The Myogenic Lineage: Evidence for Multiple Cellular Precursors during Avian Limb Development (43058)

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Among the basic issues of vertebrate development are morphogenesis, pattern formation, and the commitment, or determination, of cells during embryonic development. There is no better embryologic structure to illustrate these issues than the limb of the developing bird, particularly, the limb musculature. The fore- and hindlimb of the bird appear as outgrowth in the splanchnic mesenchyme of the developing embryo during the second day of development. The splanchnic mesenchyme is not the source of the myogenic cells that will form the musculature of the limb over the ensuing 5 or 6 days (1, 2). Cell commitment to myogenesis occurs prior to limb bud formation in the somites, and cells that form the musculature migrate into the forming limb bud. Therefore, the somites are the site of origin for all limb myogenic cells. It is not known, however, whether the cells that migrate to the limb bud are already committed to myogenesis or whether commitment occurs following arrival in the limb bud.

Early Formation of the Limb Musculature

Morphogenesis of the limb musculature occurs rapidly from the first appearance of discreet muscle primordia, the dorsal and ventral muscle masses of the limb bud. These primordia are first seen on Day 4 of chick development (stage 25) (3, 4). At the time of dorsal and ventral muscle mass formation, cells committed to myogenesis were detected and fibers appeared that express more than a single isoform of myosin heavy chain (3–5). By the sixth day of development, all of the muscles of the limb were morphologically demarcated and composed of fibers of differing functional types.

Remarkable progress has been made in analyses of the genes responsible for commitment of cells to a myogenic fate. The work of several investigators (6–13) revealed that several genes were activated during the commitment process. Any one of these genes was sufficient to restrict a cell to a myogenic fate. The myogenic fate of cells expressing these genes was evidenced by muscle-specific proteins found in the muscle fibers they formed. However, rather than there being a generic myoblast in avian limb development, it was shown that the commitment process resulted in the formation of discreet myoblast types (14–16) (Fig. 1).

At the time of dorsal and ventral muscle mass formation and the first appearance of muscle fibers, predominantly two major and one minor types of myoblasts can be isolated from the limb bud (5, 17, 18). These were designated as fast, fast/slow, and slow embryonic myoblasts. It was postulated that such myoblasts were precursors of the first skeletal fibers to form (primary muscle fibers) in the newly emerging musculature of the avian limb and that specific primary fiber types had their origins in specific embryonic myoblast types (14, 16, 19) (Fig. 1). Primary fibers of different types established specific distributions, within each anatomical muscle, that were characteristic of each particular muscle of the limb (3, 20).

The evidence that primary fibers have different origins rests on several points. When embryonic myoblasts were cloned from the limb bud at Day 4 of development, three types of colonies were formed in which all the progeny of a single myoblast formed a single type of muscle fiber (17). About 75% of the colonies only contained fibers that expressed fast isoforms of myosin heavy chain (MHC). About 25% of the colonies only contained fibers that expressed fast as well as what was operationally defined as slow MHC 1 and 2 (react with McAb S46, but not McAb S58), and an occasional colony only contained slow MHC 1 and 2 (fibers react with McAb S46 and S58). Because these antibodies only recognize an epitope, the particular isoforms expressed may not be those indicated, but it is clear that fibers formed by different myoblasts have different staining patterns by immunocytochemistry and, therefore, different MHC composition. These fiber types formed in the absence of interactions with other

Received October 12, 1989. [P.S.E.B.M. 1990, Vol 194]
Accepted January 30, 1990.

0037-9727/90/1942-0071$2.00/0

cells (stromal and connective tissue cells) and in the aneural environment of the culture dish. That these myoblasts were restricted to specific differentiated fates was demonstrated through many generations of daughter cell formation and through three subclonings of embryonic myoblast cells (18). A second basis for asserting that embryonic myoblasts formed primary muscle fibers of the limb bud was that these embryonic myoblasts disappeared (could no longer be isolated from the limb bud) at the time that the formation of primary muscle fibers ceased (5).

Formation of Secondary Muscle Fibers and Fetal Myoblasts

Following the morphologic formation of each anatomical muscle from Days 4 to 7 of chick limb development is the emergence of a new myoblast type, the fetal myoblast (5). In the chicken, the fetal myoblasts begin to appear in increasing numbers about Days 7 and 8 of limb development. This corresponds in time to the appearance of the "late" type of myoblast originally described by Hauschka and colleagues (21–23). Fetal myoblasts formed fibers in cell culture that were easily distinguished from those formed from embryonic myoblasts because these fibers were very long, contained many nuclei, and did not require conditioned medium for differentiation. Unlike fetal myoblasts, embryonic myoblasts only formed short fibers with very few nuclei and did not do so unless the medium was first conditioned by other cells.

Fetal myoblasts are not as diverse as embryonic myoblasts. When fetal myoblasts were placed in high density cell culture, they formed fibers that initially only produced fast isoforms of myosin heavy chain (24). When these newly formed fibers entered the second week of culture, they initiated the synthesis of slow MHC 1 as well as neonatal fast MHC (24, 25). The initiation of slow MHC 1 expression occurred within existing fibers. These fibers did not synthesize slow MHC 2. When cloned, fetal myoblasts first formed colonies in which all fibers synthesized only fast isoforms, but as the colonies aged, there was the initiation of slow MHC expression within some fibers.

Development of the limb musculature changes around Days 7 and 8 of chick development because new fibers, secondary fibers, appear in all anatomical sites (3, 26). Analyses showed that secondary fibers differed from primary fibers and formed in close proximity to the existing primary fibers, tending to complement the existing pattern established by the primary fibers (3). Secondary fibers in the chicken contained fast isoforms plus slow MHC 1 (react with McAb S46), but not slow MHC 2 (do not react with McAb S58). Most muscles grow markedly during the fetal stage of development (8 days to hatching at Day 20) by the formation of many new secondary fibers and the hypertrophy of existing primary and secondary fibers.

Satellite Cells and Muscle Fiber Formation

The final cell in the sequential emergence of specific myogenic precursor cells is the satellite cell (27). It is not known precisely when satellite cells (myoblasts) first appear, but most likely they first appear late in fetal life. The satellite cells are situated between the plasma membrane of the multinucleated muscle cell and the basement membrane that surrounds each muscle fiber. Satellite cells remain in this location throughout the lifetime of an organism and provide for muscle growth in the neonatal period and following injury in the adult. Comparisons of satellite cells isolated from fast-twitch muscles (pectoralis major) and from slow-twitch muscles (anterior latissimus dorsi) demonstrated that there was more than a single type of satellite cell in the adult chicken (14, 28). Satellite cells cloned from adult chicken pectoralis major muscle formed colonies composed of a single fiber type, fast fibers; whereas those colonies formed from adult anterior latissimus dorsi muscle were of two types. About 75% of colonies from adult anterior latissimus dorsi satellite cells were only composed of fast fibers and about 25% were composed of fibers, all of which contained fast and slow MHC 1 (stain with McAb S46, but not S58).

The Concept of a Myogenic Lineage

These observations indicate that myogenesis in the limb bud of the bird is accomplished by the sequential appearance of distinct myoblast types as the limb musculature forms and undergoes morphogenesis. There are at least five types of myoblasts in the developing chick limb. They differ operationally in forming fibers in cell culture that contain different combinations and isoforms of MHC and by the size of the fibers that they produce. While correlative in nature, evidence supports that each type of myoblast is responsible for the formation of fibers associated with a specific phase in the development of each anatomical muscle, i.e., embryonic myoblasts with primary fibers in early embryogenesis, fetal myoblasts with secondary fibers in fetal development, and satellite cells with neonatal and regenerative development.

Therefore, the concept of lineage in myogenesis must account for the origins of at least three groups of myoblasts (embryonic, fetal, and neonatal, as well as subsets within each of these groups). The commitment of mesenchymal cells to limb myogenesis, whether it occurs in the somites or in the developing embryonic limb, must involve the activation of a group of commitment genes that produce transacting proteins localized to the nuclei of the myoblast and muscle fiber (11, 12). The mechanism by which these commitment genes are activated is unknown, but hypomethylation is likely

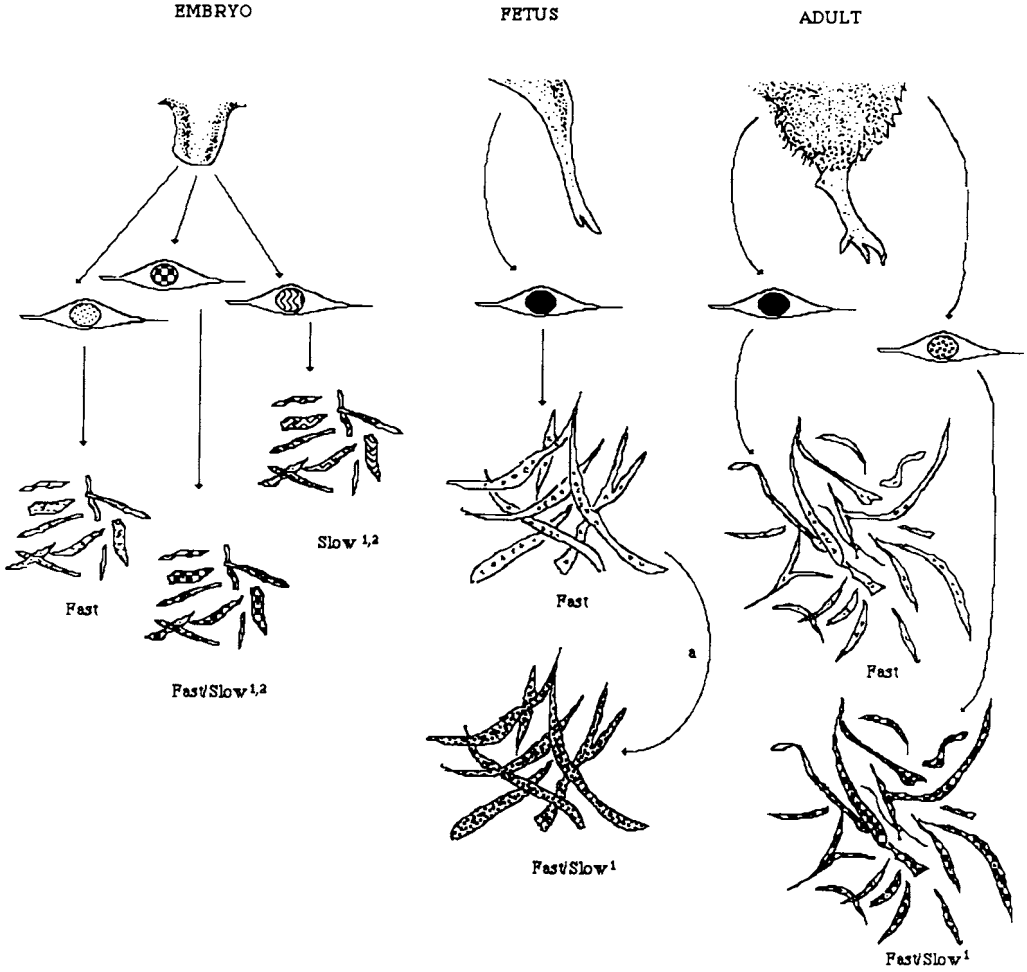

Figure 1. Type of myoblasts isolated from the three phases of chicken development. Two major and one minor types of myoblasts can be isolated from the chick embryo which, when cloned, form three types of muscle fiber colonies indicated by the types of myosin heavy chain the fibers contain. There is one type of myoblast that can be isolated from the fetal phase of chick development which forms a single type of muscle fiber colony. Such colonies undergo transitions in myosin heavy chain synthesis or modulations in phenotype (a). They change from the synthesis of only fast isoforms to the synthesis of both fast and slow myosin heavy isoforms. Myoblasts (satellite cells) isolated from the adult chicken are of two types based upon the types of muscle fiber colonies they form. Fast, fibers containing fast isoforms of myosin heavy chain; Fast/Slow, fibers containing both fast and slow isoforms of myosin heavy chain. In this diagram, the MHC are operationally defined by reaction with particular monoclonal antibodies. Those which react with McAb S46 alone are designated Slow 1; those that react with McAb S58 alone are designated Slow 2; those that react with both are designated Slow 1,2. It is acknowledged that reaction with these antibodies identifies an epitope, not a particular isoform of MHC. Therefore, the isoforms identified by antibody reaction in specific fibers may not be the same as those expressed in other fibers even though they react with the same antibody.

to be involved. Cell lines (10T1/2) and others) exposed to 5-azacytidine, an agent that can produce hypomethylation of the genome (29), resulted in activation of *Myo D1, myd*, and other commitment genes (6, 30). Recently, hypomethylation of a promoter region for the third fast myosin light chain also was demonstrated to be responsible for activation of this muscle fiber specific gene during myogenesis *in vivo* (31).

Yet to be resolved is the lineal relationship of embryonic, fetal, and satellite myoblasts (16). When embryonic myoblasts were subcultured repeatedly for as many as 30 generations, a number of generations far exceeding that undergone *in vivo*, they did not form any other type of myoblast than the embryonic type (17, 23). Seed and Hauschka (32) also showed that the migration of myogenic cells into the limb bud occurred in two phases—one responsible for embryonic (early) myoblasts and one responsible for fetal (late) myoblasts. Limb buds populated by embryonic myoblasts alone never produced fetal myoblasts. Although it appears clear that embryonic and fetal myoblasts are not direct lineal descendents of one another, it is not known if fetal myoblasts can produce satellite cells. The work of Cossu (33, 34) and Feldman and Stockdale (28) showed that satellite cells and fetal myoblasts had different properties, but this work did not directly address the issue of the origin of satellite cells. Transplantation of fetal myoblasts marked with reporter genes into developing limb buds should shed light on this question.

Conclusions

These studies indicate that there are no generic myoblasts that fuse with other genetic myoblasts to form generic skeletal muscle fibers which then can be molded by the central nervous system into fibers of a variety of types. Experiments on limbs developing aneurally (35, 36) or in which neuromuscular transmission was blocked by curare (3, 37) clearly demonstrated in both instances that muscle fiber diversity proceeded normally. This is most consistent with the diversity of the primary muscle fibers of the limb musculature being rooted in diversity of the myoblast population that produces the first muscle fibers. This is not to say that there is no role for the central nervous system in defining the definitive phenotype of a muscle fiber. Cross-reinnervation experiments as well as analysis of secondary fiber formation in the fetus showed that innervation can modify myosin isoform expression (3, 38, 39). Additional influences such as activity and thyroid hormone exposure can affect the phenotype of a muscle fiber as well (25, 40–43). Thus, there are both intrinsic and extrinsic components that define the definitive characteristics of all skeletal muscle fibers (14). Intrinsic components are important in early limb development when the primary fibers are first formed; where, especially in the bird, endocrine organs have not yet appeared and axonal outgrowth has not reached the dorsal and ventral muscle masses. Subsequently, all fibers formed from fetal myoblasts and satellite cells are subject to a variety of external influences that can produce a variety of fiber phenotypes.

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