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Original Article

Changes in the proportion and number of Pax7^{+ve} and MF20^{+ve} myoblasts during chick myogenesis in the head and limb

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ABSTRACT Previous studies suggested that all myoblasts are present in the head and limb prior to the commencement of primary myotube formation. As a consequence, these myoblasts must be in various developmental states during myogenesis, *i.e.* proliferating, differentiating or terminally differentiated. There are few *in vivo* studies investigating dynamic quantitative changes of subgroups of these myoblasts during myogenesis. In this report, using anti-Pax7 and anti-myosin heavy chain antibodies, we examined the quantitative change of proliferating (Pax7^{+ve}) and terminally differentiated (MF20^{+ve}) myoblasts during primary and secondary myogenesis in the chick head and limb. Our results show that during primary myogenesis, less than 30% of myoblasts are in the proliferating phase, but as soon as secondary myogenesis begins, over 95% of myoblasts start to proliferate. Moreover, we have found that the proportion of terminally differentiated myoblasts is maintained at a low level (less than 3%) during primary and secondary myogenesis.

KEY WORDS: *Pax7, myoblast, in vivo, myogenesis, chick, head, limb*

Introduction

Almost all vertebrate skeletal muscles originate from the dermomyotome, the dorsal portion of a somite (Wigmore and Evans, 2002). As development proceeds, the dermomyotome of each somite expands along the dorso-ventral axis, and develops specialized zones at the dorsomedial and ventrolateral lips. The dorsomedial lip (DML) provides myoblasts for all epaxial muscles while the ventrolateral lip (VLL) is the source of myoblasts for the hypaxial muscles (Stockdale *et al.,* 2000; Wigmore and Evans, 2002). The myoblasts from both lips subsequently move to a position ventral to the dermomyotome in a stereotypic manner (Cinnamon *et al.,* 2001) and form the epaxial myotome and the hypaxial myotome (Wigmore and Evans, 2002). The hypaxial myotome subsequently expands laterally into the somatopleure as an epithelium and gives rise to the body wall musculature. There are two exceptions to this general developmental process. The first is the head muscles, which originate from three sources of mesoderm: occipital somites, paraxial head mesoderm and prechordal mesoderm (Wigmore and Evans, 2002). The latter two give rise to all the craniofacial muscles, whilst the tongue and laryngeal muscles are instead derived from the dermomyotome of the occipital somites (Noden, 1983; Huang *et al.,* 1999; Wigmore and Evans, 2002). The second exception is the limb muscles, which unlike other hypaxial muscles, delaminate directly from the VLL of limb adjacent somites and undergo a long-range of migration towards the target sites in the limb (Cinnamon *et al.,* 2001; Christ and Brand-Saberi, 2002). The migrated cells then proliferate to amplify their numbers (Nikovits *et al.,* 2001) and differentiate into fusion competent myoblasts which will fuse to form multinucleated myotubes.

This multi-step myogenic process is highly regulated by various transcription factors. One of these is the multi-gene *Pax* family, which encodes a series of highly conserved DNA binding proteins and plays an important role in the development of many organs during embryogenesis (Jostes *et al.,* 1991). Two members of the *Pax* family, *Pax3* and *Pax7*, are significantly involved in the process of muscle development. During limb myogenesis, for instance, *Pax3* is initially detected in the adjacent undifferentiated somites. When the somite differentiates into dorsal and ventral compartments, *Pax3* is prominently localized in the VLL from which the cells migrate ventrolaterally to the limb bud. These migrating cells continue to express *Pax3* until they have reached

Abbreviations used in this paper: DM, depressor mandibular muscle; DML, dorsomedial lip; MA, medial adductor muscle; VLL, ventrolateral lip.

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their destination when expression of *Pax7* is upregulated (Williams and Ordahl, 1994). The expression of *Pax7* in proliferating and differentiating myoblasts, however, will be strongly downregulated during terminal differentiation (Seale *et al.,* 2000). The terminally differentiated myoblasts express genes encoding muscle structural proteins, such as myosin heavy chain (MHC), and are fusion competent (Molkentin and Olson, 1996).

The migration of myoblasts and the formation of multinucleated myotubes are phasic, rather than continuous, processes. For instance, the formation of myotubes in rats (Ontell, 1978; Ontell, 1979; Ross *et al.,* 1987) and in chicken (McLennan, 1983; Crow and Stockdale, 1986) occurs in two waves which give rise to two generations of myotubes: primary myotubes and secondary myotubes. The two waves of myotube formation are separated by a short period in which no new myotubes are formed (Ontell, 1978; Ontell, 1979; Ross *et al.,* 1987). Several studies have also demonstrated that two distinct myoblast populations sequentially migrate into avian limb buds at 3 or 4 days before the first myotube appears (Seed and Hauschka, 1984). Therefore, it would seem logical to suppose that (1) all the myoblasts must be present in the limb prior to the start of myotube formation (Edom-Vovard *et al.,* 1999); and (2) these myoblasts must be in various developmental states *i.e.,* proliferating, differentiating, or terminally differentiated.

Clonal analyses carried out on both human (Edom-Vovard *et al.,* 1999) and chick (Hauschka, 1974; Hauschka, 1994) limb muscle support this hypothesis. For example, Edom-Vovard *et al.* isolated four different types of myogenic clones from human limb muscles prior to the commencement of primary myogenesis, each having distinct morphological and biochemical properties (Edom-Vovard *et al.,* 1999). At the end of myogenesis, however, there is only one type of myogenic clone left, which indicates that the four initial populations of myoblasts undergo dynamic quantitative changes during the myogenesis (Edom-Vovard *et al.,* 1999). However, the results of these *in vitro* studies were achieved after 5-20 cell divisions and without the *in vivo* embryonic environment (Edom-Vovard *et al.,* 1999). There are very few studies investigating the *in vivo* quantitative changes of myoblasts during myogenesis. The aim of this study, therefore, was to investigate the quantitative change of proliferating and terminally differentiated myoblasts during chick head and limb myogenesis. We used anti-Pax7 antibody as a marker for proliferating myoblasts (Seale *et al.,* 2000) and anti-myosin antibody (MF20) for terminally differentiated myoblasts. In order to standardize the relative number of $Pax7^{+ve}$ or $MF20^{+ve}$ cells, an anti-desmin antibody was used as a general marker for all the myogenic cells (Tokuyasu *et al.,* 1984). Our results show that (1) during primary myogenesis (D4-D6), less than 30% of myoblasts are in the proliferating phase ($Pax7^{+ve}$) but as soon as secondary myogenesis (D8-D12) begins, over 95% of myoblasts start to proliferate; and (2) the proportion of terminally differentiated myoblasts ($MF20^{+ve}$) is maintained at a low level (less than 3%) during primary and secondary myogeneses.

Results

Chick limb and head muscles from D4 to D12 contain a mixture of $Pax7^{+ve}$ and $MF20^{+ve}$ myoblasts

The myoblasts in the limb can be subdivided into three categories: embryonic, fetal and adult myoblasts (Stockdale, 1992). Although it remains unclear whether or not they have common precursors (Christ and Brand-Saberi, 2002), these three populations of the myoblasts and/or their precursors must co-exist in the limb muscles during the formation of primary and secondary myotubes because the myogenic precursors migrate into avian limb buds at 3 or 4 days before the first myotube appears (Seed and Hauschka, 1984). Each myoblast needs to go through several discrete developmental phases, including determination, migration, proliferation, differentiation and fusion. Thus, the preliminary phase of this study was to test whether chick limb and head muscles during primary and secondary myogeneses contain a mixture of $Pax7^{+ve}$ and $MF20^{+ve}$ cells which represent the proliferating and terminally differentiated myoblasts, respectively.

Pax7 and MHC expression in myoblasts were examined in both cryostat sections and isolated cells which were harvested from D4,

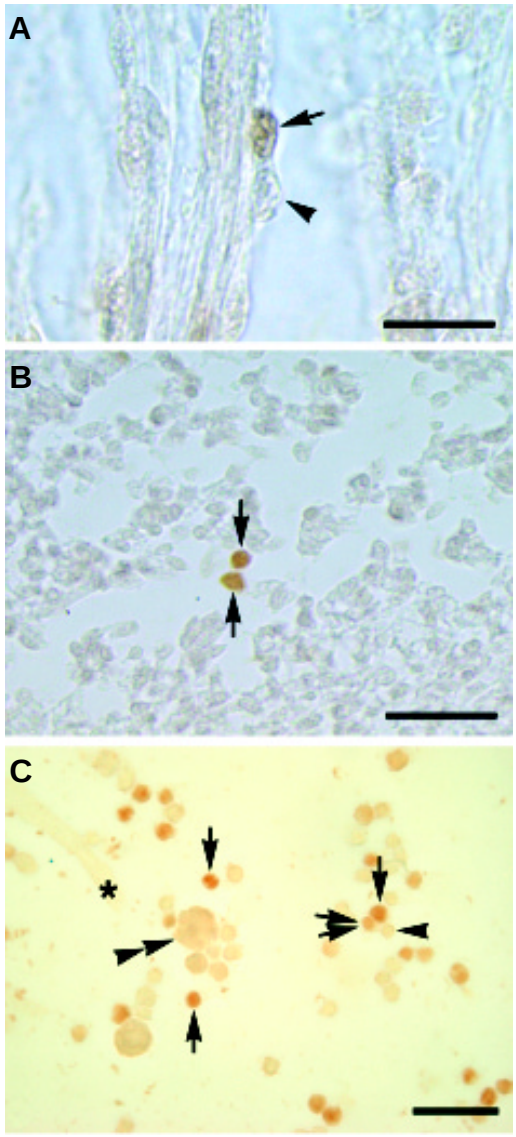

Fig. 1. Pax7 expression in longitudinal (A) and transverse (B) tissue sections, and in isolated cells (C). *The sections and cells were collected from a chick thigh muscle at D4* **(A)** *and from medial adductor muscles at D10* **(B,C)**. *Arrows and arrowheads point to $Pax7^{+ve}$ and $Pax7^{-ve}$ profiles, respectively. Myotubes are marked with an asterisk and they are $Pax7^{-ve}$. The double arrows indicate a weak $Pax7^{+ve}$ cell. The double arrowheads point to a "big cell" which is $Pax7^{-ve}$ and presumably a very young contracted myotube with two or more myonuclei. Scale bars, 10 μm.*

5, 6, 8, 10 and 12 chick limbs and heads. On cross and longitudinal sections, Pax7^{+ve} nuclei were always found outside multinucleated myotubes, with no myotube nuclei expressing Pax7 (Fig. 1A). It was common to observe Pax7^{+ve}profiles paired with another nucleus, which could be either Pax7^{+ve} (Fig. 1B) or Pax7^{-ve} (Fig. 1A). In the cell suspensions, Pax7^{+ve}cells varied slightly in their size and labeling intensity (Fig. 1C), but positive labeling was always localized to the nucleus (Fig. 1C). Fragments of myotubes and the "big cells", which were presumably very young contracted myotubes with two or more myonuclei, were Pax7^{-ve} (Fig. 1C). In contrast to Pax7 labeling, it was relatively difficult to distinguish myoblasts from young myotubes with MF20 labeling on cryostat sections, as both cell types were MF20^{+ve} (Fig. 2A). The MF20^{+ve} suspended cells varied greatly in their size and labeling intensity (Fig. 2B). The fragments of myotubes and some of the "big cells" mentioned above expressed very strong reactivity for MHC (Fig. 2B). The MF20^{+ve} staining was always distributed in the peripheral regions of the cells.

Of the Pax7/MF20 double-labeled cells (Fig. 2B), the Pax7^{+ve} and MF20^{+ve} populations were clearly distinguished as very few cells were co-expressing Pax7 and MHC. In a total of 10532 myoblasts (Pax7^{+ve} and/or MF20^{+ve}) harvested from D4-12 limb and head muscles, only about 0.7% of them (38/5180 for the limb and 35/5352 for the head) were co-expressing either strong Pax7/ weak MHC or strong MHC/weak Pax7.

Therefore, the developing limb and head muscles contain at least two populations of myoblasts which are at either the proliferation phase (Pax7^{+ve}) or the terminal differentiation phase (MF20^{+ve}). Because of the cross reactivity during the double labeling procedure used in this study, we can not confirm the existence of the third population of myoblasts which co-express Pax7 and MHC (see Experimental Procedures for the further explanation).

Sudden increase in size of the Pax7^{+ve} myoblast population following the onset of secondary myotube formation

To analyze quantitative changes of the proliferating (Pax7^{+ve}) myoblasts during primary and secondary myogenesis, we harvested mononucleated cells from the medial adductor and depressor mandibular muscles from D6 to D12 embryos. After a brief (45 minutes) differential-adhesion culture for fibroblast elimination, we were able to obtain a final cell preparation containing approximately 70% myoblasts. To further eliminate non-myogenic cells, we used Pax7/desmin double immunocytochemical labeling to estimate the ratio of Pax7^{+ve} to desmin^{+ve} cells. In order to ensure the specificity of Pax7^{+ve} cells, anti-Pax7 and anti-desmin antibodies were used as the first and second primary antibodies, respectively. Because of the cross immunoreactivity during the double labeling procedure and desmin expression of other myogenic cells, such as vascular smooth muscle cells, the number of desmin^{+ve} cells may have been slightly overestimated in this study and thus was only used for standardizing the relative number of Pax7^{+ve} cells for the comparison of their quantitative changes during myogenesis.

TABLE 1

RATIO (%) OF PAX7^{+VE} TO DESMIN^{+VE} CELLS OF THE DM AND MA AT EMBRYONIC DAYS 6, 8, 10 AND 12

	Dish 1	Dish 2	Dish 3	Means ±SEM
DM: D 6	10.53 (28/ 266)	24.69 (20/ 81)	7.83 (13/166)	14.35 ± 5.23
D 8	91.95 (137/ 149)	81.36 (48/ 59)	96.20 (152/158)	89.83 ± 4.41**
D10	98.57 (1034/1049)	96.89 (436/ 450)	97.32 (182/187)	97.60 ± 0.50**
D12	91.72 (144/ 157)	97.39 (1156/1187)	96.65 (461/477)	95.25 ± 1.78**
MA: D 6	7.61 (27/ 355)	31.25 (10/ 32)	22.11 (42/190)	20.32 ± 6.88
D 8	97.59 (243/ 249)	97.83 (135/ 138)	95.74 (45/ 47)	97.05 ± 0.66**
D10	98.83 (1013/1025)	98.94 (1584/1601)	97.43 (341/350)	98.40 ± 0.49**
D12	70.36 (197/ 280)	99.66 (2035/2042)	97.47 (308/316)	89.16 ± 9.42**

Data of dish columns are given as the ratio of Pax7^{+ve} to desmin^{+ve} cells. The number of cells detected is indicated in parentheses. The cells of each dish were harvested from 15 muscles (see Table 4 for detail). DM: the depressor mandibular muscle; MA: the medial adductor muscle. **: significantly different from D6; $p<0.01$, ANOVA (Single Factor).

The ratio of Pax7^{+ve} to desmin^{+ve} cells underwent a dynamic change from D6 to D12 in both medial adductor and depressor mandibular muscles. At D6, less than 20% of desmin^{+ve} cells are Pax7 positive, whereas at D8, the ratio suddenly increased to over 90% and remained at the same level at D10 and D12 (Table 1 and Fig. 3). The lower level of the ratio before D6 was supported by data obtained from D4, D5 and D6 (Table 2 and Fig. 3) in which tissue blocks from thigh or head, rather than individual muscles, were used. The formation of secondary myotubes in chick limb

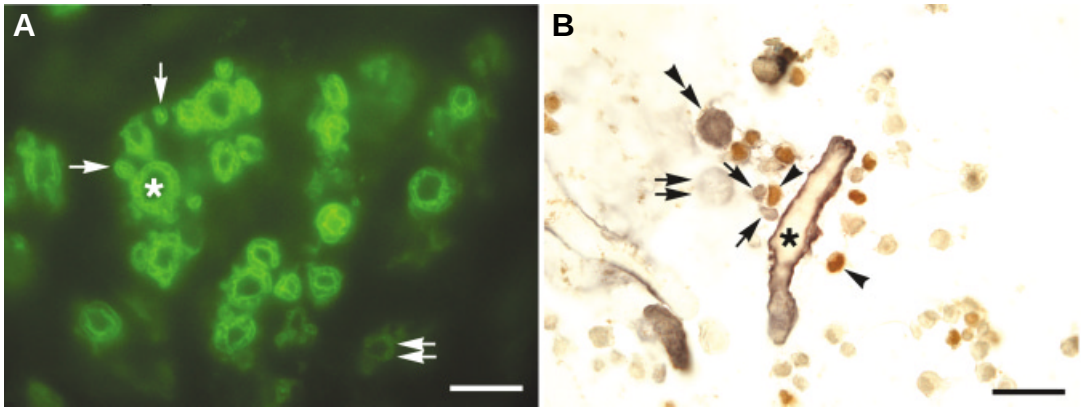

Fig. 2. MF20 expression in a transverse section from D12 chick medial adductor muscle (A) and isolated cells from D10 chick depressor mandibular muscle (B). (A) *was singly labeled with MF20 and detected with FITC, whereas* **(B)** *was double labeled with Pax7 (DAB - red/brown color) and MF20 (DAB with Nickel - purple color). Myotubes are marked with an asterisk and they are Pax7^{-ve}/MF20^{+ve}. Arrows and arrowheads point to Pax7^{+ve}/MF20^{+ve} and Pax7^{+ve}/MF20^{-ve} profiles, respectively. The double arrowheads point to a "big cell" which is presumably a very young contracted myotube with two or more myonuclei and is Pax7^{-ve}/MF20^{+ve}. Because anti-Pax7 was used as the first primary antibody, the staining of Pax7^{+ve} (arrowheads) and Pax7^{-ve}/MF20^{+ve} cells (arrows and double arrowheads in B) is specific. The pattern of MF20^{+ve} labeling in the isolated cells (B) is slightly different from that in the fragment of a myotube (marked with an asterisk). As in the isolated cells, cytoplasmic myosin may overlay the top and bottom of their nuclei. The double arrows indicate an MF20^{-ve} " big cell" (B) and a myotube (A). Scale bars, 10 μm.*

muscles starts at D8 (McLennan, 1983) and the timing of myogenesis in head muscles appears to be similar (Wigmore and Evans, 2002). Thus, the change in ratio of Pax7^{+ve} to desmin^{+ve} cells from 20% at D6 to over 90% after D8 both in a limb (medial adductor) and a head (depressor mandibular) muscle indicates a sudden increase in the population of proliferating myoblasts at the onset of secondary myogenesis.

The MF20^{+ve} myoblast population forms a constant but low proportion of total myoblasts

In order to examine quantitative changes in the population of terminally differentiated myoblasts (MF20^{+ve}) during primary and secondary myogeneses, we used MF20/desmin double immunocytochemical labeling to identify the ratio of MF20^{+ve} cells to desmin^{+ve} cells. The materials were from the same pools of suspended cells harvested in the above Pax7/desmin experiments. For the same reasons as mentioned above, the number of desmin^{+ve} cells may have been overestimated.

In all specimens examined, the number of MF20^{+ve} cells was very small and the majority of desmin^{+ve} cells were MF20^{-ve}. The ratio of MF20^{+ve} to desmin^{+ve} cells in both medial adductor and depressor mandibular muscles was maintained at a very low level of less than 3% from D4 to D12 (Fig. 3 and Tables 2 and 3), indicating that the relative number of terminally-differentiated, fusion-competent myoblasts remains constant throughout the myogenesis.

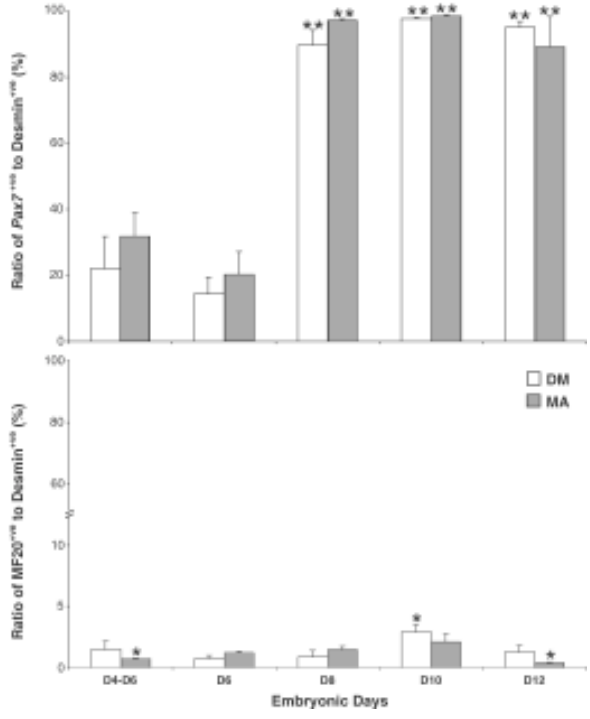

Fig. 3. Comparison of the ratio (%) of Pax7^{+ve} or MF20^{+ve} to desmin^{+ve} cells in chick head and limb muscles during primary (D4-6) and secondary (D8-12) myogeneses. *Columns D6, D8, D10 and D12 were derived from pooled dissected depressor mandibular (DM) or medial adductor (MA) muscles, whereas the D4-6 columns were derived from either the left half of the head (containing DM) or the left thigh (containing MA). The total number of cells detected is indicated in Tables 1-3. Asterisks indicate significant difference from D6; *, p<0.05; **, p<0.01, ANOVA (Single Factor).*

TABLE 2

RATIO (%) OF Pax7^{+VE} OR MF20^{+VE} TO DESMIN^{+VE} CELLS OF THE HEAD AND THIGH TISSUE BLOCK AT EMBRYONIC DAYS 4, 5 AND 6

	D4	D5	D6	Means ±SEM
Pax7/desmin:				
Hd	6.58 (67/1018)	19.14 (93/486)	40.30 (106/263)	22.01±9.84
Th	21.24 (205/965)	28.82 (117/406)	45.55 (169/371)	31.87±7.18
MF20/desmin:				
Hd	2.86 (28/979)	0.71 (4/567)	0.91 (10/1095)	1.49±0.69
Th	0.64 (8/1243)	0.86 (7/817)	0.71 (8/1131)	0.74±0.06*

Data are given as the ratio (%) of Pax7^{+ve} or MF20^{+ve} to desmin^{+ve} cells. The number of cells detected is indicated in parentheses. The number of tissue blocks of the each age is summarized in Table 4. Hd: the left bisected head tissue block without skin; Th: the left thigh tissue block without skin. *: significantly different from D6 of Table 3; $p<0.05$, ANOVA (Single Factor).

TABLE 3

RATIO (%) OF MF20^{+VE} TO DESMIN^{+VE} CELLS OF THE DM AND MA AT EMBRYONIC DAYS 6, 8, 10 AND 12

	Dish 1	Dish 2	Dish 3	Means ±SEM
DM: D 6	0.79 (3/381)	1.14 (7/ 613)	0.23 (1/430)	0.72±0.26
D 8	1.88 (7/372)	0.39 (3/ 764)	0.47 (4/855)	0.91±0.48
D10	4.03 (19/472)	2.82 (29/1030)	1.91 (9/471)	2.92±0.61*
D12	2.30 (11/478)	0.95 (8/844)	0.64 (5/780)	1.30±0.51
MA: D 6	1.06 (5/472)	1.48 (5/338)	1.15 (11/954)	1.23±0.13
D 8	1.32 (9/684)	2.02 (18/892)	1.22 (7/573)	1.52±0.25
D10	0.88 (5/566)	3.34 (30/898)	2.01 (13/646)	2.08±0.71
D12	0.35 (3/867)	0.28 (2/706)	0.50 (5/996)	0.38±0.07*

Data of dish columns are given as the ratio of MF20^{+ve} to desmin^{+ve} cells. The number of cells detected is indicated in parentheses. The cells of each dish were harvested from 15 muscles (see Table 4 for detail). DM: the depressor mandibular muscle; MA: the medial adductor muscle. *: significantly different from D6; $p<0.05$, ANOVA (Single Factor).

TABLE 4

NUMBER AND GROUPING OF THE MATERIALS USED*

	DM	MA	Hd	Th
D4			10 (1 dish)	10 (1 dish)
D5			7 (1 dish)	7 (1 dish)
D6	45 (3 dishes)	45 (3 dishes)	9 (1 dish)	9 (1dish)
D8	45 (3 dishes)	45 (3 dishes)		
D10	45 (3 dishes)	45 (3 dishes)		
D12	45 (3 dishes)	45 (3 dishes)		
Total	**180 (12 dishes)**	**180 (12 dishes)**	**26 (3 dishes)**	**26 (3 dishes)**

*: Data are given as the number of muscles or tissue blocks from each age. The number of culture dishes they were collected into is indicated in parentheses. DM: the depressor mandibular muscle; MA: the medial adductor muscle; Hd: the left bisected head tissue block without skin; Th: the left thigh tissue block without skin.

Discussion

The formation of skeletal muscle fibers is accomplished through a sequence of developmental phases, including determination, migration, proliferation, differentiation, fusion and maturation. In recent years, distinct sets of control genes underlying these processes have been revealed. At the center of the control mechanism are the members of the *MyoD* family of transcription factors, which are essential for myogenic determination and differentiation. The *MyoD* family governs the differentiation of myoblasts by controlling the withdrawal of cells from the cell cycle, activating the expression of muscle structural proteins (*e.g.,* MHC) and permitting the fusion of myoblasts into myotubes (reviewed by Molkentin

and Olson, 1996). The *MyoD* family requires transcription factors, *Pax3* and/or highly related *Pax7*, as upstream regulators and is significantly down regulated during migration and proliferation phases (reviewed by Dietrich, 1999; Mootoosamy and Dietrich, 2002). Therefore, in a developing muscle after migration, the proliferating ($Pax7^{+ve}$) and terminally differentiated (MHC^{+ve}) myoblasts represent two extreme ends of developmental stages of the myoblasts. In this study, we report that these two populations of myoblasts in chick limb and head muscles undergo a dynamic change during primary and secondary myogeneses.

Identification of proliferating and terminally differentiated myoblasts in the developing chick head and limb muscles

In this study, we used anti-Pax7 antibody as a marker for proliferating myoblasts and anti-myosin antibody (MF20) for terminally differentiated myoblasts. Pax7 is a specific myogenic marker in skeletal muscle tissue (Ziman *et al.,* 1997) and recently, on the basis of northern blot analysis, expression of Pax7 was found to be confined to proliferating myoblasts and strongly downregulated during terminal differentiation (Seale *et al.,* 2000). In this study, very few $Pax7^{+ve}$ cells co-expressed MHC. This is consistent with the $Pax7^{+ve}$ population representing myoblasts which are in a proliferating phase. Terminal differentiation involves the permanent cessation of DNA synthesis and activation of muscle-specific gene expression, including MHC. The MHC^{+ve} cells identified in this study represent terminally differentiated myoblasts. It should be pointed out that some of these might be very young multinucleated myotubes with two or more myonuclei (Figs. 1,2). Therefore, the number of MHC^{+ve} cells may have been slightly overestimated.

The purpose of using anti-desmin antibody as a second primary antibody during the double immuno-labeling was to eliminate non-myogenic cells, so that the quantitative data of $Pax7^{+ve}$ or $MF20^{+ve}$ cells can be standardized and comparable. Although in order to ensure specificity of $Pax7^{+ve}$ or $MF20^{+ve}$ cells, we used anti-Pax7 or MF20 monoclonal antibody as the first primary antibody and anti-desmin monoclonal antibody as the second primary antibody, the number of $desmin^{+ve}$ cells may have been overestimated in this study. There are at least two reasons for this assumption. First is due to the cross immunoreactivity during the double labeling procedure as the secondary antibody may recognize the first primary (anti-Pax7 or MF20) antibody. Second is because desmin is an intermediate filament sub-unit (Lazarides and Hubbard, 1976) and is expressed by all the myogenic cells, including cardiac and smooth muscles (Tokuyasu *et al.,* 1984). However, this overestimation should not affect the overall conclusions of this study, which are based on the comparison of the ratio of $Pax7^{+ve}$ or $MF20^{+ve}$ to $desmin^{+ve}$ cells. The number of $desmin^{+ve}$ cells was only used to standardize the quantitative changes of the $Pax7^{+ve}$ or $MF20^{+ve}$ cells at different developmental stages.

Rapid and significant increase in the proliferating myoblast pool following the onset of secondary myogenesis

Muscle growth is the result of a balance between undifferentiated proliferating myoblasts and differentiated myoblasts (Amthor *et al.,* 1998; Patel *et al.,* 2002). An increased proliferation rate of myoblasts results in a stimulation of muscle growth and an enlargement of muscle size (Füchtbauer, 2002). In this study, we found that the ratio of $Pax7^{+ve}$ cells to $desmin^{+ve}$ cells significantly increased from less than 30% before D6 to over 90% after D8, indicating that the proliferating myoblast pool is rapidly and significantly increased following the onset of secondary myogenesis. This dynamic change matches well with the demand for embryonic muscle growth. In chick head and limb muscles, the formation of primary myotubes starts at D4-5 and continues until about D8, by which time secondary myotube formation has been initiated (Crow and Stockdale, 1986). Secondary myogenesis produces over 90% of muscle fibers in most muscles, and during the same period, the existing myotubes continuously absorb new myonuclei from differentiated myoblasts (Evans *et al.,* 1994; Zhang and McLennan, 1995). The continuous and significant growth in the number and size of myotubes during secondary myogenesis requires the acceleration of myoblast proliferation and the expansion of the proliferating myoblast pool (Amthor *et al.,* 1998; Patel *et al.,* 2002). Therefore, a rapid and significant increase of proliferating myoblasts following the onset of secondary myogenesis will provide a sufficient source of undifferentiated myoblasts for the required rapid growth of muscles.

On the other hand, a sufficient number of undifferentiated myoblasts may also meet the demand for the patterning and shaping of individual muscles during secondary myogenesis. Unlike differentiating myoblasts which lose their migratory ability (Amthor *et al.,* 1998), the undifferentiated myoblasts can migrate to the fusion sites, either at the center of primary myotubes for forming a new secondary myotube (Duxson *et al.,* 1989) or at the ends of existing myotubes for adding new myonuclei (Zhang and McLennan, 1995). Because during the patterning and shaping process of an organ, members of *Pax* family always play an essential role (Dahl *et al.,* 1997), the result of over 90% of myoblasts expressing Pax7 during a rapid growth period of embryonic muscles correlates well with this general view.

Interestingly, we found that during primary myogenesis, less than one third of myoblasts were $Pax7^{+ve}$, suggesting that the proliferation of myoblasts during primary myogenesis may be selective. Multiple myoblast lineages in the developing muscle are well known although it remains unclear whether they are sharing the same precursors or have different origins (reviewed by Stockdale, 1992). Several studies have demonstrated that two distinct myogenic precursor populations sequentially migrate into avian limb buds at 3 or 4 days before the first myotube appears (Seed and Hauschka, 1984; Van Swearingen and Lance-Jones, 1995). These two migration waves may be selective because the cells migrating at early and late stages preferentially contribute to slow and fast muscle fibers, respectively (Van Swearingen and Lance-Jones, 1995). These migratory cells are committed to the myogenic pathway as they express the *MyoD* upstream regulatory factor, *Pax3*, and the highly related gene *Pax7* (Mansouri *et al.,* 1996; Seale *et al.,* 2000). The number of these migratory cells is very small and must be amplified before differentiation begins. It is unclear whether the migrated cells selectively enter into the proliferation phase. It has been proposed that selective proliferation of fast- or slow-fiber-committed myoblasts, in response to proliferative signals that originate in the stroma, is one mechanism by which the size, position and fiber-type pattern of individual anatomic muscles could be established (Nikovits *et al.,* 2001). The result of this study seems to indirectly support this hypothesis as only less than 30%, rather than all, of myoblasts move into the proliferation phase after both waves of migration have been completed.

What are the characteristics of those non-proliferating myoblasts ($Pax7^{-ve}$) during primary myogenesis? In addition to a very

small proportion of terminally differentiated myoblasts that expressed MHC, those non-proliferating but committed and migrated myoblasts can be either under a differentiating phase or withdrawn from the cell cycle, becoming G_0 cells. Normally the differentiating phase, a time period for a post-mitotic myoblast to become fusion-competent, is very short. In somites and in the limb bud, for example, desmin-positive post-mitotic myoblasts will express a cohort of myofibrillar proteins within a few hours (Li *et al.,* 1994). Therefore, it is logical that the proportion of post-mitotic differentiating myoblasts should not be significantly different from that of terminally differentiated myoblasts, which were less than 3% in this study. Based on this assumption, it is most likely that the majority of those non-proliferating myoblasts during primary myogenesis are in the G_0 phase. How long and why those cells are maintained in this state is still unclear. It has been reported that hypaxial muscle cells have a much longer time period (over two days) between their determination and the onset of differentiation than epaxial muscle cells (a few hours) (Carles de la Brousse and Emerson, 1990; Lin-Jones and Hauschka, 1996). During this period, the cells are maintained in an undifferentiated state by intracellular suppressor molecules, such as the regulatory factors *Id* and *Twist,* before undergoing differentiation (reviewed by Brand-Saberi and Christ, 1999).

The proportion of the terminally differentiated myoblast population remains constant throughout myogenesis

Embryonic muscle growth includes two aspects: hyperplasia (an increase in the number of muscle fibers) and hypertrophy (an increase in the diameter and length of individual fibers). They both require an addition of new nuclei by either myoblast-to-myoblast fusion (hyperplasia) or myoblast-to-myotube fusion (hypertrophy) (Landon, 1982). The only source for those newly-added myonuclei are the terminally differentiated myoblasts that express MHC. In the present study, we demonstrated that the ratio of terminally differentiated myoblasts to total myoblasts was constant at a relatively low level (less than 3%) throughout primary and secondary myogeneses. This suggests that the increase in numbers of terminally differentiated myoblasts proportionally follows the increase in the total number of myoblasts through myogenesis. In the rat and mouse, muscle growth rate is not constant (Goldspink, 1980). Thus the demand for terminally differentiated myoblasts may vary at different developmental stages. In the lumbrical muscle of the rat foot, for instance, there are approximately 100 primary and 280 secondary myotubes at birth (Ross *et al.,* 1987). By the end of primary myogenesis (on E17), each primary myotube contains approximately three nuclei and at birth, each primary and secondary has 30 and 14 nuclei, respectively (Wigmore *et al.,* 1992). One of our early studies also demonstrated that in the rat EDL and TA muscles, myonuclei birthdated at the early stages of primary (E14 to E15) and secondary (E16 to E17) myogeneses increased very rapidly whereas the growth between these two periods was slow (Table 1 from Zhang and McLennan, 1995). The variable demand for terminally differentiated myoblasts during muscle growth must be matched by a similarly variable increase in the total number of myoblasts. This is most likely achieved by regulating the size of the proliferating myoblast population. A dividing myoblast can undergo a symmetrical mitosis, yielding two daughter postmitotic or replicating myoblasts, which will decrease or increase the relative size of the proliferating population, respectively. When a myoblast undergoes an asymmetrical mitosis, generating a daughter postmitotic and a daughter replicating cell, it will maintain the existing relative size of the proliferating population. Therefore, during a rapid growth period, more myoblasts will undergo a symmetrical mitosis in order to increase the proliferating myoblast population.

In summary, this study has demonstrated that during primary and secondary myogeneses, the relative proportion of the terminally differentiated myoblast pool was maintained at a constant low level whereas that of proliferating myoblasts changed dramatically during the active phase of secondary myogenesis. This suggests that various subgroups of myoblasts in the head and limb muscles undergo different dynamic changes following the waves of myotube formation. Whether such changes are governed or regulated by various cell lineages or/and environmental signals is still a subject for further investigation.

Materials and Methods

Avian embryos

Fertilized White Leghorn chicken eggs *(Gallus)* were obtained from Winter Egg Farms, Hertfordshire, UK and incubated at 38ºC in a humidified incubator. Embryos were collected at embryonic day 4 (D4) to D12 and staged according to the criteria of Hamburger and Hamilton (1951) (Hamburger and Hamilton, 1951). Following decapitation, the depressor mandibular (DM) and medial adductor (MA) muscles of each embryo were dissected from the left side of the head and limb bud, respectively. As each individual muscle from very young embryos (D4 to D5) could not be clearly delineated, the left-bisected head and whole thigh region without skin, instead of the individual muscles, were collected from these animals.

Therefore, two types of material were used in this study: the isolated individual muscle (DM or MA) and the tissue block of the left head or thigh without skin. The muscles were collected from D6 (HH 28 to 29), D8 (HH 32 to 35), D10 (HH 35 to 37) and D12 (HH37 to 39) embryos. A total of 180 embryos (45 for each age) were used for this group (Table 4). The 45 dissected muscles (DM or MA) from each age were collected into 3 culture dishes (15 muscles for each dish) (Table 4) containing several drops of sterile Hank's Balanced Salt Solution (HBSS - Sigma) to prevent drying. A total of 26 embryos were used for the tissue block group (Table 4). They were collected from D4 (10 HH24 to 25 embryos), D5 (7 HH25 to 17 embryos) and D6 (9 HH28 to 29 embryos). The dissected head or thigh tissue blocks from each age were placed into separate culture dishes (Table 4).

Preparation of isolated myoblasts

Tissues in each dish were mechanically cut into pieces less than 1 mm^3 to aid cell dissociation. The tissues were then digested in a solution of 0.05% trypsin/EDTA (In Vitrogen, Paisley UK) for 15 minutes at 37ºC with gentle agitation every 5 minutes. Following trypsin inactivation with fresh growth media (DMEM with 10% FCS), the contents were filtered through a 150 μm pore mesh sterilized with 70% IMS into a fresh 60 x 10 mm culture dish. Following incubation at 37ºC (in 5% CO_2) for 45 minutes, unattached cells were collected and washed twice in 0.01M phosphate-buffered saline (PBS), pH7.2. After re-suspending in 0.01 M PBS, the final cell suspension was dropped onto 2% gelatin coated slides. The slides were air-dried at room temperature and then stored at -20ºC until required.

Immunocytochemistry

The following antibodies were used in this study. Primary antibodies: anti-Pax7 (Developmental Studies Hybridoma Bank, Iowa), anti-MF20 (DSHB, Iowa) and anti-Desmin antibody (BD Biosciences, Oxford, UK). Secondary antibodies: biotinylated anti-mouse IgG (Sigma), anti-mouse

FITC-conjugated IgG (Sigma), anti-mouse Texas-Red-conjugated IgG (Vector Laboratories, Burlingame, CA). Tertiary reagents: strepavidin-HRP complex (Vector Laboratories, Burlingame, CA).

Air-dried slides were rinsed in 0.01 M PBS for 5 minutes and then fixed in 2% paraformaldehyde in 0.01 M PBS for 10 minutes at room temperature, followed by a 10 minute incubation in 0.1 M glycine in 0.01 M PBS to stop fixation. After washing in 0.01 M PBS and permeablizing with 0.15 % Triton-X100 in 0.01 M PBS for 10 minutes, the slides were incubated overnight at 4ºC in a humidified chamber with primary antibody diluted in antibody diluting buffer (AbDB: 1% BSA + 0.15% Triton-X100 in 0.01 M PBS). Following three washes (each for 20 minutes) in AbDB, slides were incubated in biotinylated antimouse IgG diluted 1:300 at room temperature for 1 hour. The antibody was removed by washing two times (15 minutes each) in AbDB and once in 0.01 M PBS. Endogenous peroxidase activity was eliminated by exposure to 0.03% hydrogen peroxide in methanol for 10 minutes at room temperature after which the sections were washed twice in 0.01 M PBS for 5 minutes per wash, strepavidin-horseradish peroxidase (S-HRP) complex diluted 1:300 in AbDB for 1 hour, AbDB for 5 minutes and twice with 0.01 M PBS for 5 minutes per wash. The immunoreactivity was developed with DAB kit (Sigma). Non-specific staining was controlled for by replacing the primary antibody with an equivalent concentration of nonimmunized mouse IgG (Sigma).

When double labeling with two monoclonal antibodies was required, the immunohistochemical procedure was performed as previously described (Zhang and McLennan, 1995). Briefly, the slides were washed in three changes of 0.01M PBS and the above procedure repeated except that different primary and secondary antibodies were used, the methanol-H_2O_2 step was omitted, and an AEC kit (Sigma) was used instead of the DAB kit, or different colored DAB staining was used. The slides were rinsed in distilled water, and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Three combinations of the double labeling were performed in this study: Pax7/desmin, MF20/desmin and Pax7/MF20, in which anti-Pax7, MF20 and anti-Pax7 antibodies were used as the first primary antibodies, respectively. Therefore, having considered the possibility that the second secondary antibody may recognize the first primary antibody, we only defined the following labeled cells were specific: (1) the cells positively labeled by the first primary antibodies, and (2) the cells negative for the first primary but positive for the second primary antibodies.

To view slides with a fluorescence microscope, the biotinylated antimouse IgG was replaced by an anti-mouse Texas-Red or FITC conjugated secondary antibody for one hour at room temperature. The slides were then washed in 0.01 M PBS and mounted with Vectashield.

Cell counting and image capture

Stained slides were examined using a Leitz Laborux 12 epi-fluorescence microscope equipped with a Coolsnap digital camera (RS Photometrics, USA). Counting frame was set as a view-field with 40 times objective lens. The starting frame and the interval of counting frames were determined using the random number table. Cells from each counting frame were counted and recorded manually. Electronic images were captured using Coolsnap software (RS Photometrics, USA). The data were analyzed with the Data Analysis Tool Kit included in the Microsoft Excel and tested by ANOVA (Single Factor) analysis.

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