

CORRESPONDENCE

Methodological issues limit interpretation of negative effects of satellite cell depletion on adult muscle hypertrophy

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We are writing regarding the recent publication in *Development* entitled ‘Satellite cell depletion prevents fiber hypertrophy in skeletal muscle’ (Egner et al., 2016). Egner et al. claim to have ‘essentially repeated’ our work (McCarthy et al., 2011); however, we think that methodological differences between the two studies make it impossible to compare the results directly. More importantly, technical problems with their analyses and lack of controls make it impossible to attribute the lack of growth that they observed to the loss of satellite cells (SCs). We have not studied hypertrophy in the extensor digitorum longus (EDL) muscle, as the mechanics and function of the tibialis anterior (TA)/EDL do not support the relevance of a synergist ablation-mechanical overload model. The EDL is only activated during the ‘swing phase’ of walking, designed for velocity at the expense of force. It operates essentially unloaded, undergoing only concentric contractions to lift the weight of the foot during ambulation (Carlson-Kuhta et al., 1998), so removing the TA should have little effect on the size of EDL (Lieber and Ward, 2011). On the other hand, the gastrocnemius, soleus and plantaris muscles are designed for force production and, following removal of the gastrocnemius muscle, the plantaris experiences significant concentric and eccentric loading during walking, the latter being crucial for hypertrophy (Booth and Thomason, 1991; Gregor et al., 2006). Thus, we will limit our comments to the results reported from the plantaris following synergist ablation, a well-accepted hypertrophic model.

We have performed synergist ablation surgery on hundreds of Pax7-DTA mice treated with tamoxifen to deplete SCs or vehicle, starting when they are at least 4 months old, and consistently find no difference in the plantaris growth response, as defined by muscle mass and fiber cross-sectional area (CSA), in the first 2 weeks. We do not include tamoxifen-treated mice that are less than 90% depleted in our analyses, and we do not observe significant proliferation of the few remaining SCs at 2 weeks, or myonuclear accretion into fibers. By contrast, Egner et al. report an average of only 76% depletion, and a 420% increase in SCs in their SC-depleted, overloaded (SC-OL+) plantaris; this level of depletion should have limited effect on growth. However, the fact that they detect only about five SCs in the entire cross-section of the control SC+OL- plantaris (figure 1B in Egner et al., 2016), makes the entire quantification questionable. That SCs are not effectively depleted is most clearly demonstrated by the observation that markers of muscle regeneration [embryonic myosin heavy chain (eMyHC)-positive and centrally nucleated fibers] are significantly higher in SC- compared with SC+ plantaris with overload

(figure 2B-D in Egner et al., 2016). We reported that SC-depleted muscle showed significantly fewer eMyHC+ fibers compared with vehicle-treated OL+, as expected, and severely impaired regeneration capacity after BaCl₂ injection. The higher regenerative response in plantaris from tamoxifen- compared with vehicle-treated mice in the Egner study suggests that there is actually more SC activity in response to overload in SC- mice, making the lack of growth even more inexplicable. Without the appropriate control – the parental Pax7-CreER strain tamoxifen treated (Fry et al., 2014; Jackson et al., 2015) and subjected to overload (Fry et al., 2014) – no conclusions regarding the specific effect of SC depletion, as opposed to tamoxifen toxicity, on growth can be drawn from the Egner study.

We think a much more reasonable explanation for the lack of growth reported by Egner et al. is that their tamoxifen-treated mice were unhealthy, as evidenced by lower body weight. They report that tamoxifen-treated mice had a 13% lower body weight than vehicle-treated mice. The tamoxifen-treated mice in our 2011 study showed 4% lower body weight compared with vehicle-treated mice (vehicle, 24.5±2.7 g; tamoxifen, 23.5±2.5 g), which was not statistically different. This is also the case for tamoxifen-compared with vehicle-treated mice from our various studies in both male (Fry et al., 2014, 2015; McCarthy et al., 2011) and female (Jackson et al., 2015, 2012) mice. As stated above, the parental Pax7-CreER strain must be tamoxifen treated to rule out tamoxifen toxicity accounting for the lack of growth in the Egner study.

In addition to being unhealthy, we posit that the difference in response to tamoxifen observed between the studies is due to the fact that the mice in the Egner study were less than 4 months old at the time of tamoxifen treatment, and thus were still growing. Femur and humerus growth increases rapidly from 4 to 12 weeks, after which time longitudinal bone growth appears to cease (Ferguson et al., 2003). We only use mice that are 4 months of age or older to be sure we are past the period of long bone growth, and therefore muscle growth, as we expect different SC requirements for hypertrophic growth in full grown adult compared with growing mice. Figure 7D in Egner et al., 2016 shows a larger myonuclear domain (MND) in SC-OL- compared with SC+OL- mice, indicating that control muscles were in fact growing, without addition of myonuclei, which is counter to the premise of the paper.

There are other technical differences contributing to the different results. As stated above, we have performed surgery on hundreds of mice and are very adept at the procedure, and do not experience the various problems reported by Egner et al. We are very careful not to

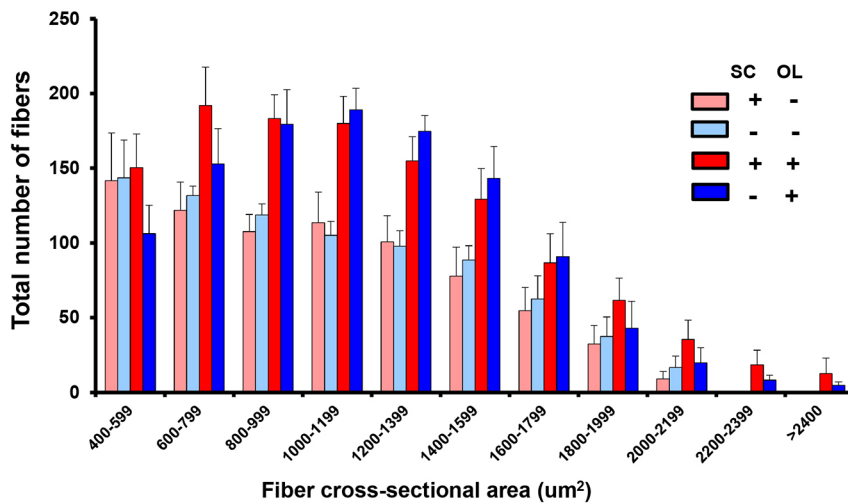


Fig. 1. Binned CSA presented as fiber frequency. Number of fibers counted per cross-section with data presented as mean \pm s.e.m. Data taken from McCarthy et al., 2011.

disturb the blood supply or the nerve to the plantaris and do not observe the ‘rupture damage’ described by Egner et al., where they eliminated 5 of 30 ablated mice because the plantaris was ‘almost completely degenerated’. We agree with Egner’s conclusion that their muscle weights are unreliable due to their inability to dissect the plantaris cleanly, resulting in overloaded plantaris muscle weights ranging from 20 to 45 mg. Given the small number of fibers quantified for CSA in their study, we also believe those values are unrepresentative. Using a grid, they only measured an average of 160 fibers (range 101-205) per cross-section, whereas we count essentially all fibers in the section (>1000). Measuring CSA with a grid could favor measuring larger fibers, because those fibers will have a higher chance of falling on a grid intersection. They report an unusually high number of very large fibers (>3000) which might be due to the fact that they counted relatively more of them, thereby skewing their results. Our results agree with those of Egner that hypertrophy is normally associated with myonuclear accretion, which is abolished in response to overload following tamoxifen treatment.

Although we do not see the extensive degeneration reported by Egner et al., we do agree that synergist ablation subjects the plantaris to significant mechanical overload that results in a regenerative response, which we documented in our 2011 Development paper (McCarthy et al., 2011). In our study, we saw a 30% increase in eMyHC⁺ fibers and centrally nucleated fibers in response to overload in the vehicle-treated plantaris. However, we saw no relationship between those two markers with each other or with fiber CSA, which also appears to be the case in the Egner et al. report (figure 2 in Egner et al., 2016), so the relationship of these markers to ‘degeneration’ is not clear. That is, as shown in the McCarthy et al. (2011) paper, some large fibers expressed eMyHC, and some large fibers were centrally nucleated, but not eMyHC⁺, which is why we chose to count all fibers in the entire cross-section in every muscle. Resident myonuclei might activate eMyHC expression as a repair response, independently of SCs, and eMyHC⁺ fibers could grow and contribute to the overall hypertrophic response, so we do not think it is appropriate to eliminate them from the analyses. Thus, as mentioned above, counting all fibers compared with counting 160 randomly selected fibers might contribute to the different results due to the heterogeneous and regional nature of the plantaris.

We reported in our 2011 paper that vehicle-treated overloaded mice had more very small, eMyHC⁺ fibers ($<300 \mu\text{m}^2$), which are

probably formed *de novo* as a result of SC-dependent regeneration, and those were quantified separately (McCarthy et al., 2011). However, they made up less than 1% of the total muscle area and did not contribute substantially to overall muscle mass. Re-analysis of all other fibers in bins in bar graph form with error bars (Fig. 1) shows that there is no significant difference in fiber size distribution between vehicle and tamoxifen groups.

We agree that on average, the increase in CSA that we observe at 2 weeks is relatively modest. We think that this overload model results in such a large increase in fiber CSA that by 2 weeks many of the largest fibers split. There is precedence for this in the literature in rats (Ho et al., 1980), mice (Vaughan and Goldspink, 1979) and humans (Larsson and Tesch, 1986; Tesch and Larsson, 1982) in response to a robust hypertrophic stimulus. As a result, although there is a rightward shift in fiber size distribution, average fiber CSA is only modestly larger, but there are more fibers and the muscle is significantly hypertrophied, evidenced by increased mass. (This might not have occurred in the Egner et al. study because they performed unilateral surgery and the mice may have favored the sham-operated limb.) Although we admit this is supraphysiological, the important point is that the response of SC-depleted muscle compared with muscle with its full complement of SCs is not different. Plantaris muscles with and without SCs grow.

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An apparent lack of effect of satellite cell depletion on hypertrophy could be due to methodological limitations. Response to 'Methodological issues limit interpretation of negative effects of satellite cell depletion on adult muscle hypertrophy'

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In their Correspondence, Charlotte Peterson's group suggests that methodological weaknesses of our paper (Egner et al., 2016) preclude the interpretation that satellite cells (SCs) are obligatory for hypertrophy after mechanical overload (OL), a finding conflicting with their own study concluding that SC depletion does not affect overload hypertrophy (McCarthy et al., 2011).

Most papers are subject to methodological strengths and weaknesses. In our paper (Egner et al., 2016), we highlight some limitations of the McCarthy study (McCarthy et al., 2011), specifically: their reliance on muscle weights as a proxy for fiber hypertrophy rather than using direct cell size measurements; the inclusion in their histological analysis of a high number of fibers with central nuclei or embryonic myosin (eMyHC); and the fact that their paper is based on an unusually small increase in fiber size ($\approx 10\%$) compared with the 25-60% increase in the existing literature on the 2-week plantaris synergist ablation model (Dearth et al., 2013; Egner et al., 2016; Huey et al., 2016; Perez-Schindler et al., 2013; Zempo et al., 2016). It is this $\approx 10\%$ hypertrophy in control muscles that they subsequently fail to blunt by SC depletion.

These limitations of the McCarthy paper (McCarthy et al., 2011) can, in our opinion, explain the discrepancies between the two papers. As we understand it, these limitations are uncontested in their Correspondence.

Another important difference between our work and that of McCarthy et al. is that we studied both the plantaris and the extensor digitorum longus (EDL) muscles. We regret that the Peterson group does not regard the EDL as a relevant overload model for hypertrophy, particularly since it has frequently been used as such for at least 40 years by various laboratories (Deveci and Egginton, 2002; Dick and Vrbová, 1993; Egginton et al., 1998; Fortes et al., 2015; Freeman and Luff, 1982; Frischknecht and Vrbová, 1991; Goldspink, 1981; Hamilton et al., 2010; Johnson and Klueber,

1991; Rosenblatt and Parry, 1993; Schiaffino et al., 1972; Young et al., 1992). It seems implausible that the load on the EDL should not be increased by ablating its synergist; the question is how much. We do not think that the electromyography data from stereotype walking experiments in cats cited by the Peterson group (Carlson-Kuhta et al., 1998) provides full justice to the varied movement patterns of free mice in cages (climbing, standing, jumping, etc.), and regardless of this, the EDL clearly undergoes hypertrophy after overload and therefore offers an independent muscle in which to evaluate the necessity of satellite cells.

It is interesting that the Peterson group now suggests that the low degree of hypertrophy in their overloaded plantaris could be due to fiber splitting. A very small increase or even reduction in cross-sectional area (CSA) accompanied by an increase in the number of fibers has previously been reported in a few studies (Ferry et al., 2014; Joanne et al., 2012; Parsons et al., 2004), but to our knowledge not for observation periods as short as 2 weeks, and even most long-term studies report a robust hypertrophy (Ballak et al., 2016, 2015; Dunn et al., 1999, 2000; Guerci et al., 2012; Ito et al., 2013; Johnson and Klueber, 1991; White et al., 2009). Although fiber splitting was not studied in either of the two papers, and is not a well-understood phenomenon, we agree that it is a reasonable speculation about the causes of the poor hypertrophy observed in the SC+OL+ muscles by McCarthy et al. (2011) (since we observed more robust hypertrophy, we think it less likely to be a significant issue in our own work). Although it is possible that an initial hypertrophy was masked by subsequent fiber splitting, we would argue that this makes the material poorly suited to unravel the importance of SCs for hypertrophy defined as a size increase of pre-existing cells.

We are pleased that the Peterson group agrees that both papers demonstrate that the SC depletion was sufficient to abolish

myonuclear accretion. The number of myonuclei is the crucial parameter because it is the myonuclei and not the SCs that are involved in the protein synthesis during hypertrophy. We therefore do not understand why a discussion about possible moderate differences in the degree of SC depletion is relevant. It also seems illogical to us that an alleged higher residual SC population in our study should lead to less hypertrophy.

We also disagree that McCarthy has demonstrated a more efficient SC ablation, and are unable to follow the authors when they state in the Correspondence that 'We do not include tamoxifen-treated mice that are less than 90% depleted in our analyses' as in their paper they report a range of 84 to 98% (fig. 1B legend in McCarthy et al., 2011). Moreover, whereas we report data from the plantaris, where the degree of depletion ranged from 66 to 84%, they reported data from the gastrocnemius. This is unfortunate as observations from the gastrocnemius are not directly relevant for studies on the plantaris. The numbers are not comparable because both the absolute number of SCs and their ability to proliferate varies between muscles (Gibson and Schultz, 1983; Lagord et al., 1998; Ono et al., 2010).

The Peterson group moves on to state 'That SCs are not effectively depleted is most clearly demonstrated by the observation that markers of muscle regeneration [embryonic myosin heavy chain (eMyHC)-positive and centrally nucleated fibers] are significantly higher in SC- compared with SC+ plantaris with overload (figure 2B-D in Egner et al., 2016)'. This description of the data is formally correct, but highly misleading. Thus, the difference between the two papers is that McCarthy et al. reported a $>15\times$ higher frequency of fibers with such markers in their OL+SC+ group compared with our data (comparing figure 3C,D in McCarthy et al., 2011 and figure 2B,C in Egner et al., 2016). We attribute this to more overload- or surgery-induced damage observed in their muscles. Both groups reported, however, the same frequency of fibers with such markers in the OL+SC- muscles. If the frequency of fibers with central nuclei or eMyHC is related to the number of SCs, this would indicate that the number of residual SCs is similar in the two studies.

In any case, the reduction in a prominent damage and repair process by SC depletion reported in the McCarthy paper has little bearing on our paper as, contrary to what is argued in the Correspondence, we observed much less damage. Thus, in the material analyzed in McCarthy et al., they reported that 30% of the fibers in the OL+SC+ population displayed central nuclei/eMyHC, whereas we report 4% and none of our experimental groups was above 13%. We also would point out that whereas McCarthy included their significant population of fibers with such markers, we excluded the relatively few we had from further analysis. Our reasoning was that hypertrophy is defined as a size increase in pre-existing cells, and that damage or repair are confounding factors in the study of this phenomenon. Although it seems likely that such markers are related to damage and repair, at this stage we do not know if they appear in pre-existing fibers, regenerating fibers, new fibers or split fibers. In any case, a high incidence of fibers with such markers is a confounding factor.

Importantly, the appearance of central nuclei/eMyHC is less of a problem in the EDL. The fraction of fibers with such markers was 1.3% or lower in all the experimental groups. The EDL model has the advantage that the overload is probably gentler than for the plantaris because the size ratio between the ablated and remaining synergist is smaller, and perhaps because the muscles on the ventral side are less loadbearing, as discussed by the Peterson group. Thus, the EDL model might be less supraphysiological than the plantaris model.

Regarding the age difference in mice used between the two studies (McCarthy et al. report using 4 month-old and we 3-4 month-old mice), although we cannot exclude that there is a critical period for SC dependence between 3 and 4 months, we are not aware of any evidence for such a notion. C57BL/6J mice are considered mature adults when they are 3-6 months old (Flurkey et al., 2007), and we do not understand why referring to rapid bone growth of mice age <3 months (Ferguson et al., 2003) is relevant to our study.

The Peterson group raises valid concerns about the effect of tamoxifen on body weight. Both groups, however, used the same dosage and our animals appeared healthy. As far as we can tell, neither the McCarthy paper (McCarthy et al., 2011), nor the other papers from the Peterson group quoted in this context in their Correspondence (Fry et al., 2014, 2015; Jackson et al., 2015, 2012), reported numbers for body weights, but by comparing muscle weights corrected and non-corrected for body weight we calculate a difference of 9% for the non-running group in Jackson et al. (2015) compared with our 13%. In their Correspondence, the Peterson group also provides hereto unpublished data on body weight for the McCarthy material, and report that the tamoxifen effect was smaller (4%).

Lower body weights are commonly observed in animals given the estrogen antagonist tamoxifen compared with controls. This seems to be due to reduced accumulation of body fat (Lampert et al., 2013; Liu et al., 2015), whereas protein is not significantly affected (Wade and Heller, 1993). We agree that controls with tamoxifen treatment of the parental Pax7-CreER strain would have been desirable, but these were not used in either of the two studies (Egner et al., 2016; McCarthy et al., 2011). Although we cannot exclude the possibility that the lower weights might have had some influence on the hypertrophic response in our case, we observed no atrophy in OL-muscles that were given tamoxifen; the fibers were similarly large with and without tamoxifen. Moreover, tamoxifen did not blunt hypertrophy after 8 weeks synergist ablation in the parental Pax7-CreER mouse strain (Fry et al., 2014).

Although randomized sampling of fibers on sections is a common method in the literature and provides good estimates of the average values for the whole population, we agree with the Peterson group that measuring all relevant fibers (but we suggest excluding damaged/regenerating fibers) rather than a randomized sample would have been an optimal strategy. It is correct that using standard grid sampling, as we did, would tend to oversample large fibers, but automatic CSA measurements (as used by McCarthy et al.) might also introduce systematic differences. Although such factors might influence absolute values, there would be a similar bias for controls and experimental groups, so we do not think these methodological differences could explain the completely different outcomes of the two studies.

We are more inclined to attribute the different outcome to the appearance and inclusion of a significant population of damaged fibers by McCarthy et al. Their cell size analysis might also be statistically underpowered because there was such a low degree of hypertrophy in the control muscles with intact SCs (Fig. S2E). A potential blunting of a $\approx 10\%$ hypertrophy by SC depletion might be hard to detect. The re-analysis published in their Correspondence seems to have increased the variance, further aggravating this problem.

Thus, based on our interpretation of the current literature, including the two papers debated here (Egner et al., 2016; McCarthy et al., 2011), we maintain our conclusion that satellite cells seems to be obligatory for a robust *de novo* hypertrophy.

While we were finalizing this Correspondence Response, this conclusion was supported by new data from a completely different genetic model, but subjected to the same overload experiment. In this study, when satellite cells were prevented from fusing to muscle fibers during overload, hypertrophy was prevented (Goh and Millay, 2017).

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