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# Embryonic and Fetal Rat Myoblasts Form Different Muscle Fiber Types in an Ectopic In Vivo Environment

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**ABSTRACT** Limb muscle development is characterized by the migration of muscle precursor cells from the somite followed by myoblast differentiation and the maturation of myotubes into distinct muscle fiber types. Previous in vitro experiments have suggested that rat limb myoblasts are composed of at least two distinct myoblast subpopulations that appear in the developing hindlimb at different developmental stages. These embryonic and fetal myoblast subpopulations are believed to generate primary and secondary myotubes, respectively. To test this hypothesis, cells obtained from embryonic day 14 (ED 14) and ED 20 rat hindlimbs were analyzed for myosin heavy chain expression after long-term differentiation in adult rat brains. Fetal myoblasts from ED 20 hindlimbs produced muscle fibers with a phenotype similar to that seen in tissue culture—predominantly fast myosin with a small proportion also coexpressing slow myosin. However, injection sites populated by embryonic myoblasts from ED 14 hindlimbs produced a different phenotype from that previously reported in culture, with fibers expressing an entire array of myosin isoforms. In addition, a subpopulation of fibers expressing exclusively slow myosin was found only in the embryonic injection sites. Our results support the existence of at least three myogenic subpopulations in early rat limb buds with only one exhibiting the capability to differentiate in vitro. These findings are consistent with a model of muscle fiber type development in which the fiber type potential of myoblast populations is established before differentiation into myotubes. This process establishes myogenic subpopulations that have restricted adaptive ranges regulated by both intrinsic and extrinsic factors. © 2002 Wiley-Liss, Inc.

**Key words:** myosin heavy chain; myoblast populations; developmental potential; muscle fiber type

## INTRODUCTION

The development of limb skeletal muscle is initiated by the migration of limb muscle precursor cells (LMPCs) from the lateral lip of the dermamyotome into the developing limb bud (Christ et al., 1977; Ordahl

and Le Douarin, 1992; Dietrich, 1999). This migration is followed by the differentiation of myoblasts to form multinucleated myotubes and the subsequent maturation of myotubes into distinct muscle fiber types (Hauschka, 1994). Muscle fibers can be classified as either slow (type I) or fast (type IIA, IIB, IIX) based on the expression of specific myosin heavy chain (MyHC) isoforms and other muscle fiber type-specific genes (Schiaffino and Reggiani, 1996), and it is now clear that the development of a normal repertoire of muscle fiber types is essential to adult muscle function (Allen et al., 2000). Although much progress has been made in elucidating the factors that control the migration (Birchmeier and Brohmann, 2000) and the differentiation of muscle precursor cells (Perry and Rudnicki, 2000), the cellular and molecular mechanisms that regulate the development of distinct muscle fiber types are less clear. Although innervation plays an important role in the modulation of fiber type-specific genes in adult muscle (Pette and Vrbova, 1985; Talmadge, 2000), the development of fast and slow fiber types in the embryo can occur in the absence of innervation (Butler et al., 1982; Butler-Browne et al., 1982; Crow and Stockdale, 1986; Condon et al., 1990b). However, there is now good evidence that the developmental origin (or lineage) of muscle precursor cells plays an important role in the differentiation of muscle fiber types in developing embryos (Stockdale, 1992, 1997).

During embryonic development, the LMPCs that migrate into the developing limb bud express several specific cell lineage markers, including Pax3, the c-Met tyrosine kinase receptor, and its ligand SF/HGF (scatter factor/hepatocyte growth factor; Dietrich, 1999). Such markers allow one to follow the migration and fate of these cells, and knocking out any one of them gives rise to embryos that lack limb muscle (Gross et

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al., 2000). Evidence from several animal models suggests that all LMPCs are not the same and that different subpopulations of LMPCs, myoblasts, or both, can be identified by clonal analyses, which have different growth requirements (White et al., 1975; Seed and Hauschka, 1984), and/or express different myosin heavy chain isoforms (Stockdale, 1992).

Culturing limb myoblasts from different stage embryos has indicated that temporally distinct populations of myoblasts are predisposed to expressing fast, slow, or fast/slow MyHC isoforms. For example, myoblasts isolated from chick embryos at early stages of limb development preferentially express slow MyHC when cultured *in vitro*, whereas fetal myoblasts from later stage embryos preferentially express fast MyHC (Miller et al., 1985). *In vivo* experiments in the chick have suggested that myoblasts which migrate into the limb early in development (*i.e.*, embryonic myoblasts) give rise mainly to slow, primary myotubes, whereas later migrants (fetal myoblasts) generate secondary myotubes expressing predominantly fast MyHC (Van Swearingen and Lance-Jones, 1995). Previously, we have shown that embryonic myoblasts obtained from hindlimbs of embryonic day 14 (ED 14) rats and fetal myoblasts from ED 20 rats express different phenotypes *in vitro* (Pin and Merrifield, 1993). In culture, embryonic myoblasts fuse to form small myotubes that express exclusively embryonic and slow MyHCs, whereas fetal myoblasts generate large myotubes that express embryonic, neonatal, and fast MyHCs. With longer time in culture, a subpopulation of fetal myotubes coexpresses slow MyHC as well (Torgan and Daniels, 2001). These observations suggest that separate myogenic populations with distinct fiber type potentials can be detected at different times during development of the hindlimb.

In the developing limb, myogenesis is characterized by the sequential appearance of primary and secondary myotubes (Rubenstein and Kelly, 1981). In the rat, primary myotubes are first detected at ED 14 and express predominantly slow MyHC. Secondary myotubes subsequently develop around primary myotubes and initially express neonatal/fast MyHCs (Condon et al., 1990a). On the basis of the phenotype of embryonic and fetal rat myoblasts *in vitro*, we have suggested that—as in the chick—rat embryonic myoblasts give rise to primary myotubes, whereas fetal myoblasts develop into secondary myotubes (Pin and Merrifield, 1993).

To test the hypothesis that embryonic myoblasts preferentially give rise to slow myotubes, muscle fibers, or both, *in vivo* while fetal myoblasts give rise to predominantly fast myotubes/fibers, we examined the long-term fiber type potential of each myogenic population after injection into an ectopic environment where their fate could be easily examined. Specifically, myoblasts obtained from ED 14 and ED 20 rat hindlimbs were injected into the brains of adult rats and examined for MyHC expression for up to 28 days after

injection. Previous experiments in which mixed populations of myoblasts were injected into ectopic sites resulted in mature muscle fibers in the absence of innervation (Jiao et al., 1993; Irntchev et al., 1998). Because the brain is devoid of muscle, studying the phenotypic potential of the myoblasts is not complicated by the necessity to label donor cells or by fusion between host and donor myoblasts. The brain can, therefore, be considered an environment that allows for the long-term analysis of the phenotypic potential of myoblasts and the muscle fibers that they form.

By using a panel of monoclonal antibodies (Mabs) specific for MyHC isoforms, we identified several classes of myotubes 28 days after injection which expressed subsets of adult MyHCs. These myotubes resembled muscle fibers in that they formed sarcomeric arrangements and down-regulated developmental MyHC isoforms. Importantly, myotubes/muscle fibers that expressed exclusively slow MyHC were only detected after injection of embryonic myoblasts derived from ED 14 hindlimb buds. This finding suggests that a myogenic population committed to a slow phenotype is present only at early stages of hindlimb development. Because myotubes expressing exclusively fast MyHCs or both slow and fast MyHCs were also detected in ED 14 injection sites, our results also suggest that other myogenic populations—which are unable to differentiate in tissue culture—are present in ED 14 hindlimbs. These fetal myoblasts are more apparent in ED 20 hindlimb, because by this stage, they are differentiation competent when analyzed either *in vitro* or *in vivo*. These results support a model for muscle fiber type development in which different populations of myoblasts are established with unique fiber type potentials.

## RESULTS

### Primary Myoblasts Exhibit Complete MyHC Maturation in a Foreign Environment

To determine the long-term potential of embryonic and fetal myoblasts, cultured cells were harvested and injected into the brains of adult rats in the region of the caudate putamen. When the phenotypic profile of myotubes derived from embryonic and fetal cultures was examined by using immunohistochemistry (IHC) with Mabs specific for the various MyHCs, very similar patterns of MyHC expression were observed for the two populations (Table 1). Seven days after injection, embryonic and neonatal fast MyHCs could be detected in the majority of myotubes. Slow MyHC was detected in embryonic and fetal-derived myotubes as well, albeit in a lower percentage of cells. At this time point, no adult fast isoforms were observed. IHC on injection sites obtained 14 days after transplantation revealed the same phenotypic profile of MyHCs, with embryonic, neonatal fast, and slow MyHCs expressed. Once again, embryonic and neonatal fast MyHC expression was widespread, whereas slow MyHC expression was limited to a smaller proportion of myotubes. This finding

**TABLE 1. Myosin Heavy Chain Expression in Myotubes/Muscle Fibers After Injection of Embryonic or Fetal Myoblasts Into the Caudate Putamen of Adult Rats<sup>a</sup>**

MyHC isoform	Days postinjection					
	7 Days		14 Days		28 Days	
	Embryonic	Fetal	Embryonic	Fetal	Embryonic	Fetal
Embryonic MyHC (47A)	+++	+++	+++	+++	++	++
Neonatal (MY-32, NN6)	++	++	+++	+++	+++	+++
Adult fast IIA (SC.71, 4A.74)	-	-	-	-	+	+
Adult fast IIB (BF.F3)	-	-	-	-	+	+
Adult fast IIX (212F)	-	-	-	-	++	++
Slow (8H8, 10D10)	+	+	+	+	++	+

<sup>a</sup>Expression calculated by scoring at least 100 random myotubes. -, no observable staining; +, less than 30% of the observed myotubes; ++, less than 90% of the observed myotubes; +++, greater than 90% of the observed myotubes. MyHC, myosin heavy chain.

was particularly evident for fetal injections in which only a minor subset of myotubes expressed slow MyHC. Twenty-eight days after injection, many myotubes still expressed embryonic and neonatal MyHCs. Slow MyHC was still expressed in a subset of fibers, but at this time point, a large number of fibers also expressed adult fast MyHCs, typical of muscle fibers.

Characterization of MyHC expression in 28 day injection sites indicated populations of myotubes expressing adult slow, IIA, IIX, and IIB MyHCs (Fig. 1). These results indicate that both embryonic and fetal-derived cultures have the potential to express all adult fast and slow MyHCs 28 days after injection—a fate not realized in tissue culture.

#### Embryonic-Derived Myotubes Obtain Full Maturation of MyHC Expression and Localization

To analyze the coexpression of developmental MyHC isoform expression in embryonic and fetal-derived myotubes, immunofluorescence (IF) by using antibodies specific for embryonic MyHC and neonatal/adult fast MyHC was performed at 14 and 28 days after injection (Fig. 2). At 14 days after injection (Fig. 2A–C), all observed myotubes derived from embryonic cultures expressed embryonic MyHC, with the majority also expressing neonatal/adult fast MyHC. At 28 days after injection, almost all of the embryonic myotubes expressed neonatal and/or adult fast isoforms (Fig. 2D–F). However, many myotubes were embryonic MyHC negative, indicating down-regulation of this isoform. Myotubes derived from fetal cultures exhibited a similar trend in expression with the eventual down-regulation of embryonic MyHC (data not shown).

To determine whether the neonatal MyHC isoform is down-regulated as well, triple IF was performed. Antibodies specific for neonatal and slow MyHCs were combined with antibodies for either neonatal/adult fast or IIB/IIX MyHC on embryonic cells 14 and 28 days after injection (Fig. 3). Confocal microscopy of these localizations revealed slow myotubes that did not express neonatal or adult fast isoforms at 14 days after injection (Fig. 3A). This finding is different from the myogenic

differentiation observed in culture, in which all myotubes expressed embryonic MyHC (Pin and Merrifield, 1993). To determine whether the developmental isoforms are also down-regulated in fast myotubes, the expression of neonatal and adult fast and slow isoforms was analyzed 28 days after injection (Fig. 3B,C). Close examination of these sites revealed myotubes that expressed both slow and fast MyHC, but not the neonatal MyHC isoform. These results indicate that the neonatal MyHC can be down-regulated to produce myotubes that express only adult MyHC isoforms. Higher magnification of the myotubes revealed well-defined sarcomeric arrangements, with distinguishable M-lines and peripheral nuclei (Fig. 3Ad). The formation of sarcomeres, in conjunction with the down-regulation of developmental MyHCs, indicates that some myotubes have matured into muscle fibers by 28 days after injection.

#### A Population of Slow MyHC Only-Expressing Myotubes Is Limited to Embryonic Cell Injections

Analysis of the embryonic myoblast injection sites indicated the presence of myotubes that express exclusively slow MyHC (Fig. 3). To compare slow MyHC expression in fetal and embryonic-derived myotubes, sections were analyzed by using double IF labeling using Mabs specific for neonatal/adult fast MyHC and slow MyHC. Characterization of fetal and embryonic injection sites 14 days after injection revealed an interesting difference between the two cell populations (Fig. 4). Immunofluorescent colocalization on fetal cell injections identified myotubes that expressed neonatal fast MyHC alone or both slow and neonatal fast MyHCs (Fig. 4A,C,E). This finding suggests that the fetal myoblasts harvested from ED 20 hindlimbs exist as two subpopulations; a larger subpopulation that gives rise to myotubes expressing exclusively fast MyHCs and a second (smaller) subpopulation that generates myotubes expressing both fast and slow MyHCs. When MyHC expression was analyzed in embryonic injection sites, a different result was obtained. As previously observed (Fig. 3), three classes of myotubes could be

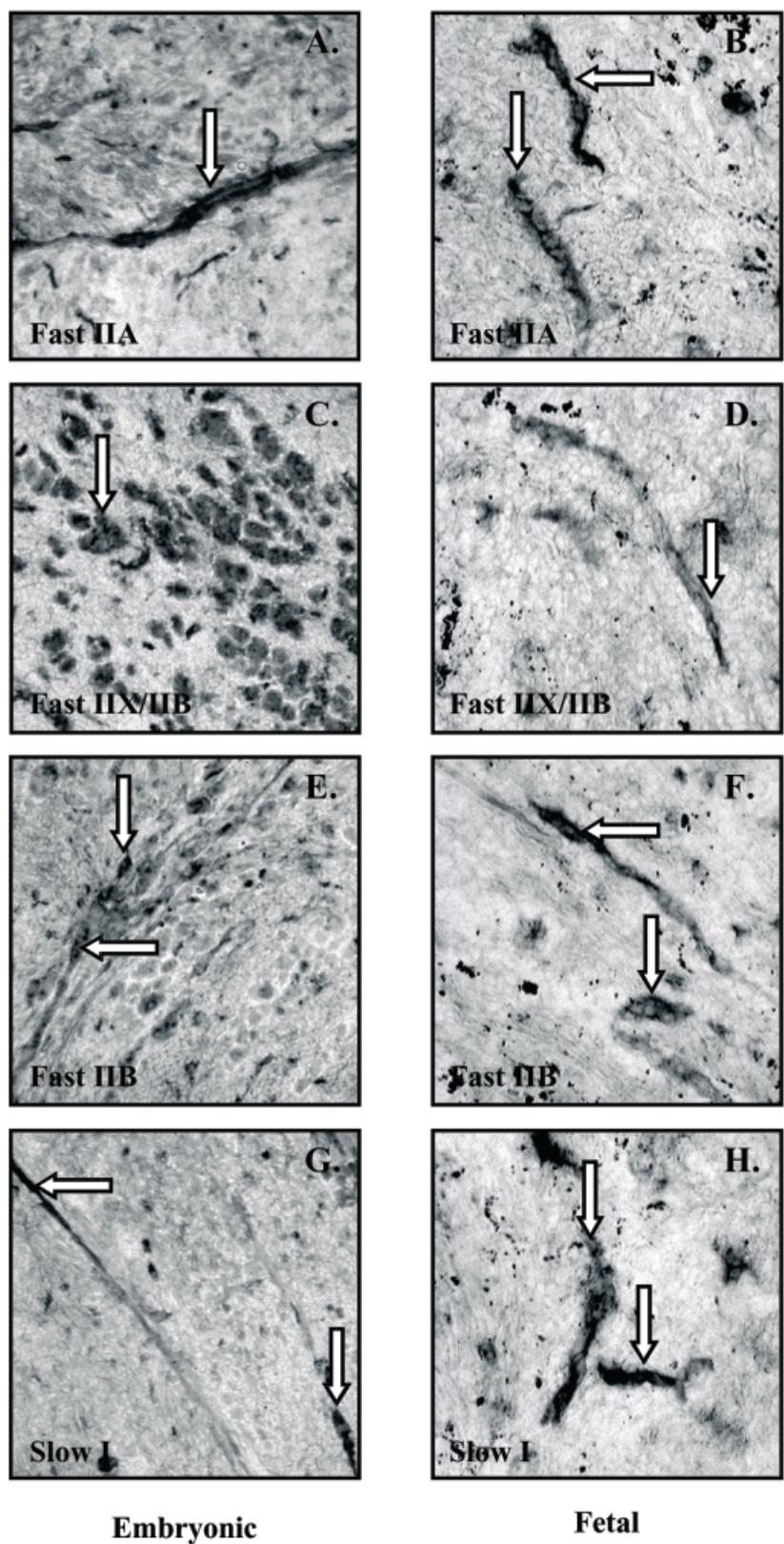
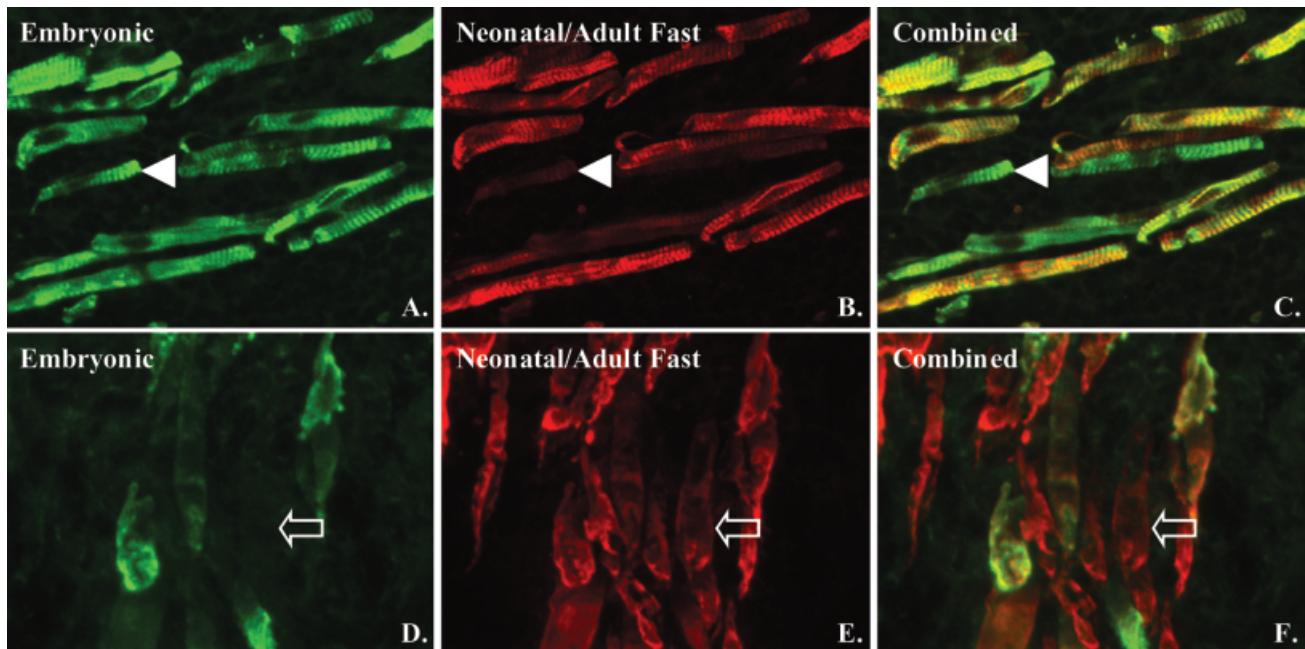


Fig. 1. All adult myosin heavy chain (MyHC) isoforms are detected in myotubes derived from injections of embryonic and fetal cell cultures. ABC-AP immunohistochemistry localization of injection sites in the caudate putamen, 28 days after injection. Tissue was analyzed by using Mabs for fast IIA (SC.71; A,B), fast IIB/IIX (212F; C,D), fast IIB (BF.F3; E,F), and slow (10D10; G,H) MyHCs. Arrows indicate myotubes expressing specific isoforms.



**Fig. 2.** Embryonic myosin heavy chain (MyHC) isoform is down-regulated in myotubes after injection into a nonmuscle environment. Colocalization of embryonic and neonatal/adult fast MyHC isoforms in embryonic myotubes 14 and 28 days after injection. Localization of embryonic MyHC (47A; **A,D**) and neonatal/adult fast MyHC (MY-32; **B,E**) was detected with a fluorescein isothiocyanate or rhodamine isothiocyanate-conjugated secondary antibody, respectively. **C,F:** Overlay of the

two fluorochromes is shown to indicate coexpression of the isoforms. At 14 days (**A–C**), although some myotubes express only embryonic MyHC (arrowheads) most coexpress embryonic and neonatal/adult fast MyHCs. At 28 days (**D–F**), embryonic MyHC expression is reduced and myotubes that express neonatal/adult fast MyHC but not embryonic MyHC can be detected (arrows). This finding suggests that myotubes are developing into mature muscle fibers.

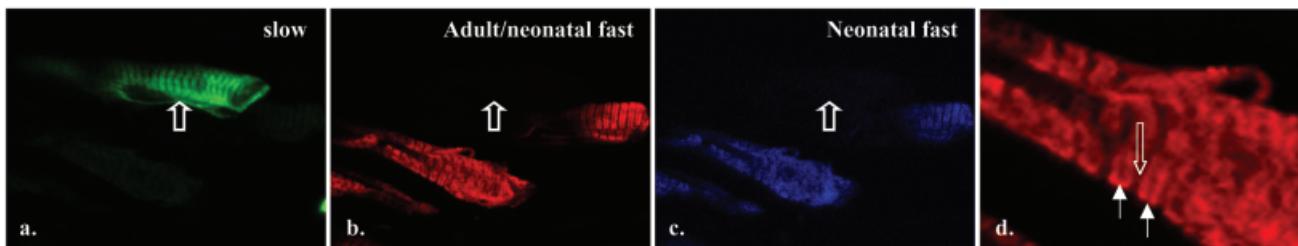
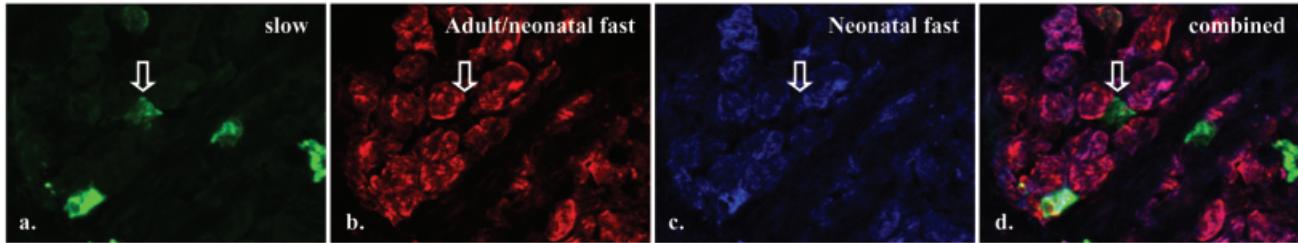
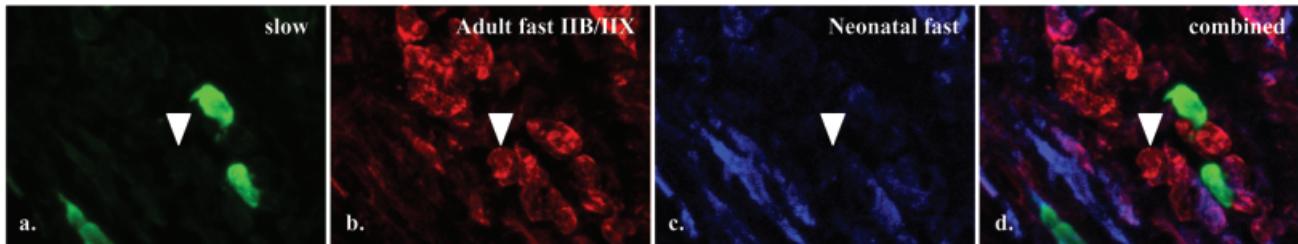
distinguished based on MyHC expression—the fast only and fast/slow populations observed in fetal injection sites, as well as a third population expressing exclusively slow MyHC (Fig. 4B,D,F). The presence of slow myotubes only in embryonic cell injection sites suggests that this third population may be formed by a myogenic population present only at early time points in development (i.e., ED 14). Characterization of grafts at 4 weeks after injection produced similar results with a slow only myotube/muscle fiber population found exclusively in embryonic cell injection sites, albeit at lower proportions (Table 2).

#### Myotubes/Muscle Fibers Exhibit Limitations in Their Ability to Coexpress Adult MyHC Isoforms

To address the coexpression of slow MyHC with adult fast MyHC isoforms, IF localization was performed by using Mabs for slow MyHC and either fast IIB/IIX or fast IIA MyHCs. Due to the absence of adult fast MyHCs at earlier time points, colocalizations were carried out on embryonic and fetal cell injections only at 28 days after injection (Fig. 5). Colabeling of embryonic cell injection sites revealed myotubes/muscle fibers that coexpressed fast IIA and slow MyHC (Fig. 5A–C). In addition, myotubes/fibers expressing only slow MyHC or only fast IIA MyHC were also observed, indicating a range in MyHC expression. In sharp con-

trast to these results, coexpression of slow MyHC with fast IIX/IIB MyHCs was not observed in embryonic-derived myotubes/fibers (Fig. 5B,D,F), indicating that slow MyHC and fast IIB/IIX MyHC may be mutually exclusive isoforms. Examination of fetal cell injections revealed an identical exclusion of MyHC coexpression with all slow myotubes/fibers also expressing fast IIA MyHC but not fast IIB/IIX (data not shown).

To determine whether the other adult fast MyHC isoforms exhibited restricted coexpression, a Mab specific for fast IIB MyHC (BF.F3) was localized with Mabs against either fast IIB/IIX (212F) or fast IIA (SC.71) MyHCs (Fig. 6). Double IF localization and confocal microscopy were used to characterize embryonic cells 28 days after injection. Labeling with BF.F3 and 212F revealed the presence of myotubes/fibers that were recognized by both Mabs or by 212F only (compare Fig. 6A with 6C). This result demonstrates the presence of IIX MyHC expression in the grafts, indicating that embryonic cell cultures have the potential to express the full range of adult MyHCs. This type of analysis was necessary because a Mab specific for IIX MyHC was not available at the time this study was carried out. Analysis of IIB and IIA MyHC expression revealed myotubes/fibers that coexpressed these isoforms (Fig. 6B,D,E), as well as myotubes/fibers that expressed only fast IIA or IIB MyHC alone. Identical results were obtained after immunolocalization on fetal

**A.****B.****C.**

**Fig. 3.** Embryonic-derived myotubes exhibit complete maturation of myosin heavy chain (MyHC) expression. **A:** Triple localization of slow (a, 10D10), fast (b, MY-32), and neonatal (c, NN6) MyHC isoforms. A population of slow only myotubes (arrows in a–c) is observed at 14 days after injection. All other myotubes express neonatal fast MyHC. At this time, well-defined sarcomeres are evident (d) with prominent A bands (open arrow), I bands and M lines (filled arrows), typical of mature muscle fibers.

**B,C:** Similar localization of slow (10D10, a), fast (b, B-MY32 or C-212F), and neonatal (c, NN6) MyHC isoforms was observed 28 days after injection of embryonic cultures. Myotubes are observed that express only slow (arrows in B) or adult fast (arrowheads in C) MyHC. Colour channels have been combined to produce a composite image indicating the myotubes of interest (d). Green myotubes are exclusively slow, whereas red myotubes are exclusively adult fast.

cell grafts, indicating that colocalization of the various adult fast MyHC isoforms is not restricted.

## DISCUSSION

To investigate the fiber type potential of myoblasts from ED 14 and ED 20 rat hindlimbs, embryonic and fetal rat hindlimb cells were injected into the brains of adult rats and the resulting myotubes analyzed for MyHC expression up to 28 days after injection. Surprisingly, differentiation of embryonic limb cells in the brain led to the development of three general classes of myotubes/fibers that expressed either slow, fast, or both slow and fast MyHCs. This finding is different from the developmental potential of ED 14 cells in vitro, where only myotubes coexpressing embryonic and slow MyHCs were observed (Pin and Merrifield, 1993). This unexpected result suggests that either the fate of embryonic myoblasts is unstable and becomes altered by long-term maintenance in the brain or that differentiation incompetent precursors of other myoblast populations are present within ED 14 hindlimbs.

Myoblast injection has been used previously to examine the stability of myoblast fate in a variety of species. When clonal populations of quail embryonic myoblasts from ED4 hindlimbs were transfected with a reporter gene and injected back into the hindlimbs of ED5 chicks, the pattern of MyHC expression seen in cultured quail myotubes was always recapitulated in nascent quail muscle fibers *in vivo* (DiMario et al., 1993). In rats, we have demonstrated that the L6 myoblast cell line represents a stable IIX fiber type-specific myoblast lineage, because L6 cells labeled with a B-galactosidase reporter gene express IIX MyHC both *in vitro* and after injection into regenerating muscles of adult rats (Pin and Merrifield, 1997a). Recently, reciprocal transplantation of somites or lateral plate mesoderm to generate chick-quail chimeras has demonstrated that the fate of avian LMPCs is not altered by signals from the surrounding limb stroma. For example, when chick fast muscle LMPCs migrated into a region of the quail limb normally populated by slow and fast muscle fibers, they always formed fast muscle

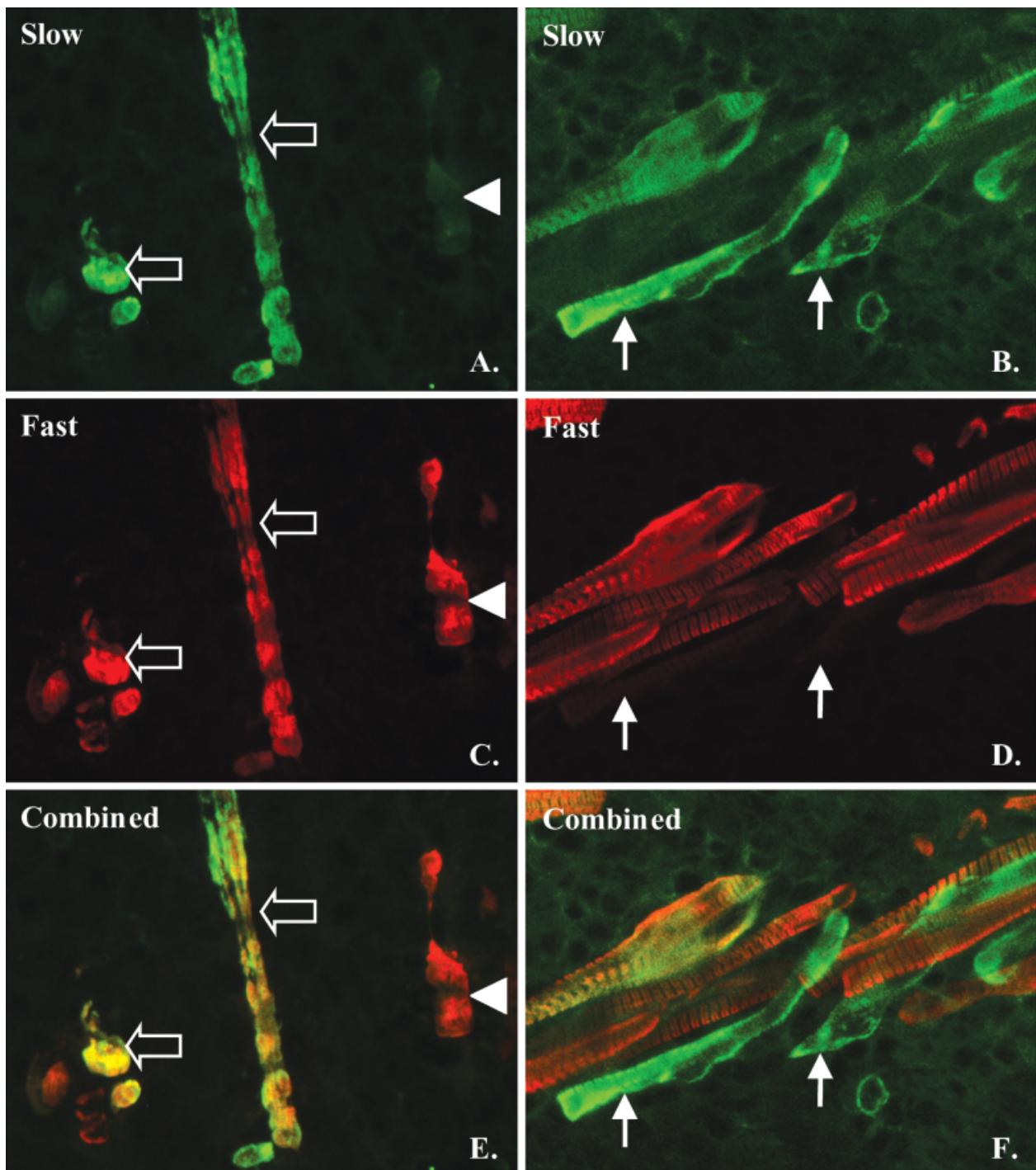


Fig. 4. Embryonic cultures contain a unique slow myosin heavy chain (MyHC) only population of myotubes. Colocalization of slow (10D10, A,B) and neonatal/adult fast (MY32, C,D) MyHCs. **A,C,E:** Confocal analysis revealed myotubes formed by fetal cells that can be classified as either fast (arrowheads) or fast/slow (arrows). **B,D,F:** Embryonic cultures pro-

duced myotubes expressing either fast/slow MyHC or fast MyHC, as well as myotubes expressing exclusively slow MyHC (arrows). Channels were combined (E and F) to document differences in staining and indicate myotubes that coexpress isoforms.

fibers (Nikovits et al., 2001). Combined, these studies demonstrate that the fate determination of avian LMPCs, embryonic myoblasts and rat myogenic cell

lines is remarkably stable after expansion and maturation *in vivo*. In contrast, clonally derived mouse satellite cells labeled *in situ* by retroviral infection and

**TABLE 2. Colocalization of Fast and Slow Myosin Heavy Chain Isoforms in Embryonic Cell Injections Into the Caudate Putamen**

Monoclonal Antibody	14 Days		28 Days	
	# of myotubes	% of myotubes	# of myotubes	% of myotubes
MY-32 only (fast)	257	76.3	337	66.1
10D10 only (slow)	60	17.8	18	3.5
MY-32/10D10	20	5.9	155	30.4
Total # of myotubes	337	—	510	—

C2C12 mouse myoblasts (derived from mouse satellite cells) can give rise to both fast and slow muscle fiber types *in vivo* (Hughes and Blau, 1992). Interestingly, when mouse fetal myoblasts destined to form fast muscle fibers were injected into the limb buds of developing chicks, they were “re-programmed” by local signals within the chick limb bud to express slow MyHC (Robson and Hughes, 1999). The best synthesis of this seemingly contradictory literature is that, although the fate of fetal myoblasts and satellite cell precursors can be modulated by environmental influences, the fate of embryonic myoblasts to form slow myotubes is relatively stable.

In light of the stability of embryonic myoblasts, the possibility that ED 14 hindlimbs contain both embryonic myoblasts and other LMPCs destined to become fetal myoblasts emerges as an attractive explanation for the diversity of muscle fiber types observed in ED 14 injection sites. Because ED 14 cells cultured *in vitro* only generate myotubes expressing slow MyHC, this argument must assume that other populations of LMPCs are not able to differentiate into myotubes *in vitro*. However, with increased time *in vivo* and/or in response to factors produced by the brain, these LMPCs become differentiation competent and develop into myotubes expressing fast MyHCs. These LMPCs are most likely proliferative precursors of fetal myoblasts but could represent a common precursor of both embryonic and fetal populations. The former possibility is supported by the observation that some of the myogenic classes in ED 14 injection sites are very similar to those observed in sites injected with fetal myoblasts from ED 20 hindlimbs. This explanation suggests that the differences that we have observed in culture are not simply due to the migration of distinct myogenic populations into the limb bud at different developmental time points—as previously suggested by others (Van Swearingen and Lance-Jones, 1995)—but may also result from differences in the degree of commitment of different myogenic cell populations that coexist within the developing limb bud at ED 14. Because our experiments involved the injection of mixed populations of cells, confirmation of this interpretation must await the results of experiments in which the fate of clonally isolated cells can be analyzed.

Our observation that injection of ED 20 cells into the brain gives rise to myotubes expressing exclusively fast MyHC or coexpressing fast and slow MyHCs is consis-

tent with *in vitro* results previously reported by us (Pin and Merrifield, 1993) and others (Torgan and Daniels, 2001). Although the majority of cultured myotubes obtained from rat fetal myoblasts express developmental and fast MyHCs, a small proportion coexpress slow MyHC after 8 days *in vitro* (Pin and Merrifield, 1993). This proportion increases with increased time in culture and in response to stimulation of the calcineurin activated NFAT signaling pathway (Torgan and Daniels, 2001). Similar to the *in vivo* results reported here, slow myotubes (expressing exclusively slow MyHC) were not observed in cultures of fetal myoblasts (Pin and Merrifield, 1993; Torgan and Daniels, 2001), presumably because the embryonic myoblasts responsible for their formation have all fused to form primary myotubes before ED 20. The heterogeneity of slow MyHC expression, both *in vitro* and *in vivo*, therefore, suggests that there are at least two subpopulations of fetal myoblasts present in ED 20 hindlimbs—one responsible for the formation of fast myotubes and one responsible for fast/slow myotubes.

The argument for subpopulations of fetal myoblasts is supported by the observation that distinct populations of fast and fast/slow fetal myoblasts with different fiber type potentials have previously been described in birds (Schafer et al., 1987). Although both populations could be generated from a common precursor by serial passage *in vitro*, the fast/slow lineage remained stable after clonal isolation *in vitro* (Schafer et al., 1987). Recent studies suggest that the fast/slow myoblast lineage present in fetal hindlimbs may actually correspond to precursors of satellite cells, because such precursors have been shown to exist in hindlimb musculature before birth in both birds (Feldman and Stockdale, 1992; Hartley et al., 1992) and mammals (DeAngelis et al., 1999; Heslop et al., 2001). Although satellite cells are first observed in mouse hindlimbs around ED 16.5 (Beauchamp et al., 2000), satellite cell precursors lacking the characteristic CD34 lineage marker could be present at earlier stages and theoretically contribute to the embryonic injection sites derived from ED 14 cells.

Although it is possible that the formation of fast/slow myotubes from ED 20 cells could result from the fusion of latent embryonic myoblasts with fetal myoblasts, the absence of slow myotubes in cultures of ED 20 cells or after injection of ED 20 cells into the brain argues strongly against this explanation. We have previously

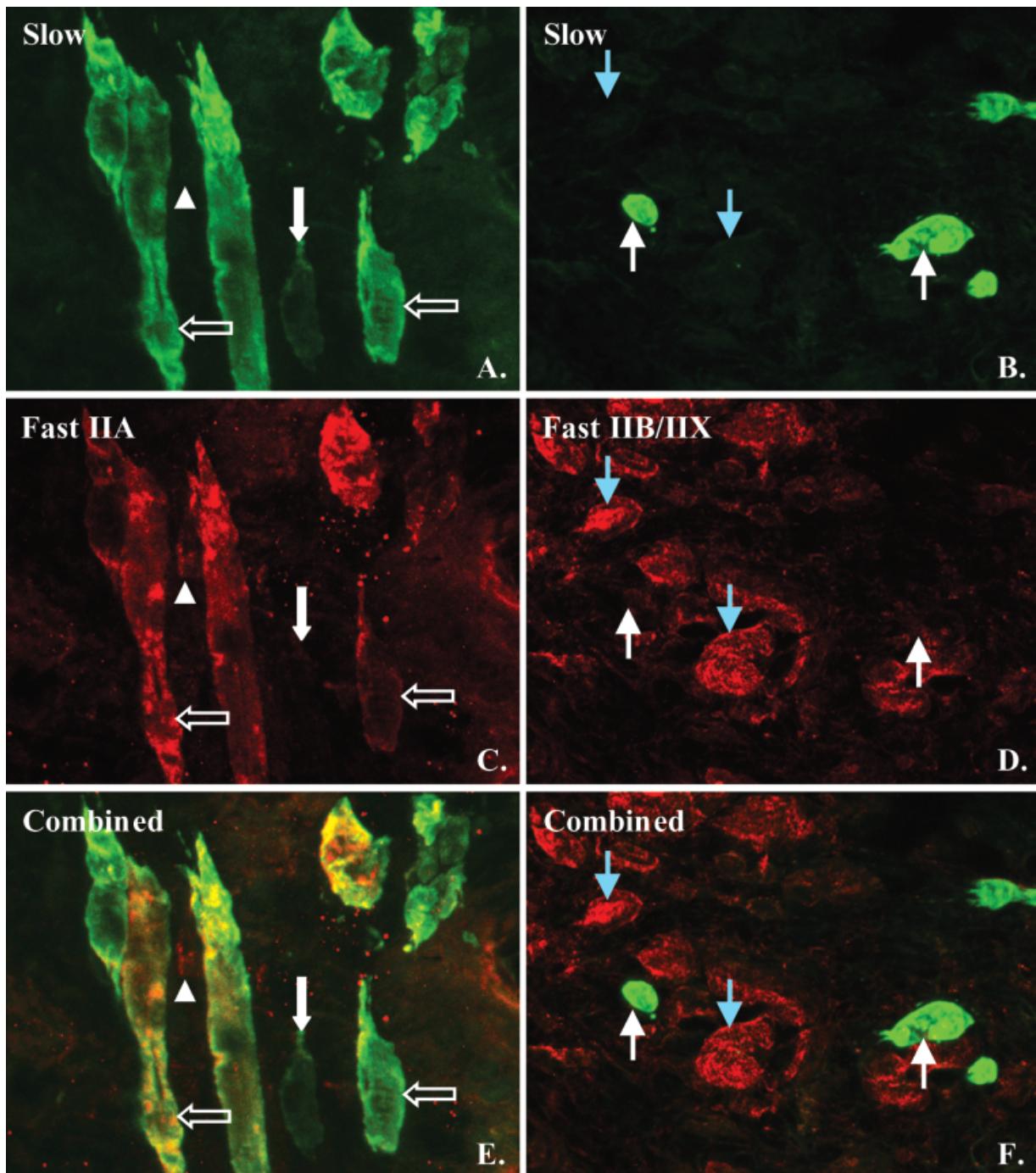


Fig. 5. The coexpression of slow and fast myosin heavy chains (MyHC) is limited to fast IIA myotubes/fibers. Colocalization of slow (A,B, 10D10) and either fast IIA (C, 4A.74) or IIB/IIX (D, 212F) was examined in embryonic cell grafts 28 days after injection. Analysis of fast/slow IIA coexpression revealed populations of myotubes expressing only slow (A,

filled arrow), only fast IIA (C, arrowhead), or both isoforms (E, open arrows). Conversely, analysis of slow (B) and fast IIB/IIX (D) expression indicates that these isoforms are not coexpressed (see combined F). Myotubes either express slow (white arrows in B,D,E) or fast IIB/IIX (blue arrows in B,D,E), but not both.

demonstrated that heterotypic myotubes can be obtained experimentally by coculturing embryonic and fetal rat myoblasts and that heterotypic myotubes accumulate fast and slow MyHCs as nuclear domains

around the appropriate donor nuclei (Pin and Merrifield, 1997b). However, nuclear domains of MyHC were never present in the fast/slow myotubes, which we observed in our brain injection sites. Rather, fast and

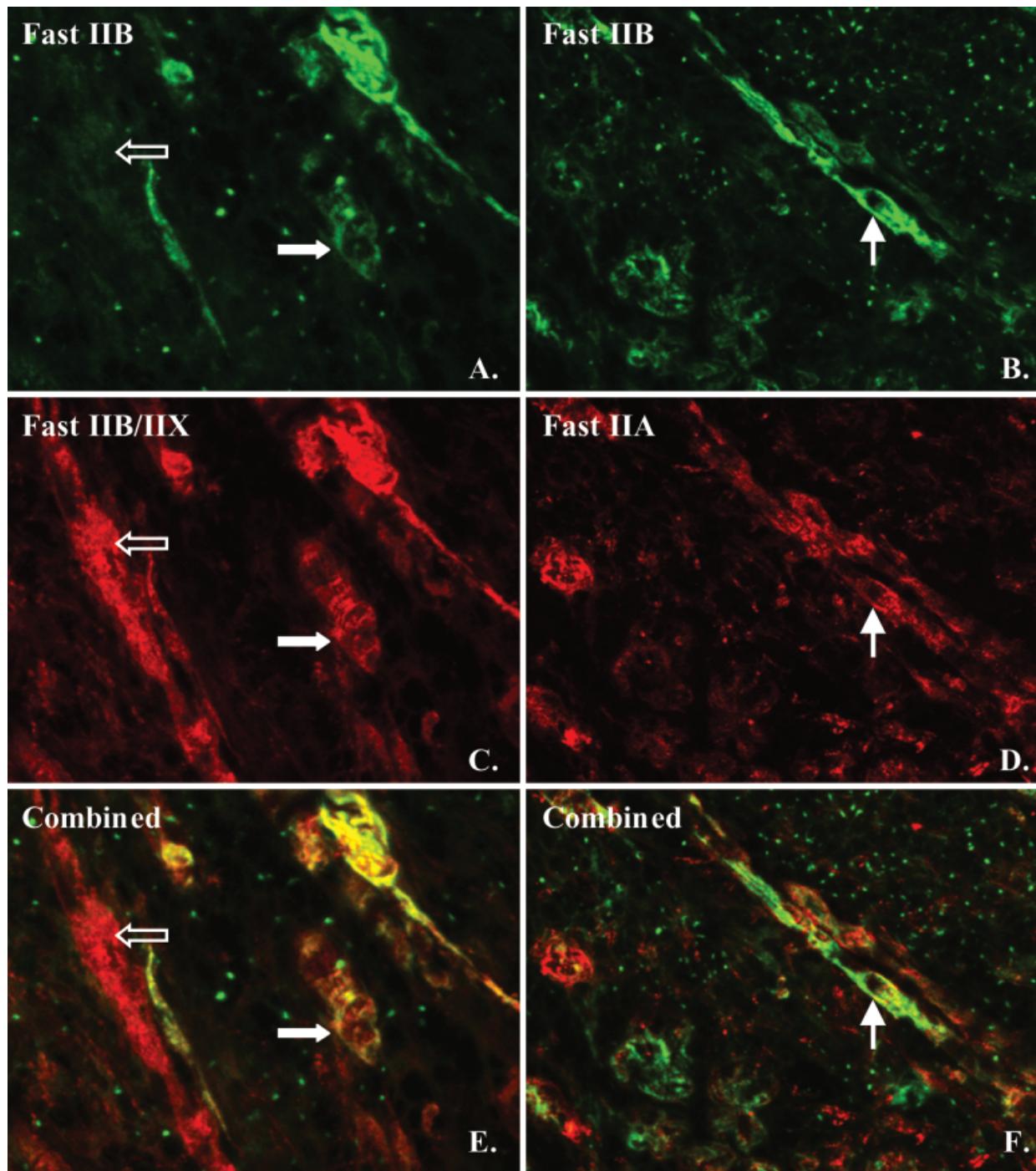


Fig. 6. Analysis of adult fast myosin heavy chain (MyHC) expression in embryonic grafts reveals several different populations of fast myotubes/fibers. Colocalization of adult fast IIB MyHC (**A**, BF.F3) with fast IIB/IIX MyHC (**C**, 212F) reveals populations of myotubes that express IIB MyHC (filled arrow) or IIX MyHC but not IIB (open arrow). Similar local-

ization of fast IIB MyHC (**B**) with fast IIA MyHC (**D**) indicates that these two isoforms can be expressed in the same myotube (arrow). In addition, myotubes expressing only IIB or only IIA MyHC can be observed. Channels were combined (**E,F**) to document differences in staining and indicate myotubes that coexpress isoforms.

slow MyHCs were coexpressed within common sarcomeres. This would be the case if fast and slow MyHCs were coexpressed by individual nuclei, and this observation argues against the fusion of embryonic and fetal

myoblasts in the brain to form fast/slow myotubes. In addition, the fast/slow and fast myogenic populations differ significantly in their expression of fast MyHC isoforms. Fast/slow myotubes express exclusively fast

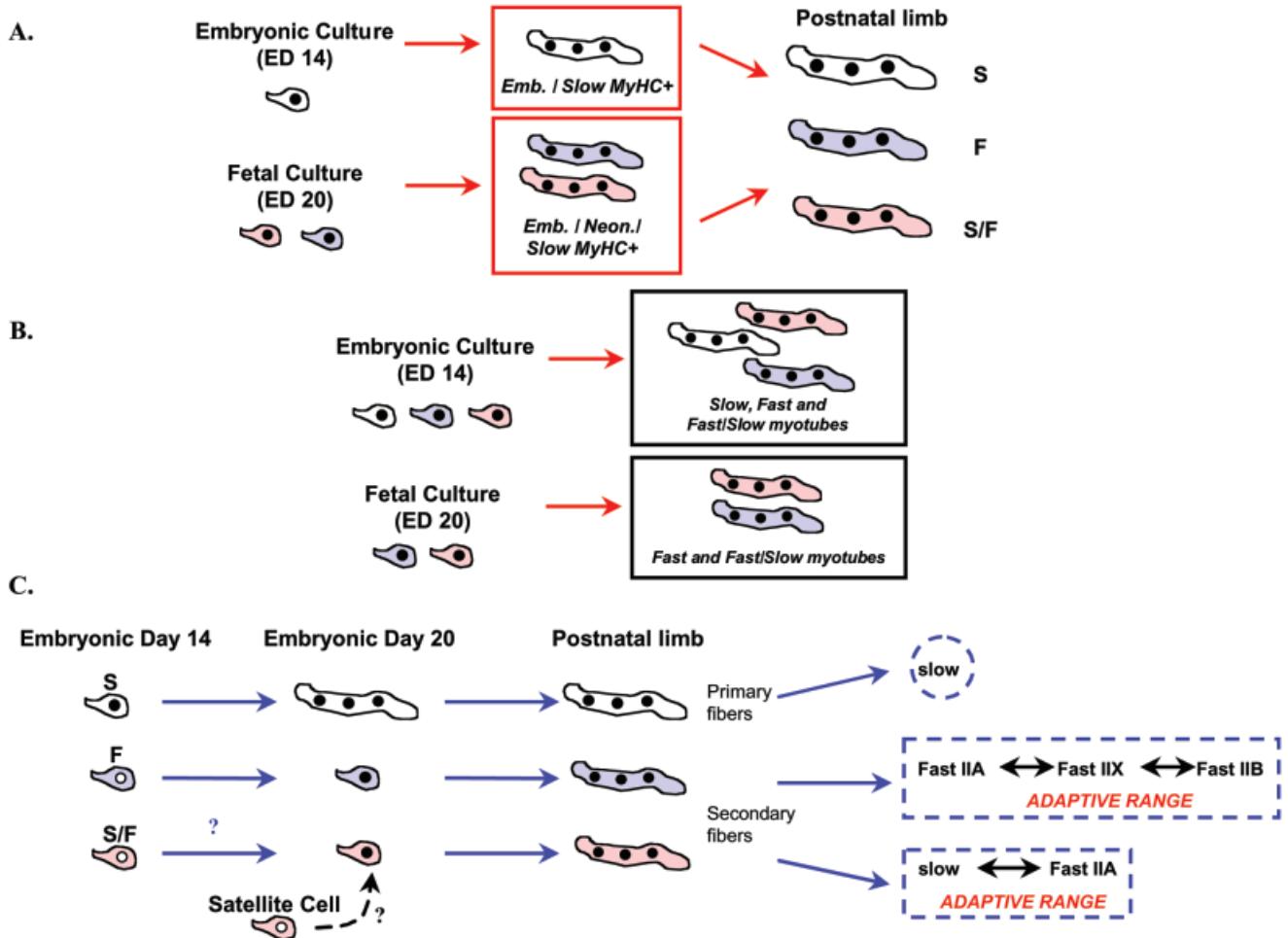


Fig. 7. The contribution of embryonic and fetal myoblasts to the development of muscle fiber types in rat hindlimbs. **A:** Previous work has demonstrated that rat myoblasts derived from embryonic day 14 (ED 14) or ED 20 hindlimbs exhibit different phenotypes when grown in culture. Embryonic myoblasts (white cytoplasm) give rise to small myotubes that express only embryonic (Emb.) and slow myosin heavy chain (MyHC), whereas most fetal (Neon.) myoblasts (blue) give rise to larger myotubes that express embryonic and neonatal/adult fast MyHCs (Pin and Merrifield, 1993; Ghosh and Dhoot, 1998a). In longer term cultures, a subpopulation of fetal myotubes (pink) also coexpresses slow MyHC (Torgan and Daniels, 2001). These three populations of cultured myotubes are similar to those known to express slow (S), neonatal fast (F), or both slow and neonatal fast (S/F) MyHCs in prenatal rat hindlimbs (Condon et al., 1990a). **B:** Experiments described in this manuscript demonstrate that, after the injection of ED 14 cells into the brain, three general classes of myotubes are generated that express slow, fast, or slow and fast MyHCs. However, injection of ED 20 myoblasts results in the development of only the fast and fast/slow myotube populations. **C:** Combined, these results suggest a model of muscle development in which all three populations of myoblasts are present in the ED 14 rat hindlimb but where the F and S/F populations are differentiation incompetent in culture (white nucleus). In ED 20 hindlimbs, mononucleated embryonic myoblasts (S) have fused

and can no longer contribute to the formation of new myotubes in culture or in the brain. However, the two fetal myoblast populations (F and S/F) are now differentiation competent (black nuclei) and can form myotubes both in vitro and in vivo. According to this model, embryonic myoblasts give rise to primary myotubes which mature predominantly into slow (type 1) fibers. Fetal myoblasts form secondary myotubes capable of maturing into all four fiber types and may contain precursors to the satellite cells of mature muscle. In light of the high proportion of muscle fibers that coexpress multiple MyHC isoforms at 28 days after the injection of ED 20 cells, we suggest that the two fetal myoblast subpopulations have different "adaptive ranges," which limit their ultimate fiber type potential. According to this model, the fast fetal myoblasts (F) are restricted to forming one of the three fast fiber types. In contrast, the fast/slow (S/F) population of fetal myoblasts can become either slow (type 1) or fast IIA fibers—depending on their innervation. Thus, both developmental history (i.e., cell lineage) and extrinsic influences cooperate in the determination of muscle fiber type. This model predicts that slow fibers are heterogeneous, because they can develop from either embryonic or fetal (fast/slow) myoblasts. Similarly, IIA fibers can be generated from either of the two fetal myoblast subpopulations. This model illustrates why the developmental history of a muscle fiber may influence how it responds to changes in environmental influences (such as hormones, exercise, or cross-innervation).

IIA MyHC, whereas the fast only myotubes express predominantly fast IIX and IIB. There are no transition fibers (i.e., fibers expressing slow and fast IIX or IIB) as observed in vivo (Mira et al., 1992). In fact, the existence of several different fast fiber types based on

MyHC expression suggests that other potential classes of myogenic cells may exist.

Our results are consistent with a model of hindlimb muscle development that involves the existence of three distinct myogenic populations characterized as

slow, fast/slow, and fast, based on their patterns of MyHC expression (Fig. 7). The slow myoblast population we believe to be analogous to the population of embryonic myoblasts that give rise to primary myotubes in vivo (Dunglison et al., 1999). During normal hindlimb development, embryonic myoblasts appear at ED 14 and form typically slow MyHC expressing myotubes. This myogenic population also gives rise to the slow myotubes observed in cultures derived from this time point and, therefore, is the only “differentiation competent” myoblast population in ED 14 cultures. The other two myogenic populations, which differentiate at later time points and presumably give rise to secondary fibers and/or satellite cells in the hindlimb, are readily observed after injection into the brain. The absence of these two myogenic populations in primary ED 14 cultures suggests that they are “differentiation incompetent” at this developmental time point in vitro. At ED 14, all myogenic classes are available for delivery, whereas at ED 20, the slow myogenic class is fully differentiated and not included in the fetal cell injections. Therefore, the *in vivo* setting provides an environment that promotes the differentiation and maturation of fetal myoblast precursors present in ED 14 rat hindlimb that is not reproduced *in vitro*.

This model is similar to one originally published by Stockdale describing the role of myoblast lineages in avian limb development (Stockdale and Miller, 1987) and revised over the years to incorporate new findings (Stockdale, 1992, 1997). Our results support recent evidence that cells committed to the myogenic lineage classes establish their program of MyHC expression early in development, before the formation of fusion competent myoblasts *in vivo* (Nikovits et al., 2001). This finding supports the existence of intrinsic mechanisms within distinct LMPCs and myoblast populations that restrict their fiber type potential at later stages of development. The myogenic populations that are differentiation incompetent in ED 14 cultures give rise to a full array of fast muscle fibers in embryonic injection sites, similar to the patterning that occurs during hindlimb myogenesis. Importantly, each MyHC is expressed in a spatially restricted pattern similar to that observed in response to denervation (Talmadge, 2000). Slow MyHC is only coexpressed with IIA and fast fibers coexpress IIA, IIB, and/or IIX MyHC. The patterns of MyHC expression in the brain suggests that these muscle fibers are expressing a range of MyHCs that would normally be further restricted by environmental influences, such as innervation. We hypothesize that the “fast” muscle fibers that express various combinations of fast MyHCs are capable of developing into IIA, IIB, or IIX (but not type I) fibers in response to appropriate extrinsic cues. Similarly, the fast/slow fetal population may develop into type I or IIA fibers, depending on whether they are innervated by fast or slow motoneurons. Changes in the electrical stimulation of the fiber can then modulate the fiber’s

phenotype, but only within the original “adaptive range” (Westgaard and Lomo, 1988).

Our model suggests that a myotube forms with a range of potential phenotypes and environmental cues *down-regulate* MyHC isoforms. However, there is a substantial body of evidence that suggests environmental cues *up-regulate* certain MyHC isoforms. A similar interplay between intrinsic and extrinsic mechanisms has previously been described for the activation of slow (SM2) MyHC expression within specific chick myoblast populations after coculture with neural tube (DiMario and Stockdale, 1997). Because the cells in this study were injected into the caudate putamen, both neuronal trophic factors as well as a plethora of other growth factors from activated astrocytes are present and could easily exert influences on the myotube population. Such a hypothesis would predict that myotubes closer to the trophic source (i.e., the surrounding neural tissue) would more likely be affected. Because the grafts developed as solid masses with a thick connective tissue layer surrounding them (data not shown), one would predict that trophic influences from the surrounding brain would cause a gradient of MyHC expression to be generated. However, no such gradient was observed with myotubes within a common fascicle often exhibiting vastly different expression patterns.

If the expression profiles of these myogenic classes are determined intrinsically before differentiation, then there must be inherent molecular differences between these cell populations. In the axial muscle of the back, sonic hedgehog signaling from the somite has been shown to promote the development of slow muscle fibers in epaxial muscle (Du et al., 1997; Blagden et al., 1997; Cann et al., 1999; Norris et al., 2000). To date, similar effects on hypaxial muscle of the limb have not been reported. Although fast and slow muscle fibers in the limb preferentially express myoD or myogenin, respectively (Hughes et al., 1997), selective expression of myogenic regulatory factors (MRFs) does not seem to play a critical role in determining the muscle fiber type. However, knocking out genes associated with LMPCs, such as *Pax3*, *met-C*, or *HGF/SC* prevents the migration of LMPCs and limb muscle development (Gross et al., 2000), whereas the targeted ablation of the *Lbx1* gene preferentially affects the formation of specific muscle groups (Brohmann et al., 2000). Current studies in our lab are aimed at elucidating the differences in gene expression between different myoblast populations to identify specific genes that determine fiber type potential.

## EXPERIMENTAL PROCEDURES

### Myoblast Injections Into the Caudate Putamen

Primary myoblasts were obtained from ED 14 hindlimb buds (embryonic cell injections) or ED 20 hindlimbs (fetal cell injections) as previously described (Pin and Merrifield, 1993). Twenty-four hours after plating, the cells were completely (embryonic cells) or selec-

tively trypsinized (fetal cells; Konigsberg, 1979), and cells were immediately added to 0.5 ml (per plate) of complete medium containing 0.5 mg/ml soybean trypsin inhibitor (Gibco/BRL, Burlington, Ontario). After trypsinization, harvested cells were washed once with cold complete medium (10% horse serum, 5% fetal bovine serum, 50 units/ml penicillin, 10 µg/ml streptomycin, and 1.25 µg/ml, fungizone in 68% Dulbecco's minimal essential medium [DMEM], and 17% Medium 199) and then twice with calcium and magnesium free Hanks' balanced salt solution (CMF-HBSS). Before injection into the caudate putamen, cells were resuspended at a concentration of 50,000 cells/µl of CMF-HBSS containing 0.1% India ink to mark injection sites. In total, 20 Sprague Dawley rats (Charles River, Montreal, Quebec), between the ages of 2 to 3 months, were used as hosts for the injection experiments. Each rat received 500,000 cells in a total volume of 10 µl, injected into the caudate putamen over a 10-min period by using either a 10- or 25-µl Hamilton syringe mounted on a stereotaxic tower. Ten rats received embryonic cell injections, with two killed at 7 days after injection, and 4 rats killed at both 14 and 28 days after injection. The 10 rats that received fetal cells were killed at 7 and 14 days (2 rats each), 28 days (4 rats), and 56 days (2 rats) after injection. At these time points, brains were flash frozen in dry ice and then embedded and sectioned as described in Pin and Merrifield (1997a).

### Characterization of Embryonic and Fetal Muscle Tissue in the Caudate Putamen

Serial sections were examined with ABC-AP IHC or indirect immunofluorescence (as described in Pin and Merrifield, 1997a). To determine a general MyHC expression profile for each population of cells, antibodies specific for embryonic MyHC (47A), neonatal MyHC (NN6), neonatal/adult fast MyHCs (MY-32), fast IIA MyHC (SC.71 and 4A.74), fast IIB MyHC (BF.F3), fast IIB/IIX MyHCs (212F), and slow MyHC (8H8 and 10D10) were used. The source and specificity of these antibodies has previously been documented (Pin and Merrifield, 1997a). Hybridoma cells producing Mabs BF-F3 (HB-283), SC-71 (HB-277), and 4A.74 (CRL-2041) were obtained from the American Type Culture Collection (ATCC; Rockville, MD).

To determine the pattern of coexpression of MyHCs within individual myotubes, double immunofluorescent colocalization similar to that previously described in Pin and Merrifield (1993) was carried out. Colocalizations were performed in a sequential manner. Primary Mabs belonging to either the IgG<sub>2A</sub> class (which included 47A, 10D10, and 8H8) or to the IgM class (BF.F3) were recognized by a fluorescein-conjugated rabbit anti-mouse (FITC-RAM) IgG<sub>2A</sub> or IgM secondary antibody. Primary Mabs belonging to the IgG<sub>1</sub> class (including MY-32, 212F, 4A.74, and SC.71) were recognized by a Cy3-conjugated sheep anti-mouse (RITC-SAM) IgG<sub>1</sub> antibody.

Triple immunofluorescent colocalization was used to correlate neonatal MyHC (by using polyclonal antibody NN6; courtesy of G. Butler-Browne) to adult MyHC expression. In these experiments, mouse monoclonal labeling was preceded by the localization of this polyclonal antibody followed by a goat anti-rabbit secondary antibody conjugated to Cy5 (GAR-Cy5; Jackson ImmunoResearch Labs, Inc., West Grove, PA). In all cases, fluorescent localizations were analyzed, and images obtained by using a Zeiss LSM 410 confocal microscope. Ten- to 15-µm sections were optically sectioned into 0.7-µm sections and then projected into one plane to preserve the focus of the images. Prints were produced by using a Phaser 440 Tektronix dye sublimation printer.

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