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The Effect of Serum Origin on Tissue Engineered Skeletal Muscle Function

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ABSTRACT

Skeletal muscle phenotype is regulated by a complex interaction between genetic, hormonal, and electrical inputs. However, because of the interrelatedness of these factors in vivo it is difficult to determine the importance of one over the other. Over the last 5 years, we have engineered skeletal muscles in the European Union (EU) and the United States (US) using the same clone of C2C12 cells. Strikingly, the dynamics of contraction of the muscles was dramatically different. Therefore, in this study we sought to determine whether the hormonal milieu (source of fetal bovine serum (FBS)) could alter engineered muscle phenotype. In muscles engineered in serum of US origin time-to-peak tension (2.2-fold), half relaxation (2.6-fold), and fatigue resistance (improved 25%) all showed indications of a shift towards a slower phenotype. Even though there was a dramatic shift in the rate of contraction, myosin heavy chain expression was the same. The contraction speed was instead related to a shift in calcium release/sensitivity proteins (DHPR = 3.1-fold lower, slow CSQ = 3.4-fold higher, and slow TnT = 2.4-fold higher) and calcium uptake proteins (slow SERCA = 1.7-fold higher and parvalbumin = 41-fold lower). These shifts in calcium dynamics were accompanied by a partial shift in metabolic enzymes, but could not be explained by purported regulators of muscle phenotype. These data suggest that hormonal differences in serum of USDA and EU origin cause a shift in calcium handling resulting in a dramatic change in engineered muscle function. J. Cell. Biochem. 115: 2198–2207, 2014.

KEY WORDS: MUSCLE PHENOTYPE; METABOLISM; EXERCISE

Skeletal muscle consists of functionally diverse fiber types that differ in contractile properties, myofibrilliar protein isoforms, metabolic enzymes, and mitochondrial density [Ranvier, 1874; Pette and Staron, 1990; Moss et al., 1995; Schiaffino and Reggiani, 1996]. In rodents, adult skeletal muscle fibers can generally be classified into four phenotypes via their metabolism and myosin heavy chain isoforms: (1) slow-twitch oxidative type I; (2) fast-twitch oxidative type IIa; (3) fast-twitch glycolytic type IIx/d; and (4) fast-twitch glycolytic type IIb [Pette and Staron, 1990; Schiaffino and Reggiani, 1996]. Humans show a similar diversity of fiber types, but lack the IIb MHC gene [Harrison et al., 2011].

Skeletal muscle phenotype is dependent on developmental state, cellular origin, and extrinsic signals. During skeletal muscle development in vivo [Kelly and Rubinstein, 1980; Condon et al., 1990; Hallauer and Hastings, 2002] and differentiation in vitro [Cooper et al., 2004], the slow and embryonic fast MHC isoforms are initially co-expressed in each myofiber. Further into development in vivo [DiMario and Stockdale, 1997; Hastings and Emerson, 1982] and in vitro [Huang et al., 2006], myofibers selectively express either the fast or slow program and repress the reciprocal program, resulting in a fast or slow phenotype. However, in vivo, adult muscle phenotype is primarily regulated by neural activity, with cross-innervation [Salmons and Sreter, 1976], denervation [Harris, 1981; DiMario and Stockdale, 1997], and denervation-electrical stimulation [Eken and Gundersen, 1988; Wehrle et al., 1994] studies demonstrating that slow muscle phenotype is driven by chronic low frequency electrical stimulation (CLFS) and fast muscle phenotype driven by infrequent high frequency electrical stimulation [Gorza et al., 1988; Westgaard and Lomo, 1988].

Fast and slow skeletal muscles differ not only in the rate of muscle tension development, but also the rate of relaxation. These functional differences are due to variation in the isoforms of contractile and regulatory proteins in the muscle [Close, 1972]. Muscle tension development, typically measured as time-to-peak tension (TPT), depends on: (1) sarcoplasmic reticulum (SR) calcium release; (2) the sensitivity of the myofibrillar apparatus to Ca^{2+}, which is regulated by troponin [Reiser et al., 1992; Fitzhugh and Marden, 1997]; and (3) the speed of actin-myosin interaction, which

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is dictated by MHC isoform and is typically the major determinant of TPT in mature muscle [Barany, 1967; Close, 1972]. Muscle relaxation, classically measured as half relaxation time (1/2RT), is determined by the rate of calcium clearance from the sarcoplasm. The rate of calcium clearance is in turn regulated by calcium sequestering proteins: Sarco(endo) plasmic reticulum Ca^{2+}-ATPase (SERCA) and parvalbumin. Two SERCA isoforms are encoded in muscle, SERCA1, preferentially expressed in adult fast-twitch skeletal fibers; and SERCA2a, in adult slow-twitch skeletal fibers [Wu and Lytton, 1993; Wu et al., 1995]. Parvalbumin is expressed only in fast-twitch muscle fibers and acts as a temporary Ca^{2+} buffer permitting more rapid calcium clearance and therefore faster relaxation [Leberer and Pette, 1986; Schwaller et al., 1999; Chen et al., 2006; Racay et al., 2006].

The molecular basis of fiber type-specific gene expression is still not clear. A number of proteins have been suggested to control slow (MEF2 [Wu et al., 2000; Potthoff et al., 2007], myogenin [Hughes et al., 1993], PGC-1α [Lin et al., 2002]), and fast (MyoD [Hughes et al., 1993], the six transcription factors six4 and six1 and the cofactors Eya1 and Vp16 [Grifone et al., 2004, 2005; Niro et al., 2010; Richard et al., 2011]) muscle protein expression. Reciprocally, the slow muscle program can be transcriptionally repressed by the transcription factor sox6, which also regulates part of the fast myogenic program [Hagiwara et al., 2005, 2007; Quiat et al., 2011]. However, whether these factors control early markers of muscle phenotype remains unclear.

Skeletal muscle tissue engineering holds much promise for both the treatment of traumatic muscle injury and the in vitro study of muscle development and physiology [Khodabukus et al., 2007]. One of the key challenges precluding the widespread use of muscle tissue engineering as a clinical tissue source or as an in vitro tool is the reproducibility of engineered muscle function both within laboratories and between different laboratories across the world. We have previously shown that engineered muscle force production and fibrinolysis varies greatly between different batches of fetal bovine serum (FBS) that come from the same supplier [Khodabukus and Baar, 2009]. Animal serum is a basic component for cell culture media, providing a wide range of growth factors, hormones, vitamins, amino acids, fatty acids, and trace elements that are required for cell growth. Despite its widespread use, animal sera exhibit very high levels of batch to batch variation which can affect cellular proliferation, differentiation, and function. In this study, we highlight this issue by determining the effect of serum from either the EU or USDA on engineered muscle function.

MATERIALS AND METHODS

2D CELL CULTURE

The C2C12 myoblast cell line (ATCC) was grown in growth media consisting of high glucose DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) until 70% confluent. All experiments were performed using the same clone of C2C12 cells between passages 6 and 10. In both the US and the UK, multiple FBS lots from at least three independent companies were tested. The contractile dynamics presented were consistent across all of the lots tested. Therefore, we are confident that the results represent a difference between what is available in the US and the UK and not lot-to-lot or company-to-company variations. The lot that demonstrated the highest maintained force (force of the constructs from week 2 to week 4) was selected both in the US and the UK. Detailed analysis of the growth factor/hormone, amino acid, fatty acid, and trace element content was not available from any of the companies. In the US, the FBS used for the experiments shown was from Hyclone (CAT# SH3039.02; LOT# AUB34149). In the UK, the FBS used for the experiments shown was from Biosera (CAT# S1810; LOT# 50567751810).

3D CELL CULTURE

Muscles were engineered using fibrin casting as reported previously [Khodabukus and Baar, 2009]. Briefly, the muscle constructs were engineered between two 6 mm long silk sutures set 12 mm apart on Sylgard (PDMS)-coated dishes. 500 μl of growth media containing 10 U/ml thrombin, 0.2 μg/ml genipin, and 0.5 μg/ml aprotinin was added to the plate and agitated until it covered the entire surface. The growth media used to make up the thrombin mixture was made with the relevant serum to ensure that the constructs were only cultured in the stated experimental conditions. 200 μl of 20 mg/ml fibrinogen was added dropwise and the gels were left to polymerise for 1 h before addition of 100,000 cells. Two days after plating cells, the constructs were switched to differentiation media previously found optimal for engineered muscle [Khodabukus and Baar, 2009], which consisted of DMEM supplemented with 7% FBS and 100 U/ml penicillin for the remainder of the experiment.

ENGINEERED MUSCLE FUNCTIONAL TESTING

Functional testing of the C2C12 constructs was performed 14 days after the onset of differentiation as described previously [Dennis et al., 2001]. Briefly, one of the anchors was freed from the Sylgard substrate and attached to a custom-made force transducer via one of the minutien pins. Rheobase (R_{50}) and chronaxie (C_{50}) were determined as described previously [Dennis et al., 2001]. Rheobase was calculated as the electric field strength (V/mm) eliciting 50% peak twitch force (P_t) with a 4 ms pulse width. Maximum tetanic force was calculated using a 1 s train at 150 Hz for each construct at four times rheobase and a 4 ms pulse width. Cross-sectional area was calculated from the measured width of each construct (at its narrowest point), assuming a rectangular cross-section and a depth of 500 μm. Specific force was calculated as kilonewtons per square meter: the force generated by the construct (kN) divided by its cross-sectional area (m^2).

WESTERN BLOT

Tissues were washed in ice-cold PBS, and then blotted dry before freezing in liquid nitrogen and storing at −80 °C. At the time of processing, samples were powdered in a 1.5 mL microcentrifuge tube on dry ice, suspended in 200 μL ice-cold sucrose lysis buffer (50 mM Tris pH 7.5, 250 mM sucrose, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM $Na_2(PO4)_2$, and 0.1% DTT) and shaken at 1,400 rpm for 1 h at 4 °C in an Eppendorf thermomixer (Hauppauge, NY). The samples were then centrifuged at 4 °C for 1 min at 10,000 g to remove insoluble material. The

supernatant was transferred to a new tube, and protein concentration was determined using the DC protein assay (Bio-Rad, CA). Equal aliquots of protein in 1× Laemmli sample buffer were boiled for 5 min before separation on a 10% acrylamide gel by SDS–polyacrylamide gel electrophoresis. After electrophoresis, proteins were electrophoretically transferred to a nitrocellulose membrane (Protran, Whatman, Piscataway, NJ) at 100 V for 1 h. The membrane was blocked for 1 h in 3% milk in Tris-buffered saline + 0.1% Tween (TBST). Membranes were incubated overnight at 4 °C with the appropriate primary antibody in TBST at 1:1,000. The membrane was then washed three times in TBST before incubation for 1 h at room temperature with the appropriate peroxidase-coupled secondary antibody in TBST at 1:10,000 (Pierce, Rockford, IL). Antibody binding was detected using an enhanced chemiluminescence horseradish peroxidase substrate detection kit (Millipore, Billerica, MA). Imaging and band quantification were carried out using a Chemi Genius Bioimaging Gel Doc System (Syngene, Cambridge, UK). The primary antibodies used in this study were MF20, F59, CaF2–5D2, M3FII, (Hybridoma Bank, Iowa), total eEF2, SERCA 2a, (Cell Signaling, MA), SDH, ATPsynthase, (Millipore, MA), LDH, PFK, GLUT4, Cytochrome C, CPT-1, PGC1-α, PRC, MEF2, MyoD, Myogenin, Myf5, Sox6, Six4, Troponin C-SS, Troponin C-FS, Troponin I-SS, Troponin I-FS, Troponin T-SS, and Troponin T-FS (Santa Cruz Biotechnology, CA).

STATISTICAL ANALYSIS

Data is presented as means ± S.E.M. Differences in mean values were compared within groups and significant differences were determined by ANOVA with post hoc Tukey-Kramer HSD test using Brightstat.com [Stricker, 2008]. The significance level was set at $P < 0.05$.

RESULTS

EFFECT OF FBS ORIGIN ON SPONTANEOUS CONTRACTILITY

One of the most striking differences we found between the FBS of different origins was the effect on spontaneous contractility (Fig. 1). When cultured in EU origin FBS, spontaneous contractions were single impulses and never fused (Fig. 1A). In the presence of USDA origin FBS, constructs were much more spontaneously contractile and formed tetanic/fused spontaneous contractions (Fig. 1B). The tetanic contractions normally lasted 2–6 s, but contractions longer than 10 s have been observed. This contractile activity was reminiscent of a developing slow muscle fiber [Eken et al., 2008].

EFFECT OF FBS ORIGIN ON CONTRACTILE PROPERTIES

Two weeks post-differentiation we tested the contractile properties of the engineered tissues. When cultured in the presence of EU-FBS the time-to-peak tension (Fig. 1C) was 40% slower in USDA (53.4 ± 1.6 ms) compared to EU-FBS (33.0 ± 0.6 ms). Half relaxation time (1/2RT; Fig. 1D) was ∼30% slower in USDA (54.8 ± 1.7 ms) compared to EU-FBS (32.3 ± 0.5 ms). The result of the slower TPT and 1/2RT was that the force-frequency relationship was shifted to the left in USDA- (Fig. 1E) compared with the EU-FBS. In USDA-FBS the force-frequency relationship was similar to that of a slow phenotype muscle, with a fully fused tetanus occurring at 20 Hz, whereas in EU-FBS the force-frequency was similar to that of a fast phenotype muscle, with a fully fused tetanus occurring above 80 Hz (Fig. 1E). In accordance with the shift to slow contractile dynamics in USDA-FBS, these constructs also showed greater fatigue resistance, losing less force during a 3 min fatigue protocol than those raised in EU-FBS (USDA-FBS = 44.9 ± 2.5%; EU-FBS = 60.6 ± 4.5% force decrease; Fig. 1F). These data suggest that serum of a different origin is sufficient to change engineered skeletal muscle phenotype from fast to slow.

EFFECT OF FBS ORIGIN ON MYOSIN HEAVY CHAIN PROTEIN EXPRESSION

To determine whether the differences in contraction dynamics could be explained by a shift in MHC isoform, we analyzed the MHC isoform profile by Western blot (Fig. 2). In spite of the dramatic difference in contractile dynamics, there was no difference in the relative amount of slow, fast, or total MHC between muscles engineered in the EU versus the USDA serum. Further, the fact that the total myosin heavy chain was not different suggests that there was no change in the degree of differentiation in the two conditions.

EFFECT OF FBS ORIGIN ON CALCIUM RELEASE AND REGULATORY PROTEINS

Since changes in MHC could not explain the differences in contractile dynamics between the muscles grown in EU versus USDA serum, we next sought to determine whether the calcium regulatory proteins, such as DHPR (a regulator of calcium release) and troponin (regulator of calcium sensitivity), could explain the increased rate of contraction. In support of a role for calcium release proteins regulating contraction, constructs grown with EU-FBS had 3.12-fold more DHPR protein than USDA-FBS (Fig. 3). Constructs grown with USDA-FBS also showed a small decrease (0.86 ± 0.04-fold) in the fast CSQ1 protein content and a large increase (3.4 ± 0.3-fold) in the slow CSQ2 compared to EU-FBS (Fig. 3). Within the troponin (Tn) complex, TnT showed a fast to slow shift with less fast TnT (0.4 ± 0.07-fold) and more slow TnT (1.9 ± 0.27-fold) in the USDA serum. The other components of the troponin complex (TnC and TnI) were all lower in serum from the USDA, but there was no fast-to-slow shift (TnC-SS = 0.3 ± 0.07-fold; TnC-FS = 0.7 ± 0.06-fold; TnI-SS = 0.3 ± 0.1-fold; TnI-FS = 0.5 ± 0.10-fold; Fig. 3).

EFFECT OF FBS ORIGIN ON CALCIUM SEQUESTERING PROTEINS

We next determined whether a shift in calcium sequestering proteins could explain the slowed relaxation rate in USDA-FBS (Fig. 4). The levels of slow SERCA (sSERCA) protein increased 1.7 ± 0.3-fold, when cultured in USDA compared to EU-FBS. In contrast, the fast SERCA (fSERCA) protein was significantly lower (0.52 ± 0.07-fold), when cultured in USDA compared to EU-FBS. The slow isoform of the calcium sequestering protein calsequestrin (sCSQ) were significantly higher (3.43 ± 0.30-fold) when cultured in USDA compared to EU-FBS. Most striking, the fast specific calcium sequestering protein, parvalbumin was 41-fold lower in USDA- compared to EU-FBS.

EFFECT OF FBS ORIGIN ON GLYCOLYTIC PROTEIN EXPRESSION

To determine why the muscles engineered from USDA-FBS were more fatigue resistant, we next analyzed metabolic proteins that

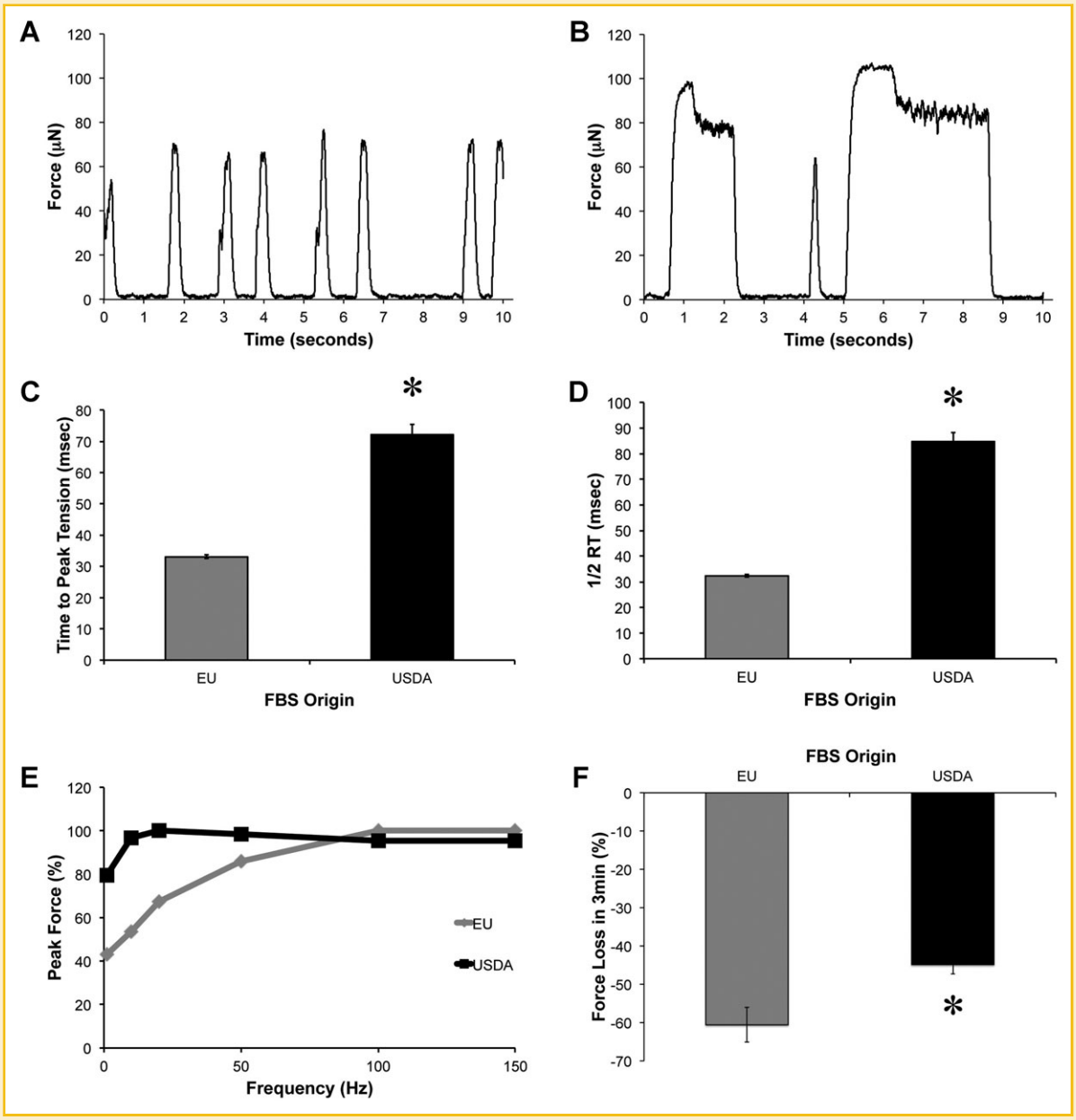

Fig. 1. Contractile properties of muscles engineered in serum from EU or USDA origin. Traces of spontaneous contractions in muscles engineered in serum of (A) EU or (B) USDA origin. The different sera were associated with shifts in (C) time-to-peak tension and (D) half relaxation rates that together resulted in a leftward shift in the (E) force-frequency curve in muscles engineered in the US. Concomitant with the differences in contractile dynamics, there was also a shift in (F) fatigue. The muscles engineered in the USDA serum lost less force over a 3-min fatigue protocol than those engineered in serum from the EU indicating improved fatigue resistance. Data are representative traces and dynamics from n = 6 with at least three independent experiments. * Indicates significantly different than EU-FBS ($P < 0.05$).

may enable greater ATP production. First, since C2C12 cells are thought to be shifted towards glycolytic metabolism, we determined phosphofructokinase (PFK), lactate dehydrogenase (LDH), and GLUT 4 (the rate limiting enzymes in glycolysis) levels. PFK (0.48 ± 0.07-fold), LDH (0.24 ± 0.05- fold), and GLUT 4 (0.60 ± 0.15) levels were all lower in USDA compared to EU-FBS, suggesting that in USDA-FBS muscles are less glycolytically active (Fig. 5).

EFFECT OF FBS ORIGIN ON MITOCHONDRIAL, FAT OXIDATION, AND FAT TRANSPORT PROTEIN EXPRESSION

With the decrease in glycolytic metabolism, we expected a reciprocal increase in oxidative/fat metabolism. Consistent with this hypothesis, the mitochondrial marker SDH was 2.04 ± 0.16-fold higher and the very long-chain acyl-CoA dehydrogenase was $3.3 \pm 0.43 =$ fold higher in serum from the USDA (Fig. 5). However, other markers of mitochondrial mass (such as cytochrome C and ATPsynthase) or fat

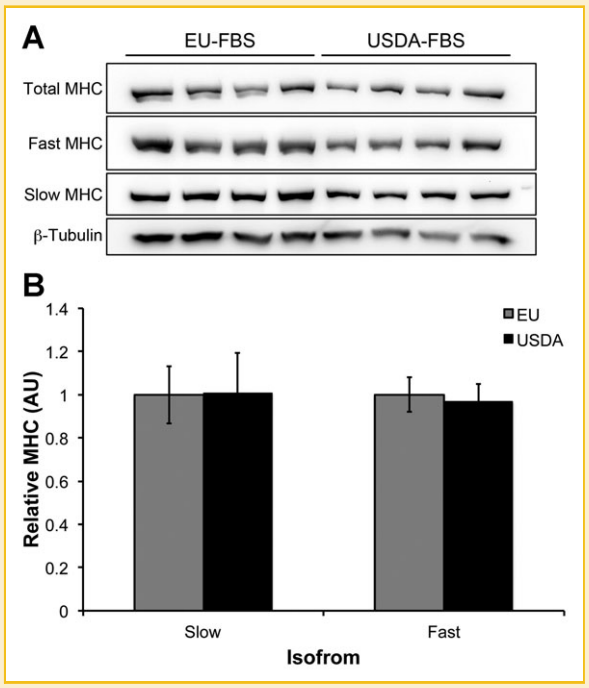

Fig. 2. Myosin heavy chain content of muscles engineered in serum from EU or USDA origin. (A) Western blots showing myosin heavy chain protein and (B) the quantification of the protein by densitometry. Data are mean ± SEM from n = 6 with at least three independent experiments.

metabolism (CPT-1, and the long- and medium-chain acyl-CoA dehydrogenase) were not upregulated in serum from the USDA. This indicates that there is not a coordinated shift in metabolism as a result of culturing in the different sera.

EFFECT OF FBS ORIGIN ON REGULATORS OF MUSCLE PHENOTYPE

A number of proteins have been suggested as controllers of muscle phenotype. We therefore measured the levels of purported drivers of the slow phenotype (Fig. 6), such as PGC-1α (82% lower in USDA), MEF2 (97% lower in USDA), and myogenin (37% lower in USDA) and those that are reported to drive muscle towards a fast phenotype such as MyoD (2.65-fold higher in USDA), Six4 (not different between the sera), and Sox6 (not different between the sera). Interestingly, none of these regulators could explain the difference in contractile dynamics or calcium signaling observed between the two groups.

DISCUSSION

In this paper we have shown that the country of origin of FBS can dramatically alter engineered muscle function. When cultured in FBS of EU origin, engineered C2C12 muscles adopt a more fast-glycolytic phenotype. In contrast, culturing with USDA approved FBS results in a slow-oxidative phenotype muscle. The phenotype shift in serum of different origin is only a partial shift since culturing in USDA-FBS does not alter the MHC isoform or many mitochondrial proteins and fat oxidation genes compared to EU-FBS fed constructs.

Classically, adult muscle fiber type has been defined by myosin heavy chain isoform. From our contractile data (i.e., force-frequency, TPT, and 1/2RT) we saw a classic fast- to-slow contractile shift when cultured in USDA-FBS. However, this occurred in the absence of a change in MHC isoform. This finding supports recent evidence calling into question the accuracy of classifying fibers by solely MHC isoform [Bottinelli, 2001; Spangenburg and Booth, 2003; Calderon et al., 2010] and demonstrates the value of functional testing muscle tissue. The lack of MHC isoform shift indicates that we did not observe a full phenotype shift but a selective shift in metabolic and calcium handling proteins. The change in contractile

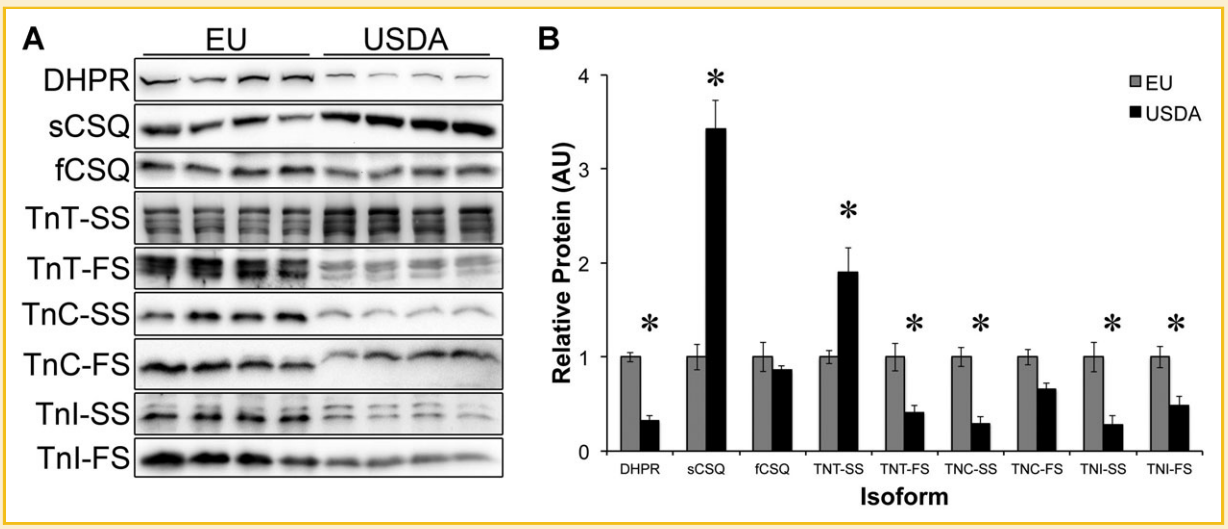

Fig. 3. Calcium release and sensitivity proteins of muscles engineered in serum from EU or USDA origin. (A) Western blots showing protein involved in the release of calcium from the SR (DHPR) and the sensitivity to calcium (TnT, TnI, and TnC) and (B) the quantification of the protein by densitometry. Data are mean ± SEM from n = 6 with at least three independent experiments. * Indicates significantly different than EU-FBS ($P < 0.05$).

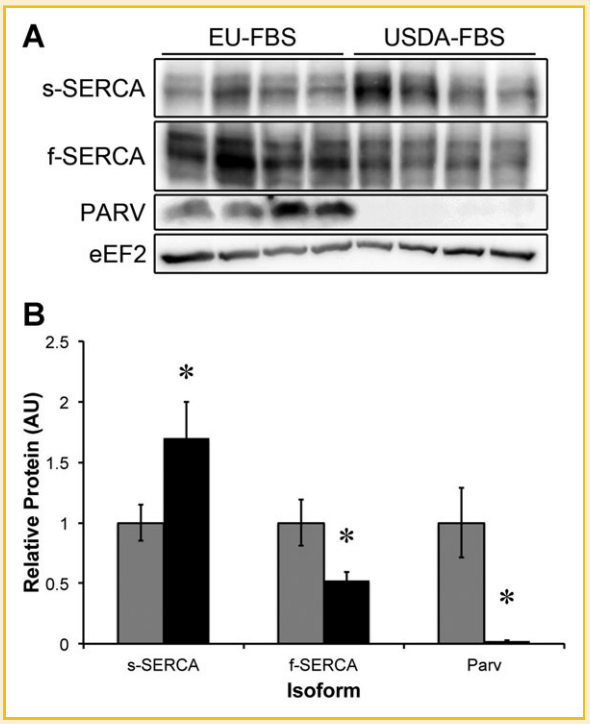

Fig. 4. Calcium uptake and sequestering proteins of muscles engineered in serum from EU or USDA origin. (A) Western blots showing proteins involved in calcium uptake (SERCA) and sequestering (CSQ and parvalbumin) and (B) the quantification of the protein by densitometry. Data are mean ± SEM from n = 6 with at least three independent experiments. * Indicates significantly different than EU-FBS ($P < 0.05$).

dynamics (i.e., TPT and 1/2RT) but not MHC further supports the finding that different signals regulate TPT, 1/2RT, and MHC content in engineered muscle [Huang et al., 2006]. The lack of correlation between TPT and MHC in our study can be explained by the expression of the SR calcium release proteins and the troponin complex. We found a decrease in the DHPR, a marker of fast fibers in vivo. The lower density of DHPR channels in the muscles engineered in USDA-FBS 1 would slow calcium release and decrease the rate of contraction [Luff and Atwood, 1971].

TPT is also regulated by the troponin complex, where Ca^{2+} binding to TnC results in a series of allosteric changes in TnI, TnT, and tropomyosin to permit contraction [Gordon et al., 2000]. Classically, TnC is thought to dictate the rate of contraction through its calcium sensitivity: slow TnC has a higher calcium sensitivity than fast [Close, 1972; Gordon et al., 2000]. Furthermore, within single fibers the isoform of TnT and TnI typically form a complex with the corresponding TnC isoform [Kischel et al., 2000; Brotto et al., 2006]. However, we found that only the TnT isoform correlated with the function of our engineered muscles, whereas both the slow and fast isoforms of TnC and TnI were lower in the USDA tissues. While this was unexpected, the differential expression of the three troponins supports findings that expression of the troponin complex members is not temporally co-ordinated [Silver and Etlinger, 1985; Campione et al., 1993; Brotto et al., 2006; Yu et al., 2007]. Additionally, it appears that of the troponin isoforms, TnT plays the dominant role in dictating contractile dynamics [Reiser et al., 1992; Fitzhugh and Marden, 1997; Huang et al., 1999]. In the heart [Huang et al., 1999], or in unloading experiments [Campione et al., 1993; Yu et al., 2007], changes in only the TnT isoform correlate both with contractile dynamics and fatigue resistance. Importantly, these

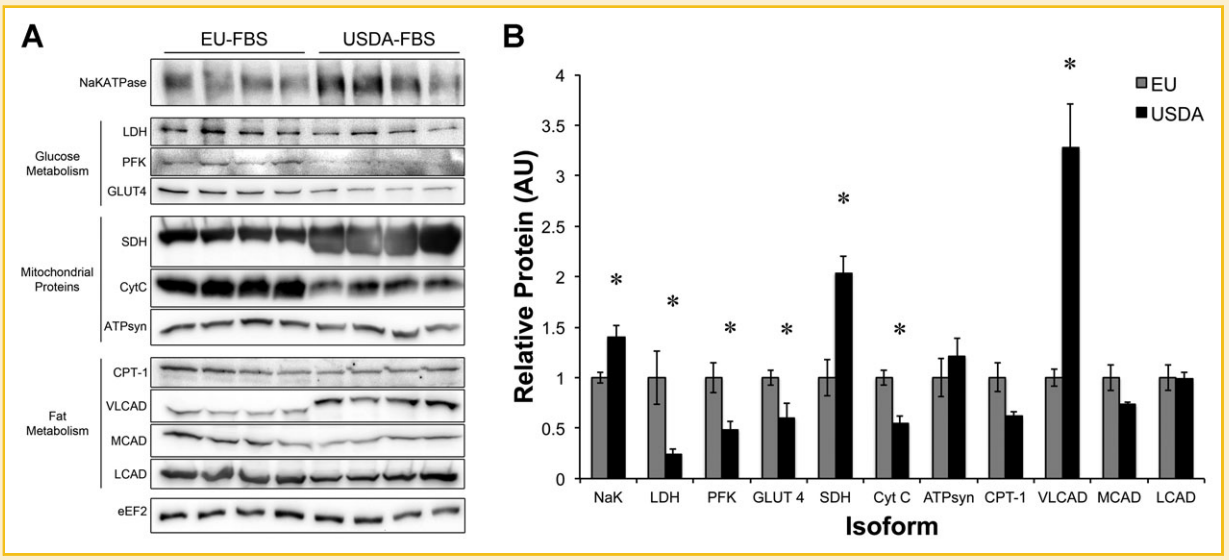

Fig. 5. Metabolic proteins from muscles engineered in serum from EU or USDA origin. (A) Western blots showing proteins involved in skeletal muscle metabolism and (B) the quantification of the protein by densitometry. Data are mean ± SEM from n = 6 with at least three independent experiments. * Indicates significantly different than EU-FBS ($P < 0.05$).

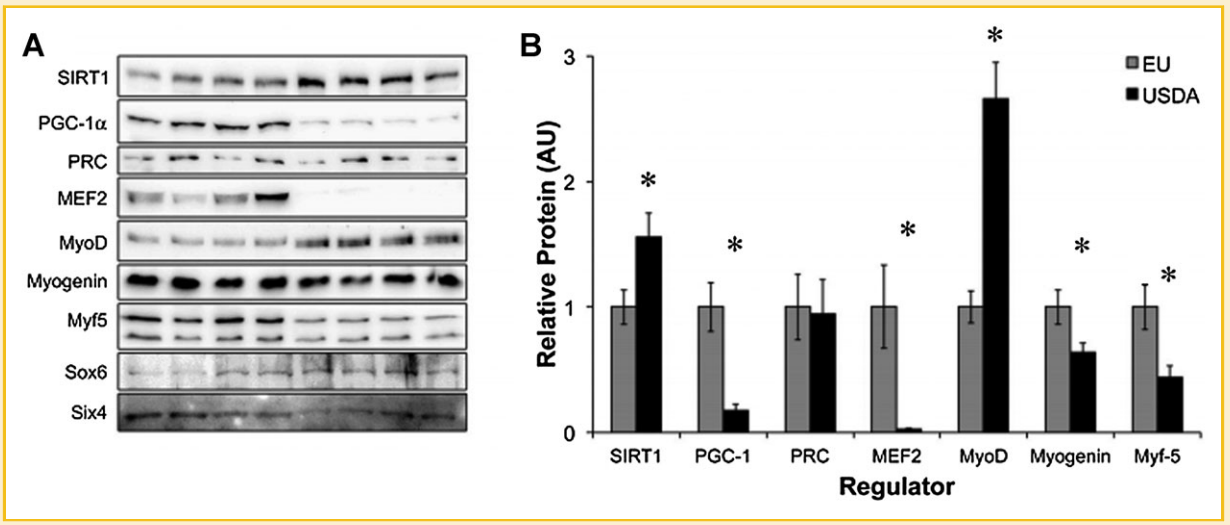

Fig. 6. Putative phenotype regulatory proteins of muscles engineered in serum from EU or USDA origin. (A) Western blots showing proteins thought to control fast (MyoD, Six4, and Sox6) or slow (MEF2, PGC-1α, and myogenin) muscle phenotype and (B) the quantification of the protein by densitometry. Data are mean ± SEM from n = 6 with at least three independent experiments. * Indicates significantly different than EU-FBS ($P < 0.05$).

functional changes occur before significant changes in either the TnC or TnI isoform [Kischel et al., 2000; Yu et al., 2007]. The relationship between TnT, dynamics and fatigue observed in vivo is completely consistent with what we observed in the shift from EU to USDA serum. The decrease in all of the TnC and TnI isoforms in serum from the USDA likely indicates elevated intracellular calcium levels, since increasing intracellular calcium increases the degradation of TnC but not TnT [Silver and Etlinger, 1985].

Skeletal muscle relaxation is controlled by the calcium uptake protein SERCA and the calcium sequestering proteins CSQ and parvalbumin [Close, 1972; Schwaller et al., 1999; Murphy et al., 2009]. The expression of the fast and slow isoforms of SERCA and CSQ correlated well with the observed changes in half-relaxation time. However, the most striking difference we found was the level of parvalbumin in the constructs.

Parvalbumin is a calcium sequestering protein that is specifically enriched in fast muscle fibers. Parvalbumin was high in the EU samples and barely detectable in the slow-like phenotype muscle engineered with USDA-FBS. Parvalbumin expression is thought to be controlled by neural activity, with fast patterned electrical activity promoting [Gorza et al., 1988] and slow patterned electrical activity decreasing its expression. Since our engineered muscles were not innervated, the decrease in parvalbumin levels could be the result of the greater spontaneous contractility and consequent elevated levels of intracellular calcium seen with the USDA-FBS. In vivo parvalbumin appears to be under the transcriptional control of the Six transcriptional complex (STC). When Six4 and Six1 are knocked out, muscle is unable to initiate the fast program and parvalbumin expression is lost [Grifone et al., 2005; Niro et al., 2010]. However, in our muscles cultured in USDA-FBS we observed no difference in Six4 protein content. PGC-1α also regulates parvalbumin expression in vitro [Mormeneo et al., 2012]. PGC-1α knockdown in human myotubes results in a dramatic decrease in parvalbumin expression. We do see a 5.85-fold decrease in PGC-1α protein levels and this could potentially explain the decrease in parvalbumin. However, it should be noted that overexpression of PGC-1α induces a slow shift in muscle including in calcium handling proteins [Summermatter et al., 2012]. This makes the dependence of parvalbumin expression on PGC-1α paradoxical and suggests a more complex interplay between several factors that regulate calcium handling.

One of the more striking differences between the two FBS groups was spontaneous contractility. In general, we have found the FBS of USDA origin is much more supportive of spontaneous contractility. Not only are the spontaneous contractions more frequent and vigorous they also form tetanic-like contractions typically lasting 3–4 s but some last as long as 10 s. During the development of a slow muscle fiber in vivo, there is a characteristic increase in the number of contractions and then an increase in the length of each contraction [Eken et al., 2008]. The spontaneous contractility in the USDA-FBS was similar to that of a more developed slow muscle fiber. However, even with batches of USDA serum which did not support vigorous spontaneous contractility, a slow phenotype-like muscle was formed based on contractile data (i.e., force-frequency relationship and contractile dynamics), suggesting that the spontaneous contractility is not solely responsible for the phenotype shift.

Together with the shift towards slower contractile dynamics, fatigue resistance was also enhanced in cultures made with USDA-FBS. The shift in fatigue resistance included both a decrease in glycolytic enzymes and an increase in mitochondrial proteins, such as SDH, and enzymes of fat metabolism, such as the fat oxidation enzyme VLCAD. However, despite the increases in select mitochondrial proteins, PGC-1α protein was markedly decreased. PGC-1α is considered to be a master regulator of mitochondrial biogenesis in response to stresses such as exercise [Baar et al., 2002] but it is not

required for basal mitochondrial biogenesis [Kraft et al., 2006]. During differentiation, mitochondrial mass is regulated in part by PGC-1α regulated coactivator (PRC) and pyruvate which can increase mitochondrial mass independent of PGC1α [Wilson et al., 2007]. If PGC-1α is not driving the metabolic shift, the mitochondrial profile seen in USDA-FBS constructs could be the result of the decrease in parvalbumin [Chen et al., 2006; Racay et al., 2006]. Parvalbumin knockout mice have increased mitochondrial mass and display increased protein levels of SDH and cytochrome *c* but no change in ATPsynthase compared to wild-type mice, identical to what we see between the two sera [Chen et al., 2006; Racay et al., 2006]. Mitochondria can function not only as a metabolic engine, but also as a calcium store with similar calcium kinetics to parvalbumin [Racay et al., 2006]. The increase in mitochondrial mass seen with parvalbumin elimination appears to be a homeostatic compensation mechanism to compensate for the loss of calcium buffering capacity [Chen et al., 2006; Racay et al., 2006]. How parvalbumin regulates mitochondrial mass remains unclear but is most likely via regulating calcium transients and consequent effects on downstream calcium signaling pathways such as CamK and calcineurin.

Currently we do not know what the root cause of the change in phenotype from FBS of different origins. The shift is likely due to the regulatory laws concerning cattle in the two regions. The US Food and Drug Administration (FDA) currently approves the use of recombinant bovine growth hormone, progesterone, trenbolone acetate and estrogen in cattle, all of which are prohibited within the EU [Stephany, 2010]. The specific growth factors or hormones responsible for the shift seen in culture are unclear. Estradiol and testosterone derivatives are known to induce intracellular calcium signals [Estrada et al., 2000; Wang et al., 2007]. Estradiol increases SERCA2 protein content and Na^+/K^+-ATPase activity, one of the main determinants of spontaneous contractility. However, the effect could equally be the result of differences in growth hormone (GH) and IGF-1. If both GH and IGF-1 levels are increased, a fast-to-slow phenotype shift is seen [Dalla Libera et al., 2004; Schuenke et al., 2008]. Importantly, both of these factors must be increased for the slow phenotype shift to occur [Schuenke et al., 2008], suggesting that GH and IGF-1 may underlie the shift towards a slow phenotype in USDA-FBS.

Tissue engineered skeletal muscle has the potential to be a powerful tool for drug discovery and studying muscle physiology and development [Khodabukus et al., 2007]. For this to be true, the in vitro milieu needs to be the same globally from laboratory to laboratory. We have previously shown that batch variability in both horse and fetal bovine serum can vary engineered muscle force production [Khodabukus and Baar, 2009]. Despite the variation between different sera we consistently see the same relative increase in force production in response to 24 h electrical stimulation [Khodabukus and Baar, 2012] and IGF-I (Khodabukus and Baar, unpublished observation) between different batches of sera from different origins. However, the effect of sera on engineered muscle function supports the need for serum-free culture media. Engineered muscle has been generated using alternative serum media such as those using Ultroser G [Gawlitta et al., 2008] or AIM-V [Fujita et al., 2010], with the serum-free media generating higher active tension than serum fed controls [Fujita et al., 2010]. However, Ultroser G is extracted from animal products and therefore is equally batch dependent and is extremely expensive, preventing its widespread use. Until a standardized media that can reproducibly support engineered muscle function is discovered, the use of these muscles as a model for muscle in vivo will remain limited.

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