The Expression of Troponin I and Troponin T Isoforms in Some Orthotopic and Cross-transplanted Chicken Skeletal Muscles.

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Abstract

Muscle regeneration of tibialis anterior and pectoralis major was studied in orthotopic and cross-transplanted muscles of 3 week old chicks to investigate the role of muscle specific myogenic lineages and extrinsic factors in determining the muscle cell phenotype. The phenotype of regenerating muscles was analysed by investigating changes in the isoforms of troponin I and troponin T using immunoblotting procedure.

The muscle phenotype in terms of both troponin I and troponin T isoforms appeared to be influenced by the extrinsic factors since the muscle properties in the longer term corresponded to the site of the muscle transplant rather than to the satellite cells of the donor muscle. The role played by the pectoral muscle type myogenic lineage in influencing the muscle cell phenotype, however, was apparent from changes observed in the muscle-specific isoforms of troponin T particularly during earlier stages of regeneration.

Keywords: muscle, phenotype, regeneration, troponin T, troponin I.

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Most adult skeletal muscles are composed of different proportions of fast and slow skeletal fibre types. Fast and slow skeletal muscle fibres contain different isoforms of a number of myofibrillar proteins that impart unique biochemical and physiological properties to these muscles. Such characteristics of adult muscles have often been shown to relate to their patterns of innervation. This view is particularly illustrated by those studies in which muscle type characteristics have been shown to change by altering the nerve supply to the muscle [1]. The role played by thyroid hormones is also considered crucial in muscle cell differentiation since hypothyroidism has been shown to block transitions to adult muscle type myosins in developing rat skeletal muscles [2,7]. Changes in thyroid hormones in the adult rats have also been shown to change the proportions of fast and slow isoforms of a number of proteins [4,12].

It is still not known, however, whether differentiation into different muscle fibre types is entirely dependent on extrinsic factors or partly dictated by programmed commitment of different myogenic lineages. The existence of distinct embryonic myogenic cell lineages identified by the expression of fast and slow myosin isoforms in muscle cell differentiation has been demonstrated in the chicken [6,15]. The existence of different myogenic cell lineages in the adult skeletal muscles, however, has remained controversial perhaps due to the use of different species, muscles or molecular markers. For example, while cultures prepared from adult chicken fast and slow skeletal muscles have been shown to express different phenotypes based on both myosin light [11] and heavy chains [6], fast and slow skeletal muscles of rat were shown to express an identical phenotype in culture [5].

The process of muscle regeneration has also been used to assess the role of various factors in determining the muscle cell phenotype. In response to injury, the muscle undergoes regeneration and new muscle fibres are formed from the satellite cells located between the basal lamina and sarcolemma. The role played by myogenic cells produced by activation of satellite cells during muscle regeneration has also produced conflicting results. For example, while the muscle phenotype of rat soleus was reported to be entirely dependent on innervation [17], the myogenic lineage was reported to determine the expression pattern of troponin T in some chicken skeletal muscles [18]. The studies by Hoh & Hughes [8] using fast, slow

and superfast myosins as markers of muscle cell specific lineages have also ascribed a role for myogenic lineages in generating muscle fibre diversity.

The present study was undertaken to investigate the role of myogenic lineages in determining the muscle cell phenotype. The muscle phenotype in these studies was defined by observing changes in fast and slow isoforms of troponin I and also developmental and muscle specific differences in troponin T. This approach clearly demonstrated the role of extrinsic factors in influencing the muscle cell phenotype. The contribution by the myogenic lineages was also indicated by low level expression of some pectoral muscle-specific isoforms of troponin T during earlier stages of regeneration.

Materials and Methods

Surgical procedures

The fertilized eggs of white leghorn chickens were incubated at 38°C and hatched in the departmental animal house. The surgery for muscle grafting experiments was carried out on 35 three week old chicks using a combination of diazepam (2.5 mg/kg) and ketamine hydrochloride (50 mg/kg) as anaesthetic. These were injected intra-muscularly in the right thigh of the chick. Muscle grafts were prepared from the tibialis anterior (TA) and the pectoralis major from the left side of the chicks. The TA was cut anteriorly from near the tibial head and posteriorly midway along the insertion tendon. A region of the pectoralis major of equivalent dimensions (15x4x3mm) to the excised TA was resected from the middle superficial aspect of the muscle such that fibres were orientated along the length of the graft. All vascular and neural connections were severed. The excised muscles were injected with 0.25ml of 0.75% bupivacaine hydrochloride (Marcain) (Astra Pharmaceuticals Ltd, Kings Langley, UK) and immersed in the same solution for 5 minutes. The Marcain-treated muscles were blotted dry and either cross-transplanted or orthotopically grafted. The muscle grafts were sutured into position and the wounds closed. Two or three chickens at each time interval were sacrificed by cervical dislocation up to 34 weeks after surgery and the grafts dissected free.

Electrophoresis and immunoblotting procedure

Muscle samples were prepared for electrophoresis as described previously [14]. The muscle graft was carefully removed to exclude surrounding undamaged muscle tissue. One half of the graft was frozen in liquid nitrogen for histological investigation and the other half homogenised individually for electrophoresis. SDS polyacrylamide (10%) gel electrophoresis was performed by the method of Laemmli [10]. Protein was transferred from gels to nitrocellulose sheets by the method of Towbin *et al.* [16] and stained with monoclonal antibodies by the immunoperoxidase procedure reported earlier [14]. Monoclonal antibody F24 was used to identify all the fast isoforms of troponin T [3]. Monoclonal antibody 42/25 was used to identify fast, slow and cardiac isoforms of troponin I [13]. Different isoforms of both troponin I and troponin T could be recognised from their different electrophoretic mobilities on immunoblots.

Results

Expression patterns of troponin I isoforms in developing and regenerating skeletal muscles.

Both fast and slow isoforms of troponin I were observed in embryonic leg and pectoral muscles (Fig. 1 and 2). The slow troponin I also persisted during post-hatch development of the TA muscle although it was undetectable or present in only small amounts in some older chickens (Fig. 1 and 3). Slow troponin I, however, was not detected during post-hatch development of the superficial parts of the pectoral muscle (Fig.2). Changes in the proportions of fast and slow troponin I were also investigated during regeneration of TA and pectoral muscles transplanted into either orthotopic or heterotopic muscle beds.

The presence of a small proportion of slow troponin I was observed in orthotopic TA grafts throughout the 34 week period investigated (Fig. 3). TA muscle transplanted in pectoral muscle bed, however, showed only trace amounts

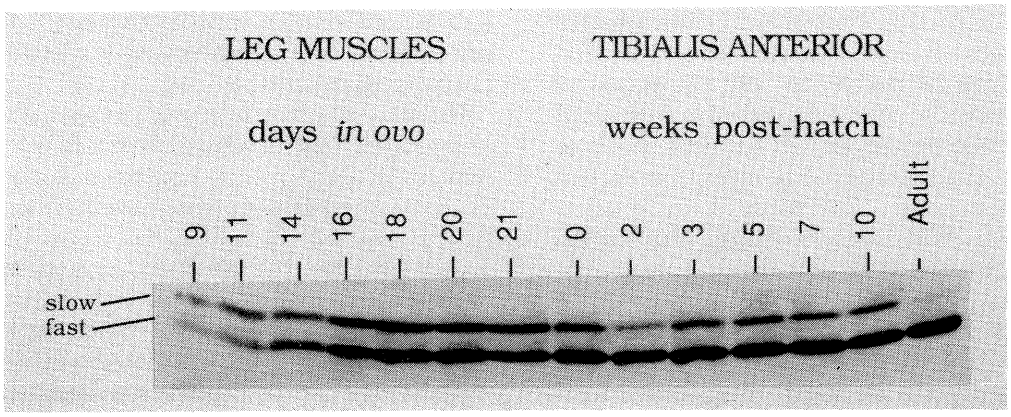

Figure 1. The pattern of fast and slow troponin I expression in developing chicken leg and tibialis anterior muscles. The Western blot of muscle extracts separated in SDS polyacrylamide gel was stained for troponin I with antibody 42/25 by immunoperoxidase procedure.

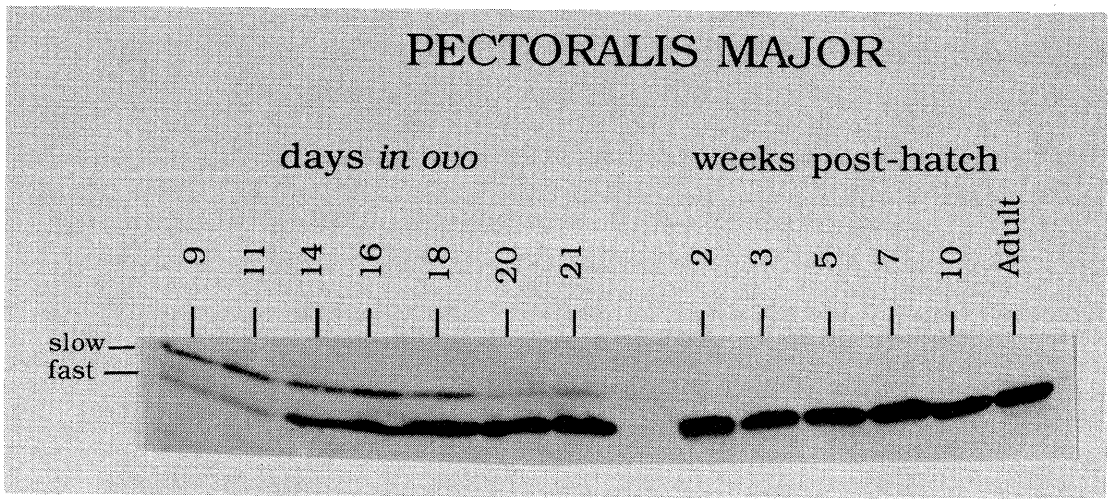

Figure 2. The expression pattern of fast and slow troponin I in the pectoralis major of developing chicken. The Western blot of muscle extracts separated in SDS polyacrylamide gel was stained for troponin I with antibody 42/25 by immunoperoxidase procedure.

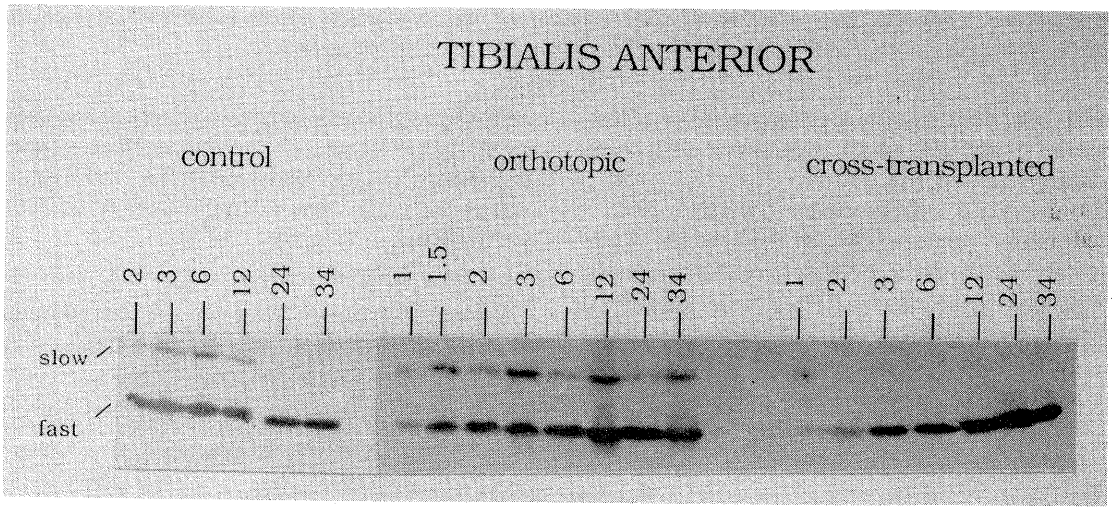

Figure 3. The expression pattern of fast and slow troponin I in contralateral control and transplanted tibialis anterior at different times, in weeks, after grafting. The Western blot of muscle extracts separated in SDS polyacrylamide gel was stained for troponin I with antibody 42/25 by immunoperoxidase procedure.

of slow troponin I although the level of fast troponin I increased gradually with time (Fig. 3).

In orthotopic pectoral muscle, slow troponin I was only detected at 1 week post-surgery whereas fast troponin I was observed throughout the 34 week period of transplantation (Fig. 4). Pectoral muscle cross-transplanted into the TA bed, however, expressed small amounts of slow troponin I at most stages except at 34 weeks post-surgery (Fig. 4).

Expression patterns of troponin T isoforms in normal and regenerating skeletal muscles

Control muscles

The immunoblotting studies showed that chicken TA muscle expressed only lower molecular weight isoforms of fast troponin T, called leg muscle type troponin T, throughout the developmental period investigated in the present study. The pectoral muscle started expressing the higher molecular weight isoforms of troponin T after hatching leading to reduction in the level of leg type troponin T isoforms associated with earlier stages of development (not shown). This study thus confirmed our earlier observations of the appearance of both neonatal (N1 and N2) and adult (B1 and B2) isoforms of troponin T during the post-hatch period of growth [3]. Similar to earlier studies [3], some minor bands with electrophoretic mobilities intermediate to pectoral and leg type troponin T were also observed.

TA muscle transplants

In orthotopic regenerating TA, very little fast troponin T was detectable at the 1 week stage although a low level expression of leg muscle type troponin T could be observed even at this stage. No pectoral muscle type isoforms

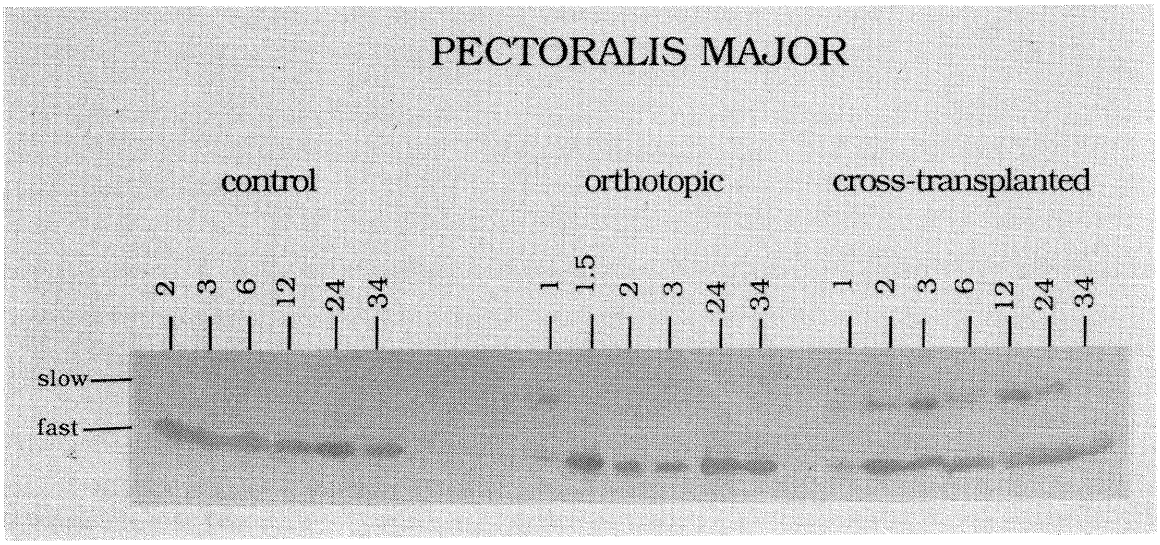

Figure 4. The expression pattern of fast and slow troponin I in contralateral control and transplanted pectoralis major at different times, in weeks, after grafting. The Western blot of muscle extracts separated in SDS polyacrylamide gel was stained for troponin I with antibody 42/25 by immunoperoxidase procedure.

of troponin T were detected.The level and number of leg muscle type troponin T isoforms increased gradually with time after transplantation (Fig. 5).

Hardly any troponin T was detected in TA muscle cross-transplanted into pectoral muscle bed for 1 week. Leg muscle type and all the pectoral muscle type troponin T isoforms including the neonatal isoforms, were detectable 1.5 weeks post-transplantation (Fig. 5). The neonatal isoforms were gradually suppressed and became undetectable by the 12 week stage. The level of leg muscle type troponin T isoforms in cross-transplanted TA, however, appeared somewhat higher than in similar regions of contralateral pectoral muscle (Fig. 5).

Pectoral muscle transplants

Very little troponin T was observed in orthotopic pectoral muscle at 1 week after transplantation. One major

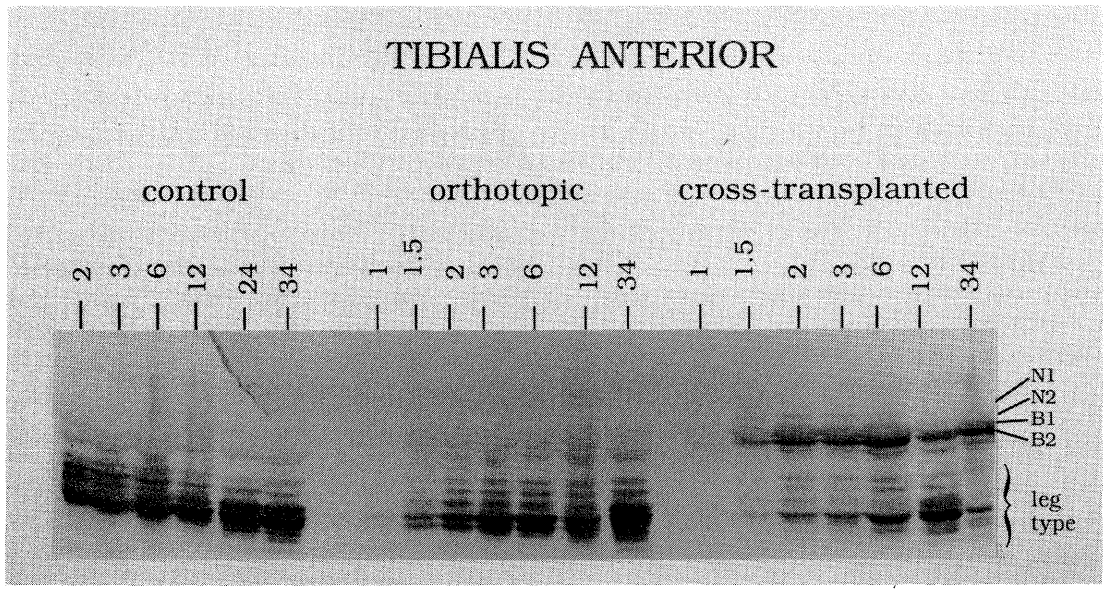

Figure 5. The expression of pectoral muscle type adult (B1 and B2), neonatal (N1 and N2) and leg type troponin T isoforms in contralateral control and transplanted tibialis anterior at different times, in weeks, after grafting. The Western blot of muscle extracts separated in SDS polyacrylamide gel was stained for fast troponin T with antibody F24 by immunoperoxidase procedure.

protein band was observed at this stage and it appeared to correspond to a pectoral muscle type troponin T, called B2 (Fig. 6). Virtually all the pectoral and leg type troponin T isoforms, however, could be observed 1.5 weeks after orthotopic grafting of this muscle (Fig. 6). The level of neonatal isoform N1 decreased after 3 weeks and the level of N2 after 24 weeks of transplantation. The orthotopic pectoral muscle at 34 weeks post-surgery appeared similar to the contralateral control (Fig.6). The level of leg type troponin T isoforms, however, was higher in these transplants than in similar regions of the contralateral controls.

Pectoral muscle transplanted into the TA muscle bed also showed very little troponin T at 1 week after transplantation and was similar to orthotopic pectoral muscle in producing mostly the B2 isoform of troponin T at this stage (Fig. 6). The level of B2 isoform in these transplants was lower than the leg muscle type troponin T isoform but it continued to be expressed at detectable levels until 12 weeks after transplantation. Trace amounts of other pectoral muscle type isoforms were also apparent in some samples during early stages of regeneration. The leg muscle type troponin T, the major component, was detected throughout regeneration of pectoral muscle transplanted in TA bed.

Discussion

There was very little protein, either troponin I or troponin T one week after surgery in both orthotopic and heterotopic transplants indicating the degeneration of most of the original fibres of muscles used as transplants. The presence of regenerating myotubes soon became apparent from the expression of not only some adult but also neonatal isoforms of troponin T in both pectoral and TA muscles transplanted in pectoral muscle beds. The degeneration of original muscle fibres and regeneration of new myotubes was also apparent from the histological investigation of these transplants (unpublished observations).

The importance of extrinsic factors in determining the muscle cell phenotype was apparent from studies of both troponin I and troponin T isoforms. For example, the appreciable amounts of slow troponin I were only observed in control TA muscles and muscles transplanted in TA muscle bed whether derived from pectoral or TA muscles. Fast troponin I was the major isoform expressed in pectoral muscle or muscles transplanted into the pectoral muscle bed. The role of extrinsic factors in determining the muscle cell phenotype was also clear using troponin T isoforms as muscle and developmental stage specific markers. For example, only the muscles transplanted in pectoral muscle bed whether orthotopic or heterotopic, produced large amounts of pectoral muscle type adult and neonatal isoforms of troponin T. The nature of extrinsic factors, however, was not established in the present study since all the transplants were allowed to be reinnervated. The appearance of all the fast isoforms of troponin T, both pectoral muscle type and leg muscle type, at only 1.5 weeks not only in orthotopic pectoral muscle but also in TA muscle transplanted in pectoral muscle bed indicates the possible role of some other factors in addition to innervation in determining the muscle cell phenotype. Although Whalen *et al.* [17] demonstrated the influence of

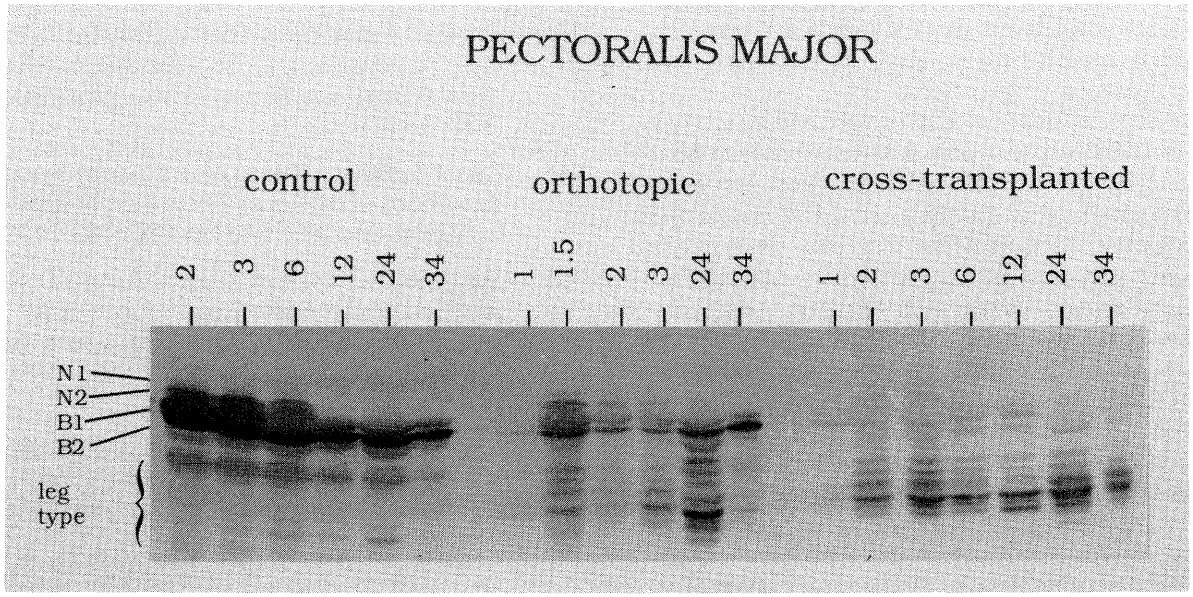

Figure 6. The expression of pectoral muscle type adult (B1 and B2), neonatal (N1 and N2) and leg type fast troponin T isoforms in contralateral control and transplanted pectoralis major at different times, in weeks, after grafting. The Western blot of muscle extracts separated in SDS polyacrylamide gel was stained for fast troponin T with antibody F24 by immunoperoxidase procedure.

nerve on regenerating muscle within only a few days of intact muscle injured with a snake toxin, the innervation in our study in which the muscle is removed completely before Marcain injury, is expected to require longer to establish. The nerves have been reported to enter Marcain-injured transplants in cat hindlimb within 9 days but functional neuromuscular junctions are not believed to be established at this stage [8]. However, the nerve may also exert its influence on transplants through some neurotrophic factors. It may take up to four weeks to establish fully functional neuromuscular junctions since no differences in aneural and innervated muscle transplants were reported by Hoh & Hughes [9] during the first four week period of transplantation.

The existence and role of myogenic cell lineages in specifying the muscle cell phenotype did not become very apparent using troponin I as a marker of muscle cell differentiation in immunoblotting studies. A small amount of slow troponin I was observed in orthotopic pectoral and only a trace amount in TA cross-transplanted into the pectoral muscle bed at one week post-surgery. The muscle cell phenotype determination by myogenic lineage would predict the presence of somewhat higher levels of slow troponin I in at least the early cross-transplanted TA muscles. Despite detecting slow troponin I during embryonic development of both TA and pectoral muscles, large amounts of slow troponin I were not detected in regenerating myotubes of either of these muscles. The presence of slow troponin I in all the early regenerating muscles would also be expected if it acted as a developmental isoform as indicated by normal developing chicken skeletal muscles. This did not appear to be the case for most of these muscle transplants since fast troponin I was the major isoform expressed during all stages of regeneration. Significant levels of slow troponin I were only detected in muscles regenerating in TA muscle bed whether orthotopic or heterotopic.

The presence of small amounts of a pectoral muscle type troponin T (B2) in only pectoral muscles transplanted in both pectoral and TA muscle beds after 1 week post-transplantation clearly points to the existence of muscle-specific myogenic lineages which may play some role in influencing the muscle cell phenotype. Although the presence of a small amount of this isoform in the cross-transplanted pectoral muscle could also result from the persistence of a few original surviving fibres, the continued presence of this protein isoform in all such transplants expressed at very similar levels until 12 weeks of transplantation, however, makes it highly unlikely to be due to carryover from the donor muscle. A very low level expression of the other pectoral muscle isoforms e.g. B1 and neonatal isoforms, could also be observed in some cross-transplanted pectoral muscles during earlier stages of regeneration. While the level of pectoral type troponin T in cross-transplanted pectoral muscle remained low, the presence of B2 nevertheless was detectable until 12 weeks and became undetectable at only 24 and 34 weeks indicating the influence of probably innervation or some other extrinsic factors overriding the residual muscle properties dictated by the myogenic lineage. Our studies thus indicated the existence of myogenic lineages and their possible role in specifying the muscle cell phenotypes but the extrinsic factors appeared to play a major role in modulating the muscle cell phenotype. Yao *et al.* [18] using a different procedure of muscle damage in newly hatched chicks reported the troponin T expression to be primarily determined by cell lineage since the transplantation of a pectoral muscle in the leg muscle bed expressed the pectoral muscle type troponin T.

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