Myosin Heavy Chain Expression Following Myoblast Transfer into Regenerating Chicken Muscle

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Abstract

Myoblasts from fetal (day 11) chicken fast pectoral (PM) and from fast/slow adductor (AD) muscles were injected into regenerating fast and slow muscles of adult chicken. We show that the injected myoblasts remain viable and contribute myonuclei to new fibers which are formed following muscle cold injury. Myosin heavy chain (MyHC) expression was studied immunocytochemically in the injected, regenerating muscles 7 and 14 days post-injection using MyHC isoform specific monoclonal antibodies. We demonstrate that MyHC expression was unaffected in the regenerating muscles containing heterologous myoblasts. However, if the endogenous population of satellite cells was first suppressed with irradiation, slow MyHC isoforms not typically found in fast muscle were expressed in the regenerating fibers in fast irradiated muscle injected with adductor myoblasts, and neonatal and adult fast MyHCs, which are not expressed in slow muscle fibers, were found in regenerating fibers of a slow muscle injected with fetal pectoral muscle myoblasts. These results are consistent with previous studies which indicate that myoblasts from presumptive fast and slow muscles are programmed to express different MyHC subsets and thus may represent different myogenic lineages. However, commitment to MyHC expression is not irreversible and may still be altered by extrinsic factors during myogenesis.

Key words: myosin, myosin heavy chain, satellite cells, myoblast transfer, myoblast lineage, muscle, muscle development, x-irradiation, muscle regeneration.

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Skeletal muscle development has been an area of intense scrutiny for many years. Describing the process by which primordial cells become committed to a myoblast lineage and the regulatory mechanisms involved in this developmental transition have led to a more general understanding of cellular differentiation.

Much of what has been postulated about myoblast lineage has been inferred from studies of myosin protein expression in developing myotubes *in vivo* and in cell culture. Both light and heavy chain myosin proteins have been studied extensively in avian and mammalian species (cf. 2 and 60 for extensive reviews). Although much diversity exists among species with respect to specific myosin protein expression, a generalized pattern of myosin heavy chain (MyHC) isoform transitions during development has emerged.

MyHCs may be classified as either fast or slow, a designation which is a reflection of its associated ATPase activity, contractile speed, and electrophoretic mobility on native and SDS-PAGE [3, 4, 28, 38, 39, 40, 53, 55, 59]. Avian fast and slow MyHCs have been further defined. A subset of 5 fast MyHC isoforms which appear during development and are the products of a multi-gene family have been termed embryonic, neonatal, and adult isoforms [42, 43, 48, 54]. Slow MyHCs are designated slow myosin heavy chain 1 (SM_1) which appears in embryonic and neonatal stages of development and disappears with age and slow myosin heavy chain 2 (SM_2) which is the predominant adult form [15, 16, 17, 28, 36]. In addition to these 7 fast and slow MyHCs, two additional cardiac isoforms have been identified in chicken. One is predominantly expressed in the adult ventricle [5, 7, 70], and the other found predominantly in the atria [69]. However, each one of these isoforms has been found to be expressed transiently in newly differentiated skeletal muscle cells both in vivo and in vitro [6, 7, 49, 61]. The atrial isoform has also been referred to as SM_3 because it appears to be expressed early in presumptive slow muscle fibers [49].

Differential expression of MyHC isoforms in myotubes and myofibers formed from presumptive myoblasts of varying sources [12, 13, 14, 18, 20, 26, 27, 37, 38, 39, 40, 41, 52, 57], combined with differing media requirements of certain populations of muscle precursor cells in culture [58, 66], has led to the hypothesis that myoblasts may be committed to several different lineages. Although the bulk of evidence supports the theory of distinct myoblast lineages for the expression of fast and slow MyHCs, some investigators have found no evidence for differential expression of MyHCs in vitro [19] or in vivo [29]. Some have proposed that myofiber diversity arises only subsequent to myotube formation and that presumptive myoblasts are not committed to distinct lineages [29, 61]. Thus a key question that remains unanswered in myogenesis is when and via what mechanism does commitment to fast or slow MyHC expression occur.

Myoblast transfer is used in this study to determine if specific fast or slow MyHC expressing myoblasts are present in fetal chicken muscles (day 11 in ovo) and whether these specific myoblast lineages would maintain their MyHC expression in a foreign, adult regenerating environment. Myoblast transfer is a protocol in which donor myoblasts, which have been harvested elsewhere, are introduced into a growing or regenerating host muscle [30, 35, 50]. If host myogenic activity is sufficient, the transferred myoblasts will fuse to endogenous myoblasts and form mosaic fibers with myonuclear domains derived from both host and donor tissue or the implanted myoblasts will fuse to each other and form homogeneous myotubes. Myofibers derived from donor cells can be traced by labeling the donor myoblasts with genetic or histological markers such as 5-Bromo-2'deoxyuridine (BrdU), which can later be visualized with immunological staining techniques [62] or by transferring cells whose phenotypic expression (eg. glucose-6-phosphate isomerase, phosphorylase kinase, etc.) is different from that of the host [23, 31, 32, 33, 34, 44, 45, 46, 47, 51, 64, 65, 68].

It has been known for some time that irradiation administered at a proper dosage is very effective in suppressing proliferation and fusion of myogenic stem cells in skeletal muscle, especially satellite cells [1, 24, 46, 63, 67]. In this study we tested the myogenic potential of injected presumptive myoblasts in both non-irradiated and in irradiated adult muscles and have found that suppression of endogenous satellite cells was required in order to detect differences in MyHC expression.

Materials and Methods

Animal model

All myoblast injection, cold injury and irradiation studies were carried out in 6 month to 1 year-old White Leghorn (Line 03) adult chickens originating from University of California, Davis flocks. A total of 6 chickens were injected in the non-irradiated studies and 4 chickens in the irradiation experiments. Myoblasts for cell culture and myoblast transfer were derived from 11 day-old fetal [25] isogenic chicken embryos. All procedures were approved and monitored by the University of California, Davis department of Environmental Health and Safety and Animal Health and Welfare's Animal Use and Care Administrative Advisory Committee.

BrdU labelling

Myoblasts were labeled in the embryonic chick via injection of 200 µl of a 10 mM BrdU (5-Bromo-2'deoxyuridine) solution in phosphate buffered saline into the airspace of each egg, 14-16 hours prior to cell harvest. BrdU labeled all cells undergoing mitosis. The presence of BrdU was detected in fixed tissue sections and cell cultures via reactivity with a commercial monoclonal anti-BrdU primary antibody.

Myoblast preparation and muscle cell culture

Myoblasts were prepared as previously described [6] and were either injected into an adult chicken host muscle or plated for cell culture. Myoblasts were derived from 11 day-old fetal, presumptive fast twitch pectoralis major (PM) and slow tonic/fast twitch adductor complex (presumptive medial and lateral adductor (AD) muscles). The fetal muscles were removed, placed in 10 mls of Ham's F-10 (Irvine Scientific) and finely minced. The media was replaced with 10 mls of 0.05% to 0.1% Trypsin in Hanks BSS and incubated at 37°C. After 4-8 minutes, trypsinization was stopped by transferring the cell suspension into a polypropylene centrifuge tube containing 30-40mls of 20% donor horse serum (Hazleton) in Ham's F-10. The cells were pelleted by centrifugation at 1200g for approximately 4-5 minutes, the supernatant discarded and the pellet resuspended in 10-12 mls of media composed of 80% Ham's F-10, 15% horse serum (Gibco), 1% $CaCl_2$ (100mM), 0.5% Glutamine (100mM) and 3% embryo extract. The suspension was filtered through 2 layers of Nitex mounted in Swinney filters. Mononucleated cell concentration (which includes fibroblasts as well as myoblasts) was determined via a Coulter Counter and the suspension was diluted with additional media to deliver approximately 100,000 cells in 0.1 ml per injection site in the non-irradiated experiments and 500,000 per 0.1 ml per injection site in the irradiation studies. The number of mononucleated cells delivered to each injection site was increased in the irradiated experiments in order to compensate for the lack of endogenous satellite cells at the injury site in these muscles. To check for viability, cells were plated for culture in collagen coated 35mm dishes at approximately 300,000 cells per plate and routinely checked for the appearance of myotubes. The cell cultures were allowed to incubate for 4 days.

Irradiation of host muscle

In some of the experiments, host muscles were irradiated just prior to cold injury to suppress regeneration of endogenous satellite cells [1]. Adult chickens were intubated and maintained by inhalation anesthesia with 0.2% isoflourane. An incision was made in the skin overlying the

muscle so that the exact location of the muscle could be verified and the field to be irradiated could be marked directly on the muscle. The muscle was exposed to a Cobalt source of gamma irradiation in a uniform field measuring 4x4cm for pectoralis major (PM), and 4x5.5cm for the anterior latissimus dorsi (ALD, another slow tonic muscle). The ALD was chosen for these experiments due to the difficulty in accessing the medial adductor (MA) with the irradiation source (See Cold Injury section below). Dosages ranging from 1500 rads to 5000 rads were applied to the PM muscle to determine the optimal radiation dose. All muscles were irradiated for 20 minutes. In some cases the irradiation source was moved varying distances from the chicken to modify the dosage. The dosage sufficient to suppress the endogenous regenerative response to cold injury was typically 4500 rads for approximately 20 minutes. Radiation doses in excess of 5000 rads damaged the non-injured, adult myofibers.

Cold injury

Cold injury was performed on host muscles in order to induce regeneration of non-irradiated muscles and encourage incorporation of injected donor myoblasts into developing myofibers. Cold injury of irradiated muscles was used in order to provide a favorable regenerative environment for the injected donor cells. In the non-irradiated birds, cold injury was performed 3 days prior to myoblast injection on the PM and Medial Adductor (MA, adult slow specific) muscles. The MA was chosen for these initial non-irradiated experiments because it is derived from the fetal AD complex, which is a larger source of fetal cells than the ALD and because the MA is a larger adult muscle than the ALD. The birds were anesthetized with Sodium Pentobarbital and the skin over the PM incised. The incision was large enough to allow access to the MA muscle as well. A metal rod, 1 cm in diameter, was cooled in liquid nitrogen and pressed lightly to the muscle surface for approximately 10 seconds. Four to 6 spots were injured on each PM muscle and 2-3 areas on each MA muscle. In the irradiated muscles, cold injury was performed at the time of irradiation (anesthesia described above). Four sites were injured on each PM muscle. The ALD muscle (used as host instead of MA, see above rationale in the Irradiation of host muscle section) had 2 injury sites on one side, the other side serving as a control. In the non-irradiated study, muscles were allowed to regenerate for 7 days post-injection for a total of 10 days post cold-injury. Irradiated injected muscle regenerated for 7 and 14 days post collective trauma.

Tissue preparation and Immunocytochemistry

Tissue preparation and cryosectioning was performed as previously described [9]. Seven to 14 days post trauma, the injured/injected portions of the host muscles, as identified by superficial round white spots of necrotic tissue, were excised and quick frozen in Freon [22] cooled in liquid nitrogen. As many of the originally cold injured sites were frozen and assayed as possible. Occasionally, tissue blocks were rejected due to extensive connective tissue infiltration or unsuccessful injection attempts as evidenced by a lack of BrdU staining at the injection site or lack of an injection bolus as visualized with hematoxylin and eosin staining in the irradiated studies. Typically 2-4 sites were assayed from the PM muscles and 1-2 sites from the MA and ALD muscles. Tissue blocks were stored at -80°C. Ten micrometers thick tissue sections were cut on a Slee cryostat and mounted on gelatin coated coverslips. Immunocytochemical staining has been previously described [6]. Sections reacted with BrdU primary antibodies (Becton-Dickinson) were initially fixed in methanol for 10 minutes and rinsed with PBS, followed by immersion in 2N HCl for 10-20 minutes to denature the DNA. All tissue sections were blocked for 15-30 minutes in a 2% horse serum in PBS solution, then incubated with primary antibodies ranging in concentration from 1:1000 to 1:5000 (1:20 for BrdU) in a 1% horse serum PBS for 1 hour at room temperature or overnight at 4°C. Incubation with biotinylated rabbit anti-mouse IgG for 30 minutes was followed by biotin-avidin-peroxidase complex in 1% horse serum PBS for an additional 30 minutes according to a Vecta Stain ABC kit (Vector Laboratories). Antibody staining was visualized with 0.2 mg diaminobenzidine (DAB) in 100mM Tris Buffer and 0.01% H_2O_2. Standard ethanol and xylene rinses allowed for permanent mounting (Permount). Cell culture plates were doubly stained with DAB and 4-Chloro-1-napthol as previously described [8]. Myonuclei were reacted with antiBrdU antibodies and were visualized with DAB as described above. DAB staining was immediately followed by treatment of the cultures with 0.1M glycine (brought to pH 2.8 with HCl) for 10 minutes in order to remove bound IgG. The cultures were reblocked with 2% horse serum, incubated with anti-my-

Table 1. The specificities reported here are based on a combination of ELISA and data from western blots and immunocytochemistry.

THE SPECIFICITY OF ANTI-MYHC MONOCLONAL ANTIBODIES	
ANTIBODY	SPECIFICITY
HV11	ventricular MyHC
EB165	embryonic and adult fast MyHC isoforms
B103	embryonic and neonatal fast MyHC isoforms
2E9	neonatal fast MyHC only
AB8	adult fast MyHC only
NA1	SM2 MyHC only
NA2	SM1 and SM2 MyHC isoforms
NA3	SM2 MyHC only
NA8	SM2 and Atrial (or SM3) MyHC isoforms

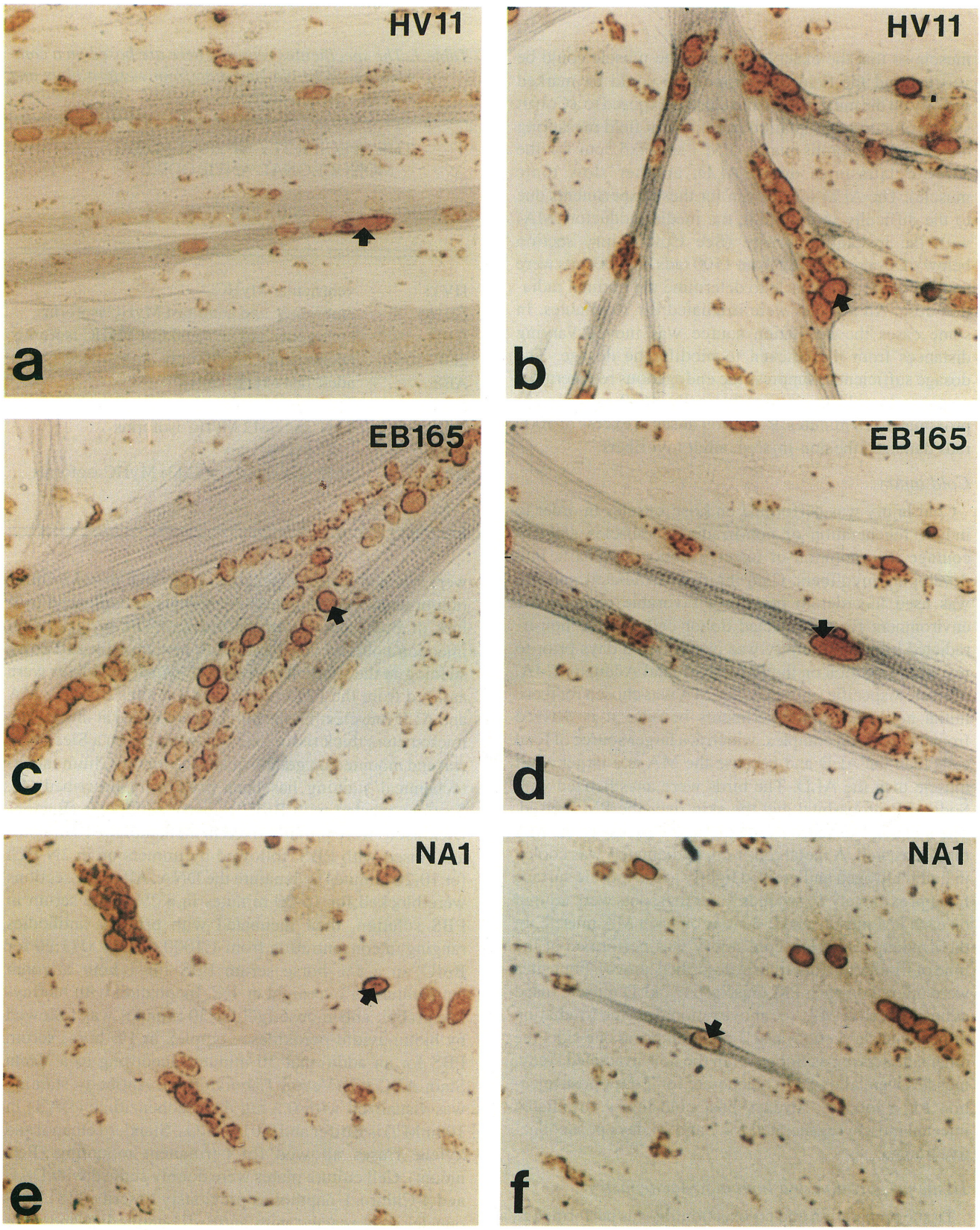

Figure 1. Muscle cell culture of day 11 fetal chick myoblasts derived from presumptive PM (a,c,e) and AD complex (b,d,f). Cultures were fixed at 4 days and were double immunostained for BrdU (nuclei, arrows a-f) and MyHCs (myofibers a-f). Cultures prepared from the PM and the AD reacted with HV11 ventricular/embryonic antibody (a,b) and EB165, embryonic fast antibody (c,d). No staining with NA1 adult slow antibody was observed in cells derived from the presumptive fast PM (e), however, some cells in the AD cultures reacted with the antibody. Magnification 450X.

osin monoclonal antibodies as described above; however, myosin staining was visualized with 4-Chloro-1-napthol in 0.1M Tris buffer ph 7.4 resulting in a light purple stain. Photomicrographs were taken on a Nikon Diaphot microscope with Tri-X pan film.

Antibody specificity

Antibody specificities have been described elsewhere [6, 8, 9]. Table 1 summarizes the specificity of the monoclonal antibodies used in this study.

Results

BrdU labelling and myoblast viability

In order to identify myoblasts injected into non-irradiated host muscle, embryos were incubated with 5-Bromo-2'Deoxyuridine (BrdU). Cells isolated from these embryos were viable and both fibroblasts and myoblasts exhibited BrdU labelling. Myoblasts from both the PM and the AD muscles of 11 day fetal chicken were able to proliferate and fuse to form myotubes which expressed MyHCs. All cultures reacted with the BrdU specific antibody (Figure 1, arrows). Myotubes formed in cultures derived from PM cells reacted with HV11 and EB165 (Figure 1a, c). No staining was observed with NA1 antibody (Figure 1e). The number of myotubes formed was similar to control cultures prepared from cells not labeled with BrdU suggesting that the level of BrdU incorporation did not have a significant impact on myogenesis. Cultures derived from adductor muscle cells displayed similar reactivity with HV11 and EB165 antibodies (Figure 1b, c) and a few fibers reacted with NA1 (Figure 1f). When cells from the PM and AD of BrdU labeled embryos were injected into cold-injured muscle, the anti-BrdU antibody reacted with the majority of the myonuclei incorporated into regenerating myofibers (Figures 2a, 3a arrows). These results indicate that the BrdU labeled cells remain viable and retain their myogenic potential.

Myoblast transfer into cold injured adult muscle

Localized cold injury stimulates endogenous satellite cells to divide mitotically and produce new myotubes [9, 56]. Adult chicken PM and MA muscles were cold injured 3 days prior to injection and muscle precursor cells harvested from fetal AD and PM muscles were injected into the adult cold injured site. Regenerated muscle sites were analyzed 7 days post injection, a total of 10 days after cold injury.

Myonuclei in regenerating PM muscle injected with BrdU labeled fetal myoblasts derived from the AD muscle complex reacted with the BrdU specific antibody (Figure 2a, arrows). In addition, fibers reacted with HV11, EB165,

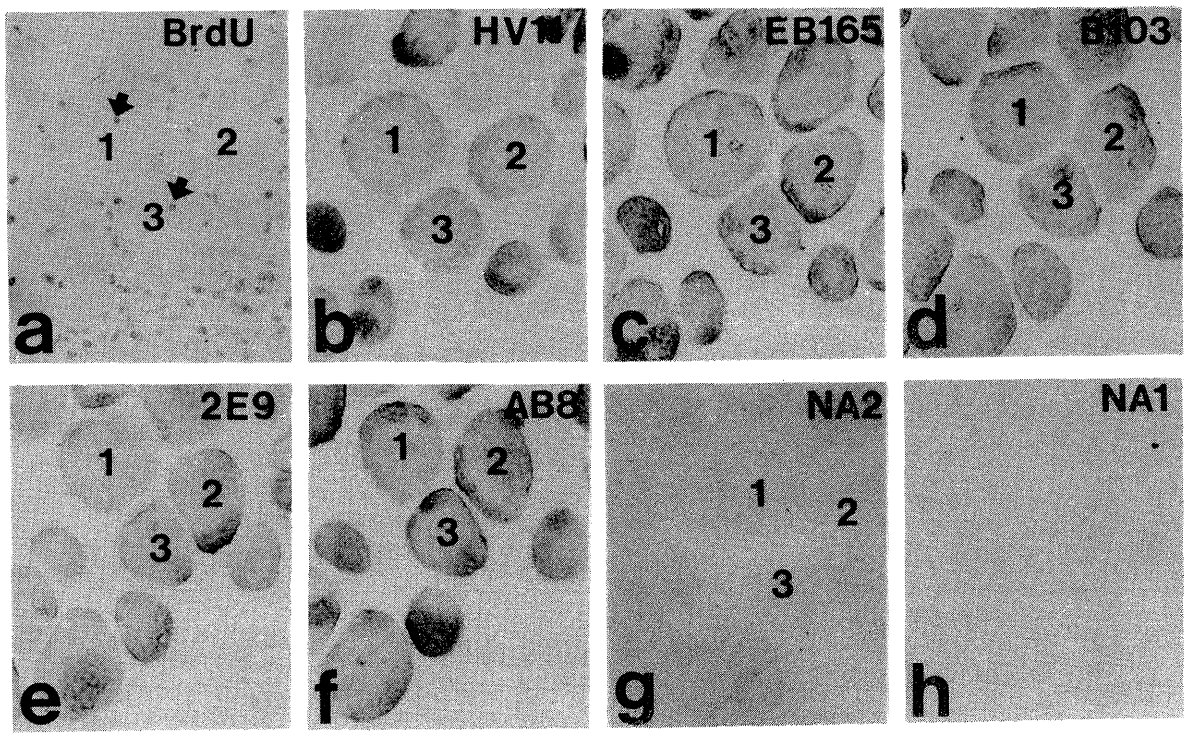

Figure 2. Serial sections of regenerating, cold injured PM muscle 7 days post-injection with fetal AD myoblasts. Centrally placed myonuclei in regenerating muscle fibers reacted with BrdU antibody (a, arrows). Myofibers (see fibers labeled 1,2,3) reacted with ventricular HV11 (b), embryonic fast EB165 (c), B103 (d), neonatal fast 2E9 (e), and adult fast AB8 (f) MyHC antibodies. No fibers were observed that reacted with slow MyHC specific antibodies NA2 (g) or NA1 (h). Magnification 250X.

B103, 2E9, and AB8 (Figures 2b-f). However, no fibers were observed that reacted with slow MyHC specific antibodies NA2 or NA1 (Figure 2g, h).

Myonuclei in regenerated MA muscle injected with BrdU labeled fetal muscle precursor cells derived from the PM reacted with the BrdU specific antibody (Figure 3a, arrows). Fibers that reacted with HV11, EB165, and B103 antibodies were observed (Figure 3b, c, d), but no fibers were found to react with 2E9 and AB8 antibodies (Figure 3e,f). Thus many regenerating fibers expressed ventricular and embryonic fast MyHCs, but none went on to express neonatal or adult fast isoforms. Most fibers reacted with slow MyHC specific NA2 and NA1 antibodies (Figure 3g,h).

Irradiation of adult muscle

In order to reduce the contribution of activated endogenous satellite cells in regenerating muscle, host muscles were irradiated prior to cold injury. Irradiated muscles showed a reduced regenerative response. Since HV11 antibody only reacts with newly formed myotubes in skeletal muscle [6], the reactivity of the antibody with myotubes following cold-injury is a marker for muscle regeneration. Cold-injured PM muscle shown at extremely low magnification 7 days after irradiation exhibited a graded response as the radiation dose increased from 0-4500 rads (Figure 4a-d, small stained cells are regenerating myofibers). It was not possible to reduce regeneration in the ALD to the same extent as in the PM (Figure 4e, f). Attempts at doses higher than 5000 rads often resulted in adult myofiber degeneration (data not shown). The suppressed regenerative response was maintained for at least 14 days after irradiation (data not shown). An optimal dose level of 4500 rads was set for all experimental muscles, although suppression of endogenous ALD satellite cells was incomplete.

Adult irradiated PM muscles injected with AD myoblasts

Irradiated adult PM muscles were injected with myogenic cells derived from the 11-day fetal AD and analyzed at 7 days post injection. Unlike uninjected controls, a strong reactivity with HV11 antibody in the cold-injured zone was observed (Compare Figures 4d and 5a). Serial sections of the cold injury site shows fibers staining with EB165 (adult fibers also stained, designated af) and B103 antibodies (Figure 5b, c) and a small population of cells in the regenerative zone reacting with 2E9 (Figure 5d). Virtually no reactivity was seen with AB8 in the regenerating zone, although adult fibers (af) showed positive staining

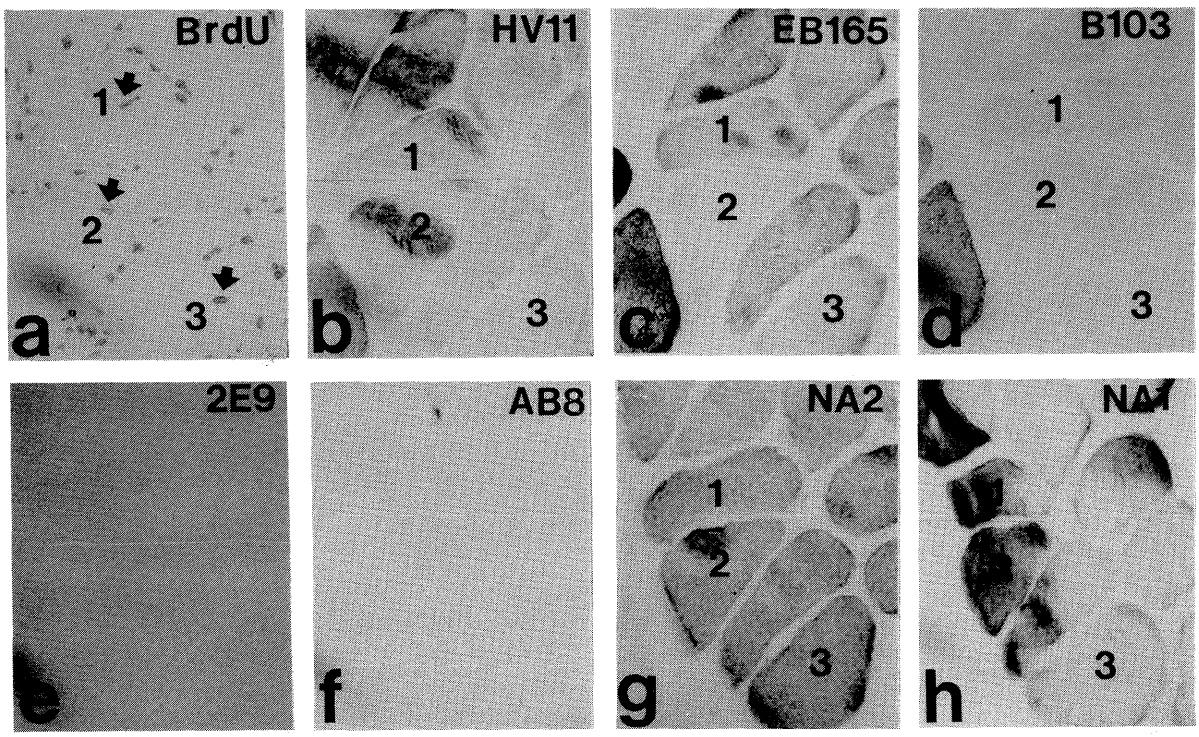

Figure 3. Serial sections of regenerating, cold injured MA muscle 7 days post-injection with fetal PM myoblasts. Centrally placed myonuclei in regenerating muscle fibers reacted with BrdU antibody (a, arrows). Myofibers (see fibers labeled 1,2,3) reacted with ventricular HV11 (b), embryonic fast EB165 (c), and a few with B103 (d) MyHC antibodies. No reactivity was observed with neonatal fast 2E9 (e) or adult fast AB8 (f) MyHC antibodies. Typical of regenerating MA, myofibers stained with slow MyHC specific antibodies NA2 (g) or NA1 (h). Magnification 250X.

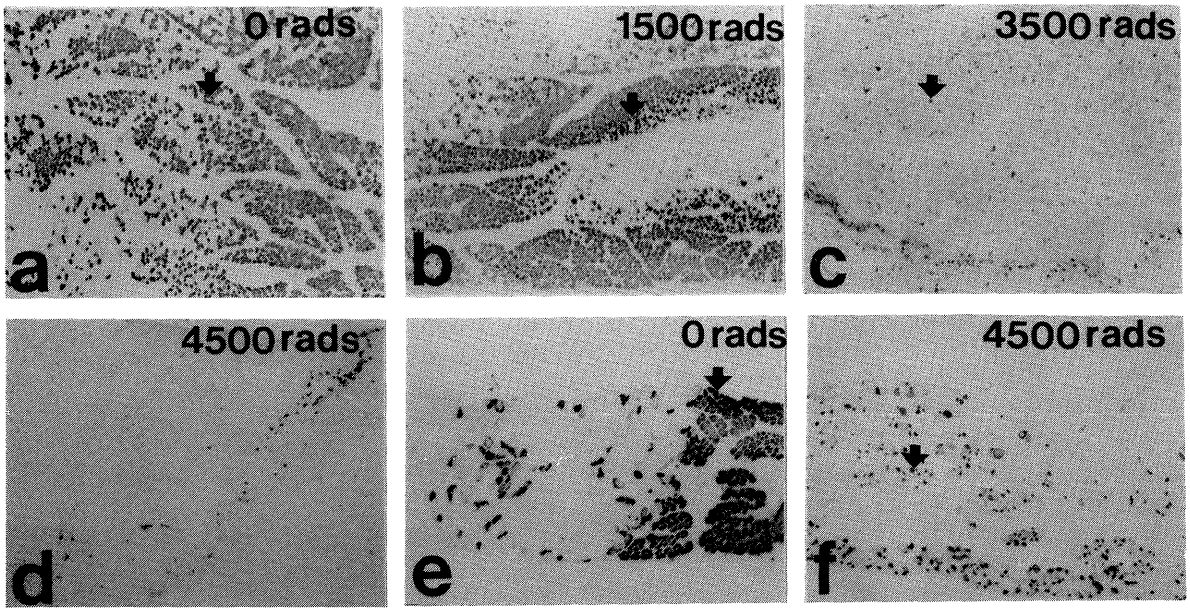

Figure 4. PM and ALD muscles examined 7 days after cold injury and irradiation. The entire regenerating zone is shown at this magnification (20X). Newly forming myotubes are visualized as small dark staining cells in the regenerating zone (arrows). Non-irradiated control PM (a) and ALD (e) muscles demonstrates typical regeneration after cold injury as visualized with HV11 antibody staining (arrows). Administration of irradiation for 20 minutes at 1500 rads (b), 3500 rads (c), and 4500 rads (d) shows increased suppression of regeneration with higher doses of radiation. Suppression of regeneration in the ALD muscle was not equivalent at 4500 rads (f).

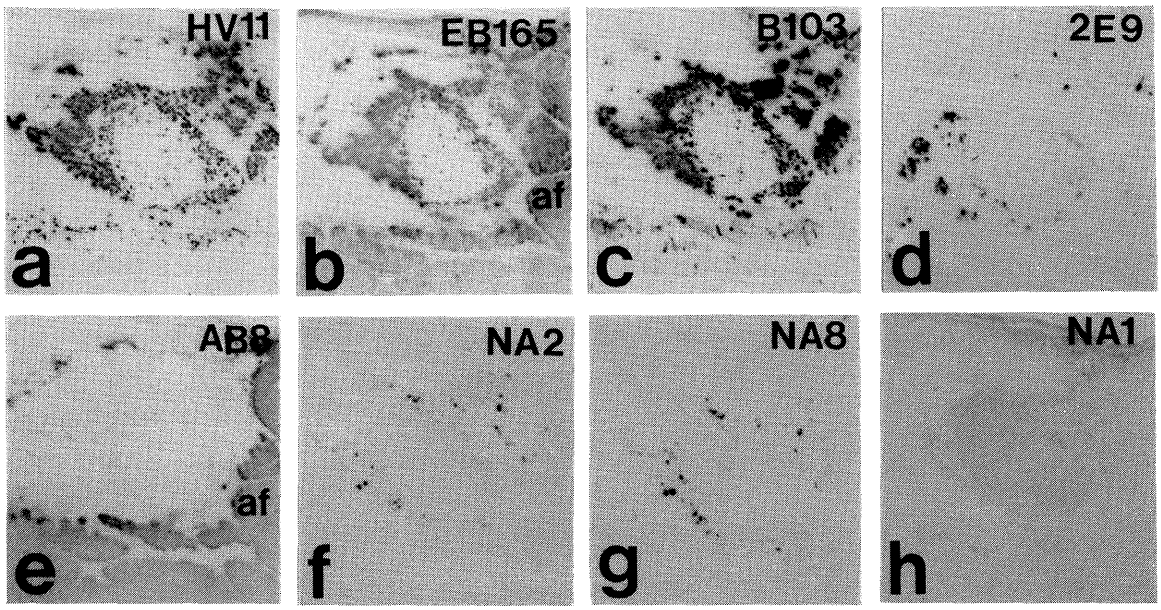

Figure 5. Serial sections of regenerating, cold injured PM muscle 7 days post-injection with fetal AD myoblasts. Regenerating myofibers reacted with ventricular HV11 (a), embryonic fast EB165 (b), B103 (c) MyHC antibodies. Sparse staining was observed with neonatal fast 2E9 (d) and no clearly labeled cells in the regenerating zone were seen at this magnification stained with adult fast AB8 (e) MyHC antibodies. Adult non-regenerating fibers (af) showed positive staining with EB165 and AB8 antibodies (b,e). However, some reactivity with slow MyHC specific antibodies NA2 (f) or NA8 (g) was observed in a small population of regenerating myofibers. No reactivity was seen with NA1 (h) at this time period. Magnification 20X.

(Figure 5e). Contrary to the results in non-irradiated muscle, there was clear labeling of regenerating myofibers with slow MyHC specific antibodies NA2 and NA8 (Figure 5f, g), but no labeling was found with NA1, an antibody to SM_2 MyHC (Figure 5h). Higher magnification shows fibers that reacted with slow MyHCs also reacted with HV11 antibody (Figure 6a,f,g); however, there was very little co-labeling of the fibers that reacted with 2E9 and slow MyHC antibodies (Figure 6d, f, g).

Irradiated adult PM muscles injected with myoblasts from fetal AD analyzed at 14 days post injection exhibited antibody reactivity similar to that shown at 7 days. Labelling with ventricular/embryonic MyHC antibodies HV11, EB165 and B103 was still apparent (Figure 7a, b, c) in the regenerating zone. Adult fibers (af) reacted with EB165 as well (Figure 7b). Reactivity with neonatal fast specific 2E9 is increased over that seen at 7 days (Figure 7d). Similar to 7 days post injection, at 14 days, very little staining is seen with AB8 adult fast MyHC specific antibody except in the non-regenerating adult fibers (af) (Figure 7e). Some reactivity is seen with slow specific MyHC antibodies NA2 and NA8 (Figure 7f, g).

Adult irradiated ALD muscles injected with PM myoblasts

Irradiated and cold injured adult ALD muscles were injected with myoblasts from the fetal PM and analyzed 7 days post injection. Because the ALD is a thin muscle, the extent of the cold injury and regenerative area often spanned the width of the muscle section (Figure 8). Regenerating myofibers reacted with ventricular/embryonic MyHC specific antibodies HV11, EB165 and B103 (Figure 8a, b, c). Some myofibers in the regeneration zone also reacted with slow MyHC specific antibodies NA2, NA8 and NA1 typical of slow muscle regeneration (Figure 8f, g, h). Most significantly, myofibers reacted with 2E9 antibody specific for the neonatal fast MyHC (Figure 8d). Although labeling is very sparse, some fibers also reacted with adult fast MyHC specific AB8 antibody as well (Figure 8e). At higher magnification, co-labeling of regenerating myofibers with HV11, EB165, B103, and 2E9 can be more clearly observed (Figure 9a,b,c,d arrows). No reactivity with AB8 is seen in this section (Figure 9e). Regenerating fibers labeling with NA8 and NA1 antibodies are shown as well (Figure 9f, g, arrows).

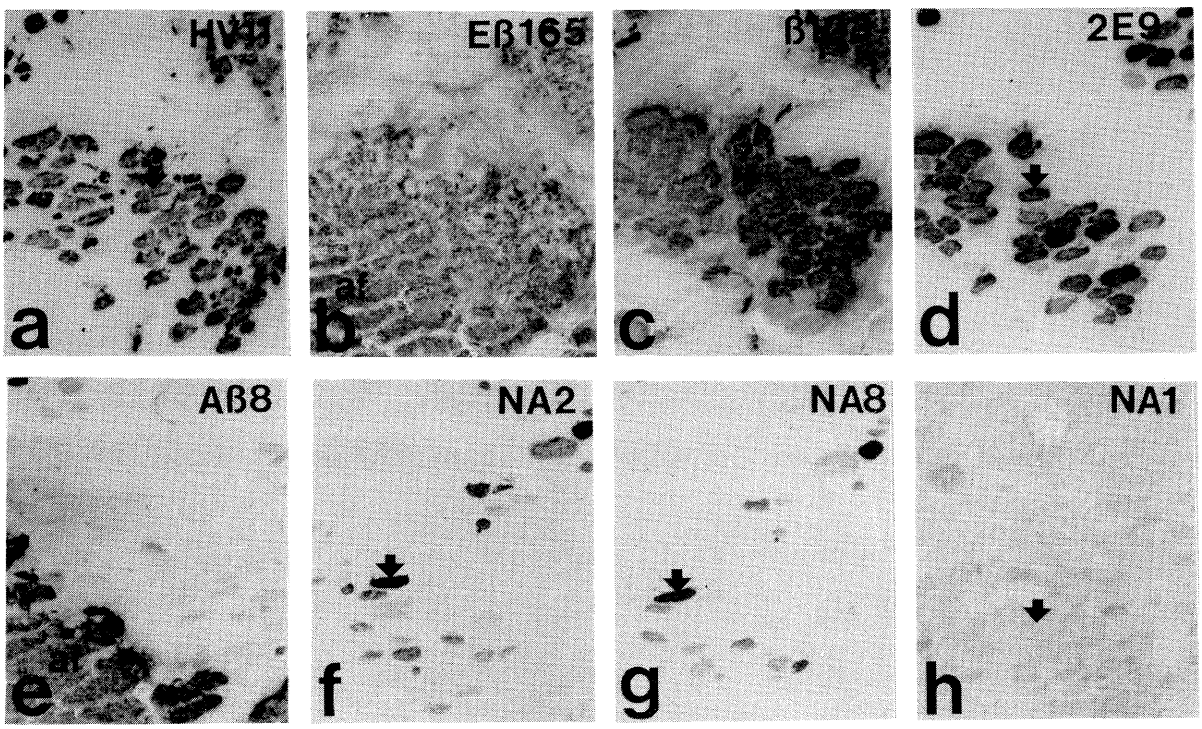

Figure 6. Serial sections of regenerating, cold injured PM muscle 7 days post-injection with fetal AD myoblasts shown at a higher magnification. Myofibers marked with arrows indicate the same fiber throughout the sections. Regenerating myofibers reacted with ventricular HV11 (a), embryonic fast EB165 (b), B103 (c) MyHC antibodies. Staining was observed in the regenerating zone with neonatal fast 2E9 (d) and slight staining with adult fast AB8 (e) MyHC antibodies. Adult non-regenerating fibers (af) showed positive staining with EB165 and AB8 antibodies (b,e). At this magnification, myofibers are clearly labeled with slow MyHC specific antibodies NA2 (f) and NA8 (g) and some co-labeling is seen with fibers also stained with HV11 and 2E9 (arrows). No reactivity was seen with NA1 (h). Magnification 100X.

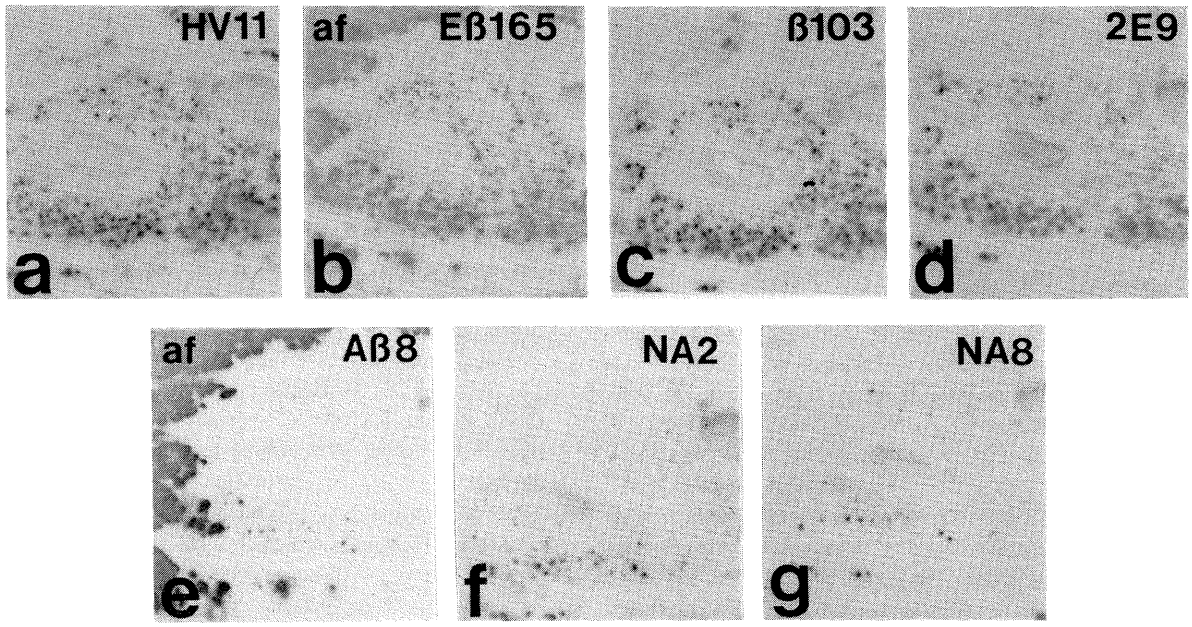

Figure 7. Serial sections of regenerating, cold injured PM muscle 14 days post-injection with fetal AD myoblasts. Regenerating myofibers reacted with ventricular HV11 (a), embryonic fast EB165 (b), B103 (c) MyHC antibodies. Reactivity with neonatal fast specific 2E9 antibody (d) is increased over that seen at 7 days (compare to Figure 5d). Few clearly labeled cells in the regenerating zone were seen at this magnification which reacted with adult fast AB8 (e) MyHC antibody. Adult non-regenerating fibers (af) showed positive staining with EB165 and AB8 antibodies (b,e). However, some reactivity with slow MyHC specific antibodies NA2 (f), NA8, (g) was observed in a small population of regenerating myofibers. Magnification 20X.

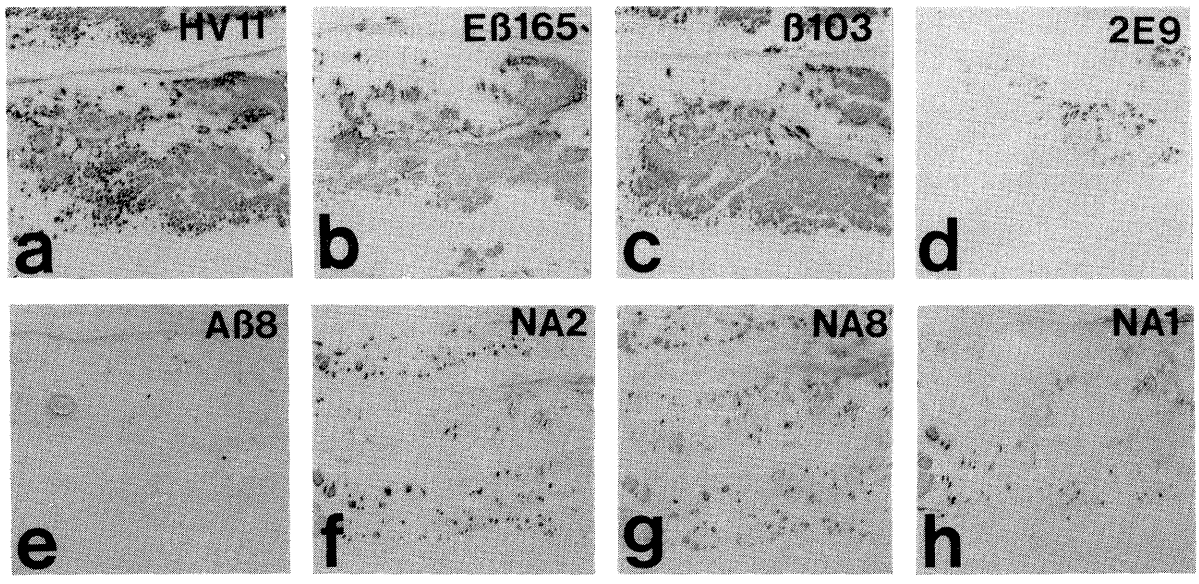

Figure 8. Serial sections of regenerating, cold injured ALD muscle 7 days post-injection with fetal PM myoblasts. Regenerating myofibers reacted with ventricular HV11 (a), embryonic fast EB165 (b), B103 (c) MyHC antibodies. Staining was observed with neonatal fast 2E9 (d) and very sparse reactivity was seen with adult fast AB8 (e) MyHC antibodies. Some reactivity with slow MyHC specific antibodies NA2 (f), NA8 (g), and NA1 was observed in a small population of regenerating myofibers and adult fibers (af). Magnification 20X.

Irradiated and cold injured adult ALD muscles were injected with myoblasts from the fetal PM and analyzed at 14 days post injection. Regenerating fibers in the ALD muscles continued to react with ventricular/embryonic MyHC antibodies (Figure 10a, b, c) and neonatal MyHC fast specific antibody, 2E9 (Figure 10d). Adult fast MyHC specific AB8 labeling was minimal, but present (Figure 10e). Slow specific myosins continued to be expressed as indicated by labeling with NA8 and NA1 (Figure 10f, g).

Discussion

In this report, we demonstrate that cells isolated from fetal chicken muscles are viable when injected into regenerating muscles in adult chickens. However the muscle origin of the injected cells had no detectable effect on MyHC expression in the regenerating muscle fibers unless the endogenous satellite cell population was first suppressed by irradiation prior to donor cell injection. The ability of donor cells to determine MyHC expression in regenerated fibers is consistent with previous studies suggesting that avian myoblasts are committed to distinct programs of MyHC expression [60].

In the experiments where muscle precursor cells were injected into host regenerating muscle that had not been previously irradiated, regenerating fibers only expressed MyHC isoforms typical of the regenerating fast or slow muscle fiber. These results are similar to other studies where the phenotypic expression of MyHC was characteristic of host muscle [29, 37]. In our studies it appears likely that the injected cells fused with endogenous satellite cells forming "mosaic" fibers [47, 65] containing donor (BrdU-labeled) and host derived (unlabeled) myonuclei. While we cannot rule out the possibility that the incorporated myonuclei labeled with BrdU are not expressing any MyHCs, BrdU-labeled myoblasts are competent to differentiate in culture and produce myotubes which express MyHCs similar to unlabeled control myoblasts. Since we were not able to detect any atypical isoforms in the injected muscles, it is possible that factors produced by the endogenous satellite cell derived myonuclei may regulate MyHC expression of the fetally derived myonuclei in the syncytia of the hybrid fibers. If this is correct, then the commitment of myoblasts to specific patterns of MyHC expression seen in vivo and in vitro can be altered. However, we can not rule out the possibility that the viability of the injected myoblasts was too low to affect MyHC expression. Despite the donor myoblasts' ability to differentiate *in vitro*, their viability may have been decreased because of immune attack at the host injection site due to their suspension in horse serum. Low viability coupled with competition from the endogenous satellite cells may have rendered any atypical MyHC expression

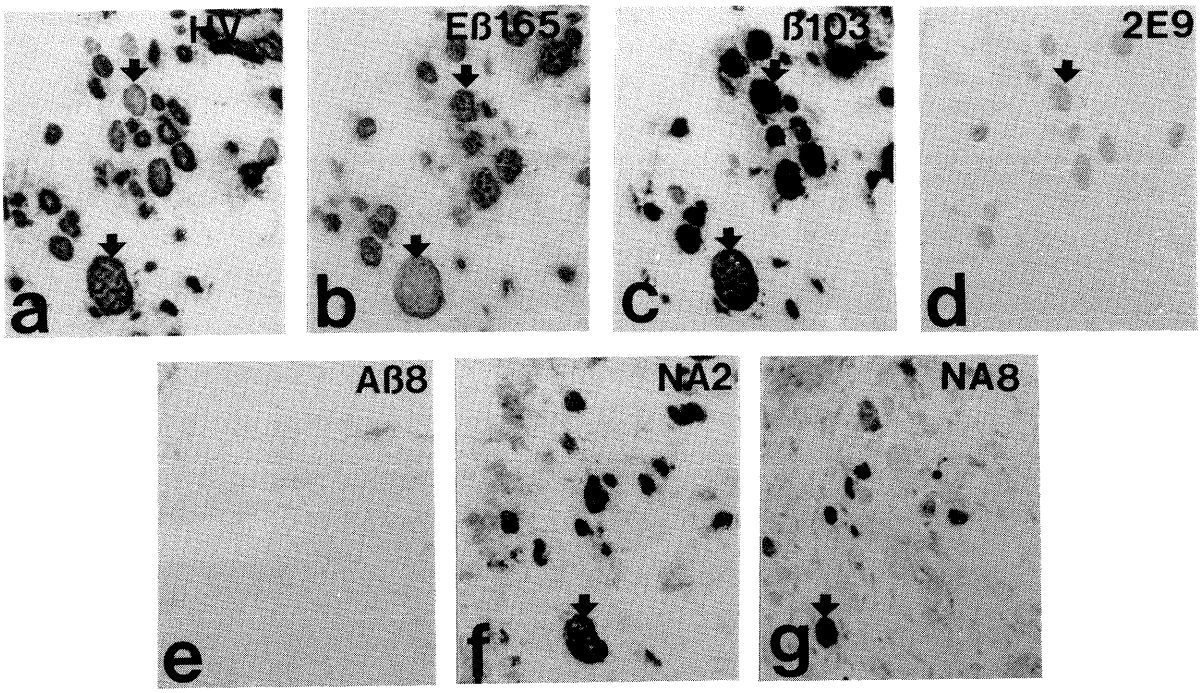

Figure 9. Serial sections of regenerating, cold injured ALD muscle 7 days post-injection with fetal PM myoblasts shown at a higher magnification. Myofibers marked with arrows indicate the same fiber throughout the sections. Regenerating myofibers reacted with ventricular HV11 (a), embryonic fast EB165 (b), B103 (c) MyHC antibodies. Slight staining was observed with neonatal fast 2E9 (d) and no reactivity with adult fast AB8 (e) MyHC antibodies in these sections. Some myofibers are also labeled with slow MyHC specific antibodies NA2 (f) and NA8 (g). Magnification 100X.

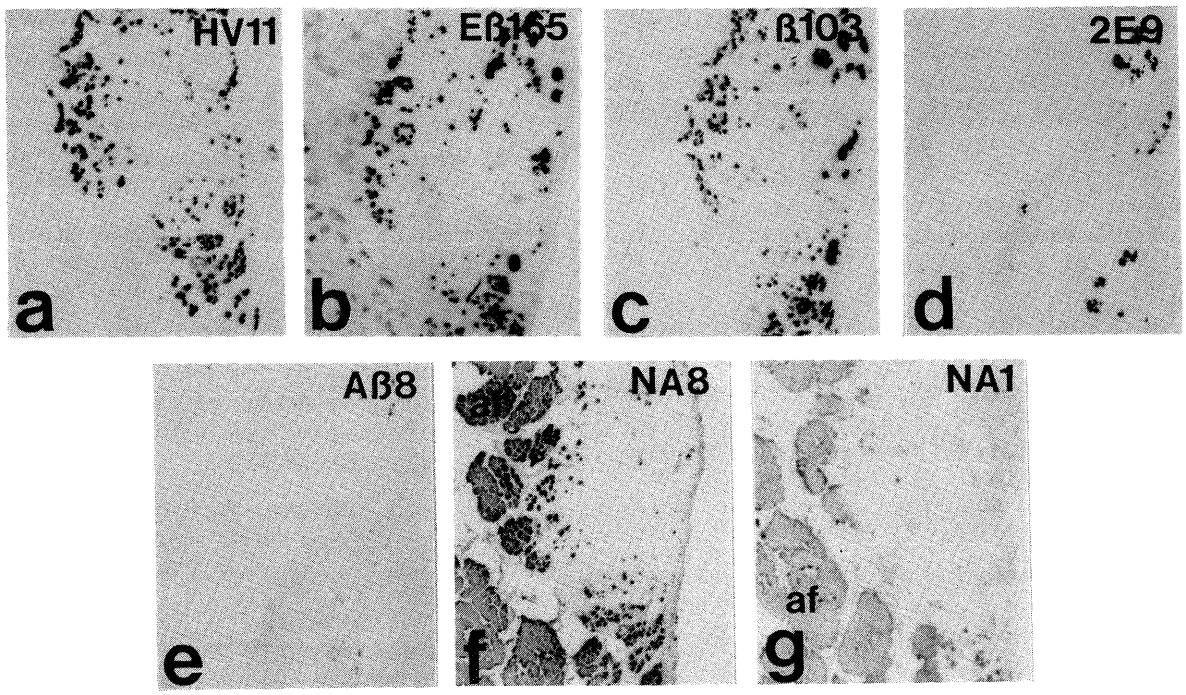

Figure 10. Serial sections of regenerating, cold injured ALD muscle 14 days post-injection with fetal PM myoblasts. Regenerating myofibers reacted with ventricular HV11 (a), embryonic fast EB165 (b), B103 (c) MyHC antibodies. Reactivity with neonatal fast specific 2E9 antibody (d) is similar to that seen at 7 days. Few cells were seen that stained with adult fast AB8 (e) MyHC antibody. Some reactivity with slow MyHC specific antibodies NA8(f), NA1, (g) was observed in a small population of regenerating myofibers and adult fibers (af). Magnification 20X.

from the injected cells impossible or undetectable with our antibodies. It may also be possible that atypical MyHCs were expressed transiently, at earlier time periods which were not examined. Clearly any early expression of foreign MyHCs did not persist to seven days post-trauma.

In contrast to the above results, injection of muscle precursor cells from the fetal AD muscle into the irradiated PM muscle resulted in fibers in the PM that expressed fast and slow MyHCs as demonstrated by immunocytochemistry with isoform-specific monoclonal antibodies. Regenerating fibers in the PM have not been shown previously to produce slow MyHCs even in the absence of innervation [9]. Since cold-injured irradiated PM muscle showed almost no regeneration, these newly formed myofibers are most likely predominantly derived from injected myoblasts. The MyHC expression pattern of these fibers is consistent with the source of the donor myoblasts, since the AD muscle contains both a slow (medial) and a fast (lateral) component, and cell cultures derived from these cells formed myotubes producing predominantly fast and a few slow myotubes. In a similar manner, injection of muscle precursor cells from the fetal PM into the irradiated ALD muscle resulted in regenerated fibers in the ALD that contained neonatal and adult fast MyHCs, fast isoforms not previously detected in regenerating slow muscle. We also observed regenerating fibers expressing slow MyHCs in the ALD. However because we were unable to completely suppress the endogenous satellite cell population, we cannot determine whether these isoforms were produced from donor or host myonuclei. In both cases, while there was extensive myogenesis as detected by ventricular MyHC expression in the cold injured areas following cell injection, the appearance of atypical MyHC isoforms derived from the donor cells was not widespread. This might suggest that fetally-derived muscle precursor cells might not readily replace adult satellite cell derived myoblasts as a source of new fibers in adult muscle. This would be consistent with recent studies indicating that satellite cells and fetal myoblasts represent distinct myogenic lineages as determined by MyHC expression in vitro [10, 11, 21, 26, 27]. The low amounts of atypical MyHC expression may also be due to low viability of the injected cells at the trauma site. However, because there is less competition with endogenous satellite cells in irradiated muscle, even a severely decreased population of myoblasts might be capable of producing a detectable amount of donor MyHCs.

Irradiation of host muscle prior to donor cell injection has previously been used to suppress endogenous satellite cells [1, 24, 46, 63, 67]. In the PM muscle we could essentially completely inhibit muscle regeneration as demonstrated by the absence of newly formed myofibers that

reacted with HV11 antibody [6]. However, in our studies we were unable to completely eliminate regeneration in the ALD muscle unless irradiation doses which resulted in damage to existing muscle fibers were used (data not shown). Although the molecular basis for the differential susceptibility of the two populations of satellite cells is unclear, it is another example of differences between satellite cells in fast and slow muscles [20, 22].

Our results demonstrate that the source of donor myoblasts can affect the expression of MyHC isoforms in the muscle fibers they form in vivo. Since transfer of normal myoblasts into diseased muscle has demonstrated the feasibility of correcting muscle genetic defects [31, 32, 33, 34, 44, 45, 46, 47, 51] it will be important to ensure that the donor cells do not alter the myosin phenotype and thus the contractile properties of the myofibers they that populate.

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References

[1] Alameddine HS, Dehaupas M, Fardeau M: Regeneration of skeletal muscle fibers from autologous satellite cells multiplied in vitro: An experimental model for testing cultured cell myogenicity. *Muscle and Nerve* 1989; 12: 544-555.

[2] Bandman E: Myosin isoenzyme transitions in muscle development, maturation, and disease. *Int Rev Cytol* 1985; 97: 97-131.

[3] Bandman E, Matsuda R, Strohman RC: Developmental appearance of myosin heavy and light chain isoforms in vivo and in vitro, in chicken skeletal muscle. *Dev Biol* 1982; 93: 508-518.

[4] Barany M: ATPase activity of myosin correlated with speed of muscle shortening. *J Gen Physiol* 1967; 50: 197-218.

[5] Bisaha JG, Bader D: Identification and characterization of a ventricular-specific avian myosin heavy chain, VMHC1: Expression in differentiating cardiac and skeletal muscle. *Dev Biol* 1991; 148: 355-364.

[6] Bourke DL, Wylie SR, Wick M, Bandman E: Differentiating skeletal muscle cells initially express a ventricular myosin heavy chain. *Basic and Appl Myol* 1991; 1: 13-21.

[7] Camoretti-Mercado B, Dizon E, Jakovcic S, Zak R: Differential expression of ventricular-like myosin heavy chain mRNA in developing and regenerating avian skeletal muscles. *Cell and Mol Biol Res* 1993; 39: 425-437.

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

[9] Cerny LC, Bandman E: Contractile activity is required for the expression of neonatal myosin heavy chain in embryonic chick pectoral muscle cultures. *J Cell Biol* 1986; 103: 2153-2161.

[10] Cossu G, Eusebi F, Grassi F, Wanke E: Acetylcholine receptor channels are present in undifferentiated satellite cells but not in embryonic myoblasts in culture. *Dev Biol* 1983; 123: 43-50.

[11] Cossu G, Molinaro M, Pacifici M: Differential response of satellite cells and embryonic myoblasts to a tumor promotor. *Dev Biol* 1987; 98: 520-524.

[12] Cho M, Webster SB, Blau HM: Evidence for myoblast-extrinsic regulation of slow myosin heavy chain expression during muscle fiber formation in embryonic development. *J Cell Biol* 1993; 121: 795-810.

[13] Crow MT, Stockdale FE: Myosin expression and specialization among the earliest muscle fibers of the developing avian limb. *Dev Biol* 1986a; 113: 238-254.

[14] Crow MT, Stockdale FE: The developmental program of fast myosin heavy chain expression in avian skeletal muscles. *Dev Biol* 1986b; 118: 333-342.

[15] Dhoot GK: Changes in the distribution of slow skeletal myosin heavy chain SM1 in developing avian muscle fibres. *J Mus Res Cell Motil* 1988a; 9: 120-31.

[16] Dhoot GK: Identification and changes in the pattern of expression of slow skeletal-muscle-like myosin heavy chains in a developing fast muscle. *Differentiation* 1988b; 37: 53-61.

[17] Dhoot GK: Evidence for the presence of a distinct embryonic isoform of myosin heavy chain in chicken skeletal muscle. *Differentiation* 1989; 40: 76-83.

[18] DiMario JX, Fernyak SE, Stockdale FE: Myoblasts transferred to the limbs of embryos are committed to specific fibre fates. *Nature* 1993; 362: 165-167.

[19] Dusterhoft S, Yablonka-Reuveni Z, Pette D: Characterization of myosin isoforms in satellite cell cultures from adult rat diaphragm, soleus and tibialis anterior muscles. *Differentiation* 1990; 45: 185-191.

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

[21] Feldman JL, Stockdale FE: Temporal appearance of satellite cells during myogenesis. *Dev Biol* 1992; 153: 217-226.

[22] Gibson MC, Schultz E: The distribution of satellite cells and their relationship to specific fiber types in soleus and extensor digitorum longus muscles. *Anat Rec* 1982; 202: 329-337.

[23] Grounds M, Partridge TA, Sloper JC: The contribution of exogenous cells to regenerating skeletal muscle: An isozyme study of muscle allografts in mice. *J Path* 1980; 132: 325-341.

[24] Gulati AK: The effect of X-irradiation on skeletal muscle regeneration in the adult rat. *J Neurol Sci* 1987; 78: 111-120.

[25] Hamburger V, Hamilton HL: A series of normal stages in the development of the chick embryo. *J Morphol* 1951; 88: 49-92.

[26] Hartley RS, Bandman E, Yablonka-Reuveni Z: Myoblasts from fetal and adult skeletal muscle regulate myosin expression differently. *Dev Biol* 1991; 148: 249-260.

[27] Hartley RS, Bandman E, Yablonka-Reuveni Z: Skeletal muscle satellite cells appear during late chicken embryogenesis. *Dev Biol* 1992; 153: 206-216.

[28] Hoh JFY: Light chain distribution of chicken skeletal muscle myosin isozymes. *FEBS Lett* 1978; 90: 297-300.

[29] Hughes SM, Blau HM: Muscle fiber pattern is independent of cell lineage in postnatal rodent development. *Cell* 1992; 68: 659-671.

[30] Jones PH: Implantation of cultured regenerate muscle cells into adult rat muscle. *Exp Neurol* 1979; 66: 602-610.

[31] Law PK: Beneficial effects of transplanting normal limb-bud mesenchyme into dystrophic mouse muscles. *Muscle and Nerve* 1982; 5: 619-627.

[32] Law PK, Goodwin TG, Li HJ: Histoincompatible myoblast injection improves muscle structure and function of dystrophic mice. *Transplantation Proceedings* 1988a; 20: 1114-1119.

[33] Law PK, Goodwin TG, Wang, MG: Normal myoblast injections provide genetic treatment for murine dystrophy. *Muscle and Nerve* 1988b; 11: 525-533.

[34] Law PK, Yap JL: New muscle transplant method produces normal twitch tension in dystrophic muscle. *Muscle and Nerve* 1979; 2: 356-363.

[35] Lipton BH, Schultz E: Developmental fate of skeletal muscle satellite cells. *Science* 1979; 205: 1292-1294.

[36] Matsuda R, Bandman E, Strohman RC: The two myosin isozymes of chicken latissimus dorsi muscle contain different myosin heavy chains encoded by separate mRNAs. *Differentiation* 1982; 23: 36-42.

[37] Matsuda R, Spector DH, Strohman RC: Regenerating adult chicken skeletal muscle and satellite cell cultures express embryonic patterns of myosin and tropomyosin isoforms. *Dev Biol* 1983; 100: 478-488.

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

[39] Miller JB, Stockdale FE: Developmental origins of skeletal muscle fibers: Clonal analysis of myogenic cell lineages based on expression of fast and slow myosin heavy chains. *Proc Nat Acad Sci* 1986a; 83: 3860-3864.

[40] Miller JB, Stockdale FE: Developmental regulation of the multiple myogenic cell lineages of the avian embryo. *J Cell Biol* 1986b; 103: 2197-2208.

[41] Miller JB, Stockdale FE: Multiple cellular processes regulate expression of slow myosin heavy chain isoforms during avian myogenesis in vitro. *Dev Biol* 1989; 136: 393-404.

[42] Moore LA, Arrizubieta MJ, Tidyman WE, Herman LA, Bandman E: Analysis of the chicken fast myosin heavy chain family. Localization of isoform-specific antibody epitopes and regions of divergence. *J Mol Biol* 1992; 225: 1143-1151.

[43] Moore LA, Tidyman WE, Arrizubieta MJ, Bandman E: The evolutionary relationship of avian and mammalian myosin heavy-chain genes. *J Mol Evol* 1993; 36: 21-30.

[44] Morgan JE, Coulton GR, Partridge TA: Muscle precursor cells invade and repopulate freeze-killed muscles. *J Muscle Res Cell Motil* 1987; 8: 386-396.

[45] Morgan JE, Coulton GR, Partridge TA: MDX muscle grafts retain the mdx phenotype in normal hosts. *Muscle and Nerve* 1989; 12: 401-409.

[46] Morgan JE, Hoffman EP, Partridge TA: Normal myogenic cells from newborn mice restore normal histology to degenerating muscles of the mdx mouse. *J Cell Biol* 1990; 111: 2437-2449.

[47] Morgan JE, Watt DJ, Sloper JC, Partridge TA: Partial correction of an inherited biochemical defect of skeletal muscle by grafts of normal muscle precursor cells. *J Neurol Sciences* 1988; 86: 137-147.

[48] Nguyen H, Bubits R, Wydro R, Nadal-Ginard B: Sarcomeric myosin heavy chain is coded by a highly conserved multigene family. *Proc Nat Acad Sci* 1982; 79: 5230-5234.

[49] Page S, Miller JB, DiMario JX, Hager EJ, Moser A, Stockdale FE: Developmentally regulated expression of three slow isoforms of myosin heavy chain: Diversity among the first fibers to form in avian muscle. *Dev Biol* 1992; 154: 118-128.

[50] Partridge TA, Grounds M, Sloper JC: Evidence of fusion between host and donor myoblasts in skeletal muscle grafts. *Nature* 1978; 273: 306-308.

[51] Partridge TA, Morgan JE, Coulton GR, Hoffman EP, Kunkel LM: Conversion of mdx myofibres from dystrophin-negative to positive by injection of normal myoblasts. *Nature* 1989; 337: 176-179.

[52] Pin CL, Merrifield PA: Embryonic and fetal rat myoblasts express different phenotypes following differentiation in vitro. *Dev Gen* 1993; 14: 356-368.

[53] Reiser PJ, Moss RL, Giulian GG, Greaser ML: Shortening velocity in single fibers from adult rabbit soleus muscles is correlated with myosin heavy chain composition. *J Biol Chem* 1985; 260: 9077-9080.

[54] Robbins J, Horan T, Gulick J, Kropp K: The myosin heavy chain gene family. *J Biol Chem* 1986; 261: 6606-6612.

[55] Rushbrook JI, Stracher A: Comparison of adult, embryonic and dystrophic myosin heavy chains from chicken muscle by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and peptide mapping. *Proc Nat Acad Sci* 1979; 76: 4331-4334.

[56] Saad AD, Obinata T, Fischman DA: Immunochemical analysis of protein isoforms in thick myofilaments of regenerating skeletal muscle. *Dev Biol* 1987; 119: 336-349.

[57] Schafer DA, Miller JB, Stockdale FE: Cell diversification within the myogenic lineage: In vitro generation of two types of myoblasts from a single myogenic progenitor cell. *Cell* 1987; 48: 659-670.

[58] Seed J, Hauschka SD: Temporal separation of the migration of distinct myogenic precursor populations into the developing chick wing bud. *Dev Biol* 1984; 106: 389-393.

[59] Sheetz MP, Chasan R, Spudich JA: ATP-dependent movement of myosin in vitro: Characterization of a quantitative assay. *J Cell Biol* 1984; 99: 1867-1871.

[60] Stockdale FE, Miller JB: The cellular basis of myosin heavy chain isoform expression during development of avian skeletal muscles. *Dev Biol* 1987; 123: 1-9.

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

[62] Tapscott SJ, Lassar AB, Davis RL, Weintraub H: 5-Bromo-2'-deoxyuridine blocks myogenesis by extinguishing expression of MyoDl. *Science* 1989; 245: 532-536.

[63] Wakeford S, Watt DJ, Partridge TA: X-irradiation improves mdx mouse muscle as a model of myofiber loss in DMD. *Muscle and Nerve* 1991; 14: 42-50.

[64] Watt DJ, Lambert K, Morgan JE, Partridge TA, Sloper JC: Incorporation of donor muscle precursor cells into an area of muscle regeneration in the host mouse. *J Neurol Sci* 1982; 57: 319-331.

[65] Watt DJ, Morgan JE, Partridge TA: Use of mononuclear precursor cells to insert allogenic genes into growing mouse muscles. *Muscle and Nerve* 1984; 7: 741-750.

[66] White NK, Bonner PH, Nelson DR, Hauschka SD: Clonal analysis of vertebrate myogenesis. IV. Medium dependent classification of colony-forming cells. *Dev Biol* 1975; 44: 346-361.

[67] Wirtz P, Loermans H, Rutten E: Effects of irradiation on regeneration in dystrophic mouse leg muscles. *Br J Exp Path* 1982; 63: 671-679.

[68] Yao SN, Kurachi K: Implanted myoblasts not only fuse with myofibers but also survive as muscle precursor cells. *J Cell Science* 1993; 105: 957-963.

[69] Yutzey Ke, Rhee JT, Bader D: Expression of the atrial-specific myosin heavy chain amhc1 and the establishment of anteroposterior polarity in the developing chicken heart. *Development* 1994; 120: 871-883.

[70] Zhang Y, Shafiq SA, Bader D: Detection of a ventricular-specific myosin heavy chain in adult and developing chicken heart. *J Cell Biol* 1986: 102: 1480-1484.