Quantitative evaluation of mammalian skeletal muscle as a heterologous protein expression system

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Abstract

The production of mammalian proteins in sufficient quantity and quality for structural and functional studies is a major challenge in biology. Intrinsic limitations of yeast and bacterial expression systems preclude their use for the synthesis of a significant number of mammalian proteins. This creates the necessity of well-identified expression systems based on mammalian cells. In this paper, we demonstrate that adult mammalian skeletal muscle, transfected in vivo by electroporation with DNA plasmids, is an excellent heterologous mammalian protein expression system. By using the fluorescent protein EGFP as a model, it is shown that muscle fibers express, during the course of a few days, large amounts of authentic replicas of transgenic proteins. Yields of ~1 mg/g of tissue were obtained, comparable to those of other expression systems. The involvement of adult mammalian cells assures an optimal environment for proper protein folding and processing. All these advantages complement a methodology that is universally accessible to biomedical investigators and simple to implement.

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A general problem in biology is the design of protein expression systems able to express authentic mammalian proteins in large enough quantities to satisfy the necessities of various biomedical applications. Prokaryotic expression systems, though powerful in their ability to generate massive quantities of recombinant proteins, have severe limitations for the expression of properly folded (and processed) full-length replicas of a large number of eukaryotic proteins [1]. Although these limitations pertain to both cytosolic and membrane proteins, they have become a critical deficiency for the study of integral membrane proteins such as ion channels and transporters [2]. Thus, alternative expression systems able to approximate the yield of bacteria, but that overcome some of their limitations, have been developed. Of these, yeast and baculovirus expression systems have been considered favorable hosts for the expression of foreign eukaryotic proteins for research, industrial or medical use [1,3,4]. However, since biological activity depends critically on the proper folding and authenticity of proteins, their expression in bona fide mammalian systems has become a necessity. Cultured mammalian cells have been utilized as expression systems to fulfill this role, but issues related to the creation of stable cell lines, their relatively low yield, and the requirement of balanced delicate nutrients weaken their effective use for large-scale protein production [5–7].

Important advances in gene manipulation, plasmid design, and in vivo transfection methodologies during the past decade have led to the proposal that skeletal muscles can be used as a potential factory of proteins for gene therapy [8–12]. In this paper, we explore the alternative use of mammalian skeletal muscle as a transient expression system for mass production of eukaryotic heterologous proteins. Salient features that make skeletal muscle amenable for this purpose are that: they represent a large proportion

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of the body mass; they are easily accessible for transfection procedures; and, they are composed of large syncytial postmitotic cells with large capacity to synthesize proteins [12,13]. However, up until now, most experimental evidence seems to suggest that skeletal muscle tissue may be unable to synthesize recombinant proteins at a rate, and in quantities (on a scale of \( \text{mg of protein/g wet weight of tissue} \)), compatible with its potential use as an effective protein expression system [9,14–16]. This paper aims to dispel these qualms. To this end, we used: (a) in vivo electroporation as the method of choice to deliver controlled amounts of plasmids into well-identified limb muscles of the mouse; (b) pDNA vectors to encode for a genetically engineered derivative of the green fluorescent protein that have enhanced fluorescence in the green (EGFP) region of the visible spectrum as an example of a medium sized (27 kDa) transgenic cytosolic protein; (c) two-photon laser scanning confocal fluorescence microscopy (TPLSCM)\(^1\) to monitor the expression of fluorescent proteins in live tissue at the organ (whole muscles), cellular (single muscle fibers), and sub-cellular (intra-sarcomeric distribution) levels; and finally, (d) standard biochemical techniques to isolate and characterize the fluorescent proteins synthesized by muscle tissue, and spectrofluorometry to quantitate the expression yield. Altogether, our data demonstrate that mammalian skeletal muscle is a strong heterologous protein expression system, which due to the simplicity of its use may become an important biological tool in the future.

Materials and methods

Animal model

Male C57BL mice 1.5–3 months of age, weighting 20–30 g, were used. Plasmid transfection was performed in either FDB muscles or a group of “lower limb muscles” which predominantly included the soleus, tibialis anterior, and extensor digitorum muscles. Experiments were carried out according to the guidelines laid down by the UCLA Animal Care Committee.

Plasmids amplification

The plasmid encoding for EGFP (pEGFP-N2) was obtained from Clontech. It was amplified in OneShot TOP10 (Clontech) bacteria and were isolated using Qiagen Endo-Free Kits (Qiagen, Valencia, CA, USA) following the procedures of the manufacturer.

Muscle transfection with DNA plasmids

Muscle transfection was achieved by injection with pDNA, followed by in vivo electroporation, in anesthetized animals (isoflurane). The protocols used in FDB muscles differed slightly from those in “lower limb” muscles. In the case of FDB, 5 \( \mu \)l of 2 mg/ml hyaluronidase [12,17] was dissolved in sterile saline and injected subcutaneously into the foot pads of the animal using a 33-gauge needle. An hour later, \(-20 \mu\)g of pDNA, dissolved in buffer (10 mM Tris–Cl, pH 8, 1 mM EDTA) at concentrations of \( 2–5 \mu\)g/\( \mu\)l, was injected. After 10 min, two electrodes (200 \( \mu\)m gold plated stainless steel needles) were placed subcutaneously close to the proximal and distal tendons of the muscles in order to deliver electrical pulses for muscle electroporation. The pulse protocol was: 20 pulses of 100 V in amplitude, 20 ms in duration, applied at a frequency of 1 Hz. Pulses were generated by a Grass S88 medical stimulator (Grass, Quincy, MA, USA). For lower limb muscles, the protocol was the same except that the injections of hyaluronidase and pDNA were applied intramuscularly at three positions (equidistant locations between the ankle and the knee) of the lower limb muscles. Stimulating electrodes were placed parallel to the leg axis and inserted subcutaneously at both sides of the leg. In general, right muscles were transfected while left muscles were used as contra-lateral controls.

Two-photon laser scanning confocal microscopy

FDB muscles were fixed to Sylgard-bottomed Petri dishes and placed on the stage of an upright microscope (Olympus, BX51WI) equipped with an adjustable wavelength Chameleon Ti/Sapphire laser system (Coherent) and a Radiance 2000 Scanning Head (Bio-Rad, UK). EGFP was excited at 880 nm [18] and its fluorescence detected through a 495-515/30 dichroic-emission filter combination. Low- and high-magnification TPLSCM images were obtained with a 10\( \times \), NA 0.45 (Olympus) and a 20\( \times \), NA 0.95 (Olympus XLMPLANFL). Image stacks were constructed from TPLSCM sections spaced at 5 \( \mu\)m and at 2 \( \mu\)m, for low and high magnification, respectively. Images were analyzed using commercial and public domain imaging software packages (LaserSharp 2000, Confocal Assistant, and ImageJ).

Muscle homogenization and fractionation

Muscles were blot dried and weighed. Tendons were trimmed and muscles minced into small pieces using razor blades. Homogenization buffer, consisting of 150 mM KCl, 5 mM MgSO\(_4\), 20 mM Mops (pH 7.00), protease inhibitor cocktail 1:50, and 0.1 mM PMSF, was added to the tissue at a ratio of 4\( \mu\)l/mg. Homogenization was performed with a glass tissue grinder. Supernatant fractions of muscle homogenates were obtained following two steps of centrifugation at 1500 rpm (Eppendorf, 5415C), and one at 20,000 rpm (Beckman Coulter, Avanti J20 XP).

Protein quantitation

Protein concentration was determined using a commercial kit (Quick Start Bradford Dye Reagent, Bio-Rad) and

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1 Abbreviations used: TPLSCM, two-photon laser scanning confocal fluorescence microscopy; FDB, flexor digitorum brevis.
BSA as a standard. Absorbance measurements were done in an HP8453 UV–visible System (Hewlett–Packard, Palo Alto, USA). Purified bacterial EGFP (rEGFP, Clontech) was utilized to calibrate the concentrations of EGFP in the supernatants. To this end, the fluorescence of solutions at protein concentration ranging from 0.625 to 20 μg/ml was measured using a dual beam spectrofluorimeter (Jobin Yvon Fluorolog, FL3-21, Edison, NJ, USA). Fluorescence values at 508 were plotted as a function of the protein concentration and regression lines were fitted to the data. The EGFP concentrations in muscle supernatants were obtained by interpolation.

SDS–PAGE and Western blotting

Prior to the analytical characterization of proteins by gel electrophoresis, endogenous muscle soluble proteins were partially removed from supernatant fractions by heat shock treatments that did not affect their EGFP contents [19]. The supernatant was heated at 70 °C for 5 min and concentrated 3-fold in a refrigerated evaporator (Speed Vac, SVC100, Savant Instruments). The treated supernatants were mixed 1:1 with sample buffer (62.5 mM Tris, pH 6.8, 0.1% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol blue, and 50 mM DTT) and denatured by boiling for 5 min [20]. Aliquots of this solution were loaded in duplicated 16% polyacrylamide–SDS gels. Molecular weight markers (See-Blue Plus2 Pre-Stained Standard) were obtained from Invitrogen (Carlsbad, CA, USA). Protein bands were stained with Imperial Protein Staining (Pierce, Rockford, IL, USA). One replica of the SDS gel was used for Western blotting as described elsewhere [21]. The primary antibody was EGFP/anti-GFP (Clontech), and the secondary antibody was anti-mouse Ig (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were incubated with a chemiluminescent substrate (Inmun-Star HRP Chemiluminescent Kit, Bio-Rad) at room temperature for 5 min. Signals were detected with a chemiluminescent imaging system (ChemiDoc System EQ, Bio-Rad) and stored digitally.

Results

Our initial approach was to investigate the expression of transgenic proteins in flexor digitorum brevis (FDB) muscles of the mouse (Fig. 1) because of their importance in physiological and structural studies [22], and because they have been previously used in muscle electro-transfection studies [23]. Some advantages of these muscles for the latter purposes are their accessibility for pDNA solutions injected subcutaneously in the feet pads, and the possibility to attain uniform electric fields between two parallel subcutaneous electrodes. Panel A2 in Fig. 1 shows that in vivo electroporation of FDB muscles with pEGFP-N2 results in the expression of EGFP which confers a relatively uniform green fluorescence (4 days after transfection). This fluorescence is readily visible with the naked eye when the muscle is illuminated with blue color light.

Transfected muscles were routinely tested for their mechanical response upon electrical stimulation. We confirmed that protein expression did not alter the muscle contractile properties since all transfected branches contracted vigorously in response to electrical stimulation, irrespective of the level of fluorescence observed (data not shown).

Microscopic evaluation of the expression of EGFP in FDB muscles

A more precise evaluation of the pattern of expression of EGFP was obtained by in vivo fluorescence imaging using TPLSCM. Panels B1 and B2 in Fig. 1 show low-magnification TPLSCM images acquired from FDB muscles dissected 12 h and 5 days after the electroporation procedure (see Materials and methods), respectively. Under these conditions it is possible to observe that, in every branch of the FDB muscle, EGFP fluorescence was confined inside the intact fibers. Fig.1B1 illustrates that few hours after transfection, the levels of fluorescence intensity observed in the population of fibers are uneven, and that only a small proportion of them show bright fluorescence. At longer times (e.g., 5 days), the overall intensities are significantly larger and less variability is observed among the fibers, indicating that virtually all the fibers attained high levels of EGFP expression (Fig.1B2). Fluorescence image sections obtained at a higher magnification through a small bundle of fibers give a detailed depiction of the pattern of massive expression of EGFP within the individual muscle fibers (Fig.1C). It can be observed that while the fluorescent protein fills the volume of the fibers, there is a periodic arrangement of bands of high fluorescence where presumably the protein is more concentrated. We have determined that the peaks and valleys in fluorescence intensity (Fig.1C, inset) do not differ by more than 25% and that the location of the high intensity bands corresponds to the A bands of the sarcomere, where the contractile protein myosin is found [24]. For all the expression times assessed (6 h to 31 days), the fluorescence was always found confined within the fibers, and never detected in the extracellular space. (The efficiency of EGFP transfection is further documented with additional image sections in the supplementary material.)

Biochemical and spectroscopic characterization of EGFP isolated from FDB muscles

The observation of large fluorescence intensities in live FDB muscle fibers shortly after in vivo transfection with pDNA, though a necessary condition, does not sufficiently prove that mammalian skeletal muscle is an effective expression system for transgenic proteins. Several questions can be raised: Do the biochemical properties of EGFP expressed in muscle matches those of the same protein generated by bacteria? What is the time course of protein expression? And more importantly, how much EGFP can be extracted per unit weight of FDB muscle? To answer these questions we performed standard biochemical tests in supernatant
fractions from muscle homogenates and verified the presence of fluorescent proteins. The first biochemical evidence that skeletal muscles transfected with pEGFP-N2 are able to synthesize sizable amounts of EGFP is seen in Fig. 2A, where supernatant fractions from FDB muscle homogenates were run in SDS-PAGE gels (see Materials and methods). Lanes 1 and 2 correspond to samples from control (electroporated with vehicle only), and pEGFP-transfected muscles, respectively, obtained 5 days after electroporation. Fig. 2A illustrates that a protein band corresponding to EGFP is readily visible with the Commassie blue staining; its position coincides with that observed in the Western blot of Fig. 2B. The molecular weight of the protein is calculated to be 26.6 kDa, which agrees superbly with the 26.8 kDa predicted from the amino acid sequence of the protein [25]. It should be added that no recombinant EGFP could be detected in the microsomal fractions of muscle homogenates (data not shown), as expected for soluble cytoplasmic proteins.

The spectral characteristics of recombinant fluorescent proteins provide a fingerprint of their molecular identity and correct folding. This has been extensively documented in studies showing that even point mutations in the original wtGFP amino acid sequence generate proteins which differ widely in their spectra, quantum efficiency, and thermal sensitivity [26–29]. Bearing this in mind, we compared the spectral properties of muscle-generated EGFP with those of commercially available protein purified from bacterial sources (Clontech). The results are shown in Fig. 2C where the fluorescence emission spectrum of 10 μg/ml of commercial EGFP (trace b) is compared with a 1:20 dilution of supernatants obtained from a muscle 5 days after transfection (trace a) and from a non-transfected muscle (trace c). It can be observed that the latter shows negligible fluorescence. Moreover, the emission spectrum of the supernatant from the transfected muscle (trace a) is similar to that of the pure EGFP standard, a feature that is further emphasized...
by the almost perfect overlap of the scaled spectra shown in Fig. 2D. This key evidence shows that muscle- and bacteria-generated EGFP fluorophores are bona fide replicas of each other.

Quantitative evaluation of efficiency of protein expression in FDB muscle

Commercial recombinant EGFP was used to build calibration curves correlating the peak fluorescence with the protein concentration (see Materials and methods). For the specific data shown in Fig. 2C, we calculated the supernatant EGFP concentration to be 249 μg/ml. When this value was normalized to the muscle wet weight, the EGFP expression was 1.6 mg/g wet weight, a value that compares favorably with those reported for heterologously expressed proteins per gram of pellet in bacterial systems [30,31]. Nevertheless, though the efficiency of protein expression by FDB muscles is large, the total amount of recombinant protein that can be extracted from them is relatively small due to their little mass (~15 mg). For example, the total amount of protein recovered from the FDB muscle shown in Fig. 2C was only 19 μg, a quantity sufficient for analytical purification protocols, but not large enough to satisfy the needs of crystallography or other biotechnological applications.

Scaling up protein expression: lower limb muscles

The high levels of expression attained in FDB muscles encouraged us to investigate if the yield could be maintained in larger muscle masses, and how long does it take for the muscle system to attain significant levels of heterologous protein expression. To this end, we transfected a group of lower limb muscles (see Materials and methods) with the pEGFP-N2 plasmid. Supernatant fractions from lower limb muscle homogenates were run in SDS–PAGE gels (see Materials and methods). Lanes 1–8 of Fig. 3A correspond to supernatants from muscles transfected for 12 h, and 1, 2, 4, 8, 16, 24 and 31 days, respectively; they demonstrate that EGFP bands are readily visible with the Comassie blue staining after the second day of protein expression. The progress of EGFP expression within the period of transfection can be better appreciated in the Western blot of Fig. 3B. It can be seen that the presence of EGFP is detectable 1 day after transfection, and that the
size and intensity of the blots increases with time, reaching a peak at day 8 and slightly decaying thereafter.

We estimated the concentration of heterologous proteins in supernatant fractions from several lower limb extracts after various periods of expression of EGFP and the results are summarized in Table 1. It should be noted that although the presence of recombinant protein is detectable 6 h after transfection, the amount of EGFP collected from the muscles increases steadily from 1 to 8 days and later decays slightly. Interestingly, at the peak of heterologous protein expression, the amount of EGFP extracted in the supernatant of transfected muscles represented approximately 2% of the total soluble proteins, comparable to that of a major cytosolic muscle protein such as parvalbumin [32].

To compare the efficiency of muscle with that of other expression systems, it was important to quantitate the yield of heterologous protein per wet weight of transfected muscle tissue. Fig. 4 shows a bar plot of data obtained from muscle duplicates as a function of the expression period. It can be observed that the levels of expression reach a peak of ~0.9 mg of EGFP/g wet weight, 8 days after transfection and remain significant after 31 days. It is important to note that the average mass of transfected lower limb muscles per mouse was 0.9 ± 0.4 g (means ± SD, n = 9); thus, on average, at the time of peak yield (between 8 and 16 days) approximately 0.8 mg of transgenic EGFP can be obtained from one animal if both lower limb muscles are transfected.

**Discussion**

The main goal of this paper is to validate the use of mammalian skeletal muscle as a transient heterologous protein expression system. Although previous work from other laboratories demonstrates that muscle can be readily transfected by electroporation with DNA plasmids for the purpose of gene therapy [8–12], to our knowledge the use of this organ as a source for the production and extraction of recombinant proteins has not been previously proposed. Furthermore, our comprehensive approach consisting of
the visualization of protein expression in situ, the characterization of the biochemical properties of extracted proteins, and the quantitation of the levels of protein production per unit mass of muscle tissue, is novel.

To expedite the evaluation of skeletal muscle’s efficiency in expressing heterologous proteins, the fluorescent protein EGFP was chosen as a model because its location and concentration is readily determined by optical methods. This protein has been extensively used before as a practical tool to evaluate potential expression systems [30]. Using TPLSCM, we first showed that EGFP is consistently confined in the myoplasm. As suggested by the banded fluorescence pattern, although EGFP distributed throughout the fiber volume, it seemed to be more concentrated at the A band, but to an extent not larger than 25%. Based on the fact that the space among thick filaments is smaller than that among thin filaments, this distribution suggests a preferential binding of EGFP to A band proteins. Additionally, TPLSCM studies showed that inclusion bodies known to occur in heterologous protein expression systems [33] were never detected, regardless of the level of expression.

Several independent evidences support the authenticity of the muscle-generated EGFP. First, its apparent molecular weight was close to the prediction from the amino acid sequence, e.g., ~26.6 vs. 26.8 kDa, respectively (Fig. 2A). Second, muscle-generated EGFP was accurately recognized by commercial polyclonal antibodies raised against EGFP epitopes, which did not show cross-reaction with muscle native proteins (Fig. 2B). Finally, although the detection of fluorescence within the muscle fibers (using TPLSCM) already suggested that EGFP was correctly folded, the ultimate confirmation that EGFP expressed in muscle is indistinguishable from that obtained from bacteria (using an identical cistron) is provided by the fact that the emission spectra of both proteins are identical (Fig. 2D).

Most of previously published data regarding the expression of heterologous proteins in mammalian skeletal muscle are not rendered in units amenable to quantitative comparison of the yields [9,14,16,34]. In contrast, we chose to express the yield in terms of the protein production normalized with respect to the mass of muscle tissue (mg of protein/g wet weight). These units allow for easy comparison between our data and that from other expression systems, and set a comparative standard for future reports on the expression of other proteins. We found that, 5 days after transfection, FDB muscles exhibit a large EGFP expression yield of ~1.6 mg/g wet tissue, which is comparable to the ~1 mg/g of pellet generated by bacteria [30]. Altogether, these values exceed by orders of magnitude the 20 ng/g wet weight of muscle reported by Umeda et al. [15]. Since the amount of protein isolated from FDB muscles was constrained by its small mass, we proved that larger muscle masses, like lower limb muscles, could also be effectively transfected. We found that although the yield is approximately one-half of that found in FDB muscles, the scaling up process was successful because the total amount of EGFP collectable per animal reached significant levels, on the order 0.8 mg. These results are encouraging since additional muscles (e.g., quadriceps and hamstrings) can be readily transfected, while using identical transfection protocols, in order to increase the total protein yield by transfesting muscle masses of up to 3 g per mouse. Furthermore, they pave the road for using equivalent muscles in larger animals, like rats (already tested preliminarily), which automatically will increase the expected yield of total protein by 10-fold.

Besides the appropriate yield at relatively short times after transfection, our approach is quite easy to implement and relatively inexpensive. Since the protein expression occurs in live animals, the complex requirements for tissue culturing and the incubating machinery that other mammalian expression systems utilize, are not necessary. In addition, the use of naked DNA plasmids, instead of viral vectors, allows expressing large transcripts in a biologically safe fashion. Altogether these features make muscle an ideal expression system for applications in the biotechnological industry in which it is imperative to express functional proteins rapidly and efficiently with the proper post-translational modifications, including protein folding and glycosylation [6]. The expression system described here intrinsically complies with these requirements since it is based on the use of mammalian cells.

Although in this paper we have used EGFP as a prototype to test the ability of the muscle system to generate large amounts of protein, we already obtained evidence that it is also competent for the synthesis of functionally relevant soluble proteins like ECFP-tagged β1a subunit of the dihydropyridine receptor channel (Nèco et al., Biophysical Society Abstracts, 2005) and, most importantly, membrane proteins like GFP-tagged α1S subunit of the dihydropyridine receptor channel, EGFP-tagged Shaker K+ channel, and EYFP-tagged ryanodine receptor Ca2+ release channel (Vergara et al., Biophysical Society...
Abstracts, 2005). These results, in turn, give further assurances that post-translational modifications, trafficking, and targeting of the proteins to the right cellular compartment, are intrinsically present in our expression system.

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Appendix A. Supplementary data


References