# Myosin Expression in Heterotopic Autografts of Avian Skeletal Muscle Following Short-Term and Long-Term Regeneration

Terrence W. Miller and Robert S. Hikida[(1)]

*University of California, Los Angeles, Department of Physiology, School of Medicine, 405 Hilgard Avenue, Los Angeles, CA 90024-1751 USA and (1) Department of Biological Sciences, Ohio University, Athens, Ohio 45701 USA.*

**Abstract**

Myosin isoforms synthesized by avian slow-tonic (STo) and fast-twitch (FT) skeletal muscle were studied in heterotopic autografts following short-term (3 weeks) and long-term (7-17 months) regeneration. Autografts of the pigeon STo anterior latissimus dorsi (ALD) to the contralateral FT extensor digitorum communis site (EDCX), and the reciprocal grafts (ALDX grafts), were performed. Myosins were analyzed by histochemical adenosine triphosphatase (ATPase) activities and electrophoresis of myosin heavy and light chains. Objectives were to distinguish satellite cell lineages and their initial patterns of regeneration and determine the influence of foreign innervation on long-term muscle grafts.

*Short-term* graft results indicate that the FT fiber satellite cells are less likely to survive initial phases of degeneration/regeneration. FT and STo fiber satellite cells represent distinct lineages, and myosin synthesis in early phases of regeneration corresponds to that of the developing donor muscle. However, specific developmental stages were not discernible; instead results were indicative of overlapping, extended stages. In long-term grafts, many regenerated fibers exhibited the donor fiber type, surviving for an extended period in a developmental state. Fiber immaturity suggests a lack of functional innervation. A portion of regenerating fibers in the ALDX was in various stages of transformation to STo fibers, and we hypothesize that a subpopulation of satellite cells from FT fibers is capable of accepting STo neural inputs.

**Key words:** pigeon muscle, muscle graft, muscle regeneration, fiber types, myosin ATPase.

*BAM 2 (3): 221- 234, 1992*

Innervation is necessary for maintaining the structural and functional integrity of adult muscle fibers, but the precise role of innervation during muscle fiber type differentiation is not clear (reviewed by Miller [32]). After disruption of initial innervation, myotube differentiation occurs independently of innervation [3, 36]. Correspondingly, when embryonic muscle receives foreign innervation, initial fiber type differentiation is unaffected [46].

Crow and Stockdale [12] have distinguished three populations of early myotubes in the chick embryo using monoclonal antibodies to adult fast and slow myosin heavy chains (MHCs), and these same myotube populations develop from cultured embryonic chick myoblasts [33]. Colonies formed from cloned embryonic day 4 to 6 chick myoblasts are always composed of one of the three myotube types [34]. Miller and Stockdale [34] proposed that myoblasts are committed to one of three cell lineages prior to myotube formation, and differences in the phenotype of primary myotubes (and eventually of mature myofibers), arise from intrinsic differences among the earliest myoblasts. Others have suggested that myoblasts represent a plastic population of cells guided in their development by external factors [4,45]. Both Butler et al. [4] and Sweeney et al. [45] hypothesize that all muscle fibers arise from an homogeneous population of myoblasts. At a very early point in development, these myoblasts are guided into different lineages by some extrinsic factors. Sweeney et al. attribute the three myoblast lineages cloned by Miller and Stockdale [34] to "extrinsic input received in vivo prior to culture".

Muscle regeneration provides an excellent model for examining muscle fiber type differentiation, since the sequence of events during regeneration parallels that of developing muscle [6]. Mammalian fast twitch (FT) and slow twitch muscles express a developmental MHC isoform in the first week following cold-injury, and this expression diminishes in the second week [38]. When mammalian muscle is denervated and destroyed by

myotoxins [7] or minced and placed back into its muscle bed [28], the recapitulation of early development is spread over a longer period. Cold-injured avian FT and slow tonic (STo) muscles also express developmental isoforms of contractile proteins in the initial stages of regeneration, but in a much narrower time frame than during embryonic development [8, 14, 30, 43]. By the end of the second week following cold-injury, the regenerating fibers are producing nearly the same complement of protein isoforms as the normal adult fibers [8, 14, 37].

Innervation appears to be the factor that ultimately determines the fiber type of regenerated mammalian muscle [7, 16]. The primary factors determining the mature fiber types in free grafted, regenerated avian muscle are less clear. Heterotopic autografts of chicken FT muscles [27] or pigeon mixed muscles [48] to the site of the STo anterior latissimus dorsi (ALD) resulted in regenerated muscles with histochemical profiles more similar to the donor muscle through approximately the first four months of regeneration, although variability among grafts was high. Conversely, heterotopic grafts of the chicken ALD to the site of the FT posterior latissimus dorsi (PLD) had relatively few histochemically STo fibers in the same period [27].

Heterotopic grafts to the site of the pigeon ALD which regenerated for periods longer than five months seemed to be approaching the histochemical characteristics of the ALD, suggesting that the long-term innervation of the host site gradually transformed the fibers [48]. However, regenerated fibers in heterotopic grafts to the pigeon ALD site retained a high percentage of their original fiber type even after 11 months, and the inappropriate fiber types were not innervated [24]. These results suggest that transitional forms may be present, but complete transformation may not occur.

In the present study, heterotopic muscle grafts were performed on pigeon muscles of homogeneous fiber type. The STo anterior latissimus dorsi (ALD) was grafted to the site of the FT extensor digitorum communis (EDC) and, conversely, the EDC was grafted to the ALD site. Muscles were injected with the myotoxin, Marcaine, to ensure uniform degeneration, and the fiber types and myosins were analyzed in the regenerating myoblasts. This protocol offers a unique combination of advantages for defining the factors influencing satellite cell commitment: a) the grafted muscle and the muscle originally in the host site are of homogeneous and distinct fiber types; b) original innervation is completely severed; and c) fiber degeneration occurs uniformly throughout the muscle before regeneration commences.

Myosin expression was examined both histochemically and biochemically following short-term (three weeks) and long-term (seven or more months) muscle regeneration. Myosins expressed in the short-term, uninnervated grafts were compared to determine if satellite cells from the two fiber types initially developed into distinct myoblasts lineages. This early myosin expression was then compared to expression in comparable grafts regenerated for periods of seven or more months. The relative importance of the satellite cell genetic program versus exogenous factors such as foreign innervation are discussed in light of the shifts in myosin expression observed in the long-term grafts.

## Materials and Methods

Adult pigeons, *Columba livia*, Carneaux breed, were obtained from either Bowman-Gray School of Medicine, Winston-Salem North Carolina, or the Palmetto Pigeon Plant, Sumter, South Carolina. The muscles used for the grafts were the ALD and EDC.

Two heterotopic muscle grafts were performed on each of 19 sodium pentobarbital-anesthetized pigeons; one ALD muscle was grafted into the site of the contralateral EDC and, conversely, that EDC muscle was grafted into the ALD site. Prior to grafting, muscles were injected and soaked in 0.75% bupivacaine (Marcaine) (Winthrop Pharmaceutical Co.) to ensure complete muscle fiber degeneration [5]. The undisturbed ALD and EDC served as controls. Throughout this text, the ALD grafted into the EDC site is termed the EDCX, and the EDC grafted into the ALD site is termed the ALDX. The ALD and EDC controls are referred to as ALDC and EDCC, respectively.

Grafts were removed either three weeks (short-term grafts) or 7 to 17 months (long-term grafts) following the operation. Birds were anesthetized as above and the grafted muscles, contralateral control muscles, and the posterior biventer cervicis (PBVC, composed of FT and slow twitch fibers arranged in mosaic pattern) were mounted together for cross sectioning beginning from the midbelly region, and frozen in methylbutane cooled in liquid nitrogen.

Muscles were sectioned in a cryostat at -20°C. The muscle portions remaining after sectioning were minced and put into pre-weighed, cooled containers and stored at -100°C for subsequent myosin isolation.

### *Histochemistry*

Fiber types were distinguished based on the stability of the myosin ATPase activity after exposure to acid or alkaline conditions following the procedures of Guth and Samaha [15] modified for pigeon muscle; the ATPase activity of STo fibers is moderate following either acid or alkaline preincubation, while that of FT fibers is acid-labile/alkaline-stable. Some sections were preincubated in a buffered pH 4.2 solution and adjacent sections in a buffered pH 10.4 solution.

Cross-sectional areas of all fibers in each grafted muscle, and approximately 200 fibers from randomly chosen fascicles from each control muscle were measured using a digitizer and the Bioquant System IV software program (R&M Biometrics, Nashville, TN). The total number of fibers was counted for all muscles having complete cross sections. Other sections were examined for the presence of nerve endings and motor endplates using a combined silver

nerve stain and acetylcholinesterase histochemical staining method (Ag-AChase) [1].

*Biochemistry*

The portion of each muscle which had been frozen was weighed and the procedure of Ianuzzo et al. [26] was used to isolate the myosin. Muscles were homogenized in 11.5ml of buffer (10mM KCl, 50mM Tris, pH 6.6)/gram muscle. The homogenate was extracted in 590mM KCl, 2mM EDTA, 2mM dithiothreitol, 20mM ATP, 20mM $MgCl_2$, pH 6.6 (11.5ml/gm muscle) for 10 min, then centrifuged 1 hr at 150,000g. An $(NH_4)_2SO_4$ solution was added to the supernatant with constant stirring until the resulting solution was 34% saturated with $(NH_4)_2SO_4$, and this was centrifuged at 20,000g. The $(NH_4)_2SO_4$ solution was again added to the supernatant until 45% saturation was reached, and this was centrifuged at 20,000g. The resulting pellet of myosin was dissolved in 600mM KCl, 50mM Tris, pH 6.5 (5ml/gm original muscle) and dialyzed overnight against 1mM EDTA, 10mM KCl, 1mM Tris, 0.05 mM DTT, pH 7.2. The volume of this dialysate containing purified myosin was measured and its myosin concentration determined colorimetrically using a Bio-Rad protein assay (Bio-Rad Labs, Richmond, CA), and comparing to an actin standard curve. Pellets of myosin (2/muscle) were obtained by centrifuging 15 min at 20,000g, then resuspended in SDS or urea buffers for subsequent SDS-PAGE or 2D-PAGE, respectively; myosin in concentrations of 8μg/μl (in urea) and 2μg/μl or 0.15μg/μl (in SDS) were stored at -100°C.

Molecular weight differences among MHCs and light chains (LCs) were detected using SDS-PAGE. Protein was loaded approximately 3μg per lane and run at 120V constant voltage for approximately 20 hours on 5% acrylamide gels. The gels were then stained with silver [35]. Myosin samples were run on 20% acrylamide Phastsystem gels (Pharmacia Inc., Piscataway, NJ) to distinguish LCs. Lanes were loaded with 0.3 to 1.0μl of the 2μg/μl protein sample. Gels were stained with silver using the Pharmacia Phastsystem development procedure.

When more than one type of LC or MHC was present in one sample, the myosin band densities were analyzed with an X-ray scanner. The areas of the resulting myosin band peaks were measured using the Bioquant System IV software program, and proportions were calculated from these measurements. The proportions of fast versus slow LCs in each graft were determined by comparing band densities of the two DTNB-LCs, LC2s and LC2f. In addition, LC2f:LC3f ratios were calculated and averaged for each experimental group. Some LC samples were separated by two-dimensional gel electrophoresis (2D-PAGE). Ampholytes were removed from Phastsystem gels, dried, and rehydrated in buffer containing urea and ampholytes with a pI range of 4-6.5. The myosin samples were then run on a 2-D gel, and stained as above.

*Statistical Analysis*

One-way ANOVA was used to compare the group means from the total fiber number data. Group means for individual fiber type cross-sectional areas were compared with one-way, two-level nested ANOVAs in order to eliminate variance due to variation among birds and not attributable to experimental effects. Significant differences between pairs of means were detected with the least significant difference test, with significance accepted at the 5% confidence level.

Comparisons were made between the EDCC and long-term ALDX groups in the mean number of STo fibers per muscle each possessed, and between the ALDC and long-term EDCX groups in their mean number of FT fibers per muscle. The Mann-Whitney U test was used for the analyses, with significance accepted at the 95% confidence level.

## Results

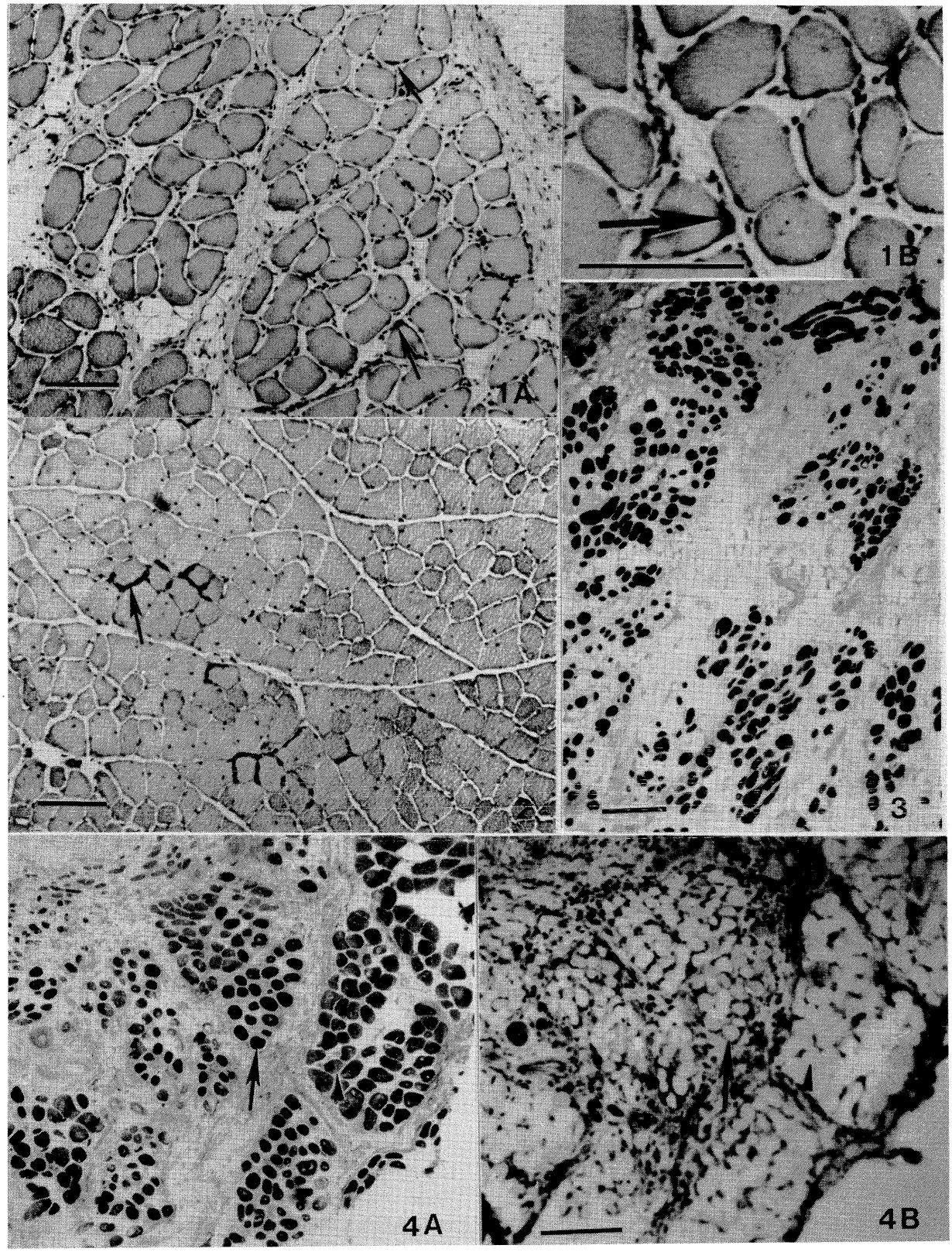
*Control muscles*

The morphology of the ALDC was consistent with descriptions in earlier studies [22, 23]. The 3223.1 ± 128.9 muscle fibers in cross sections of the ALDC were divided into well defined fascicles, and nearly all were large STo fibers. Fast twitch fibers were not observed in eight of the 11 ALDC muscles in which fibers were counted, and in the other three muscles they accounted for only 0.1 to 3.4% of the total fibers. The STo fiber mean cross-sectional area was 2651.0 ± 24.3$\mu m^2$. Most myonuclei were peripherally located (Fig.1). Many motor endplates were observed in the Ag-AChase preparations, and were highly variable in size (Fig. 1).

The EDCC had less endomysium and perimysium than the ALDC. Fiber counts were made on 11 EDCC muscles and only four had STo fibers, making up 0.05 to 2.4% of their total number of fibers. The 4628 ± 371.6 fibers in the EDCC was significantly higher than in the ALDC. The FT fibers of the EDCC had a mean cross-sectional area of 1755.6 ± 18.2$\mu m^2$, significantly lower than that of the ALDC STo fibers. Most EDCC myonuclei were peripherally located, and many intensely stained motor endplates of variable size were observed in the Ag-AChase preparations (Fig. 2). These were larger and more variable than in the ALD because the latter consist of small "en grappe" terminations [22] while the EDCC had single large endplates.

Myosins isolated from the ALDC and EDCC differed in their MHC composition, but only one MHC was distinguished for each of these muscles. The FT MHC isolated from the EDCC migrated slightly faster than the STo MHC on the 5% acrylamide gels.

The STo ALDC muscles had a 24kD LC and a 20.5kD LC, hereafter referred to as LC1s and LC2s, respectively. Three LCs were associated with the FT EDCC myosin: LC1f, LC2f, and LC3f, with molecular weights of 24, 18.5, and 14kD, respectively.


1A
1B
3
4A
4B

The LC1f and LC1s had identical molecular weights, but 2D-gel electrophoresis revealed differences in their IEF points (data not shown).

*Three week grafts*

Five grafts were successful and three unsuccessful for each three week group. Failure was caused by the graft pulling away from the sutured point of attachment.

Both EDCX and ALDX grafts were surrounded by a large amount of connective tissue, and the endomysium was also thicker and denser than in control muscles (Figs. 3 and 4). Myonuclei were located centrally and peripherally within the myofibers. No endplates were observed in any of the Ag-AChase preparations.

EDCX

The mean number of fibers in the EDCX, 1951.2 ± 474.7, was not significantly different than that for the ALDC. The number varied widely among the five grafts, as did the fiber cross-sectional areas and proportions of different fiber types (Table 1).

Three of the grafts were homogeneous in their histochemical fiber type. Two of these were composed of fibers histochemically identical to adult FT fibers (fig 3). The third had myofibers with an unusual ATPase histochemical profile; the activity was high following alkaline preincubation and moderate following acid preincubation (i.e., alkaline-stable/acid-moderate).

Alkaline-stable/acid-moderate fibers accounted for approximately 90% of the fibers and cross-sectional area in another graft. The remaining fibers of this graft were FT. Fibers with the histochemical activity of adult STo fibers (i.e., acid and alkaline moderate) were present in just one EDCX graft, but they accounted for 68% of the fibers and cross-sectional area.

Myosin heavy chains from four EDCX grafts were analyzed, and three different MHCs were present in each case (Fig. 5). The two slowest migrating MHCs comigrated with adult STo and FT MHCs. The fastest migrating MHC was not observed in any control muscles. The migratory pattern of this MHC in relation to adult STo and FT MHCs suggests that it corresponds to the developmental SM1 MHC described in developing chicken ALD muscles [25]; therefore it will be referred to as "SM1" while the adult STo MHC will be referred to as "SM2".

The MHC proportions for individual grafts are presented in Table 1. The four grafts had a relatively consistent proportion of SM2 (between 25 and 30% of the total MHC). The graft with a majority of STo fibers had 62% SM1 MHC and just 13% FT MHC (Fig. 5). Two grafts, one composed entirely of FT fibers and the other of alkaline-stable/acid-moderate fibers, had virtually identical proportions of the MHCs (approximately 50% FT MHC and 25% of both SM1 and SM2 MHCs). The fourth graft (>90% alkaline-stable/acid-moderate fibers) had the most even distribution of MHCs, each MHC accounting for 30% to 40% of the total.

Slow and fast LCs were present in all EDCX grafts (Table 1; Fig. 6). As was the case for the MHCs, the graft composed predominantly of STo fibers had predominantly STo LCs; the graft with an even distribution of MHCs also had a high proportion of STo LCs. In the other three grafts, LC2f comprised 58 to 65% of the DTNB-LC (Fig. 6). All five grafts had comparable LC2f:LC3f ratios of approximately 4:1. Myosin samples from these grafts run on 2D-gels did not reveal any unique LCs.

ALDX

The mean number of myofibers in the ALDX grafts was only 940.0 ± 244.2, significantly lower than the number for the EDCC. All regenerating fibers of the largest graft (1658 fibers) were histochemically FT. In the other four grafts, 67% to 100% of the fibers had low activity following acid preincubation, but following alkaline preincubation their activity was moderate (Table 1; Fig. 4). These fibers were assumed to be regenerating FT fibers (see discussion). All other fibers in these grafts had a histochemical profile typical of control FT fibers. The mean cross-sectional area of these fibers (698.9 ± 9.8$\mu m^2$) was significantly lower than FT fibers of the EDCC.

*Fig. 1A. Cross section of ALDC stained using combined Ag-AChase technique, counter-stained with toluidine blue. Arrows point to small sites of endplate AChase activity. Myonuclei are peripherally located. 135x. Bar = 100 μm.*

*Fig. 1B. Higher magnification of 1A showing sites of AChase activity. 300x. Bar = 100μm.*

*Fig. 2. Cross-section of EDCC stained using combined Ag-AChase technique, counter-stained with toluidine blue. Intense endplate AChase activity (arrow) is prevalent. Myonuclei are peripherally located. 130x. Bar = 100 μm.*

*Fig. 3. Cross section of three-week EDCX graft stained for myosin ATPase activity following alkaline preincubation. Fibers in this graft stain like FT fibers. Fibers are small and variable in size and are separated by much connective tissue. 60x. Bar = 200 μm.*

*Fig. 4A and B. Adjacent cross sections of a three-week ALDX graft stained for myosin ATPase activity. All fibers stained like FT fibers following acid preincubation (A). However, following alkaline preincubation (B), some fibers stained like FT fibers (arrow) while others stained less intensely (arrowhead). Fibers are highly variable in size. 75x. Bar = 200 μm.*

*Table 1. Fiber types and myosin composition of individual three week grafts**

| Graft No. | Fiber Type | No. Fibers | xs Area | Whole Muscle Myosin Proportions Light Chains LC2s | LC2f | Heavy Chains SM2 | FT | SM1 |
|---|---|---|---|---|---|---|---|---|
| **EDC-X Grafts** | | | | | | | | |
| 1 | FT | 117 | 960.4 ± 52.7 | 0.77 | 0.23 | 0.30 | 0.39 | 0.32 |
| | AS/AM | 1230 | 844.3 ± 14.8 | | | | | |
| 2 | FT | 688 | 465.6 ± 17.8 | 0.35 | 0.65 | -- | -- | -- |
| 3 | AS/AM | 3397 | 986.5 ± 14.2 | 0.42 | 0.58 | 0.26 | 0.50 | 0.24 |
| 4 | FT | 1421 | 647.3 ± 12.3 | 0.35 | 0.65 | 0.26 | 0.50 | 0.24 |
| 5 | FT | 130 | 814.6 ± 47.1 | 0.80 | 0.20 | 0.26 | 0.13 | 0.62 |
| | STo | 410 | 1237.5 ± 29.4 | | | | | |
| | AS/AM | 541 | 1191.4 ± 26.1 | | | | | |
| **ALD-X Grafts** | | | | | | | | |
| 1 | FT | 120 | 481.5 ± 24.8 | 0.33 | 0.67 | 0.00 | 1.00 | 0.00 |
| | AM/AL | 451 | 259.8 ± 7.7 | | | | | |
| 2 | FT | 1658 | 807.0 ± 15.3 | 0.11 | 0.89 | 0.00 | 1.00 | 0.00 |
| 3 | FT | 177 | 614.2 ± 36.6 | 0.00 | 1.00 | 0.00 | 1.00 | 0.00 |
| | AM/AL | 1073 | 482.1 ± 12.9 | | | | | |
| 4 | FT | 326 | 954.6 ± 33.9 | 0.13 | 0.87 | 0.00 | 1.00 | 0.00 |
| | AM/AL | 536 | 1184.4 ± 44.5 | | | | | |
| 5 | AM/AL | 243 | 621.8 ± 32.1 | 0.23 | 0.77 | 0.00 | 1.00 | 0.00 |

*For the control values, see text.
Fiber type: FT - fast twitch; STo - slow tonic; AS/AM - alkaline stable and acid moderate ATPase activity; AM/AL - alkaline moderate and acid labile ATPase activity.
XS Area: Mean ± Standard Error in μm2

In contrast to the EDCX grafts, only one MHC was detected in each of the five ALDX grafts (Fig. 7; Table 1). This comigrated with the adult FT MHC.

Exclusively FT LCs were observed in one graft, but LC2s made up 11% to 33% of the DTNB-LC in the other four grafts (Table 1; Fig. 6). The mean LC2f:LC3f ratio was approximately 5:1. No unique LCs were observed in 2D-gels from these grafts.

*Long-term grafts*

Of the grafts attempted on 11 pigeons, four EDCX and three ALDX grafts were successful. The unsuccessful grafts were the result of the grafted muscle detaching from the sutured origin or insertion.

EDCX

The four successful long-term EDCX grafts were removed at 12, 14, 15, and 17 months. Myonuclei were prominent centrally and peripherally within muscle fibers. Endplates were observed in all grafts, but no judgment could be made regarding the extent or specificity of the innervation (Fig. 8).

The mean fiber number in successful EDCX grafts was 2195.3 ± 345.7, not significantly different from the ALDC. One graft was composed entirely of STo fibers (1469 fibers). Two of the other grafts had over 80% STo fibers and less than 2% FT fibers; the fourth graft had 48% STo and 20% FT fibers. The remainder of the fibers in these three grafts (between 15% and 32%) were alkaline-stable/acid-moderate fibers, the highest percentage occurring in the graft with the least number of STo fibers (Table 2). The graft in Fig. 9 contains all three fiber types.

The alkaline-stable/acid-moderate fibers had a mean cross-sectional area of only 778.1 ± 20.2μm$^2$ and the STo fiber mean cross-sectional area was 1240.5 ± 12.0μm$^2$. Both were significantly lower than control STo fibers of the ALDC (2651.0 ± 24.3μm$^2$). The FT fibers had a mean

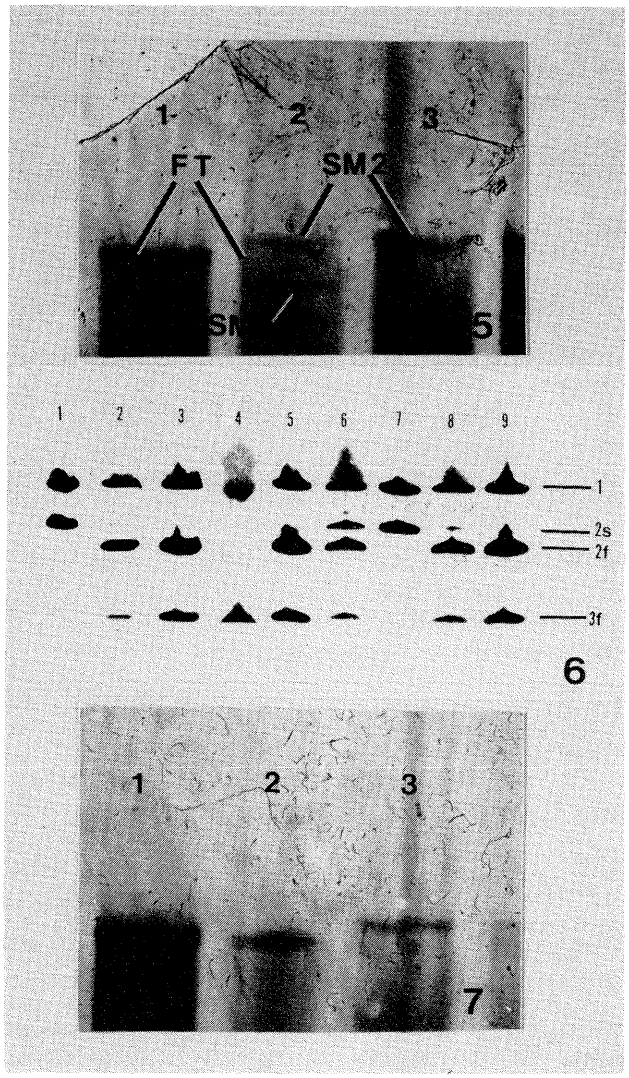



*Fig. 5. Three-week EDCX MHCs (lane 2) separated on a 5% acrylamide gel. The SM2, FT, and SM1 MHCs are present. Lane 1 is FT MHC from an EDCC and lane 3 is the MHC from an ALDC (SM2 only).*

*Fig. 6. Three-week EDCX (lane 6) and ALDX (lanes 2 and 8) myosin samples run on 20% acrylamide gels. Lanes 1 and 7 are STo LCs from an ALDC, and lanes 3, 5, and 9 are FT LCs from an EDCC. Protein standards were run in lane 4; the 26kD band (top) and the 12.4kD band (bottom) are present. The band labeled 1 consists of either LC1f or 2s, both having a molecular weight of 24kD. In this EDCX sample, LC2f is somewhat more prominent than LC2s, and LC3f is present. Both ALDX samples have considerably more LC2f than LC2s, and both have LC3f.*

*Figure 7. Three-week ALDX (lane 2) MHC separated on a 5% acrylamide gel. Only FT MHC was observed. Samples of EDCC and ALDC myosins were run in lanes 1 and 3, respectively.*

cross-sectional area ($2082.0 \pm 54.5 \mu m^2$) not significantly different from that of the EDCC FT fibers. The actual number (not percentage) of FT fibers in these grafts was not significantly different from that of ALDC muscles.

The MHC proportions for individual long-term grafts are presented in Table 2. The graft with 100% STo fibers was the only one without FT MHC; 95% of the MHC was SM2 and the other 5% was SM1. The SM2 MHC also predominated in the other three grafts, accounting for 55% to 76% of the total MHC, and the SM1 MHC was responsible for 3% to 18% of the total (Fig. 10). Between 21% and 30% was FT MHC, but only one of these grafts had a similar proportion of FT fibers (25% of the cross-sectional area), while FT fibers made up less than 2% of the cross-sectional area in the other two grafts.

Light chain 2s made up 66% to 100% of the DTNB-LC, although the correlation with the MHCs and histochemistry was inconsistent (Table 2). The proportions of LC2f and LC3f were variable, but the mean LC2f:LC3f ratio (approximately 1.6:1) was similar to the EDCC ratio.

*Table 2. Fiber types and Myosin Composition of Individual Long-term Grafts*

| Time (Mos) | Fiber type | No. Fibers | xs Area | Whole Muscle Myosin Preparations: Light Chains LC2s | Light Chains LC2f | Heavy Chains SM2 | Heavy Chains FT | Heavy Chains SM1 |
|---|---|---|---|---|---|---|---|---|
| **EDCX Grafts** | | | | | | | | |
| 12 | FT | 483 | 2136.6 ± 56.6 | 0.66 | 0.34 | 0.55 | 0.27 | 0.18 |
| | STo | 1153 | 1880.2 ± 40.4 | | | | | |
| | AS/AM | 762 | 1021.0 ± 32.7 | | | | | |
| 14 | STo | 1469 | 1249.9 ± 22.4 | 0.96 | 0.04 | 0.95 | 0.00 | 0.05 |
| 15 | FT | 5 | 1460.6 ± 337.1 | 1.00 | 0.00 | 0.76 | 0.21 | 0.03 |
| | Sto | 2285 | 790.7 ± 11.6 | | | | | |
| | AS/AM | 260 | 401.1 ± 14.7 | | | | | |
| 17 | FT | 28 | 1479.9 ± 195.4 | 0.73 | 0.27 | 0.64 | 0.30 | 0.06 |
| | STo | 1456 | 1438.3 ± 20.9 | | | | | |
| | AS/AM | 260 | 665.1 ± 32.7 | | | | | |
| **ALDX Grafts** | | | | | | | | |
| 7 | FT | 2479 | 244.7 ± 5.2 | 0.38 | 0.62 | 0.42 | 0.58 | 0.00 |
| | STo | 181 | 1335.7 ± 62.3 | | | | | |
| | AS/AM | 64 | 491.1 ± 48.3 | | | | | |
| 15 | FT | 429 | 1995.8 ± 82.0 | 0.52 | 0.48 | 0.55 | 0.41 | 0.04 |
| | STo | 732 | 1654.4 ± 11.6 | | | | | |
| | AS/AM | 338 | 208.2 ± 14.2 | | | | | |
| 17 | FT | 227 | 246.7 ± 12.5 | 0.33 | 0.67 | 0.44 | 0.56 | 0.00 |
| | STo | 17 | 2761.8 ± 285.1 | | | | | |
| | AS/AM | 3 | 459.2 ± 977.1 | | | | | |

Fiber type: FT - fast twitch; STo - slow tonic; AS/AM - alkaline stable and acid moderate ATPase activity.
XS Area: Mean ± standard error ($\mu m^2$)

ALDX

Long-term ALDX grafts were removed at 7, 15, and 17 months. Central and peripheral myonuclei were observed in the FT fibers of these grafts. However, central nuclei were much less prevalent in the STo fibers of the long-term ALDX grafts than in myofibers of the other grafts; a large proportion of the myonuclei were peripheral in location (Fig. 11). Endplates were observed in sections from all grafts (Fig. 11).

The three successful ALDX grafts had a mean fiber number significantly lower than the EDCC (1561.7 ± 717.0), but the number varied tremendously. Two grafts had similar percentages of FT (~91%) and STo (~7%) fibers. The third graft had 29% FT and 49% STo fibers. The mean number of STo fibers in the grafts was significantly higher than the mean number observed in the EDCC muscles. The FT and STo fiber mean cross-sectional areas (484.5 ± 16.1$\mu m^2$ and 1612.6 ± 46.1$\mu m^2$, respectively) were significantly lower than their control counterparts. The graft in figure 12 has FT and STo fibers.

Alkaline-stable/acid-moderate fibers were present in all three grafts, comprising 1% to 23% of the fibers (Table 2). The alkaline-stable/acid-moderate fiber mean cross-sectional area was just 262.2 ± 26.5$\mu m^2$, significantly smaller than control fibers.

The long-term ALDX grafts did not show as much variability between MHCs, LCs, and histochemistry either within or among grafts (Table 2). Only one graft had a small amount of SM1 MHC (4%); 55% of the MHC in this graft was SM2 MHC and 57% of the cross-sectional area was occupied by STo fibers. The SM2 MHC (and STo fiber cross-sectional area) were in the minority in the other two grafts, one having 44% SM2 MHC and STo fiber cross-sectional area and the other 42% SM2 MHC and 26% STo fiber cross-sectional area (Fig. 13).

Light chain 2s comprised 33%, 38%, and 52% of the DTNB-LCs, the largest proportion belonging to the graft with the most STo fibers. The LC2f:LC3f ratio (approximately 2.4:1) approached the EDCC ratio.

## Discussion

The pattern of regeneration prior to innervation was analyzed to clarify the influence of innervation on fiber type expression. The focus was on the relationship between regenerating myotubes and the origin of the satellite cells; in particular, whether characteristics of regenerating myotubes indicated that satellite cells were unique for each fiber type or were an homogeneous pool of cells. In addition, parallels between regenerating and normally developing myotubes in their myosin expression were of interest.

### *Control Muscles*

Myosin from adult pigeon STo and FT fibers has not been studied previously. The MHC and LC composition paralleled that of adult chicken muscle in several respects, but some differences were found. The MHC composition of both adult pigeon and chicken muscle includes one predominant STo MHC and one slightly lower molecular weight FT MHC [29]. The SM1 MHC, which accounts for up to 5% of the MHC in the adult chicken ALD [29], was not detected in the pigeon ALDC. The 24 and 20.5kD LCs isolated from STo fibers were considered the pigeon muscle equivalent to the chicken muscle LC1s and LC2s, respectively. The three LCs isolated from FT fibers had molecular weights of 24, 18.5, and 14kD, and were assumed to correspond to chicken muscle LC1f, LC2f, and LC3f, respectively.

### *Three Week Grafts*

No evidence of reinnervation was observed in any sections of three week grafts stained for motor endplates and nerve-endings. Pigeon heterotopic grafts are not reinnervated even after six weeks [24].

The small cross-sectional areas and centrally-located myonuclei are characteristics of developing or denervated muscle fibers. The alkaline-moderate/acid-labile fibers of the ALDX grafts were considered to be developing FT fibers, comparable to the type II embryonic chick myotubes described by McLennan [31] which have alkaline-moderate ATPase activity through embryonic day (ED) 13.

Embryonic and/or neonatal MHC probably comprises at least a portion of the MHC in the three week ALDX grafts, since embryonic, neonatal, and adult FT MHCs have the same molecular weights. Studies of FT avian muscle regeneration induced by cold-injury have consistently reported embryonic MHC production through approximately the first week of regeneration [8, 14, 30, 37]. The neonatal MHC is subsequently produced for a short period [8]. Although these MHCs could not be distinguished, the MHC production in the ALDX grafts was similar to FT fibers and distinct from developing STo fibers.

Both the coproduction of FT and STo LCs and the low levels of LC3f observed in the three week ALDX grafts are characteristic of FT myotubes during embryonic development. The proportion of slow LCs detected in the three week ALDX grafts is comparable to that reported for developing chick FT muscles, in which STo LCs reach a maximum of about 12% of the total LCs by ED12 [11].

The range of results for EDCX ATPase histochemistry and myosin analysis could indicate the presence of either different fiber (i.e. satellite cell) types or different stages of a single fiber type. Results tend to support the latter: A) some alkaline-moderate/acid-labile fibers would be expected if developing FT fibers were present; B) grafts #3 and #4 displayed one histochemical fiber type and fibers are therefore co-producing all three MHCs, unlike developing FT fibers of ALDX grafts. However, the results clearly cannot rule out the possibility that some fibers were expressing FT MHC exclusively. The array of developmental stages among grafts could be related to the tension originally placed on individual grafts, since increased tension yields greater fiber size and number [9].

Myofibers with the same ATPase activity as found in the EDCX grafts have been described in developing chicken STo muscle. Alkaline-stable/acid-labile myosin ATPase activity is typical of stage 25 (ED 4.5) myotubes from both future FT and STo muscles [2]. Alkaline-stable/acid-moderate ATPase activity has been reported from stage 29 (ED 6) until hatching for myotubes that will develop into STo fibers of the hindlimb [31].

Developing STo myotubes simultaneously synthesize the three MHCs - SM1, SM2, and embryonic - in early stages of development [30]. The embryonic MHC is predominant through ED10, but by ED13 approximately 80% of the MHC is SM1 and 20% SM2 [25, 30]. The embryonic ALD also cosynthesizes FT and STo LCs. The ED12 ALD produces FT LCs in a 3.5 fold excess over STo LCs, but the STo LCs predominate by ED16 [11]. The EDCX LC synthesis parallels this period of development in which the shift from fast to slow LCs occur, while the MHC profile is typical of earlier development. Myosin production is unlike that of either developing FT fibers or the ALDX grafts.

Grafts #3 and #4 had nearly identical MHC and LC distributions, yet their histochemical ATPase activities differed. Myotubes coproducing fast and slow myosins would be expected to demonstrate some degree of acid-stable ATPase activity, as observed in graft #3. The reason for the acid-labile activity in graft #4 is not known.

A significant number of fibers did not survive in the ALDX grafts. The EDCX grafts exhibited a smaller loss of fibers than the ALDX, but this was not significant due to high variability. The most critical determinants of regenerating fiber survivability are manifested early on, since fiber loss does not increase with longer periods of regeneration (this study, [20, 24]). Our results and those of [24] indicate that regenerating avian FT fibers are inherently less robust than STo fibers. This may be due to a greater dependence on innervation by developing FT fibers, as suggested by the finding that only STo fibers persist following long periods of denervation in embryonic chick musculature [3].

## Discussion

The pattern of regeneration prior to innervation was analyzed to clarify the influence of innervation on fiber type expression. The focus was on the relationship between regenerating myotubes and the origin of the satellite cells; in particular, whether characteristics of regenerating myotubes indicated that satellite cells were unique for each fiber type or were an homogeneous pool of cells. In addition, parallels between regenerating and normally developing myotubes in their myosin expression were of interest.

### *Control Muscles*

Myosin from adult pigeon STo and FT fibers has not been studied previously. The MHC and LC composition paralleled that of adult chicken muscle in several respects, but some differences were found. The MHC composition of both adult pigeon and chicken muscle includes one predominant STo MHC and one slightly lower molecular weight FT MHC [29]. The SM1 MHC, which accounts for up to 5% of the MHC in the adult chicken ALD [29], was not detected in the pigeon ALDC. The 24 and 20.5kD LCs isolated from STo fibers were considered the pigeon muscle equivalent to the chicken muscle LC1s and LC2s, respectively. The three LCs isolated from FT fibers had molecular weights of 24, 18.5, and 14kD, and were assumed to correspond to chicken muscle LC1f, LC2f, and LC3f, respectively.

### *Three Week Grafts*

No evidence of reinnervation was observed in any sections of three week grafts stained for motor endplates and nerve-endings. Pigeon heterotopic grafts are not reinnervated even after six weeks [24].

The small cross-sectional areas and centrally-located myonuclei are characteristics of developing or denervated muscle fibers. The alkaline-moderate/acid-labile fibers of the ALDX grafts were considered to be developing FT fibers, comparable to the type II embryonic chick myotubes described by McLennan [31] which have alkaline-moderate ATPase activity through embryonic day (ED) 13.

Embryonic and/or neonatal MHC probably comprises at least a portion of the MHC in the three week ALDX grafts, since embryonic, neonatal, and adult FT MHCs have the same molecular weights. Studies of FT avian muscle regeneration induced by cold-injury have consistently reported embryonic MHC production through approximately the first week of regeneration [8, 14, 30, 37]. The neonatal MHC is subsequently produced for a short period [8]. Although these MHCs could not be distinguished, the MHC production in the ALDX grafts was similar to FT fibers and distinct from developing STo fibers.

Both the coproduction of FT and STo LCs and the low levels of LC3f observed in the three week ALDX grafts are characteristic of FT myotubes during embryonic development. The proportion of slow LCs detected in the three week ALDX grafts is comparable to that reported for developing chick FT muscles, in which STo LCs reach a maximum of about 12% of the total LCs by ED12 [11].

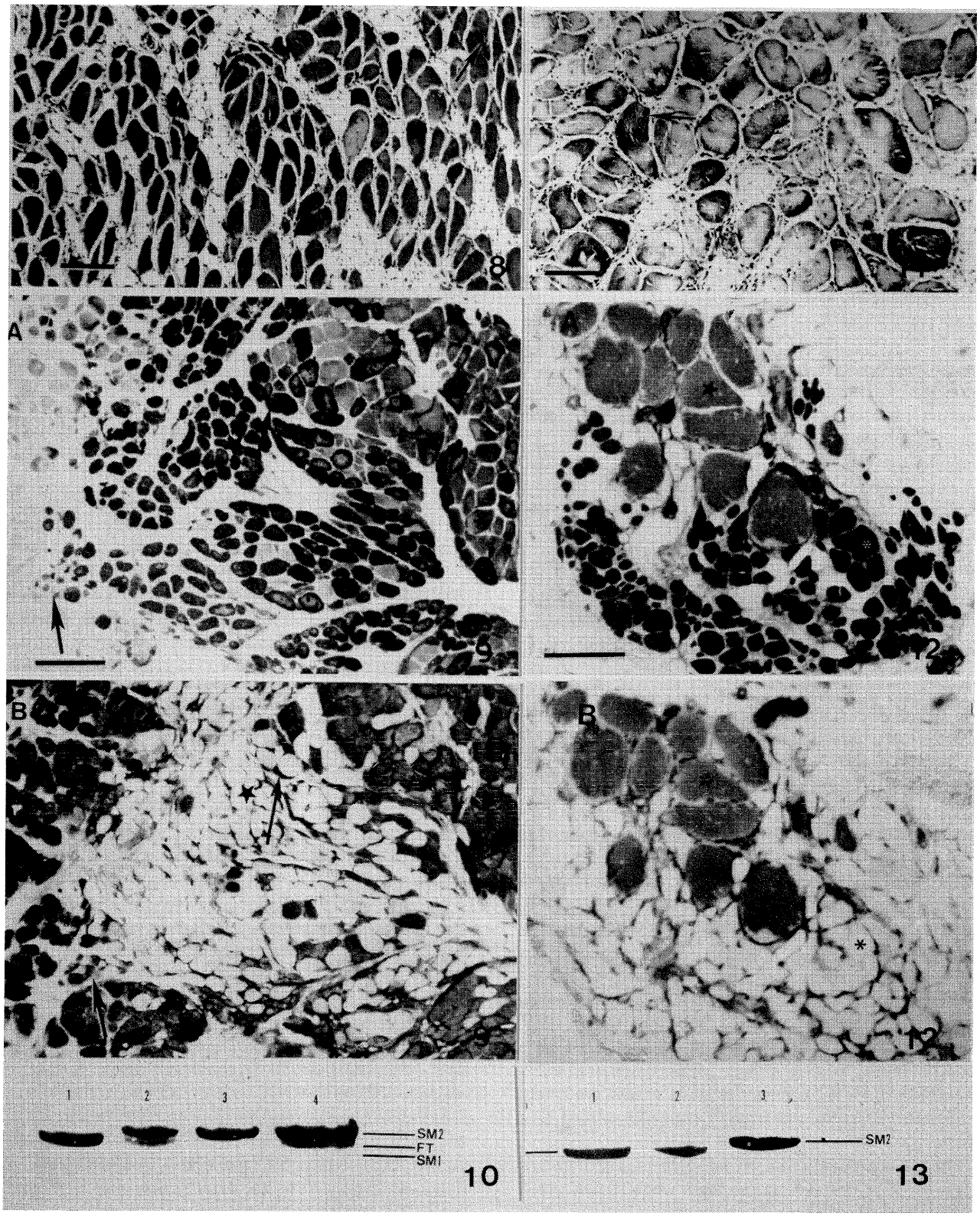
The range of results for EDCX ATPase histochemistry and myosin analysis could indicate the presence of either different fiber (i.e. satellite cell) types or different stages of a single fiber type. Results tend to support the latter: A) some alkaline-moderate/acid-labile fibers would be expected if developing FT fibers were present; B) grafts #3 and #4 displayed one histochemical fiber type and fibers are therefore co-producing all three MHCs, unlike developing FT fibers of ALDX grafts. However, the results clearly cannot rule out the possibility that some fibers were expressing FT MHC exclusively. The array of developmental stages among grafts could be related to the tension originally placed on individual grafts, since increased tension yields greater fiber size and number [9].

Myofibers with the same ATPase activity as found in the EDCX grafts have been described in developing chicken STo muscle. Alkaline-stable/acid-labile myosin ATPase activity is typical of stage 25 (ED 4.5) myotubes from both future FT and STo muscles [2]. Alkaline-stable/acid-moderate ATPase activity has been reported from stage 29 (ED 6) until hatching for myotubes that will develop into STo fibers of the hindlimb [31].

Developing STo myotubes simultaneously synthesize the three MHCs - SM1, SM2, and embryonic - in early stages of development [30]. The embryonic MHC is predominant through ED10, but by ED13 approximately 80% of the MHC is SM1 and 20% SM2 [25, 30]. The embryonic ALD also cosynthesizes FT and STo LCs. The ED12 ALD produces FT LCs in a 3.5 fold excess over STo LCs, but the STo LCs predominate by ED16 [11]. The EDCX LC synthesis parallels this period of development in which the shift from fast to slow LCs occur, while the MHC profile is typical of earlier development. Myosin production is unlike that of either developing FT fibers or the ALDX grafts.

Grafts #3 and #4 had nearly identical MHC and LC distributions, yet their histochemical ATPase activities differed. Myotubes coproducing fast and slow myosins would be expected to demonstrate some degree of acid-stable ATPase activity, as observed in graft #3. The reason for the acid-labile activity in graft #4 is not known.

A significant number of fibers did not survive in the ALDX grafts. The EDCX grafts exhibited a smaller loss of fibers than the ALDX, but this was not significant due to high variability. The most critical determinants of regenerating fiber survivability are manifested early on, since fiber loss does not increase with longer periods of regeneration (this study, [20, 24]). Our results and those of [24] indicate that regenerating avian FT fibers are inherently less robust than STo fibers. This may be due to a greater dependence on innervation by developing FT fibers, as suggested by the finding that only STo fibers persist following long periods of denervation in embryonic chick musculature [3].


8
A
9
12
B
B
*
1
2
3
4
SM2
FT
SM1
10
1
2
3
SM2
13

The number of STo fibers in the control ALD and ALD orthografts is comparable [20]. Thus, the success of ALD STo fiber regeneration when grafted to the EDC site is limited by factors associated with the *site*. The EDC is a spindle-shaped muscle while the ALD is a larger flap-like muscle; the snug fit of the ALD in the muscle bed may have physically impeded reinnervation and revascularization of EDCX grafts.

The uninnervated EDCX and ALDX grafts are undergoing distinct patterns of regeneration. In both cases, the biochemical and histochemical characteristics of the regenerating muscles are representative of early stages in development of the donor muscle. Myotubes within a developing muscle undergo synchronized transitions in myosin production at relatively precise stages, and these transitions are reflected histochemically as well as biochemically. This was not the case for the regenerated muscle. Developmentally discrete stages in myosin transitions were extended and overlapped, indicating that mechanisms responsible for coordinating myosin transitions differed from those in normal development.These results support the hypothesis that satellite cells of avian muscle are committed to distinct developmental lineages related to the fiber with which they are associated [30, 44].

*Long-term Grafts*

Long-term grafts of seven to 17 months were studied to determine the success of fiber regeneration in a foreign site and to assess the effects of innervation on the resulting fiber types. Although grafts displayed marked AChase staining, regenerated FT fibers in heterotopic grafts to the ALD site may have AChase activity but lack nerve endings [24]. There have been other examples of sites of high AChase activity [10, 18, 42, 19] and other postsynaptic specializations [39,41] on developing fibers in the absence of innervation. Muscle fiber atrophy and central myonuclei are both characteristic of denervated muscle (reviewed by Vrbova et al., [47]), and therefore it is likely that many fibers in the grafts remained uninnervated.

While FT fibers were very small with cross-sectional areas of less than 250μm in two ALDX grafts, those of the third graft matched EDCC fiber areas. It is possible that the original ALD in this bird had at least one FT motor unit [21], and this fast motor neuron may have provided the proper innervation to a portion of the regenerating fibers.

The EDCC muscles occasionally have a small number of STo fibers, but the number in the ALDX grafts was significantly higher than that expected for EDCC muscles. The high number of STo fibers may have arisen from two sources: 1) regenerating FT fibers transformed by the STo innervation, or 2) satellite cells from STo fibers of the donor muscle which differentiated into more than one fiber. Previous studies have reported that several fibers can develop within the basal lamina of a single fiber during mammalian muscle regeneration [17, 40], but results from the three week ALDX grafts preclude this as a source of the STo fibers in the long-term grafts. None of the three week ALDX grafts had detectable levels of either SM1 or SM2 MHC, nor were any fibers observed with ATPase activities unique to developing or mature STo fibers. This suggests that FT fibers were transformed into STo fibers due to the influence of the STo innervation.

This transformation in the long-term ALDX grafts appears to be an ongoing process even after more than a year of regeneration. Both the fiber size and presence of central nuclei indicated that the alkaline-stable/acid-moderate and FT fibers were not mature and possibly not innervated. The apparent immaturity of the fibers and the presence of low levels of developmental SM1 MHC in one graft suggest that the alkaline-stable/acid-moderate fibers are either in an arrested state of transformation or only recently started

*Figure 8. Cross section of an EDCX graft stained with the combined Ag-AChase technique and counter-stained with toluidine blue. Arrow points to endplate AChase activity. Central myonuclei are still prevalent. 100x. Bar =100 μm.*

*Figs. 9. A and B. Adjacent cross sections of an EDCX graft stained for myosin ATPase activity following alkaline (A) and acid (B) preincubation. Three fiber types are present in this graft: small hybrid fibers (arrows); a group of FT fibers (star); and a STo fiber (asterisk). 65x. Bar = 200 μm.*

*Fig. 10. Long-term EDCX MHCs separated on a 5% acrylamide gel. Lane 1 is an EDCC myosin sample with FT MHC only; lane 2 is an EDCX sample with SM2, FT, and SM1 MHCs; lane 3 is an ALDC sample with SM2 MHC as well as a small amount of FT MHC. Lane 4 contains both the ALDC and EDCX myosin samples.*

*Fig. 11. Cross section of an ALDX graft stained with the combined Ag-AChase technique and counter-stained with toluidine blue. Arrow points to endplate AChase activity. A higher proportion of myonuclei are peripherally located than in other grafts. 100x. Bar = 100 μm.*

*Figs. 12. A and B. Adjacent cross sections of an ALDX graft stained for myosin ATPase activity following alkaline (A) and acid (B) preincubation. Slow-tonic (star) and small FT (asterisk) fibers are present. 155x. Bar = 100 μm.*

*Fig. 13. Long-term ALDX MHCs separated on 5% acrylamide gels. Lane 1 is an EDCC myosin sample with FT MHC only; lane 2 is the ALDX sample with SM2 and FT MHCs; and lane 3 is an ALDC sample with SM2 MHC only. Line on the left indicates position of FT MHC band.*

to transform. Conversely, the STo fibers of the long-term ALDX grafts had the ATPase activity and peripheral myonuclei typical of fully developed fibers.

The reason(s) why such a wide variety of developmental states are present in individual long-term grafts is not known. Three potential factors which could influence the rate of transformation are: 1) the number of slow motor neurons contacting a myotube; 2) the proximity of the section to a slow endplate (i.e., the motor neuron may only induce regional transformation); and 3) the time it takes for slow motor neurons to contact a myotube. Orthotopic ALD grafts regenerated for periods of 11 months still contained immature STo fibers as determined by fiber size and presence of central nuclei [20], suggesting that STo fibers regenerate slowly. However, these orthotopic grafts were primarily composed of rather well developed STo fibers, with neither FT nor alkaline-stable/acid-labile fibers present. The contrast to the results for heterotopic grafts illustrates the overwhelming significance of factors responsible for "recognition" between motor neuron and muscle in promoting the regeneration process.

One EDCX graft had an unusually high number of fibers which appeared to be FT. However, many of these fibers may actually have been in very early stages of regeneration like those alkaline-stable/acid-labile fibers in the three week EDCX grafts, since the cross-sectional area of many fibers was small and this graft had considerably more SM1 MHC than the others. The other FT fibers in this graft, as well as the small number in two of the other three grafts, probably regenerated from satellite cells originating from FT fibers present in the donor ALD muscles.

The presence of relatively high numbers of alkaline-stable/acid-moderate fibers in three EDCX grafts and SM1 MHC in all four indicates that many regenerating fibers were still expressing the developmental program. Part of the FT MHC may also be a developmental form produced by the alkaline-stable/acid-moderate fibers and some of the STo fibers, since the proportion of FT MHC in two grafts is much higher than expected based on the proportion of FT and alkaline-stable/acid-moderate fibers present.

## Conclusions

The current results support the view that the satellite cells from STo and FT fibers represent cell lineages which can begin development independently of innervation, although proper innervation is necessary to orchestrate the normal developmental sequence from myoblast to mature fiber. Cultured satellite cells isolated from adult chicken muscle have been shown to belong to separate lineages [13, 44]. Satellite cells from the chicken ALD formed myoblasts which produced either FT MHC or a mixture of FT and STo MHC, while those from the FT pectoralis muscle synthesized only FT MHC. These lineages paralleled myoblast lineages reported for chick and quail embryos, with the exception that up to 3% of embryonic myoblasts developed from a STo-only lineage [34].

While myotubes appear homogeneous in three week regenerated muscle of ALDX grafts or FT satellite cell cultures [44], differences become apparent in long-term grafts. The STo fibers may have arisen from a unique population of satellite cells capable of accepting the non-matching STo innervation, and their control STo ATPase activity and peripheral myonuclei indicate that they probably have received multiple neural inputs. The impairment of transformation in alkaline-stable/acid-moderate fibers is most likely a function of fewer or later-forming neuromuscular junctions. The small FT fibers also result from a lack of innervation. Given the low number of muscle fibers in the ALDX grafts and the long period of regeneration, it is unlikely that innervation was prevented by an inability to access or accommodate the fibers. This suggests that the small FT fibers descend from a cell lineage which cannot accept the STo innervation.

The presence of fibers in our grafts which are still in a developmental state may offer an explanation for the variable and sometimes conflicting results of other studies on avian heterotopic graft histochemistry. Classifying these fibers based on adult histochemical ATPase criteria can lead to misclassification and incorrect conclusions.

A better understanding of the number of satellite cell lineages and their characteristics would come from investigations of the myosin histochemistry and biochemistry of individual fibers within heterotopic grafts. In addition, the hypothesis that fibers descended from some satellite cells that do not accept foreign innervation is based on indirect evidence, and this requires a thorough study concerning the pattern and specificity of heterotopic graft reinnervation.

**Acknowledgments:**

The authors thank Drs. R. Staron, P. Johnson, and W. Costello for their helpful suggestions. This work was supported by NIH grant 5RO1AM26992.

**Address correspondence to:**

Dr. T.W. Miller, University of California, Los Angeles, Department of Physiology, School of Medicine, 405 Hilgard Avenue, Los Angeles, CA 90024-1751

## References

[1] Beerman D and Cassens R: A combined silver and acetylcholinesterase method for staining intramuscular innervation. *Stain Tech* 1976; 51: 173-177.

[2] Butler J and Cosmos E: Differentiation of the avian latissimus dorsi primordium: analysis of fiber type expression using the myosin ATPase histochemical reaction. *J Exp Zool* 1981; 218: 219-232.

[3] Butler J, Cosmos E and Brierly J: Differentiation of muscle fiber types in aneurogenic brachial muscles of the chick embryo. *J Exp Zool* 1982; 224: 65-80.

[4] Butler J, Cosmos E and Cauwenbergs P: Positional signals: evidence for a possible role in muscle

fibre-type patterning of the embryonic avian limb. *Development* 1988; 102: 763-772.

[5] Carlson B: A quantitative study of muscle fiber survival and regeneration in normal, predenervated, and Marcaine-treated free muscle grafts in the rat. *Exp Neurol* 1976; 52: 421-432

[6] Carlson B, Hansen-Smith F and Magon D (1979). The life history of a free muscle graft. In "Muscle Regeneration" (A. Mauro, Ed.), pp. 493-507. Raven Press, New York.

[7] Carraro U, Dalla Libera L. and Catani C: Myosin light and heavy chains in muscle regenerating in absence of the nerve: transient appearance of the embryonic light chain. *Exp Neurol* 1983; 79: 106-117.

[8] Cerny L and Bandman E: Expression of myosin heavy chain isoforms in regenerating myotubes of innervated and denervated chicken pectoral muscle. *Devel Biol* 1987; 119: 350-362.

[9] Coan M and Tomanek R (1979). Regeneration of transplanted muscle with reference to overload. In: "Muscle Regeneration" (A. Mauro, Ed.), pp. 509-522. Raven Press, New York.

[10] Creazzo T and Sohal G: Neural control of embryonic acetylcholine receptor and skeletal muscle. *Cell Tiss Res* 1983; 228: 1-12.

[11] Crow M Olson P and Stockdale F: Myosin light-chain expression during avian muscle development. *J Cell Biol* 1983; 96: 736-744.

[12] Crow M and Stockdale F: Myosin expression and specialization among the earliest muscle fibers of the developing avian limb. *Devel Biol* 1986; 113: 238-254.

[13] Feldman JL and Stockdale FE: Skeletal muscle satellite cell diversity: satellite cells form fibers of different types in cell culture. *Devel Biol* 1991; 143: 320-334.

[14] Gorza L, Sartore S, Triban C and Schiaffino S: Embryonic-like myosin heavy chains in regenerating chicken muscle. *Exp Cell Res* 1983; 143: 395-403.

[15] Guth L and Samaha F: Qualitative differences between actomyosin ATPase of slow and fast mammalian muscle. *Exp Neurol* 1969; 25: 138-152.

[16] Gutmann E and Carlson B: Transposition of the soleus into the bed of the extensor digitorum longus muscle in the rat. *Neurosci Letters* 1978; 9: 1-6.

[17] Hansen-Smith FM and Carlson B: Cellular responses to free grafting of the extensor digitorum longus muscle of the rat. *J Neurol Sci* 1979; 41: 149-173.

[18] Harris A (1981a): Embryonic growth and innervation of rat skeletal muscles. II. Neural regulation of muscle cholinesterase. *Phil Trans R Soc Lond Ser B* 293: 279-286.

[19] Harris A (1981b): Embryonic growth and innervation of rat skeletal muscles. III. Neural regulation of junctional and extrajunctional acetylcholine receptor clusters. *Phil Trans R Soc Lond Ser B* 293: 287-314.

[20] Hather B and Hikida RS: Properties of standard avian slow muscle grafts following long-term regeneration. *J Exp Zool* 1988; 246: 115-123.

[21] Hikida RS: Tenotomy of the avian anterior latissimus dorsi muscle. II. Can regeneration from the stump occur in the pigeon? *Am J Anat* 1981; 160: 409-418.

[22] Hikida RS: Quantitative ultrastructure of histochemically identified avian skeletal muscle fiber types. *Anat Rec* 1987; 218: 128-135.

[23] Hikida RS and Bock W: Effect of denervation on pigeon slow skeletal muscle. *Z Zellforsch* 1972; 128: 1-18.

[24] Hikida RS, Hather B and Wang N: Properties of the donor muscle determine the characteristics of regenerated avian skeletal muscle. *Monogr Devel Biol* 1992; 23: 175-188.

[25] Hoh J: Developmental changes in chicken skeletal myosin isoenzymes. *FEBS Letters* 1979; 98: 267-270.

[26] Ianuzzo C, Patel P, Chen V and O'Brien P (1980). A possible thyroidal trophic influence on fast and slow skeletal muscle myosin. In "Plasticity of Muscle" (D. Pette, Ed.), pp. 594-605. Walter DeGruyter, Berlin, New York.

[27] Kikuchi T, Akiba T and Ashmore C: Conversion of muscle fiber types in regenerating chicken muscles following cross-reinnervation. *Acta Neuropathol (Berlin)* 1986; 71: 197-206.

[28] Marechal G, Schwartz K, Beckers-Bleukx G and Ghins E: Isozymes of myosin in growing and regenerating rat muscles. *Eur J Biochem* 1984; 138: 421-428.

[29] Matsuda R, Bandman E and Strohman R: The two myosin isoenzymes of chicken anterior latissimus dorsi muscle contain different myosin heavy chains encoded by separate mRNA's. *Differentiation* 1982; 23: 36-42.

[30] Matsuda R, Spector D and Strohman R: Regenerating adult chicken skeletal muscle and satellite cell cultures express embryonic patterns of myosin and

tropomyosin isoforms. *Devel Biol* 1983; 100: 478-488.

[31] McLennan I: Differentiation of muscle fiber types in the chicken hindlimb. *Devel Biol* 1983; 97: 222-228.

[32] Miller J: Myoblast diversity in skeletal myogenesis: how much and to what end? *Cell* 1992; 691: 1-3.

[33] Miller J, Crow M and Stockdale F: Slow and fast myosin heavy chain content defines three types of myotubes in early muscle cell cultures. *J Cell Biol* 1985; 101: 1643-1650.

[34] Miller J and Stockdale F: Developmental origins of skeletal muscle fibers: clonal analysis of myogenic cell lineages based on expression of fast and slow myosin heavy chains. *Proc Natl Acad Sci USA* 1986; 83: 3860-3864.

[35] Oakley B, Kirsch D and Morris N: A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal Biochem* 1980; 105: 361-363.

[36] Phillips W, Everett A and Bennett M: The role of innervation in the establishment of the topographical distribution of primary myotube types during development. *J Neurocytol* 1986; 15: 397-405.

[37] Saad A, Obinata T and Fischman D: Immunochemical analysis of protein isoforms in thick myofilaments of regenerating skeletal muscle. *Devel Biol* 1987; 119: 336-349.

[38] Sartore S, Gorza L and Schiaffino S: Fetal myosin heavy chains in regenerating muscle. *Nature* 1982; 298: 294-297.

[39] Silberstein L, Inesstrosa N and Hall Z: Aneural muscle cell cultures make synaptic basal lamina components. *Nature* 1982; 295: 143-145.

[40] Snow M: Myogenic cell formation in regenerating rat skeletal muscle injured by mincing. II. An autoradiographic study. *Anat Rec* 1977; 188: 201-218.

[41] Sohal G: Development of postsynaptic-like specializations of the neuromuscular synapse in the absence of motor nerve. *Int J Devel Neurosci* 1988; 6: 553-565.

[42] Sohal G and Wrenn R: Appearance of high-molecular-weight acetylcholinesterase in aneural muscle developing in vivo. *Devel Biol* 1984; 101: 229-234.

[43] Stewart A, Kennedy J, Bandman E and Zak R: A myosin isoform repressed in hypertrophied ALD muscle of the chicken reappears during regeneration following cold injury. *Devel Biol* 1989; 135: 367-375.

[44] Stockdale F, Miller J, Feldman J, Lamson G and Hager J (1989). Myogenic cell lineages: commitment and modulation during differentiation of avian muscle. In "Cellular and Molecular Biology of Muscle Development" (L. Kedes and F. Stockdale, Ed.), pp. 3-13. Alan R. Liss, New York.

[45] Sweeney L, Kennedy J, Zak R, Kokjohn K and Kelley S: Evidence for expression of a common myosin heavy chain phenotype in future fast and slow skeletal muscle during initial stages of avian embryogenesis. *Devel Biol* 1989; 133: 361-374.

[46] Vogel M and Landmesser L: Distribution of fiber types in embryonic chick limb muscles innervated by foreign motoneurons. *Devel Biol* 1987; 119: 481-495.

[47] Vrbova G, Gordon T and Jones R (1978). In "Nerve-Muscle Interaction" , pp. 81-104. Halsted Press, New York.

[48] Walro J, Hikida RS and Frangowlakis T: Patterns of avian muscle fiber type regeneration. *J Neurol Sci* 1982; 55: 327-338.