

Comparative expression analysis of Pax3 and Pax7 during mouse myogenesis

DAVID HORST^{1,4, #}, SVETLANA USTANINA², CONSOLATO SERGI³, GREGOR MIKUZ³, HERBERT JUERGENS¹, THOMAS BRAUN² and EUGENE VOROBYOV^{4,*}

¹Klinik und Poliklinik für pädiatrische Hämatologie/Onkologie, Universitätsklinikum Münster, Germany, ²Max Planck Institut für Herz- und Lungenforschung, Bad Nauheim, Germany, ³Institut für Pathologie, Medizinische Universität Innsbruck, Austria and ⁴Institut für Humangenetik, Universitätsklinikum Münster, Germany

ABSTRACT *Pax3* and *Pax7* are closely related transcription factors involved in the commitment of myogenic precursors in the developing trunk. However, it is not yet clear whether these genes are required for myogenic cell specification in the head and for post-somitic myogenesis *per se*. In part, this uncertainty is due to the scarce information about their normal time course and pattern of expression. Here, we present a systematic immunohistochemical *in situ* analysis of spatiotemporal characteristics of Pax3 and Pax7 protein expression in comparison to that of MyoD and myogenin in the developing trunk and head muscles. The observed patterns of expression suggest that Pax3 is not involved in myogenesis in the head and its post-somitic expression in the trunk and limb muscles is mostly repressed after stage E13.5. In contrast, Pax7 expression is shared among all striated muscles and exhibits a uniform pattern. Pax7 is expressed only in mononucleated cells that either differentiate into myotubes or later form satellite cells. During development of head muscles, expression of Pax7 follows expression of MyoD and myogenin, implying that Pax7 is not required to induce the initial steps of the myogenic program in the head. In Pax7 homozygous mutants, in which muscle development proceeds normally, expression of Pax3 is indistinguishable from its wild-type pattern (i. e. absent), suggesting that after stage E13.5 myogenesis does not require Pax3 and Pax7. These data challenge the concept that Pax3 and Pax7 determine a persistent lineage of myogenic precursors in pre-natal and post-natal muscle development.

KEY WORDS: *Pax3*, *Pax7*, myogenesis, development

Introduction

Myogenesis is a multi-step process consisting of the determination of mesodermal precursor cells, myoblast migration and proliferation, cell fusion and terminal differentiation of myotubes. In vertebrates, all these steps are governed by sequential and compound activation of four myogenic regulatory factors (MRFs) that belong to the *MyoD* gene family: *Myf5*, *MyoD*, *Mrf4* and *myogenin* (see Arnold and Braun, 1996). Functions of these genes have been defined by analyses of their single, double and triple mutants in mice. Recent data show that *Myf5* and *Mrf4* play partially non-redundant roles in the determination of muscle identity and act upstream of *MyoD* (Kassar-Duchossoy *et al.*, 2004). In turn, function of *MyoD* significantly overlaps with functions of *Myf5* and *Mrf4*. Double mutants of *MyoD* with either *Myf5* or *Mrf4* are characterized by an absence of skeletal muscles at

birth (Rudnicki *et al.*, 1993; Kassar-Duchossoy *et al.*, 2004). In mice lacking *myogenin*, myoblasts are specified and positioned correctly, but their fusion capacity is greatly impaired. This indicates that *myogenin* is critical for terminal differentiation of myoblasts (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). Analysis of *Myf5* *MyoD* double mutants with an intact *Mrf4* gene revealed that skeletal myogenesis was absent in the head and, at later stages, severely compromised in the trunk, thus excluding a role for *Mrf4* in the development of head muscles (Kassar-Duchossoy *et al.*, 2004). Apparently, *Myf5*, *MyoD* and *myogenin* are essential activators of the myogenic program that are required in virtually all striated muscles. In contrast to the well established role of MRFs for muscle development, much less is known about genes which have been proposed to act upstream of MRFs to determine early

Abbreviations used in this paper: MRF, myogenic regulatory factor.

*Address correspondence to: Dr. Eugene Vorobyov, Institut für Humangenetik, Universitätsklinikum, Vesaliusweg 12-14, 48149 Münster, Germany.
Fax: +49-251-83-56995. e-mail: vorobie@uni-muenster.de

#Present address: Institut für Pathologie, Medizinische Universität Innsbruck, Austria.

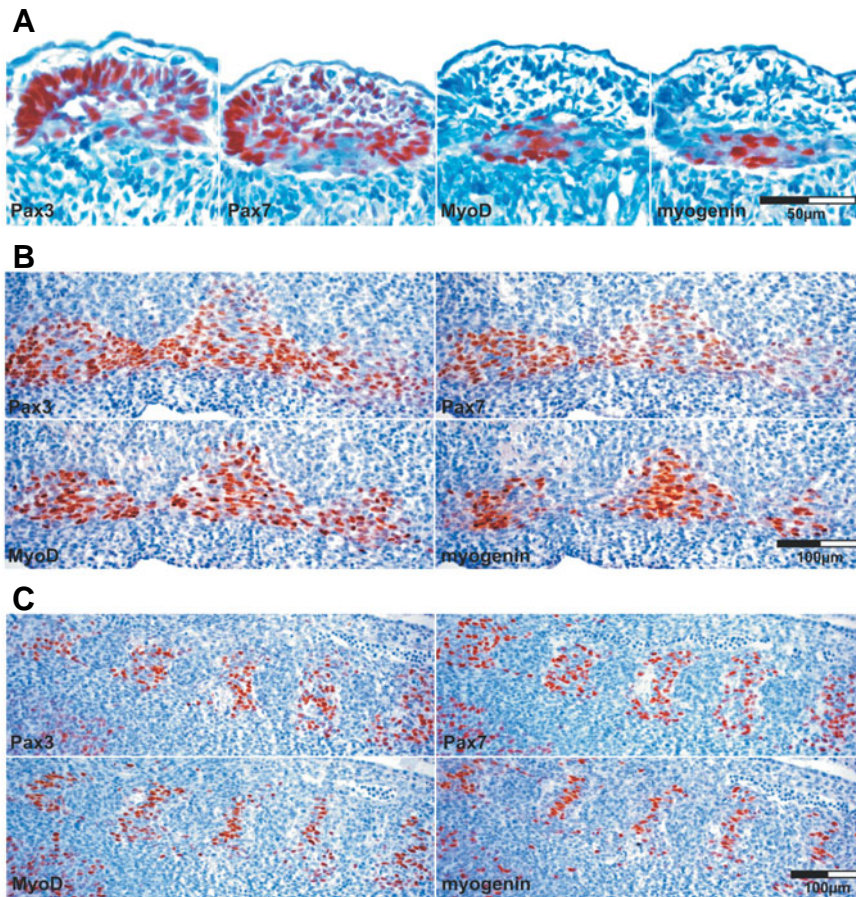


Fig. 1. Expression of Pax3, Pax7, MyoD and myogenin in somites. (A) *The dermatome and myotome at E11.5 and the myotome at E11.5 (B) and E12.5 (C). The location of sections is shown in Figure 9.*

steps of myogenic lineage commitment.

Two members of the *Pax* family of transcriptional factors, *Pax3* and *Pax7*, are supposed to be involved in the myogenic cell specification. Both genes are known as early markers of the forming dermomyotome, which is the source of future myoblasts (Goulding *et al.*, 1991; Jostes *et al.*, 1990). The spontaneous *Pax3* mutation, *Spotch*, when homozygous, is associated with the inability of myogenic precursors to migrate from somites into the growing limbs and the developing tongue and results in the complete absence of the respective muscles (Bober *et al.*, 1994; Goulding *et al.*, 1994; Tremblay *et al.*, 1998). Homozygous *Pax7* knockout mice develop normally but the newborns show a slightly reduced muscle mass and most mutants survive only a few weeks (Mansouri *et al.*, 1996; Oustanina *et al.*, 2004). Analysis of the affected muscles revealed a huge decrease of the number of satellite cells after birth indicating a role for *Pax7* in satellite cell self-renewal (Seale *et al.*, 2000; Oustanina *et al.*, 2004). Double knockouts of *Pax3* and *Pax7* resulted in a compromised development of somites and embryonic lethality at mid-gestation, suggesting a redundancy in functions of both genes during somitogenesis. In these compound mutant mice myotomal myogenesis is initiated but resident muscle progenitor cells do not follow the myogenic program (Relaix *et al.*, 2005). Evidence for a potential

involvement of *Pax3* in myogenesis was also demonstrated by analysis of compound mutants of *Pax3* and *Myf5* (Tajbakhsh *et al.*, 1997). In the double homozygous embryos, skeletal muscle development in the trunk was missing, although the head muscles were essentially unaffected. The latter finding might be explained by a compensative role of *Pax7* in the head myogenesis, although this possibility was excluded in the same study since expression of both genes in the head mesoderm was not detected at stage E10.5. In chicken embryos, however, *Pax7* is expressed in at least one developing head muscle, the lateral rectus (Mootoosamy and Dietrich, 2002). In this study, the rudiment of the largest extrinsic eye muscle was found to co-express *Pax7* and an early somitic mesoderm marker *Paraxis* suggesting that in the cranial paraxial mesoderm *Pax7* may work upstream of MRFs and substitute for *Pax3*.

Currently, it is not clear whether and to what extent *Pax3* and/or *Pax7* contribute to the progression of the myogenic process or whether they are involved in the development of head muscles. Skeletal muscles in the head and trunk are distinct in their origin. Most craniofacial muscles, except the tongue and laryngeal muscles, derive from the non-segmented pre-chordal and pre-otic mesoderm. Recent data indicate that distinct regulatory cascades operate in the head and trunk myogenesis that rely on either *Pax3* or *Pax7* (Hacker and Guthrie, 1998; Mootoosamy and Dietrich, 2002). However, little is known about the normal time course and pattern of expression of these *Pax* genes in relation to the expression of MRFs in post-

somatic and head myogenesis. Here, we present a systematic *in situ* analysis of spatiotemporal characteristics of *Pax3* and *Pax7* gene expression on the protein level during development of skeletal muscles of the trunk and head.

Results

Expression patterns of the *Pax3*, *Pax7*, *MyoD* and *myogenin* genes were analysed during mouse embryogenesis starting at stage E11.5 and in post-natal muscles. The respective proteins were immunohistochemically detected on adjacent paraffin sections using monoclonal antibodies, which were previously characterized as highly specific to the corresponding antigens. Expression of *MyoD* was considered as a marker of determined myoblasts, whereas *myogenin*-positive cells were judged as myoblasts that are more advanced in their differentiation (see introduction). In this study, we focused on the time course and spatial expression of *Pax3* and *Pax7* in respect to myogenic cells identified by their expression of MRFs.

Expression of *Pax3* in developing muscles

Previously it was demonstrated on the RNA level that expression of *Pax3* in the trunk region starts at E8.5, coinciding with

segmentation of the mesoderm and begins to disappear at E11.5 during somite disintegration (Goulding *et al.*, 1991). To achieve a better understanding of the role of *Pax3* at later stages of myogenesis we have focused our analysis on the expression of *Pax3* from E11.5 onwards. At E11.5 Pax3-positive cells in the somites constituted the dermomyotome and delaminating myotome. However, only cells located within the myotome expressed *MyoD* and *myogenin* (Fig. 1 A,B and data not shown). Not all cells in these structures were positive for either of these proteins. *Pax3* was expressed in 79% ($\pm 5.5\%$) of all cells in the MyoD-positive areas. At E12.5, staining intensity for Pax3 did not decline, but Pax3-positive cells were found in a more diffuse allocation, constituting 56% ($\pm 4.6\%$) (Fig. 1C). This pattern of distribution of Pax3-positive cells was observed in all MyoD-positive regions, which at this stage include pre-muscle masses in different positions in the trunk and in the limbs. These muscle anlagen are known to be formed by cells actively migrating out of the myotome. In addition, *Pax3* was expressed in the hypoglossal cord, consisting of myoblasts migrating from the occipital trunk somites to the tongue (Fig. 2). At E13.5, some differentiated muscle fibers can be already distinguished by the tandem locations of the *myogenin*-positive nuclei. Starting at this time, most of the MyoD-positive areas were Pax3-negative, including muscles of the tongue and limbs (Fig. 3 A,B and data not shown). However, in some muscle groups, e.g. those surrounding the sternum and located ventrally to the brown adipose tissue, a weak staining for Pax3 was observed in a few nuclei until E16.5 (Fig. 3C). At later stages, we did not detect Pax3-specific nuclear signals above background even after extended staining (data not shown). No Pax3 expression was detected at any stage in the MRFs-positive areas in the head (Figs. 2,4 and data not shown). In summary, the post-myotomal expression of *Pax3* in the trunk was characterized by a restriction in cell number and complete disappearance in most of the differentiating muscles. Furthermore, expression of

Pax3 was not detected in newborn and adult muscle tissues of the leg, trunk and head (Figs. 5,6 and data not shown). Similar results were obtained using polyclonal anti-Pax3 antibodies (data not shown).

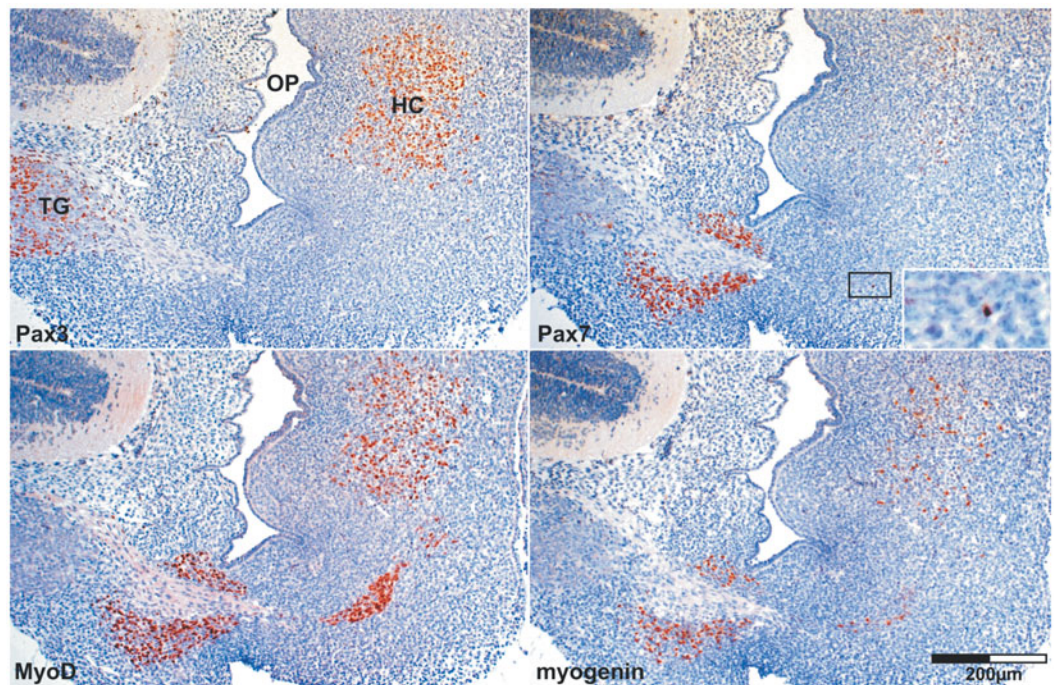
To investigate a putative redundancy between *Pax3* and *Pax7* we also have analysed the expression of *Pax3* in *Pax7* knockout mice. *Pax7*^{-/-} embryos were examined at E11.5 and E15.5, when *Pax3* is expressed and not expressed in wild type embryos, respectively. The expression of *Pax3* in the mutant embryos did not differ from its expression in wild type littermates (Fig. 6 A,B and data not shown). In addition, we also did not find Pax3 protein in skeletal muscles of newborn and adult *Pax7*^{-/-} mice (Fig. 6C and data not shown).

Expression of Pax7 in developing muscles

It is well known that between E9-E11 the expression of *Pax7* in the trunk largely overlaps with the expression of *Pax3*. During somitogenesis *Pax7* is activated in the dermomyotome after *Pax3* but before the onset of expression of MRFs (Jostes *et al.*, 1990). However, little is known about expression of *Pax7* during myogenesis in the head. Previously, it has been reported that *Pax7* was not expressed in the head mesenchyme at E10.5 (Tajbakhsh *et al.*, 1997). We also did not observe Pax7-positive cells in the head at this stage (data not shown). At E11.5, we detected Pax7 in cells of the MRFs-positive areas in the trunk and head mesenchyme. In the trunk, Pax7-positive cells marked the dermomyotome and myotome in a pattern very similar to Pax3 (Fig. 1A). During the next stages the spatio-temporal expression of Pax7 resembled that of Pax3. In particular, we noted a decrease of the number of the Pax7-positive cells (Fig. 1B). At E13.5, however, in contrast to Pax3, Pax7 was expressed in 35% ($\pm 3.3\%$) of all cells in MyoD-positive areas (Fig. 3A). At later stages, the number of Pax7-positive cells declined further (Figs. 3B,4 and data not shown). However, it was difficult to quantify the

Fig. 2. Expression of Pax3 and Pax7 in the cranial region including the third branchial arch at stage E11.5.

Pax3 is expressed in migrating myoblasts, which constitute the hypoglossal cord but not in the muscle anlagen of the branchial arch. In contrast to *Pax3*, *Pax7* is expressed in the developing muscles of the branchial arch. Note, that the pre-muscle mass penetrated by the trigeminal nerve is in a more advanced developmental stage compared to the distal muscle mass (a Pax7 positive nucleus shown in the magnified area) and to the extrinsic eye muscles (Fig. 7) and hence contains more Pax7-positive nuclei. HC, hypoglossal cord; OP, oropharynx; TG, trigeminal (V) ganglion.



remaining Pax7-positive cells due to their irregular distribution along the differentiated myofibers, which are not fully represented on the sections. A reverse pattern of *Pax7* expression was observed during myogenesis in the head. In contrast to somites, where activity of *Pax7* preceded expression of MRFs, in the head the MRFs were expressed prior to *Pax7*. At E11.5, MyoD and myogenin were found in small areas corresponding to the future extrinsic eye muscles (Fig. 7) and in muscle anlagen of the branchial arches, which are penetrated by nerves of the fifth cranial ganglion (Fig. 2). Myogenin was present in fewer cells than MyoD within the respective areas, reflecting a later function in myogenesis. Expression of *Pax7* just started at this stage and was found in a very small number of cells. Figure 7 shows that only few Pax7-positive nuclei were present on section No.2, compared to several MyoD and myogenin positive nuclei on sections No.1 and No.3, respectively. The order of sections excluded the possibility that fewer Pax7-positive nuclei were detected due to a marginal position of the section in respect to the MRFs-positive area. After E15.5, the spatial pattern of *Pax7* expression in the developing cranial muscles was very similar to the situation in the trunk, where Pax7 was present in only a few cells within the MyoD-positive areas (Fig. 4 and data not shown). Pax7 was found only in mononucleated cells, the number of which declined with ongoing development. Co-staining with anti-laminin antibodies revealed that Pax7 was present exclusively in the satellite cells of head and body muscles of newborn and adult mice (Figs 5,8 and data not shown).

Discussion

Pax3 is not associated with myogenic development after E13.5

The observed expression pattern of Pax3 suggests that the function of this gene is not associated with myogenesis in the head but transiently correlates with the beginning of muscle development in the trunk and limbs. In the head only the hypoglossal cord did contain cells that were positive for MRFs and Pax3. This structure, however, does not originate from the head but is formed by myoblasts that actively migrate from the occipital trunk somites to the tongue anlage. Muscles of the developing tongue were Pax3-negative. The post-somitic expression of Pax3 in the trunk and limbs was repressed in most muscles after E13.5. A very weak Pax3 signal, detected until E16.5 in rare nuclei of distinct muscle groups, might be explained by the different timing of the development of individual muscles. The negative staining for Pax3 in muscles at later stages was also corroborated using polyclonal anti-Pax3 antibodies. Since polyclonal antibodies recognize multiple

epitopes, it can be excluded that the negative staining using the monoclonal antibody was due to masking of the epitope by a posttranslational modification.

Our observation that *Pax3* is expressed only transiently during muscle development is not in agreement with recently published data, which postulate expression of *Pax3* during pre-natal and post-natal myogenesis and even in satellite cells of adult mice (Relaix *et al.*, 2005). This apparent inconsistency might be explained by the different approaches used to detect Pax3 expression. We used a monoclonal antibody that recognizes Pax3 with a high affinity and produces no background allowing very sensitive antigen detection. The study by Relaix *et al.* (2005) is based on the detection of the green fluorescent protein (*GFP*) reporter targeted to the *Pax3* gene. Obviously the artificial *Pax3-GFP* allele does not faithfully recapitulate expression of the endogenous *Pax3* gene. It is possible that the integration of the *GFP* reporter gene resulted in silencing of control elements that are normally required for the down-regulation of *Pax3*. Alternatively, it might be envisioned that the GFP protein used to detect Pax3 expression is more stable than the actual Pax3 protein.

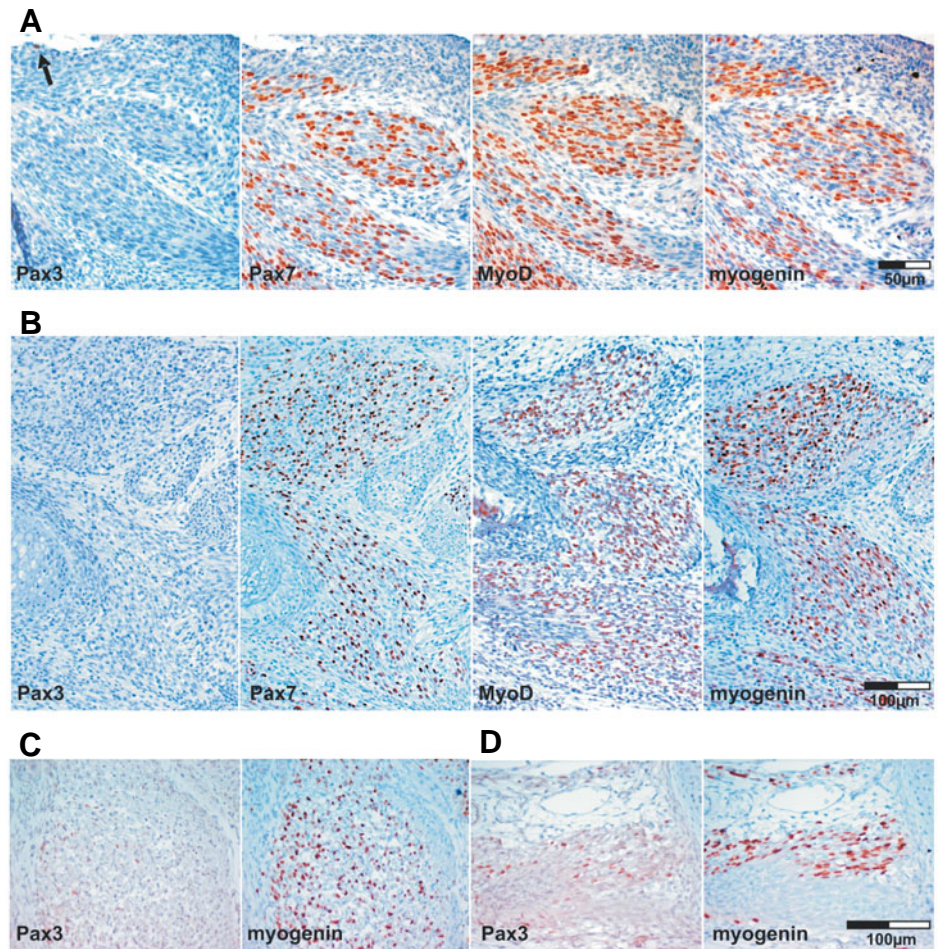


Fig. 3. Expression of Pax7 and MRFs in the developing limb muscles. (A) Stage E13.5, (B) E15.5 and (C and D) E16.5. At stage E13.5 most of the muscle groups are Pax3-negative. (A,B) represent muscle groups in a region of the humerus. (C,D) represent the Pax3-positive muscles located under the brown adipose tissue and surrounding the sternum, respectively. Note the expression of Pax3 in melanocyte precursor cells (arrow) within the dermal layer of the skin (disrupted during sectioning), which serve as a positive control.

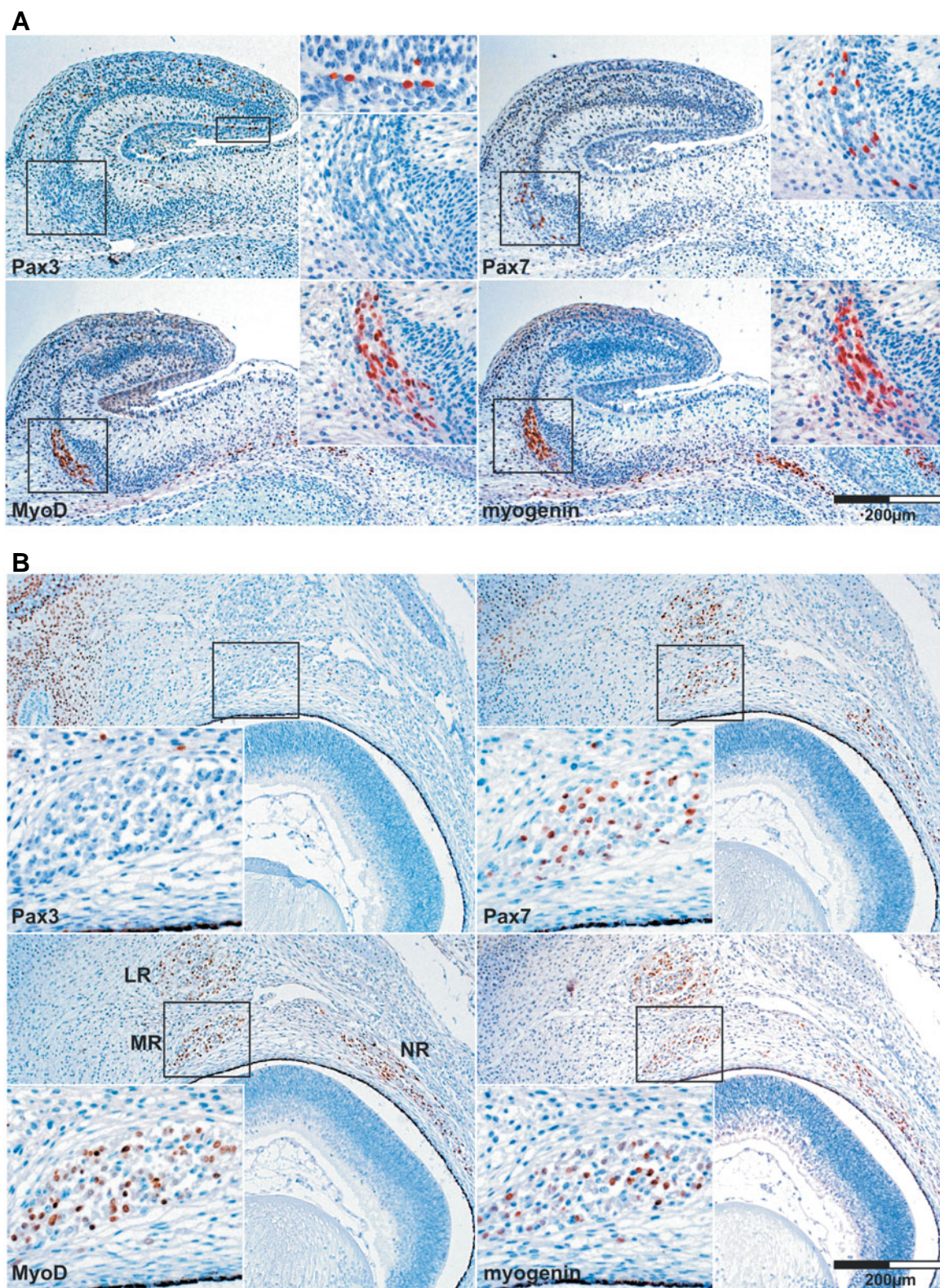


Fig. 4. Expression of Pax7 and MRFs at stage E15.5 in the developing ear (A) and extrinsic muscles of the eye (B). The muscle anlagen are Pax3 negative. LR, lateral rectus; MR, medial rectus; NR, nasal rectus. The framed areas are displayed at higher magnification.

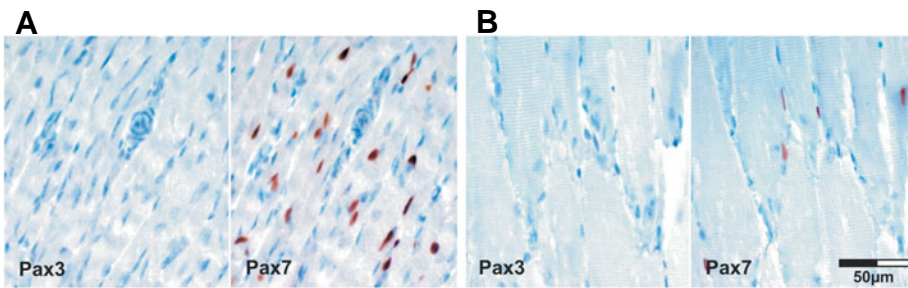
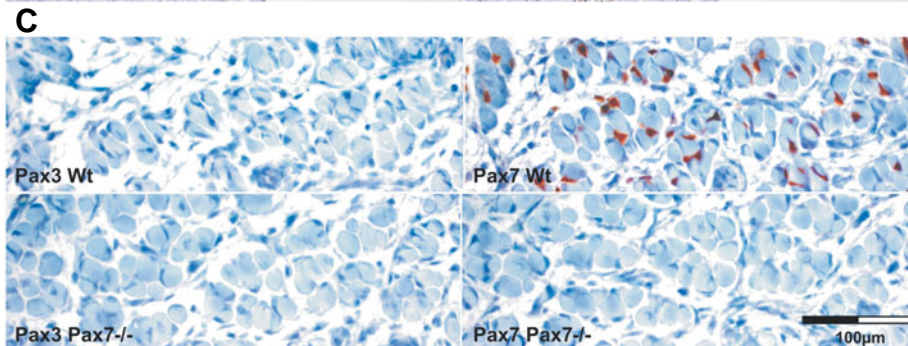
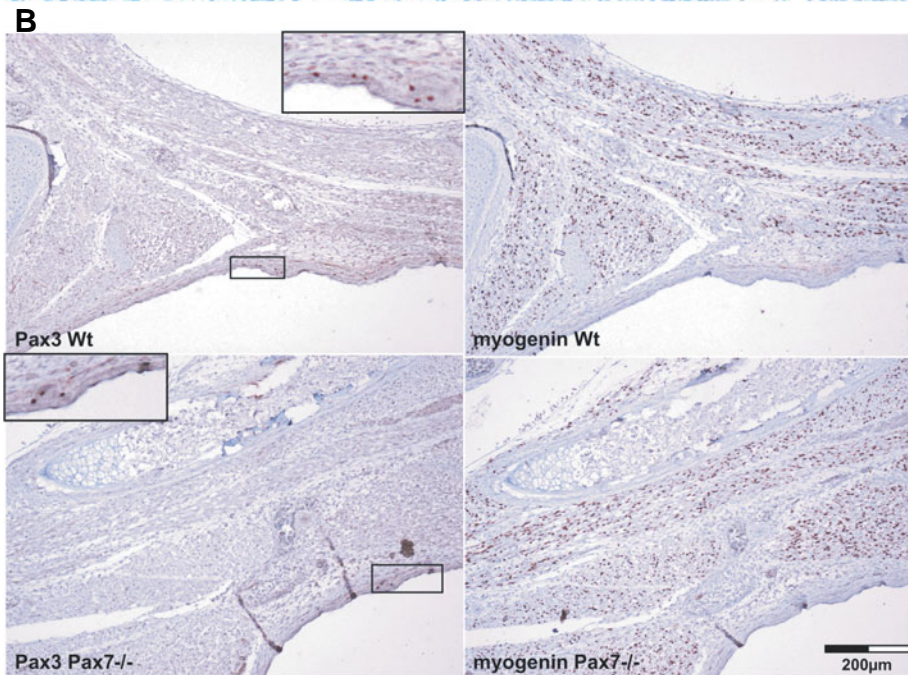
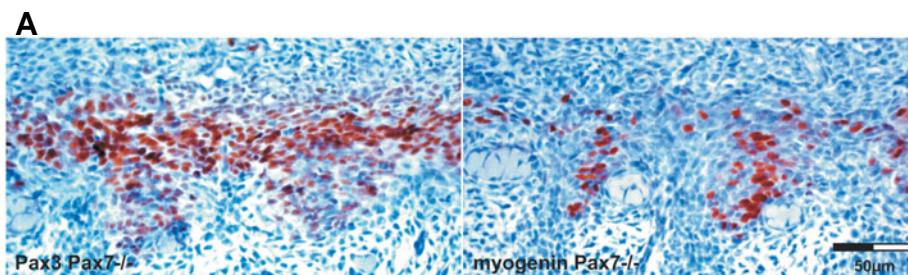


Fig. 5. Expression of Pax3 and Pax7 in the intercostal muscles of newborn (A) and adult (B) mice. Pax3 is not detected in the differentiated muscle. Pax7 is expressed in the satellite cells of both newborn and adult mice.



Pax3 is not de-repressed or re-activated in muscles of Pax7^{-/-} mice

Pax3^{-/-} mutant embryos die at E13.5, due to multiple malformations including deformities of the heart, failure of the neural tube closure and lack those muscles, which develop from the migrating myoblast precursors (Tremblay *et al.*, 1998). Due to the early embryo lethality it is not possible to analyse whether *Pax7* compensates for the absence of *Pax3* in other developing muscles. On the other hand, muscle development in *Pax7*^{-/-} mutants was unaffected (Mansouri *et al.*, 1996). Although this finding might suggest a possible compensative role of *Pax3* we did not find a compensatory up-regulation of *Pax3* in muscles of *Pax7* mutants making this possibility less likely.

Pax7 is uniformly expressed in muscles of the head and trunk

In contrast to *Pax3*, *Pax7* is expressed in developing head muscles. Head and trunk muscles differ in their developmental origin. Muscles of the trunk derive from segmented somitic mesoderm. Cranial muscles (with the exception of the muscles of the tongue, which have a somitic origin) develop from pre-chordal and pre-otic non-segmented mesoderm. There is experimental evidence that different regulatory cascades drive the initiation of myogenesis in the head and trunk (Hacker and Guthrie, 1998; Mootoosamy and Dietrich, 2002; Lu *et al.*, 2002; Tzahor *et al.*, 2003). On the other hand, a recent transplantation study established that the head environment is able to support progressive differentiation of several distinct myogenic progenitor populations from the trunk (Borue and Noden, 2004). In light of these data it was important to determine whether *Pax7* is expressed in head muscle. As shown in Figure 5, the onset of *Pax7* expression followed the expression of MRF genes suggesting that *Pax7* is not directly associated with the onset of myogenesis in the head

Fig. 6. Comparison of Pax3 expression in muscles of Pax7^{-/-} and wild type mice.

(A) *Pax3* is expressed in the myotome of *Pax7* mutant mice at E11.5 (see also Fig. 1B). *Pax3* is not de-repressed or re-activated in muscles of this mutant at E15.5 (B) and in newborns (C). Magnified areas show *Pax3*-positive melanocyte precursors, which serve as an internal positive control. Myogenic cells on adjacent sections are indicated by myogenin staining. (B,C) represent the thoracic and hind leg muscles, respectively.

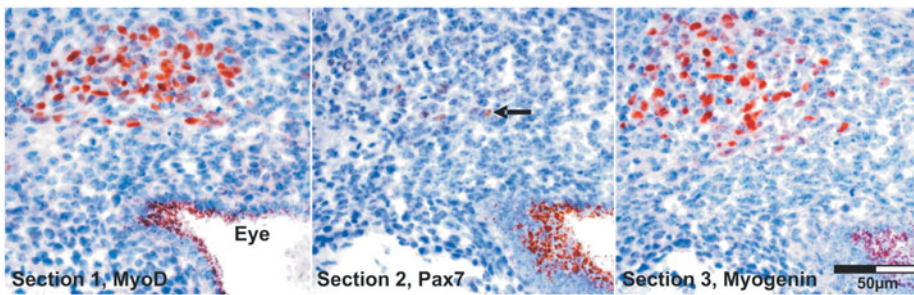


Fig. 7. Expression of Pax7 and MRFs in the lateral rectus rudiment at E11.5. *Pax7* is activated later than MRFs in head muscles. *Pax7* is expressed in only a few cells within the MRFs-positive area (a positive cell is shown by arrow). The order of sections ascertains that *Pax7* positive cells are located inside the MRF-positive area.

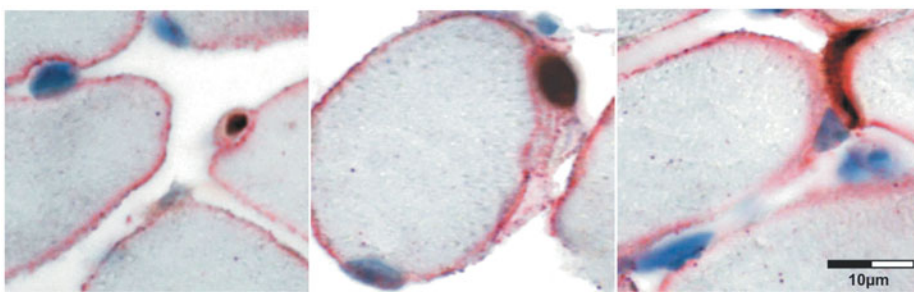


Fig. 8. Co-staining for Pax7 (brown) and laminin (red) reveals that Pax7 is expressed in the satellite cells of head muscles. Pictures represent fragments of the masseter and two facial muscles of newborn mice.

region. At later developmental stages a uniform pattern of *Pax7* expression was observed in the head and body muscles. *Pax7* was present exclusively in satellite cells suggesting a universal role in striated muscles.

Pax3, Pax7 and myogenesis

Several studies suggested a direct involvement of *Pax3* and *Pax7* in the myogenic process. A direct involvement of *Pax3* in myogenesis has been suggested previously by experiments based on the ectopic expression of *Pax3* in chicken embryos and the generation of *Pax3/Myf5* double knockout mice (Maroto *et al.*, 1997; Tajbakhsh *et al.*, 1997). The latter study demonstrated the absence of myogenesis in the trunk. However, re-examination of the original *Pax3/Myf5* double knockout mice revealed that the double mutant was in fact a triple mutant, in which expression of the *Mrf4* gene was additionally compromised. In fact, double knockouts of *Pax3* and *Myf5*, in which *Mrf4* is expressed regularly, seem to undergo normal myogenesis in the trunk (Kassar-Duchossoy *et al.*, 2004). At present it is unclear whether *Pax3* is directly required to activate the *MyoD* gene in the absence of *Myf5* as originally postulated (Tajbakhsh *et al.*, 1997) or whether *Pax3* provides a permissive environment for the enlargement of muscle precursor cell populations, which might increase the bias for myogenic differentiation and thus enable myogenic cells to respond to environmental cues (Mennerich and Braun, 2001). *Pax3* has also been shown to direct the formation of limb muscles (Bober *et al.*, 1994). However, *Pax3* is not directly linked to myogenesis in these cells. Transplantation experiments revealed that somites from *Spotch* embryos taken at stage E9.25 undergo

muscle differentiation when grafted into the limb bud of chick host embryos (Daston *et al.*, 1996). In double knockouts of *Pax3* and *Pax7* myotomal myogenesis is initiated and essentially normal until stage E13.5 further supporting the idea that *Pax3* and *Pax7* are not required for the induction of myogenesis (Relaix *et al.*, 2005). Our results also argue for a limited or indirect role of *Pax* genes for muscle development.

Striated muscles are known to exhibit identical histology and physiology in the head and trunk, suggesting that realization of the myogenic programs is similar. A main difference between head and trunk muscles, however, is the mode of induction. Here, we have demonstrated that neither *Pax7* nor *Pax3* are likely to serve a role in the initiation of myogenesis in head muscles: (i) *Pax3* is not expressed in head muscles; (ii) head muscle development is not affected in *Pax7*^{-/-} and *splotch* mice; (iii) *Pax7* is expressed only after expression of MRFs in head muscles. Moreover, *Pax3* is not de-repressed in *Pax7*^{-/-} mice, in which muscle development proceeds normally, suggesting that after E13.5 myogenesis does not require *Pax3* and *Pax7*. Taken together it seems not likely

that the core program of striated muscle development depends on *Pax* genes. Given the relatively short period of *Pax3* expression in the trunk until stage E13.5 (in some muscles - until E16.5) it seems likely that the *Pax3/7*-cell population of muscle progenitors (Relaix *et al.*, 2005) exists only transiently and does not constitute a persistent cell lineage.

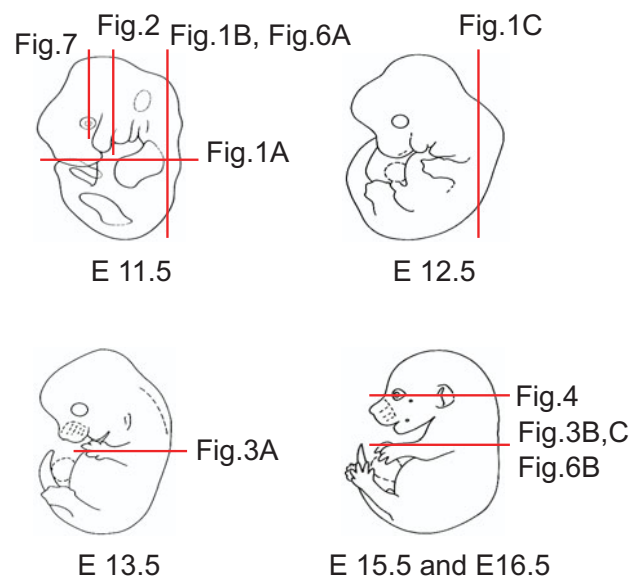


Fig. 9. Schematic representation of embryos and positions of the sections shown in the respective figures.

Based on these arguments it is tempting to speculate that *Pax3* and *Pax7* allow the formation of provisory myogenic structures, in which the myogenic process itself is initiated as a distinct program independent of *Pax* genes. Further experiments are required to reveal specific instructive roles of *Pax3* and *Pax7* for myogenesis.

Materials and Methods

Genotyping of the *Pax7* knockout embryos

The generation of the *Pax7-LacZ* mutant mice has been published (Mansouri et al., 1996). The mutant mice were maintained on a mixed 129Sv/C57/Bl6 background. Embryos were genotyped by PCR and Southern blot analysis, the absence of *Pax7* protein was also corroborated by immunohistochemical stainings.

Immunohistochemistry

Embryos of wild type Balb/c mice were fixed in 4% paraformaldehyde and embedded into paraffin according to standard protocols. Adjacent 7 μ m sections were used for comparative analysis. Endogenous peroxidase was inactivated in 0.3% H₂O₂ in methanol. For antigen unmasking, the slides were autoclaved in 1 mM citrate buffer, pH 6.0. Immunodetection was performed with the primary mouse monoclonal antibodies anti-MyoD (DakoCytomation, Clone 5.8A), anti-myogenin (DakoCytomation, Clone F5D), anti-*Pax3* developed by C. P. Ordahl and anti-*Pax7* developed by A. Kawakami. The *Pax3* and *Pax7* antibodies were obtained from the Developmental Studies Hybridoma Bank (NICHD; The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242). Rabbit polyclonal anti-*Pax3* antibody was kindly provided by J. Epstein, University of Pennsylvania, Philadelphia, PA. The antibodies were applied with the following dilutions: 1:50 for anti-MyoD, 1:100 for anti-myogenin, 1:1000 for anti-*Pax3* and anti-*Pax7*, 1:1000 for pre-absorbed polyclonal anti-*Pax3*. For detection of primary antibodies we used biotinylated rabbit-anti-mouse antibody (DakoCytomation) and the avidin-biotin-complex (ABC, Vector). Anti-laminin rabbit polyclonal antibodies (L-9393, Sigma) were applied (1:200) after an additional unmasking treatment with proteinase K (Sigma), 100 μ g/ml, for 8 min at 37°C. The anti-laminin antibodies were detected by donkey anti-rabbit-alkaline phosphatase conjugates (Dianova) and subsequent staining with the Fast Red alkaline phosphatase substrate (Sigma). Finally, the sections were counterstained with hematoxylin (DakoCytomation).

Cell quantification

Two embryos per stage were analysed. Cell counting was achieved using the Adobe Photoshop program. On a high magnification image of a selected area, positively stained nuclei were marked by dots and counted. The counting results were tested for consistency using a Chi-Square test ($n=12$, $p\alpha = 0.01$). The differences between E11.5 and E12.5 and between E12.5 and E13.5 were shown to be significant (p -Test of binomial distributions; $p\alpha = 0.01$), mean values were taken.

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