Transdifferentiation of esophageal smooth to skeletal muscle is myogenic bHLH factor-dependent

Boris Kablar1, Shahragim Tajbakhsh2 and Michael A. Rudnicki1,*

1Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario, Canada L8S 4K1
2Department of Molecular Biology, CNRS URA1947, Pasteur Institute, 25 rue du Dr Roux, 75724 Paris, Cedex 15, France

*Author for correspondence (e-mail: rudnicki@mcmaster.ca)

Accepted 4 February; published on WWW 21 March 2000

SUMMARY

Previously, coexpression of smooth and skeletal differentiation markers, but not myogenic regulatory factors (MRFs), was observed from E16.5 mouse fetuses in a small percentage of diaphragm level esophageal muscle cells, suggesting that MRFs are not involved in the process of initiation of developmentally programmed transdifferentiation in the esophagus. To investigate smooth-to-skeletal esophageal muscle transition, we analyzed Myf5lacZ knock-in mice, MyoD-lacZ and myogenin-lacZ transgenic embryos with a panel of the antibodies reactive with myogenic regulatory factors (MRFs) and smooth and skeletal muscle markers. We observed that lacZ-expressing myogenic precursors were not detected in the esophagus before E15.5, arguing against the hypothesis that muscle precursor cells populate the esophagus at an earlier stage of development. Rather, the expression of the MRFs initiated in smooth muscle cells in the upper esophagus of E15.5 mouse embryos and was immediately followed by the expression of skeletal muscle markers. Moreover, transdifferentiation was markedly delayed or absent only in the absence of Myf5, suggesting that appropriate initiation and progression of smooth-to-skeletal muscle transdifferentiation is Myf5-dependent. Accordingly, the esophagus of Myf5+/−:MyoD+/− embryos completely failed to undergo skeletal myogenesis and consisted entirely of smooth muscle. Lastly, extensive proliferation of muscularis precursor cells, without programmed cell death, occurred concomitantly with esophageal smooth-to-skeletal muscle transdifferentiation. Taken together, these results indicate that transdifferentiation is the fate of all smooth muscle cells in the upper esophagus and is normally initiated by Myf5.

Key words: Myf5, MyoD, Esophagus, Transdifferentiation, Skeletal muscle

INTRODUCTION

Transdifferentiation is a relatively rare phenomenon that denotes a switch in the phenotype of a cell that has acquired specific differentiated characteristics (Okada, 1991). The best known examples of experimentally induced transdifferentiation are changes in subclasses of neural crest-derived neurons upon transplantation (Anderson, 1989; Patterson, 1990; Okada, 1991), amphibian limb regeneration upon injury (Okada, 1991; Brockes, 1994) and lens regeneration upon lentectomy (Okada, 1991; Juric-Lekic et al., 1991; Hitchcock and Raymond, 1992). By contrast, smooth-to-skeletal muscle transdifferentiation in the esophagus takes place during normal mouse development (Patapoutian et al., 1995b) and does not need to be induced by experimental manipulations or injury. Therefore, esophageal transdifferentiation resembles the normally occurring switch in the phenotype of sympathetic nerves to sweat gland (Landis, 1990), and that of the iris sphincter muscle in the chicken (Volpe et al., 1993; Link and Nishi, 1998).

Unlike the rest of the gastrointestinal tract, the muscularis externa of the entire cervical segment and the greater part of the thoracic segment of the adult mouse esophagus consists of striated muscle (Samarasinghe, 1972; Sang and Young, 1997). Smooth muscle cells begin to appear in the muscularis externa at the lower end of the thoracic segment, just proximal to the diaphragm, gradually replacing the striated muscle in a caudal direction. As a consequence, the entire muscularis externa in the caudal part of the abdominal esophageal segment is made up of smooth muscle cells (Samarasinghe, 1972). In contrast, during early stages of embryonic development, the outer layer of the entire mouse esophagus consists of differentiated smooth muscle cells (Patapoutian et al., 1995b; Sang and Young, 1997). Cells exhibiting striations are first detected in the outer layer of the most rostral parts of the esophagus at E15.5 (Sang and Young, 1997). The external muscle layer of the mouse esophagus undergoes smooth-to-skeletal muscle transdifferentiation in a craniocaudal direction, coexpressing both smooth (myosin light chain) and skeletal (myosin heavy chain) differentiation markers within individual cells (Patapoutian et al., 1995b). This process of transdifferentiation continues through the first 2 weeks of esophageal postnatal development (Patapoutian et al., 1995b), at which point the esophageal muscularis externa acquires its adult appearance.
The evident switch of phenotypes from smooth to skeletal in the muscularis of the esophagus is a remarkable phenomenon that raises questions about the underlying molecular mechanisms. A number of extrinsic and intrinsic factors are known to have an effect on transdifferentiation (reviewed by Okada, 1991). For instance, the formation of potential pigment cells by transdifferentiation from glia-like cells of embryonic chicken neural retina was stimulated by the effects of tricarboxylic acid (Pritchard, 1981). Moreover, growth factors, such as the basic fibroblast growth factors (bFGF), and extracellular matrix factors, are found to be involved in different steps of the transdifferentiation process (reviewed by Okada, 1991).

Understanding the function of genes involved in skeletal muscle differentiation is essential for studies on the molecular basis of esophageal transdifferentiation. The myogenic regulatory factors (MRFs), a group of basic helix-loop-helix (bHLH) transcription factors consisting of Myf5, MyoD, myogenin and MRF4, play important regulatory functions in development of the skeletal muscle (Weintraub et al., 1991; Rudnicki and Jaenisch, 1995; Orldahl and Williams, 1998). Null mutations in Myf5, MyoD, myogenin and MRF4 have revealed hierarchical relationships and apparent functional overlap within the MRF regulatory network (Rudnicki et al., 1992; Braun et al., 1992; Rudnicki et al., 1993; Hasty et al., 1993; Nabeshima et al., 1993; Patapoutian et al., 1995a; Zhang et al., 1995). The four MRFs are divided into two functional groups: the primary MRFs, MyoD and Myf5, are required for the determination of skeletal myoblasts, whereas the secondary MRFs, myogenin and MRF4, act later in the program as differentiation factors (reviewed by Rudnicki and Jaenisch, 1995; Megeney and Rudnicki, 1995). Therefore, it seems likely that esophageal transdifferentiation should indeed be subject to regulation by the MRFs. Moreover, it is well known that experimentally induced ectopically expressed MRFs have capacity to convert terminally differentiated non-muscle cells to skeletal muscle (Weintraub et al., 1989; Choi et al., 1990; Miner et al., 1992; Blau, 1992).

By birth many of the esophageal muscularis cells have transdifferentiated into skeletal muscle. However, previous studies failed to detect expression of Myf5, MyoD, myogenin and MRF4 until shortly after birth (Patapoutian et al., 1995b). Therefore, smooth-to-skeletal esophageal muscle transition was suggested to be MRF-independent. Consequently, it has remained unclear whether the mechanisms that regulate muscle gene expression during esophageal muscularis transdifferentiation are analogous to those in the other parts of the body. In addition, it is also unclear whether the smooth-to-skeletal muscle transition is cell-autonomous or occurs in response to external signals (e.g. signaling molecules or innervation).

To understand the underlying mechanisms regulating smooth-to-skeletal esophageal muscle transition, we examined esophageal muscle development in lacZ knock-in and transgenic embryos that contained different permutations of null mutations in Myf5 and MyoD with a panel of immunohistochemical markers. We report that: (1) the myogenic precursor cells from extra-esophageal sources did not appear to colonize the developing esophagus at early times; (2) the expression of MRFs preceded the expression of skeletal muscle markers; (3) early transdifferentiation was abrogated in the absence of Myf5, whereas it was not affected by the absence of MyoD; (4) the smooth esophageal muscle of Myf5−/−:MyoD−/− embryos did not transdifferentiate, proliferate or undergo programmed cell death; (5) the entire cervical segment of the wild-type esophagus undergoes extensive proliferation as well as a complete transdifferentiation. Taken together, our findings suggest that appropriate smooth-to-skeletal muscle transdifferentiation requires Myf5 and is the fate of all smooth muscle cells in the upper esophagus.

MATERIALS AND METHODS

Interbreeding, collection and genotyping of embryos

We employed four different lacZ reporter mice: (1) the Myf5lacZ knock-in mice, in which the lacZ reporter gene is introduced into the Myf5 locus by homologous recombination (Tajbakhsh et al., 1996); (2) the MD6.0-lacZ transgenic mice that carry a transgene in which 6.0 kb of MyoD sequence upstream from the transcription start site is linked to the bacterial lacZ gene (Tapscott et al., 1992); (3) the 258/-5lacZ transgenic mice that carry a construct in which the 258 bp core of the −20 kb MyoD enhancer is linked to 2.5 kb of MyoD sequence upstream of the transcription start site and the lacZ gene (Goldsammer et al., 1995); and (4) the transgenic Myo1565lacZ mice, generated with 1.565 kb of 5′ myogenin sequence linked to the lacZ transgene (Cheng et al., 1993; kindly provided by E. N. Olson).

All four lacZ transgenic mice were used to generate embryonic day (E)11.5-E14.5 embryos, while Myf5lacZ, 258/-5lacZ and MD6.0-lacZ were also used to generate E18.5 fetuses, from which the esophagus was isolated. Myf5lacZ knock-in mice were also bred with MyoD−/− mice (Rudnicki et al., 1992) to generate Myf5lacZ−/−:MyoD−/− mice. Myf5lacZ−/−:MyoD−/− mice were interbred to obtain embryos of nine different genotypes at E18.5. Embryos (E11.5-E14.5) and isolated esophagi (E15.5-E18.5) were fixed, stained for β-galactosidase activity, paraffin-infiltrated and serially sectioned for histological analysis and immunohistochemistry of the esophagus.

In addition, MyoD−/− mice (Rudnicki et al., 1992) and Myf5−/− mice (Braun et al., 1992) were bred in order to generate Myf5−/−:MyoD−/− mice, which were subsequently interbred to obtain embryos of nine different genotypes at E14.5, E15.5, E16.5 and E17.5. Embryos were fixed, embedded in paraffin, sectioned and immunostained. Embryos and fetal placentas were collected at different embryonic days by Cesarean section. DNA isolated from placentas, as described by Laird et al. (1991), was used to genotype embryos by Southern analysis (Sambrook et al., 1989). Southern analysis of placental DNA was performed using Myf5-, MyoD- and lacZ-specific probes as described previously (Braun et al., 1992; Rudnicki et al., 1992).

In order to study esophageal muscle development in the absence of Myf5, we also employed transgenic mice in which myogenin is knocked-in into the Myf5 locus (Myf5myg-ki/myg-ki mice; Wang et al., 1996; kindly provided by R. Jaenisch). Care of animals was in accordance with institutional guidelines.

β-galactosidase staining

β-galactosidase staining was performed as described by Kablar et al. (1997). Embryos and esophagi were fixed for 1 hour in fixative containing 2% paraformaldehyde, 0.2% glutaraldehyde, 0.1 M phosphate buffer, pH 7.4, and 2 mM MgCl2. After two washes for 30 minutes, first in solution A (0.1 M phosphate buffer, pH 7.4, 2 mM MgCl2, 0.1% sodium deoxycholate, 0.2% Nonidet P-40) and then in solution B (0.1 M phosphate buffer, pH 7.4, 2 mM MgCl2, 0.01% sodium deoxycholate, 0.2% Nonidet P-40), β-galactosidase was detected by incubation for 1 hour to overnight at 37°C in 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), 5 mM

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Potassium ferricyanide and 5 mM potassium ferrocyanide. After two washes in phosphate-buffered saline (PBS) and post-fixation, embryos and esophagi were dehydrated in ethanol, infiltrated in paraffin, embedded, serially sectioned and stained with Eosin or used for immunohistochemistry. Cryostat sections were prepared and X-gal stained after sectioning (Tajbakhsh et al., 1996).

Immunohistochemistry and TUNEL detection

Immunohistochemistry was performed as previously described (Kablar et al., 1997) on paraffin sections with mouse monoclonal anti-Skeletal Fast Myosin (SFM) antibody (1:100; Sigma), mouse monoclonal anti-α-Sarcomeric Actin (SA) antibody (1:600; Sigma), mouse monoclonal anti-α-Smooth Muscle Actin (SMA) antibody (1:100; Sigma), mouse monoclonal anti-Myosin Light Chain Kinase (MLCK) antibody (1:800; Sigma), mouse monoclonal anti-desmin antibody D33 (1:100; DAKO), mouse monoclonal anti-myosin heavy chain antibody MF20 (1:10; Bader et al., 1982), mouse monoclonal anti-PCNA antibody (1:400; DAKO), rabbit polyclonal anti-Myf5 antibody C-20 (1:100; Santa Cruz), rabbit polyclonal anti-MyoD antibody M-318 (1:50; Santa Cruz) and rabbit polyclonal anti-myogenin antibody m-225 (1:20; Santa Cruz).

To detect apoptotic nuclei in situ by TUNEL (Gavrieli et al., 1992), we employed the ApopTag detection system (Oncor and Genzyme), according to the manufacturer’s instructions.

Fig. 1. The local origin of esophageal striated muscle. The myogenic precursors expressing Myo1.565lacZ (A), Myf5nlacZ (B), 258/-2.5lacZ (C) and MD6.0-lacZ (D) were not detected in the Eosin-counterstained transverse sections of the esophagus at E11.5 (A), E12.5 (B), E13.5 (C) and E14.5 (D) of transgenic (A,C,D) and heterozygous Myf5nlacZ knock-in (B) embryos. The wall of the E11.5 esophagus is mesenchymal (arrowhead in A), sandwiched between an inner lining of mucosal epithelium (short arrow in A) and an outer lining of serosal epithelium (long arrow in A). Demarcation of layers within the mesenchyme became apparent from E12.5, when the highly condensed cells formed the circular layer (arrowhead in B) and separated the submucosal from the subserosal mesenchyme. At E13.5 and E14.5 it was possible to distinguish two layers of esophageal muscularis, an inner layer (lamina muscularis interna, arrowheads in C and D) and an outer layer (lamina muscularis externa, arrows in C and D). Magnification, ×400.

Fig. 2. Transdifferentiation coincides with the expression of MRFs. Serial transverse sections of the rostral part of the E15.5 wild-type esophagus were immunostained with anti-SMA (A), anti-Myf5 (B), anti-MyoD (C), anti-myogenin (D) and anti-SFM (E). SMA expression was found in smooth muscle cells of the lamina muscularis mucosae (arrowhead in A), the lamina muscularis interna (short arrow in A) and the lamina muscularis externa (long arrow in A). The detection of antibodies reactive to Myf5 (arrows in B), MyoD (arrows in C), myogenin (arrows in D) and SFM (arrows in E) was first evident in the outer layer of the esophageal muscularis only. Magnification, ×400.
RESULTS

The local origin of esophageal striated muscle

To examine whether the esophageal striated muscle originated from sources other than in situ smooth-to-skeletal muscle transdifferentiation, we employed Myf5\(^{nlacZ}\) knock-in mice, MyoD\(^{-}\)\(^{lacZ}\) transgenic mice harboring the bacterial lacZ gene under the transcriptional control of truncated MyoD gene promoters (either 258/-2.5lacZ or MD6.0-lacZ), and myogenin\(^{-}\)\(^{lacZ}\) transgenic mice (Myo1565lacZ). As previously reported, the expression pattern of the MD6.0-lacZ and Myo1565lacZ transgenes appears to closely follow myogenic differentiation in differentiated embryonic myocytes (Asakura et al., 1995; Kablar et al., 1997; unpublished observations). By contrast, the 258/-2.5lacZ and Myf5\(^{nlacZ}\) are expressed upon myogenic determination (Tajbakhsh et al., 1996; Kablar et al., 1999). Therefore, the MD6.0-lacZ and Myo1565lacZ transgenes served as muscle-specific markers in the analysis of the skeletal muscle differentiation program during early stages of the esophageal development, while Myf5\(^{nlacZ}\) and 258/-2.5lacZ served as in vivo markers of determined myogenic progenitor cells.

Transverse sections were prepared to represent the upper part of the esophagus from embryos of all four mouse lines (Fig. 1A-D). Heterozygous embryos from Myf5\(^{nlacZ}\) knock-in mice (or Myf5\(^{nlacZ/+}\)) and embryos from both types of MyoD\(^{-}\)\(^{lacZ}\) transgenic mice (258/-2.5lacZ and MD6.0-lacZ) were analyzed at every embryonic age between E11.5 and E14.5, while embryos from Myo1565lacZ transgenic mice were analyzed at ages E11.5 and E14.5. Although some adjacent structures (e.g. arythenoid swelling or thymus primordia; not shown), myotomes and other muscular locations (Tajbakhsh et al., 1996; Kablar et al., 1997, 1998, 1999; not shown) were strongly expressing lacZ, the esophageal wall did not contain lacZ-expressing cells (Fig. 1A-D). At E11.5, the esophagus consisted of a hollow tube, the wall of which was mesenchymal, sandwiched between an inner lining of mucosal and an outer lining of serosal epithelium (Fig. 1A). Demarcation of layers within the mesenchyme became apparent from E12.5, when the highly condensed cells formed the circular layer and separated the submucosal from the subserosal mesenchyme. Similar changes have previously been reported for other locations within the gastrointestinal tract (Gershon et al., 1981; Kablar, 1995). Myo1565lacZ was not activated at E11.5 (Fig. 1A) and E14.5 (not shown) in the condensation of cells in the esophageal wall. Consistently, Myf5\(^{nlacZ}\), 258/-2.5lacZ and MD6.0-lacZ were also absent in the wall of the esophagus between E11.5 and E14.5 (Fig. 1A-1D; data not shown). However, cells exhibiting striations are first detected in the outer layer of the most rostral part of the esophagus at E15.5 (Fig. 2E; Sang and Young, 1997). Therefore, because myogenic precursor cells expressing nlacZ also expressed smooth muscle actin at E15.5, we conclude that the appearance of skeletal muscle in the esophagus is likely to be a consequence of transdifferentiation from smooth muscle.

![Fig. 3. Transdifferentiation is initially dependent on Myf5. Serial transverse sections of the rostral part of the E16.5 wild-type (A,E,I,M,Q), Myf5\(^{-}\)/ (B,F,J,N,R), MyoD\(^{-}\)/ (C,G,K,O,S) and Myf5\(^{-}\)/MyoD\(^{-}\)/ (D,H,L,P,T) esophagus were immunostained with anti-SMA (A-D), anti-desmin (E-H), anti-myogenin (I-L), anti-SFM (M-P) and anti-SA (Q-T). SMA and desmin were similarly localized in the esophageal muscularis in embryos of all four genotypes (A-H). The expression of myogenin, SFM and SA were clearly absent in Myf5\(^{-}\)/ (J,N,R) and Myf5\(^{-}\)/MyoD\(^{-}\)/ (L,P,T) embryos, while it was normally localized in the outer layer of esophageal muscularis of wild type (arrows in I,M,Q) and MyoD\(^{-}\)/ (arrows in K,O,S). Magnification, ×400.](image-url)
cells, rather than via colonization by migratory precursors from somites, or other structures.

**Transdifferentiation coincides with the expression of MRFs**

To examine the expression pattern of MRFs at the time of the first appearance of striated muscle cells in the esophagus (Sang and Young, 1997), we performed immunohistochemistry with antibodies reactive to Myf5, MyoD and myogenin on serial sections that represent the rostral part of the esophagus at E15.5. Immunohistochemical analysis of antibody reactive specifically with smooth muscle actin (anti-SMA) revealed that smooth muscle cells were localized in three different layers of the esophageal wall. These were situated in the mucosal muscular layer called lamina muscularis mucosae, as well as in the inner (lamina muscularis interna) and outer (lamina muscularis externa) layer of the esophageal muscularis (Fig. 2A). Importantly, the anti-SMA antibody did not stain cardiac tissue or skeletal muscle, indicating that the specificity of the antibody was smooth muscle-specific (not shown).

The expression of MRFs also initiated in the upper esophagus of E15.5 mouse embryos. Expression of Myf5 (Fig. 2B), MyoD (Fig. 2C) and myogenin (Fig. 2D) protein was first immunodetected in the outer layer of the esophageal muscularis, and was immediately followed by the expression of the differentiation-specific skeletal fast myosin (SFM) as detected with anti-SFM antibody (Fig. 2E). Taken together, these data support the assertion that esophageal smooth muscle transdifferentiates into skeletal muscle and also establishes that the direction of transdifferentiation occurs from the outer towards the inner layer of esophageal muscularis.

**Transdifferentiation is dependent on Myf5**

To investigate any requirement for Myf5 and MyoD during transdifferentiation, we used immunohistochemistry with a panel of antibodies that react with muscle proteins and MRFs, using wild-type, Myf5+/−, MyoD+/− and Myf5−/−:MyoD−/− embryos at E14.5-E17.5. The expression pattern of smooth muscle actin (as detected with anti-SMA and anti-MLCK), as well as desmin (a marker expressed in both smooth and skeletal muscle), was distributed normally in the esophageal muscularis (Fig. 2A). The antibody was smooth muscle-specific (not shown).

**Transdifferentiation in the absence of Myf5 is postponed and subsequently initiated by MyoD**

To confirm and extend this analysis we isolated esophagi from wild-type, heterozygous Myf5nlacZ+/−, MyoD+/−, Myf5−/− and Myf5−/−:MyoD−/− embryos at E18.5 and stained for β-galactosidase (Fig. 4), followed by immunohistochemistry on sections with a muscle-specific panel of antibodies (Fig. 5).

Whole-mount β-galactosidase staining revealed that Myf5nlacZ and both MyoD enhancers (258/−2.5lacZ and MD6.0-lacZ) were appropriately expressed in the heterozygous (Fig. 4A) and transgenic esophagus (Fig. 4B,C), respectively, in a rostrocaudal gradient. The highest number of lacZ-expressing cells were evident in the pharyngeal side of the esophagus, while the lowest were towards the diaphragm. The nature of the cells expressing lacZ was further confirmed by

**Fig. 4.** Transdifferentiation in the absence of Myf5 is postponed and subsequently initiated by MyoD only in the pharyngeal segment. Whole-mount β-galactosidase staining of esophagi isolated from heterozygous (A) and wild-type (B,C) E18.5 embryos revealed that the number of cells expressing Myf5nlacZ (A), 258/−2.5lacZ (B) and MD6.0-lacZ (C) were located at the pharyngeal (or rostral) side of the esophagus (arrowheads in A-C), while the lowest number of cells was towards the diaphragm (short arrows in A-C). Whole-mount β-galactosidase staining of E18.5 esophagi isolated from MyoD−/− (D), Myf5−/− (E) and Myf5−/−:MyoD−/− (F) Myf5nlacZ embryos revealed that Myf5nlacZ was appropriately expressed only in the absence of MyoD (D). In the absence of Myf5, Myf5nlacZ was expressed in the pharyngeal segment of the esophagus only (E), whereas in the absence of both genes, Myf5nlacZ was not detectable at all (F).

However, E16.5 Myf5−/− esophagi expressed Myf5nlacZ (G) in a similar manner to that observed in the heterozygous embryos (H). Long arrows in all panels indicate the trachea. Magnification, ×50 (A-F), ×400 (G,H).
immunohistochemistry with anti-SMA (Fig. 5A-C), anti-desmin (Fig. 5G-I), anti-myogenin (Fig. 5M-O) and anti-SFM (Fig. 5S-U), showing that nuclear expression of lacZ coincided with SMA, desmin and SFM cytoplasmic expression, as well as with nuclear expression of myogenin. The muscularis externa of the entire pharyngeal segment and the majority of the thoracic segment of the adult mouse esophagus consisted of striated muscle (Samarasinghe, 1972; Sang and Young, 1997). Smooth muscle cells were apparent at the lower end of the thoracic segment, just proximal to the diaphragm, and in the entire muscularis externa of the distal part of the abdominal esophageal segment (Samarasinghe, 1972). Therefore, the expression pattern of lacZ closely coincided with the distribution of skeletal muscle in the esophagus.

Consistently, in the MyoD−/− esophagus, the expression pattern of Myf5lacZ (Fig. 4D), SMA (Fig. 5D), desmin (Fig. 5J), myogenin (Fig. 5P) and SFM (Fig. 5V) were identical to that observed in the heterozygous and wild-type esophagus. However, in the absence of Myf5, the coexpression of Myf5lacZ and skeletal muscle markers was observed for the first time only in the pharyngeal segment of the E18.5 esophagus (Fig. 4E). In the pharyngeal segment, the expression of SMA (Fig. 5E), desmin (Fig. 5K), myogenin (Fig. 5Q) and SFM (Fig. 5W) were identical to that observed in the heterozygous, wild-type and MyoD−/− esophagus. At earlier developmental ages, in the absence of Myf5, the expression of Myf5lacZ coincided with the expression of SMA and desmin (compare Figs 3B,F and 4G), but did not coincide with the expression of myogenin, SFM and SA (compare Figs 3J,N,R and 4G). By contrast, smooth and skeletal muscle markers were coexpressed with Myf5lacZ in heterozygous and wild-type esophagus (compare Figs 3A,E,I,M,Q and 4H). Taken together, these results indicate that the process of transdifferentiation in the absence of Myf5 was initiated by MyoD after E18.5 only in the pharyngeal segment.

In Myf5−/−:MyoD−/− embryos in the absence of both primary MRFs, expression of Myf5lacZ (Fig. 4F), myogenin (Fig. 5R) and SFM (Fig. 5X) was not observed even in the rostral E18.5

Fig. 5. Absence of transdifferentiation in the Myf5−/−:MyoD−/− esophagus. Immunostaining of transverse sections of heterozygous (A,G,M,S) and wild-type (B,C,H,I,N,O,T,U) E18.5 esophagi revealed that cells expressing Myf5lacZ (A,G,M,S), 258/-2.5lacZ (B,H,N,T) and MD6.0-lacZ (C,I,O,U) coexpressed SMA (A-C), desmin (G-I), myogenin (M-O) and SFM (S-U). Immunostaining of transverse sections of MyoD−/−(D,J,P,V) and Myf5−/− (E,K,Q,W) E18.5 esophagi revealed that cells expressing Myf5lacZ also coexpressed SMA (D,E), desmin (J,K), myogenin (P,Q) and SFM (V,W). Finally, immunostaining of transverse sections of Myf5−/−:MyoD−/− (F,L,R,X) E18.5 esophagi, whose cells did not express Myf5lacZ, expressed SMA (F) in all three muscular layers of the esophageal wall, whereas desmin (L) was localized mostly in the outer muscularis layer. Myogenin (R) and SFM (X) were not detected in Myf5−/−:MyoD−/− E18.5 esophagi. Immunohistochemical signal is brown-red (nuclear or cytoplasmatic), β-galactosidase staining is turquoise, nuclear (usually larger nuclei) and counterstaining is light blue, nuclear (Hematoxylin). Note the smaller size of the Myf5−/−:MyoD−/− E18.5 esophagi. Magnification, ×400.
esophagus, while the expression of SMA (Fig. 5F) and desmin (Fig. 5L) were almost identical to what was observed in heterozygous, wild-type or MyoD\(^{-/-}\) and Myf5\(^{-/-}\) esophagi. Therefore, these data suggest both the absence of smooth muscle actin-negative myogenic precursor cells (i.e. absence of Myf5\(^{nlacZ}\) expression) in the esophagus and the absence of smooth muscle cell transdifferentiation (i.e. absence of MRF and skeletal muscle marker expression). However, there is a difference between the expression pattern of SMA and desmin in transgenic (or heterozygous Myf5\(^{nlacZ/+}\), MyoD\(^{-/-}\) and Myf5\(^{-/-}\)) and double mutant (Myf5\(^{-/-}\):MyoD\(^{-/-}\)) embryos. SMA, a specific marker for smooth muscle cells (Patapoutian et al., 1995b; Link and Nishi, 1998), was clearly expressed in both wild-type and double mutant esophagi in an identical pattern. There were three locations of the esophageal wall expressing SMA: the lamina muscularis mucosae, the mucosal layer of smooth muscle cells, and the inner and the outer layer of esophageal muscularis (compare wild-type, Fig. 5C, and mutant, Fig. 5F, expression). The same expression pattern was observed for desmin in wild-type esophagus (Fig. 5I, the expression in the lamina muscularis mucosae in this section is slightly lower). However, in the mutant esophagus desmin was expressed only in the outer layer of esophageal muscularis, suggesting that these smooth muscle cells are different from the smooth muscle cells in the inner layer of the esophageal muscularis. The observed difference between smooth muscle cells situated in the outer and inner layers of the esophageal muscularis, in the absence of both Myf5 and MyoD, suggests that these layers are differently affected by the absence of skeletal muscle transdifferentiation.

In conclusion, using markers for smooth muscle (anti-SMA), for both smooth and skeletal muscle (anti-desmin), for MRFs (anti-myogenin) and for skeletal muscle cells (anti-SFM), we demonstrated that in the absence of Myf5, transdifferentiation initiates just before the end of gestation (E18.5, in the upper esophagus). In the absence of both Myf5 and MyoD, only smooth muscle markers can be observed in the esophageal muscularis and smooth-to-skeletal muscle transdifferentiation does not occur. Therefore, transdifferentiation is completely dependent on the primary MRFs, Myf5 and MyoD.

Transdifferentiation is the fate of all smooth muscle cells of the rostral esophagus

Previously, the appearance of a mixed (smooth and skeletal) muscle phenotype in the diaphragm level esophagus was reported to be very rare (Patapoutian et al., 1995b). In an adult mouse, the esophageal muscularis at the level of the diaphragm consists of both smooth and skeletal muscle, whereas the rostral esophagus (also called cervical or pharyngeal) consists entirely of skeletal muscle (Samarasinghe, 1972). Therefore, if transdifferentiation is the sole mechanism of esophageal skeletal myogenesis, the appearance of a mixed (smooth and skeletal) muscle phenotype should be widespread in the developing rostral esophagus.

To examine the importance of transdifferentiation for development of skeletal muscle in the esophagus, we isolated esophagus from heterozygous embryos expressing Myf5\(^{nlacZ}\) and characterized the identity of lacZ-expressing cells using a panel of antibodies (Fig. 6). Importantly, we found that the vast majority of cells expressing Myf5\(^{nlacZ}\) (Fig. 6A-I) also clearly expressed the smooth muscle marker SMA and MLCK (Fig. 6A and not shown), the smooth and skeletal muscle marker desmin (Fig. 6B), as well as the skeletal muscle markers SFM (Fig. 6F) and SA (Fig. 6G). These cells also exhibited the nuclear expression of MRFs, such as Myf5 (Fig. 6C), MyoD (Fig. 6D) and myogenin (Fig. 6E). Therefore, these data support the assertion that smooth muscle is the source of
skeletal muscle in the esophagus. In addition, a high number of cells expressing Myf5nlacZ also expressed a marker of cellular proliferation, proliferating cell nuclear antigen (PCNA) (Fig. 6H). Moreover, no cells expressing Myf5nlacZ were detected undergoing programmed cell death by TUNEL analysis (Fig. 6I). Taken together, these data suggest that cells expressing the mixed phenotype proliferate while undergoing transdifferentiation.

It has been previously shown that targeting myogenin into the Myf5 locus (Myf5myg-ki) rescues the truncatated rib phenotype in the Myf5 null mutant, resulting in viable Myf5myg-ki/myg-ki mice and demonstrating apparent functional overlap between Myf5 and myogenin (Wang et al., 1996). Therefore, to assess whether myogenin can substitute for the role of Myf5 in esophageal transdifferentiation, we investigated whether transdifferentiation was delayed in Myf5myg-ki/myg-ki embryos. The immunohistochemical analysis of the cervical esophageal segment from E15.5 (Fig. 7A,B), E16.5 (Fig. 7C,D) and E17.5 (Fig. 7E,F) Myf5myg-ki/myg-ki embryos, using anti-SMA (Fig. 7A,C,E,G) and anti-SFM (Fig. 7B,D,F,H) antibodies, revealed a 2-3 day delay in transdifferentiation, as indicated by the absence of SFM expression at E15.5/16.5 (Fig. 7B,D) and low levels of SFM expression at E17.5 (Fig. 7F, arrowheads) in Myf5 myg-ki/myg-ki embryos. In addition, the immunohistochemical analysis of the cervical esophageal segment from adult Myf5myg-ki/myg-ki revealed the absence of SMA expression (Fig. 7G) and the exclusive expression of SFM (Fig. 7H) in the well-defined outer and inner layers of the esophageal muscularis. Taken together, these experiments confirm that the process of transdifferentiation is significantly delayed when myogenin is substituted for Myf5, but the delay is more prominent in Myf5 null embryos (compare Fig. 3J,N,R with Fig. 7). Moreover, these data indicate that all smooth muscle cells, detected at earlier embryonic stages, eventually transdifferentiate into skeletal muscle by adulthood, in the uppermost level of the esophagus. Therefore, myogenin can functionally substitute for Myf5 in the development of a morphologically normal and functional esophagus.

Myf5−/−:MyoD−/− esophageal smooth muscle cells do not proliferate, die or transdifferentiate

To assess whether cell proliferation or cell death are important mechanisms during smooth-to-skeletal muscle transdifferentiation in the esophagus, we compared the expression patterns of smooth and skeletal markers with the expression of markers for cell proliferation (anti-PCNA) and cell death (TUNEL) in heterozygous (Myf5nlacZ/+) and double mutant (Myf5−/−:MyoD−/−) embryos. Consistently, the esophagus of double mutant embryos did not express skeletal differentiation markers such as myosin heavy chain (MHC) and SA (MF20 and anti-SA, compare Fig. 8C,E with D,F), and exclusively expressed SMA (compare Fig. 8A with B). The pattern of expression of SMA was almost identical in the two layers (the inner and the outer) of both wild-type and mutant muscularis, as well as in the mucosal lamina muscularis mucosae. In addition, the expression pattern of skeletal muscle markers observed exclusively in the wild-type esophagus resembled the pattern of the smooth marker in the wild-type and mutant esophagus, suggesting that smooth-to-skeletal muscle transition is the fate of all cells in the uppermost level of the esophagus. Surprisingly, it was also noted that myogenic

Fig. 7. Transdifferentiation in Myf5myg-ki/myg-ki esophagi is delayed. Immunohistochemistry against anti-SMA (A,C,E,G) and anti-SFM (B,D,F,H) antibodies of the cervical esophageal segment from E15.5 (A,B), E16.5 (Fig. C,D) and E17.5 (Fig. E,F) Myf5myg-ki/myg-ki embryos, revealed a 2-3 day delay in transdifferentiation, as indicated by the absence of SFM expression at E15.5/16.5 (B,D) and low levels of SFM expression at E17.5 (F, arrowheads) in Myf5myg-ki/myg-ki embryos. Immunostaining with anti-SMA (G) and anti-SFM (H) of transverse sections of the rostral segment of Myf5myg-ki/myg-ki adult esophagi revealed an exclusive skeletal muscle phenotype and therefore normal esophageal muscularis in Myf5myg-ki/myg-ki mice. Magnification, ×400.
cells of the muscularis layers were extensively proliferating only in the wild-type esophagus (Fig. 8G) during the process of transdifferentiation, while, in the double mutant esophagus very few muscularis cells (and those in the epithelial layer) exhibited evidence of proliferation (Fig. 8H). In heterozygous (Fig. 9A,C,E,G) and mutant (Fig. 9B,D,F,H) esophagi, programmed cell death was a very rare event between E15.5 and E18.5. Therefore, these results are consistent with the hypothesis that cell proliferation, and not cell death, is necessary for the process of esophageal transdifferentiation.

**DISCUSSION**

In this report we provide evidence that MRFs control smooth-to-skeletal muscle transdifferentiation in the developing mouse esophagus. By employing Myf5nlacZ knock-in mice, MyoD-lacZ transgenic mice (258/-2.5lacZ or MD6.0-lacZ), and myogenin-lacZ trangenic mice (Myo 1565lacZ), together with a panel of the antibodies reactive with smooth muscle markers, MRFs and skeletal muscle markers, we found that (1) the appearance of skeletal muscle in the esophagus resulted from smooth muscle cell transdifferentiation and was not due to colonization by the myogenic precursors; (2) the expression of MRFs started in the upper esophagus of E15.5 mouse embryos and was immediately followed by the expression of skeletal muscle markers; (3) the smooth-to-skeletal muscle transdifferentiation was a Myf5-dependent event, that could be initiated by MyoD at late times only in the pharyngeal segment; (4) the smooth-to-skeletal muscle transdifferentiation in the esophageal muscularis was not possible without both Myf5 and MyoD, and some differences between smooth muscle cell layers could be found in their absence; (5) the extensive proliferation of muscularis cells without programmed cell death, occurred concomitantly with transdifferentiation.

It has been well documented that vertebrate skeletal muscle is derived from multipotential cells in the prechordal, paraxial head and somitic mesoderm that give rise to committed myogenic precursor cells, which become the skeletal muscle of the head, trunk and limbs (reviewed by Hauschka, 1994). It is also believed that the dermamyotome is the source of all skeletal myogenic precursor cells within the segmented region of the developing embryo and each somite gives rise to between 30 and 100 migratory myogenic precursor cells (Ordahl and Le Douarin, 1992; reviewed by Hauschka, 1994; Christ and Ordahl, 1995). However, the origin of muscle cells in the esophagus has received little attention. In view of the observations from the recent study describing developmentally programmed transdifferentiation in mouse esophageal muscle (Patapoutian et al., 1995b), studies to...
investigate the origin of myogenic progenitors in the esophagus, including somitic, head and lateral plate mesoderm, have become very important. Therefore, we employed Myf5\textsuperscript{nlacZ} knock-in mice, MyoD-\textsuperscript{lacZ} transgenic mice harboring the bacterial \textit{lacZ} gene under the transcriptional control of truncated MyoD gene promoters (either 258/-2.5\textit{lacZ} or MD6.0-\textit{lacZ}), and myogenin-\textit{lacZ} transgenic mice (Myo1565\textit{lacZ}) to examine the developing mouse esophagus before the onset of transdifferentiation (E15.5; Sang and Young, 1997; this report). Surprisingly, myogenic precursors expressing \textit{lacZ} were not detected in the esophagus between E11.5 and E14.5. Therefore, we conclude that the appearance of skeletal muscle in the esophagus is likely a direct consequence of transdifferentiation from smooth muscle cells in the esophageal wall into skeletal muscle.

However, it is technically very difficult to completely exclude the possibility that some skeletal muscle of the esophageal muscularis arise from extra-esophageal sources. For example, esophageal skeletal muscle may have two or more embryonic origins. In support of this idea are the findings from the avian iris, which represents another candidate for muscle conversion (Volpe et al., 1993), similar to the one described for the esophagus (Patapoutian et al., 1995b). For the avian iris and ciliary body, it has been proposed that striated muscle differentiation is initiated in cells derived from different embryonic origins, including the head mesoderm and the neuroectoderm via transdifferentiation of smooth muscle cells (Yamashita and Sohal, 1987; Nakano and Nakamura, 1985; Link and Nishi, 1998). Therefore, it remains possible that some precursor cells of esophageal skeletal muscle arise from the somite (Epstein et al., 1994). Further, splotch mice mutated in the \textit{Pax3} gene, which is required for muscle progenitor cell migrations from the somite (Tajbakhsh et al., 1997), have striated muscle in the esophagus at E18.5 (S. T., unpublished results).

Previously, the restriction of expression to the postnatal period of MyoD and myogenin (Patapoutian et al., 1995b), provided support for the hypothesis that the initiation of smooth-to-skeletal muscle transdifferentiation and its propagation throughout embryonic development of the esophagus was not dependent on MRFs. Considering the fact that skeletal muscle differentiation is only possible in the presence of primary MRFs (Rudnicki et al., 1993), the appearance of skeletal muscle in the esophagus before induction of MRFs (Patapoutian et al., 1995b), raised the possibility that the mechanisms regulating muscle gene expression during esophageal muscularis transdifferentiation are not analogous to those in other parts of the body. By contrast, our immunohistochemical analysis revealed that smooth muscle cells were localized in all three layers of the esophageal wall, while the expression of MRFs and skeletal muscle markers initiated in the outer layer of the esophageal muscularis only. Therefore, the expression of smooth muscle actin preceded both the expression of MRFs and other skeletal muscle markers. Taken together, our results indicate that the initiation of smooth-to-skeletal muscle transdifferentiation in the esophagus is MRF-driven.

Fig. 9. \textit{Myf5}\textsuperscript{−−}/\textit{MyoD}\textsuperscript{−−} esophageal smooth muscle cells do not die by apoptosis in the absence of transdifferentiation. Transverse sections of wild-type (A,C,E), heterozygous E18.5 esophagus expressing \textit{Myf5}\textsuperscript{nlacZ} (turquoise staining in G) and \textit{Myf5}\textsuperscript{−−}/\textit{MyoD}\textsuperscript{−−} E18.5 esophagus (B,D,F,H), were analyzed by TUNEL at E15.5 (A,B), E16.5 (C,D), E17.5 (E,F) and E18.5 (G,H). The number of apoptotic cells was not increased at any of the examined stages in the absence of transdifferentiation. Apoptotic cells in the muscularis layer are indicated by arrowheads. Magnification, ×400.
Therefore, the function of the MRFs in regulating skeletal muscle determination during esophageal muscularis transdifferentiation appears to be analogous to the molecular mechanisms underlying skeletal myogenesis in other parts of the body.

Immunohistochemical analysis has revealed that the direction of transdifferentiation is from the outer towards the inner layer of esophageal muscularis. This expression pattern tightly resembles the developmental pattern of nicotinic receptor clusters, which are typical for skeletal muscle innervation in the mouse esophagus (Sang and Young, 1997, 1998). The initiation of transdifferentiation coincides with the penetration of typically striated nicotinic innervation. This correlation raises an interesting possibility that the innervation influences the smooth-to-skeletal muscle transition in the esophagus. Therefore, esophageal muscularis transdifferentiation may occur in response to an external signal and is thus not a cell-autonomous process. Alternatively, the fact that the expression of Myf5nlacZ, MRFs and skeletal muscle markers initiates in the rostral tip of the esophagus, but does not spread in the caudal direction when Myf5 is absent, is suggestive of a necessity for cell-cell interactions that perpetuate the rostral-caudal spread of transdifferentiating cells in the esophagus.

During skeletal muscle development, Myf5 and MyoD are found to play unique roles in the development of epaxial and hypaxial muscle, respectively (Kablar et al., 1997, 1998). Therefore, it was of interest to investigate whether one of the primary MRFs, either Myf5 or MyoD, would play a unique role during esophageal transdifferentiation. Indeed, our analysis revealed that the expression of myogenin and other skeletal muscle proteins was clearly absent in the rostral segment of the esophagus of Myf5−/− and Myf5−/−:MyoD−/− embryos, while it was normal in wild-type and MyoD−/− embryos between E15.5 and E17.5. Therefore, we conclude that smooth-to-skeletal muscle transdifferentiation in the esophagus is normally initiated and controlled by Myf5, suggesting that the smooth-to-skeletal muscle transition in the esophagus shares similar molecular mechanism with epaxial skeletal myogenesis in the somite.

MyoD controls the development of all skeletal muscle derived from migratory precursor cells, namely tongue, diaphragm, branchial arches and limb muscle (Kablar et al., 1997, 1998). By contrast, the appropriate initiation and propagation of esophageal transdifferentiation appears to be Myf5-dependent initially as in the somite (Tajbakhsh et al., 1996). Taken together, our data support the hypothesis that esophageal skeletal muscle directly originates from the Myf5-dependent transdifferentiation of smooth muscle, rather than from MyoD-dependent process.

Our analysis of myogenesis in MyoD−/− embryos has previously revealed that development of skeletal muscle in the limb buds is delayed by 2.5 days and is subsequently initiated by Myf5 (Kablar et al., 1997). By analogy, our analysis of β-galactosidase and immunostained esophagi revealed that the initiation of transdifferentiation in the absence of Myf5 was delayed for 3 days, and was subsequently initiated by MyoD: such a delay was also evident in the somite (Tajbakhsh et al., 1997). However, MyoD-mutant embryos display normal migration of Pax3-expressing cells into the limb buds and normal subsequent induction of Myf5 in these cells (Kablar et al., 1997). By contrast, during the process of transdifferentiation in Myf5 null embryos, the expression of MRFs or skeletal muscle markers was not observed before E18.5, while an expression of Myf5nlacZ was observed even before E18.5 and therefore before the expression of skeletal muscle markers. The expression of Myf5nlacZ was also observed in E15.5 Myf5−/−:MyoD−/− esophagus (B. K. and M. A. R., unpublished results). These findings indicate that Myf5 is cell-autonomously activated in esophageal skeletal muscle progenitors (e.g. smooth muscle cells). Moreover, the amount of cell death by apoptosis in the muscularis layers of both the wild-type and mutant esophagi was similarly low. In addition, the expression pattern of SMA between the wild-type and mutant esophagi did not significantly differ. Taken together, these results indicate that progenitors for esophageal skeletal muscle cells were not eliminated by programmed cell death. Instead, these progenitors retain the smooth muscle phenotype in the absence of both primary MRFs.

In the absence of primary MRFs (Myf5 and MyoD), myogenic determination cannot occur (Rudnicki et al., 1993; B. Kablar and M. A. Rudnicki, unpublished). Consistently, it was not possible to detect skeletal muscle markers in the Myf5−/−:MyoD−/− esophagus. However, an interesting difference between the expression pattern of smooth muscle actin and desmin was observed between esophagi where transdifferentiation took place (wild-type, MyoD−/− or Myf5−/− esophagi) and the esophagus where transdifferentiation did not take place (Myf5−/−:MyoD−/− esophagi). While SMA in the Myf5−/−:MyoD−/− esophagi was expressed in both the inner and the outer layer of esophageal muscularis, desmin was only expressed in the outer layer of the esophageal muscularis. Therefore, even in the absence of transdifferentiation in Myf5−/−:MyoD−/− esophagi, the smooth muscle cells situated in the outer layer were different from the smooth muscle cells in the inner layer of the esophageal muscularis. Taken together, these data suggest the existence of effectors, upstream of Myf5 and MyoD, that are involved in the process of smooth-to-skeletal muscle transition in the esophagus. For example, three recent papers report on defects in esophageal genesis or even agenesis in the absence of zing-finger transcription factors Gli2 and Gli3 (Motoyama et al., 1998), in the absence of a secreted glycoprotein Sonic Hedgehog (Shh; Litingtung et al., 1998) and in the absence of a homeodomain transcription factor Nkx2.1 (Minoo et al., 1999). Gli genes encode transcription factors implicated in Shh signaling (Motoyama et al., 1998; Litingtung et al., 1998), whereas Nkx2.1 is suggested to act via Bone morphogenetic protein 4 (Bmp-4; Minoo et al., 1999). Shh and Bmp-4 are important signaling molecules implicated in the patterning of divergent embryonic structures including somite derivatives, such as skeletal muscle (Tajbakhsh and Spörrle, 1998 and references therein). Taken together, it appears possible that Gli genes, Nkx2.1, Shh and Bmp-4 are involved in the process of skeletal muscle formation even in the esophagus, acting prior or simultaneously with primary MRFs.

By monitoring the expression pattern of smooth and skeletal muscle markers, Patapoutian et al. (1995b) demonstrated that only a small percentage of cells at the diaphragm level of the esophagus were seen to express the mixed phenotype at any given time during development or after birth. Therefore, they suggested an alternative model in which cells expressing the
mixed phenotype were not destined to transdifferentiate, but instead, were destined to die or proliferate. However, they did not observe programmed cell death or significant levels of proliferation within the population of cells expressing the mixed phenotype (Patapoutian et al., 1995b). Of note, Patapoutian et al. (1995b) studied the esophagus at the diaphragm level, which contains smooth muscle even in the adult (Samarasinghe, 1972) and, therefore, observed only a small number of esophageal muscularis cells coexpressing smooth and skeletal markers. Considering the fact that the muscularis externa of the entire cervical part of the adult esophagus entirely consists of striated muscle (Samarasinghe, 1972; Sang and Young, 1997), and that smooth muscle cells are apparent at the lower end of the thoracic segment (from the level of diaphragm), gradually replacing the striated muscle in a caudal direction, we concentrated our analysis to the cervical level of diaphragm, which contains smooth muscle even in the adult (Samarasinghe, 1972) and, therefore, observed only a small number of esophageal muscularis cells coexpressing smooth and skeletal muscle markers, as well as with the nuclear expression of MRFs and PCNA, and not with TUNEL. Taken together, these data suggest that cells expressing the mixed phenotype are not destined to die, but that they do proliferate as they change their phenotype, in agreement with Patapoutian et al. (1995b).

The current view is that the conversion of cells from one phenotype to another without first undergoing one or more cell divisions (also called a direct transdifferentiation) is extremely rare (Holtzer et al., 1975; Beresford, 1990). Importantly, we found that myogenic cells of the wild-type esophageal muscularis extensively proliferated during the process of transdifferentiation. In the double mutant esophagus, where the process of transdifferentiation did not take place, virtually no cell proliferation occurred. Taken together, these results are consistent with the hypothesis that cell proliferation is an obligatory step in transdifferentiation. However, cell proliferation and transdifferentiation could also be just two parallel processes without any mechanistic relationship. Future experiments with cultured esophageal smooth and skeletal muscle should elucidate this issue.

M.A.R. is a Research Scientist of the Medial Research Council of Canada, and a member of the Canadian Genetic Disease Network. We thank Margaret Buckingham for critical reading of the manuscript and Chuyan Ying for expert technical assistance. This work was supported by a grant from the Medical Research Council of Canada to M.A.R. and by a grant from the Human Frontiers to M.A.R. and S.T.

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