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Research Article

A Study on Ovine Muscle Development [#]

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ABSTRACT

The ontogeny of ovine muscle was studied. 23 single fetuses aged between 48 and 125 days of gestation (dg) were collected from abattoirs. The weight and crown-rump length of fetuses were measured and gestational age was estimated. Semitendinosus muscle (ST) or hind limbs (for smallest fetuses) were dissected and stained for alkali-stable ATPase and slow myosin heavy chain (MHC) antibody and also examined under the electron microscope (EM). Based on histochemical, immunohistochemical and electron microscopy study, qualitative findings were obtained. The results suggested that some secondary fibres early express slow MHC. These small, slow expressing secondary fibres adjacent to the large, slow expressing primary fibres began to occur at 52 dg and these increased in size at around day 69 and then migrated around 87 dg to act as a scaffold for the next generation of secondary (tertiary) fibres. We conclude that some of the large, central, slow expressing (also less intensely stained with alkali-ATPase) fibres at near term sheep fetus might be secondary fibres.

Key Words: Muscle, sheep, ontogeny, myogenesis

ÖZET

KOYUNDA KAS GELİŞİMİ ÜZERİNE BİR ÇALIŞMA

Koyunda kas ontojenisi üzerine çalışıldı. Gebelik yaşları (gy) 48 ile 125 arasında değişen 23 adet tekiz fötus mezbahadan toplandı. Fötusların ağırlıkları ve baş-kuyruk mesafeleri ölçülerek gebelik yaşları tahmin edildi. Semitendinosus kası (ST) veya çok küçük olan fötuslardan tüm arka bacak disekte edilerek alkali- adenozin trifosfataz (ATPaz) ve yavaş-miyozin ağır zincir (MHC) antikoru ile boyandı. Ayrıca, kesitler elektron mikroskopta (EM) incelendi. Histokimyasal, immunohistokimyasal ve EM çalışmalarına dayanarak kalitatif bulgular elde edildi. Sonuçlar, bazı sekonder liflerin çok erken dönemde yavaş MHC ekspresyonu yaptıklarını gösterdi. Büyük, yavaş MHC eksprese eden primer liflerin çevresinde oluşan bu küçük, yavaş MHC ekspresyonu yapan sekonder lifler 52. günde oluşmaya başlar; yaklaşık 69. günde çapları iyice artar ve yaklaşık 87. günde göç ederek ikinci generasyon sekonder liflerin (tersiyer) oluşumu için çerçeve görevi görürler. Gelişmiş geç dönem koyun fötusunda görülen büyük, merkezi ve yavaş MHC eksprese eden (alkali ATPaz ile düşük yoğunlukta da boyanan) liflerin bir kısmının aslında sekonder lifler olabileceği sonucuna varıldı.

Anahtar Kelimeler: Kas, koyun, ontojeni, miyogenez

[#] This study is a part of PhD thesis of the first author.

Introduction

Muscle fibre number is a major determinant of muscle mass. Muscle fibre hyperplasia occurs during the fetal period and is completed by birth in many agricultural animals such as sheep (Ashmore et al., 1972), cattle (Russell and Oteruelo, 1981) and pigs (Karunaratne et al., 2005; Rehfeldt, 2005; Stickland and Goldspink, 1973).

Muscle fibres form by fusion of mononucleated myoblasts into multinucleated myofibres. In small animals, muscle fibre generation is biphasic. Thus, prenatally muscle fibres develop as two distinct populations. Fibres which form during the initial stages of myoblast fusion are primary fibres which provide a structural framework for the subsequent formation of secondary myofibres (Novakofski et al., 2004). Secondary myofibres are created by fusion of two mononucleated myoblasts in the vicinity of the endplate of primary myofibres (Wilson et al. 1992).

In large animals, biphasic development of myogenesis in sheep (Ashmore et al., 1972), pig (Ashmore et al., 1973, Swatland and Cassens, 1973) and cattle (Russell and Oteruelo, 1981) has been observed. Data from the sheep presented by Maier et al. (1992) and Wilson et al. (1992) support the idea that there may be qualitative difference in the mechanisms of muscle formation between small and large animals including sheep having at least three generations of myotubes.

There are more conflicting ideas between scientists who examined the early myogenesis in sheep. Sheep is a precocial mammal and the prenatal development of muscle tissue in the lamb is completed by 5 days prior to birth (Ashmore et al., 1972). Ashmore et al. (1972) examined the prenatal development of muscle fibres histochemically, using ATPase alkali staining in the fetal lamb from 50-145 days of gestation. They observed biphasic development of muscle fibres and suggested that all fibres destined to be primary fibres were formed during the initial stages of fusion and were followed by the development of secondary fibres. They observed that primary fibres formed in a short duration relatively to the secondary fibres and served as a structural framework around which secondary fibres developed. At no time after 50 days of gestation were new primary fibres observed to form, whereas secondary fibres begin to form around 60 days of gestation at first rapidly then slower up to 140 days of gestation. Therefore, primary fibres are large and uniform in their size whereas secondary generation of myofibres are not uniform in size having a wide variation in diameter (Ashmore et al., 1972; Wilson et al., 1992).

Wilson et al. (1992) examined the generation of myotubes in fetal tibials cranialis muscle between 32 and 76 days of gestation. Using EM they observed that primary myotubes were first seen on embryonic day 32 (E32) and reached their maximum number by embryonic day 38. Secondary fibres began immediately after completion of primary myotube formation which is around E38. The majority of secondary fibres could be recognised by their smaller size, less rounded profile and protruding folds into primary motubes. However, a more difficult problem in classification arises from immunehistochemical observations. In mammalian muscle all primary fibres express initially slow MHC but secondary fibres express fast MHC isoforms (Novakofski et al., 2004; Wigmore and Dunglison, 1998). Primary myotubes identified in this manner continued to increase in number at least 76 days whereas in EM they reached their maximum number by E38. Thus, there is some confusion regarding primary fibre formation between EM and immunohistochemical methods. Whether later formed slow MHC positive fibres could be identified as primary or secondary should be made clear. Maier et al. (1992) examined developing sheep tibialis cranialis muscle immunohistochemically from E76 to postnatal day 20 (PN20) and from adult animals. They observed that large, central, expressing slow MHC fibres increased in number between E76 and E100. It was also observed that peripheral fibres stained positively with anti slow MHC antibody after

E110 and postnatal periods as a result of smaller peripheral fibre transformation.

The aim of this study is to highlight some conflicting ideas on muscle development of sheep fetus and clarify the relationship between primary and secondary fibre population with fibre type profile.

Materials and Methods

In this study, 23 single fetuses aged between 48 and 125 dg were collected from abattoirs in London, UK. The weight and crown-rump length of fetuses were measured and gestational age was estimated using the illustrated growth curve given by Evans and Sack (1973). ST or hind limbs (for smallest fetuses) were collected. Midbelly slices of ST muscle were rapidly frozen in liquid nitrogen. Adjacent parts of each muscle were fixed in Karnovsky fixative (4% parafolmaldehyde, 5% glutaraldehyde in 0.1 sodium cacodylate buffer; pH 7.4) for further electron microscopic study. Blocks were rinsed in 0.1 M cacodylate buffer, post fixed for 1 hour in 1% osmium tetroxide in cacodylate buffer, dehydrated in increasing concentrations of ethanol (30-100%), rinsed in propylene oxide and embedded in resin (epoksi resin kit medium; TAAB). Resin-embedded sections were cut using a microtome (Reichert, UK). Semithin sections (1.5 μm) were stained with 0.1% toluidine blue solutions and then thin transverse sections (90 nm) were placed on copper grids and stained with uranyl acetate and lead citrate. Samples were examined by EM.

10 μm frozen sections were cut from the frozen tissue and stained for alkali-stable ATPase at pH 10.4 (Guth and Samaha, 1970) and slow MHC antibody. For immunohistochemistry, sections were incubated with primary anti-slow MHC (NCL-MHCs; Novocastra, UK) antibody in 1% goat serum for 2 hours at room temperature. The sections were washed in 3 changes of PBS with 0.1% Tween 20. The secondary antibody (B7264; Sigma) was applied in PBS for 1 hour at room temperature.

Primary fibre numbers were counted in 60 day old fetus. In this specimen, large, slow fibres with central vacuoles were counted as primary. For 125 day old fetus, all the large, central and slow fibres were also counted. For all analyses a Kontron image analysis system (KS300, Zeiss, UK) was used.

All procedures were carried out with local ethics approval of Royal Veterinary College and in accordance with the regulations of UK Home Office Animals (Scientific Procedures) Act, 1986.

Results

Serial sections were either incubated with slow MHC antibody or stained for myosin ATPase (Figure 1). The antibody staining was consistent with the ATPase staining. Figure 1a demonstrates alkali-stable myosin ATPase activity. The intensity of myosin ATPase reaction of primary fibres is significantly lower than that of secondary fibres at 94 dg. Figure 1b shows that these primary fibres react strongly with slow-MHC antibody.

The pattern and progression of muscle development is detailed in a number of figures (Figures 2-7) based on histochemical, immune-histochemical and electron microscopy studies.

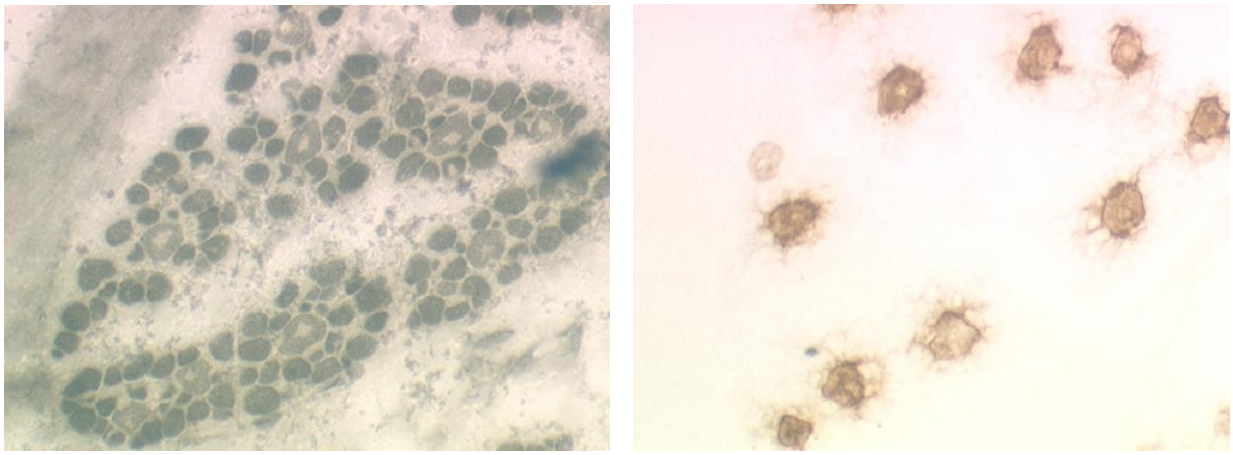
At embryonic day 48- primary myofibres already exist in clusters. Primay fibres are large and there are vacuoles in their center. They express slow MHC (Figure 2ai and 2aii).

At embryonic day 52- the large primary myofibres become surrounded by smaller secondary fibres some of which express slow MHC (Figure 2bi and 2 bii).

At embryonic 56- secondary myofibres are closed to the vicinity of primary fibres so as to form clusters as seen in EM picture (Figure 4). Small secondary fibres express slow MHC (Figure 2 cii).

At embryonic day 60- the number of secondary fibres increases (Figure 2 di). The small slow expressing secondary fibres can be seen in Figure 2 dii. They are still small in size. They are closed to the vicinity of primary fibres to form clusters as seen in EM picture (Figure 5).

At embryonic day 69- secondary fibres expressing slow MHC has increased in diameter (Figure 3-eii) and still are around the primary fibres as seen in EM picture (Figure 6).



(a) **(b)**

Figure 1. **a**. ATPase alkali staining in 94 day old sheep
b. Slow MHC-antibody staining in the same section (×40 objektive).
Şekil 1. **a.** 94 günlük koyun fötusunda ATPaz alkali boyama
b. Aynı kesitte yavaş MHC-antikor ile boyama (×40 objektif).

At embryonic day 87- the large, expressing slow MHC secondary myofibres moved out under the basement membrane and have started to support smaller secondary fibres (tertiary). All large, slow fibres are separated as seen in Figure 3 fii. The new small secondary fibres (tertiary) probably express only fast MHC. The increased number of newly formed secondary fibres (tertiary) makes all myofibres more closely apposed (Figure 3 fi).

At embryonic day 112- all the myofibres appear to have formed. The fascicles look more compact (Figure 3 gi) and all the fibres are getting to the same size. All large, central and slow MHC fibres look separated from each other (Figure 3 gii).

At Embryonic 125- the large, central and slow MHC expressing fibres stain less intensely with ATPase (Figure 7).

The total number of primary fibres assessed at 60 dg was 13647 whereas the number of apparent primaries (large, central, slow) at 125 dg was about 28000.

Based on these figures the qualitative findings from the ontogeny study are depicted in Figure 8.

Discussion

Our ontogeny study showed that primary fibres which are large, slow and uniform in size already existed at 48 dg (Figure 2ai, 2aii). Primary fibres that are large, central, slow MHC expressing fibres form till the first trimester of gestation (Ashmore et al., 1972; Wilson et al., 1992).

Small secondary fibres begin to occur around 52 dg around the large, slow primary fibres (Figure 2bi, 2bii). Our ontogeny study showed that some secondary fibres may express slow MHC at the onset of their formation (Figure 2bii). In mammalian muscle all primary fibres express initially slow MHC and secondary fibres express fast MHC isoforms (Cho et al., 1993; Condon et al., 1990; Nova-kofski et al., 2004; Pin and Merrifield, 1993; Vivarelli et al., 1988; Wigmore and Dunglison, 1998). However, there is some evidence that secondary fibres can express different MHC (Pin et al., 2002). Embriyonic myoblasts form primary fibres all of which are initially slow and produce only slow fibres (Cho et al., 1993; Pin and Merrifield, 1993; Vivarelli et al., 1988), whereas fetal myoblasts form secondary fibres (Stockdale, 1992).

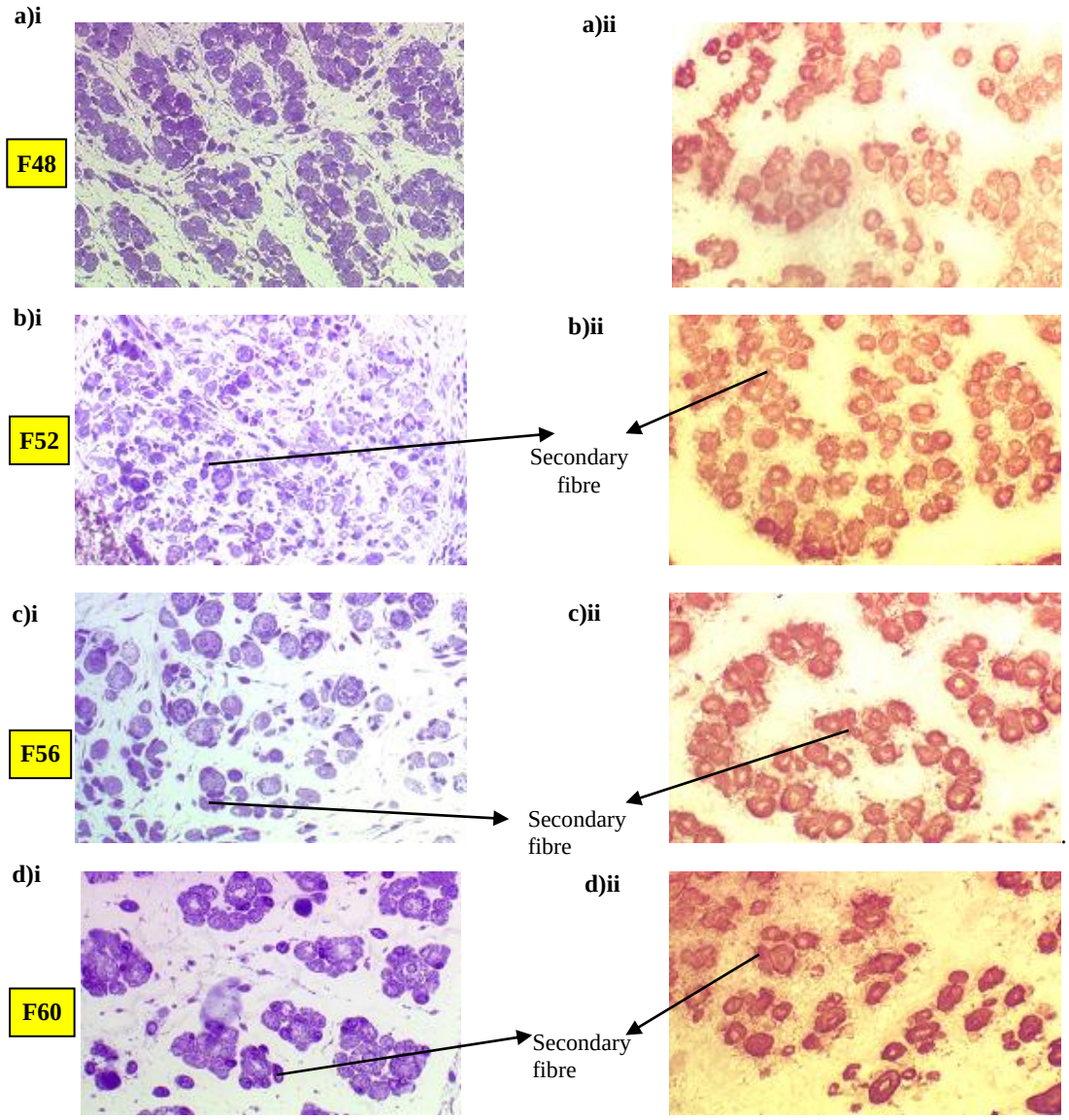


Figure 2. Semitendinosus muscle from 48 dg, 52 dg, 56 dg, 60 dg fetus
i) toulidine blue staining in resin embedded section (ai, bi, ci: ×20 objektive; di: ×40 objektive)
ii) slow MHC antibody staining in frozen section (aii, bii, cii, dii: ×40 objektive)
dg: days of gestation; **F48:** from 48 dg fetus; **F52:** from 52 dg fetus; **F56:** from 56 dg fetus; **F60:** from 60 dg fetus.

Şekil 2. Fötusun 48, 52, 56, 60. günlerinde semitendinosus kasından alınan kesitler
i) Rezin blok kesitlerinde toluidin-mavisi ile boyama (ai, bi, ci: X20 objektif; di: X40 objektif)
ii) Dondurulmuş kesitlerde yavaş MHC antikor ile boyama (aii, bii, cii, dii: X40 objektif)
dg: gebelik günü; **F48**: 48 günlük fötus; **F52**: 52 günlük fötus; **F56**:56 günlük fötus; **F60**: 60 günlük fötus.

Furthermore, some authors have shown that single clones derived from fetal mouse or human cells can produce a mixture of fast and slow fibres in culture (Cho et al., 1993; Robson and Hughes, 1997). In another study it has been observed that embryonic rat myoblasts form slow expressing primary fibers whereas fetal myoblasts form both slow and fast expressing secondary fibres in culture (Torgan and Daniels, 2001). Interestingly, Maier et al. (1992) found that slow expressing large, central myofibres increased in number till 100 dg. Our

results are similar to these observations. Since completion of primary fibre formation and beginning of secondary fibre formation is around 60 dg (Ashmore et al., 1972) primary fibre number was counted at 60 dg and 125 dg. The total number of primary fibres assessed at 60 days was 13647 whereas the number of apparent primaries (all the large, central and slow fibres) at 125 dg was about 28000. Our qualitative study showed that some small, slow expressing secondary fibres adjacent to the large, central, slow expressing primary fibres began to occur at 52 dg; these increased in size to around day 69 and then migrated around 87 dg to act as a scaffold for the next generation of secondary fibres (tertiary) (Figure 8). In sheep tibialis cranialis muscle there appears to be the third generation of developing muscle, tertiary myofibres. A small population of secondary fibres move away from primaries and then support the formation of third generation of myofibres (Maier et al., 1992; Wilson et al., 1992). Some authors also claim that the pig may show some tertiary myofibre formation (Lefaucher et al., 1995).

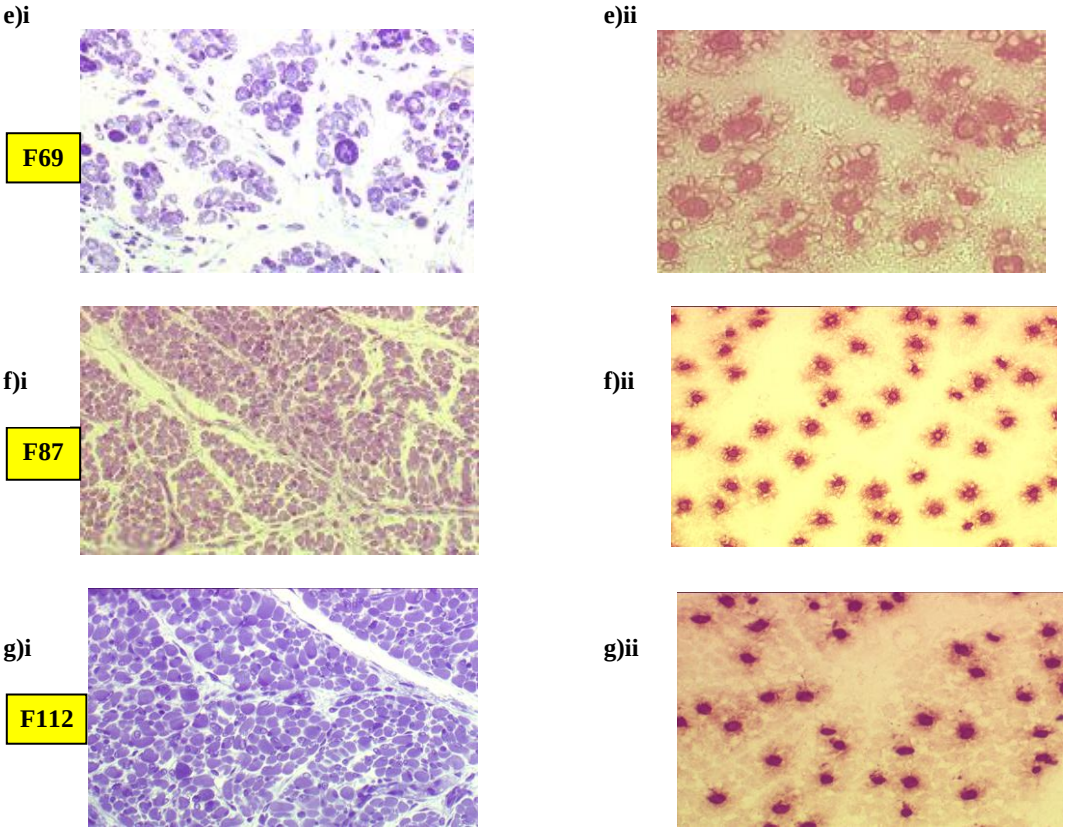


Figure 3. Semitendinosus muscle from 69 dg, 87 dg, 112 dg fetus

i) toulidine blue staining in resin embedded section (ei, fi, gi: ×20 objektive)

ii) slow MHC antibody staining in frozen section (eii: ×40; fii, gii, ×20 objektive**)**

dg: days of gestation; **F69:** from 69 dg fetus; **F87:** from 87 dg fetus; **F112:** from 112 dg fetus.

Şekil 3. Fötusun 69, 87, 112. günlerinde semitendinosus kasından alınan kesitler

i) Rezin blok kesitlerinde toluidin-mavisi ile boyama (ei, fi, gi: X20 objektif)

ii) Dondurulmuş kesitlerde yavaş MHC antikor ile boyama (eii: X40; fii, gii: X20 objektif)

dg: gebelik günü; **F69**: 69 günlük fötus; **F87**: 87 günlük fötus; **F112**: 112 günlük fötus.

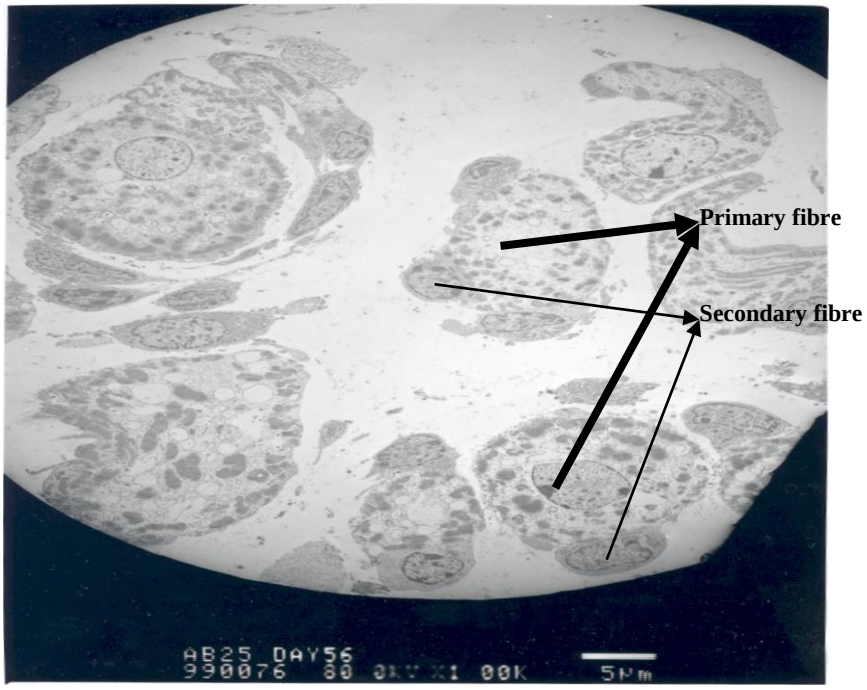


Figure 4. EM section of semitendinosus muscle from 56 dg fetus.
Şekil 4. EM'de 56 günlük fötal semitendinosus kas kesiti.

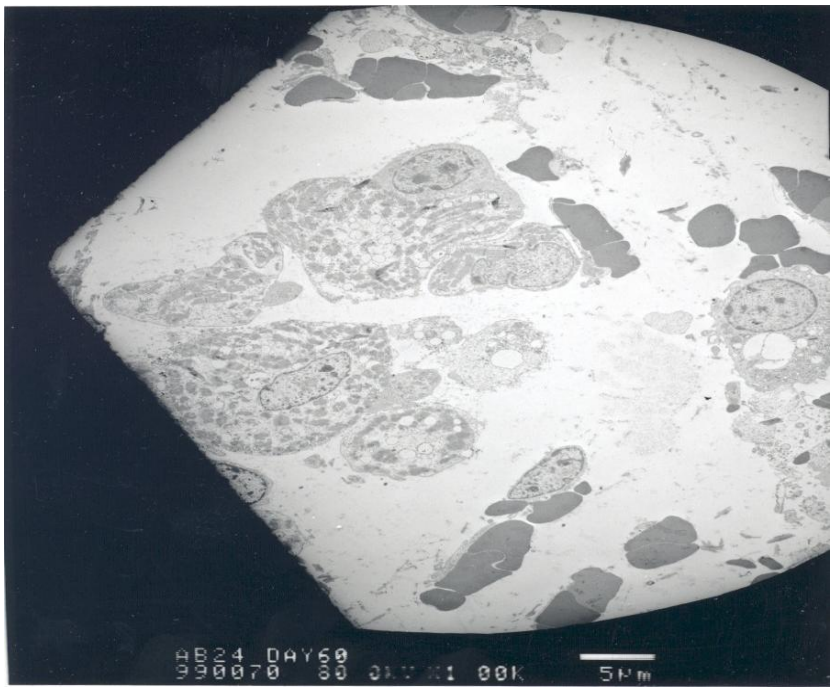


Figure 5. EM section of semitendinosus muscle from 60 dg fetus.
Şekil 5. EM'de 60 günlük fötal semitendinosus kas kesiti.

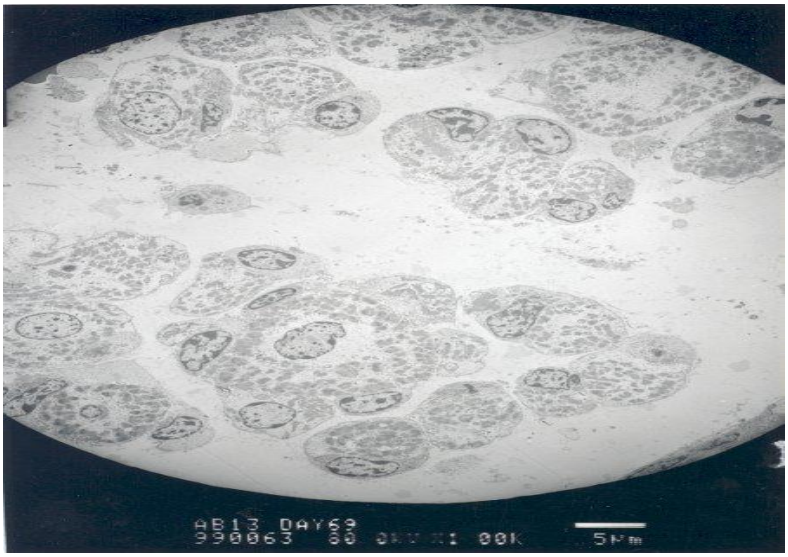


Figure 6. EM section of semitendinosus muscle from 69 dg fetus.
Şekil 6. EM'de 69 günlük fötal semitendinosus kas kesiti.

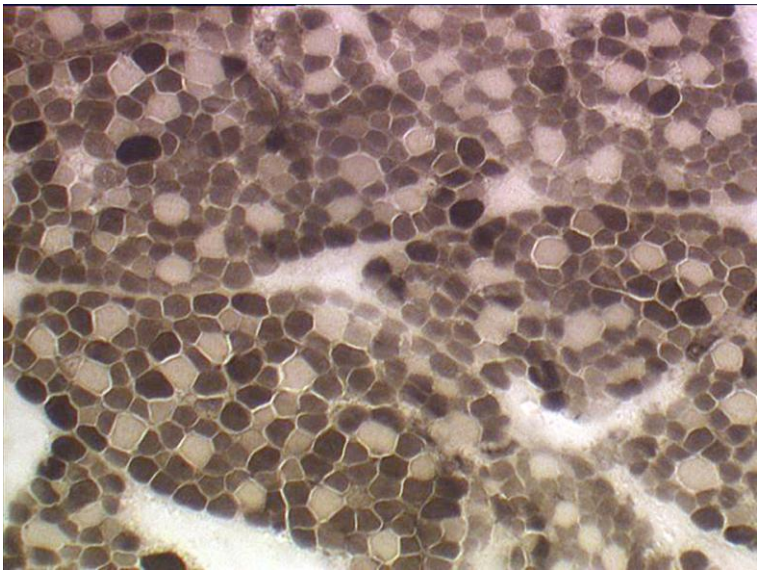
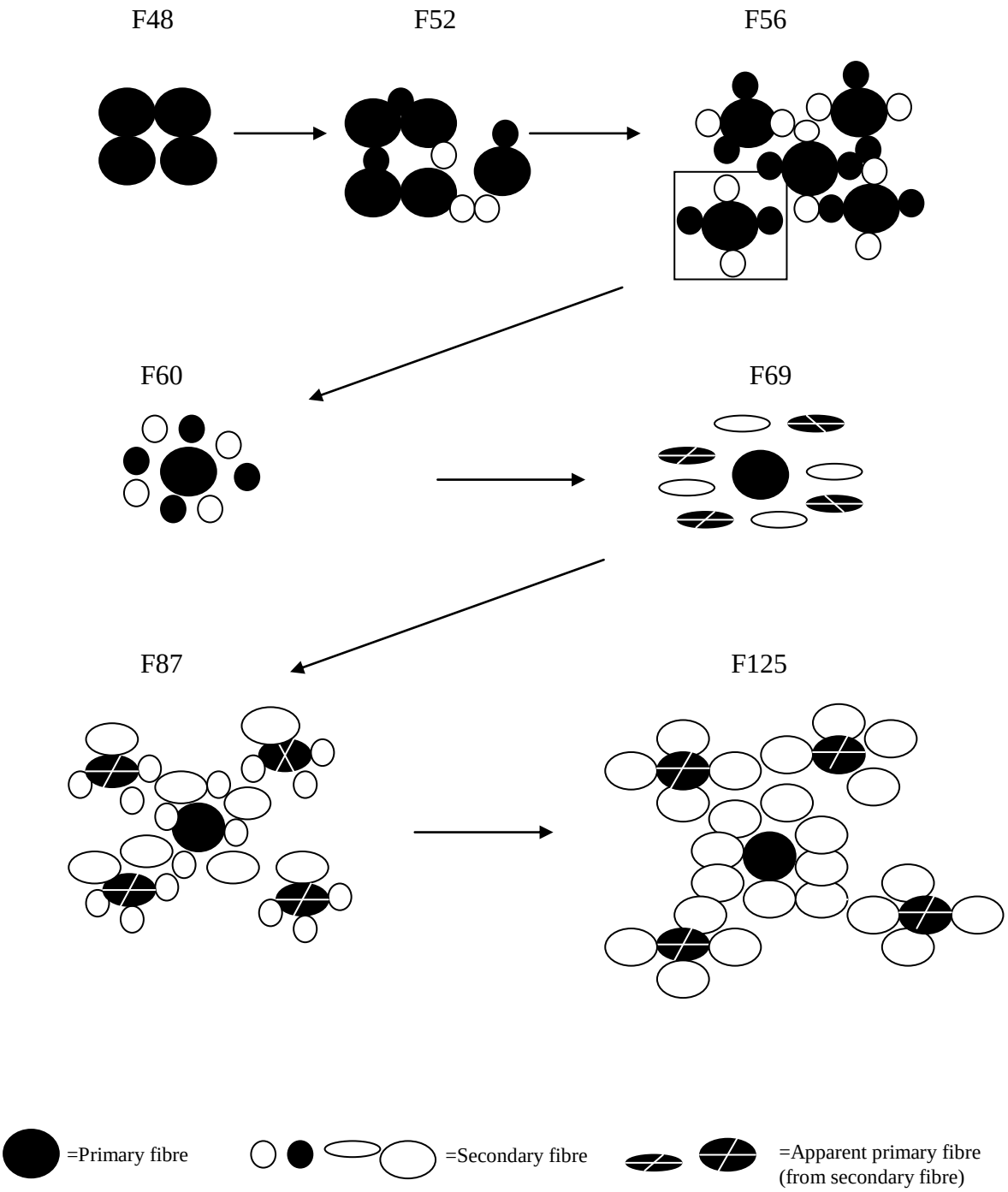


Figure 7. ATPase alkali staining of fetal semitendinosus muscle from 125 dg (×20 objektive).
Şekil 7. 125 günlük fötal semitendinosus kasında ATPaz alkali boyama (×20 objektif).

In the highlights of these observations the large, central and slow myosin expressing cells at near term (125 dg) originate from both primaries and secondaries. We cannot distinguish these large, central, slow expressing secondary fibers in late gestation from real primaries by ATPase (Figure 7) or slow myosin heavy chain antibody staining. Since some secondaries at this stage look like primaries (slow, large, central), we called them as apparent primaries in Figure 8.

We therefore conclude that some of the large, central, slow MHC expressing fibres at near term sheep fetus are probably secondary fibres. Some secondary fibres may express slow MHC at the onset of their formation.



Sometimes the secondaries forming on the apparent primaries are called tertiary fibres

Figure 8. Process of myogenesis in sheep.
Şekil 8. Koyunda miyogenez tasviri.

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