INVESTIGATION OF MURINE ESOPHAGEAL MYOGENESIS

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Mark Rishniw

May 2009

INVESTIGATION OF MURINE ESOPHAGEAL MYOGENESIS

Mark Rishniw, BVSc, MS, Ph. D.

Cornell University 2009

This work focuses on the development of the muscular layers of the murine esophagus. Considerable controversy surrounds the manner in which the *muscularis externa* of the esophagus develops, ever since investigators proposed the rare phenomenon of transdifferentiation as a mechanism for virtually complete replacement of smooth muscle by striated muscle that is observed in the murine esophagus.

I found, using several independent levels of evidence, that transdifferentiation is an unlikely explanation for esophageal myogenesis. First, by fate-mapping smooth muscle cells, I failed to observe expression of the fate-mapping marker in any esophageal striated muscle. Second, I failed to co-localize expression of smooth-muscle-specific and striated-muscle-specific proteins in any cell undergoing differentiation into a striated muscle lineage. Finally, selective deletion of a critical striated muscle transcription factor (myogenin) in esophageal smooth muscle cells prior to the appearance of any striated muscle failed to arrest subsequent esophageal striated myogenesis.

To address prior concerns of the fate of esophageal smooth muscle, I examined smooth muscle morphology in transgenic mice expressing smooth-muscle-specific eGFP, and identified 3 possible fates: (A) the incorporation of smooth muscle cells into the striated muscle stroma as “remnant” smooth muscles; (B) compaction and persistence of smooth muscle cells in the pre-

gastric region; and (C) occasional apoptosis of smooth muscle cells. Using exhaustive co-immunolabeling methods, I demonstrated that remnant smooth muscle cells are indeed a previously unidentified cell population resident within the striated esophageal musculature.

Finally, I examined the origin of striated muscle precursors in a series of ex vivo experiments. I was able to maintain a functional esophageal phenotype in culture for up to 7 days, and determined that striated muscle precursors most likely populate the esophageal muscularis externa early in development, and are subsequently induced to express a myogenic program in a cranio-caudal direction.

Together, these findings extend the understanding of esophageal myogenesis and redress fundamental concepts of myogenesis, namely, transdifferentiation, by providing evidence of a more simple, classical myogenic program in this unique muscular organ.

BIOGRAPHICAL SKETCH

Mark Rishniw was born in Melbourne, Australia, in 1964, and graduated with honors from University of Melbourne, in December 1987 with a Bachelor of Veterinary Science degree. After 4 years in mixed veterinary practice, he was accepted into a clinical residency in small animal internal medicine at Washington State University, where he simultaneously obtained a Master of Science (Veterinary Science) degree in 1994, studying myocardial perfusion under the mentorship of the late Grant G Knowlen and subsequently, Brian K Slinker. Subsequently, he was accepted into, and, in 1996, completed a residency in veterinary cardiology at the University of California, Davis. He attained board certification by the American College of Veterinary Internal Medicine in small animal medicine in 1996 and in cardiology in 1997. In 1996 Mark was employed as a Registrar in Small Animal Medicine at the University of Melbourne, and in 1997 he accepted a position as staff cardiologist at the Cornell University Hospital for Animals. In 2000, he was accepted into a doctoral program in the field of physiology at Cornell University under the mentorship of Michael I Kotlikoff. After a 4 year leave-of-absence (2005-2009) he completed his doctoral degree. Mark is currently employed by the Veterinary Information Network.

Dedicated to Maya, Mum, Grant, Paul and Mike.

ACKNOWLEDGMENTS

I would like to acknowledge my doctoral committee (Drew Noden, Stephen Barr, John Ludders, Robert Gilmour) for understanding my perspective and my needs. I would especially like to thank my advisor Mike Kotlikoff for indulging me in this endeavor, and for patiently, quietly, but always supportively tolerating my exceptionally slow ride to completion.

I would like to thank the entire Kotlikoff laboratory: Bob Doran, Jane Lee, Yvonne Tallini, and Kai Sue Greene, Ke Yu Deng, Hong Bo Xin, Bo Shui, Shaun Reining, Jason Wilson, Michael Craven and Maurice Feldman for their assistance during the 8-or-so years that I took to get through this.

I would especially like to thank Patricia Fisher for her incredible help with immunohistochemistry, and the pathology technicians at Cornell University College of Veterinary Medicine for their unbelievable patience and assistance with sectioning tissues. Without them, I'd still be breeding mice and wondering what was going on with their tissues.

I would like to thank Paul Pion for employing me at the Veterinary Information Network with the full knowledge that I would be devoting considerable time and effort to this endeavor. His understanding and empathy have allowed me to finally conclude this small chapter of my life.

Finally, I would like to thank my wife, Maya, who has prodded, cajoled, coerced, threatened, invoked GFD rules and simply waited for me to finally get this over with and get a real job. No such luck, dear! After all, I'm "faculty".

This research was funded in part by NIH grants RO1 DK065992 and NIH training grant T32RR07059-09

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

c-kit:	transmembrane tyrosine kinase encoded by ckit proto-oncogene
CO_2:	Carbon dioxide
dpc:	Days Post-Conception
ED:	Embryonic Day
EDTA:	Ethylenediaminetetraacetic acid
eGFP:	Enhanced Green Fluorescent Protein
FGF-2:	Fibroblast Growth Factor 2
FITC:	Fluorescein isothiocyanate
GFP:	Green Fluorescent Protein
hr-FGF2:	Human recombinant Fibroblast Growth Factor 2
ICC:	Interstitial Cells of Cajal
LacZ:	gene coding for -galactosidase
MEK:	Map-erk Kinase
MLCK:	Myosin Light Chain Kinase
MRF:	Myogenic Regulatory Factor
NFDM:	Non-fat Dry Milk
PBS:	Phosphate-Buffered Saline
PCR:	Polymerase Chain Reaction
PN:	Post-Natal Day
PTAH:	Phosphotungstic acid-hematoxylin
$RyR3^{-/-}$:	Ryanodine Receptor subtype 3 null genotype
TBS:	Tris-buffered Saline
VIP:	Vasoactive Intestinal Peptide
vWF:	vonWillebrand Factor

CHAPTER 1

INTRODUCTION

Esophageal Anatomy and Development

The esophagus is a unique organ in mammals, with a seemingly limited function – propulsion of food and liquid from the oropharynx to the stomach via peristalsis, and, occasionally (or in some species, commonly), propulsion of gastric contents to the oropharynx (regurgitation, eructation or vomiting) via reverse peristalsis.

Mammalian esophagi are all comprised of similar tissue components – an epithelial layer, a submucosa, a thin muscular layer of smooth muscle (*muscularis mucosae*) that is only a few cells thick, two orthogonally oriented muscle layers of smooth and striated muscle (*muscularis externa*) and the outer adventitial layers – consistent with the rest of the post-glossal gastrointestinal tract (Figure 1.1) (Achildi & Grewal 2007).

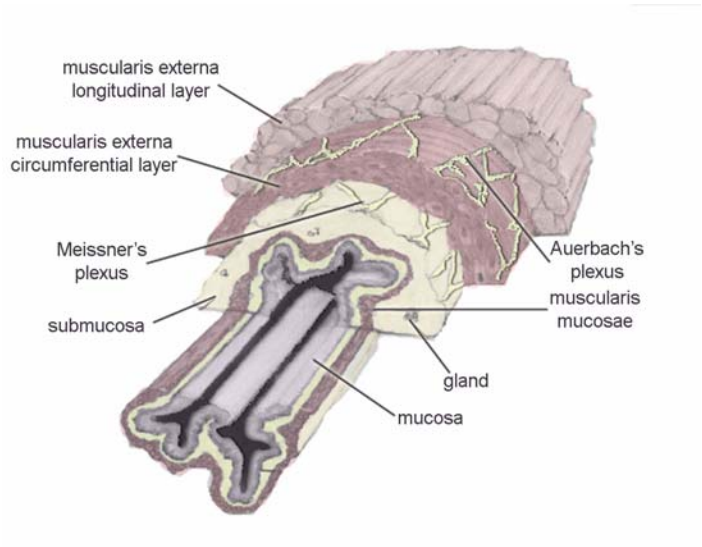

Figure 1.1 Esophageal anatomy

Despite the similar structure and common functionality across most species, several features make this organ interesting from both an anatomical

and functional or teleological standpoint. First, it is the only striated muscle organ of the gastrointestinal tract distal to the oropharynx. As such it is the only striated muscle organ that is under purely autonomic neural control – voluntary contraction of the esophagus is not possible. Swallowing, which is voluntary, is initiated within the oropharynx – the degree of swallowing, however, is outside the control of the swallower – once initiated, it is an all-or-none response. Second, it is one of few striated muscles (along with lingual muscles and elephantine trunk muscles) that do not have a tendino-skeletal attachment at one end or the other – both ends of the esophagus attach to non-skeletal organs, although rostrally, the longitudinal esophageal striated muscles merge with pharyngeal striated muscles and anchor via a membrane-like fascia to the thyroid cartilage (Wang et al. 2007). Third, the esophageal musculature comprises of both smooth and skeletal muscle portions which intermingle in a "transition" zone, but which, more importantly, occupy different proportions of the muscular layer in different species (see below).

Esophageal innervation

Innervation of the esophagus is complex. The *muscularis mucosae* is innervated predominantly by stimulatory cholinergic vagal efferent neurons, with smaller numbers of adrenergic neurons (Christensen & Percy 1984, Kamikawa & Shimo 1979). The response of the *muscularis mucosae* to adrenergic stimulation varies between species – in some species, contraction results, while in others, relaxation occurs. Non-adrenergic-non-cholinergic innervation appears to be absent in the *muscularis mucosae*, but nitrergic and peptidergic neurotransmitters do inhibit vagally mediated contraction of the *muscularis mucosae*, presumably by ganglionic inhibitory actions.

Additionally, endothelin mediates contraction of muscularis mucosae through endothelin receptors (Huang & Chang 2004, Uchida et al. 1998). Much of the innervation of the muscularis mucosae is found in the submucosal plexus, also known as Meissner's plexus.

The *muscularis externa* has much more complex motor innervation, consisting of intrinsic (enteric) neurons and extrinsic (parasympathetic and sympathetic) neurons. Intrinsic nitrergic neurons (nitric-oxide-mediated) and peptidergic neurons (VIP-mediated, substance P-mediated) have been shown to innervate the striated muscle of the esophageal *muscularis externa*, although the function of these intrinsic enteric neurons remains incompletely understood (Leander et al. 1982, Storr et al. 2000, Storr et al. 2001, Uddman et al. 1980). The two muscle layers of the *muscularis externa* are separated by a neural layer – the myenteric plexus, also known as Auerbach's plexus – which is composed of interneurons, enteric neurons and extrinsic neurons. Enteric neurons from the myenteric plexus co-innervate motor endplates of the striated musculature of the myenteric plexus (Wörl & Neuhuber 2005). Differences exist in innervation of the striated and smooth muscle segments of the *muscularis externa,* with vagal neurons from the nucleus ambiguus innervating the striated muscle portion, and vagal neurons from the dorsal motor nucleus innervating the smooth muscle portion (Diamant 1989, Neuhuber et al. 1998, Park & Conklin 1999). In addition to neuronal innervation, the esophageal *muscularis externa* also contains interstitial cells of Cajal (ICC), which are thought to function as enteric pacemaker cells (Berezin et al. 1994, Takaki 2003), although recent evidence suggests that they may act as stretch receptors for sensory vagal neurons (Huizinga et al. 2008, Wu et al. 2003). Somewhat surprisingly, the ICC are not limited to the

smooth muscle component of the muscularis externa, but have been found in the striated muscle portion as well (Burns et al. 1997, Rumessen et al. 2001, Wu et al. 2003).

Sensory innervation to the esophagus is less well defined, but appears to consist of mechanoreceptors and thermoreceptors that function through sensory vagal nerves, although adrenergic sensory inputs can also be observed (Christensen & Percy 1984, Neuhuber et al. 1998).

Esophageal muscular anatomy

The esophageal anatomy of the *muscularis externa* covers the full spectrum of smooth and striated muscle phenotypes, ranging from a completely striated organ in rodents (mice, rats, guinea pigs), ruminants and dogs, to an organ with variably mixed cellular composition in humans, cats, pigs, and opossums and an exclusively smooth muscle organ in birds and alligators (Bremner et al. 1970, Christensen & Lund 1969, Hudson 1993, Jamdar & Ema 1982, Shiina et al. 2005, Uriona et al. 2005). Several anatomists have recognized this anatomical diversity in the face of functional homogeneity and have questioned the evolutionary basis for such diversity. It does not appear to depend on posture (bipedal versus quadripedal stance), eructation or physiological regurgitation (both dogs and ruminants have striated esophageal *muscularis externa*).

In the conventional view the esophageal *muscularis externa* consists of two muscle layers irrespective of whether the cells are striated or smooth – an inner circumferential layer, and an outer longitudinal layer, separated by the myenteric plexus. However, examination of data presented by Kaufmann (Kaufmann et al. 1968) suggests that the orientation of muscle layers in the

esophagus is more complex, and often adopts an orthogonally diagonal orientation. My unpublished observations of the striated muscle layers in adult mice support this hypothesis.

The striated muscle cells are thought to be predominantly type IIA or type IIB subtypes (Hudson 1993, Shedlofsky et al. 1982, Whitmore 1982), although some investigators identified a novel subtype (II_{oes}), a finding that has not been subsequently confirmed (Mascarello et al. 1984).

Esophageal Development

Esophageal fetal development in the mouse (and other mammals) is a complex process. In the mouse, after the formation of the gut tube from the endoderm at approximately embryonic day (ED) 8-8.5, the developing trachea, with its lung buds, separates from the esophagus by septation and elongation. This septation process begins around ED9.5, and is, at least in part, directed by sonic hedgehog (*Shh*) gene expression within the endoderm. Septation is largely completed by ED11.5. Mice deficient in *Shh* fail to separate the trachea and esophagus, resulting in a tracheo-esophageal fistula phenotype. Additionally, the esophageal endodermal-to-mesodermal *Shh* signaling allows for development of the muscular layers – mice deficient in the *Shh*-transduced transcription factor *Gli2* fail to develop any esophageal smooth muscle (van den Brink 2007)

The esophageal endoderm (epithelial layer) is already differentiated into a layer 2-to-3 cells thick by ED15 (Raymond 1991). Initially, it comprises both squamous and ciliated epithelial cells, but postnatally, the ciliated (columnar) cells regress, while the squamous cells undergo keratinization (a process absent in humans) (van den Brink 2007).

Esophageal myogenesis

The murine esophageal muscle forms 3 concentric layers – a thin mucosal layer (the *muscularis mucosae*), and 2 external layers (the *muscularis externa*). The *muscularis mucosae* remains a smooth muscle layer throughout life, however, the *muscularis externa* initially develops as a smooth muscle structure that is subsequently replaced by striated muscle. The extent to which this process occurs varies between species, as highlighted earlier. The process by which this occurs is controversial, and not completely understood. Conflicting studies have argued for markedly different models of esophageal muscular development (Patapoutian et al. 1995, Wörl & Neuhuber 2005), and the conflict remains unresolved. The controversy originated with the suggestion that esophageal striated myogenesis uniquely involves a process of transdifferentiation (see below for more details on this process), whereby differentiated smooth muscle cells transform into striated muscle cells (Patapoutian et al. 1995). These authors suggested transdifferentiation as a means of esophageal myogenesis because they could not detect striated myogenic precursors in the esophagus, and they could not account for the fate of the smooth muscles, which constitute 100% of the esophageal *muscularis externa* when it first forms. They failed to observe apoptosis of smooth muscle cells, and therefore hypothesized that smooth muscle cells *became* striated muscle cells. Their observations were challenged by Zhao and Dhoot in several studies (Zhao & Dhoot 2000a,b,c), who claimed that myosin-light-chain kinase (MLCK) antibody, used by Patapoutian and colleagues to identify smooth muscle cells, was an inappropriate marker of fetal smooth muscle because MLCK was not specific to smooth muscles, but was also expressed in developing striated muscles. Zhao and Dhoot instead suggested that

myogenic precursor cells could be identified along the esophagus as a separate and distinct population of cells as early as ED12.5. At the same time, an ultrastructural study identified esophageal muscle cells that were thought to be expressing both smooth and striated muscle proteins simultaneously, supporting Patapoutian's transdifferentiation hypothesis (Stratton et al. 2000), although the authors did not consider the possibility of transient developmental coexpression of heterologous proteins, which is known to occur in other developing organs and tissues. Additionally, Kablar and colleagues argued for transdifferentiation, suggesting that Zhao and Dhoot's observations of MRF expression (by *in situ* hybridization) were incorrect, and that the earliest expression of MRFs in the esophagus was at ED15.5, rather than ED12.5 (Kablar et al. 2000). Reddy and Kablar followed up this study with another that invoked transdifferentiation as the mechanism of esophageal myogenesis (Reddy & Kablar 2005). However, their data could be plausibly explained by conventional myogenesis. More recently, a study by Wörl & Neuhuber suggested that striated muscle precursor cells are resident within the esophagus, although how these cells come to populate the superficial zone of the mesoderm is unknown (Wörl & Neuhuber 2005). These investigators also identified smooth muscle apopotosis by ultrastructural examination (electron-microscopy), suggesting that this process accounted for the loss of smooth muscle cells during development of the esophageal *muscularis externa*.

It is apparent that at least some of the discrepancies between these studies, such as the earliest time of onset of MRF expression, could be a function of different methodologies. While Zhao and Dhoot identified *Myf5* mRNA as early as ED12.5, they only observed structural skeletal muscle

protein on ED14, consistent with observations by Kablar and colleagues (Kablar et al. 2000, Zhao & Dhoot 2000c).

A common feature and limitation of all these studies is the static nature of the investigations – all the studies involved imaging of esophageal tissue at different stages of development, and therefore, necessarily from different mice. Thus, it is impossible to determine from these experiments if any particular cell changes its phenotype, as no cell can be imaged at 2 different points in time to demonstrate its phenotype or change in phenotype.

Several unusual features of esophageal myogenesis and esophageal anatomy argue against traditional models of myogenesis. First, as explained earlier, the esophagus does not have a tendino-skeletal attachment at either end. Rostrally, esophageal muscles are integrated with the pharyngeal striated muscle, and are jointly incorporated through thin fascia into the laryngeal cartilage. Caudally, esophageal *muscularis externa* myofibers blend into the gastric *muscularis externa*. Second, the striated esophageal myogenic phenotype appears relatively late in development, compared with appendicular, lingual and pharyngeal myogenesis. Murine esophageal striated myogenesis can be first detected at ED11.5-12.5, and progresses in an apparent rostro-caudal direction throughout gestation, continuing throughout the first 2 weeks after birth, much later than striated myogenesis in other regions, where *Myf5*-expressing precursor cells are evident by day 10. However, once striated myogenesis begins in the esophagus, a typical striated myogenic program is invoked, involving the standard panel of myogenic regulatory factors: *Myf5*, *MyoD*, *Myf6* (*MRF4*) and myogenin. This myogenic program has been suggested to be absolutely *Myf5*-dependent, but *MyoD*-independent, as demonstrated by loss-of-function studies, where

deletion of *Myf5* resulted in loss of striated myogenesis, while loss of *MyoD* had no effect (Kablar et al. 2000). However, it is possible that the *Myf5*$^{-/-}$ mice used in this study also possessed a non-functional *Myf6* gene, as the targeting construct was similar to the *Myf5*^{nLacZ} construct used by other investigators (Kassar-Duchossoy et al 2004). These investigators showed that the integrity of *Myf6* was dependent on the type of targeting construct used to inactivate *Myf5*. However, they did not investigate esophageal myogenesis in any of the three *Myf5*$^{-/-}$ lines they created to determine if esophageal striated myogenesis was in fact dependent on expression of *Myf5* or whether *Myf6* inactivation was responsible for the myogenic failure that Kablar et al observed.

Defects in esophageal myogenesis have been occasionally, and somewhat randomly, observed in various phenotyping experiments of transgenic mice, or mice with specific genetic loss of function (knockout mice). Randelia and colleagues first described esophageal myogenic dysplasia in ICRC-HiCri mice (an inbred strain that developed various neoplasms and was maintained at the Cancer Research Institute) that had spontaneously developed megaesophagus, which was shown to be a recessive genetic trait (Randelia & Lalitha 1988,Randelia et al. 1990). These mice failed to complete striated myogenesis, with the caudal portion of the esophagus retaining its smooth muscle phenotype. Subsequently, Wang and colleagues identified an esophageal dysplasia in mice with a deleted *frizzled*-4 gene (Wang et al. 2001). These mice failed to undergo striated myogenesis, presumably due to a loss of pro-myogenic Wnt signaling, although other somitic myogenesis that is dependent on Wnt signaling proceeded uninterrupted. This suggests that *frizzled*-4 may be the Wnt receptor expressed in esophageal striated myogenic progenitor cells but not in other somitic progenitor cells.

Unpublished data from our laboratory identified esophageal dysplasia in a colony of inbred *RyR3*$^{-/-}$ mice (mice in which the Ryanodine Receptor 3 gene had been deleted). These mice had a partial failure of striated myogenesis similar to that seen by Randelia and colleagues and developed megaesophagus (Figure 1.2). However, after development of a congenic *RyR3*$^{-/-}$ strain, the frequency of the dysplasia decreased to 25% of offspring, despite carrying two mutant *RyR3* alleles. This suggested that the esophageal defect was a recessive characteristic, associated with a gene other than *RyR3*, or that *RyR3* effects were modified by other, unidentified, genes.

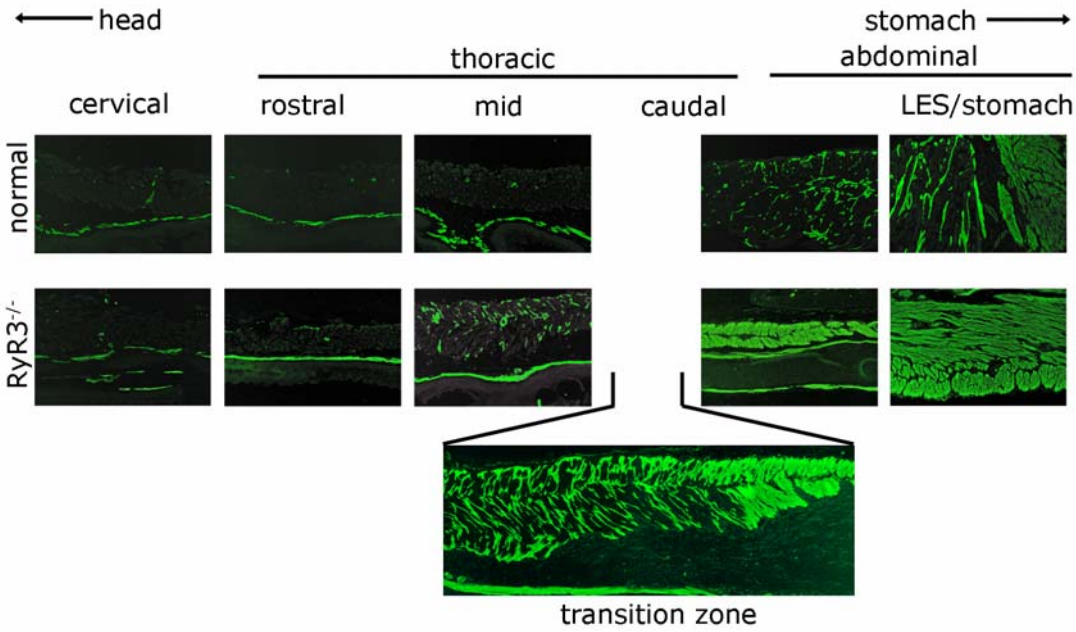

Figure 1.2 Esophageal dysplasia in *RyR3*$^{-/-}$ mice. Immunofluorescent staining of smooth muscle in normal and *RyR3*$^{-/-}$ mice reveals a myodysplasia, with failure of striated myogenesis in *RyR3*$^{-/-}$ mice arising midway along the esophagus.

Sumiyoshi et al (Sumiyoshi et al. 2004) showed that a lack of Collagen XIXα1 in mice arrested the myogenic transformatory process at birth,

suggesting a role for extracellular matrix in normal esophageal myogenesis. In a study examining the effect of *Tbx1 and FGF8* in cardiac development, researchers observed what was considered a defect of esophageal smooth muscle development in *FGF8-deficient* mice (Brown et al. 2004). However, the examination (performed on new-born mice) did not specify the region of esophagus being examined; therefore it is possible that the authors unknowingly sectioned esophagus that had already undergone striated myogenesis.

It is interesting to note that most other studies of factors regulating cranial or somitic myogenesis have consistently ignored the effects on esophageal myogenesis. Kassar-Duchossoy et al (Kassar-Duchossoy et al. 2004) examined several *Myf5*$^{-/-}$ models, but ignored the esophagus. Vasyutina and colleagues (Vasyutina et al. 2005) examined myogenesis using an *Lbx1*^{GFP} mouse, which labels all hypaxial musculature, but ignored the esophagus. However, in a previous study, Gross and colleagues, using a mouse with an inactivated *Lbx1* gene, demonstrated that *Lbx1* deficiency did not affect development of the tongue and larynx, despite the fact that the myogenic progenitor cells that form these muscles express *Lbx1* during migration from the occipital somites (Gross et al. 2000). Like Vasyutina et al, they failed to examine the esophagus. Nevertheless, if esophageal striated muscle is of somitic origin, it is unlikely to require *Lbx1* for expression. Burgess and colleagues (Burgess et al. 1996) inactivated Paraxis, a gene that is instrumental in somite morphogenesis, but did not examine the esophagus. However, axial and appendicular musculature, while abnormal in these mice, was present. Takahashi and colleagues more recently examined Mesp2/Paraxis double-null mice, but also ignored esophageal myogenesis

(Takahashi et al. 2007). Similarly, I could find no studies examining esophageal myogenesis in the spontaneous *Pax3* mutant Splotch mouse. However, *Pax3* null mice appear to have normal esophageal myogenesis (Tajbakhsh S, personal communication 2003), which might suggest that esophageal myogenic progenitor cells are not of somitic origin.

The paucity of esophageal evaluation in studies of cranial myogenic programs is equally apparent. Kelly et al examined *Tbx1* in cranial muscle development but failed to examine the esophagus (Kelly et al. 2004). A study by Lu et al examining the role of *Msc* (*MyoR*) and Tcf21(capsulin) in cranial myogenesis also ignored the esophagus (Lu et al. 2002). It is intriguing to speculate that esophageal myogenic progenitor cells originate in the unsegmented cranial paraxial, or possibly even prechordal mesoderms, but evidence is lacking.

Thus, the exact source(s) of esophageal striated myofibers remains mostly undetermined.

Striated Myogenesis

Features of axial, appendicular and cranial myogenesis may help identify the process of esophageal myogenesis. It is interesting to note that virtually no review of striated myogenesis discusses the esophagus. Additionally, most studies of mammalian myogenic lineages (cranial paraxial mesoderm versus somitic paraxial mesoderm) ignore esophageal myogenesis.

Head and body muscles have different embryonic origins

Two distinct and separate myogenic programs control the development of striated musculature in mammals. The muscles of the body develop from the dermomyotome, a collection of cells found in paraxial mesodermal somites that lie beside the neural tube from the occipital region to the tail. Several comprehensive reviews detail the process of somitically-derived axial and appendicular myogenesis (Biressi et al. 2007, Bryson-Richardson & Currie 2008, Buckingham 2006, Grefte et al. 2007, Shih et al. 2007). Most skeletal muscles in the head originate in the cranial paraxial and splanchnic mesoderms. Myogenic primordia expand into the branchial (pharyngeal) arches, where they undergo final lineage commitment and subsequently move peripherally to their various cranio-facial locations. The exception to this are the muscles of the tongue and neck (non-masticatory, non-ocular muscles), which originate from somites, and most of the extraocular muscles, which arise from myogenic domains located originally in both paraxial and prechordal mesoderms (Tzahor 2009). Muscles of the pharynx, unlike the hypoglossal muscles, originate from the cranial paraxial mesoderm. Cranial myogenesis is less extensively reviewed than axial myogenesis (Grifone & Kelly 2007, Noden & Francis-West 2006, Tzahor 2009).

Three myogenic phases or waves are described in somitic myogenesis: embryonic, fetal and post-natal; also known as primary, secondary and tertiary myogenic waves. Primary myogenesis involves early formation of myotomal myoblasts and mononucleated myocytes. These *Myf5/Myf6*-expressing cells (see below for further details of molecular sequences in myogenesis) delaminate from the dermomyotome to form the early embryonic myotome, which will later give rise to all axial, appendicular and body wall musculature.

However, within this population of cells are a subset of myogenic precursor cells that express *Pax3* and *Pax7*, which do not undergo rapid differentiation into myoblasts, but are maintained as a population of proliferating myogenic precursors. These myogenic precursors provide the bulk of myoblasts in the fetal wave of myogenesis, when axial and appendicular hyperplasia is markedly accelerated.

Simultaneously with the formation of the mature myotome, myogenic precursors delaminate from the hypaxial lip of the dermomyotome of certain somites, and migrate to appendicular locations – these precursors form the limb muscles, as well as the tongue and neck muscles.

During the later, fetal, phase of development, *Pax3/Pax7*-expressing progenitor cells, which have migrated with the primary myoblast pool to their ultimate location (e.g., limbs, diaphragm, etc.), begin to proliferate and expand the resident myoblast population. This expansion is regulated by expression of Tgfβ and other antagonists of bone morphogenic protein (BMP), which acts to promote myoblast proliferation.

Finally, a third, post-natal, phase of myogenesis can be observed, both post-natally, as part of normal myogenesis, and subsequently during muscle regeneration following muscle injury. This involves the differentiation of satellite cells (adult striated muscle stem cells), which populate muscles, and reside within muscle bundles (under the basal lamina of myocytes) as undifferentiated, *Pax7*-expressing mononuclear cells. Following muscle injury, these cells are induced to undergo myodifferentiation and proliferate as myoblasts to ultimately form myotubes, replacing damaged and killed myotubes.

The muscles of the head originate in prechordal, paraxial, and lateral mesoderms. Cells from the prechordal mesoderm stream laterally and contribute to those extra-ocular muscles innervated by the oculomotor (IIIrd) cranial nerve. Paraxial mesoderm, which in the head fails to form epithelial somites, forms the other extra-ocular muscles and all proximal pharyngeal (branchial) arch muscles (Evans and Noden 2006). Recent studies in avian and mammalian embryos revealed an *islet-1*-positive population of cells located adjacent to paraxial mesoderm, in a location analogous to lateral mesoderm, that contributes to the distal branchial muscles, and also extrinsic laryngeal muscles (Nathan et al. 2008). These authors reported that the esophagus is also labeled in their *islet-1-LacZ* transgenic mice, but did not show the data or discuss which cell populations are labeled (and did not imply that muscle was labeled). Interestingly, this *islet-1*-expressing cell population also contains cardiac myocytes that contribute to the outflow tract of the heart.

Head and body muscles have different myogenic programs

Not only does the origin of striated muscle differ, but the genetic and molecular pathways involved in myogenesis of these two groups of muscles also differs.

Somitic myogenesis is induced by expression of *Pax3* and *Wnt* in the dermomyotome. Additionally *c-Met* is thought to play a role in directing migration of somite-derived appendicular myogenic precursors, while Notch signaling prevents onset of commitment and maintains a progenitor state (Vasyutina et al. 2007). Recently, other early embryonic genes, such as *Six1, Six4, Eya1* and *Eya2* and SRF (Serum Response Factor) have been shown to play a role in myogenesis, in part by affecting *Pax3* expression and function

(Buckingham 2006). Under the influence of *Pax3* and *N-cadherin*, myogenic progenitor cells delaminate from the dermomyotome and induce the classically recognized myogenic pathway involving activation of a series of transcription factors, known as myogenic regulatory factors, in a particular sequence.

In the classic model of myogenesis, the basic helix-loop-helix MRFs, *Myf5* and *MyoD,* are activated first, with *Myf5* slightly preceding and inducing *MyoD* activation. These two MRFs have been considered the early "commitment" MRFs – cells expressing these MRFs are committed to becoming myoblasts. *MyoD* and *Myf5* then induce expression of the differentiating MRFs, *Myf6* (*MRF4*) and myogenin (*myog*), which in turn initiate expression of muscle-specific functional proteins and downstream transcriptional regulators, such as *Mef2* (Figure 1.3).

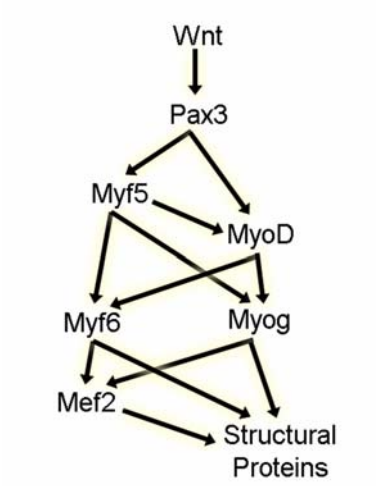

Figure 1.3 Classic Striated Myogenic Program. Pax3, under the influence of Wnt signaling from the neural tube and ectoderm, induces expression of the myogenic determination (commitment) basic-helix-loop-helix transcription factors, Myf5 and MyoD in myogenic progenitor cells. Additionally, Myf5 activates MyoD expression. Together, these factors induce expression of Myf6 and Myogenin, which trigger myodifferentiation into myoblasts, with subsequent expression of Mef2 and sarcomeric proteins.

This classical myogenic pathway has undergone considerable evolution since it was initially described and proposed. Complex studies of the roles of *Myf5, MyoD* and *Myf6* revealed a substantially more complex interaction of these MRFs than had been previously thought. First, investigators identified largely redundant, but not completely interchangeable roles for *Myf5* and *MyoD*. Deletion of either of these MRFs resulted in marginally altered myogenesis, and an essentially normal muscle phenotype (Braun et al. 1992, Rudnicki et al. 1992). Deletion of both of these MRFs resulted in complete absence of axial and appendicular musculature (Rudnicki et al. 1993). However, further studies revealed that *Myf6* shares the same locus as *Myf5* (it resides within the enhancer/promoter region of *Myf5*) and *Myf5* deletion in the early experiments also inactivated *Myf6*. Subsequent experiments that inactivated *Myf5*, but preserved *Myf6* function, revealed that *Myf6* could induce axial, but not appendicular myogenesis in the absence of both *Myf5* and *MyoD* (Kassar-Duchossoy et al. 2004). Thus, the role of *Myf6* appears to be more complex than originally thought – it functions in both commitment and differentiation.

Additionally, recent studies have suggested that rather than having redundant roles in myogenic commitment, *Myf5* and *MyoD* regulate initially separate populations of myogenic precursors (Haldar et al. 2008). Myogenin, while considered essential for myodifferentiation prenatally (*myog*$^{-/-}$ mice fail to develop muscle), is not essential for the third (postnatal) wave of myogenesis (temporally conditional *myog*$^{-/-}$ mice have essentially normal muscles), even though it is essential for differentiation of *Pax7*-positive satellite cells that contribute to the third myogenic wave (Knapp et al. 2006). Thus, the exact role of myogenin is not completely defined (Figure 1.4).

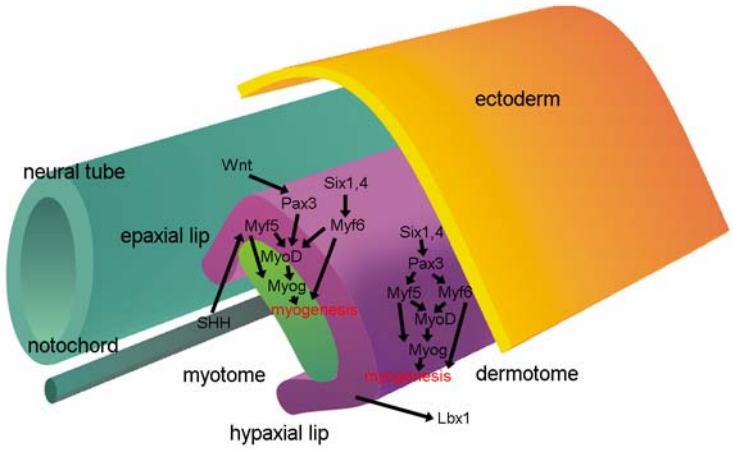

Figure 1.4 Striated myogenic program. Epaxial myotome expresses a slightly different myogenic program from hypaxial myotome. Additionally, Lbx1-positive myogenic progenitors separate from the hypaxial lip and migrate to appendicular sites.

Somitically-derived myogenic precursors migrating peripherally into the body wall and appendicular regions activate additional molecular programs to those expressed in axial muscles. Translocating myogenic cells express Lbx1 and *CXCR4* during the migration process and are regulated by *Wnt* signaling (Vasyutina et al 2005). (Figure 1.4)

The muscles of the head express a fundamentally different myogenic program (Figure 1.5). First, *Wnt* signaling, which initiates somitic myogenesis, inhibits myogenesis in head muscle progenitor cells. Upstream regulators of MRF expression in head muscle progenitor cells also differ. Through loss-of-function experiments, transcription factors such as *Tbx1*, *Pitx2*, *Msc* (*MyoR*) and *Tcf21* (*Capsulin*) have been demonstrated to regulate cranial myogenesis, without affecting axial/somitic myogenesis, despite the observation that several of these factors [*Pitx2*, *Msc*] are expressed in somitic subdomains or after somitic myodifferentiation (Dong et al. 2006, Kelly et al. 2004, Lu et al. 2002). Specifically, lack of *Msc* and *Tcf21* inhibits myogenesis in the proximal

part of the first pharyngeal arch, resulting in altered masticatory muscle development (Lu et al. 2002).

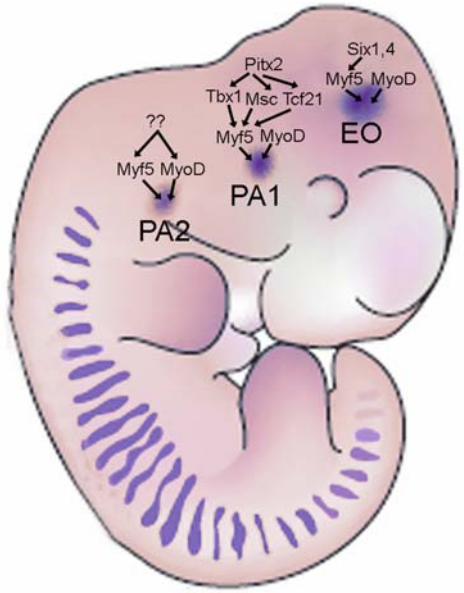

Figure 1.5 Cranial myogenic program. Pharyngeal arch (PA1 & PA2) and extraocular (EO) myogenesis is regulated differently from somitic myogenesis and differs even between cranial myogenic loci. Additionally, less is known about specific upstream regulators and regulatory interactions of Myf5 and MyoD.

Deletion of *Tbx1* results in patchy and sporadic myogenesis of masticatory muscles by altering myogenesis most notably in the 1st arch (Kelly et al. 2004). However, since *Tbx1* is also expressed in tissues adjacent to these muscles, it is not clear from knockout studies whether this negative effect is direct or indirect. Similar alterations in myogenesis are observed with *Pitx2* mutants, which have reduced *Tbx1*, *Mcf*, *Tcf21*, and, consequently, Myf5 activation in the pharyngeal arches and periocular tissues (Dong et al. 2006, Shih et al. 2008). Deficiencies in these transcription factors manifest most severely in the 1st arch and oblique extraocular muscles, suggesting that

other, as yet undiscovered, myogenic regulators affect branchiomeric and extra-ocular myogenesis. For example, the role of *Tlx1*, a homeobox-encoding transcription factor expressed in arch-derived myogenic precursors, but not somitic precursors, is not defined (Grifone & Kelly 2007).

On the other hand, *Pax3* is not expressed in head muscle progenitor cells, and *Pax3*$^{-/-}$::*Myf5*$^{-/-}$ mice develop normal head muscles, while lacking body muscles (Tajbakhsh et al. 1997). This is presumably due to the independent myogenic rescuing effects of *MyoD* in head muscles, but not in body muscles, where *MyoD* expression is *Pax3*-dependent. Further differences in myogenic programs are evidenced by different requirements for *Myf6*. Mice deficient in *Myf5* and *MyoD*, but with a functional *Myf6* gene, develop axial, somitically derived muscles (but not limb muscles) (Kassar-Duchossoy et al. 2004). However, head muscles fail to form, suggesting that *Myf6* is unable to rescue head myogenesis in the absence of *Myf5* and *MyoD*.

As stated earlier, which of these early myogenic programs, if any, is expressed in the developing esophageal *muscularis externa*, is currently unknown.

Transdifferentiation and Cellular Plasticity

In 1995, Patapoutian and colleagues suggested that esophageal striated myogenesis occurs through a process known as transdifferentiation, rather than originating from distinct progenitor subpopulations. Transdifferentiation describes the transformation of one differentiated (or committed) cell type into another (Patapoutian et al. 1995). Recently, the term has been used less discriminantly, to describe plasticity of stem cells, namely, the directed or induced differentiation of stem cells into particular cellular

phenotypes. However, since stem cells are not necessarily committed to a particular lineage, but can maintain a certain degree of pluripotency even if apparently lineage-restricted, the term is inappropriate in this context. To that end, Eguchi and Kodama suggested that 2 criteria should be met to use the term “transdifferentiation” (Eguchi & Kodama 1993): first, ***that the differentiated state before and after transdifferentiation can be reliably described and distinguished*** and second, ***that a direct ancestor-descendent relationship between cells before and after the phenotype switch can be demonstrated***.

Transdifferentiation was first coined as a term for this process by Eguchi and Okada in 1973 (Eguchi & Okada 1973), but interest in this process grew in the 1990s with the potential for stem-cell directed therapies. Understanding transdifferentiation could allow scientists to use abundant cell types to replace injured or diseased cell types by inducing them to transdifferentiate into replacement cells. For example, hepatocytes could be induced to transdifferentiate into pancreatic cells in the treatment of diabetes mellitus.

Two models of transdifferentiation are currently considered possible, and both may occur in different situations. The first model requires a differentiated cell to de-differentiate into a primordial cell that is then induced to re-differentiate into the new phenotype. In the second model the differentiated cell simply changes phenotype without an intermediary step (Burke & Tosh 2005) (Figure 1.6).

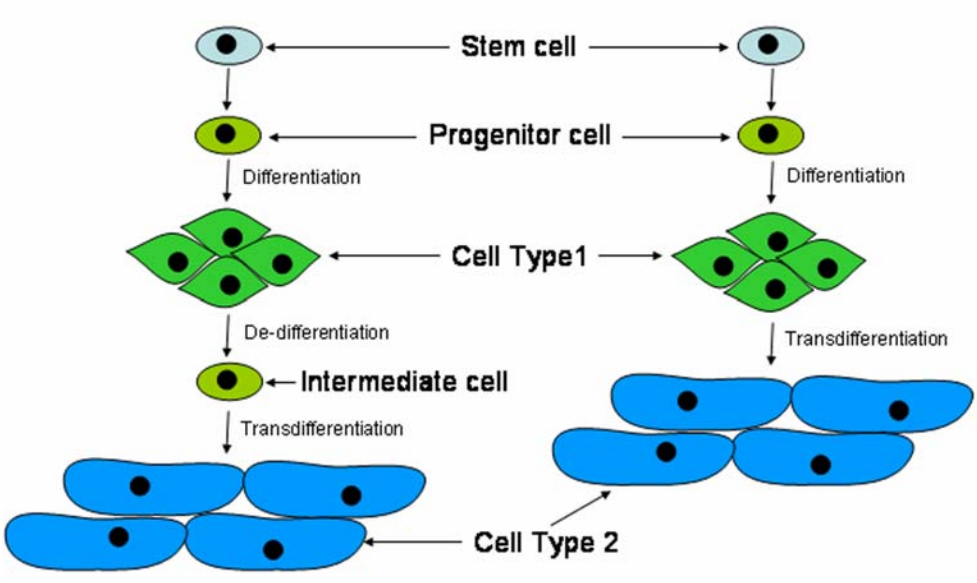

Figure 1.6 Models of transdifferentiation. Transdifferentiation is thought to occur either by dedifferentiation into an intermediate primordial cell, or directly without involvement of a de-differentiation step.

Several examples of transdifferentiation are currently accepted as likely events. Many of these appear to be restricted to *in vitro* manipulation of committed or differentiated cells. Xie et al transdifferentiated B lymphocytes into macrophages by adding specific transcription factors (C-EBPα and C-EBP-β) (Xie et al. 2004). The authors provided compelling evidence for transdifferentiation and proposed a model by which such a process might occur: they found that these transcription factors suppressed B lymphocyte-specific gene expression while simultaneously inducing macrophage-specific gene expression. Importantly, however, the B lymphocytes were committed progenitor cells, rather than differentiated mature B lymphocytes. Nevertheless, the process of B lymphocyte-to-macrophage transdifferentiation was induced both *in vivo* and *in vitro*. Heyworth and colleagues showed similar plasticity of lineage-restricted hemopoietic progenitor cells *in vitro* by modulating transcription factors in these cell lines (Heyworth et al. 2002).

In vitro pancreatic cell-to-hepatocyte transdifferentiation has been demonstrated by several groups of investigators (Shen et al. 2000, Tosh et al. 2002). These investigators, like Xie et al, found that C-EBPα and C-EBPβ were responsible for this phenotype switching (Westmacott et al. 2006). Likewise, Roach and colleagues induced a phenotype change in embryonic bone cultures where hypertrophic chondrocytes transdifferentiated into osteoblasts (Roach 1992, Roach et al. 1995).

Transdifferentiation of muscle cells has also been observed, or postulated. Cultured G8 myoblasts have been transdifferentiated into adipocytes in vitro by expression of C-EBPα and (PPAR)-γ (Hu et al. 1995), while another myoblast cell line C2C12 was transdifferentiated into adipocytes by inhibiting Wnt signaling (Ross et al. 2000). More recently, these events have been explained by demonstrating the common origin of brown fat cells and skeletal muscle cells from *Myf5*-expressing progenitors (Seale et al 2008). Bobryshev suggested that chondrocytes found in atherosclerotic plaques transdifferentiated from smooth muscle cells (Bobryshev 2005), although these findings have not been confirmed by other investigators. Several investigators have demonstrated the ability of striated muscles in some jellyfish (*Podocoryne carnea*) to transdifferentiate into smooth muscle and sensory nerve cells via a de-differentiated intermediary smooth muscle stem cell when manipulated in cell culture (Alder & Schmid 1987, Galle et al. 2005, Schmid & Alder 1984, Schmid et al.1993). Weintraub showed MyoD expression forces many cell types to become striated muscle cells (Weintraub et al. 1989), although the relevance of this to in vivo processes is uncertain. Finally, multiple investigators have demonstrated a transdifferentiation of striated muscle in urodele amphibian limb regeneration (see below).

Transdifferentiation *in situ* has also been proposed, although evidence of this event is limited. Additionally, virtually all putative observations of transdifferentiation have been restricted to regenerative processes, rather than normal development, and most often observed in non-mammalian species. The original observations of transdifferentiation were made by Wolff at the turn of the last century, although the event was not termed “transdifferentiation” at that time. Wolff noted that new lens tissue in amphibians formed from pigmented iridal epithelial cells after enucleation. Hans Spemann made similar observations and experimented with lens regeneration at around the same time (Spemann 1901). These observations were extended to other species (chickens, humans) (Eguchi & Okada1973, Eguchi 1988), and subsequently shown to be commonly inducible *in vitro* events with cells of diencephalic origin (possibly associated with the formation of the “parietal eye”) (Jurić-Lekić et al. 1991).

The other, well-recognized, model of natural transdifferentiation is that of striated muscle cells and other cells during limb regeneration in urodele amphibians. This phenomenon has been long recognized (Thornton 1938), and intensively investigated (Echeverri et al. 2001, Hay 1961, Kumar et al. 2000, Lo et al. 1993, Simon et al. 1995, Tanaka et al. 1997, Velloso et al. 2000, Velloso et al. 2001). The commonly held view in newt limb regeneration is that myocytes enter the cell cycle, dedifferentiate into pluripotent mononucleated blastema cells, and then differentiate into various tissues. This cell-cycle entry and dedifferentiation is mediated by a humoral factor found both in serum and in blastema extracts which is thought to activate *Msx-1*, a homeobox transcription factor that can induce dedifferentiation of myotubes and maintain a dedifferentiated state (Odelberg et al. 2000).

Echeverri and Tanaka additionally demonstrated transdifferentiation of ectodermally derived neural stem cells into mesodermal tissues – muscle and chondrocytes – in axolotls undergoing tail regeneration after amputation (Echeverri & Tanaka 2002). However, Gargioli and Slack showed a lack of transdifferentiation in tail regeneration in Xenopus tadpoles, and argued that a more traditional "healing" response was invoked (Gargioli & Slack 2004). Thus, the field of muscle transdifferentiation still remains incompletely defined.

In 1995, Patapoutian and colleagues published a paper claiming the first instance of transdifferentiation in a mammal as part of the normal development process, rather than as a response to regeneration or injury (Patapoutian et al. 1995). Specifically, these authors proposed that the smooth muscle cells in the fetal murine esophagus transdifferentiated into striated muscle during development, resulting in the final striated phenotype observed in adult mice. By co-immunostaining for smooth-muscle and striated-muscle markers, they showed co-expression in cells they considered to be in the process of transdifferentiation. This observation excluded the dedifferentiation/redifferentiation model as a possible mechanism, and even more astoundingly, argued for transdifferentiation of cells that are only primitively related embryologically (both cell types do originate from the dermomyotome and express *Pax3* early in their progenitor stage), a notion that flies in the face of accepted transdifferentiation theory (Tosh & Slack 2002). These results were variably supported (Kablar et al. 2000, Reddy & Kablar 2004, Stratton et al. 2000) or challenged (Zhao & Dhoot 2000a,2000b, 2000c, Wörl & Neuhuber 2005).

Hypothesis

The literature reviewed and summarized above reveals several gaps in the understanding of esophageal myogenesis and raises several questions. First, can transdifferentiation be more conclusively demonstrated as a mechanism for striated myogenesis in the murine esophagus? Second, if transdifferentiation does not occur, what is the fate of the smooth muscle population that constitutes 100% of the muscle cell type in early esophageal development? And third, if striated muscle cells do not arise by transdifferentiation, where do they originate?

The following chapters detail several studies of esophageal myogenesis that aim to answer some of these questions. Specifically, I hypothesized that murine esophageal striated myogenesis does not occur by transdifferentiation of esophageal smooth muscle cells, and that fate-mapping and conditional gene inactivation experiments would confirm this. Further, I hypothesized that the smooth muscle cell population did not require marked apoptosis or transdifferentiation to explain its apparent disappearance, but that growth and organ remodeling could account for the smooth muscle cells. Finally, I hypothesized that striated muscle precursors were resident within the *muscularis externa* prior to their initial expression of myogenic regulatory factors, and that the onset of myogenesis could be demonstrated *ex-vivo*.

REFERENCES

Achildi O, Grewal H (2007) Congenital anomalies of the esophagus. Otolaryngol Clin North Am. 40:219-44, viii

Alder H, Schmid V (1987) Cell cycles and in vitro transdifferentiation and regeneration of isolated, striated muscle of jellyfish. Dev Biol. 124:358-69

Berezin I, Daniel EE, Huizinga JD (1994) Ultrastructure of interstitial cells of Cajal in the canine distal esophagus. Can J Physiol Pharmacol. 72:1049-59

Biressi S, Molinaro M, Cossu G (2007) Cellular heterogeneity during vertebrate skeletal muscle development. Dev Biol. 308:281-93

Bobryshev YV (2005) Transdifferentiation of smooth muscle cells into chondrocytes in atherosclerotic arteries in situ: implications for diffuse intimal calcification. J Pathol. 205:641-50

Braun T, Rudnicki MA, Arnold HH, Jaenisch R (1992) Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. Cell. 71:369-82

Bremner CG, Shorter RG, Ellis FH Jr (1970) Anatomy of feline esophagus with special reference to its muscular wall and phrenoesophageal membrane. J Surg Res. 10:327-31

Brown CB, Wenning JM, Lu MM, Epstein DJ, Meyers EN, Epstein JA (2004) Cre-mediated excision of Fgf8 in the Tbx1 expression domain reveals a critical role for Fgf8 in cardiovascular development in the mouse. Dev Biol. 267:190-202

Bryson-Richardson RJ, Currie PD (2008) The genetics of vertebrate myogenesis. Nat Rev Genet. 9:632-46

Buckingham M (2006) Myogenic progenitor cells and skeletal myogenesis in vertebrates. Curr Opin Genet Dev. 16:525-32

Burgess R, Rawls A, Brown D, Bradley A, Olson EN (1996) Requirement of the paraxis gene for somite formation and musculoskeletal patterning. Nature. 384:570-3

Burke ZD, Tosh D (2005) Therapeutic potential of transdifferentiated cells. Clin Sci (Lond). 108:309-21

Burns AJ, Herbert TM, Ward SM, Sanders KM (1997) Interstitial cells of Cajal in the guinea-pig gastrointestinal tract as revealed by c-Kit immunohistochemistry. Cell Tissue Res. 290:11-20

Christensen J, Lund GF (1969) Esophageal responses to distension and electrical stimulation. J Clin Invest. 48:408-19

Christensen J, Percy WH (1984) A pharmacological study of oesophageal muscularis mucosae from the cat, dog and American opossum (Didelphis virginiana). Br J Pharmacol. 83:329-36

Diamant NE (1989) Physiology of esophageal motor function. Gastroenterol Clin North Am. 18:179-94

Dong F, Sun X, Liu W, Ai D, Klysik E, Lu MF, Hadley J, Antoni L, Chen L, Baldini A, Francis-West P, Martin JF (2006) Pitx2 promotes development of splanchnic mesoderm-derived branchiomeric muscle. Development. 133:4891-9

Echeverri K, Clarke JD, Tanaka EM (2001) In vivo imaging indicates muscle fiber dedifferentiation is a major contributor to the regenerating tail blastema. Dev Biol 236:151–164

Echeverri K, Tanaka EM (2002) Ectoderm to mesoderm lineage switching during axolotl tail regeneration. Science. 298:1993-6

Eguchi G, Okada TS (1973) Differentiation of lens tissue from the progeny of chick retinal pigment cells cultured in vitro: a demonstration of a switch of cell types in clonal cell culture. Proc Natl Acad Sci U S A. 70:1495-9

Eguchi G (1988) Cellular and molecular background of wolffian lens regeneration. Cell Differ Dev. 25 Suppl:147-58

Eguchi G, Kodama R (1993) Transdifferentiation. Curr Opin Cell Biol. 5:1023-8

Evans DJR, Noden DM (2006) Spatial relations between avian craniofacial neural crest and paraxial mesoderm cells. Dev Dyn 235:1310-25

Galle S, Yanze N, Seipel K (2005) The homeobox gene Msx in development and transdifferentiation of jellyfish striated muscle. Int J Dev Biol. 49:961-7.

Gargioli C, Slack JM (2004) Cell lineage tracing during Xenopus tail regeneration. Development. 131:2669-79

Grefte S, Kuijpers-Jagtman AM, Torensma R, Von den Hoff JW (2007) Skeletal muscle development and regeneration. Stem Cells Dev. 16:857-68

Grifone R, Kelly RG (2007) Heartening news for head muscle development. Trends Genet. 23:365-9

Gross MK, Moran-Rivard L, Velasquez T, Nakatsu MN, Jagla K, Goulding M (2000) Lbx1 is required for muscle precursor migration along a lateral pathway into the limb. Development. 127:413-24

Haldar M, Karan G, Tvrdik P, Capecchi MR (2008) Two cell lineages, myf5 and myf5-independent, participate in mouse skeletal myogenesis. Dev Cell. 14:437-45

Hay ED (1959) Electron microscopic observations of muscle dedifferentiation in regenerating Amblystoma limbs. Dev Biol 1:555–585

Heyworth C, Pearson S, May G, Enver T (2002) Transcription factor-mediated lineage switching reveals plasticity in primary committed progenitor cells. EMBO J. 21:3770-81

Hu E, Tontonoz P, Spiegelman BM (1995) Transdifferentiation of myoblasts by the adipogenic transcription factors PPAR gamma and C/EBP alpha. Proc Natl Acad Sci U S A. 92:9856-60

Huang SC, Chang BS (2004) Endothelin causes contraction of human esophageal muscularis mucosae through interaction with both ETA and ETB receptors. Regul Pept. 117:179-86

Hudson LC (1993) Histochemical identification of the striated muscle of the canine esophagus. Anat Histol Embryol. 22:101-4

Huizinga JD, Reed DE, Berezin I, Wang XY, Valdez DT, Liu LW, Diamant NE (2008) Survival dependency of intramuscular ICC on vagal afferent nerves in the cat esophagus. Am J Physiol Regul Integr Comp Physiol. 294:R302-10

Jamdar MN, Ema AN (1982) The submucosal glands and the orientation of the musculature in the oesophagus of the camel. J Anat. 135:165-71

Jurić-Lekić G, Bulić-Jakus F, Kablar B, Svajger A (1991) The ability of the epithelium of diencephalic origin to differentiate into cells of the ocular lens. Int J Dev Biol. 35:231-7

Kablar B, Tajbakhsh S, Rudnicki MA (2000) Transdifferentiation of esophageal smooth to skeletal muscle is myogenic bHLH factordependent. Development 127:1627–1639

Kamikawa Y, Shimo Y (1979) Cholinergic and adrenergic innervations of the muscularis mucosae in guinea-pig esophagus. Arch Int Pharmacodyn Ther. 238:220-32

Kassar-Duchossoy L, Gayraud-Morel B, Gomès D, Rocancourt D, Buckingham M, Shinin V, Tajbakhsh S (2004) Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. Nature. 431:466-71

Kaufmann P, Lierse W, Stark J, Stelzner F (1968) Die Muskelanordnung in der Speiseröhre (Mensch, Rhesusaffe, Kaninchen, Maus, Ratte, Seehund) [Muscle arrangement in the esophagus (man, rhesus monkey, rabbit, mouse, rat, seal)]. Ergeb Anat Entwicklungsgesch 40:3-33

Kelly RG, Jerome-Majewska LA, Papaioannou VE (2004) The del22q11.2 candidate gene Tbx1 regulates branchiomeric myogenesis. Hum Mol Genet. 13:2829-40

Knapp JR, Davie JK, Myer A, Meadows E, Olson EN, Klein WH (2006) Loss of myogenin in postnatal life leads to normal skeletal muscle but reduced body size. Development. 133:601-10

Kumar A, Velloso CP, Imokawa Y, Brockes JP (2000) Plasticity of retrovirus-labelled myotubes in the newt limb regeneration blastema. Dev Biol 218:125–136

Leander S, Brodin E, Håkanson R, Sundler F, Uddman R (1982) Neuronal substance P in the esophagus. Distribution and effects on motor activity. Acta Physiol Scand. 115:427-35

Lo DC, Allen F, Brockes JP (1993) Reversal of muscle differentiation during urodele limb regeneration. Proc Natl Acad Sci U S A 90:7230–7234

Lu JR, Bassel-Duby R, Hawkins A, Chang P, Valdez R, Wu H, Gan L, Shelton JM, Richardson JA, Olson EN (2002) Control of facial muscle development by MyoR and capsulin. Science. 298:2378-81

Mascarello F, Rowlerson A, Scapolo PA (1984) The fibre type composition of the striated muscle of the oesophagus in ruminants and carnivores. Histochemistry. 80:277-88

Nathan E, Monovich A, Tirosh-Finkel L, Harrelson Z, Rousso T, Rinon A, Harel I, Evans SM, Tzahor E (2008) The contribution of Islet1-expressing splanchnic mesoderm cells to distinct branchiomeric muscles reveals significant heterogeneity in head muscle development. Development. 135: 647-57

Neuhuber WL, Kressel M, Stark A, Berthoud HR (1998) Vagal efferent and afferent innervation of the rat esophagus as demonstrated by anterograde DiI and DiA tracing: focus on myenteric ganglia. J Auton Nerv Syst. 70:92-102

Noden DM, Francis-West P (2006) The differentiation and morphogenesis of craniofacial muscles. Dev Dyn. 235:1194-218

Odelberg SJ, Kollhoff A, Keating MT (2000) Dedifferentiation of mammalian myotubes induced by msx1. Cell. 103:1099-109

Park H, Conklin JL (1999) Neuromuscular control of esophageal peristalsis. Curr Gastroenterol Rep. 1:186-97

Patapoutian A, Wold BJ, Wagner RA (1995) Evidence for developmentally programmed transdifferentiation in mouse esophageal muscle. Science. 270:1818-21

Randelia HP, Lalitha VS (1988) Megaoesophagus in ICRC mice. Lab Anim. 22:23-6

Randelia HP, Panicker KN, Lalitha VS (1990) Megaoesophagus in the mouse: histochemical and ultrastructural studies. Lab Anim. 24:78-86

Reddy T, Kablar B (2004) Evidence for the involvement of neurotrophins in muscle transdifferentiation and acetylcholine receptor transformation in the esophagus of Myf5(–/–): MyoD(–/–) and NT-3(–/–) embryos. Dev Dyn 231:683–692

Roach HI, Erenpreisa J, Aigner T (1995) Osteogenic differentiation of hypertrophic chondrocytes involves asymmetric cell divisions and apoptosis. J Cell Biol. 131:483-94

Roach HI (1992) Trans-differentiation of hypertrophic chondrocytes into cells capable of producing a mineralized bone matrix. Bone Min 19:1-20

Ross SE, Hemati N, Longo KA, Bennett CN, Lucas PC, Erickson RL, MacDougald OA (2000) Inhibition of adipogenesis by Wnt signaling. Science. 289:950-3

Rudnicki MA, Braun T, Hinuma S, Jaenisch R (1992) Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell. 71:383-90

Rudnicki MA, Schnegelsberg PN, Stead RH, Braun T, Arnold HH, Jaenisch R (1993) MyoD or Myf-5 is required for the formation of skeletal muscle. Cell. 75:1351-9

Rumessen JJ, de Kerchove d'Exaerde A, Mignon S, Bernex F, Timmermans JP, Schiffmann SN, Panthier JJ, Vanderwinden JM (2001) Interstitial cells of Cajal in the striated musculature of the mouse esophagus. Cell Tissue Res. 306:1-14

Schmid V, Alder H (1984) Isolated, mononucleated, striated muscle can undergo pluripotent transdifferentiation and form a complex regenerate. Cell. 38:801-9

Schmid V, Baader C, Bucciarelli A, Reber-Müller S (1993) Mechanochemical interactions between striated muscle cells of jellyfish and grafted extracellular matrix can induce and inhibit DNA replication and transdifferentiation in vitro. Dev Biol. 155:483-96

Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, Scimè A, Devarakonda S, Conroe HM, Erdjument-Bromage H, Tempst P, Rudnicki MA, Beier DR, Spiegelman BM (2008) PRDM16 controls a brown fat/skeletal muscle switch. 454:961-67

Shedlofsky-Deschamps G, Krause WJ, Cutts JH, Hansen S (1982) Histochemistry of the striated musculature in the opossum and human oesophagus. J Anat. 134:407-14

Shen CN, Slack JM, Tosh D (2000) Molecular basis of transdifferentiation of pancreas to liver. Nat Cell Biol. 2:879-87

Shih HP, Gross MK, Kioussi C (2007) Cranial muscle defects of Pitx2 mutants result from specification defects in the first branchial arch. Proc Natl Acad Sci U S A. 104:5907-12

Shih HP, Gross MK, Kioussi C (2008) Muscle development: forming the head and trunk muscles. Acta Histochem. 110:97-108

Shiina T, Shimizu Y, Izumi N, Suzuki Y, Asano M, Atoji Y, Nikami H, Takewaki T (2005) A comparative histological study on the distribution of striated and smooth muscles and glands in the esophagus of wild birds and mammals. J Vet Med Sci. 67:115-7

Simon HG, Nelson C, Goff D, Laufer E, Morgan BA, Tabin C (1995) Differential expression of myogenic regulatory genes and Msx-1 during dedifferentiation and redifferentiation of regenerating amphibian limbs. Dev Dyn. 202:1-12

Spemann H (1901) Der Korrelation in der Entwicklung des Auges. Verh Anat Ges. 15: 61-79

Storr M, Geisler F, Neuhuber WL, Schusdziarra V, Allescher HD (2000) Endomorphin-1 and -2, endogenous ligands for the mu-opioid receptor, inhibit striated and smooth muscle contraction in the rat oesophagus. Neurogastroenterol Motil. 12:441-8

Storr M, Geisler F, Neuhuber WL, Schusdziarra V, Allescher HD (2001) Characterization of vagal input to the rat esophageal muscle. Auton Neurosci. 91:1-9

Stratton CJ, Bayguinov Y, Sanders KM, Ward SM (2000) Ultrastructural analysis of the transdifferentiation of smooth muscle to skeletal muscle in the murine esophagus. Cell Tissue Res 301:283–298

Sumiyoshi H, Mor N, Lee SY, Doty S, Henderson S, Tanaka S, Yoshioka H, Rattan S, Ramirez F (2004) Esophageal muscle physiology and morphogenesis require assembly of a collagen XIX-rich basement membrane zone. J Cell Biol. 166:591-600

Tajbakhsh S, Rocancourt D, Cossu G, Buckingham M (1997) Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell. 89:127-38

Takahashi Y, Takagi A, Hiraoka S, Koseki H, Kanno J, Rawls A, Saga Y (2007) Transcription factors Mesp2 and Paraxis have critical roles in axial musculoskeletal formation. Dev Dyn. 236:1484-94

Takaki M (2003) Gut pacemaker cells: the interstitial cells of Cajal (ICC). J Smooth Muscle Res. 39:137-61

Tanaka EM, Gann AA, Gates PB, Brockes JP (1997) Newt myotubes reenter the cell cycle by phosphorylation of the retinoblastoma protein. J Cell Biol 136:155–165

Thornton CS (1938) The histogenesis of muscle in the regenerating forelimb of larval Ambystoma punctatum. J Morphol 62:219–235

Tosh D, Slack JM (2002) How cells change their phenotype. Nat Rev Mol Cell Biol. 3:187-94

Tosh D, Shen CN, Slack JM (2002) Differentiated properties of hepatocytes induced from pancreatic cells. Hepatology. 36:534-43

Tzahor E (2009) Heart and craniofacial muscle development: A new developmental theme of distinct myogenic fields. Dev Biol (Epub ahead of print)

Uchida K, Yuzuki R, Kamikawa Y (1998) Pharmacological characterization of endothelin-induced contraction in the guinea-pig oesophageal muscularis mucosae. Br J Pharmacol. 125:849-57

Uddman R, Alumets J, Håkanson R, Sundler F, Walles B (1980) Peptidergic (enkephalin) innervation of the mammalian esophagus. Gastroenterology. 78:732-7

Uriona TJ, Farmer CG, Dazely J, Clayton F, Moore J (2005) Structure and function of the esophagus of the American alligator (Alligator mississippiensis). J Exp Biol. 208:3047-53

Van den Brink GR (2007) Hedgehog signaling in development and homeostasis of the gastrointestinal tract. Physiol Rev 87:1343-1375

Vasyutina E, Stebler J, Brand-Saberi B, Schulz S, Raz E, Birchmeier C (2005) CXCR4 and Gab1 cooperate to control the development of migrating muscle progenitor cells. Genes Dev. 19:2187-98

Vasyutina E, Lenhard DC, Birchmeier C (2007) Notch function in myogenesis. Cell Cycle. 6:1451-4

Velloso CP, Kumar A, Tanaka EM, Brockes JP (2000) Generation of mononucleate cells from postmitotic myotubes proceeds in the absence of cell cycle progression. Differentiation 66:239–246

Velloso CP, Simon A, Brockes JP (2001) Mammalian postmitotic nuclei reenter the cell cycle after serum stimulation in newt/mouse hybrid myotubes. Curr Biol 11:855–858

Wang Q, Xu S, Tu L, Liu Y, Zhang M (2007) Anatomic continuity of longitudinal pharyngeal and esophageal muscles. Laryngoscope. 117:282-7

Wang Y, Huso D, Cahill H, Ryugo D, Nathans J (2001) Progressive cerebellar, auditory, and esophageal dysfunction caused by targeted disruption of the frizzled-4 gene. J Neurosci. 21:4761-71

Weintraub H, Tapscott SJ, Davis RL, Thayer MJ, Adam MA, Lassar AB, Miller AD (1989) Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc Natl Acad Sci U S A. 86:5434-8

Westmacott A, Burke ZD, Oliver G, Slack JM, Tosh D (2006) C/EBPalpha and C/EBPbeta are markers of early liver development. Int J Dev Biol. 50:653-7

Whitmore I (1982) Oesophageal striated muscle arrangement and histochemical fibre types in guinea-pig, marmoset, macaque and man. J Anat. 134:685-95

Wörl J, Neuhuber WL (2005) Enteric co-innervation of motor endplates in the esophagus: state of the art ten years after. Histochem Cell Biol. 123:117-30

Wu M, Majewski M, Wojtkiewicz J, Vanderwinden JM, Adriaensen D, Timmermans JP (2003) Anatomical and neurochemical features of the extrinsic and intrinsic innervation of the striated muscle in the porcine esophagus: evidence for regional and species differences. Cell Tissue Res. 311:289-97

Xie H, Ye M, Feng R, Graf T (2004) Stepwise reprogramming of B cells into macrophages. Cell. 117:663-76

Zhao W, Dhoot GK (2000a) Both smooth and skeletal muscle precursors are present in foetal mouse oesophagus and they follow different differentiation pathways. Dev Dyn 218:587–602

Zhao WF, Dhoot GK (2000b) Skeletal muscle precursors in mouse esophagus are determined during early fetal development. Dev Dyn 219:10–20

Zhao W, GK Dhoot (2000c) Development and composition of skeletal muscle fibres in mouse oesophagus. J Muscle Res Cell Motil 21:463-473

CHAPTER 2

Skeletal Myogenesis in the Mouse Esophagus Does Not Occur Through Transdifferentiation*

* Originally published as: Rishniw M, Xin H-B, Deng K-Y, Kotlikoff MI. Skeletal Myogenesis in the Mouse Esophagus Does Not Occur Through Transdifferentiation. Genesis 2003;36:81-82. Reproduced with permission.

Introduction, Results and Discussion

Transdifferentiation is the conversion of cells from one differentiated phenotype to another (Tosh and Slack, 2002), a process that implies substantial cellular plasticity and could allow regeneration or repair of tissues or organs with cells committed to dissimilar lineages. The developmental program of the mouse esophagus, in which the esophageal musculature is converted from smooth to skeletal muscle, is perhaps the most commonly cited example of transdifferentiation during normal mammalian development (Patapoutian et al., 1995). The external muscle layer of the fetal mouse esophagus initially develops as smooth muscle and is gradually and almost completely converted to skeletal muscle by the second week of postnatal life. Here we show conclusively that skeletal myocytes arise from a distinct lineage, rather than through the transdifferentiation of smooth muscle cells.

To permanently lineage-map smooth muscle cells we crossed transgenic mice expressing Cre recombinase and eGFP under control of the smooth muscle myosin heavy chain promoter (Xin et al., 2002) with R26R LacZ reporter mice. In these double transgenic mice, all differentiated smooth muscle cells express eGFP, whereas cells that were of smooth muscle phenotype at any point in development constitutively express β-galactosidase through Cre recombinase-mediated excision of an inhibitory cassette at the ROSA26 locus.

In 15-day post-conception (dpc) fetuses, eGFP expression and X-gal staining marked the muscle layers along the entire esophagus, identifying them as being predominately composed of smooth muscle, and demonstrating tissue-specific activation of the LacZ gene (Figure 2.1, left panels).

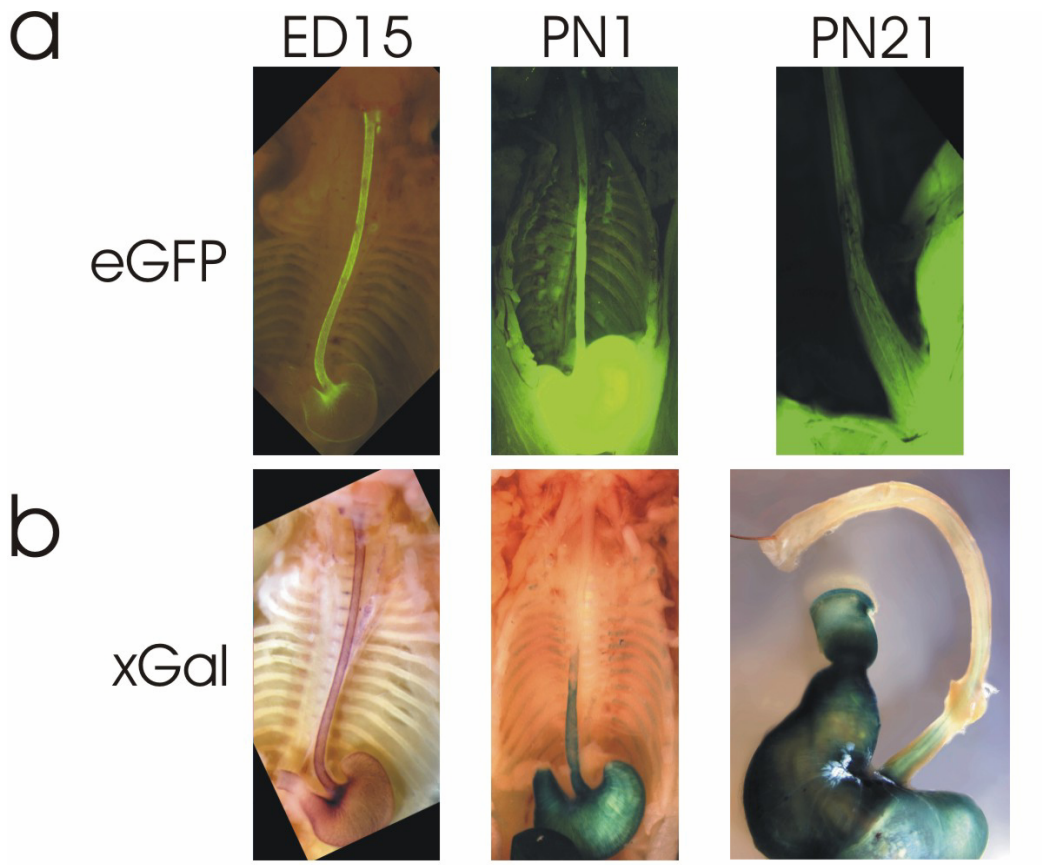

Figure 2.1 Lineage mapping of murine esophageal smooth muscle. a. eGFP fluorescence of ED 15 (left), PN 1 (middle), and PN 21 (right) transgenic mice, showing cranio-caudal regression of smooth muscle in the esophagus with maturation. b: LacZ expression at same stages indicates parallel regression of smooth muscle, fate-mapped cells.

If esophageal smooth muscle transdifferentiated into striated muscle, this X-gal staining pattern would be anticipated to be retained in spite of a cranio-caudal regression of eGFP fluorescence associated with the regression of smooth muscle during development. Conversely, replacement of smooth muscle through skeletal myogenesis from non-smooth muscle cells would show parallel regression of X-gal staining and eGFP fluorescence in the *muscularis externa*, but not the *muscularis mucosae* (which remains smooth muscle throughout adult life).

We found that both the X-gal staining pattern and eGFP expression in the external muscle layers regressed cranio-caudally as the esophagus matured. In 1-day-old mice (at which time the external esophageal muscle is approximately 30–50% transformed from smooth to striated), only the caudal two-thirds of the esophagus showed X-gal staining, completely concordant with eGFP expression (Figure 2.1, middle panels). At 21 days (when the murine esophagus is fully developed), only the terminal esophagus showed X-gal staining, identical to eGFP fluorescence (Figure 2.1, right panels).

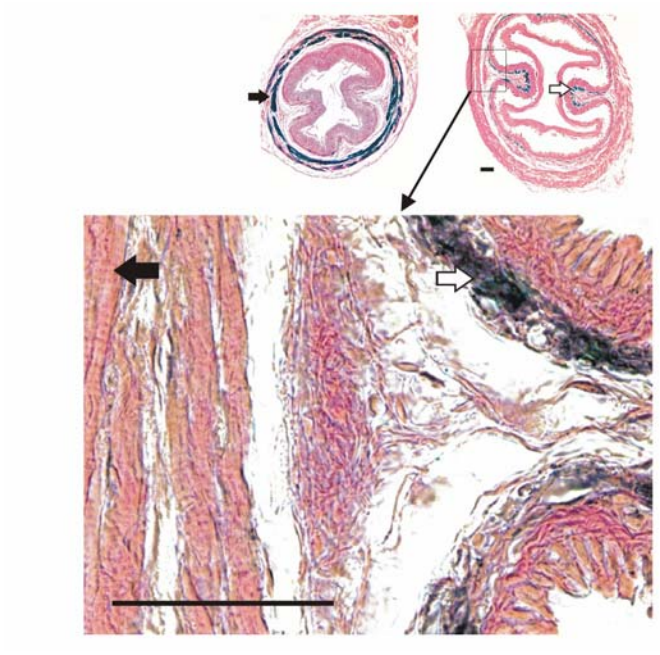

Figure 2.2 LacZ expression is limited to esophageal smooth muscle cells. Above, LacZ expression in section of caudal esophagus showing staining of *muscularis externa* at PN 1 (left, filled arrow) and loss of smooth muscle fate-mapped cells in this layer at PN 21 (right). Smooth muscle in the *muscularis mucosae* (open arrow) is stained. Below, Inset showing LacZ-negative, skeletal myocytes in *muscularis externa* (filled arrow), as well as LacZ-positive cells in *muscularis mucosae* (open arrow), at PN 21. Scale bar = 50 μm.

Histologic sections demonstrated that skeletal fibers were completely free of X-gal staining, whereas smooth muscle in the *muscularis mucosae* exhibited X-gal staining in the same sections (Figure 2.1b and Figure 2.2). To exclude the possibility that the failure of any skeletal fibers to express LacZ results from the differentiation of skeletal myocytes from a subpopulation of smooth muscle cells that do not express the transgene, we examined eGFP and smooth muscle α-actin staining in rostral esophageal sections from 15 dpc embryos. eGFP and smooth muscle α-actin expression in the *muscularis externa* were concordant, with cells expressing both proteins in two layers, separated by a single cell layer of nonsmooth muscle cells (Figure 2.3).

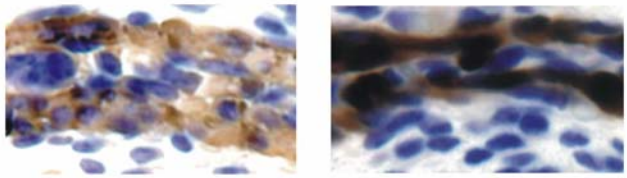

Figure 2.3 Smooth muscle α-actin (left) and eGFP (right) antibody staining of the esophageal *muscularis externa* of 15 day-post-conception fetuses. Both layers of the *muscularis externa* are stained with each antibody. A population of nonstaining cells is present between the two layers.

Thus, the coordinate regression of eGFP and X-gal-positive cells seen throughout the esophagus in the fetus to the most caudal esophagus in mature mice is definitive evidence that striated esophageal myocytes do not transdifferentiate from smooth muscle cells, but arise through skeletal myogenesis of a separate pool of skeletal precursor cells (Zhao and Dhoot, 2000). The evidence against transdifferentiation is particularly compelling given the expression of LacZ along the complete length of the embryonic

esophagus, indicating Cre recombinase activity sufficient to produce recombination in all embryonic smooth muscle.

These results highlight the unique myogenic program in the mammalian esophagus and, more importantly, place additional constraints on the notion of transdifferentiation during normal development in mammals and the plasticity of distinct muscle lineages.

Materials and Methods

R26R LacZ reporter mice were crossed with male smMHC/Cre/IRES/eGFP (Xin et al., 2002) mice from a single line (SMCG2). ED (embryonic day) 15 embryos and 1- and 21-day postnatal mice were analyzed. Embryos and day-old mice were dissected to expose the esophagus and stomach by removing all other viscera. In postnatal day (PN) 21 mice the esophagus and stomach were removed for fluorescence imaging, fixation, and staining. eGFP fluorescence in mice or tissues was visualized by epifluorescence microscopy and tissues were stained for β-galactosidase activity using standard methods. Briefly, mice or tissues were fixed for 20 minutes to 1 hour in 4% formaldehyde at 4ºC, rinsed once in PBS, then permeabilized in permeabilization buffer (100 mM sodium phosphate (pH 7.3), 2 mM magnesium chloride, 0.01% sodium deoxycholate, 0.02% NP-40 (by volume)), once at 4ºC and twice at room temperature for 30 minutes, and stained in staining solution (permeabilization buffer plus: 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1mg/mL X-gal) for 18 hours at room temperature in the dark.

X-gal-stained tissues from day-old and 21-day old mice were processed by standard histological methods, with the exception that the pre-embedding

infusion of the dehydrated tissues was performed with an isopropanol-paraffin solution rather than xylene. Sections were stained with eosin only.

Smooth-muscle α-actin and eGFP immunohistochemistry was performed on formalin-fixed, paraffin-embedded embryos, processed identically to tissues described above. Sections were counterstained with hematoxylin.

Acknowledgments

The authors thank Dr. Drew Noden for reviewing the manuscript and Jason Wilson for help in tissue processing.

REFERENCES

Patapoutian A, Wold BJ, Wagner RA. 1995. Evidence for developmentally programmed transdifferentiation in mouse esophageal muscle. Science 270:1818–1821.

Tosh D, Slack JM. 2002. How cells change their phenotype. Nat Rev Mol Cell Biol 3:187–194.

Xin H-B, Deng K-Y, Rishniw M, Ji G, Kotlikoff MI. 2002. Smooth muscle expression of Cre recombinase and eGFP in transgenic mice. Physiol Genomics 10:211–215.

Zhao W, Dhoot GK. 2000. Both smooth and skeletal muscle precursors are present in foetal mouse oesophagus and they follow different differentiation pathways. Dev Dyn 218:587–602.

CHAPTER 3

Smooth muscle persists in the *muscularis externa* of developing and adult mouse esophagus *

* Originally published as: Rishniw M, Fisher PJ, Doran RM, Meadows E, Klein WH & Kotlikoff MI. Smooth muscle persists in the *muscularis externa* of developing and adult mouse esophagus. J Muscle Res Cell Motil 2007;28:153-165. Reproduced with permission.

Abstract

Following initial patterning as differentiated smooth muscle cells, the *muscularis externa* of the murine esophagus is replaced by skeletal muscle, but the mechanism underlying this process is controversial. The hypothesis that committed smooth muscle cells transdifferentiate into striated muscle is not consistent with fate mapping studies. Similarly, apoptosis does not fully explain the process. Using immunohistochemical techniques and transgenic mice that express eGFP and Cre recombinase exclusively in smooth muscle, we have identified a population of remnant smooth muscle cells that persist throughout the developing and mature murine esophagus. These cells display an atypical phenotype, are not associated with microvasculature, but are often apposed to c-Kit positive, interstitial cells of Cajal. The absolute length of the smooth muscle component of the developing esophagus remains constant during a period when total esophageal length increases 4-fold, resulting in a small maintained distal segment of smooth muscle. Esophageal smooth muscle cells fail to express myogenin during development, and striated muscle cell precursors expressing myogenin fail to express specific smooth muscle cell markers, indicating that they did not transdifferentiate from smooth muscle cells. Moreover, smooth muscle-specific myogenin inactivation has no effect on esophageal skeletal myogenesis.

Taken together, our results provide an alternative hypothesis regarding the fate of smooth muscle cells in the developing murine esophagus, which does not invoke apoptosis or transdifferentiation.

Introduction

The murine esophagus begins development at approximately embryonic day 11.5, as a tube ensheathed by three layers of smooth muscle cells—the muscularis interna (*muscularis mucosae*), and two layers of the *muscularis externa*. Over a 3-week period (until about postnatal day 14), the smooth muscle of the *muscularis externa* is progressively replaced by striated muscle in a cranio-caudal direction. The mature murine esophagus has a small smooth muscle segment just cranial to the lower esophageal sphincter, but striated muscle composes >95% of the *muscularis externa*.

The mechanism underlying the process of muscular substitution is controversial. Initial studies of murine esophageal development suggested that committed smooth muscle cells transdifferentiated into striated muscle cells (Patapoutian et al. 1995; Kablar et al. 2000; Stratton et al. 2000), however, several lines of evidence raise fundamental doubts with respect to the transdifferentiation hypothesis of the origin of esophageal skeletal muscle. First, esophageal striated muscle cells fail to develop in mice lacking Myf5 and MyoD myogenic regulatory factors (Kablar et al. 2000; Reddy and Kablar 2004), indicating that if transdifferentiation occurs, the process requires the expression of factors that normally regulate mesenchymal myogenesis. Second, striated muscle precursors are present during the initial stages of esophageal development (Zhao and Dhoot 2000a, b). Third, immunocytochemical studies have failed to find unambiguous evidence of the simultaneous expression of smooth and skeletal muscle restricted proteins in transdifferentiating myocytes (Zhao and Dhoot 2000a, b), which remains the major evidence for this hypothesis. Finally, and perhaps most definitive, fate mapping studies in which smooth muscle cells were lineage-tagged through

expression of the lacZ gene demonstrated unequivocally that esophageal skeletal myocytes show no evidence of a prior smooth muscle phenotype (Rishniw et al. 2003). One argument advanced in favor of muscle transdifferentiation has been the lack of evidence of apoptosis of smooth muscle cells during the period of skeletal muscle development (Patapoutian et al. 1995) concomitant with the apparent disappearance of the *muscularis externa* (Breuer et al. 2004; Sumiyoshi et al. 2004). However, this hypothesis has been recently challenged by exhaustive ultrastructural analysis that identified apoptotic cells within developing esophageal muscle (Wörl and Neuhuber 2005). Here we address the issue of the fate of smooth muscle cells in the embryonic esophagus. We show that the absolute length of the smooth muscle component of murine esophagus remains virtually unchanged during the first 2 weeks of development; and that smooth muscle cells remain scattered throughout the developing and mature striated *muscularis externa*, but adopt atypical smooth muscle morphology. Further, we show that myogenin expression occurs in a population of cells distinct from cells expressing a smooth muscle specific transgene. We propose that a loss of smooth muscle through apoptosis is not an inevitable consequence of esophageal striated muscle development and that striated muscle myogenesis simply displaces and dilutes at least a portion of the pre-existing smooth muscle cells.

Materials and methods

Tissue preparation

The smMHCCre-IRES-eGFP (SMCG2) mice and wildtype C57B6J mice used in this study were bred at the Core Transgenic Mouse Facility at Cornell University College of Veterinary Medicine. The day at which a vaginal plug was detected was designated embryonic day 0 (ED0) and parturition date was designated as postnatal day 0 (PN0). Mice were killed by CO_2 inhalation at various embryonic and post-natal stages. Conditional myogenin-null (*Myog*$^{flox/flox}$) mice were obtained from the University of Texas. Embryonic and neonatal esophagi, along with stomachs and pharynges were dissected out in ice-cold phosphate-buffered saline (PBS, pH 7.4), and immediately imaged for fluorescence and measured using a dissecting scope (Leica MZ FL3, Leica Microsystems Bannockburn, IL) coupled through a digital camera (Optronics Magnafire, Optronics Inc., Goleta CA) to image acquisition software (Magnafire 2.0, Optronics Inc., Goleta CA). Immediately after imaging, tissues were placed on Costar Transwell® filters (6.5 mm diam, 3 μm pore size, Corning Inc., Corning NY) and fixed in 10% formalin for 24 hours at room temperature. Fixed esophagi were then embedded in Histogel® (Richard- Allen Scientific, Kalamazoo MI) on a flat surface, and processed for routine immunohistochemistry. Adult esophagi were handled in a similar fashion with the exception of embedding and fixation: the duodenum was ligated, the stomach and esophagus was filled with Histogel, and then ligated at the pharynx. Once the Histogel had set, the stomach was trimmed, and the esophagus was placed in 10% formalin for 24 hours at room temperature in a processing cassette.

For c-Kit immunostaining, adult esophagi and stomachs were dissected in ice-cold PBS, embedded in OCT medium (Tissue-Tek OCT Compound, Sakura Finetek, The Netherlands) and immediately frozen in semi-liquid isopentane, chilled in liquid nitrogen.

Genotyping

Conditional myogenin null mice (*Myog*$^{flox/flox}$) were mated with SMCG2 mice to produce SMCG2*Myog*$^{flox\ /+}$ offspring. Male SMCG2MyogfloxD/+ offspring were backcrossed to *Myog*$^{flox/flox}$ females to produce the target population SMCG2*Myog*$^{flox\ /flox}$ that had myogenin deleted from smooth muscle. Myogenin status was identified by PCR genotyping that was developed to identify *Myog*^{WT}, *Myog*^{flox}, and *Myog*^{flox} . Cre recombinase status was identified by PCR genotyping for Cre recombinase.

Immunohistochemistry

Esophagi were sectioned sagittally either at 4 μm (for standard fluorescence microscopy) or 10 μm (for confocal imaging) and mounted on poly-lysine coated slides for immunostaining. Immunostaining procedures were optimized for each antibody and antibody combination. For c-kit and smooth muscle α-actin co-immunostaining, frozen sections were blocked in Mouse IgG blocking reagent (M.O.M., Vector labs, Burlingame, CA), TBS triton, 10% goat serum, 10% horse serum and 10% non-fat dry milk (NFDM) for 20 minutes; incubated at 37ºC for 20 minutes, then at room temperature for 20 minutes simultaneously with mouse anti-human smooth muscle α-actin (1:20, M0851, clone 1A4, Dako-Cytomation Inc., Carpinteria, CA) and rat anti-mouse c-kit (1:5, RM6200, Caltag Laboratories, Burlingame, CA) antibodies; followed by FITC-conjugated horse anti-mouse IgG (1:50, Vector Labs) to detect smooth muscle α-actin. Subsequently, biotinylated goat-anti-rat (1:30,

minimum cross-reaction mouse, 112-065-167, Jackson ImmunoResearch, Westgrove, PA) and Texas Red Avidin DCS (1:50, A-2016, Vector Labs) were applied for 20 minutes at room temperature to detect c-kit positive interstitial cells of Cajal (ICC) cells.

For CD31 (or vWF), and smooth muscle α-actin co-immunostaining, fixed sections were microwaved 2 X 10 minutes in citrate (pH 6.0) (CD31) or treated with pronase for 20 minutes at 37ºC (vWF); blocked in mouse IgG blocking reagent as above, TBS triton, 10% rabbit serum (CD31) or 10% goat serum (vWF) and 10% horse serum and 10% NFDM for 20 minutes; incubated at 37ºC for 20 minutes, then at room temperature for 20 minutes, simultaneously with mouse anti-human Smooth muscle α-actin (1:30, as above) and goat anti-rat CD31 (1:50, SC1506, Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-human vWF (1:1,500, A0082, Dako-Cytomation Inc., Carpinteria, CA) antibodies; detected with FITC-conjugated horse anti-mouse IgG (as above) to detect smooth muscle α-actin; biotinylated rabbit anti-goat IgG (undiluted, 50-232Z Invitrogen Corp., Carlsbad, CA) to detect CD31 or biotinylated goat anti-rabbit IgG (1:50, BA-1000,Vector Labs) for 20 minutes at room temperature, then Texas Red Avidin DCS(1:100, A-2016, Vector Labs, Burlingame, CA) to detect vWF.

For smooth muscle α-actin and Concavalin A immunostaining, fixed sections were incubated in pronase for 15 minutes at 37ºC; blocked in mouse IgG blocking reagent as above and 10% goat serum for 20 minutes; incubated at 37ºC for 2 hours with mouse-antihuman anti-smooth muscle α-actin (1:30, M0851, clone 1A4, Dakocytomation Inc., Carpintaria, CA) antibody; detected with Texas Red goat anti-mouse IgG (1:100, Molecular Probes, Eugene, OR). The sections were then incubated with biotinylated Concavalin A (1:450,

B1005, Vector Labs, Burlingame, CA) for 20 minutes at room temperature; then streptavidin-conjugated Alexafluor 488 (1:100, Molecular Probes, Eugene, OR).

For Pax-7 and eGFP, Cre recombinase or smooth muscle α-actin co-immunostaining, fixed sections were steamed in 0.01 M citrate buffer (pH 6.0) for 20 minutes. After permeabilization with TBS 0.05% Triton X for 5 minutes, a Mouse-on-Mouse (M.O.M.) labeling kit (Vector Labs, Burlingame, CA) was used as per instructions for Pax-7 staining (1:2, Developmental Studies Hybridoma Bank, Iowa City, IA), substituting Texas Red Avidin (or Streptavidin Alkaline phosphatase/DAKO Permanent Red) for the final ABC step. Stained sections were held in distilled water overnight, then blocked with 10% goat serum. Incubation with rabbit polyclonal GFP antibody (1:4, AB3080, Chemicon International Inc., Temecula, CA) or rabbit polyclonal Cre recombinase antibody (1:100, PRB-106C, Covance Research Products, Berkley, CA) was followed by FITC goat anti-rabbit secondary antibody (1:50, FI-1000, Vector Labs, Burlingame, CA). Controls were included, substituting normal mouse IgG or rabbit IgG for primary antibodies at appropriate dilutions. Additional blocking steps were required for co-immunostaining with two mouse primary antibodies (Pax-7 and a-SM actin). Following Pax-7 staining with DAKO Permanent Red, samples were blocked in 10% normal horse serum and normal mouse IgG blocker (M.O.M., Vector Labs, Burlingame, CA) for 1 hour, followed by Fab fragment goat anti-mouse IgG (1:50, 115-006-006, Jackson Immunoresearch Laboratories, West Grove, PA) for 20 minutes at 37°C. Sections were then incubated with mouse-antihuman anti-smooth muscle α-actin (1:40, M0851, clone 1A4, Dakocytomation Inc., Carpintaria,

CA) antibody, and FITC horse antimouse secondary antibody (1:50, FI-2000, Vector Labs, Burlingame, CA).

Sections used for CD31 and smooth muscle α-actin or eGFP and Pax-7 staining were additionally mounted in DAPI Vectashield (H-1200, Vector Labs, Burlingame, CA) for nuclear visualization.

For myogenin and Cre recombinase co-immunostaining, fixed sections were steamed for 15 minutes in 0.01 M citrate buffer (pH 6.0); blocked in mouse IgG blocking reagent as above, TBS triton, 10% goat serum and 10% NFDM for 20 minutes; incubated at 37ºC for 2 hours with mouse anti-rat myogenin (1:30, M3559, DakoCytomation Inc., Carpinteria, CA), then with biotinylated anti-mouse (M.O.M., Vector Labs, Burlingame, CA) for 20 minutes at room temperature; followed by streptavidin-conjugated Alkaline phosphatase (1:200, Vector Labs, Burlingame, CA), and finally, myogenin was detected with Permanent Red (K0640, DakoCytomation Inc., Carpinteria, CA). The slides were then incubated with rabbit anti-Cre recombinase (1:100, PRB-106C, Covance Research Products, Berkley, CA) for 2 hours at 37ºC and detected with FITC-labeled goat anti-rabbit IgG (1:50, 65-6111, Zymed Laboratories, Carlsbad, CA). Apoptosis was detected by staining fixed sections stained with Hoechst 33342 (1:1,000, H3570, Invitrogen Corp., Carlsbad, CA); sections were incubated at room temperature for 5 minutes, washed and mounted in Vectashield (Vector Labs, H-1000, Burlingame, CA). The Pax-7 monoclonal antibody developed by Atsushi Kawakami was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

Imaging

Fluorescent microscopy was performed using a Fluorescent microscope (AX70 or BX51, Olympus America, Melville, NY). Confocal microscopy was performed with a inverted microscope (Nikon TE300, Nikon USA or Olympus IX81) with a confocal scanhead attachment (Radiance 2000, Bio-Rad Laboratories, Hercules, CA or FV1000, Olympus America, Melville, NY). All fluorescent images were acquired with filters optimized for the emission spectra of the fluorophores used. Three-dimensional models of SM cells and adjacent tissues were created using Volocity 3.0 (Improvision Inc., Lexington, MA).

Measurements and calculations

$$Area = \pi\left(\frac{d_o}{2}\right)^2 - \pi\left(\frac{d_i}{2}\right)^2$$

To estimate cross-sectional area of the *muscularis externa* at E14.5 and adult, digital images of sagittal sections were acquired. The inner and outer diameters of the *muscularis externa* were obtained and the cross-sectional area was calculated as

where d_o is the outer diameter of *muscularis externa* and d_i is the inner diameter of *muscularis externa*. Length measurements of the esophagi were obtained from digital image files using ImageJ software (ImageJ 1.33U, NIH, Bethesda, MD) with measuring tools developed in our laboratory. Length of the fluorescent portion of whole-mount esophagi was determined using ImageJ using the measuring tool and examining the profile plot of the pixel intensity along the measurement line. The point at which pixel intensity (grey

value) decreased by 50% of the pre-gastric esophageal pixel intensity was assigned as the limit of fluorescent portion of the esophagus. Esophagi were laid out on Petri dishes for imaging, which slightly stretched them compared to their in vivo lengths.

Results

Absolute length of the smooth muscle portion of the muscularis externa remains unchanged during a 2-fold increase in esophageal length. To investigate the fate of esophageal smooth muscle cells, we isolated esophagi from ED14.5, PN2-3, PN8-10, and PN21 SMCG2 transgenic mice, in which the expression of Cre recombinase and eGFP is restricted to smooth muscle by the smooth muscle myosin heavy chain promoter (Xin et al. 2002; Rishniw et al. 2003), and measured the length of the fluorescent portion of the esophagus and total esophageal length (Figure 3.1A–D). Whole-mount esophagi from ED14.5 mice measured 4.7 mm (n = 6) and smooth muscle fluorescence extended along the entire length of the esophagus, consistent with our previously published results (Rishniw et al. 2003) (Figure 3.1A and Figure 3.2). Whole-mount esophagi from PN2-3 mice measured 9.6 mm (n = 13) and fluorescence was largely restricted to the caudal 50% of the esophagus and the length of the fluorescent smooth muscle portion measured 4.8 mm (Figure 3.1B and Figure 3.2). In PN8-10 mice, the esophagus measured 15 mm (n = 4), and smooth muscle fluorescence was observed along the distal 4 mm of the esophagus (Figure 3.1C and Figure 3.2). In PN21 mice, at which time esophageal development has been completed, gross fluorescence was limited to a small region (approximately 1.5 mm) at the lower esophageal sphincter (Figure 3.1D).

We next obtained full-length sagittal histological sections of esophagi from SMCG2 mice, labeled them with anti-smooth muscle α-actin or anti-GFP antibodies, and measured the length of the smooth muscle portion of the *muscularis externa*. The results were similar to those obtained with gross fluorescent measurements (Figure 3.1E–H). Esophagi from ED14.5 mice measured 4.2 mm with a smooth muscle portion of 4.2 mm; those from PN3 mice measured 7.8 mm, with a smooth muscle portion of 4.3 mm; those from PN8 mice measured 11 mm with a smooth muscle portion of 3 mm and those from PN21 or adult mice measured 25–35 mm with a smooth muscle portion of approximately 1–1.5 mm (Figure 3.2).

Thus while the esophagus undergoes a substantial change in length (from 3 mm to 10–15 mm) (Figure 3.2), the linear dimension of the smooth muscle portion remains relatively unchanged, even though the proportion of its contribution to the total esophageal *muscularis externa* decreases by approximately 75%.

These findings suggest that marked smooth muscle apoptosis is unlikely to occur over this period, particularly in light of the likelihood of tissue compaction during this period. To further investigate the possibility of apoptosis, which has recently been proposed as a mechanism by which esophageal developing smooth muscle disappears (Wörl and Neuhuber 2005), we stained full-length 4 μm thick sagittal esophageal sections of PN8 mice, where approximately 70% of the esophagus is striated, with Hoechst 33342, which identifies apoptotic nuclei. We could only identify occasional apoptotic cells in these sections.

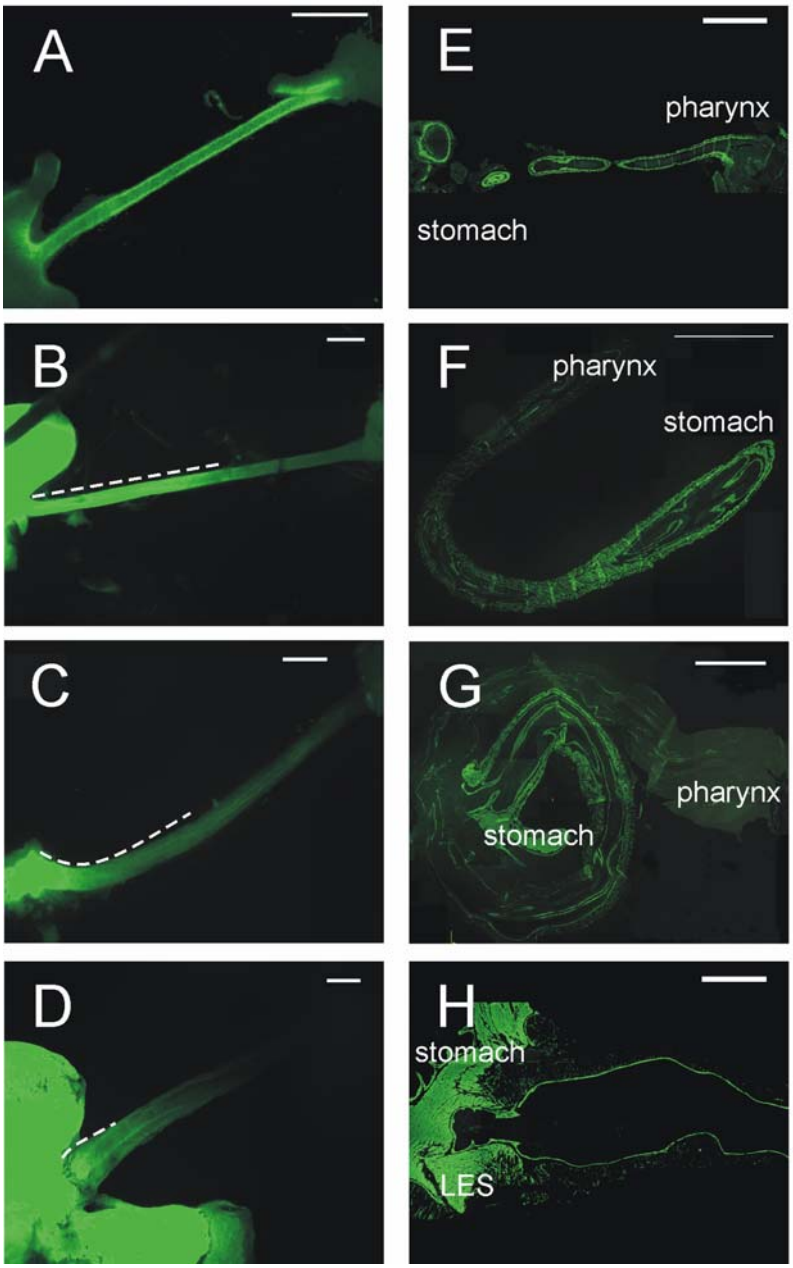

Figure 3.1 Smooth muscle expression is progressively restricted during development to the caudal esophagus. Extent of the smooth muscle component of esophagi from ED14.5 (A, E), PN2-3 (B, F), PN8-10 (C, G) and PN21 (D, H) smMHC-Cre-IRES-eGFP (SMCG2) transgenic mice. Fluorescence of fresh whole-mount esophagi from SMCG2 mice (A-D), wide-field fluorescent smooth muscle α-actin labeling (E-H), shows a progressive restriction of smooth muscle to the caudal esophagus during esophageal maturation. Dashed line in panels B-D represents the approximate portion of the esophagus measured as fluorescent. LES = Lower Esophageal Sphincter. Scale bar = 1mm.

The general conservation and progressive confinement of smooth muscle tissue to the caudal esophagus, suggest that marked smooth muscle apoptosis is unlikely to occur over this period. However, a progressive decrease in the length of the smooth muscle segment of the *muscularis externa* by adulthood suggests that simple compaction of the initial muscle might not fully explain smooth muscle cell fate, and gradual apoptotic loss may account for some of the decrease in smooth muscle tissue.

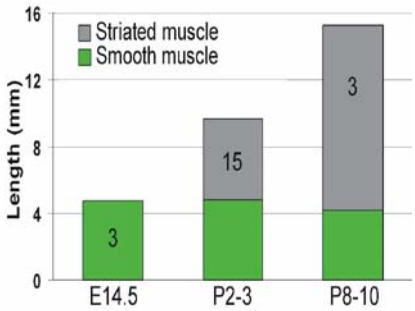

Figure 3.2 Extent of smooth muscle expression is constant during early esophageal growth. The absolute length of the smooth muscle portion between ED14.5 and PN8-10 does not change substantially despite a 3-fold increase in total esophageal length.

Remnant smooth muscle cells persist throughout the developing and mature striated muscle component of the muscularis externa. Examination of esophagi from PN8 SMCG2 mice revealed discrete, linear areas of fluorescence within the skeletal *muscularis externa* in the subpharyngeal esophagus, similar to that exhibited by smooth muscle cells in a more dense pattern near the lower esophageal sphincter. These smooth muscle cells were also observed with confocal imaging of freshly isolated whole esophagus (Figure 3.3A). Sparse fluorescent cells were also observed subpharyngeally in the developing stages PN2-3 (not shown). The oblique

orientation of these fluorescent cells matched the orientation of smooth muscle cells in the pregastric esophagus and the orientation of striated muscle fibers throughout the esophagus. We could not identify fluorescent cells in adult esophagi using widefield fluorescent microscopy, because of background fluorescence. However, similar fluorescent cells were observed with confocal imaging in freshly isolated whole adult esophagi (Figure 3.3B). These findings suggested a persistence of smooth muscle cells near the serosal surface of the *muscularis externa*. We consequently performed a series of immunohistochemical studies to determine the nature of these apparent smooth muscle cells.

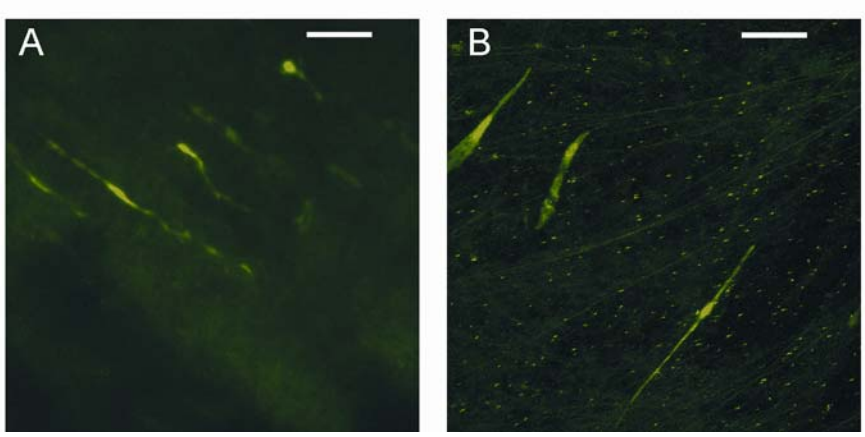

Figure 3.3 Remnant smooth muscle cells in the sub-pharyngeal portion of the mouse esophagus. Confocal z-projections through fresh whole-mount esophagi from PN8-10 (A) and adult (B) smCG2 mice showing linear endogenously expressed GFP fluorescence in the subserosal *muscularis externa*. Scale bar = 50μm.

Remnant smooth muscle cells express smooth-muscle actin and demonstrate an atypical morphology. Sagittal sections of developing and adult esophagus and stomach were labeled with anti-smooth muscle actin, which is restricted to smooth muscle in adult mice and is transiently expressed in atrial muscle, as well as expressed in committed smooth muscle, during

development (Babai et al. 1990; Nehls and Drenckhahn 1991; Borirakchanyavat et al. 1997). To verify this, we examined gastric smooth muscle cells from SMCG2 mice for co-expression of eGFP and smooth muscle α-actin. Typical smooth muscle cells showed eGFP fluorescence throughout the cytoplasm, with periplasmic labeling of smooth muscle α-actin (data not shown). We then examined the striated muscle portion of the esophageal *muscularis externa* for smooth muscle α-actin labeled cells. Occasional small and elongated smooth muscle cells were observed tightly associated with striated muscle fibers, and these cells increased in frequency along the *muscularis externa* nearer the lower esophageal sphincter (Figure 3.4A, B, D). At the transition zone, where striated muscle is replaced by smooth muscle over a small region, there were mixtures of smooth muscle cells that were similar in appearance to the remnant smooth muscle cells found more cranially, as well as more typical smooth muscle cells. In contrast to pre-gastric smooth muscle cells, the remnant smooth muscle cells contained very little cytoplasm. Confocal images and 3D reconstructions suggested that these remnant smooth muscle cells were nucleated and flattened (Figure 3.4E). Additionally, examination of appendicular striated muscle (semimembranosus and semitendinosus) revealed only vascular SM cells, further supporting our hypothesis that remnant smooth muscle cells occur only in esophageal striated muscle.

Remnant smooth muscle cells are different from vascular smooth muscle cells or pericytes. To determine if the remnant smooth muscle cells were vascular smooth muscle cells, we labeled sagittal esophageal sections with anti-smooth muscle α-actin antibodies and endothelial markers (anti-vonWillebrand Factor-related Antigen or anti-CD31 antibodies).

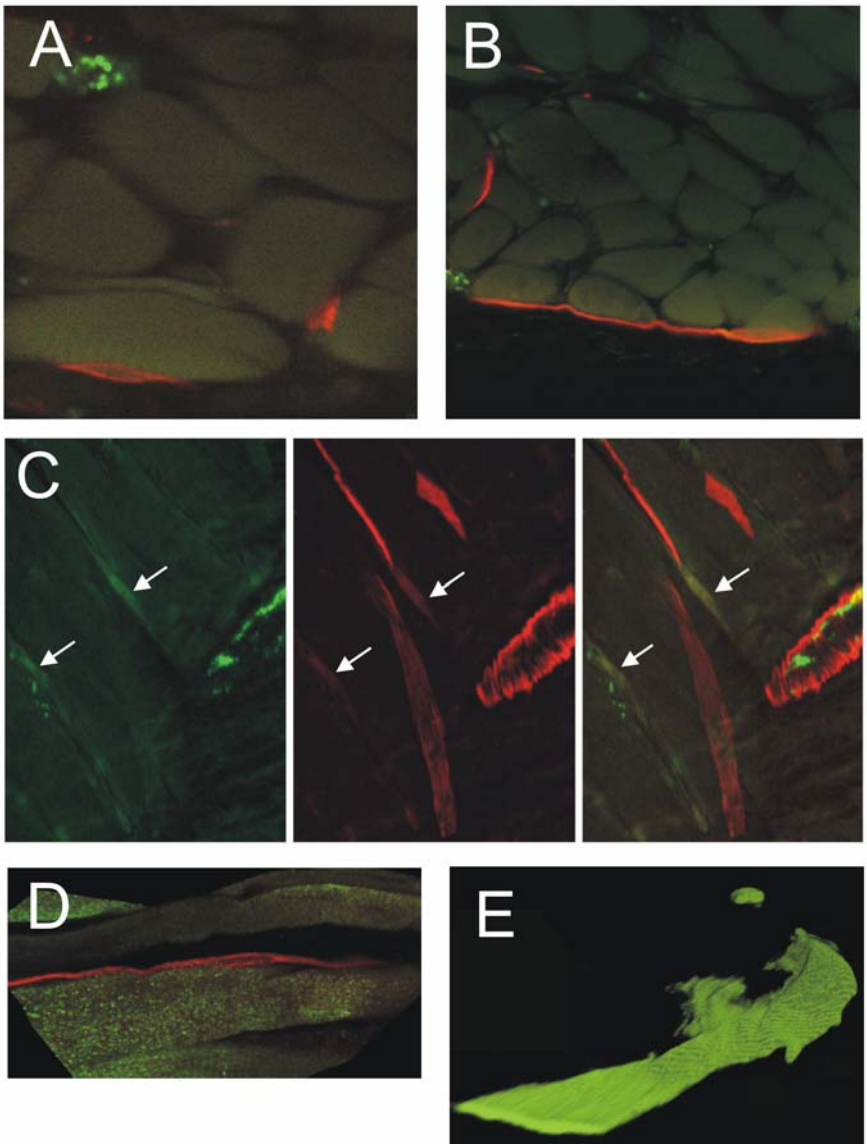

Figure 3.4 Remnant smooth muscle cells are intimately associated with striated muscle in esophageal *muscularis externa*, and are not vascular SM cells. Confocal z-projections of *muscularis externa* (A-C) from adult SMCG2 mice showing SM cells (red) and vascular endothelium (green). Vascular smooth muscle cells co-localize with vascular endothelium as an irregular circumferential network (C), however remnant smooth muscle cells are single, nucleated cells that are separate from any vascular labeling. Some remnant smooth muscle cells have faint green cytoplasmic GFP fluorescence (arrows, C). They are intimately associated with striated muscle (A, B, D), and have a flattened and irregular phenotype, as evidenced by 3D reconstruction (E). Magnification for A-D is 40x.

While some smooth muscle α-actin-positive staining of vascular smooth muscle cells was observed along small blood vessels in close association with endothelial markers (Figure 3.4C), we observed remnant smooth muscle cells separate from any vascular staining (Figure 3.4A, B). Further, vascular smooth muscle cells were oriented circumferentially around blood vessels (Ushiwata and Ushiki 1990; Bizuneh et al. 1991; Pannarale et al. 1996; Higuchi et al. 2000) and appeared almost as a thin syncytium or mesh of smooth muscle α-actin-staining cells, whereas the remnant smooth muscle cells often lay parallel to the microvasculature, and were discrete, well-defined cells.

Similarly, the morphology of remnant smooth muscle cells differed from the reported morphology and localization of vascular pericytes (Nehls and Drenckhahn 1991; Pannarale et al. 1996; Hughes and Chan-Ling 2004). Finally, as stated previously, no such cells were observed in appendicular striated muscles, where only vascular smooth muscle cells were observed. Thus, remnant smooth muscle cells appear to be distinct from vascular smooth muscle cells or pericytes.

Remnant smooth muscle cells are not satellite cells and are separate from striated muscle cells. To determine if the remnant smooth muscle cells were satellite cells, we examined the *muscularis externa* of smCG2 transgenic mice for co-expression of eGFP or Cre recombinase, which are selectively expressed in smooth muscle cells of SMCG2 mice, or smooth muscle α-actin in wildtype and SMCG2 mice, and Pax7, which is expressed in adult striated muscle satellite cells (Seale et al. 2000). We used striated appendicular muscle as a control. Satellite cells were identified throughout the

muscularis externa, as well as in appendicular muscle sections (Figure 3.5A, B), but these cells did not co-express eGFP, Cre recombinase or smooth muscle α-actin.

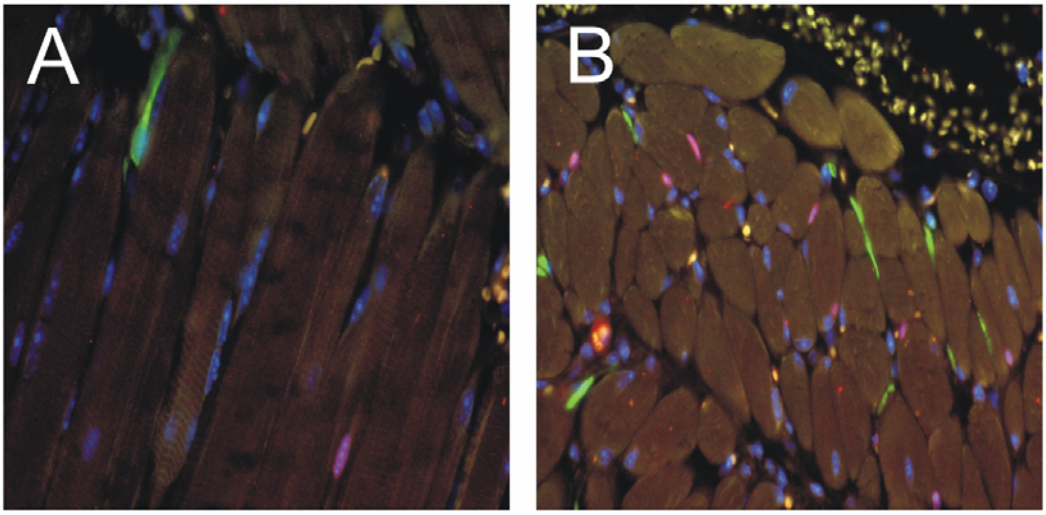

Figure 3.5 Remnant smooth muscle cells are not satellite cells. Fluorescent wide-field images showing Pax7-labeled cell nuclei (pink) and eGFP-labeled (A) or Smooth muscle α-actin (B) SM cells (green) in adult SMCG2 mice. Pink satellite cell nuclei can be seen in esophageal *muscularis externa*, separate from remnant smooth muscle cells, which have blue-staining nuclei (A).

In contrast to satellite cells, cytoplasmic projections from the remnant smooth muscle cells spanned striated muscle fibers, and diverged from the striated muscle fibers, indicating that they were not encapsulated by the same basement membrane as the fiber (Figure 3.6A, B). These findings indicate that remnant smooth muscle cells are not satellite cells.

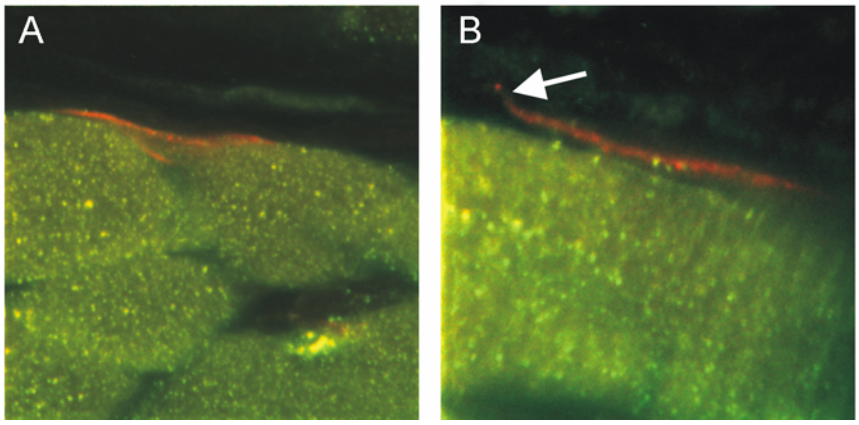

Figure 3.6 Remnant smooth muscle cells span multiple striated muscles. Confocal Z-scan images showing remnant smooth muscle cells (red) spanning multiple Concavalin A-labeled striated muscle fibers (green)(A), and diverging from adjacent striated muscle fibers (B, arrow). Magnification 60x.

Remnant smooth muscle cells are different from ICC. There is speculation that some smooth muscle develops from a c-kit expressing precursor cell, which matures into either ICC or smooth muscle (Ward and Sanders 2001). Additionally, ICC are scattered throughout the adult murine esophagus (Vanderwinden et al. 2000; Rumessen et al. 2001) but the expression of smooth muscle α-actin by ICC has not been investigated. Therefore, to determine if remnant smooth muscle cells are ICC, we labeled sagittal sections of developing and adult stomach (Figure 3.7A) and esophagus (Figure 3.7B, C) with anti-smooth muscle α-actin and anti-c-kit antibodies. Remnant smooth muscle cells were often (but not always) intimately associated with ICC within the striated muscle portion of the *muscularis externa* (Figure 3.7B, C) but we did not observe any co-staining, indicating that remnant smooth muscle cells are not ICC. Gastric ICC failed to label with anti-smooth muscle α-actin antibodies, indicating that ICC do not express smooth muscle actin (Figure 3.7A).

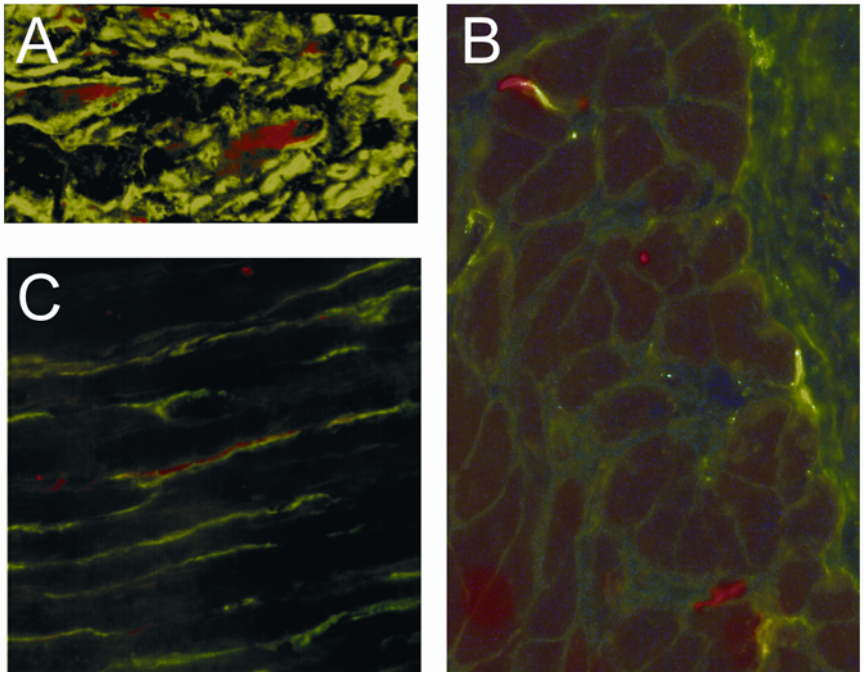

Figure 3.7 Remnant smooth muscle cells are not interstitial cells of Cajal. Confocal Z-projection of gastric *muscularis externa* (A) shows separate labeling of ICC (red) and smooth muscle cells (green) in adult SMCG2 mice. Fluorescent wide-field image of adult murine esophagus shows remnant smooth muscle cells intimately associated with c-kit positive ICC (B, upper left), as well as isolated ICC (B, red, lower right) and isolated remnant smooth muscle cells (B, green, middle right). Confocal Z-projection shows intimate association of ICC and remnant smooth muscle cells in esophageal *muscularis externa* (C). Magnification 40x (B), 120x (A, C).

Remnant and typical esophageal smooth muscle cells do not transdifferentiate into striated muscle cells. As the principal evidence for smooth muscle transdifferentiation is the coexpression of smooth muscle specific proteins and skeletal myogenic factors, (Patapoutian et al. 1995) we examined the co-expression of a marker of striated myogenesis (myogenin) and a transgene driven by the smooth muscle-specific myosin heavy chain promoter in PN2-3 and PN8-10 SMCG2 transgenic mice. Sagittal esophageal sections were labeled with antibodies directed at myogenin and Cre recombinase, which contains a nuclear localization signal. The myogenin antibody labeled nuclei along the length of the developing esophagus, with a

concentration of stained nuclei at the ‘‘transition zone’’ between smooth and striated muscle, but also more dilute staining of nuclei along the caudal esophagus, ‘‘ahead’’ of the myogenic wave-front. The Cre recombinase antibody labeled nuclei of all smooth muscle cells within the stomach, and both esophageal layers (*muscularis mucosae* and *muscularis externa*). Nuclear colocalization of the two antibodies was never observed in embryonic esophagi, at a time when esophageal smooth muscle is being progressively replaced by skeletal myocytes (Figure 3.8A, B).

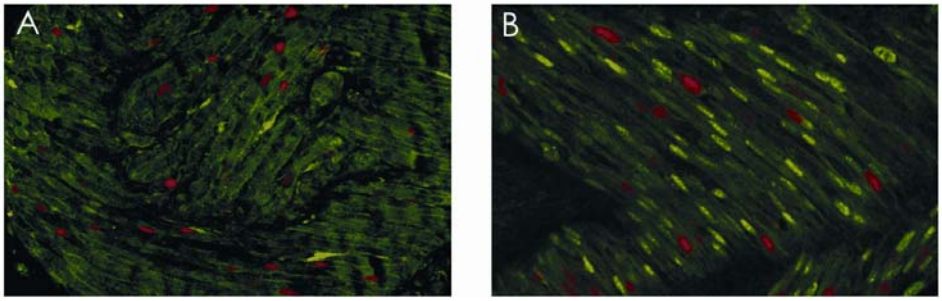

Figure 3.8 No evidence of transdifferentiation in murine *muscularis externa*. Confocal z-projections from cranial (A) and caudal (B) regions of esophageal *muscularis externa* from a PN8-10 SMCG2 mouse show no colocalization of nuclear Cre-recombinase labeling (green) and nuclear myogenin labeling (red). Inset panels show higher detail of myogenin staining at both ends of the esophagus. Magnification 60X (A,B).

These results further suggest that smooth muscle cells do not transdifferentiate into striated muscle.

Myogenin is a myogenic transcription factor that is essential for striated myogenesis but not smooth muscle or cardiac muscle development and inactivation of the myogenin gene results in striated muscle deficiency and neonatal death (Hasty et al. 1993).

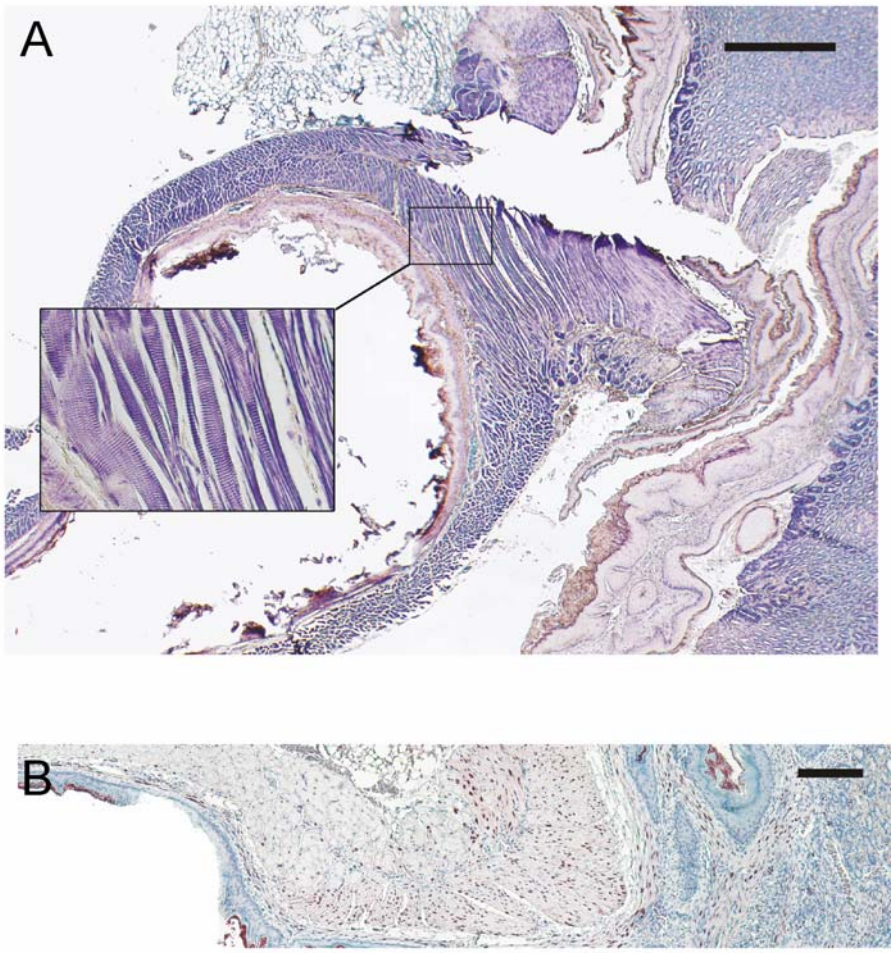

Figure 3.9 Fetal smooth-muscle-specific myogenin inactivation fails to affect striated esophageal myogenesis. (A) PTAH-stained longitudinal section of the lower esophageal sphincter of a PN21 SMCG2*Myog*$^{flox\Delta/flox\Delta}$ shows normal extension of striated muscle to the perigastric border. (B) Immunohistochemical staining for Cre-recombinase shows robust expression in gastric smooth muscle. Magnification 4X (A), 20X (B), scale bar = 1mm.

We reasoned that if transdifferentiation of esophageal *muscularis externa* occurred, deletion of myogenin from smooth muscle cells during development would disrupt striated esophageal myogenesis, resulting in either a complete or partial failure of striated esophageal myogenesis. We tested this hypothesis by deleting myogenin from smooth muscle in conditional Myogenin knockout (*Myog*$^{flox/flox}$) mice by mating them with SMCG2 mice (Knapp et al. 2006).

Esophageal myogenesis was unaffected by smooth muscle specific gene inactivation (Figure 3.9),which occurs prior to significant esophageal striated myogenesis (Madsen et al. 1998; Rishniw et al. 2003), further substantiating our hypothesis that striated esophageal muscle do not transdifferentiate from smooth muscle cells.

Discussion

We have identified a persistent population of non-vascular smooth muscle cells in developing and adult murine esophagus within the striated muscle portion of the *muscularis externa* and have shown that, while the proportion of smooth muscle contributing to the total *muscularis externa* decreases with development, its absolute length changes very little for much of the developmental period. These findings offer evidence of the fate of a portion of esophageal smooth muscle cells which complement the recent observations of apoptosis, and provide a valid alternative hypothesis to transdifferentiation. Moreover, we have shown that protein expressed by committed smooth muscle cells (expressing Cre recombinase under control of the smMHC promoter) is not co-expressed with the striated muscle myogenic regulatory factor (MRF), myogenin, as would be expected with an overlap of smooth and skeletal muscle phenotypes, as previously suggested (Patapoutian et al. 1995). Taken together, these results lend further support to the hypothesis that skeletal myogenesis occurs independent of pre-committed smooth muscle cells and that embryonic smooth muscle persists in both a compacted form (near the lower esophageal sphincter) and as a group of cells that have been diluted by skeletal myogenesis and persist throughout the length of the adult esophagus.

Remnant smooth muscle cells in mouse esophagus. Remnant esophageal smooth muscle cells appear to be distinct from vascular smooth muscle, pericytes, ICC, or satellite cells. Their morphology is markedly distorted from normal smooth muscle cells, such as those found near the lower esophageal sphincter or within the gastric *muscularis externa*, but is similar to some of the smooth muscle cells found in the ‘‘transition zone’’ where striated muscle begins to encroach on the smooth muscle layer. Remnant smooth muscle cells appear to be constricted by surrounding striated muscle fibers, often with thin sheet-like cytoplasmic projections that follow the contours of the striated muscle sarcolemma. In several instances, remnant smooth muscle cells appear to be closely associated with ICC that have been previously identified within the striated muscle portion of the esophageal *muscularis externa* (Ward et al. 1996; Vanderwinden et al. 2000; Rumessen et al. 2001). It is tempting to speculate that the remnant smooth muscle cells provide a supportive function for these ICC, since ICC exist in an exclusively smooth muscle environment in the rest of the body. How the remnant smooth muscle cells come to be surrounded by striated muscle remains unknown, however these findings argue against a discrete myogenic zone in which striated muscle cells develop. While myogenin staining of developing esophagus shows a region of higher myogenin expression that progresses caudally at incremental developmental stages, myogenin expression appears to be more diffuse than previously reported, with discrete striated muscle precursors being visible caudal to the ‘‘myogenic zone’’ (Figure 3.10). This is consistent with observations by Wörl and Neuhuber, who identified striated muscle precursor cells throughout the smooth muscle portion of developing esophagi (Wörl and Neuhuber 2005). Sagittal sectioning of fetal and neonatal

esophagus allowed unambiguous identification of such occasional myogenin-positive cells, whereas previous studies relied on transverse whole-embryo sections. While this observation does not rule out transdifferentiation, it does argue against a discrete ‘‘myogenic zone’’. We suggest that as striated muscle precursors activate their myogenic program and proliferate as myoblasts to finally form myotubes, they surround occasional SM cells, and sequester them from their smooth muscle cell neighbors.

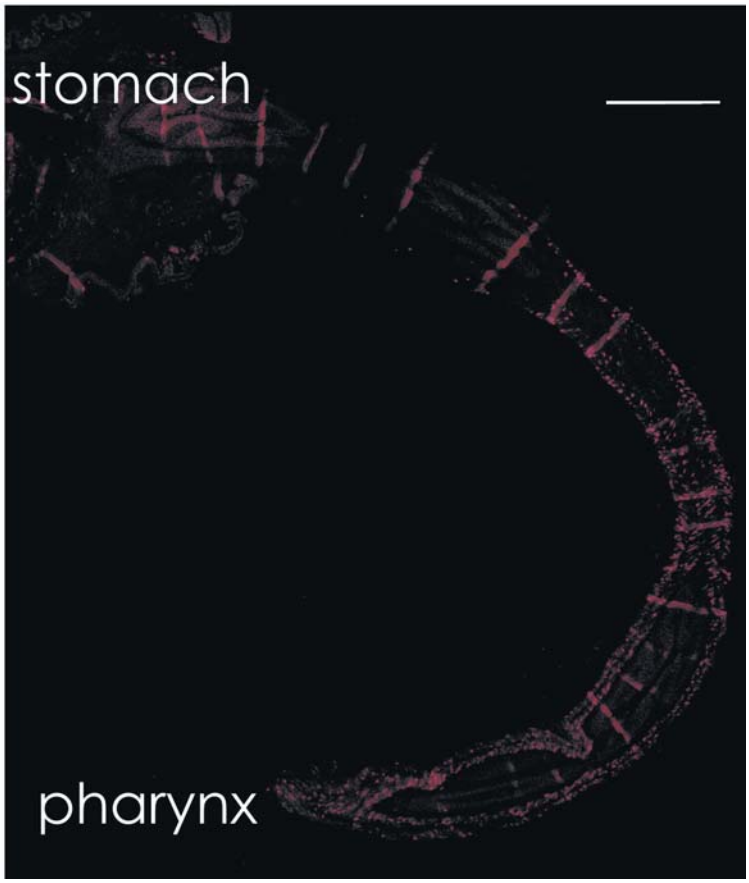

Figure 3.10 Myogenin expression proceeds craniocaudally in a diffuse manner, rather than as a discrete zone. Fluorescent wide-field image of a sagittal section of esophagus from a PN2 mouse showing a diffuse pattern of myogenin expression (red punctuate fluorescence), rather than a discrete zone of active myogenesis with myogenin expression extending to within 1mm of the lower esophageal sphincter. Scale bar = 500 µm

The relatively large subsequent expansion of the striated muscle cells separates these remnant smooth muscle cells, much as two dots on the surface of an inflating balloon move further and further apart. This process appears to be occurring at the very earliest stages of esophageal myogenesis, because remnant smooth muscle cells can be found immediately below the pharynx. While the remnant smooth muscle cells are sparsely scattered throughout the *muscularis externa*, and it is not possible to estimate their number in adult mice in this study, they could account for a substantial portion of the smooth muscle cells present at ED14.5.

Sagittal sections of ED14.5 esophagus show that the two layers of the *muscularis externa* are cumulatively 2–4 cells thick (Figure 2.3), so the total number of smooth muscle cells at this stage is relatively small. With a >10-fold increase in esophageal length between ED14.5 and adults (3–30 mm), and a >10-fold increase in cross-sectional area (0.03 to >0.3 mm^2), the volume of the *muscularis externa* increases 100-fold. If the smooth muscle mass present at ED14.5 is preserved, or even decreases slightly (as is suggested by the shapes of the remnant smooth muscle cells), it would only account for 1% of the total volume of the *muscularis externa* in adulthood. Thus, it is feasible that many of the smooth muscle cells, present as a continuous organ at day ED14.5, become dispersed remnant smooth muscle cells in adulthood. Previous investigators have hypothesized that skeletal myogenesis ‘‘dilutes’’ the pre-existing smooth muscle cells, (Zhao and Dhoot 2000a), although the persistence of these cells has not been previously demonstrated.

Preservation of smooth muscle portion of mouse esophagus. We previously demonstrated, through fate mapping experiments using a Cre-LoxP system and an irreversibly activated Cre recombinase-dependent reporter

(β-galactosidase), that committed smooth muscle cells expressing smooth muscle myosin heavy chain, did not transdifferentiate into striated muscle cells in esophageal *muscularis externa* (Rishniw et al. 2003). However, several investigators have questioned these results on the basis of a lack of demonstration of an alternative fate of smooth muscle cells other than as conversion to skeletal myocytes (Breuer et al. 2004; Sumiyoshi et al. 2004), since Patapoutian et al. had previously shown no evidence of smooth muscle apoptosis (Patapoutian et al. 1995). The current results provide an alternative model regarding the developmental fate of esophageal smooth muscle cell. If smooth muscle cells accounted for the development of skeletal myocytes that ultimately fuse to form the *muscularis externa*, one would expect to see a distinct reduction in the size of the smooth muscle portion early in development, as the smooth muscle cell pool is depleted at the expense of a growing striated muscle pool. Even in the first 7 days of development when the mouse esophagus more than doubles in length, however, the smooth muscle portion of the esophagus remains virtually unchanged. This is supported by findings of Reddy and Kablar (2004), who demonstrated that in mice lacking essential MRFs (Myf5 and MyoD), esophageal smooth muscle remained static, but did not ‘‘disappear’’. While these authors interpreted their results as indicating a failure of transdifferentiation, an identical outcome would be expected if skeletal myogenesis occurred independently via mesenchymal precursors. Although our results do not discount the recent observations of apoptosis (Wörl and Neuhuber 2005), we did not observe the same extent of apoptosis in developing esophagi. Indeed, the results of Reddy and Kablar suggest that apoptosis is not a major fate of esophageal smooth muscle cells, although factors that might trigger apoptosis could be absent in mice lacking

MRFs (Reddy and Kablar 2004). Data from other investigators also support the persistence of smooth muscle in the adult esophagus. Zhao and Dhoot temporally mapped four MRFs in developing mouse esophagus from ED13 to PN1, demonstrating the pattern and extent of MRF activation along the entire esophageal length (Zhao and Dhoot 2000b). Interestingly, the nonactivated region of tissue, which remained caudal as the esophagus grew, did not shrink substantially over this period—it measured 2–3 mm at ED13 and 2–3 mm at PN1. This region corresponds to the smooth muscle portion of the developing esophagus. Our findings support these data, suggesting that smooth muscle disappearance is not a pre-requisite for esophageal development. We suspect that the smooth muscle cells simply become dispersed throughout the striated muscle, or re-organized in the pre-gastric region to accommodate the substantial increase in esophageal size.

No transdifferentiation in mouse esophagus. Finally, we failed to observe any nuclear colocalization of smooth muscle protein and striated muscle MRF in developing esophagi and failed to arrest or disrupt striated myogenesis with targeted deletion of myogenin from embryonic esophageal smooth muscle. This contradicts the findings of Patapoutian et al. who observed MRF (MyoD) expression in cells that expressed smooth muscle cell markers (Myosin Light Chain Kinase, MLCK) (Patapoutian et al. 1995). However, other investigators have subsequently noted that MLCK is not an appropriate marker of smooth muscle cells in developing muscle, because it is transiently expressed in striated muscle (DallaLibera et al. 1997; Zhao and Dhoot 2000a). Conversely, smooth muscle myosin heavy chain is exclusively restricted to smooth muscle cells (with the exception of transient expression in

the developing atrium, Regan et al. 2000; Xin et al. 2002), and therefore serves as a specific marker of smooth muscle cells. We had previously created a Cre recombinase expressing transgenic mouse where Cre recombinase was under the control of the smooth muscle myosin heavy chain promoter (smCG2) (Xin et al. 2002) and used this mouse in our fate mapping experiments (detailed above) (Rishniw et al. 2003). Therefore, Cre recombinase expression would be anticipated in any smooth muscle cell, or cell that had recently transdifferentiated from a smooth muscle cell and was expressing myogenin. It is possible that expression of these two proteins would not overlap temporally, (the half-life of expressed Cre recombinase is unknown). However, in a region of high myogenin expression, we would expect some co-expression of Cre recombinase (persisting from prior smooth muscle cell phenotype) and myogenin (newly expressed in a transdifferentiating cell). We failed to observe any such colocalization, which strongly argues against transdifferentiation of smooth muscle cells in mouse esophagus and supports our previous study. While the immunohistochemical data may fail to detect temporally dissociated events, such as expression of Cre recombinase and myogenin, targeted deletion of myogenin in smooth muscle would prevent smooth muscle cells from transdifferentiating into striated muscle. Had myogenin been inactivated in smooth muscle that subsequently transdifferentiated into striated muscle, we would expect to observe arrested, failed or abnormal striated esophageal myogenesis. We have previously demonstrated robust Cre recombinase expression in developing mouse esophageal smooth muscle at a time when striated muscle is virtually undetectable, indicating that any conditionally deletable allele will be inactivated (Rishniw et al. 2003; Madsen et al. 1998). However, we failed to

observe any abnormalities of esophageal anatomy in mice lacking myogenin in smooth muscle. Together, these data offer strong evidence that esophageal striated muscle does not arise by transdifferentiation from smooth muscle. While some studies have demonstrated the plasticity of vascular smooth muscle cells (Wang et al. 2006), these studies have not definitively demonstrated transdifferentiation rather than differentiation from a common mesenchymal stem cell pool. Moreover, no studies of which we are aware have unequivocally demonstrated transdifferentiation into striated muscle, although one study has suggested the ability of striated myocytes to transdifferentiate into smooth muscle cells in a jellyfish species (Galle et al. 2005).

An alternate model of esophageal myogenesis. Taken together, our data, and those of previous investigators, suggest that striated myogenesis arises from a distinct population of myogenic precursors, rather than via transdifferentiation from smooth muscle cells, and their apparent ‘‘disappearance’’ can be explained morphometrically. We suggest that the fetal esophageal smooth muscle cells adopt one of three fates during the expansion of the striated muscle mass: (1) a small subset of smooth muscle cells are ‘‘trapped’’ within the expanding striated muscle tissue, giving rise to the remnant smooth muscle cells we have described in this study; (2) a subset undergoes apoptosis, as identified by other investigators (Wörl and Neuhuber 2005); and (3) the large majority of smooth muscle cells are compacted distally to give rise to the lower esophageal sphincter and the junctional region that can be clearly identified in adult mice, as is suggested by this study. In conclusion, our data provide evidence for persistence of smooth muscle cells

within the developing and adult esophagus and are supported by findings of previous studies. Taken together, these findings provide an alternative model for esophageal development that does not require transdifferentiation to account for the apparent ‘‘disappearance’’ of smooth muscle cells.

Acknowledgments

This study was supported in part by NIH grant RO1 DK065992 (Kotlikoff), NIH training grant T32RR07059-09 (Rishniw), Muscular Dystrophy Association grant MDA4289 and Robert A. Welch Foundation grant G-0010 (Klein).

REFERENCES

Babai F, Musevi-Aghdam J, Schurch W, Royal A, Gabbiani G (1990) Coexpression of alpha-sarcomeric actin, alpha-smooth muscle actin and desmin during myogenesis in rat and mouse embryos I. Skeletal muscle. Differentiation 44:132–142

Bizuneh M, Bohlen HG, Connors BA, Miller BG, Evan AP (1991) Vascular smooth muscle structure and juvenile growth in rat intestinal venules. Microvasc Res 42:77–90

Borirakchanyavat S, Baskin LS, Kogan BA, Cunha GR (1997) Smooth and striated muscle development in the intrinsic urethral sphincter. J Urol 158:1119–1122

Breuer C, Neuhuber WL, Wörl J (2004) Development of neuromuscular junctions in the mouse esophagus: morphology suggests a role for enteric coinnervation during maturation of vagal myoneural contacts. J Comp Neurol 475:47–69

DallaLibera L, Podhorska Okolow M, Martin B, Massimino ML, Brugnolo R, Cantini M (1997) Smooth muscle myosin light chain kinase is transiently expressed in skeletal muscle during embryogenesis and muscle regeneration both in vivo and in vitro. J Muscle Res Cell Motil 18:295–303

Galle S, Yanze N, Seipel K (2005) The homeobox gene Msx in development and transdifferentiation of jellyfish striated muscle. Int J Dev Biol 49:961–967

Hasty P, Bradley A, Morris JH, Edmondson DG, Venuti JM, Olson EN, Klein WH (1993) Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature 364:501–506

Higuchi K, Hashizume H, Aizawa Y, Ushiki T (2000) Scanning electron microscopic studies of the vascular smooth muscle cells and pericytes in the rat heart. Arch Histol Cytol 63:115–126

Hughes S, Chan-Ling T (2004) Characterization of smooth muscle cell and pericyte differentiation in the rat retina in vivo. Invest Ophthalmol Vis Sci 45:2795–2806

Kablar B, Tajbakhsh S, Rudnicki MA (2000) Transdifferentiation of esophageal smooth to skeletal muscle is myogenic bHLH factordependent. Development 127:1627–1639

Knapp JR, Davie JK, Myer A, Meadows E, Olson EN, Klein WH (2006) Loss of myogenin in postnatal life leads to normal skeletal muscle but reduced body size. Development 133:601–610

Madsen CS, Regan CP, Hungerford JE, White SL, Manabe I, Owens GK (1998) Smooth muscle-specific expression of the smooth muscle myosin heavy chain gene in transgenic mice requires 5'-flanking and first intronic DNA sequence. Circ Res 82:908–917

Nehls V, Drenckhahn D (1991) Heterogeneity of microvascular pericytes for smooth muscle type alpha-actin. J Cell Biol 113:147–154

Pannarale L, Onori P, Ripani M, Gaudio E (1996) Precapillary patterns and perivascular cells in the retinal microvasculature. A scanning electron microscope study. J Anat 188(Pt 3):693–703

Patapoutian A, Wold BJ, Wagner RA (1995) Evidence for developmentally programmed transdifferentiation in mouse esophageal muscle. Science 270:1818–1821

Reddy T, Kablar B (2004) Evidence for the involvement of neurotrophins in muscle transdifferentiation and acetylcholine receptor transformation in

the esophagus of Myf5(–/–): MyoD(–/–) and NT-3(–/–) embryos. Dev Dyn 231:683–692

Regan CP, Manabe I, Owens GK (2000) Development of a smooth muscle-targeted cre recombinase mouse reveals novel insights regarding smooth muscle myosin heavy chain promoter regulation. Circ Res 87:363–369

Rishniw M, Xin HB, Deng KY, Kotlikoff MI (2003) Skeletal myogenesis in the mouse esophagus does not occur through transdifferentiation. Genesis 36:81–82

Rumessen JJ, de Kerchove d'Exaerde A, Mignon S, Bernex F, Timmermans JP, Schiffmann SN, Panthier JJ, Vanderwinden JM (2001) Interstitial cells of Cajal in the striated musculature of the mouse esophagus. Cell Tissue Res 306:1–14

Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA (2000) Pax7 is required for the specification of myogenic satellite cells. Cell 102:777–786

Stratton CJ, Bayguinov Y, Sanders KM, Ward SM (2000) Ultrastructural analysis of the transdifferentiation of smooth muscle to skeletal muscle in the murine esophagus. Cell Tissue Res 301:283–298

Sumiyoshi H, Mor N, Lee SY, Doty S, Henderson S, Tanaka S, Yoshioka H, Rattan S, Ramirez F (2004) Esophageal muscle physiology and morphogenesis require assembly of a collagen XIX-rich basement membrane zone. J Cell Biol 166:591–600

Ushiwata I, Ushiki T (1990) Cytoarchitecture of the smooth muscles and pericytes of rat cerebral blood vessels. A scanning electron microscopic study. J Neurosurg 73:82–90

Vanderwinden JM, Gillard K, Timmermans JP, Schiffmann SN, Panthier JJ (2000) Kit-expressing interstitial cells in the striated muscle of the mouse esophagus. Gastroenterology 118:A867

Wang H, Yan S, Chai H, Riha GM, Li M, Yao Q, Chen C (2006) Shear stress induces endothelial transdifferentiation from mouse smooth muscle cells. Biochem Biophys Res Commun 346:860–865

Ward SM, Sanders KM (2001) Physiology and pathophysiology of the interstitial cell of Cajal: from bench to bedside. I. Functional development and plasticity of interstitial cells of Cajal networks. Am J Physiol Gastrointest Liver Physiol 281:G602–G611

Ward SM, Bayguinov J, Stratton CJ, Burns AJ, Sanders KM (1996) Developmental changes in interstitial cells of Cajal in the murine oesophagus. J Physiol (Lond) 495P:P94

Wörl J, Neuhuber WL (2005) Ultrastructural analysis of the smoothto-striated transition zone in the developing mouse esophagus: Emphasis on apoptosis of smooth and origin and differentiation of striated muscle cells. Dev Dyn 233:964–982

Xin HB, Deng KY, Rishniw M, Ji G, Kotlikoff MI (2002) Smooth muscle expression of Cre recombinase and eGFP in transgenic mice. Physiol Genomics 10:211–215

Zhao W, Dhoot GK (2000a) Both smooth and skeletal muscle precursors are present in foetal mouse oesophagus and they follow different differentiation pathways. Dev Dyn 218:587–602

Zhao WF, Dhoot GK (2000b) Skeletal muscle precursors in mouse esophagus are determined during early fetal development. Dev Dyn 219:10–20

CHAPTER 4

Striated myogenesis and peristalsis in the fetal murine esophagus occurs without cell migration or interstitial cells of Cajal

* Originally published as: Rishniw M et al. Striated myogenesis and peristalsis in the fetal murine esophagus occurs without cell migration or interstitial cells of Cajal. Cells Tissues Organs 2008 (epub ahead of print). Reproduced with permission.

Abstract

Esophageal striated myogenesis progresses differently from appendicular myogenesis, but the mechanism underlying this process is incompletely understood. Early theories of transdifferentiation of smooth muscle into striated muscle are not supported by transgenic fate-mapping experiments, however, the origin of esophageal striated muscle remains unknown. To better define the process of striated myogenesis, we examined myogenesis in murine fetal cultured esophageal whole-organ explants. ED14.5 esophagi maintained a functional contractile phenotype for up to 7 days in culture. Striated myogenesis, as evidenced by myogenin expression, proceeded in a cranio-caudal direction along the length of the esophagus. Esophageal length did not change during this process. Complete, but not partial, mechanical disruption of the rostral esophagus inhibited myogenesis distally. Addition of FGF-2 to the culture media failed to inhibit striated myogenesis, but attenuated smooth-muscle actin expression and reduced peristaltic activity. Inhibition of c-kit failed to inhibit peristalsis. These results suggest that striated myogenic precursors are resident along the entire length of the esophagus by ED14.5 and do not migrate along the esophagus after ED14.5. Induction of myogenesis cranio-caudally appears to require physical continuity of the esophagus and is not inhibited by FGF-2. Finally, peristalsis in ED14.5 esophagi appears not to be regulated by Interstitial Cells of Cajal.

Introduction

Esophageal striated myogenesis is a complex process that differs from striated myogenesis elsewhere in the body. The *muscularis externa* of the murine esophagus initially develops as a smooth muscle tube, apparent by day ED9-10, but then transforms into an almost exclusively striated muscle tube over the following 3 weeks (Zhao and Dhoot 2000a; Zhao and Dhoot 2000b; Zhao and Dhoot 2000c; Rishniw et al., 2003; Wörl and Neuhuber 2005; Rishniw et al., 2007).

Approximately 50% of this smooth-to-striated muscle transformation occurs post-natally (Rishniw et al., 2007). Although initially hypothesized to occur via transdifferentiation of smooth muscle cells into striated muscle cells (Patapoutian et al., 1995; Kablar et al., 2000; Stratton et al., 2000), more recent studies have provided compelling evidence that striated muscle cells arise from a distinct and separate population of precursors (Zhao and Dhoot 2000a; Zhao and Dhoot 2000b; Zhao and Dhoot 2000c; Rishniw et al., 2003; Wörl and Neuhuber 2005; Rishniw et al., 2007). Studies have shown three independent fates of esophageal smooth muscle cells: apoptosis (Wörl and Neuhuber 2005), persistence within the striated muscle layer as remnant smooth muscle cells (Rishniw et al., 2007), and compaction into the lower esophageal sphincter (Rishniw et al., 2007).

The origin of striated muscle cells and the means by which they populate the esophagus remain largely undetermined. Skeletal myoblasts could differentiate from pluripotent mesenchymal stem cells within the esophagus, although this seems unlikely given that other striated muscle precursors arise from neural crest cells that have migrated either to form somites or to other regions (e.g. facial muscles). Alternatively, neural crest

cells similar to those that give rise to pharyngeal and lingual muscles could migrate to the esophagus during early esophageal development, forming a resident population of myogenic precursors. Lingual and pharyngeal striated muscles are apparent by ED10.5, while esophageal striated muscle cells are not detected before ED11.5-12.5, indicating that if simultaneous migration occurs, myodifferentiation must be slightly delayed relative to other non-somatic muscle. Finally, it is possible that a later migration of precursors derived from somites or neural crest could occur, giving rise to striated myogenesis in a rostral to caudal pattern.

To address the myogenic potential of the embryonic mouse esophagus and further define this process, we examined striated myogenesis ex vivo. Using wild-type and SMCG2 transgenic fetuses, we cultured esophageal explants in toto for up to 7 days, and examined myogenesis and esophageal functional integrity by immunohistochemical, fluorescent microscopic and pharmacological means, using fibroblast growth factor (FGF-2) and neutralizing anti-ckit antibodies to alter either myogenesis or peristalsis. Our study demonstrates that striated myogenesis occurs in the explanted ED14.5 esophagus maintained in organ culture in a rostro-caudal direction and is dependent on the physical integrity of the esophagus and unaffected by exposure to FGF-2. Finally, we show that cultured fetal esophagi display prominent peristaltic contractions for up to 7 days in culture and this peristalsis is not dependent on interstitial cells of Cajal.

Materials and Methods

Organ culture

Female C57BL/6J mice were mated with male SMCG2$^{+/WT}$ transgenic mice (these mice are maintained as hemizygous transgenic mice and express Cre recombinase and eGFP under the control of a smooth-muscle myosin heavy chain promoter – thus the aforementioned mating would produce, on average, 50% wildtype fetuses, and 50% hemizygous transgenic fetuses) and euthanatized by CO_2 asphyxiation 14.5 days post coitus as approved by the Institutional Animal Care and Use Committee. Uteri were immediately excised and placed in sterile Petri dishes on ice, and fetuses removed and placed in ice-cold PBS (pH7.4) on ice. Fetal esophagi were explanted *in toto*, with tongue, pharynx/larynx and stomach attached in ice-cold PBS using a dissecting microscope (magnification 18X-30X), transferred immediately onto 6.5 mm diameter 3 μm pore size polycarbonate membrane filters (Transwells, Corning, Inc., Corning, New York), and incubated in 270 μL organ culture media (BGJb media, 10mg/ml Ascorbic acid, 20% heat-inactivated fetal calf serum, penicillin/streptomycin/amphotericin) at 37ºC in 5%CO_2 for up to 7 days. Media were exchanged every 12 hours. Motility was assessed every 24 hours by observing each of the esophagi in culture on an inverted dissecting scope for up to 2 minutes each and timing contraction intervals. For esophagi that were transected or partially transected, a razor blade was gently pressed across the esophagus approximately 25% of the length caudal to the pharynx. Disruption of muscular continuity was determined by loss of propagation of peristalsis across the disrupted region and loss of fluorescent continuity along the *muscularis externa*. Esophageal smooth muscle fluorescence (which provided an indirect measure of smooth muscle cellular differentiation) was

examined with a dissecting microscope coupled to a magnesium arc lamp and a GFP filter.

To determine if striated myodifferentiation could be inhibited by a known inhibitor of myodifferentiation that acts through the MAP kinase pathway (basic fibroblast growth factor) (Tortorella et al., 2001), hrFGF-2 (233-FB, R&D systems, Minneapolis, MN), MEK inhibitor PD98059 (513000, Calbiochem, La Jolla, CA) (Tortorella et al., 2001), or both, were added to achieve final concentrations of 25 μg/ml hrFGF-2 and/or 50μM PD98059. Fresh hrFGF-2 and/or PD98059 were added every 12 hours, when media were exchanged. To determine if fetal esophageal peristalsis was regulated by interstitial cells of Cajal (ICC), esophagi were cultured as described above, but with 5μg/ml ACK2 antibody (Gibco BRL, Gaithersburg, MD), which is a neutralizing antibody directed against c-kit (which, in turn, is essential in maintaining ICC phenotype), added to the media, and exchanged every 12 hours (David et al., 2005).

Esophagi used for immunohistochemical analysis were fixed in the culture filters for 24 hours in 10% formalin, mounted in specimen processing gel (Histogel, Richard Allan Scientific, Kalamazoo, MI) and then embedded in paraffin and sectioned at 4μm.

We performed each series of esophageal culture experiments at least 6 times. For baseline culture experiments, all fetuses in a litter were used (8-12 fetuses/litter). When performing experiments with hrFGF-2 and/or PD98059, litters were divided into 3 groups – control group, hrFGF-2 group and PD98059/hrFGF-2 group, with an average of 3 fetuses per group. Thus each experimental condition was repeated on at least 18 fetuses.

We estimated the extent of myogenesis at each time point from composite digital images of esophagi that were obtained at identical magnifications. Using a calibrated scale bar, we were able to estimate the total esophageal length and the percentage of the esophagus displaying striated myogenesis at each time point.

Immunohistochemistry

For myogenin immunostaining, fixed sections were steamed for 15 minutes in 0.01M citrate buffer (pH 6.0), blocked in mouse IgG blocking reagent (M.O.M., Vector Labs, Burlingame, CA) for 1 hour with TBS triton, 10% goat serum and 10% non-fat dry milk (NFDM) for 20 minutes, and incubated at 37ºC for 20 minutes and at room temperature for 40 minutes with mouse anti-rat myogenin (1:30, M3559, DakoCytomation Inc, Carpinteria, CA). Sections were then incubated with biotinylated anti-mouse (M.O.M., Vector Labs, Burlingame, CA) for 20 minutes at room temperature, followed by streptavidin-conjugated horse-radish peroxidase (Zymed Laboratories, San Francisco, CA) and visualized with AEC (Zymed Laboratories, San Francisco, CA).

For smooth muscle α-actin immunostaining, fixed sections were blocked in mouse IgG blocking reagent as above and incubated at 37ºC for 20 minutes and at room temperature for 40 minutes with mouse-antihuman anti-smooth muscle α-actin (1:60, M0851, clone 1A4, Dakocytomation Inc, Carpintaria, CA). Secondary antibody staining was as above.

To determine ACK2 antibody penetration into cultured organs, fixed slides were blocked for 20 minutes with 10% goat serum (Zymed Laboratories, San Francisco, CA) and incubated with biotinylated goat anti-rat IgG (Jackson

Immunoresearch Labs, Westgrove PA) for 20 minutes at room temperature, followed by Texas-Red Avidin DCS (Vector Labs, Burlingame CA).

Positive and negative controls were performed with each immunohistochemical experiment, using either smooth muscle tissue for anti-smooth muscle α-actin or ACK2 antibodies and replacing these primary antibodies from the sections with mouse or rat IgG as a nonspecific negative control.

Immunoblotting

Esophagi cultured with FGF-2 and with FGF-2 plus PD98059 were disrupted by several freeze-thaw cycles in a radioimmunoprecipitation assay (RIPA) buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, 2 mM EDTA, 5 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine. After centrifugation to remove cell debris, the protein concentrations in the whole-cell lysates were determined. Proteins (30 μg) from individual samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes by electroblotting. The blots were blocked with 5% nonfat dry milk (NFDM) in TBST (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.05% Tween 20) and then probed with anti-phospho-ERK1/2 antibody (1:1000 dilution; Cell Signaling, Beverly MA) or anti-ERK antibody (1:500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Species-specific secondary antibodies coupled to horseradish peroxidase (1:5000 dilution in 5% NFDM/TBST; Santa Cruz Biotechnology) were used to detect the primary antibodies. Protein bands

were visualized using enhanced chemiluminescence (PerkinElmer, Boston, MA).

Results

Striated myogenesis occurs in fetal esophageal muscularis externa in culture. Embryonic day 14.5 esophagi were explanted and immunostained for myogenin, a specific transcription factor involved in striated myogenesis, to examine the process of esophageal myogenesis. Over the 7 day period of examination, myogenin positive cells first appeared in the rostral esophagus, with myogenin expression progressing caudally along the length of the esophagus over the 7 day period (Figure 4.1). Myogenin positive cells could be detected along approximately 25-30%, 50% and 70% of the esophagus after 3, 5 and 7 days of culture respectively, but not at culture day 0, consistent with previous observations (Zhao and Dhoot 2000c). We could not detect any myogenin-positive cells near the lower-esophageal sphincter, in the stomach or in any esophageal layer other than the *muscularis externa* at any time, but myogenin-positive cells were detected in the pharynx and lingual musculature as early as culture day 0 (Figure 4.1). Myogenin expression appeared to progress slightly more rapidly in the external (longitudinal) layer of the *muscularis externa* than the internal layer. No differences were apparent between wild-type and SMCG2$^{+/WT}$ transgenic fetuses in any experiments, so results were pooled.

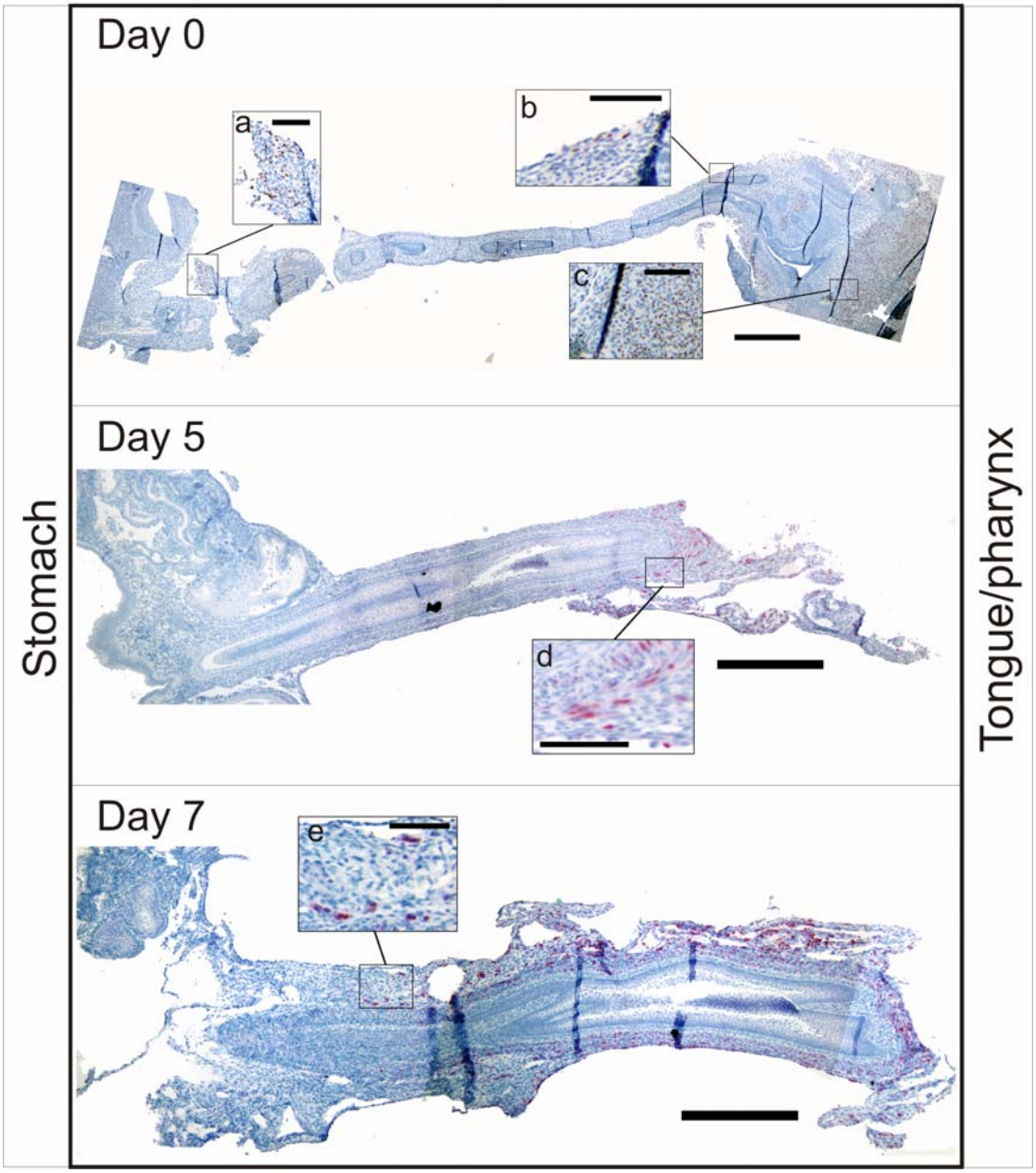

Figure 4.1 Sagittal sections of murine esophagi from ED14.5 fetuses show progressive myogenin expression over 7 days of culture. Inset (a) shows myogenin expression (red nuclear staining) in the diaphragm; (b) shows myogenin expression in 3-4 subpharyngeal nuclei; (c) shows robust myogenin expression in the tongue; (d) shows myogenin expression extending caudally along the rostral muscularis externa layers; (e) shows myogenin expression in the muscularis externa approaching the stomach. Scale bar for large panels = 500μm; scale bar for inset panels = 100μm.

Striated myogenic precursors are resident within the smooth muscle muscularis externa. Our previous studies, and those of other investigators, have refuted the hypothesis that smooth muscle cells in the esophageal *muscularis externa* transdifferentiate into striated muscle cells (Zhao and Dhoot 2000a; Zhao and Dhoot 2000b; Zhao and Dhoot 2000c; Rishniw et al., 2003; Rishniw et al., 2007), however, the origin of the striated muscle cells remains unknown. Recently, investigators have suggested that esophageal striated muscle cells arise from a resident population of precursor cells (Wörl and Neuhuber 2005). However, that study did not rule out the possibility that these striated muscle precursor cells actively migrate into the smooth muscle layers from a subpharyngeal location. To examine this hypothesis, we partially or completely transected fetal esophagi at harvest (ED14.5) just caudal to the larynx/pharynx. In partially transected esophagi the serosal and mucosal layers remained intact, but the *muscularis externa* was completely separated (Figure 4.2A), as demonstrated by an interruption of fluorescence of the smooth muscle layers (Figure 4.2B) and a failure of propagation of peristaltic waves through this zone (Supplemental data video 1). If muscle cell precursors migrate caudally to their locations in the *muscularis externa* during development, we would expect to observe a lack of myogenin staining caudal to the partially transected zone, because the migratory path would be disrupted.

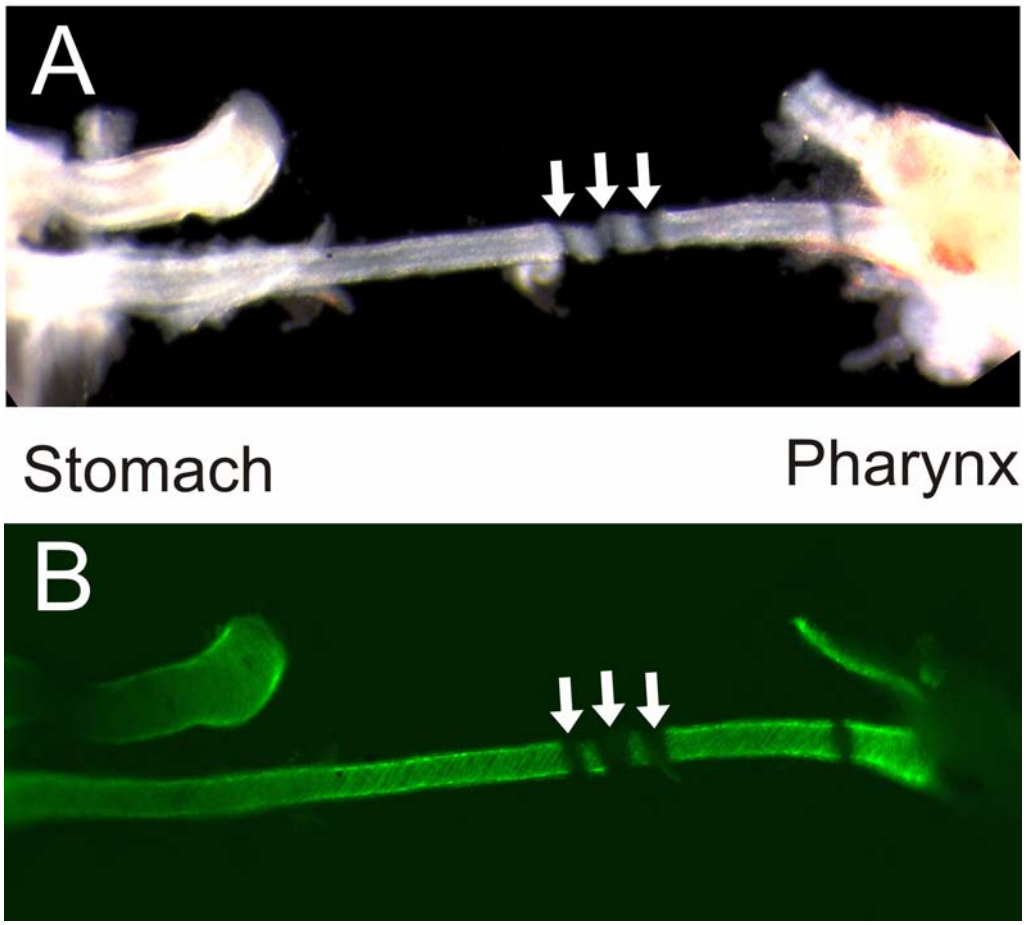

Figure 4.2 Mechanical disruption of the esophageal *muscularis externa* results in discontinuity of smooth muscle fluorescence. (A) Fresh whole mount ED14.5 esophagus explanted from SMCG2 transgenic mice (which express eGFP in smooth muscle) showing 3 adjacent zones of *muscularis externa* disruption (arrows). (B) Fluorescent imaging shows loss of smooth muscle fluorescence through the disrupted zones (arrows).

However, we found robust myogenin expression in the caudal esophageal segment at culture day 7 (Figure 4.3A), indicating that striated muscle precursors are already resident within the developing *muscularis externa* at ED14.5. On the other hand, in completely transected esophagi, where there was no serosal continuity (and therefore no proximity of the rostral and caudal esophageal segments), we could not detect striated myogenesis in

the caudal esophageal segment, despite continued robust peristalsis of both segments, and myogenin expression in the proximal segment (Figure 4.3B).

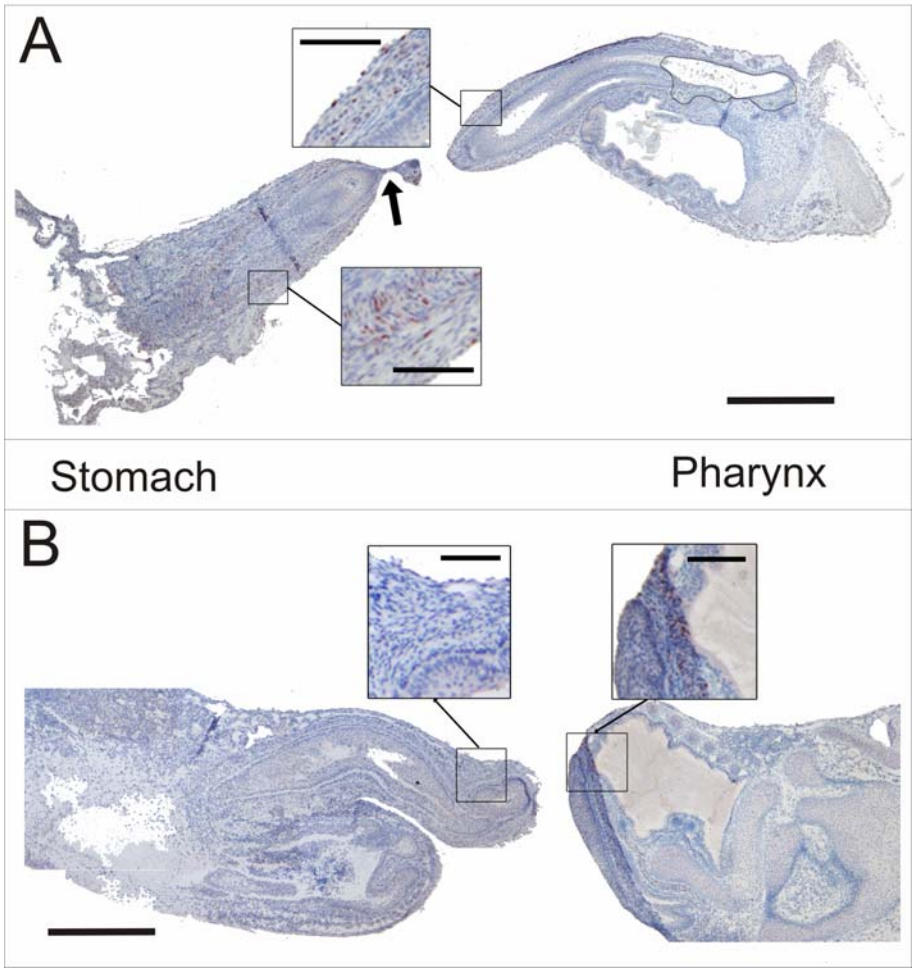

Figure 4.3 Striated myogenesis occurs in esophagi with mechanically disrupted *muscularis externa*. (A) Sagittal section of ED14.5 esophagus with a disrupted *muscularis externa* (arrow) but contiguity of the rostral and caudal segments after 7 days of culture shows myogenin expression on both sides of the zone of disruption (inset panels). The apparent dissociation to the right of the arrow (rostral) is a sectioning artifact, where a section of esophagus was positioned outside of the sectioning plane. (B) Myogenin expression is visible only in the subpharyngeal segment in a completely transected E14.5 esophagus after 7 days of culture, but is absent in the caudal segment (inset panels). Scale bar for large panels = 500μm; scale bar for inset panels = 100μm.

Together, these findings suggest that myogenic precursors do not migrate from the pharyngeal musculature at the time of myodifferentiation, but are resident in the proximal esophagus. Moreover, these results suggest that local factors are responsible for the activation of resident precursor cells, as segments that maintain close proximity show evidence of a progressive myodifferentiation. During striated myogenesis the smooth muscle component of the esophagus remains essentially unaltered, and remains considerably larger than the striated muscle cell component.

Fetal esophagi remain functional and viable for up to 7 days in culture. Esophageal explants maintained a functional phenotype for up to 7 days in culture. They failed to grow (length and width remained unchanged), but demonstrated vigorous spontaneous peristalsis throughout this period. Peristaltic waves occurred approximately every 20-30 seconds when examined at room temperature on an inverted dissecting scope, and propagated rostrally and caudally. Some of these waves were coordinated with gastric contractions, but not every gastric contraction propagated a retrograde peristaltic wave (Supplemental data video 2). Histologically, Smooth muscle α-actin could be identified in 3 distinct muscle layers along the length of the esophagus, corresponding to the 2 layers of the *muscularis externa* and the inner layer of the *muscularis mucosae* (Figure 4.4A), indicating that the smooth muscle cells maintained their phenotype throughout this period.

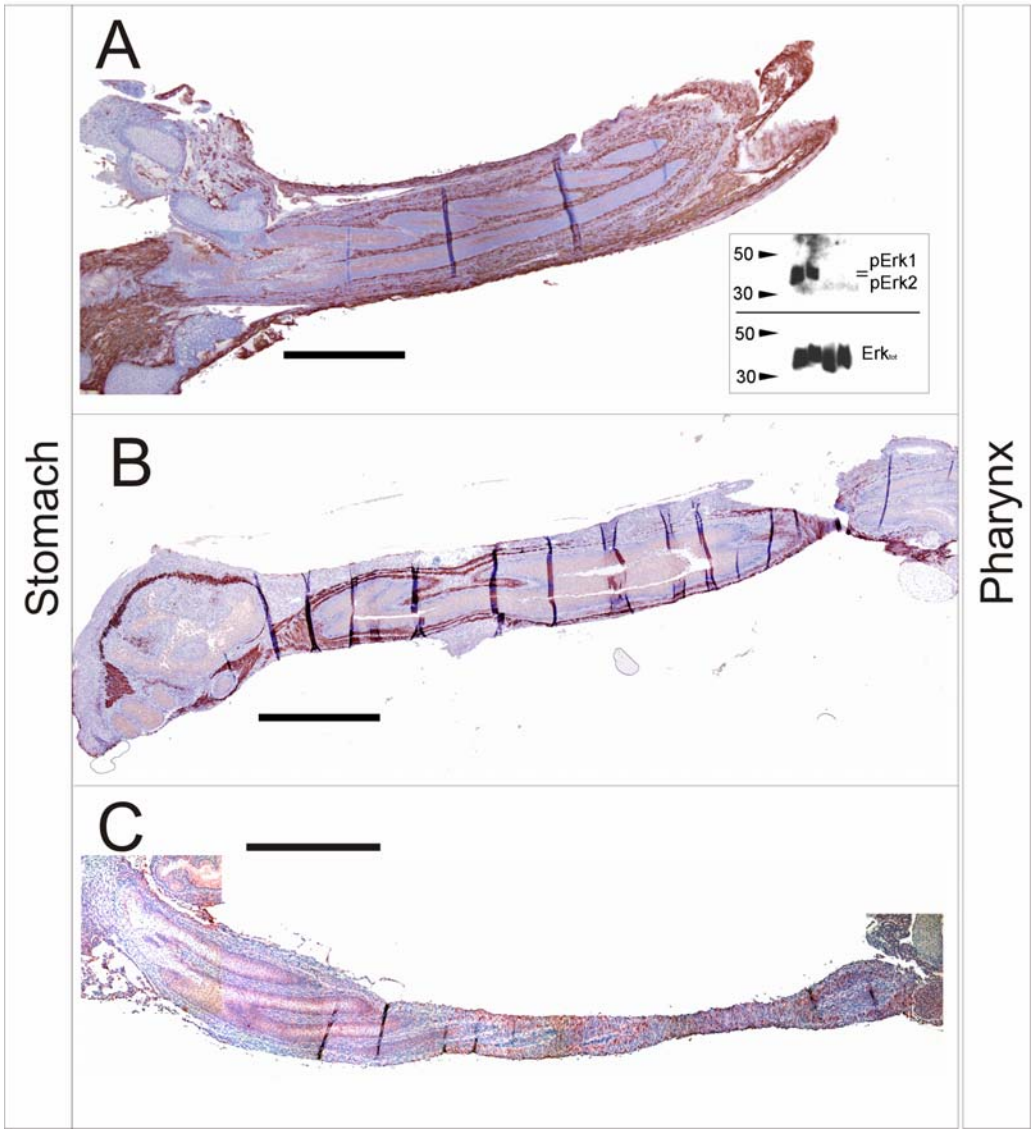

Figure 4.4 Smooth muscle maintains smooth muscle actin expression in culture for 7 days, but is attenuated by FGF-2. (A) Sagittal section of ED14.5 esophagus after 7 days of culture shows robust expression of smooth muscle actin in all 3 smooth muscle layers of the esophagus. (B) Smooth muscle actin expression is attenuated after 7 days of culture when FGF-2 is added to the culture media. Note the loss of actin in the outer gastric muscle layers. (C) Myogenin expression remains unaffected by culture in FGF-2. Scale bar = 500μm Inset: PD98059 inhibits ERK1/2 phosphorylation by FGF-2. ED14.5 esophageal lysates, immunoblotted with anti-phospho ERK1/2 or anti-pan-ERK antibodies, show complete inhibition of ERK1/2 phosphorylation by FGF-2 after concurrent treatment with MEK inhibitor PD98059 (upper panel, lanes 3 and 4). Esophagi treated with FGF-2 alone show robust phosphorylation of ERK1 and ERK2 (upper panel, lanes 1 and 2). Total ERK expression is similar for all samples (lower panel).

Esophageal striated myogenesis is not fibroblast growth factor dependent. Striated myogenesis is modulated by fibroblast growth factor 2 (FGF-2), which inhibits the expression of the differentiation markers MyoD and myogenin (Weyman and Wolfman 1998; Jones et al., 2001; Tortorella et al., 2001). Similarly, SMCs cultured in FGF-2 undergo de-differentiation or fail to express smooth muscle cell contractile proteins (Thyberg 1996; Kato et al., 1998; Kawai-Kowase et al., 2004). To determine if esophageal striated myogenesis was modulated through an FGF-2 dependent pathway, esophageal explants were cultured in 25μg/ml hrFGF-2 for 7 days. Under these conditions spontaneous peristalsis was markedly diminished over the culture period. A decrease in both frequency and amplitude of peristaltic waves was first observed after 3 days of culture, with FGF-2 treated esophagi contracting every 45-60 seconds, compared to control esophagi that contracted every 20-30 seconds. Inhibition of MEK with PD98059 prevented this attenuation of contractility.

Histologically, smooth muscle α-actin expression decreased in the *muscularis externa* after 7 days of culture in FGF-2 augmented media (Figure 4.4B), consistent with the observed loss of function. Inhibition of MEK with PD98059 resulted in attenuated phospho-ERK1/2 expression (Figure 4.4A inset) and prevented the decrease in smooth muscle α-actin expression, consistent with previous observations. (Kawai-Kowase et al., 2004) However, striated myogenesis continued in the presence of FGF-2, with little or no attenuation of myogenin expression (Figure 4.4C). These findings suggest that myogenin expression may not be FGF-2 dependent in the esophagus or in vivo and may be a phenomenon observed only in cell culture. This is consistent with observations that mice overexpressing FGF-2 develop normal

striated appendicular muscle, although in that study the FGF-2 may have been unable to interact with FGF receptors (Coffin et al., 1995).

c-kit-neutralizing antibody, ACK2, fails to inhibit peristalsis in cultured fetal esophagi. To determine if the peristalsis was induced by c-kit-positive interstitial cells of Cajal (ICC), we immunostained esophageal and gastric sections from ED14.5 uncultured fetuses with an anti-CD117 antibody. We could detect occasional immunopositive cells within the esophageal and gastric *muscularis externa*. Based on previous studies that documented inhibition of peristalsis in cultured fetal ureters after c-kit blockade (David et al., 20050, we cultured fetal esophagi in media containing 5µg/ml ACK-2 c-kit-neutralizing antibody. This failed to inhibit peristalsis over the 7 day culture period, despite strong penetration of the antibody into the *muscularis externa* (Figure 4.5), indicating that spontaneous peristalsis within the fetal esophagus is not ICC-dependent.

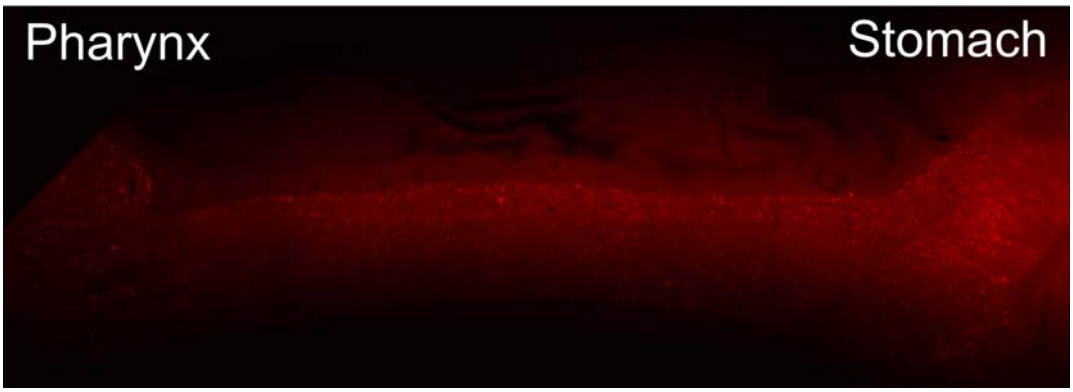

Figure 4.5 ACK2 antibody penetrates esophagi in culture but fails to inhibit peristalsis. Sagittal section of ED14.5 esophagus labeled with a biotinylated goat anti-rat antibody and detected with Texas Red avidin. Fluorescence can be detected throughout the esophagus, indicating complete penetration of the antibody during the culture period.

Discussion

We have provided additional evidence that esophageal striated myogenesis occurs from a resident population of myogenic precursors and that the striated myogenic program may be resistant to FGF-2 inhibition. While we could not determine the early embryonic origin of esophageal striated muscle cells, our data support recently published studies showing resident myogenic precursors (Wörl and Neuhuber 2005) and suggest that the progression of myogenesis depends on local factors. Additionally, we have, for the first time, cultured fetal murine esophageal explants, and maintained a functional phenotype for 7 days. The contractile behavior of explanted esophagi appears to be independent of ICCs.

Myogenin expression proceeded ex vivo in a rostro-caudal direction (from pharynx to stomach), consistent with previous observations that striated myogenesis is initially detected in the subpharyngeal regions and proceeds caudally along the length of the esophagus (Zhao and Dhoot 2000a; Zhao and Dhoot 2000b). Expression of myogenin at day ED14.5 was absent in any part of the esophagus, but progressed rostro-caudally over the 7 days in culture, similar to previous observations (Zhao and Dhoot 2000c). Similarly, the finding that myogenic precursors exist along 70% of the length of the esophagus by day ED14.5 is consistent with previous observations that Myf5 and MyoD expression was observable along most of the esophagus by this stage of development (Zhao and Dhoot 2000a) Our study demonstrates that these precursors did not migrate from a subpharyngeal location to the distal esophagus, but were resident in that tissue by day ED14.5. Had these precursors migrated from the subpharyngeal region along a smooth muscle lattice, mechanical disruption of the lattice should have prevented myogenin

from being expressed in the distal segment. However, we found robust expression in partially disrupted esophagi, indicating that myogenic precursors were present in the distal esophagus prior to mechanical disruption. We cannot, however, rule out the possibility that these precursor cells migrate through the esophagus prior to ED14.5. On the other hand, complete separation of the esophagi into 2 independent segments resulted in failure of myogenin expression in the distal (pre-gastric) segment, suggesting that either humoral or mechanical factors are involved in propagating a wave of myogenic activation and myodifferentiation. Additionally, we cannot conclude that the myodifferentiation observed ex vivo precisely mirrors events that occur in vivo. For example, cultured fetal esophagi failed to elongate appreciably over the 7 day culture period, whereas our previous studies of esophageal development in vivo demonstrate that the esophagus grows in length during the last week of gestation (Rishniw et al., 2007). It is likely that mechanical factors (distraction of the esophagus by a growing thorax), which are absent in our culture model, contribute to the normal development of the esophagus.

The persistence of esophageal peristalsis in the presence of the c-kit neutralizing antibody, ACK2, differs from the results obtained with fetal ureteral explants, where peristalsis was abolished within a few days of culture (David et al., 2005). While we identified occasional c-kit positive cells in ED14.5 esophagi, and ACK2 penetration into tissues appeared sufficient to inhibit c-kit receptors, peristalsis remained unaffected. This suggests that peristalsis is propagated in explanted esophagi by spontaneous smooth muscle depolarizations, rather than ICCs.

Peristalsis occurred both antegrade and retrograde in explanted esophagi. Peristalsis in adults is predominantly antegrade, but retrograde

peristalsis can occur (eructation and regurgitation in ruminants and birds). The direction of peristalsis is dependent, in part, on the site of initiation – in adults, swallowing initiates antegrade peristalsis. In our fetal culture experiments, gastric peristalsis appeared to trigger many of the retrograde peristaltic waves. Whether this occurred because of a continuity of electrical coupling between the stomach and esophagus, that later disappears, or due to mechanical initiation of a peristaltic wave (stretch-induced depolarization of esophageal muscle), is not known. Furthermore, whether bidirectional peristaltic activity occurs in utero in mice is unknown.

The failure of FGF-2 to inhibit myogenin expression was unanticipated. An effect of FGF-2 was observed, namely, a dedifferentiation of smooth muscle cells (characterized by loss of smooth muscle actin) and a concomitant attenuation of spontaneous contractile function, indicating that FGF-2 concentrations in the media were sufficient to produce a physiological effect. However, myogenin expression continued unabated in the presence of FGF-2, suggesting that in this system, striated myogenesis is not completely inhibited by FGF-2.

In conclusion, we have developed an ex vivo fetal esophageal culture model that remains functional for at least 7 days. Striated myogenesis in this model proceeds with a rostro-caudal activation of resident myogenic precursor cells that are resistant to FGF-2 inhibition of differentiation, but are dependent on structural integrity of the esophagus.

Acknowledgments

This study was supported in part by NIH grant RO1 DK065992 (Kotlikoff), NIH training grant T32RR07059-09 (Rishniw)

REFERENCES

Coffin, J.D., R.Z. Florkiewicz, J. Neumann, T. Mort-Hopkins, G.W. Dorn 2nd, P. Lightfoot, R. German, P.N. Howles, A. Kier, B.A. O'Toole (1995) Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice. Mol Biol Cell *6:*1861-1873

David, S.G., C. Cebrian, E.D. Vaughan Jr, D. Herzlinger (2005) c-kit and ureteral peristalsis. J Urol *173:*292-295

Jones, N.C., Y.V. Fedorov, R.S. Rosenthal, B.B. Olwin (2001) ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion. J Cell Physiol *186:*104-115

Kablar, B., S. Tajbakhsh, M.A. Rudnicki (2000) Transdifferentiation of esophageal smooth to skeletal muscle is myogenic bHLH factor-dependent. Development *127:*1627-1639

Kato, S., A. Muraishi, T. Miyamoto, J.C. Fox (1998) Basic fibroblast growth factor regulates extracellular matrix and contractile protein expression independent of proliferation in vascular smooth muscle cells. In Vitro Cell Dev Biol Anim *34:*341-346

Kawai-Kowase, K., H. Sato, Y. Oyama, H. Kanai, M. Sato, H. Doi, M. Kurabayashi (2004) Basic fibroblast growth factor antagonizes transforming growth factor-beta1-induced smooth muscle gene expression through extracellular signal-regulated kinase 1/2 signaling pathway activation. Arterioscler Thromb Vasc Biol *24:*1384-1390

Patapoutian, A., B.J. Wold, R.A. Wagner (1995) Evidence for developmentally programmed transdifferentiation in mouse esophageal muscle. Science *270:*1818-1821

Rishniw, M., H.B. Xin, K.Y. Deng, M.I. Kotlikoff (2003) Skeletal myogenesis in the mouse esophagus does not occur through transdifferentiation. Genesis *36:*81-82

Rishniw, M., P.W. Fisher, R.M. Doran, E. Meadows, W.H. Klein, M.I. Kotlikoff (2007) Smooth muscle persists in the *muscularis externa* of developing and adult mouse esophagus. J Muscle Res Cell Motil *28:*153-165

Stratton, C.J., Y. Bayguinov, K.M. Sanders, S.M. Ward (2000) Ultrastructural analysis of the transdifferentiation of smooth muscle to skeletal muscle in the murine esophagus. Cell Tissue Res *301:*283-298

Thyberg, J. (1996) Differentiated properties and proliferation of arterial smooth muscle cells in culture. Int Rev Cytol *169:*183-265

Tortorella, L.L., D.J. Milasincic, P.F. Pilch (2001) Critical proliferation-independent window for basic fibroblast growth factor repression of myogenesis via the p42/p44 MAPK signaling pathway. J Biol Chem *276:*13709-13717

Weyman, C.M., A. Wolfman (1998) Mitogen-activated protein kinase kinase (MEK) activity is required for inhibition of skeletal muscle differentiation by insulin-like growth factor 1 or fibroblast growth factor 2. Endocrinology *139:*1794-1800

Wörl, J., W.L. Neuhuber (2005) Ultrastructural analysis of the smooth-to-striated transition zone in the developing mouse esophagus: Emphasis on apoptosis of smooth and origin and differentiation of striated muscle cells. Dev Dyn *233:*964-982

Zhao, W., G.K. Dhoot (2000a) Both smooth and skeletal muscle precursors are present in foetal mouse oesophagus and they follow different differentiation pathways. Dev Dyn *218:*587-602

Zhao, W., G.K. Dhoot (2000b) Development and composition of skeletal muscle fibres in mouse oesophagus. J Muscle Res Cell Motil *21:*463-473

Zhao, W., G.K. Dhoot (2000c) Skeletal muscle precursors in mouse esophagus are determined during early fetal development. Developmental Dynamics *219:*10-20

CHAPTER 5

SUMMARY & FUTURE DIRECTIONS

The preceding studies have focused on the development of the murine esophageal musculature. Ever since the initial proposal by Patapoutian and colleagues in the mid-1990s that esophageal smooth muscle cells within the *muscularis externa* might transdifferentiate into striated muscles during perinatal esophageal development, several groups of investigators have tried to confirm or refute this hypothesis. The subsequent interest in this initial observation lies not only in esophageal development *per se*, but rather, if accurate, would provide a model for cellular plasticity, that has far broader implications. Committed or even differentiated cell lines would no longer necessarily be restricted to one phenotype, but could, with appropriate (and as yet undefined) stimuli, transform into other phenotypes. Understanding this transdifferentiation process in a validated, easily manipulated model, such as murine esophageal musculature, would be paramount to investigating this process in other, more clinically relevant cell lines, such as cardiac cells, neurons etc.

My studies demonstrated that transdifferentiation does not occur in murine esophageal smooth muscle. Rather, esophageal smooth muscle cells remain true to their original phenotype while undergoing several terminal pathways: segregation within the striated musculature as “remnant” smooth muscle cells, compaction around the lower esophageal sphincter, and apoptosis. In contrast, striated muscle cell development progresses craniocaudally, most likely from a resident population of striated muscle precursors – a process that can be observed in explanted esophageal whole-organ cultures.

Esophageal smooth muscle does not transdifferentiate into striated muscle.

I provided three independent levels of evidence refuting the original hypothesis of transdifferentiation. In Chapter 2, I devised a simple fate-mapping experiment, whereby any cell expressing smooth muscle myosin heavy chain at *any point in that cell's existence* would "unlock" a constitutively expressed marker (LacZ). Thus, if an esophageal striated muscle cell had previously expressed smooth muscle myosin heavy chain, the LacZ gene would be activated within that striated muscle cell, resulting in β-galactosidase staining regardless of that cell's ultimate fate. My results revealed an absence of β-galactodisase activity in esophageal striated muscles, indicating that these striated muscles had never activated the smooth muscle heavy chain promoter.

In Chapter 3, I examined the possibility of smooth-to-striated muscle transdifferentiation by searching for co-expression of nuclear proteins in cells undergoing striated myodifferentiation. Specifically, I searched for a smooth-muscle restricted Cre recombinase in cells expressing the striated muscle transcription factor myogenin, but could find no evidence of co-expression of these 2 lineage-dependent proteins. However, as I pointed out in that chapter, a temporally dissociated expression of each of these proteins could not be ruled out.

Finally, in Chapter 4, I devised and conducted a further experiment that examined the possibility of transdifferentiation. Using a recently constructed conditional myogenin knockout mouse, I deleted the myogenin gene in smooth muscle cells by crossing this mouse with a mouse that expressed Cre recombinase in smooth muscle. Since myogenin is absolutely required for

striated muscle differentiation, smooth muscle cells lacking the myogenin gene that were undergoing transdifferentiation into striated muscles would fail to complete the differentiation process, resulting in esophageal myodysplasia. However, we observed normal esophageal development despite successful deletion of the myogenin gene in smooth muscle.

Taken together, my results convincingly demonstrate that esophageal myogenesis does not involve transdifferentiation.

Esophageal smooth muscle has variable fates

One quandary for investigators examining esophageal myogenesis has been the fate of smooth muscle cells, where an organ that is initially composed entirely of smooth muscle develops into an organ with virtually no smooth muscle. Substantial apoptosis was not detected by Patapoutian, although subsequent investigators suggested that apoptosis may occur more frequently than initially proposed.

In Chapter 3, I examined the potential fate of esophageal smooth muscle cells, after a serendipitous observation, where I had noted linear fluorescence in the rostral esophagus of adult mice that expressed eGFP in smooth muscle. Detailed investigation of this observation led me to conclude that the adult murine esophagus, which is almost exclusively composed of striated muscle, harbors what I termed “remnant” smooth muscle cells amidst the striated stroma. These remnant cells are misshapen, express smooth muscle-specific proteins, and serve an as yet unknown function. Their frequent proximity to interstitial cells of Cajal within the striated muscle layers suggests that they may be involved in maintaining integrity of the esophageal pacemaker cells. Additionally, I demonstrated that the absolute length of the

smooth muscle component remains virtually unchanged during a 3-fold increase in esophageal length, suggesting that apoptosis is unlikely to be occurring over this period. Finally, staining for apoptotic nuclei revealed a discrete population of cells undergoing apoptosis, however, this was a relatively infrequent event.

Thus, I have provided a biologically plausible and simple explanation for the fate of smooth muscle cells in the esophageal *muscularis externa*, without the need to invoke a complex process such as transdifferentiation.

Esophageal striated muscle development progresses *ex vivo*.

In Chapter 4 I examined esophageal striated myogenesis in an isolated system. I successfully cultured fetal esophagi for up to 7 days, during which time they retained a functional phenotype. Serial examination of the myogenic development in explanted esophagi confirmed a cranio-caudal appearance of myoblasts, as defined by myogenin expression. Partial mechanical disruption of esophageal integrity rostrally at the time of harvesting the esophagi did not alter the myogenic pattern over the following 7 days suggesting that the striated muscle precursors reside within the smooth muscle layer and are sequentially induced to myodifferentiate. Complete disruption arrested myodifferentiation in the caudal esophageal segment, suggesting that either structural integrity is required to induce myodifferentiation, or a paracrine factor is involved and is prevented from passing in a cranio-caudal direction when the esophagus is divided into 2 separate segments.

Finally, I showed that fetal esophageal peristaltic function is not dependent on interstitial cells of Cajal, which have been shown to induce peristaltic activity in other developing organs.

Future Directions

Despite having resolved the issue of transdifferentiation as a mechanism for esophageal myogenesis in the mouse, many questions remain unanswered. Most striated muscle in the body arises from somites, with striated muscle precursors migrating in several waves to their final location. An exception to this are branchiomeric (head) and certain extra-ocular muscles, which have been shown to arise from separate precursor populations. Lingual and pharyngeal muscles, however, are not included in this group, but have been shown to have a somitic origin.

Where do the striated muscle precursors that populate the esophageal *muscularis externa* originate? One possibility is that they migrate along with the somitic precursors that populate the pharynx to the developing throat, and are seeded from these cells as the esophageal primitive tube develops and elongates, lying dormant until they are induced to myodifferentiate. This process has been documented for the extrinsic laryngeal muscles. Relatively simple experiments could potentially answer whether esophageal striated muscle precursors are derived from this population.

As detailed in the introductory chapter, several genes are expressed exclusively in one embryonic muscular precursor population or another. For example, somitically derived striated muscle precursors express *Paraxis*, *Lbx1*, *Pax3*, and *MRF4*, whereas head and pharyngeal muscle precursors generally do not. Conversely, most head muscles, which originate from the cranial paraxial mesoderm, exclusively express *Tbx1*, *Tcf21*, *FGF10* and *Tlx1*.

Conditional null mice and reporter mice exist for several of these genes. For example *Pax3* null mice exist as a spontaneous mutation (*Splotch* mice), and have also been created *de novo* by targeted deletion of *Pax3*.

Examination of esophageal muscularis externa in *Pax3* null mice at birth should reveal whether striated myogenesis is affected. If unaffected, this would suggest that myogenesis is independent of *Pax3*, and therefore unlikely to be of somitic origin. Similarly, a *Tbx1*-Cre reporter mouse exists. Since *Tbx1* is required for striated myogenesis of muscles derived from branchial (pharyngeal) arches, and is not expressed in somitically derived muscles, simple examination of *Tbx1-Cre*::R26R mice at 2 weeks after birth would determine if esophageal striated muscle expresses b-galactosidase, confirming that this muscle is branchiomeric. A complimentary experiment, involving examining a *Tbx1*$^{-/-}$ mouse after birth would help confirm whether Tbx1 is essential for esophageal myogenesis.

A recent study demonstrated that *Myf5*$^{-/-}$::*MyoD*$^{-/-}$ mice with a functional *Myf6* (*MRF4*) gene failed to develop head muscles or limb muscles, but developed epaxial and hypaxial muscles. Examination of these mice for esophageal development at birth could help support the hypothesis that esophageal muscle precursors are or are not dependent on *Myf6* (*MRF4*) expression. Previous studies have shown *Myf6* (*MRF4*) expression in developing esophagus, but this may be through the classical downstream activation by *Myf5/MyoD* pathway.

There are at least 2 transcription factors expressed exclusively in the somites and somitically derived cells (and the pre-otic paraxial mesoderm that gives rise to the lateral rectus muscle) during early fetal development: Paraxis and Lbx1. By constructing a Paraxis promoter-driven Cre recombinase transgene, and using the R26R reporter mouse (that constitutively expresses LacZ in tissues that co-express, even transiently, Cre recombinase), it should be possible to fate map all cells that express Paraxis. Subsequently, staining

the esophageal striated muscle for β–galactosidase activity would determine if these cells are of somitic origin (with the exception of the lateral rectus). Similar experiments could be conducted using the Lbx1 promoter, which is activated in migrating striated muscle precursors of somitic origin. An alternative approach, using available genetically modified mice, would involve examining the *Lbx1*^{GFP} mouse, which expresses GFP under control of the Lbx1 promoter (this mouse was developed through a "knock-in" strategy). Examining *Lbx1*$^{GFP/+}$ heterozygotes would permit "tracking" of cells expressing *Lbx1* to the pharynx/tongue region and, if present, in the esophagus. Alternatively, *Lbx1*$^{GFP/GFP}$ mice would fail to express *Lbx1*, allowing examination of esophageal myogenesis in the absence of *Lbx1*.

A further peculiarity about esophageal striated myogenesis remains – the myogenic program in this organ is implemented considerably later than other striated myogenic programs. By the time the myogenic precursors begin to express early myogenic regulatory factors in the mouse (embryonic day 11-12), the tongue and pharynx are thoroughly populated with myoblasts and myocytes, as are limbs and torso. Secondary and tertiary waves of myogenesis occur in other tissues, but nothing is known about such events in the esophagus. The cues that drive these additional myogenic waves are not well defined. Esophageal myogenesis could serve as a model for the delayed myogenic programs that occur throughout the body. Regional genomic analysis of esophageal tissue at various stages of myogenesis could allow factors to be identified that instigate myodifferentiation along the developing esophagus. Using the *ex vivo* culture model I developed, conditions could be controlled to allow gene expression profiling at different timepoints in an attempt to elucidate myogenic triggers. For example, esophageal myogenic

precursors could be examined for expression of Pax3 and Pax7, which are characteristically expressed by satellite cells, and myogenic progenitor cells involved in the third phase of myogenesis.

A final teleological issue is that of the interspecies variability of esophageal anatomy – why are their multiple designs for a tube that connects the pharynx and the stomach, and propels food or fluid boluses predominantly in one direction? In humans, the esophagus remains almost exclusively a smooth muscle organ, in mice and ruminants it is almost exclusively striated in nature, while other species have variable contributions of each muscle type. There does not appear to be any rhyme-nor-reason to this variation in anatomy, since esophageal function between species is not markedly different. Does the extent of striated myogenesis simply reflect the time in fetal development at which striated muscle precursors migrate to the rostral esophago-pharyngeal junction? Assuming that esophageal striated muscle precursors "seed" from this rostral location as the smooth muscle esophagus develops, the extent of this "seeding" would depend on the degree of esophageal development at the time of arrival of precursor cells. Once the origin of these cells is identified in the mouse, it could be feasible to examine this hypothesis in other species by comparing the earliest detectable precursors and the level of esophageal development at the time of detection.

Despite marked advances in understanding myogenesis in mammals, both through the body, and in the head, esophageal myogenesis remains poorly understood, and, to some extent, ignored by physiologists investigating these processes. However, better understanding of this unique myogenic process could allow better understanding of muscle formation elsewhere in the body.