administered via intraperitoneal injection 30 min prior to surgery (0.5 mg/kg, Meloxicam; Therapon, Burwood, VIC, Australia). Animals were allowed to recover for 1 week, at which time they underwent unilateral immobilization of the right hindlimb (or free movement as non-immobilized controls) for 10 days to induce muscle atrophy (7 rats for Sham+Non-immobilization, 8 rats for Sham+Immobilization, 7 rats for Castration+Non-immobilization, 9 rats for Castration+Immobilization). Hindlimb immobilization was conducted under 2% to 4% isoflurane anesthesia, tape stirrups were attached to the top and bottom of the foot, and the leg was then wrapped in cast padding and compression tape. The leg was immobilized by a thermoplastic splint (3’ Vet-lite casting material;

Therapon, Burwood, VIC, Australia) attached to the outside of the leg. The splint was secured in place using strapping tape with the foot in a neutral position. The splint was inspected and repaired daily, as required. Animals were also inspected daily by laboratory and veterinary staff, and their general level of activity, responsiveness, and appearance was assessed. After the immobilization/non-immobilization period, animals were killed in blood collection via heart puncture after ex vivo muscle contraction (see below).

Ex vivo muscle contraction and sample collection

After the 10-day immobilization/non-immobilization period, animals were deeply anaesthetized with sodium pentobarbital (60 mg/kg; Therapon, Burwood, VIC, Australia) via intraperitoneal injection. The EDL and soleus muscles from both legs were excised tendon to tendon and ex vivo contraction was conducted as described.[37] After the completion of ex vivo contraction experiments, the EDL and soleus were removed from the bath, blotted dried, cut free of tendon, and weighed. The muscles were then snap-frozen in liquid nitrogen. Blood samples were then collected via heart puncture and left on ice for 30 min, at which time they were spun in a centrifuge at 16,000*g* at 4°C for 10 min for serum samples. Serum was stored at –80°C until analysis.

Measurement of the level of serum hormones

Total osteocalcin was measured using ELISA kit purchased from Immunotopics (San Clemente, CA, USA) according to the manufacturer's instructions. ucOC level was detected following hydroxyapatite binding as described[38] using the same ELISA kit. Serum testosterone and insulin were measured using ELISA kits (in duplicate) purchased from Crystal Chem (Downers Grove, IL, USA) based on kit instructions.

Western blotting

Muscle samples (~15 mg) were lysed in ice-cold radioimmunoprecipitation assay [RIPA] buffer (Cell Signaling, Danvers, MA, USA) with Inhibitor Cocktail (Cell Signaling) and 25 mM dithiothreitol (Sigma Aldrich, St. Louis, MO, USA) using TissueLyser II

(QIAGEN, Hilden, Germany) followed by gentle rocking at 4°C for 1 hour. Protein homogenates were collected in the supernatant following centrifugation at 16,000*g* at 4°C for 15 min. Protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were subjected to electrophoresis on Criterion Stain-Free precast gels (10%; Bio-Rad) and then transferred electrophoretically using Trans-Blot Turbo Transfer System (Bia-Rad) onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). Then a stain-free blot image was taken using ChemiDoc Imaging System (Bio-Rad) for total protein measurement in each sample lane. Immunoblotting was performed at optimum conditions for each antibody. Bands were identified using ChemiDoc Imaging System, using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo, Waltham, MA, USA). Band densities of both stain-free blot and immunoblotting were measured by densitometry using Image Lab Software (Bio-Rad). Values of immunoblotting bands were normalized using total protein values before statistical analysis. p-mTOR (Ser2448), mTOR, IRS-1, p-ULK1 (Ser757), ULK1, p-FOXO3a (Ser253), FOXO3a, p-FOXO1 (Ser256), FOXO1, p-P70S6K (Thr389), P70S6K, p-AMPK (Thr172), AMPK, p-AKT (Ser473), AKT, p-ERK (Thr202/Tyr204), and ERK antibodies were purchased from Cell Signaling; GPRC6A antibody was provided by AVIVA (San Diego, CA, USA); and Fbx32 and MuRF1 antibodies were purchased from Bioss (Woburn, MA, USA).

Statistical analysis

All values are expressed as the mean fold-change (normalized to non-immobilization group) ± SE. To analyze the effects of castration and immobilization within non-immobilized animals and immobilized animals on serum hormone levels, two-way ANOVA was applied. To analyze the effects of castration and immobilization within non-immobilized animals and immobilized legs on the muscle mass/body weight ratio, muscle force, and expression/activity of signaling proteins, two-way ANOVA was

applied. To analyze the effects of immobilization within immobilized legs and the contralateral legs on the muscle mass/body weight ratio, muscle force, and expression/activity of signaling proteins, two-way ANOVA with repeated measure was applied. Spearman's correlation was performed between the variables within all groups unless other ranges are described. All figures and analyses were performed using GraphPad 6 (GraphPad Software, La Jolla, CA, USA).

Results

Impact of immobilization and castration on circulating hormones

Both immobilization and castration had no significant effect on the level of total OC (Fig. 1*A*). ucOC was reduced by ~30% ($p < 0.05$) after immobilization but not changed after castration (Fig. 1*B*). Testosterone was lower by more than 90% ($p < 0.001$) following castration (Fig. 1*C*) and –40% ($p < 0.001$) following limb immobilization. Circulating serum insulin was ~50% higher ($p < 0.05$) in the immobilization group compared to non-immobilized animals. In contrast, compared with sham surgery, insulin level was significantly lower (~50%) after castration. Circulating testosterone did not correlate with either OC or ucOC (Fig. 1*E*, *F*).

<Insert Figure 1>

Impact of immobilization and castration on EDL and soleus mass and strength

In the EDL, hindlimb immobilization but not castration led to a small, but significant reduction in muscle mass (Fig. 2*A*). Immobilization resulted in lower muscle force compared with the non-immobilized contralateral leg ($p < 0.05$, Fig. 2*C*). Immobilization resulted in a profound reduction in soleus muscle mass and force in comparison with either non-immobilized animals (~30%; $p < 0.001$, $p < 0.05$) or the contralateral leg (~50%; $p < 0.001$) (Fig. 2*B*, *D*). Castration had no significant effect on either mass or strength in either muscle.

<Insert Figure 2>

Higher ucOC level was associated with a higher EDL and soleus mass (Fig. 2*E* and Fig. 2*F*, respectively) and also higher strength (Fig. 2*G* and Fig. 2*H*, respectively). Higher insulin level was associated with lower muscle mass in soleus among all animals ($r = -0.46$, $p = 0.01$).

Impact of immobilization and castration on postulated ucOC signaling proteins

There was no alteration of GPRC6A protein expression in the EDL (Fig. 3*A*). In contrast, GPRC6A expression in the soleus was significantly lower following immobilization (Fig. 3*B*).

<Insert Figure 3>

p-AMPKα levels were lower (~50%, $p < 0.001$), in both EDL and soleus following immobilization compared immobilization to non-immobilized rats (Fig. 3*C, D*). p-AMPKα as well as phospho/total ratio of AMPKα in EDL was lower in castrated animals compared to sham counterparts ($p < 0.05$) whereas its total expression was higher (Fig. 3*C*).

ERK1/2 phosphorylation and phospho/total ratio of ERK1/2 were lower than non-immobilized animals following immobilization in EDL (Fig. 3*E*). In soleus, higher phospho/total ratio of ERK1/2 was observed in immobilized leg ($p < 0.05$) (Fig. 3*F*).

There was no significant correlation between ucOC level and the expression of GPRC6A in either muscle type ($p > 0.05$). In soleus but not EDL, GPRC6A was correlated with muscle mass ($r = 0.51$, $p < 0.001$). ucOC level was correlated with p-ERK1/2 ($r = 0.49$, $p < 0.01$) and p-AMPKα ($r = 0.42$, $p < 0.05$) in EDL. In soleus, ucOC tended to be correlated with p-AMPKα ($r = 0.34$, $p = 0.072$). Higher soleus GPRC6A expression was associated with higher soleus p-AMPKα ($r = 0.32$, $p < 0.05$). Higher insulin levels were correlated with lower soleus p-AMPKα ($r = –0.48$, $p < 0.01$).

Impact of immobilization and castration on IRS-1 and AKT

IRS-1 levels following immobilization were significantly lower compared to either non-immobilized animals or the contralateral leg in both EDL and soleus (Fig. 4*A* and Fig. 4*B*, respectively). In EDL, castration led to lower p-AKT/AKT ratio compared to sham control mainly due to an increased total AKT expression. In soleus, p-AKT/AKT was significantly lower following immobilization compared to the contralateral leg mainly due to a higher total protein levels. Soleus AKT expression was higher following castration in comparison to sham animals.

<Insert Figure 4>

ucOC level were correlated with IRS-1 levels in EDL ($r = 0.48$, $p < 0.01$). EDL IRS-1 was also correlated with p-ERK ($r = 0.30$, $p = 0.04$) and p-AMPKα ($r = 0.54$, $p < 0.001$). Similarly, IRS-1 expression in soleus muscle correlated with GPRC6A ($r = 0.41$, $p < 0.01$), and p-AMPKα ($r = 0.54$, $p < 0.001$). No correlations between IRS-1 and p-AKT were observed in both types of muscle. EDL p-AKT was correlated with p-ERK in EDL muscle ($r = 0.34$, $p < 0.05$).

Impact of immobilization and castration on mTORC1 signaling proteins

In the EDL, p-mTOR/mTOR ratio was lower following immobilization compared with both non-immobilized animals ($p < 0.001$) and the contralateral legs ($p < 0.05$) (Fig. 5*A*). Castration led to enhanced total mTOR, but attenuated p-mTOR and p-mTOR/mTOR ratio in EDL. mTOR expression in immobilized soleus was significantly lower than in non-immobilized rats (Fig. 5*B*). In soleus muscle, p-mTOR and expression was lower in castrated animals compared to sham controls (Fig. 5*B*).

<Insert Figure 5>

The mTORC1 substrates P70S6K, both phosphorylation and expression, were lower ($p < 0.05$) in immobilized EDL compared to the contralateral muscle (Fig. 5*C*). In immobilized soleus, p-P70S6K and phospho/total ratio of P70S6K were higher than the contralateral leg whereas total P70S6K level was markedly lower than either control (Fig.

5*D*). p-ULK1 was significantly lower following immobilization compared to non-immobilization rats in both the EDL (~70%, Fig. 5*E*) and the soleus (~50%, $p < 0.001$, Fig. 5*F*) immobilization.

In EDL, ucOC level was correlated with p-ULK1 ($r = 0.54$, $p < 0.01$). p-ERK had correlations with p-mTOR ($r = 0.28$, $p = 0.05$), and p-ULK1 ($r = 0.38$, $p < 0.01$). Lower EDL p-AMPKα was associated with lower EDL p-mTOR ($r = 0.31$, $p < 0.05$), and EDL p-ULK1 ($r = 0.53$, $p < 0.001$). EDL p-mTOR was associated with EDL p-ULK1 ($r = 0.50$, $p < 0.001$). EDL p-AKT was correlated with EDL p-mTOR ($r = 0.33$, $p < 0.05$), and p-ULK1 ($r = 0.44$, $p < 0.01$). In soleus, higher soleus p-AMPKα had an association with higher soleus p-ULK1 ($r = 0.46$, $p = 0.001$).

Impact of immobilization and castration on FOXO signaling proteins

p-FOXO3a levels were significantly lower following immobilization than the contralateral legs in EDL (Fig. 6*A*). In castrated animals, total FOXO3a in EDL was higher than sham controls (Fig. 6*A*). Castrated rats also had higher levels of total FOXO3a in soleus (Fig. 6*B*).

<Insert Figure 6>

Both phosphorylation and expression of FOXO1 were unchanged in EDL after immobilization or castration (Fig. 6*C*). There was a marked reduction in the phosphorylation level of FOXO1 following immobilization compared to non-immobilized animals in soleus (Fig. 6*D*). Castration led to an increase in total FOXO1 but a reduction in p-FOXO1/FOXO1 in soleus muscle (Fig. 6*D*).

Limited alterations were observed in the expression of E3 ligase Fbx32 in EDL after both immobilization and castration whereas the immobilized leg showed significant increase of Fbx32 in soleus comparing with non-immobilization rats (Fig. 6*E*, *F*). Following immobilization, Murf1 was significantly lower in EDL but higher in soleus

compared to non-immobilization animals (Fig. 6*G*, *H*). There was a higher (~40%, $p < 0.01$) Murf1 in soleus in the castration group.

In soleus, a higher GPRC6A expression was correlated with lower Fbx32 ($r = –0.29$, $p < 0.05$). Higher soleus p-AMPKα was associated with higher soleus p-FOXO1 ($r = 0.38$, $p < 0.01$). A higher soleus p-AMPKα/AMPKα ratio was correlated with a lower Fbx32 ($r = –0.31$, $p < 0.05$) and was tended to be correlated with a lower MuRF1 ($r = –0.26$, $p = 0.07$). Furthermore, soleus p-FOXO1 ($r = 0.33$, $p < 0.05$) and soleus p-FOXO3a ($r = 0.32$, $p < 0.05$) was associated with p-ULK1. Lower p-FOXO1/FOXO1 ratio was tended to be associated with higher Fbx32 ($r = –0.27$, $p = 0.062$) and was associated with higher MuRF1 ($r = –0.48$, $p < 0.001$).

Discussion

We report that lower circulating ucOC following hindlimb immobilization, but not testosterone depletion (via castration), was associated with lower muscle mass and force in both fast-twitch (EDL) and slow twitch (soleus) muscles (Fig. 2*E–H*). This association was not evident following castration. Finally, a higher serum ucOC and a higher GPRC6A expression were related to higher ERK1/2 and AMPKα phosphorylation in a muscle-specific manner. The reduced activity of these putative ucOC signaling pathways was also related to decreased activity of mTORC1 activity in EDL and activation of FOXO pathway in soleus following immobilization.

It appears that skeletal muscle is one of the target organs of ucOC.[6–8,23,37] Previous studies mainly focused on the insulin-sensitizing effects of ucOC in skeletal muscles,[37,39,40] but recently, it has been suggested that ucOC may play a role in muscle mass maintenance and physiological functions,[6,7,37] Here we report that lower circulating levels of ucOC correlates with lower muscle mass and strength in both EDL and soleus muscle following immobilization, suggesting a link between ucOC and muscle mass and function.

ucOC promotes glucose metabolism by increasing the secretion of insulin and improving insulin sensitivity.[5,40] Interestingly, insulin signaling in osteoblasts stimulates ucOC production in bone,[41] indicating a positive feedback between ucOC and insulin that is likely to affect whole-body glucose metabolism. Because ucOC was reported to contribute to male reproductive capacity by enhancing testosterone production in testis[2] and testosterone has long been known to be involved in bone remodeling and function,[19] we hypothesized that reducing testosterone would lead to a reduction in ucOC. In contrast to our hypothesis, serum ucOC levels were not different in castrated rats compared to sham controls. In addition, ucOC did not correlate with testosterone levels. We also observed that testosterone depletion following castration surgery had limited effects on muscle mass and muscle strength. A potential limitation of the current study is the relative short duration of the intervention of testosterone depletion via castration. Indeed, in other rat studies, a moderate decrease in soleus mass/body weight ratio was found in animals following 5 weeks of castration surgery.[42,43] In a mice study, 10 weeks of castration time was needed to have moderate decrease in soleus mass/body weight ratio but not in fast twitch muscles.[44] It is thus possible that a longer duration post-castration is required to observe significant effects on circulating ucOC levels, muscle mass, and muscle strength. Lower phosphorylation of AMPK, AKT, and mTOR in EDL as well as lower phosphorylation of mTOR and FOXO1 but higher expression of MuRF1 in soleus was observed, in response to testosterone deprivation, which was similar to what was previously reported.[45] As such, it is likely that, in the short time frame of castration intervention in our study, molecular changes that favor muscle protein loss were already underway. However, these changes had not yet led to any apparent reductions in muscle mass and force. Indeed, in mice with long-term testosterone depletion (7 weeks post-castration surgery) both molecular and functional changes, suggesting muscle atrophy, were reported.[45] It is also plausible that the testosterone effect on skeletal muscle is

muscle-specific because higher testosterone levels correlated with higher soleus muscle weight within sham controls. This indicates testosterone might play a role in maintaining muscle mass more predominantly in slow twitch muscles.

Following hindlimb immobilization, muscle mass/body weight ratio of soleus, but not EDL, from the non-immobilized legs was significantly higher (11%, $p < 0.05$) compared with non-immobilized rats. This finding is similar to a previous studies that reported that soleus mass/body weight ratio in the non-immobilized leg gradually increased during a 7-day hindlimb immobilization in rats while the ratio of non-immobilized gastrocnemius (consist mostly of glycolytic fibers) remained unchanged.[46] Furthermore, it was also reported that compared with non-immobilized rats, the rate of protein synthesis in non-immobilized soleus was moderately higher after 3-day immobilization. As such, it appears that oxidative muscles, but not glycolytic muscles, in the non-immobilized leg may exhibit some "mild overload" that resulted in increased mass.

To date, the downstream targets of ucOC-GPRC6A cascade in skeletal muscle cells have not yet been identified. However, it is possible that they involve ERK and AMPK, because ucOC treatment in C2C12 myotubes led to ERK1/2 phosphorylation, which can be inhibited by the administration of the inhibitor of the upstream kinase of ERK.[23] Moreover, in primary myotubes, ucOC activated AMPK/mTOR/P70SK6 kinase axis via GPRC6A, implicating AMPK as a key protein responsible for the anabolic effects of ucOC in skeletal muscle.[7] Our results demonstrate that lower ucOC level was associated with lower ERK1/2 phosphorylation in EDL. In contrast, soleus ERK1/2 phospho/total ratio was elevated post-immobilization compared with both non-immobilized rats and non-immobilized legs, despite more profound muscle wasting. It is possible that this increase in ERK activity in the soleus is a compensatory response to the

greater muscle loss as was reported previously.[47] Hence, our results may indicate that ERK involvement in the ucOC cascade in skeletal muscle is muscle-specific.

Although lower ucOC was associated with lower p-AMPKα in both EDL and soleus muscle, only in soleus did lower p-AMPKα correlate with lower GPRC6A expression compared to both non-immobilized rats and the contralateral leg. This lower p-AMPKα in atrophic oxidative muscle is consistent with a number of other studies. For instance, 10-day limb immobilization in rats led to a significant reduction in p-AMPK expression in red gastrocnemius muscle compared to the contralateral leg.[48] Furthermore, 2-week tail suspension led to a significant reduction in p-AMPK mainly in soleus and to a lesser degree in EDL in rats.[49] In humans, patients with critical myopathy showed decreased AMPK activity in skeletal muscles compared with healthy controls.[50] Accordingly, the attenuation of the ucOC signaling cascade may be related to soleus muscle wasting attributed to lower AMPK activity. However, unchanged or even a higher AMPK activity during muscle atrophy has also been reported.[46,51] These incompatible results fit the contradictory role of AMPK in cell fate in skeletal muscles. On one hand, high activity of AMPK induces membrane GLUT4 expression as well as mitochondrial biogenesis, benefiting muscle glucose metabolism and cell growth.[52,53] On the other hand, AMPK activation leads to cell autophagy via enhancing the activity of cell autophagy pathways.[54,55] Notably, the majority of findings showing reduced AMPK activity in atrophic muscle were obtained either in animals subject to inactive conditions for at least 10 days or in inactive patients, whereas short time activation or inhibition of AMPK was mainly employed in studies investigating its role in protein degradation. Therefore, impaired cellular glucose metabolism and energy production in muscle cells due to low AMPK activity can only be observed after a relatively long duration. Indeed, people with short-term leg immobilization and patients with myopathy are characterized by lower insulin sensitivity.[50,56] Intriguingly, patients with T2DM have lower circulating

ucOC,[57,58] which is of interest because low serum ucOC is also linked with impaired insulin sensitivity in animals.[59] Moreover, we observed that hyperinsulinemia induced by limb immobilization (Fig. 1) was associated with loss of muscle mass and lower p-AMPKα in the soleus. Given that patients with T2DM are at a high risk for muscle atrophy,[29,60] glucose dysmetabolism due to low AMPK activity in slow twitch muscle during disuse atrophy may be a plausible scenario. However, this hypothesis should be tested in future studies.

We observed decreased expressions of IRS-1 in both muscles following immobilization, and these reductions of IRS-1 expression were associated not only with muscle mass loss but also with alterations of several signaling proteins. However, we did not find any significant correlations between IRS-1 level and the phosphorylation of its downstream kinase AKT. These results might indicate that the reductions of IRS-1 level were likely the consequence of protein degradation, but were not able to exert significant impact on insulin signaling pathway. Although phosphorylated AKT did not exhibit apparent decrease in EDL, its level was correlated with p-ERK, p-mTOR, and p-ULK1. These correlations were not observed in soleus. Recently, it has been reported that in C2C12 myotubes, the enhancement of AKT phosphorylation induced by osteocalcin treatment was abolished by U0126, an inhibitor of ERK kinase (MEK).[23] As such, in immobilized EDL, AKT phosphorylation could be affected by decreased activity of ucOC/ERK cascade, thus leading to reduced mTORC1 activity.

Higher ULK1 activity was correlated with lower p-mTOR in EDL only. In soleus, p-ULK1 was associated with p-FOXO1 and p-FOXO3a, which is consistent with the results that FOXO proteins can lead to muscle autophagy by interacting with ULK1.[54] Ubiquitin E3 ligases Fbx32 and MuRF1 were higher only in soleus compared with non-immobilized rats following immobilization, and they were also correlated with muscle loss, GPRC6A level, and/or p-FOXO1/FOXO1 ratio. This suggests that protein

degradation caused by FOXO/Fbx32 (MuRF1) is, at least partly, responsible for soleus muscle loss.

Previous studies have shown that muscle atrophy caused by immobilization or suspension occurs in a muscle-specific manner, with muscle mass loss predominantly found in oxidative slow twitch muscles and to a lesser extent in glycolytic fast twitch muscles.[36,49,61] However, even though signaling pathways involved in muscle atrophy have been widely reviewed,[28,29,33,55] the molecular mechanism for the selectivity of muscle type atrophy still remains unresolved. Our data not only confirmed that hindlimb-immobilization does lead to more severe muscle atrophy and weakness in soleus compared with EDL, but also highlighted a potential molecular mechanisms. The potential mechanisms are illustrated in Fig. 7. We propose that lower circulating ucOC and an attenuated ucOC signaling, caused by immobilization, reduces muscle mass in a muscle type–specific manner. In EDL, it leads to attenuated activity of AMPK and ERK, resulting in increased ULK1 activity and reductions in AKT activity and mTOR activity leading to muscle atrophy. In soleus, reduced ucOC and decreased GPRC6A expression results in larger reductions in AMPK activity, leading to more profound muscle atrophy via enhanced activity of ULK1 and increased expression of Fbx32 and MuRF1 through amplified activity of FOXO proteins, and potentially glucose dysmetabolism. These hypotheses need to be fully explored in future studies.

<Insert Figure 7>

In conclusion, hindlimb immobilization, but not testosterone depletion via castration, leads to a significant reduction in ucOC, and lower ucOC was correlated with lower muscle mass and force in both fast-twitch (EDL) and slow-twitch (soleus) muscles. In addition, the putative ucOC/GPRC6A/ERK (AMPK) signaling cascade was affected by immobilization, and the expression and activity of proteins in ucOC signaling pathway were also associated with the expression and activity of a number of proteins in the

mTORC1 and FOXO signaling pathways in a muscle type–specific manner. However, whether ucOC and its putative signaling pathway play a role in muscle anabolism in addition to its insulin sensitizing effects will need to be explored in detail in future studies involving methods of ucOC deprivation and restoration. Additional investigations are also required to explore the role of ucOC in both muscle gene expression, as well as whether the alteration of gene expression is responsible for the reduced mass and strength following castration and immobilization.

Disclosures

All authors state that they have no conflicts of interest.

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Authors' roles: Study design: EH, AH, ACB, XL, and IL. Study conduct: EH, AH, and IL. Data collection: XL, EH, and ACB. Data interpretation: XL, EH, AH, and TCB. Drafting manuscript: XL. Revising manuscript: XL, IL, TCB, EH, ACB, and AH. XL, EH, and IL take responsibility for the integrity of the data analysis.

References

1. Schwetz V, Pieber T, Obermayer-Pietsch B. Mechanisms in endocrinology: the endocrine role of the skeleton: background and clinical evidence. Eur J Endocrinol. 2012;166(6):959–67.

2. Oury F, Sumara G, Sumara O, et al. Endocrine regulation of male fertility by the skeleton. Cell. 2011;144(5):796–809.

3. Ferron M, Hinoi E, Karsenty G, Ducy P. Osteocalcin differentially regulates beta cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice. Proc Natl Acad Sci U S A. 2008;105(13):5266–70.

4. Zoch ML, Clemens TL, Riddle RC. New insights into the biology of osteocalcin.

Bone. 2016 Jan;82:42–9.

5. Brennan-Speranza TC, Conigrave AD. Osteocalcin: an osteoblast-derived polypeptide hormone that modulates whole body energy metabolism. Calcif Tissue Int. 2015;96(1):1–10.

6. Shen H, Grimston S, Civitelli R, Thomopoulos S. Deletion of connexin43 in osteoblasts/osteocytes leads to impaired muscle formation in mice. J Bone Miner Res. 2015;30(4):596–605.

7. Mera P, Laue K, Karsenty G. Osteocalcin regulates muscle function and mass. J Bone Miner Res. 2014;29:S50.

8. Levinger I, Scott D, Nicholson GC, et al. Undercarboxylated osteocalcin, muscle strength and indices of bone health in older women. Bone. 2014;64:8–12.

9. Mônico-Neto M, Antunes H, Dattilo M, et al. Resistance exercise: a non-pharmacological strategy to minimize or reverse sleep deprivation-induced muscle atrophy. Med Hypotheses. 2013;80(6):701–5.

10. Wu Y, Collier L, Pan J, Qin W, Bauman W, Cardozo C. Testosterone reduced methylprednisolone-induced muscle atrophy in spinal cord-injured rats. Spinal Cord. 2012 Jan;50(1):57–62.

11. Smith MR, Saad F, Egerdie B, et al. Sarcopenia during androgen-deprivation therapy for prostate cancer. J Clin Oncol. 2012;30(26):3271–6.

12. Sakuma K, Yamaguchi A. Sarcopenia and age-related endocrine function. Int J Endocrinol. 2012;2012:127362.

13. Boissonneault G. Evidence of apoptosis in the castration-induced atrophy of the rat levator ani muscle. Endocr Res. 2001;27(3):317–28.

14. Kitahara S, Winters SJ, Attardi B, Oshima H, Troen P. Effects of castration on luteinizing hormone and follicle-stimulating hormone secretion by pituitary cells from male rats. Endocrinology. 1990;126(5):2642–9.

15. Jansson J-O, Ekberg S, Isaksson O, Mode A, Gustafsson J-Å. Imprinting of growth hormone secretion, body growth, and hepatic steroid metabolism by neonatal testosterone. Endocrinology. 1985;117(5):1881–9.
16. Colvard DS, Eriksen EF, Keeting PE, et al. Identification of androgen receptors in normal human osteoblast-like cells. Proc Natl Acad Sci U S A. 1989;86(3):854–7.
17. Herbison A. Neurochemical identity of neurones expressing oestrogen and androgen receptors in sheep hypothalamus. J Reprod Fertil Suppl. 1994;49:271–83.
18. Corbishley TP, Iqbal MJ, Wilkinson ML, Williams R. Androgen receptor in human normal and malignant pancreatic tissue and cell lines. Cancer. 1986;57(10):1992–5.
19. Chamouni A, Oury F. Reciprocal interaction between bone and gonads. Arch Biochem Biophys. 2014;561:147–53.
20. Oury F. A crosstalk between bone and gonads. Ann N Y Acad Sci. 2012;1260(1):1–7.
21. Oury F, Ferron M, Huizhen W, et al. Osteocalcin regulates murine and human fertility through a pancreas-bone-testis axis. J Clin Invest. 2013;123(6):2421–33.
22. Pi M, Quarles LD. Multiligand specificity and wide tissue expression of GPRC6A reveals new endocrine networks. Endocrinology. 2012;153(5):2062–9.
23. Tsuka S, Aonuma F, Higashi S, et al. Promotion of insulin-induced glucose uptake in C2C12 myotubes by osteocalcin. Biochem Biophys Res Commun. 2015;459(3):437–42.
24. Mounier R, Théret M, Lantier L, Foretz M, Viollet B. Expanding roles for AMPK in skeletal muscle plasticity. Trends Endocrinol Metab. 2015;26(6):275–86.
25. Shefer G, Oron U, Irintchev A, Wernig A, Halevy O. Skeletal muscle cell activation by low-energy laser irradiation: a role for the MAPK/ERK pathway. J Cell Physiol. 2001;187(1):73–80.
26. Jo C, Kim H, Jo I, et al. Leukemia inhibitory factor blocks early differentiation of skeletal muscle cells by activating ERK. Biochim Biophys Acta. 2005;1743(3):187–97.
27. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell.

2012;149(2):274–93.

28. Brooks NE, Myburgh KH. Skeletal muscle wasting with disuse atrophy is multi-dimensional: the response and interaction of myonuclei, satellite cells and signaling pathways. Front Physiol. 2014 Mar 17;5:99.

29. Perry B, Caldow M, Brennan-Speranza T, et al. Muscle atrophy in patients with type 2 diabetes mellitus: roles of inflammatory pathways, physical activity and exercise. Exerc Immunol Rev. 2015;22:94–109.

30. Acosta-Jaquez HA, Keller JA, Foster KG, et al. Site-specific mTOR phosphorylation promotes mTORC1-mediated signaling and cell growth. Mol Cell Biol. 2009;29(15):4308–24.

31. Karlsson HK, Nilsson P-A, Nilsson J, Chibalin AV, Zierath JR, Blomstrand E. Branched-chain amino acids increase p70S6k phosphorylation in human skeletal muscle after resistance exercise. Am J Physiol Endocrinol Metab. 2004;287(1):E1–7.

32. Castets P, Rüegg MA. MTORC1 determines autophagy through ULK1 regulation in skeletal muscle. Autophagy. 2013;9(9):1435–7.

33. Bonaldo P, Sandri M. Cellular and molecular mechanisms of muscle atrophy. Dis Model Mech. 2013;6(1):25–39.

34. Hirose M, Kaneki M, Sugita H, Yasuhara S, Martyn JJ. Immobilization depresses insulin signaling in skeletal muscle. Am J Physiol Endocrinol Metab. 2000;279(6):E1235–41.

35. Fitts RH, Riley DR, Widrick JJ. Physiology of a microgravity environment invited review: microgravity and skeletal muscle. J Appl Physiol. 2000;89(2):823–39.

36. Wineski LE, von Deutsch DA, Abukhalaf IK, Pitts SA, Potter DE, Paulsen DF. Muscle-specific effects of hindlimb suspension and clenbuterol in mature male rats. Cell Tissue Organ. 2002;171(2–3):188–98.

37. Levinger I, Lin X, Zhang X, et al. The effects of muscle contraction and recombinant

osteocalcin on insulin sensitivity ex vivo. Osteoporos Int. 2016 Feb;27(2):653–63.
38. Gundberg CM, Nieman SD, Abrams S, Rosen H. Vitamin K status and bone health: an analysis of methods for determination of undercarboxylated osteocalcin 1. J Clin Endocrinol Metab. 1998;83(9):3258–66.
39. Ferron M, McKee MD, Levine RL, Ducy P, Karsenty G. Intermittent injections of osteocalcin improve glucose metabolism and prevent type 2 diabetes in mice. Bone. 2012;50(2):568–75.
40. Lee NK, Sowa H, Hinoi E, et al. Endocrine regulation of energy metabolism by the skeleton. Cell. 2007;130(3):456–69.
41. Ferron M, Wei J, Yoshizawa T, et al. Insulin signaling in osteoblasts integrates bone remodeling and energy metabolism. Cell. 2010;142(2):296–308.
42. Gao W, Reiser PJ, Coss CC, et al. Selective androgen receptor modulator treatment improves muscle strength and body composition and prevents bone loss in orchidectomized rats. Endocrinology. 2005;146(11):4887–97.
43. Öner J, Öner H, Sahin Z, Demir R, Üstünel İ. Melatonin is as effective as testosterone in the prevention of soleus muscle atrophy induced by castration in rats. Anat Rec. 2008;291(4):448–55.
44. Axell A-M, MacLean HE, Plant DR, et al. Continuous testosterone administration prevents skeletal muscle atrophy and enhances resistance to fatigue in orchidectomized male mice. Am J Physiol Endocrinol Metab. 2006;291(3):E506–16.
45. White JP, Gao S, Puppa MJ, Sato S, Welle SL, Carson JA. Testosterone regulation of Akt/mTORC1/FoxO3a signaling in skeletal muscle. Mol Cell Endocrinol. 2013;365(2):174–86.
46. Kelleher AR, Kimball SR, Dennis MD, Schilder RJ, Jefferson LS. The mTORC1 signaling repressors REDD1/2 are rapidly induced and activation of p70S6K1 by leucine is defective in skeletal muscle of an immobilized rat hindlimb. Am J Physiol Endocrinol

Metab. 2013;304(2):E229–36.

47. Yoo Y-M, Park JH, Seo D-H, et al. Activation of mTOR for the loss of skeletal muscle in a hindlimb-suspended rat model. International Journal of Precision Engineering and Manufacturing. 2015;16(5):1003–10.

48. Yoshihara T, Machida S, Kurosaka Y, et al. Immobilization-induced rat skeletal muscle atrophy enhances histone modification through HDAC4. FASEB J. 2015;29(1 Supplement):877.5.

49. Han B, Zhu MJ, Ma C, Du M. Rat hindlimb unloading down-regulates insulin like growth factor-1 signaling and AMP-activated protein kinase, and leads to severe atrophy of the soleus muscle. Appl Physiol Nutr Metab. 2007;32(6):1115–23.

50. Weber-Carstens S, Schneider J, Wollersheim T, et al. Critical illness myopathy and GLUT4: significance of insulin and muscle contraction. Am J Respir Crit Care Med. 2013;187(4):387–96.

51. Xu P-T, Song Z, Zhang W-C, Jiao B, Yu Z-B. Impaired translocation of GLUT4 results in insulin resistance of atrophic soleus muscle. Biomed Res Int. 2015;2015:291987.

52. Paulsen S, Rubink D, Winder W. AMP-activated protein kinase activation prevents denervation-induced decline in gastrocnemius GLUT-4. J Appl Physiol. 2001;91(5):2102–8.

53. Lee WJ, Kim M, Park H-S, et al. AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPARα and PGC-1. Biochem Biophys Res Commun. 2006;340(1):291–5.

54. Sanchez AM, Csibi A, Raibon A, et al. AMPK promotes skeletal muscle autophagy through activation of forkhead FoxO3a and interaction with Ulk1. J Cell Biochem. 2012;113(2):695–710.

55. Sandri M. Signaling in muscle atrophy and hypertrophy. Physiology.

2008;23(3):160–70.
56. Richter E, Kiens B, Mizuno M, Strange S. Insulin action in human thighs after one-legged immobilization. J Appl Physiol. 1989;67(1):19–23.
57. Alfadda AA, Masood A, Shaik SA, Dekhil H, Goran M. Association between osteocalcin, metabolic syndrome, and cardiovascular risk factors: role of total and undercarboxylated osteocalcin in patients with type 2 diabetes. Int J Endocrinol. 2013;2013:197519.
58. Levinger I, Zebaze R, Jerums G, Hare DL, Selig S, Seeman E. The effect of acute exercise on undercarboxylated osteocalcin in obese men. Osteoporos Int. 2011;22(5):1621–6.
59. Brennan-Speranza TC, Henneicke H, Gasparini SJ, et al. Osteoblasts mediate the adverse effects of glucocorticoids on fuel metabolism. J Clin Invest. 2012;122(11):4172.
60. Andersen H, Gjerstad MD, Jakobsen J. Atrophy of foot muscles: a measure of diabetic neuropathy. Diabetes Care. 2004;27(10):2382–5.
61. Sato S, Suzuki H, Tsujimoto H, Shirato K, Tachiyashiki K, Imaizumi K. Casted-immobilization downregulates glucocorticoid receptor expression in rat slow-twitch soleus muscle. Life Sci. 2011;89(25):962–7.

Fig. **1.** Impact of immobilization and castration on circulating OC, ucOC, testosterone and insulin levels. Serum levels of total OC (*A*), ucOC (*B*), testosterone (*C*), and insulin (*D*) were detected in NR/ImmR with sham/castration surgery. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ in two-way ANOVA analysis. Symbols of *p* value of Castration main effect are illustrated on top of columns; symbols of *p* value of Immobilization main effects ("NR vs ImmR") are illustrated on the right side of each panel. OC = osteocalcin; ucOC = undercarboxylated osteocalcin; NR = non-immobilized rats; ImmR = immobilized rats.

Fig. **2.** Impact of immobilization and castration on EDL and soleus mass and strength. EDL MW/BW (*A*), soleus MW/BW (*B*), EDL muscle force (*C*), and soleus muscle force (*D*) were examined for muscles isolated from NR/ImmL and the CoL of immobilized rats with sham/castration surgery. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ in two-way ANOVA analysis. The correlation between ucOC and EDL MW/BW (*E*), EDL force (*F*), soleus MW/BW (*G*), or soleus force (*H*) was detected among all six groups. Symbols of *p* value of Castration main effect are illustrated on top of columns; symbols of *p* value of Immobilization main effects ("NR vs ImmL" and "ImmL vs CoL") are illustrated on the right side of each panel. MW/BW = muscle weight/body weight; NR = non-immobilized rats; ImmR = immobilized rats; CoL = contralateral leg.

Fig. **3.** Impact of immobilization and castration on postulated ucOC signaling proteins. GPRC6A expression (*A*, *B*), p-AMPKα,AMPKα, and p-AMPKα/AMPKα levels (*C*, *D*), as well as p-ERK1/2, ERK1/2, and p-ERK1/2/ERK1/2 levels (*E*, *F*) of EDL and soleus isolated from NR/ImmL and the CoL of immobilized rats with sham/castration surgery were examined. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ in two-way ANOVA analysis. Symbols of *p* value of Castration main effect are illustrated on top of columns; symbols of *p* value of Immobilization main effects ("NR vs ImmL" and "ImmL vs CoL") are illustrated on the right side of each panel. NR = non-immobilized rats; ImmR = immobilized rats; CoL = contralateral leg.

Fig. **4.** Impact of immobilization and castration on IRS-1 and AKT. IRS-1 expression (*A*, *B*), p-AKT, AKT, and p-AKT/AKT levels (*C*, *D*) of EDL and soleus isolated from NR/ImmL and the CoL of immobilized rats with sham/castration surgery were examined. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ in two-way ANOVA analysis. Symbols of *p* value of Castration main effect are illustrated on top of columns; symbols of *p* value of Immobilization main effects ("NR vs ImmL" and "ImmL vs CoL") are illustrated on the

right side of each panel. NR = non-immobilized rats; ImmR = immobilized rats; CoL = contralateral leg.

Fig. **5.** Impact of immobilization and castration on mTORC1 signaling proteins. p-mTOR, mTOR and p-mTOR/mTOR levels (*A*, *B*), p-P70S6K, P70S6K, and p-P70S6K/P70S6K levels (*C*, *D*), and p-ULK1, ULK1 and p-ULK1/ULK1 levels (*E*, *F*) of EDL and soleus isolated from NR/ImmL and the CoL of immobilized rats with sham/castration surgery were examined. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ in two-way ANOVA analysis. Symbols of *p* value of Castration main effect are illustrated on top of columns; symbols of *p* value of Immobilization main effects ("NR vs ImmL" and "ImmL vs CoL") are illustrated on the right side of each panel. NR = non-immobilized rats; ImmR = immobilized rats; CoL = contralateral leg.

Fig. **6.** Impact of immobilization and castration on the expression and activity of FOXO signaling proteins. p-FOXO3a, FOXO3a, and p-FOXO3a/FOXO3a levels (*A*, *B*), p-FOXO1, FOXO1, and p-FOXO1/FOXO1 levels (*C*, *D*), Fbx32 expressions (*E*, *F*), and MuRF1 expressions (*G*, *H*) of EDL and soleus isolated from NR/ImmL and the CoL of immobilized rats with sham/castration surgery were examined. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ in two-way ANOVA analysis. Symbols of *p* value of Castration main effect are illustrated on top of columns; symbols of *p* value of Immobilization main effects ("NR vs ImmL" and "ImmL vs CoL") are illustrated on the right side of each panel. NR = non-immobilized rats; ImmR = immobilized rats; CoL = contralateral leg.

Fig. **7.** Reduced level of circulating ucOC and GPRC6A expression caused by hindlimb immobilization might lead to enhanced muscle atrophy in a muscle type–specific manner.

Figure 1

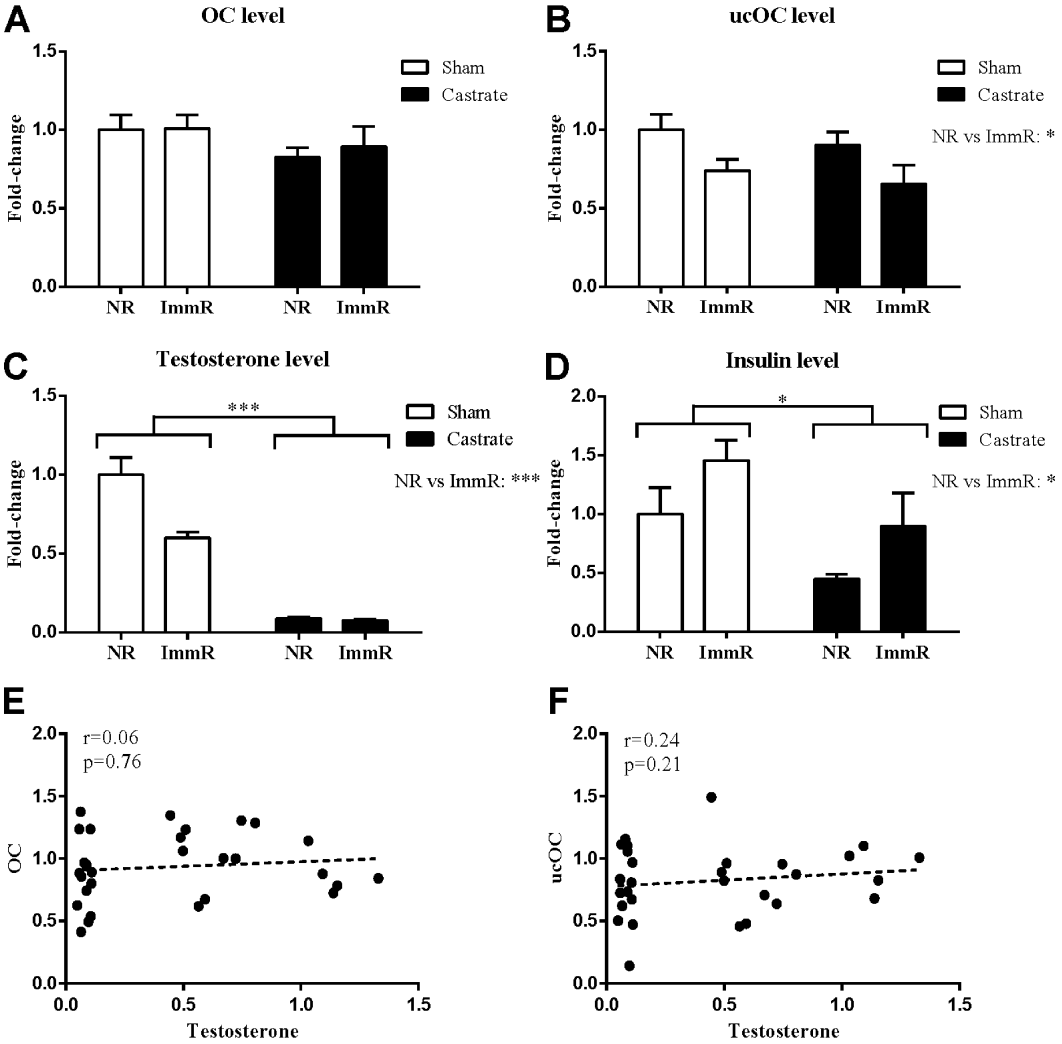

Figure 1 .

Figure 2

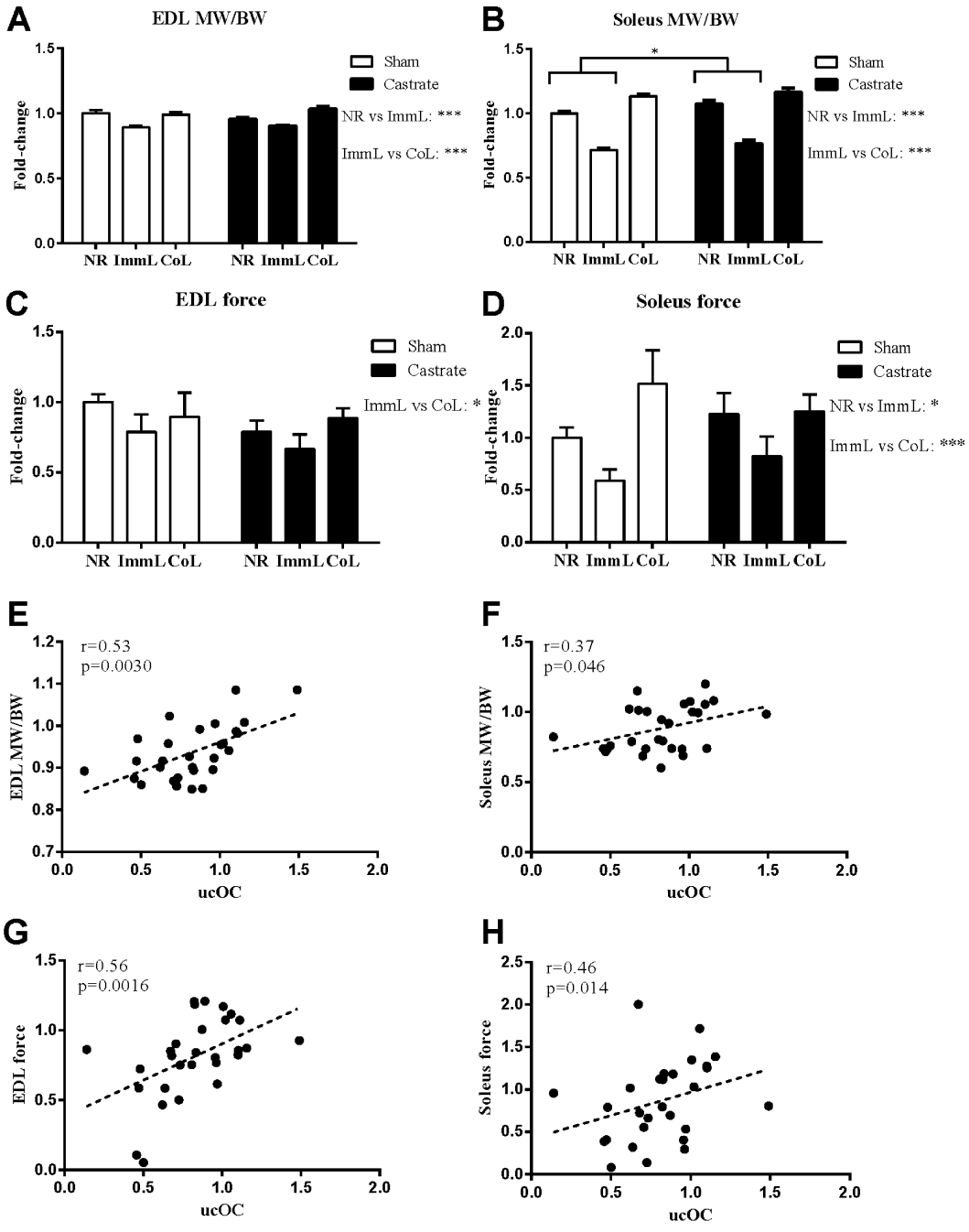

Figure 2 .

Figure 3

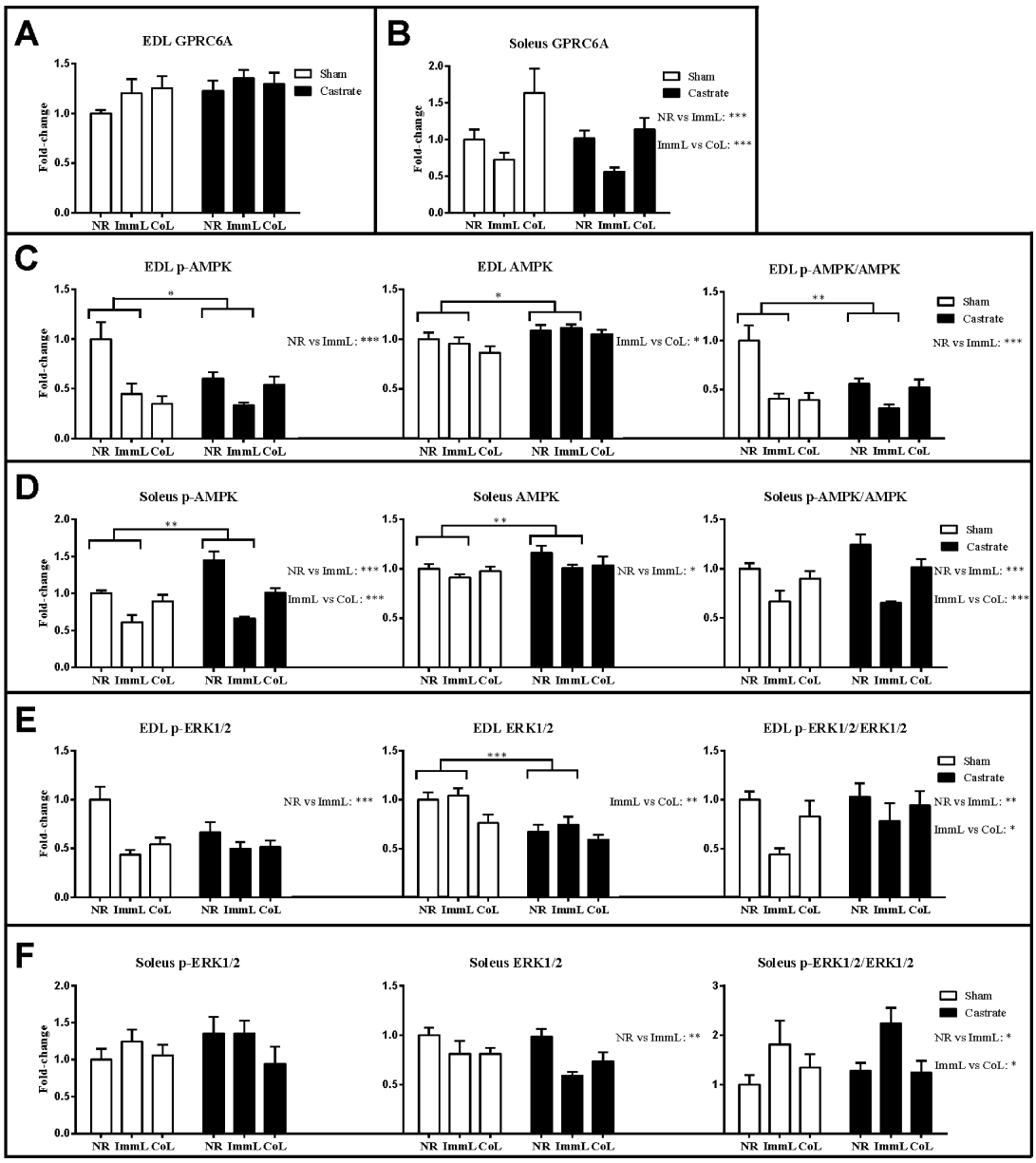

Figure 3 .

Figure 4

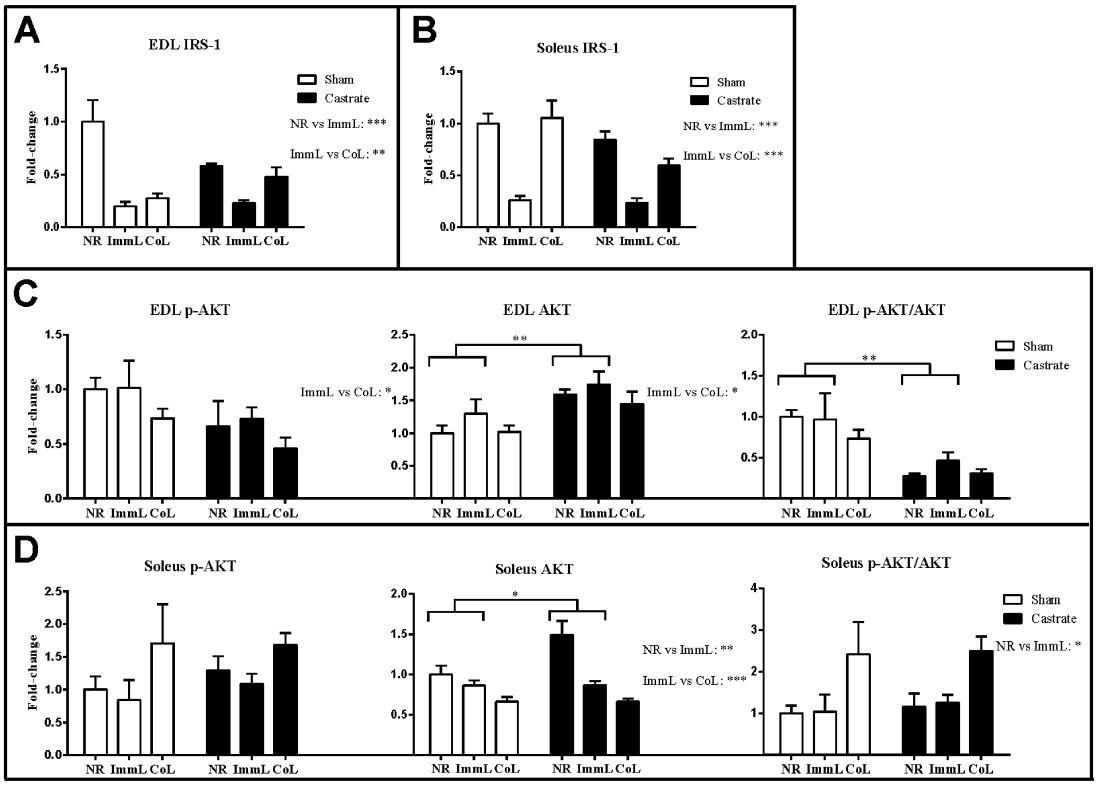

Figure 4 .

Figure 5

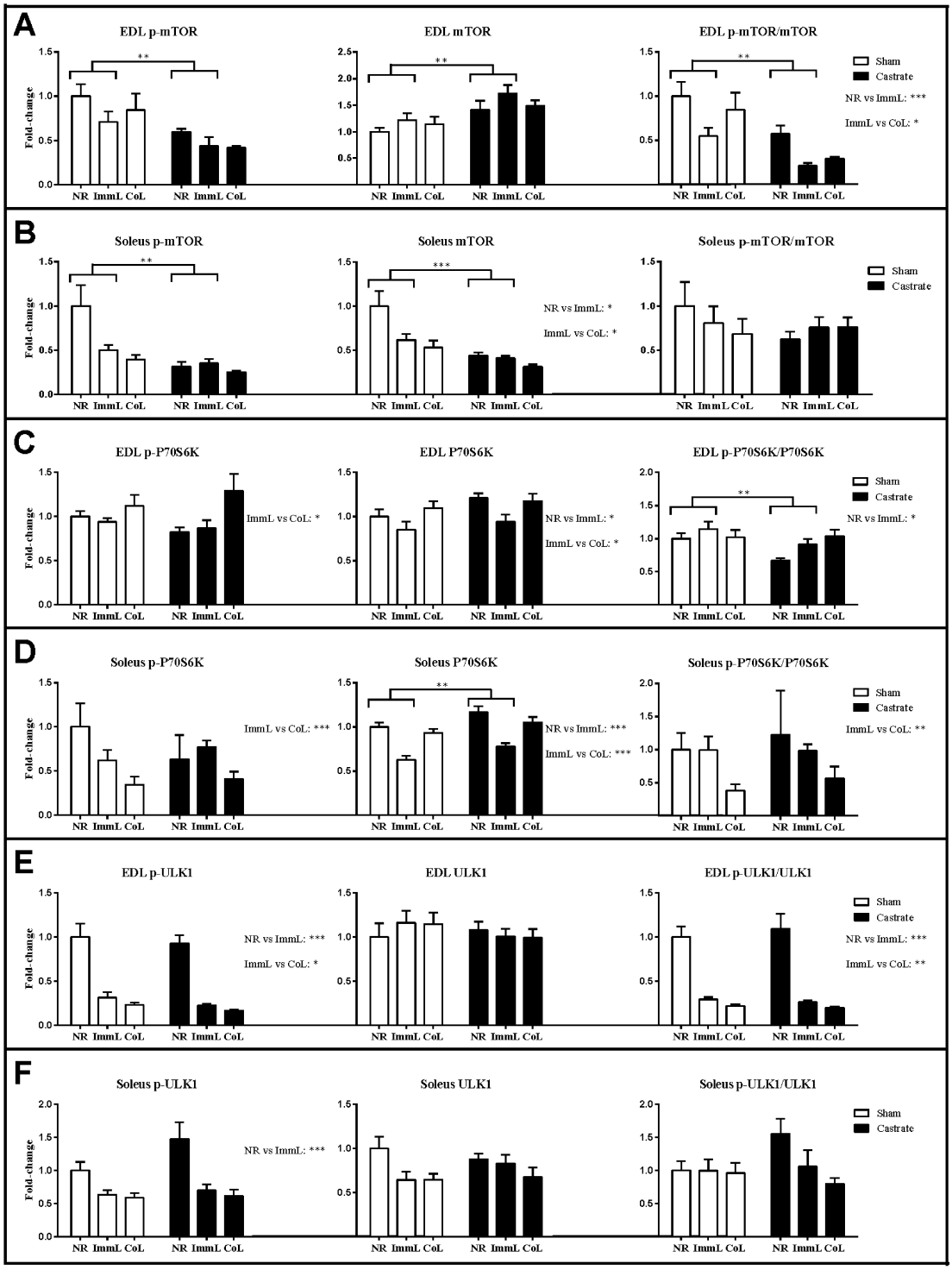

Figure 5 .

Figure 6

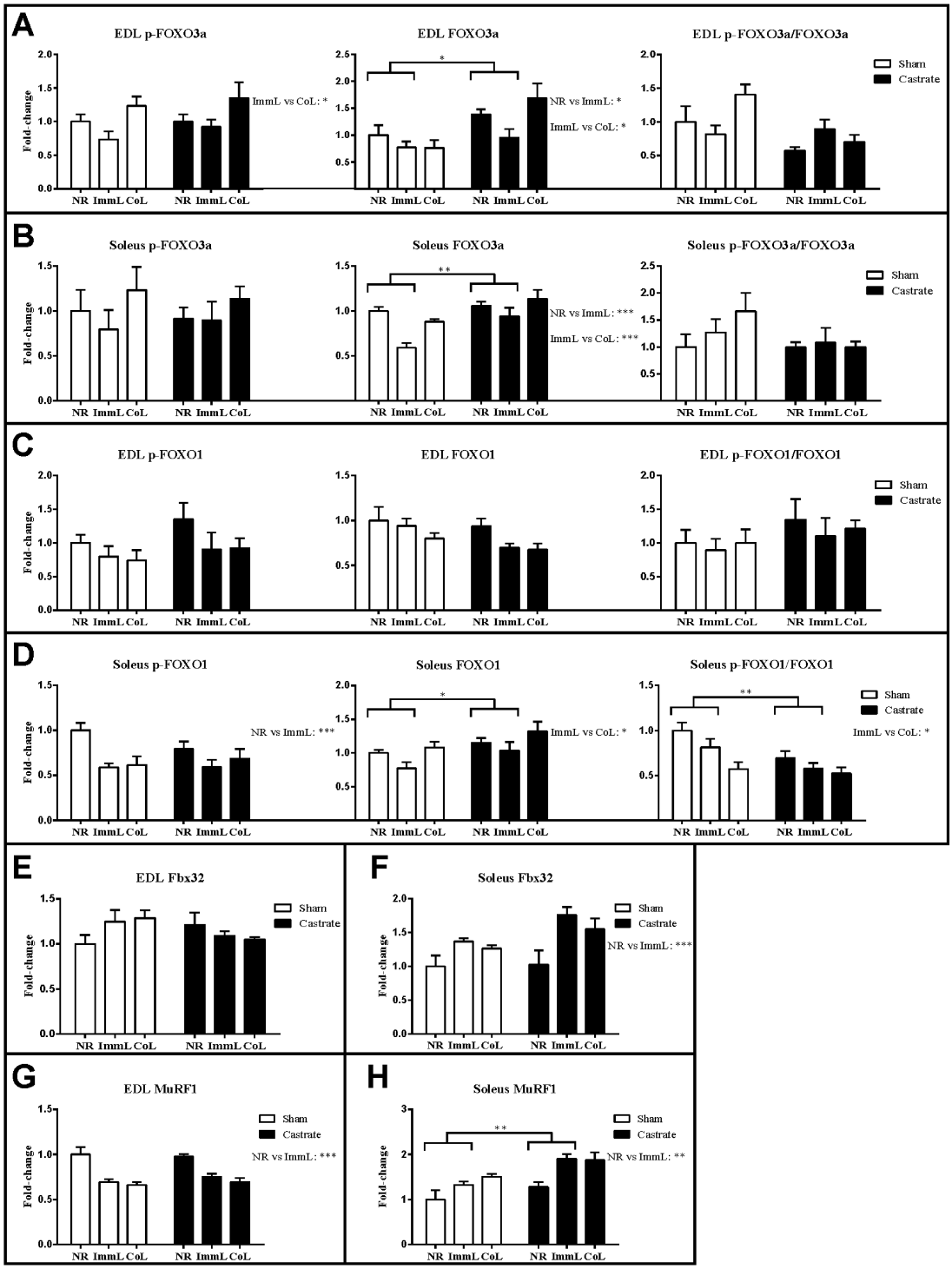

Figure 6 .

Figure 7 revised .

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