WHOLE OR HOLE? DEVELOPMENT OF THE DIAPHRAGM AND CONGENITAL DIAPHRAGMATIC HERNIAS

by

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A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Human Genetics

The University of Utah

August 2014

The University of Utah Graduate School

STATEMENT OF DISSERTATION APPROVAL

The following faculty members served as the supervisory committee chair and members for the dissertation of Allyson Jean Merrell.

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ABSTRACT

The mammalian diaphragm is the most critical skeletal muscle and defects in the development of the diaphragm give rise to congenital diaphragmatic hernias (CDH), which are common and frequently lethal birth defects. The diaphragm has been proposed to develop from multiple embryonic sources, but how these sources are integrated to form the diaphragm and how defects in diaphragm development induce CDH is poorly understood. Using mouse genetics we demonstrate that the pleuroperitoneal folds (PPF), transient developmental structures, will give rise to connective tissue fibroblasts throughout the diaphragm. Furthermore, we show that the PPFs non-cell autonomously regulate the somitically-derived muscle of the diaphragm and control the morphogenesis of the diaphragm. Deletion of candidate CDH genes, *Gata4* and *Porcn,* specifically within the PPF generates CDH with 100% penetrance, demonstrating that the PPFs are the cellular source of CDH defects. Deletion of *Gata4* in the PPF causes early defects in muscle progenitor number and localization, creating amuscularized regions that will form CDHs. Interestingly, PPF-specific deletion of *Porcn,* which is required for secretion of Wnt ligand, produces late defects in diaphragm development and prevents the diaphragm muscle from spreading completely to the most ventral region of the

diaphragm. This demonstrates that Wnt signals from the PPF are critical for the formation of the ventral muscle of the diaphragm. Furthermore, we propose that signals from the PPF may be critical for inducing muscle precursors to migrate from the somites to the diaphragm and acquisition of these signals may have allowed evolution of the muscularized mammalian diaphragm. This work demonstrates the essential roles of the PPF-derived connective tissue fibroblasts in all steps of diaphragm development.

This dissertation is dedicated to my family for their constant support and to everyone who helped me make it to this point. Thanks!

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ACKNOWLEDGMENTS

My family has been incredibly supportive of my time in graduate school. My parents spent hours talking on the phone with me when I felt overwhelmed and stressed, even when I am sure they could have been doing other things. Their enthusiasm when I have had success has made that success even more exciting. When I finally generated mice with diaphragmatic hernias after years of failed experiments, I was so excited to tell my parents about it because I knew how happy they would be for me. I have always known that I could turn to them whenever I need anything. My brothers have also provided lots of advice to me over the years, both in college and grad school, and I am grateful for their guidance over the years. I would also like to thank my sister, Brittany, for always being a positive influence in my life.

I have spent a lot of hours in lab, and these were made very memorable by the fun people in the Kardon lab. We've had a great time talking science, cracking jokes, planning creative ways to dispose of things, and generally having a great time while working. I could not have asked for a better group of people alongside whom to work. Jenn has genotyped countless mice for me, and this help has been invaluable for my experiments over the years. I would also like to thank the former members of the Kardon lab who helped me get started in the

lab. I would especially like to thank Malea Murphy for her tireless efforts as the senior graduate student. She put in countless hours helping me practice for talks and training me to be a better student. Malea was also a great friend, both in and out of lab. Most of my favorite graduate school memories involve making Malea laugh.

None of my success in graduate school would have been possible without Gab. She has invested huge amounts of energy into making sure that I could succeed in grad school. Gab has listened to me practice the same talk multiple times, all to make sure that I learned the right way to give a talk. The best part about discovering something new from an experiment is being able to tell Gab about it, because no one is more excited than she. She was incredibly supportive in the years before I had interesting results, and I appreciate her faith in me. I could not have asked for a better mentor.

CHAPTER 1

INTRODUCTION

The mammalian diaphragm is the most critical skeletal muscle in the human body. The diaphragm is a thin, domed sheet of muscle that separates the thoracic and abdominal cavities and is highly conserved in all mammals. A muscularized diaphragm is critical for respiration and as a barrier between the thoracic and abdominal cavities, and defects in this barrier function have severe health consequences. Defects in diaphragm development produce congenital diaphragmatic hernias (CDH), which arise frequently (1:3000) and cause high mortality and morbidity (Pober, 2007). Despite the critical role of the diaphragm in human health, there is a limited understanding of the cellular and morphogenetic processes regulating diaphragm development, both normally and during herniation. In this dissertation, I review the previous research on diaphragm development and herniation, establish the critical role of connective tissue during normal diaphragm development and the formation of CDH, and discuss how mammals may have evolved a muscularized diaphragm.

The diaphragm muscle is composed of crural and costal domains, which contain muscle fibers, muscle connective tissue, tendon, and nerve (Pearce, 2009) (Fig. 1.1). The thicker crural region, located posteriorly, surrounds the

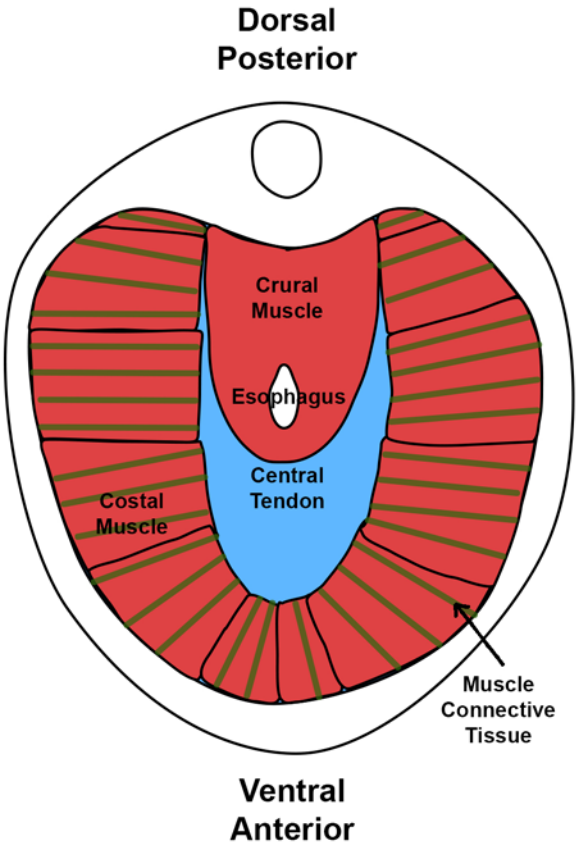

Fig. 1.1 Major components of an adult diaphragm.

esophagus and aorta and attaches to the vertebrae. The costal diaphragm consists of a radial array of muscle fibers, which connect laterally to the ribs and medially to a central tendon. The central tendon is a sheet of connective tissue consisting of extracellular matrix secreted by the resident tendon cells. Surrounding each muscle fiber is muscle connective tissue, which is secreted primarily by interstitial muscle connective tissue fibroblasts. Finally, the diaphragm muscle is innervated by the left and right phrenic nerves, which insert on the left and right halves of the diaphragm and spread posteriorly and anteriorly to innervate both crural and costal myofibers (Allan and Greer, 1997).

The diaphragm muscle is critical for inspiration and as a barrier between the thoracic and abdominal cavities. During inspiration, contraction and lowering of the diaphragm muscle expands the volume of the thoracic cavity, which reduces air pressure and allows air to flow into the lungs (Campbell EJM, 1970). The diaphragm also serves a critical role as a barrier. Developmental defects in this barrier result in congenital diaphragmatic hernias, characterized by the passage of liver and intestine through the diaphragm, inhibiting lung development (Pober, 2007).

Diaphragm Development

Despite the functional importance of the diaphragm and the frequency of defects in diaphragm development, the processes by which the muscularized diaphragm develops are not well understood. This is largely due to a lack of molecular tools and reagents to visualize the development of the diaphragm. Previous studies have proposed that the diaphragm develops from the septum

transversum, the pleuroperitoneal folds, and the somites (Fig. 1.2). However, the origin and fate of these tissues during diaphragm development have not been fully investigated. Furthermore, the morphogenetic processes that drive diaphragm development are unclear. The fate and interactions of these tissues during diaphragm development are examined in Chapter 3 of this dissertation.

The septum transversum is the first structure present in the developing diaphragm and has been proposed to contribute to the central tendon of the diaphragm. The septum is a thin sheet of tissue separating the heart and liver (Perry et al., 2010), and can be detected at the 9-12 somite stage in mice by expression of *Cited2* (Dunwoodie et al., 1998). During foregut invagination, folding causes *Cited2*+ tissue, which originates as the rostral-most mesoderm in the embryo, to ultimately lie in a position caudal to the heart and form the septum transversum. Although the septum is present early in diaphragm development, it is unclear what function the septum actually performs. Previous theories have suggested that the septum gives rise to the central tendon of the diaphragm (Iritani, 1984). However, due to the lack of markers and genetic tools to follow the fate of the septum transversum, it is unclear whether the septum actually contributes to the fully formed diaphragm.

The pleuroperitoneal folds (PPF) have been proposed to be critical for diaphragm development, although little is known about their fate or function. The PPFs are two transient, pyramidal-shaped structures that protrude from the dorsal body wall between the pleural and peritoneal cavities. These structures have been described both histologically in section (Allan and Greer, 1997; Iritani,

1984) and through scanning electron microscopy (Kluth et al., 1996; Kluth et al., 1993). The PPFs, which can be identified by expression of several transcription factors including *Gata4* (Clugston et al., 2008), are first present in the mouse at embryonic day (E)11 and then proliferate to become most prominent at E12.5 (Iritani, 1984) (Fig. 1.2). The PPFs initially form at the level of the cervical somites, then descend caudally during development to reside near the thoracic/lumbar boundary (Allan and Greer, 1997). From previous work, it is unclear whether the PPFs are simply transient, developmental structures, or whether this tissue gives rise to cells in the adult diaphragm. Previous studies have not directly tested the fate of these cells, although studies using scanning electron microscopy have suggested that the PPFs spread to fuse with the septum transversum (Allan and Greer, 1997; Iritani, 1984). In Chapter 3, I discuss the origin, fate, and critical function of the PPFs during diaphragm development.

The diaphragm muscle precursors originate in the somites, migrate to the PPF, and then spread ventrally and fuse to form the diaphragm muscle. Diaphragm muscle precursors delaminate from cervical somites and migrate to the PPFs along a track of hepatocyte growth factor (HGF) expression (Dietrich et al., 1999). From the PPFs, muscle precursors spread ventrally and dorsally to give rise to the characteristic radial array of muscle fibers in the diaphragm (Babiuk et al., 2003). From previous work, it is unclear what controls the proper expansion of muscle throughout the diaphragm, what surface the muscle moves

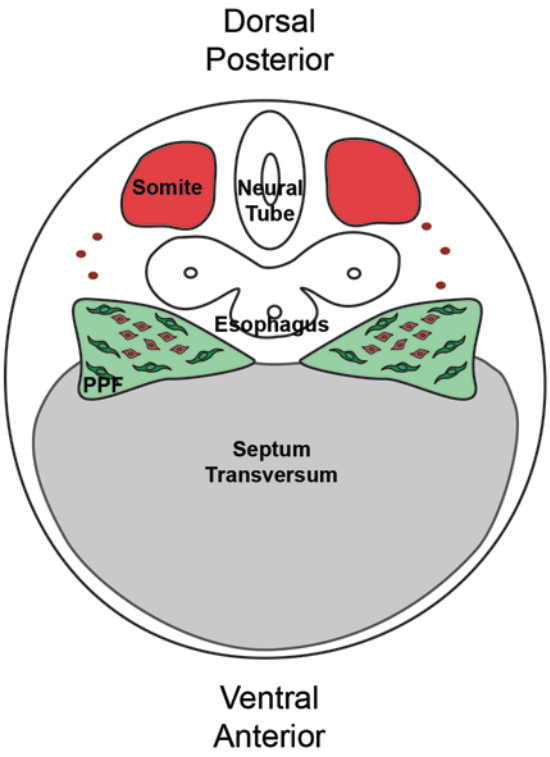

Figure 1.2 Embryonic sources of the diaphragm at E11.5 Muscle precursors (red) migrate from the somite to the pleuroperitoneal folds (PPF, green).

on, and how the myofibers are patterned and aligned. In Chapter 3, I examine the role of the connective tissue in the patterning of the diaphragm muscle. Factors that regulate migration of muscle precursors from the somite to the PPF, a critical step for the evolution of the mammalian diaphragm, are discussed in Chapter 5.

Congenital Diaphragmatic Hernias

Congenital diaphragmatic hernias (CDH) are common (1:3000) and severe birth defects that arise from defects in diaphragm morphogenesis (Pober, 2007). Weakened or incomplete regions of the diaphragm allow abdominal contents to herniate through the diaphragm into the thoracic cavity, inhibiting lung development. This causes lung hypoplasia and subsequent respiratory failure, which is the main cause of the high morbidity and mortality associated with CDH. Even with medical intervention, the CDH mortality rate is 50% (Ackerman and Pober, 2007; Harrison et al., 1994; Keller, 2007). Diaphragmatic hernias vary in the region in which they form, although most will form on the left side. The majority of hernias (90%) form posteriorly (dorsally), with the remainder forming either anteriorly (ventrally) or within the central tendon (Harrison et al., 1994; Torfs et al., 1992).

Most CDH research has focused primarily on identifying the genetic causes of CDH. Cytogenetic studies have frequently identified copy number variants (CNVs) in particular chromosomal regions associated with CDH (Holder et al., 2007; Pober, 2007; Veenma et al., 2012). The transcription factor GATA binding protein 4 (*GATA4)*, located in chromosomal region 8p23.1, is among the

candidate genes identified in these studies (OMIM 22240) (Holder et al., 2007). Recent studies have identified two noncoding variants and two missense mutations in *GATA4* in patients with CDH (Arrington et al., 2012; Longoni et al., 2012; Yu et al., 2012). However, the functional role of Gata4 during diaphragm development and how deletion of a transcription factor produces localized defects is unclear.

Mouse models of CDH have provided some insights into the mechanism of herniation, but have been limited by a lack of genetic tools. Many of the candidate CDH genes are homozygous lethal early in development, precluding study of the diaphragm in these mutants. For example, mice with a homozygous *Gata4* deletion die by E10, prior to diaphragm development. Analysis of mice heterozygous for a *Gata4* deletion demonstrated a small percentage (14%) of minor hernias, which were all located ventrally (Jay et al., 2007). The low penetrance of this phenotype makes it difficult to determine how mutations in *Gata4* produce CDH. In Chapter 3, I analyze mice with conditional deletions of *Gata4* to bypass lethality and determine the mechanism by which hernias form.

A major question regarding the formation of congenital diaphragmatic hernias is which cells and tissues behave aberrantly to give rise to diaphragmatic hernias. Although defects in the PPFs have been suggested to cause CDH, the role of specific tissues in CDH formation has not been tested. Many of the candidate CDH genes, including *Gata4*, are expressed specifically in the non-muscle PPF cells (Clugston et al., 2008), indicating the PPFs may be the source of the defects causing CDH. Previous studies of teratogen-induced CDH models

have also suggested early defects in the PPFs as the cause of CDH. This is based on analysis of sections indicating misshapen PPFs, and it was proposed that these defects in the PPF correspond to holes in the tissue that will form hernias (Clugston and Greer, 2007; Greer et al., 2000). Should hernias arise from a failure of the PPF to spread properly as proposed, it would be expected that PPF-derived cells would be absent from the herniated region. In Chapter 3, I test the tissue-specific requirement of *Gata4* in diaphragm development, and the ability of mutant PPF cells to spread throughout the diaphragm.

Wnt Signaling in Diaphragm Development

Studies of patients with CDH have suggested that the Wnt signaling pathway may be required for diaphragm development. CDH is found in some patients with focal dermal hypoplasia (FDH), a syndrome also characterized by limb malformations and regions of absent or thin dermis (Dias et al., 2010; Han et al., 2000; Kunze et al., 1979; Maas et al., 2009; Smigiel et al., 2011). Studies have indicated that FDH is caused by mutations in Porcupine (*PORCN)* (OMIM 305600) (Grzeschik et al., 2007; Maas et al., 2009; Wang et al., 2007). *PORCN* is located on the X chromosome, and patients are predominantly heterozygous females with mutant mosaic patches generated through random X-inactivation. *PORCN* is required for secretion of both canonical and noncanonical Wnt signaling ligands (Barrott et al., 2011), so homozygous (in females) or hemizygous (in males) mutations of *PORCN* cause very early embryonic lethality. Although these studies suggest a role for Wnt signaling in diaphragm formation, they do not indicate how inhibition of Wnt signaling produces

developmental defects that cause CDH or identify which tissues are required for secretion or reception of Wnt ligands.

The role of Wnt signaling in diaphragm development can be ascertained by generating mice with mutations in components of the Wnt pathway. Female mice heterozygous for a null allele of *Porcn* exhibit phenotypes similar to those of FDH patients (Barrott et al., 2011). Using a conditional allele of *Porcn,* the secretion of Wnt signals from specific tissues can be ablated, testing the requirement for secretion of Wnt ligands from specific tissues. The necessity for reception of canonical Wnt signaling can be tested in specific tissues by manipulation of the central mediator of Wnt signaling, β-catenin. In the absence of canonical Wnt signaling, β-catenin is targeted for destruction. When Wnt signals are present, β-catenin can accumulate, translocate to the nucleus, and induce transcription by binding to Tcf/Lef transcription factors (Niehrs, 2012). By either deleting or stabilizing β-catenin, the canonical Wnt pathway can be inhibited or ectopically activated. In Chapter 4, I test the requirement for secretion and reception of Wnt signaling in tissues of the developing diaphragm.

Diaphragm Evolution

The diaphragm muscle is a unique, highly conserved structure present in all mammals. It is unclear what evolutionary events led to the appearance of a fully muscularized diaphragm in mammals, although it is likely that this occurred through the acquisition of developmental structures of the diaphragm. To develop a muscularized diaphragm, muscle precursors need to delaminate from cervical somites and receive signals to migrate to the developing diaphragm. These

signals may originate from the PPFs, as they are the initial target of diaphragm muscle (Allan and Greer, 1997; Babiuk et al., 2003). It is unknown if the PPFs are present in the outgroup of mammals (e.g., birds) or evolved specifically within mammals to allow diaphragm muscularization. Cervical somites in chick embryos express the HGF receptor Met (Thery et al., 1995) and Lbx1, a marker of muscle precursors migrating to the limb, tongue, and diaphragm in mouse (Dietrich et al., 1998). This suggests that muscle precursors in the chick may be specified as migrating muscle precursors and would be capable of receiving signals to direct their migration towards the diaphragm. HGF is expressed in the mouse by non-muscle cells on the track along which muscle will migrate (Dietrich et al., 1999) and has been proposed to regulate muscle motility and proliferation (Leshem et al., 2002; Maina et al., 1996; Scaal et al., 1999). It is possible that acquisition of HGF expression permitted evolution of the muscularized diaphragm. Evolution of the mammalian diaphragm will be discussed in Chapter 5.

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CHAPTER 2

DEVELOPMENT OF THE DIAPHRAGM – A SKELETAL MUSCLE ESSENTIAL FOR MAMMALIAN RESPIRATION

Reprint of: Merrell, AJ and Kardon, G. (2013) Development of the diaphragm – a skeletal muscle essential for mammalian respiration. FEBS Journal 280 (17), 4026-35.

the FEBS Journal

REVIEW ARTICLE

Development of the diaphragm – a skeletal muscle essential for mammalian respiration

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Keywords
CDH; congenital diaphragmatic hernia; development; diaphragm; muscle; tendon

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(Received 17 January 2013, revised 27 March 2013, accepted 28 March 2013)

doi:10.1111/febs.12274

The mammalian diaphragm muscle is essential for respiration, and thus is one of the most critical skeletal muscles in the human body. Defects in diaphragm development leading to congenital diaphragmatic hernias (CDH) are common birth defects and result in severe morbidity or mortality. Given its functional importance and the frequency of congenital defects, an understanding of diaphragm development, both normally and during herniation, is important. We review current knowledge of the embryological origins of the diaphragm, diaphragm development and morphogenesis, as well as the genetic and developmental aetiology of diaphragm birth defects.

Introduction

Of all the skeletal muscles in the human body, the diaphragm is one of the most essential. It is a domed muscle separating the thoracic and abdominal cavities and is critical for respiration. Birth defects and diseases that affect diaphragm structure and function reveal its critical function. Congenital diaphragmatic hernias (CDH) result from defects in diaphragm development and are both common and frequently lethal [1]. In addition, the lethality associated with many myopathies, such as Duchenne muscular dystrophy, and is the result of a failure of the diaphragm and other respiratory muscles [2]. However, despite its critical function, knowledge of the molecular and cellular mechanisms regulating diaphragm development, both normally and during herniation, is limited. The present review describes current knowledge about diaphragm development, reviews the genetic and developmental aetiology of diaphragm birth defects and, compares the developmental processes regulating limb and diaphragm muscle morphogenesis.

Diaphragm structure, function and evolution

The diaphragm muscle is composed of two domains [3]. The costal diaphragm is a thin domed sheet of muscle composed of a radial array of myofibres extending laterally from the ribs and medially to a central tendon (Fig. 1). The crural diaphragm is thicker and located more posteriorly (dorsally), where it attaches to the vertebrae and surrounds the oesophagus and aorta (Fig. 1). Medially, the myofibres of both the costal and crural muscles insert into the central tendon. The central tendon is located at the apex of the domed diaphragm, holding the diaphragm muscle domains together. Caudally, it attaches to the liver via

Abbreviations
CDH, congenital diaphragmatic hernia; E, embryonic day; HGF, hepatocyte growth factor; RA, retinoic acid.

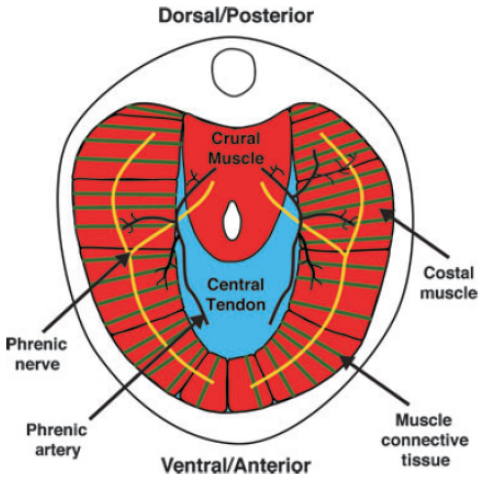

Fig. 1. Major components of the adult diaphragm.

the falciform and coronary ligaments. The tendon is a connective tissue sheet composed of extracellular matrix and the tendon cells that secrete it [3]. Although less visible in whole mount, each of the myofibres of the costal and crural muscles is surrounded by muscle connective tissue. The right and left halves of the diaphragm are innervated by the right and left phrenic motor nerves [4]. These nerves originate from the cervical nerves (C3–C5), descend along the interior of the vertebrae, pierce the left and right diaphragm, and spread posteriorly (dorsally) and anteriorly (ventrally) to innervate myofibres of the crural and costal muscles. The diaphragm is vascularized by the phrenic, internal thoracic and intercostal arteries [5].

The diaphragm has multiple functions, with its principle function being its critical role in respiration. During respiration, the actions of the diaphragm are central for inspiration. Contraction of the diaphragm muscle flattens the dome-shaped diaphragm and central tendon, which in turn expands the volume of the thoracic cavity, reduces thoracic pressure, and allows air to flow into the lungs [6]. Interestingly, the diaphragm is not strictly required for respiration when humans or animals are resting. However, analyses of rats, dogs and humans with bilateral diaphragmatic paralysis (in which the phrenic nerves are severed) demonstrate that the diaphragm is required for respiration and lung ventilation during supine posture (particularly during rapid-eye-movement sleep) and vigorous activity [7–9]. In addition to respiration, the diaphragm (particularly the crural domain) has functional roles in swallowing and emesis [10]. Finally, the diaphragm also has a passive functional role. The diaphragm serves as a barrier between the thoracic and abdominal cavities. The importance of this barrier function is dramatically apparent in newborns with CDH, whereby a weak or incompletely formed diaphragm allows abdominal contents to herniate into the thoracic cavity and impair lung development [1].

The presence of a muscularized diaphragm is a unique and defining characteristic of mammals [11,12]. Although a muscularized diaphragm is unique to mammals, the presence of a septum separating the lungs from the abdominal viscera is an ancient character, and some variant of this septum is present in reptiles and birds (but not in fish and amphibians) [12,13]. In mammals, this septum becomes muscularized to form the diaphragm. It has been proposed that the diaphragm evolved in mammals as a stabilizer of the abdominal viscera and an inspiratory muscle [12]. Together, these functions of the diaphragm allowed mammals to evolve as high-performance homeotherms, capable of concomitant respiration and locomotion. Thus, the question of how the mammalian diaphragm evolved is an important question. Presumably, developmental innovations were critical for the development of a muscularized diaphragm, although the molecular and cellular nature of these innovations is currently unknown.

Embryological sources of the diaphragm

The diaphragm develops from multiple embryonic sources. The muscle and its associated connective tissue and central tendon develop from three sources: the septum transversum, the pleuroperitoneal folds and the somites.

The septum transversum is the first structure present in the developing diaphragm and serves as the initial barrier between the thoracic and abdominal cavities (Fig. 2). In all vertebrates, the septum transversum is a thin, mesodermal sheet of tissue that separates the heart from the liver [12]. In many reptiles and all birds and mammals, septa separate the heart and lungs from the liver and the rest of the abdominal contents [12]. Expression analysis of *Cited2* (originally called *Mrg1*), which is expressed in the mouse septum transversum, suggests that the septum originates as the rostral-most mesoderm in the embryo [14]. During the process of foregut invagination, folding causes the *Cited2*+ tissue to lie in a position caudal to the heart and form the septum transversum, which can be detected by the 9–12 somite stage in mice. Other genes, such as *α4-integrin* [15] and *Pbx3* [16], are also expressed in the mouse

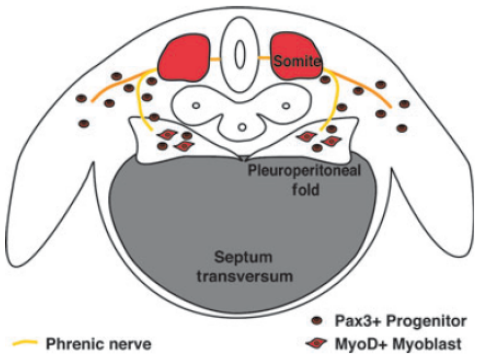

Fig. 2. Embryonic sources of the diaphragm. Mouse developing diaphragm at E12.5. The diaphragm develops from the septum transversum, pleuroperitoneal folds and the somites, and is innervated by the phrenic nerve.

septum transversum by embryonic day (E)9. It is unclear what the septum gives rise to (if anything) in the adult, although some studies have suggested that the septum gives rise to the non-muscle and central tendon components of the diaphragm [17]. However, without markers or genetic tools to follow the fate of the septum, it is still unclear whether or what the septum contributes to in the adult diaphragm. Although not explicitly tested, during development, the septum is likely to provide a scaffold for diaphragm morphogenesis.

The pleuroperitoneal folds (also referred to as the posthepatic mesenchymal plate) are the second important component of the developing diaphragm. The pleuroperitoneal folds are two transient, pyramidal-shaped structures lying on either side of the esophagus that protrude from the body wall between the pleural and peritoneal cavities (Fig. 2). The pleuroperitoneal folds have been identified both histologically in sections [4,17] and through scanning electron microscopy [18,19]. Molecularly, the cells comprising the pleuroperitoneal folds express the nuclear receptor *NR2F2* (also known as *Coup-TFII*) and the transcription factors *Wt1* and *Gata4* [20]. In mouse, the pleuroperitoneal folds are first present at E11 [17], proliferate (to be most prominent at E12.5), then appear to spread ventrally, and, eventually are assumed to fuse with the septum [4,17]. It is currently unclear whether the pleuroperitoneal folds are simply transient embryonic structures with no adult derivatives, or whether they give rise to cells or tissues of the adult diaphragm.

Similar to trunk and limb muscles, the somites are the source of the diaphragm's muscle cells (Fig. 2) [4,21,22]. Evidence that the diaphragm's muscle originates from the somites comes from analysis of the transcription factor *Pax3* and the receptor tyrosine kinase *Met* (also known as *c-Met*). Both of these genes are strongly expressed in the muscle progenitors within the somites [23–25]. Furthermore, these genes are required for the muscle progenitors to migrate from the somites to form muscles in the limb and trunk. Strikingly, mice with null mutations in either *Pax3* or *Met* have no limb or diaphragm muscles (including both the costal and crural muscle domains) [21,26–28]. Thus, the diaphragm derives from migratory muscle progenitors originating from the somites. Expression studies [4,21,22] show that the diaphragm's muscle progenitors originate from the cervical somites, likely C3–C5.

Migration of muscle and nerve to the developing diaphragm

The muscle progenitors and the phrenic nerve axons migrate from the somites and neural tube, respectively, to the developing diaphragm (Fig. 2). The target of their migration is the pleuroperitoneal folds [4,22]. In mice, the phrenic nerve axons and muscle progenitors migrate toward the folds by E10.5 [4,21,22]. The phrenic nerve migrates after the muscle progenitors and initially migrates with nerves of the brachial plexus before separating and targeting the folds [29]. When the nerves and muscle progenitors reach the folds, the folds are located at the level of cervical somites. However, gradually the folds and the developing diaphragm with its nerves and muscle descend caudally, ultimately to lie at the thoracic/lumbar boundary [4].

The pleuroperitoneal folds are likely to be an important source of signals guiding the migration of nerves and muscle to the developing diaphragm. Our knowledge of the molecular nature of these signals is limited. Neural cell adhesion molecule and low-affinity nerve growth factor receptor are expressed along the path from the neural tube to the pleuroperitoneal folds and may guide outgrowth of the phrenic nerve [4]. In addition, hepatocyte growth factor (HGF), the ligand for the *Met* receptor, is expressed along the migratory path for both the muscle progenitors and the nerves [21]. The finding that *HGF* null mice have no diaphragm muscle strongly suggests that *HGF* is important for guidance of the myogenic progenitors to the developing diaphragm [30]. *HGF* may also be critical for phrenic nerve development [31,32].

Diaphragm morphogenesis

To produce a functional diaphragm, the morphogenesis of the muscle, muscle connective tissue and tendon

derived from these different embryonic sources must not only be coordinated with each other, but with the nerves and vasculature.

After reaching the pleuroperitoneal folds, the muscle progenitors undergo the processes of myogenesis and morphogenesis. During myogenesis, the muscle progenitors differentiate into multinucleate myofibres. Similar to trunk and limb muscle, diaphragm progenitors initially express the transcription factors Pax3 and Pax7. These progenitors become committed myoblasts expressing the transcription factors Myf5 and MyoD, differentiate into Myogenin+ myocytes and, finally, fuse to form multinucleate myofibres [22,33]. Pax3/7+ muscle progenitors and MyoD+ myoblasts are present in the pleuroperitoneal folds by E12.5 in mice, as well as by E13.5 in rats [22]. Subsequently the progenitors differentiate into myoblasts and then fuse into myofibres, which are assembled into the costal and crural muscles. The morphogenesis of the costal diaphragm has been described in more detail [22]. Myofibres first begin differentiating in posterolateral regions of each left and right hemi-diaphragms. Between E12.5 and E15.5, a wave of differentiation expands both ventrally and dorsally, as well as medially (towards the central tendon) and laterally (towards the ribs) of each hemi-diaphragm. Morphogenesis of the costal diaphragm is largely complete by E15.5. It is still unclear what drives this morphogenetic process, what orients the myofibres to form a radial array, and why the central tendon region is devoid of muscle.

The morphogenesis of the diaphragm's muscle connective tissue and central tendon and their relationship to the transverse septum and the pleuroperitoneal folds remain poorly understood. Based on histological analysis of sections through developing mouse embryos, it has been proposed that the septum transversum, initially present on the cranial surface of the liver, remains in place and gives rise to the central tendon [17]. The pleuroperitoneal folds have a complicated morphogenesis. After E12.5, the folds, initially located adjacent to the cervical somites, descend caudally (along with the phrenic nerves and muscle progenitors) to reach the thoracic/lumbar boundary [4]. Using scanning electron micrographs of a series of developing embryos, the pleuroperitoneal folds then appear to spread to cover the cranial surface of the liver and fuse with the septum transversum [18,19]. The molecular and cellular nature of the interactions between the folds and the septum is not known. Also, it is unclear what the ultimate fate of the folds is: the folds may simply be transient embryonic structures or they may give rise to the non-muscular parts of the diaphragm. Thus, it is currently unknown whether the muscle connective tissue and central tendon arise from the septum transversum, the pleuroperitoneal folds, or some other embryonic source.

The close proximity of the muscle and non-muscle cells, which presumably give rise to the muscle connective tissue and central tendon, suggest that cell–cell interactions between these tissues may be critical for proper diaphragm development. Interestingly, analysis of *Met* null mice (in which muscle progenitors do not migrate into the developing diaphragm) demonstrate that the connective tissue is present even in the absence of muscle [34]. Thus, the connective tissue develops, at least initially, independent of muscle, although it is unclear whether distinct tissues such as the central tendon still form. Whether the diaphragm's muscle requires signals from the developing muscle connective tissue and/or central tendon has not yet been tested.

Innervation of the diaphragm is critical for the development of a fully functional diaphragm. After reaching the pleuroperitoneal folds, the phrenic nerves must spread, branch and innervate the developing diaphragm by forming neuromuscular junctions with differentiated myofibres. After reaching the pleuroperitoneal folds, by E13.5 the phrenic nerve splits into three branches: sternocostal, dorsocostal and crural branches [35]. The sternal branches extend and cross past the midline of the diaphragm before retracting to reach their final positions [22]. Subsequently, the three major branches send out short secondary branches, arborize and then form neuromuscular junctions with the costal and crural muscle. The molecular signals and cellular interactions controlling axon outgrowth, branching and formation of neuromuscular junctions are beginning to be elucidated. The receptor protein tyrosine phosphatases σ and δ are required for phrenic nerve branching as the phrenic nerves reach the pleuroperitoneal folds, but fail to extend and branch appropriately in mice mutant for these phosphatases [35]. Additionally, Hoxa5 and Hoxc5 are required for proper secondary branching of the phrenic nerve because the deletion of these genes in the motor neurones results in a severe reduction of branching, as well as limited synapse formation with the muscle [36]. Interactions between muscle and nerve are also critical for secondary branching and neuromuscular junction formation [37]. Conditional mutagenesis experiments in mice found that secondary branching and arborization of the phrenic nerve is regulated via β-catenin within the muscle, demonstrating that muscle-derived signals regulate phrenic nerve development [38,39]. In addition, a multitude of studies have shown that formation of phrenic nerve neuromuscular junctions

involves a complex interplay of muscle–nerve retrograde and anterograde signalling [40,41].

Finally, vascularization of the diaphragm is also critical for diaphragm development. This is an area of research that has received little attention. However, recent research using *XLacZ4* transgenic mice, which label the nuclei of vascular smooth muscle cells, shows the vascularization of the diaphragm by the phrenic, intercostal and internal thoracic arteries. The use of *XLacZ4* transgenic mice permits visualization of the complex branching structures of both the arteries and veins. This work provides the first detailed description of diaphragm vascularization and holds promise for future research [5].

CDH

CDH is a common birth defect (1 : 3000) that often has severe medical consequences [1]. CDH occurs from a failure of the diaphragm to form properly, resulting in weak or incomplete regions of muscle. Through these weakened or incomplete regions, the abdominal contents herniate into the thoracic cavity. In turn, the herniated abdominal contents impede lung development, leading to hypoplastic lungs. Although it is generally considered that lung hypoplasia simply results from a physical impedance of lung growth by the herniated tissue, some genetic defects associated with CDH directly affect both diaphragm and lung development [42,43]. The lung hypoplasia accompanying CDH is the main cause of the high morbidity and mortality associated with CDH. Despite medical intervention, the mortality rate for CDH is 50% and results from respiratory failure [1,44–46]. For patients that survive, chronic respiratory and neurodevelopmental problems are common [42].

Diaphragmatic hernias vary both in the region of the diaphragm in which they form and in size. In the majority of cases (90%), hernias form in the posterior lateral diaphragm, and these posterolateral hernias (termed Bochdalek hernias) develop predominantly on the left side of the diaphragm (Fig. 3A) [1,46,47]. Posterolateral hernias are the form of CDH most commonly associated with lung hypoplasia because the abdominal contents herniate into the posterior pleural cavity where the developing lungs are forming. Hernias also form in anterior regions (termed Morgagni hernias) or in the central tendon (termed central hernias) (Fig. 3B,C) [1]. However, hernias in these regions generally have less severe consequences [48]. Diaphragmatic hernias also vary in size [49], with larger hernias having more critical impacts on health, whereas smaller hernias may be asymptomatic.

Diaphragmatic hernias can develop in isolation or in association with other developmental abnormalities [1]. CDH that arises in association with other abnormalities is often associated with recognized syndromes, commonly including Fryns syndrome, Denys–Drash syndrome, Cornelia de Lange syndrome, Donnai–Barrow syndrome, Wolf–Hirschhorn syndrome, Ehlers–Danlos syndrome and focal dermal hypoplasia [50–56].

Diaphragm morphogenesis requires muscle progenitors to migrate to the developing diaphragm and differentiate into muscle. The deletion of genes required for the delamination and migration of muscle precursors from the somite, such as *HGF* and *Met*, results in a failure of muscle to migrate into the diaphragm from the somite and leads to an amuscularized diaphragm

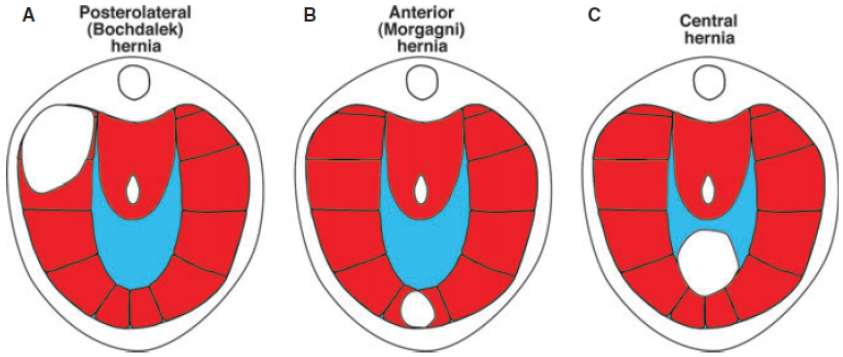

Fig. 3. Major types of congenital diaphragmatic hernias: (A) posterolateral (Bochdalek) hernia; (B) anterior (Morgagni) hernia; and (C) central hernia.

[21,26]. Similarly, the deletion of genes required for myogenesis, such as *MyoD* or *Myogenin*, results in thinner or absent diaphragm muscle [57,58]. Interestingly, the complete loss of muscle in mouse *Met* mutants does not result in herniation of abdominal content through the diaphragm [34]. Thus, the lack of diaphragm muscle is not sufficient to allow herniation of abdominal content through the diaphragm.

Formation of the diaphragm also requires that the connective tissue forms with proper structural integrity, and mutations that inhibit the development of the extracellular matrix result in CDH. Mice with null mutations for *lysyl oxidase*, an enzyme responsible for cross-linking collagen, develop central tendon hernias [59]. The central tendon of these mice is unable to withstand pressure from the growing liver and allows herniation of abdominal content through the weakened connective tissue. Similarly, patients with mutations in *Collagen 3a1* develop CDH [54]. In these patients, the reoccurrence of herniation can occur because of the weakened state of the diaphragm. Together, the *lysyl oxidase* and *Collagen 3a1* mutations indicate that the loss of connective tissue integrity can be a cause of CDH.

The molecular pathway most frequently associated with CDH is the retinoic acid (RA) signalling pathway. Much of the initial work investigating the development of CDH in rodent models used the teratogen nitrofen, which inhibits RA signalling [17,60,61]. Administration of nitrofen, as well as other teratogens that inhibit RA signalling, to pregnant mice or rats induces the development of CDH in their fetuses [62]. Furthermore, simultaneous administration of RA with these teratogens is sufficient to rescue CDH [62]. This confirms that the production of RA is critical for the developing diaphragm. Further evidence for the importance of RA signalling comes from the finding that mutations in *Stra6*, which is involved in retinol uptake and the production of retinoic acid, are present in some humans with CDH [63,64]. Also, mutations in the retinoic acid receptors *RARa* and *RARb* result in CDH in a small percentage of mice [65]. Production of RA requires processing by retinal dehydrogenase. RALDH2, the only retinal dehydrogenase enzyme present in the diaphragm, has been localized by immunofluorescence to the non-muscle cells of the pleuroperitoneal folds [62]. This suggests that the non-muscle cells are the source of RA in the developing diaphragm. An intriguing hypothesis is that RA signals from the non-muscle cells to the muscle cells are important for proper diaphragm morphogenesis.

In addition to mutations associated with RA signalling, other genetic causes underlying CDH have been identified by examining chromosomal abnormalities in CDH patients. Three of the frequently identified chromosomal regions are 8p23.1, 8q22-23 and 15q26.1-26.2, with the most commonly suggested candidate genes in these regions being *Gata4*, *Zfpm2* (also known as *Fog2*) and *Nr2f2* (also known as *Coup-TFII*), respectively. Significantly, these three genes can interact with one another and have also been proposed to interact with the RA signalling pathway [66,67]. Although *Gata4* has long been implicated in the formation of CDH, until recently, no coding variants had been identified within this gene in CDH patients. Whole exome sequencing of a familial case of CDH has identified a novel variant in a highly conserved arginine residue in the zinc finger domain of *Gata4* that is predicted to be pathogenic [68]. Additional enhancer and intronic variants in *Gata4* in CDH have also been identified [69,70]. Functional analysis of the role of *Gata4* in the formation of CDH in mice is limited because mice with homozygous null mutations of *Gata4* die before the formation of the diaphragm as a result of heart defects. However, in a study of mice heterozygous for a deletion of *Gata4*, 14% of the mice developed anterior congenital diaphragmatic hernias, and 29% of mice had some type of diaphragm defect [43], providing further evidence for *Gata4* being a candidate gene in the 8p23.1 region. *Zfpm2* has also been implicated as being important for CDH and is a binding partner of Gata4 (Fog2 is an abbreviation for Friend of Gata 2) [71]. Mice with null mutations of *Zfpm2* develop CDH [42]. Further evidence for a role of *Zfpm2* in CDH comes from the finding of a *de novo* mutation resulting in a premature stop in *Zfpm2* in a child with CDH [42]. Finally, *Nr2f2* is located in the 15q26.1-26.2 chromosomal region that is frequently deleted in CDH patients [72]. Although no causal variants within *Nr2f2* have been reported, mice with a conditional deletion of *Nr2f2* in the foregut mesentery develop CDH [73]. In addition to these genes, a multitude of other genes have been implicated by human genetic studies [1]. Also, a recent screen of genes expressed in the developing diaphragm in mouse has identified a list of 27 candidate CDH-causing genes [74].

Despite the frequency with which CDH occurs and extensive research identifying genes implicated in the formation of CDH, little is known about the molecular and cellular mechanisms by which these genetic mutations cause the diaphragm to form aberrantly. First, it is unclear in which tissues of the developing diaphragm mutations in the CDH genes are causative. Immunofluorescence of candidate CDH genes shows that these genes are predominantly expressed in non-muscle cells of the pleuroperitoneal folds [20]. This suggests that expression of CDH genes in these cells is critical. However, conditional deletion of these genes

in particular cells (e.g. the non-muscle cells of the folds) will be necessary to determine in which cells CDH genes are important. Second, although CDH is undoubtedly caused by defects in diaphragm morphogenesis, what particular cells or tissues behave aberrantly is not known. The incomplete development of the diaphragm could be a result of defects in the proliferation and/or survival of myogenic progenitors, aberrant migration of myogenic cells, or defects in muscle differentiation or morphogenesis. Alternatively, the incomplete development of the diaphragm could result from aberrant development of the septum transversum or defects in the formation or spread of the pleuroperitoneal folds. Finally, another unknown aspect of diaphragmatic herniation is the question of why hernias only form in local regions of the diaphragm, as opposed to throughout the entire diaphragm muscle. The local and variable nature of the defects suggests that local changes in levels, timing or the site of gene expression during diaphragm development may regulate the size and location of hernias.

Comparison of diaphragm and limb muscle development

A comparison of diaphragm and limb muscle development reveals many similarities in their regulation, a few differences, and suggests avenues of future research. Both diaphragm and limb muscles derive from populations of migratory muscle progenitors. These progenitors delaminate from the somites and migrate into either the pleuroperitoneal folds or the limb buds. Migration of muscle precursors to both sites relies on the signalling of HGF to Met+ muscle precursors to induce delamination and migration [26]. However, although *Lbx1* marks progenitors migrating either into the developing diaphragm or limb [21], unexpectedly, null mutations in *Lbx1* affect only the migration of limb progenitors and not diaphragm progenitors [75,76]. Once in the developing diaphragm or limb, myogenic progenitors go through a similar process of myogenesis whereby progenitors become committed myoblasts, differentiate into myocytes and fuse into myofibres. In the limb, the processes regulating muscle morphogenesis and patterning are beginning to be elucidated. Lateral plate-derived muscle connective tissue is critical for determining the pattern of individual limb muscles [77,78]. How the pattern of costal and crural diaphragm muscles is established is currently unknown. If diaphragm muscle morphogenesis is similar to that in the limb, the diaphragm's muscle connective tissue may be an important determinant of the diaphragm's muscle pattern.

Conclusions

The diaphragm is a unique mammalian muscle, essential for respiration. Unfortunately, defects in diaphragm development, leading to congenital diaphragmatic hernias, are common birth defects and result in severe morbidity and mortality. Given its functional importance and the frequency of congenital defects, an understanding of the genetic, cellular and morphogenetic mechanisms regulating diaphragm development, both normally and during herniation, is critical. However, many fundamental questions about diaphragm development remain unanswered. Embryonically, the diaphragm derives from three sources: the septum transversum, the pleuroperitoneal folds and the somites. Currently, it is unclear whether the septum and folds are only transient embryonic structures or whether they contribute to tissues of the mature diaphragm. Also unknown is whether the muscle connective tissue and central tendon arise from these structures. Muscularization of the diaphragm requires muscle progenitors to migrate from the somites to the developing diaphragm, proliferate and differentiate, and form costal and crural muscles. Although a few molecular signals (e.g. HGF/Met) have been identified that regulate progenitor migration, the signals and cell–cell interactions regulating muscle differentiation and morphogenesis have not been established. Finally, although a multitude of genes are identified as likely being important in CDH, how mutations in these genes mechanistically lead to hernias has not been determined. Identifying the molecular, cellular and morphogenetic processes that CDH genes regulate should reveal both how the diaphragm develops normally and how defects in diaphragm development lead to these devastating birth defects.

Acknowledgements

Diaphragm research in the G. Kardon's laboratory is supported by March of Dimes and NICHD/NIH grants to G.K. A.J.M. is supported by a University of Utah Graduate Research Fellowship.

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CHAPTER 3

CONNECTIVE TISSUE GENETIC MOSAICISM CAUSES CONGENITAL DIAPHRAGMATIC HERNIAS

Abstract

The diaphragm is the most essential mammalian skeletal muscle, and defects in diaphragm development are the cause of congenital diaphragmatic hernias (CDH), a common and often lethal birth defect. The diaphragm is derived from multiple embryonic sources, but how these give rise to an integrated diaphragm is unknown and, despite the identification of many CDH-associated genes, the etiology of CDH is poorly understood. Using mouse genetics, we show that the pleuroperitoneal folds (PPFs), transient embryonic structures, are the source of the diaphragm's connective tissue, regulate development of somitically-derived muscle, and their striking migration controls diaphragm morphogenesis. Furthermore, *Gata4* somatic mosaic mutations in early PPF-derived connective tissue cause CDH, leading to lung hypoplasia and neonatal lethality. Thus, although previously unappreciated, the PPFs and connective tissue are critical for diaphragm development and the source of CDH. Our finding that CDH arises from somatic mosaic mutations explains the notable incomplete

penetrance and variable expressivity of many CDH-associated genetic mutations.

Introduction

The muscularized diaphragm is not only a unique and defining character of all mammals (Perry et al., 2010), but is the most essential mammalian skeletal muscle. Diaphragm contraction drives inspiration and is critical for respiration (Campbell et al., 1970). In addition, the diaphragm has a significant passive functional role, since it serves as a barrier between the thoracic and abdominal cavities (Perry et al., 2010). These important functions are carried out by the costal part of the diaphragm: a radial array of myofibers, surrounded by muscle connective tissue (MCT), that extend from the ribs to the central tendon (Fig. 3.1y). Development of a functional diaphragm therefore requires the coordinated morphogenesis of muscle, MCT, and tendon, and these tissues have been suggested to develop from multiple embryonic sources (Merrell and Kardon, 2013). However, our knowledge of how this essential muscle develops has been limited by the inaccessibility of mammalian embryos to classic experimental embryological techniques and lack of genetic reagents to manipulate key embryonic sources.

Defects in diaphragm development are the cause of congenital diaphragmatic hernias (CDHs), a common (1/3000 of total births) and costly (exceeding $250 million/year in the USA) birth defect (Raval et al., 2011; Torfs et al., 1992). In CDH, weaknesses in the developing diaphragm allow abdominal contents to herniate into the thoracic cavity and impede lung development. The

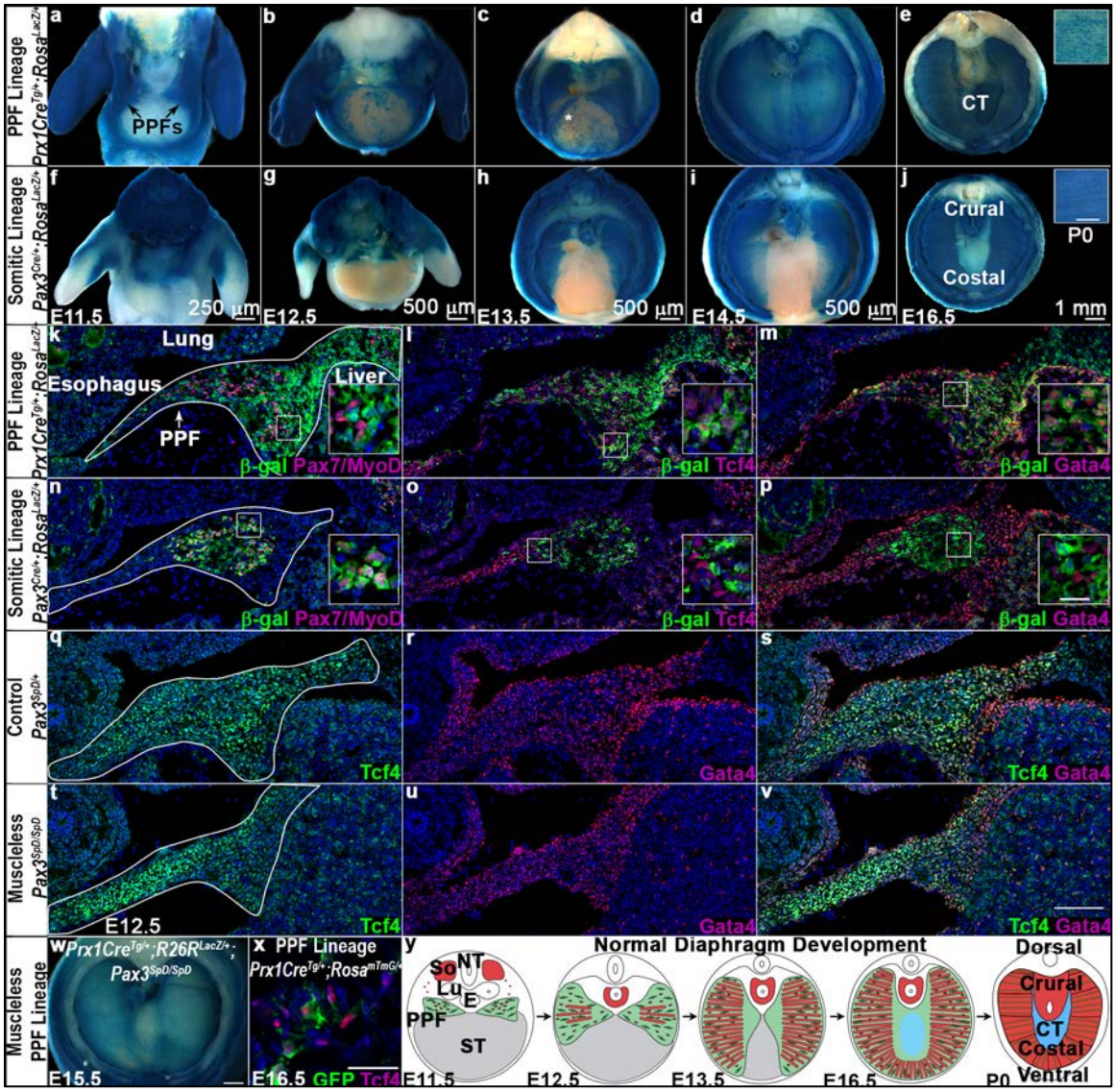

Fig. 3.1: PPFs are composed of Gata4+ fibroblasts, which migrate independently in advance of myogenic cells. a-e, *Prx1Cre* genetically labels E11.5 PPFs (a), which expand throughout the diaphragm by E14.5 (d) and contribute to the central tendon (e) and fibroblasts (inset e). f-j, *Pax3Cre* labels E11.5 myogenic cells in the PPF (f) and these expand to give rise to the muscle (j). k-m, *Prx1Cre* does not label Pax7+ muscle progenitors or MyoD+ myoblasts (k), but labels Tcf4+ (l) and Gata4+ (m) fibroblasts in the PPFs. n-p, *Pax3Cre* labels Pax7+/MyoD+ myogenic cells (n), but not Tcf4+ (o) or Gata4+ cells (p). q-v, Gata4+ cells are Tcf4+ fibroblasts (q-s) and present in the absence of muscle (t-v). w-x, Prx1-derived cells expand in the absence of muscle (w) and are Tcf4+ fibroblasts (x). y. Model of diaphragm development. a-j, w, Whole-mount β-gal staining. k-v, Sections through E12.5 PPF. x, Section through E16.5 diaphragm. Asterisk in c shows location of Supplemental Video 1. Scale bar = 100 um (k-v), 20um insets (k-p), 500 um (w), 50 um (x). CT, central tendon; E, esophagus; Lu, lung; NT, neural tube; PPFs, pleuroperitoneal folds; So, somite, ST, septum transversum

resulting lung hypoplasia is the cause of the 50% neonatal mortality and long-term morbidity associated with CDH (Pober, 2007). Despite the prevalence and severity of CDH, the genetic and cellular etiology of this birth defect is poorly understood. The majority of CDH cases appear as isolated defects (Pober, 2007), and the low incidence of sibling/familial recurrence suggests that CDH generally arises from *de novo* genetic mutations (Pober et al., 2005). Molecular cytogenetic analyses of CDH patients have identified copy number variants (CNVs) in multiple chromosomal regions strongly associated with CDH (Holder et al., 2007; Pober, 2007; Veenma et al., 2012b), and detailed analyses of these regions and a limited number of mouse studies have identified over 50 candidate CDH-causative genes (Holder et al., 2007; Russell et al., 2012). A region that contains recurrent CDH-associated CNVs is 8p23.1 (OMIM 22240) (Holder et al., 2007), and within this region, variants in the GATA binding protein 4 (Gata4) transcription factor have recently been shown to strongly correlate with CDH (Arrington et al., 2012; Longoni et al., 2012; Yu et al., 2013). Nevertheless, it is unclear in which tissue Gata4 or any candidate CDH genes are functioning and how these mutations mechanistically cause CDH. Functional analyses of mouse mutants have been severely limited by early embryonic lethality (Kuo et al., 1997; Molkentin et al., 1997), low or variable incidence of CDH (Jay et al., 2007; Mendelsohn et al., 1994; You et al., 2005), and lack of conditional mutants (Ackerman et al., 2005; Coles and Ackerman, 2013). Furthermore, the notable incomplete penetrance and variable expressivity of CDH-associated CNVs and

genetic mutations (Arrington et al., 2012; Longoni et al., 2012; Pober, 2007; Yu et al., 2013) suggest that the genetic architecture underlying CDH may be complex.

Diaphragm Development

The diaphragm has been proposed to develop primarily from two embryonic sources. The somites, segmental mesoderm adjacent to the neural tube, are well documented to be the source of the diaphragm's muscle (Allan and Greer, 1997; Babiuk et al., 2003; Dietrich et al., 1999). Less understood, the pleuroperitoneal folds (PPFs) are two pyramidal-shaped mesodermal structures located between the thoracic (pleural) and abdominal (peritoneal) cavities and hypothesized to be critical for diaphragm development (Babiuk et al., 2003). However, without genetic reagents to label and manipulate the PPFs, it has been unclear what function they play in diaphragm development. A third embryonic structure, the septum transversum, has been suggested to be a source of the central tendon (Babiuk et al., 2003; Greer et al., 2000).

To test the contribution of the PPFs to diaphragm development and structure, we identified the first genetic reagent to label the PPFs. We find that *Prx1Cre* (Logan et al., 2002), originally created to drive Cre-mediated recombination in the limb lateral plate mesoderm, robustly labels the PPFs when crossed with the Cre-responsive reporter $Rosa^{LacZ/+}$ (Soriano, 1999) (Fig. 3.1a, k-m). The PPFs are present at embryonic day (E) 11.5 and expand across the surface of the liver to give rise to cells throughout the diaphragm at E14.5 (Fig. 3.1a-d). PPF cells are distinct from somite-derived muscle progenitors and myoblasts and do not differentiate into myofibers (Fig. 3.1e, k). Instead, the PPFs

give rise to two nonmyogenic tissues. First, in contrast to the previous hypothesis that the central tendon derives from the septum transversum (Babiuk et al., 2003; Greer et al., 2000), the PPFs give rise to the central tendon (Fig. 3.1e). Second, the PPFs give rise to MCT fibroblasts. *Prx1*-derived cells express the MCT-specific marker Tcf4 (Tcf7l2) (Kardon et al., 2003; Mathew et al., 2011) early in the E12.5 PPF (Fig. 3.1l) and in the fully developed diaphragm (Fig. 3.1x) and ultimately reside interstitial to the costal (but not crural) myofibers (Fig. 3.1e). Importantly, the PPF cells also strongly express the CDH-implicated gene Gata4; at E12.5, most *Prx1*-derived cells express Gata4 (Fig. 3.1m), and all Tcf4+ fibroblasts are Gata4+ (Fig. 3.1q-s). Thus, we demonstrate that the PPFs are not simply transient developmental structures, but ultimately give rise to the central tendon and the MCT fibroblasts. Furthermore, the strong expression in PPF cells of CDH-implicated Gata4 suggests that the PPFs may be important in the etiology of CDH.

To trace the contribution of myogenic cells to diaphragm morphogenesis and compare their spatiotemporal relationship to PPF cells, we genetically labeled myogenic cells using $Pax3^{Cre/+};Rosa^{LacZ/+}$ mice (Engleka et al., 2005), in which Cre-mediated recombination in the somite labels all myogenic cells. Myogenic cells migrate from the somite and enter the PPFs by E11.5 (Fig. 3.1f, n), then spread ventrally and dorsally (Fig. 3.1g-i), differentiate into myofibers (Fig. 3.1h-i), and form a completely muscularized diaphragm by E16.5 (Fig. 3.1j). Comparison of muscle and PPF morphogenesis reveals that the PPFs expand ventrally in advance of the muscle (Fig. 3.1d versus i). Strikingly, labeled

myogenic cells do not express either Tcf4 or Gata4, but instead are surrounded by nonmyogenic Tcf4+Gata4+ cells (Fig. 3.1o-p). The myogenic cells' migration into the PPFs and their subsequent expansion within, but behind the leading edge of the PPF cells suggest that the PPFs may guide the morphogenesis of the diaphragmatic muscle.

We next tested the necessity of cell-cell interactions between myogenic and PPF-derived cells for diaphragm development. First, we attempted to test whether muscle morphogenesis depends on the PPFs by genetically ablating PPF cells via *Prx1*$^{CreTg/+}$*;Rosa* $^{DTA/+}$ mice (Wu et al., 2006), in which Prx1+ cells are killed via diphtheria toxin A. Three of 25 neonates had mispatterned or missing muscle (Fig. 3.2a, b), but most developed normally because PPF cells were incompletely ablated in these mice (data not shown). Second, if PPFs are critical for morphogenesis of the diaphragm's muscle, we expected that formation and morphogenesis of the PPFs should occur independently of muscle. To test this, we analyzed *Pax3*$^{SpD/SpD}$ mice (Vogan et al., 1993), which have a muscleless diaphragm. We found that indeed, even in the absence of muscle, PPF cells are present and express both Tcf4 and Gata4 (Fig. 3.1t-v) at E12.5 and subsequently undergo normal morphogenetic expansion (Fig. 3.1w).

The expansion of PPF cells, even in the absence of muscle, suggests that the morphogenetic movement of the PPF cells drives normal diaphragm morphogenesis. While the static images of PPF cells suggest that these cells migrate across the surface of the liver, it is formally possible that this simply reflects the dynamic activation of *Prx1Cre* and not cell movement. To test this,

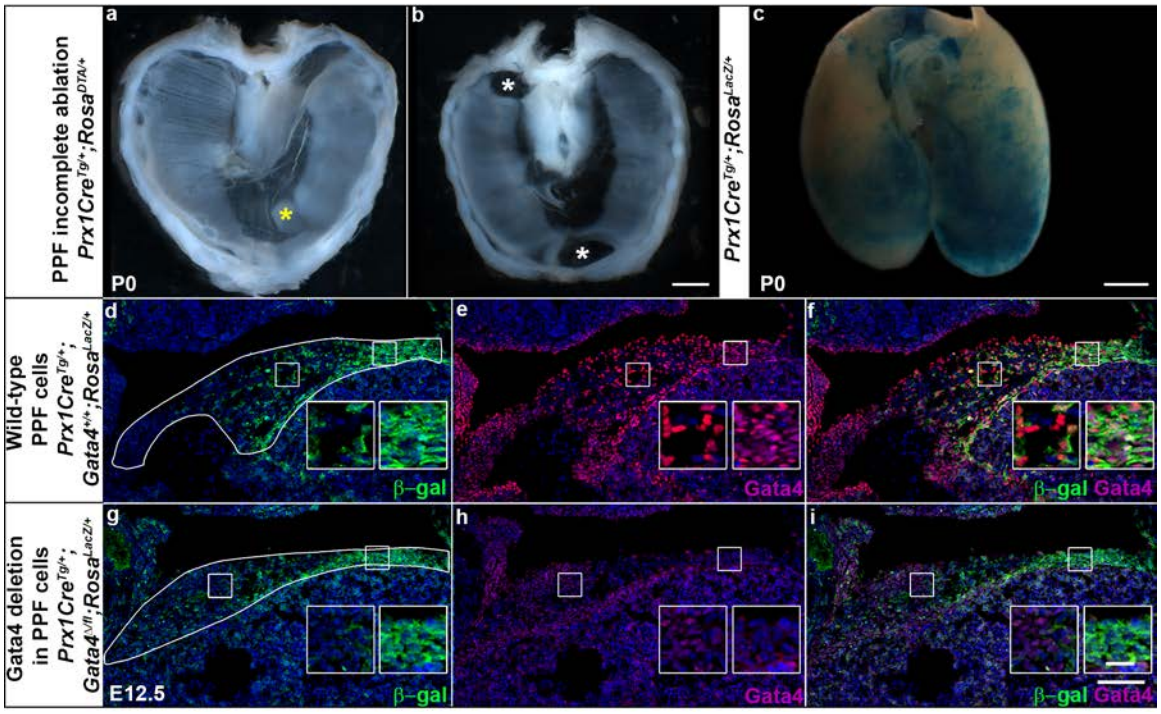

Fig. 3.2: Genetic labeling and ablation with *Prx1Cre* transgene. a-b, Incomplete ablation of PPF cells, via *Prx1Cre*$^{Tg/+}$;*Rosa*$^{DTA/+}$, leads to mispatterned (asterisk in a) or missing (asterisk in b) muscle. c, *Prx1Cre* transgene labels some cells in the lungs. d-I, *Prx1Cre* transgene causes incomplete deletion of *Gata4* in the PPFs at E12.5. Left insets show incomplete *Prx1Cre*-mediated recombination of *LacZ* and *Gata4* and right insets show more complete *Prx1Cre*-mediated recombination of *LacZ* and *Gata4*. c, Whole-mount β-galactosidase staining. d-i, Section immunofluorescence. Scale bar = 1 mm (a-b), 1mm (c), 100 um (d-i), 20 um (insets d-i).

we cultured *ex vivo* E13.5 diaphragms (leaving ribs and underlying liver intact) and imaged genetically labeled PPF cells at the leading edge via 2-photon microscopy. We found that PPF cells dynamically extend and retract filopodia and actively migrate across the liver surface (at the rate of 8 um/hour); although a few cells migrate singly, most appear to migrate collectively (Fig. 3.1c, Supplemental Video 3.1). This collective PPF cell migration likely drives diaphragm morphogenesis.

In summary, we demonstrate for the first time that the PPFs are critical for diaphragm development: PPFs are the target for migratory myogenic cells, PPF cell migration precedes independently of and likely controls muscle morphogenesis, and the PPFs are the source of the diaphragm's central tendon and MCT (Fig. 3.1y). Furthermore, the expression in PPF cells of CDH-implicated Gata4 suggests that PPF defects may cause CDH.

Cellular Origin of CDH

Human genetic studies have shown two noncoding variants and two missense mutations of *Gata4* are correlated with CDH (Arrington et al., 2012; Longoni et al., 2012; Yu et al., 2013), but explicit tests of the function of *Gata4* in development of the diaphragm and CDH have been limited by the early embryonic lethality of *Gata4* null mice (Kuo et al., 1997; Molkentin et al., 1997) and the reported low penetrance of CDH in *Gata4* heterozygous mice (Jay et al., 2007). The strong Gata4 expression in the PPFs (Fig. 3.1 and (Clugston et al., 2008; Longoni et al., 2012)), suggests that Gata4 functions in this tissue. To test this, we generated $Prx1Cre^{Tg/+};Gata4^{\Delta/fl}$ mice (using $Gata4^{fl}$ mice (Watt et al.,

2004)). Strikingly, we found that 100% of mutant mice (n = 33/33) develop multiple hernias throughout the diaphragm (Fig. 3.3b). In humans, the size and location of hernias varies, with 90-95% in the dorsal/posterior lateral diaphragm (Bochdalek hernias), a smaller number in the ventral/anterior region (Morgagni hernias), and even fewer in the central tendon (Ackerman et al., 2012; Pober, 2007). Similarly, in *Prx1Cre*$^{Tg/+}$*;Gata4*$^{\Delta/fl}$ mice, we found that the size and location of hernias varies: most (68%) form in the dorsal/posterior lateral diaphragm and the rest (32%) develop in the ventral/anterior diaphragm (Fig. 3.3b). The finding that hernias occur in only muscle-associated regions and not in the central tendon shows that, although the PPFs give rise to both MCT and central tendon, it is defects in the PPF-derived MCT that cause CDH in these mice. Unlike previous reports (Jay et al., 2007), we never observed diaphragm defects in heterozygous *Gata4*$^{\Delta/+}$ mice (n = 66, Fig. 3.3a). In addition, we tested whether *Gata4* was required in myogenic cells for diaphragm development by analyzing *Pax3*$^{Cre/+}$*;Gata4*$^{\Delta/fl}$ mice. As predicted based on the lack of Gata4 expression in myogenic cells, diaphragms develop normally in these mice (n = 28, Fig. 3.3c). Thus, we definitively demonstrate for the first time that *Gata4* null mutations cause CDH. Moreover, CDH originates with defects in the PPFs and MCT.

Mechanism of Herniation

Our results demonstrate that, with complete penetrance, loss of *Gata4* in PPF-derived MCT fibroblasts results in diaphragmatic hernias, but what mechanistically causes herniation? The most commonly proposed mechanism is that hernias are caused by a morphogenetic defect in the PPFs; this defect

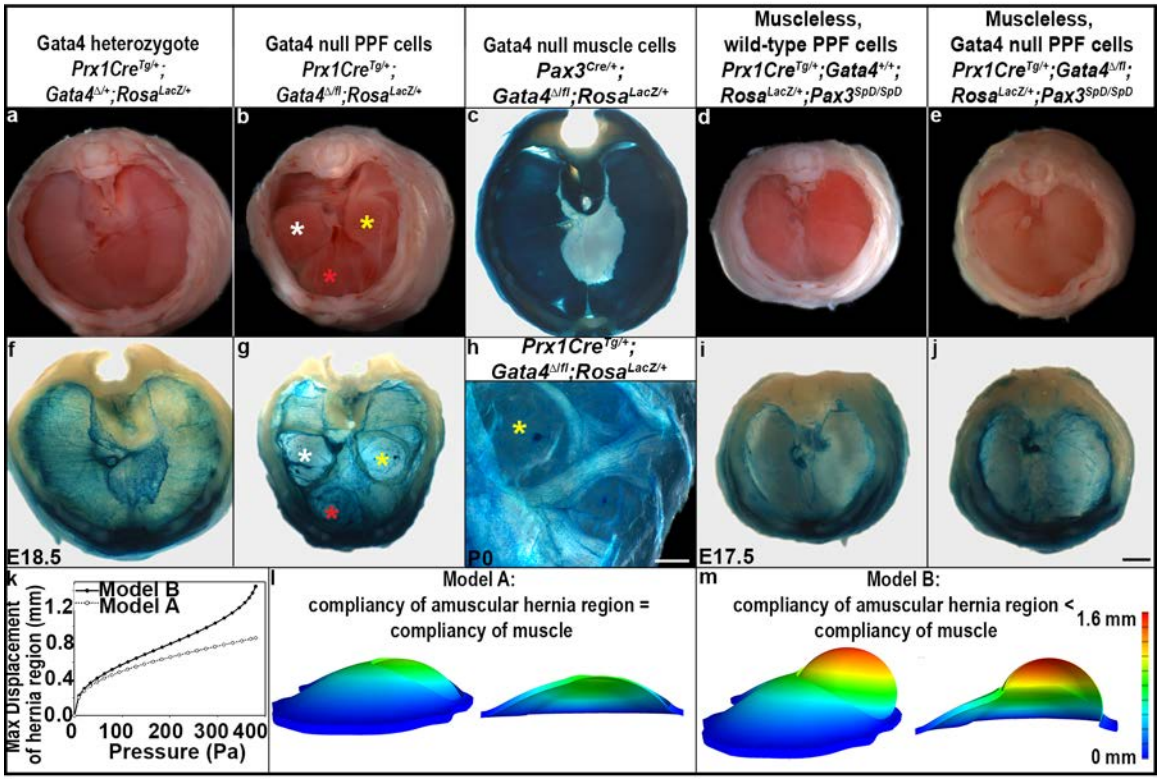

Fig. 3.3: Deletion of Gata4 in the PPFs produces localized amuscular regions that are weaker than juxtaposed muscular regions and results in CDH. a-d, CDH develops in mice with Gata4 null PPF cells (Bochdalek hernias labeled with white and yellow asterisks and Morgagni hernia labeled with red asterisks in b), but not in Gata4 heterozygotes (a), mice with Gata4 null muscle (c), or mice with muscleless diaphragms (d). Loss of muscle in diaphragms with Gata4 null PPF cells rescues herniation (e, j), indicating that juxtaposition of amuscular with muscular regions is required for CDH. g-h, In herniated regions PPF cells are present, but muscle is not (g-h). k-m, Finite element modeling shows that a hernia only develops when the amuscular region is more compliant than the muscular region. c, f-j, Whole-mount β-galactosidase staining. Scale bar = 1 mm (a-g, i-j), 0.5 mm (h).

results in a localized loss of PPF-derived tissue, a consequent absence of muscle normally associated with this tissue, formation of a hole in the diaphragm, and herniation of growing abdominal tissue through this hole (Clugston and Greer, 2007; Greer et al., 2000). If this hypothesis is true, PPF-derived cells, as well as associated muscle, should not be present in herniated regions. To test this, we examined *Prx1Cre*$^{Tg/+}$*;Gata4*$^{\Delta/fl}$*;Rosa*$^{LacZ/+}$ mice, in which PPF-derived *Gata4* null fibroblasts are β-galactosidase+ (β-gal+) and muscle is β-gal-. Surprisingly, we found that β-gal+ fibroblasts are present throughout the herniated regions, and thus these regions are not simply holes in this tissue (Fig. 3.3g-h). Yet, the herniated regions are devoid of muscle, and muscle surrounding the hernias is aberrantly patterned (Fig. 3.3g-h). Therefore, although muscle is absent in herniated regions, hernias are not caused by a morphogenetic failure of the expansion of the PPFs and the formation of holes in the diaphragm.

A second hypothesis for how CDH develops is that PPF-derived connective tissue alone, without muscle, is weak and allows herniation of the abdominal contents through the weaker tissue. We tested this by examining *Prx1Cre*$^{Tg/+}$*;Rosa*$^{LacZ/+}$*;Pax3*$^{SpD/SpD}$ diaphragms, in which muscle is absent but PPF-derived connective tissue is present (Fig. 3.1w). However, in these mice with muscleless diaphragms, hernias never formed ($n > 10$, Fig. 3.3d, i). This demonstrates that PPF-derived connective tissue alone, even in the absence of muscle, is sufficiently strong to prevent herniation of abdominal tissues.

These data suggest two additional hypotheses as to the mechanism underlying herniation. First, the connective tissue produced by *Gata4* null

fibroblasts may be more compliant (deforms more in response to an applied force) than connective tissue made by wild-type fibroblasts and this more compliant tissue allows for herniation of abdominal tissue. Alternatively, it may be that formation of relatively weak regions of amuscular connective tissue juxtaposed to relatively strong muscularized regions allows abdominal contents to herniate through the localized weak regions. To test this, we generated *Prx1Cre*$^{Tg/+}$*;Gata4*$^{\Delta/fl}$*;Pax3*$^{SpD/SpD}$ mice, in which diaphragms are muscleless and the PPF-derived MCT fibroblasts are *Gata4* null. If the first hypothesis is correct, then hernias should form in these mice, while if the second hypothesis is correct, then hernias should be absent. Strikingly, no hernias develop in these mice (n = 3/3), and the diaphragms are indistinguishable from *Pax3*$^{SpD/SpD}$ mice (Fig. 3.3d-e, i-j). Thus, the loss of muscle rescues the herniation phenotype of *Prx1Cre*$^{Tg/+}$*;Gata4*$^{\Delta/fl}$ mice. This demonstrates that the connective tissue produced by *Gata4* null fibroblasts is not inherently more compliant than wild-type connective tissue. Instead, hernias only develop when localized regions of amuscular connective tissue develop in juxtaposition with muscularized regions; through these amuscular regions that are relatively weaker than muscularized regions, abdominal tissue is able to herniate into the pleural cavity.

To gain further insight into the biomechanics governing herniation, we turned to finite element modeling. The amuscular regions of E16.5 diaphragms, prior to overt herniation of abdominal tissue, are 25% of the thickness of the muscular regions. This suggests that the relative weakness of the amuscular compared to the muscular regions could simply be due to its decreased

thickness. Alternatively, the amuscular regions may be both thinner and composed of a more compliant material than muscle. We tested this by creating a finite element model. The geometry of the diaphragm was based on the dimensions of an E16.5 mutant diaphragm, and a uniform physiologically reasonable pressure was applied to the diaphragm to simulate the pressure of the growing liver (see Supplemental Methods). Using the FEBio nonlinear finite element solver (Maas et al., 2012), when the muscle material behavior is represented by an isotropic hyperelastic constitutive equation, based on (Strumpf et al., 1993), and the amuscular region is made significantly more compliant than the muscle, a pressure of 380 Pa induced a bulge in the amuscular region that matched the geometry of the hernias in the experimental mice (Model B, Fig. 3.3k, m, Supplemental Methods and Videos 3.2-3.3). In contrast, when the thinner amuscular region was assigned the same material properties as the muscle, pressures up to 380 Pa were unable to generate a bulge in the amuscular region (Model A, Fig. 3.3 k-l). Together, these results indicate that the weakness in the amuscular regions is due to both its decreased thickness and increased compliance as compared with the muscularized regions.

Lung Hypoplasia Associated with CDH

The neonatal mortality and long-term morbidity of CDH patients is caused by secondary lung hypoplasia, which is thought to arise from the physical impedance of lung growth by the herniated abdominal tissue. However, a “dual-hit hypothesis” has also been proposed (Keijzer et al., 2000), whereby lung hypoplasia results from both physical impedance by herniated tissue and cell-

autonomous effects on lung development by CDH-associated genes. Consistent with this hypothesis, mutations in *Gata4* can cell-autonomously affect lung development (Ackerman et al., 2007). Similar to human patients, *Prx1Cre*$^{Tg/+}$*;Gata4*$^{\Delta/fl}$ mice have low O_2 blood saturation (as measured by pulse oximetry; data not shown) and generally die within the first few days after birth. Defects in the lung lobar structure were found in all mice examined (n = 7, Fig. 3.4 a-b, e-f). Although there is some recombination in the lungs induced by *Prx1Cre* (Fig. 3.2c), the tight correlation of lung defects with hernias in all mice strongly suggests that the herniated tissue physically impedes lung growth. Furthermore, the absence of lung defects in *Prx1Cre*$^{Tg/+}$*;Gata4*$^{\Delta/fl}$*;Pax3*$^{SpD/SpD}$ mice (Fig. 3.4 c-d, g-h), which have some *Prx1Cre*-induced *Gata4* deletion in the lungs but no hernias, argues that herniation precedes and causes lung hypoplasia. Thus, we demonstrate that *Prx1Cre*$^{Tg/+}$*;Gata4*$^{\Delta/fl}$ mice not only develop CDH, but also the lung hypoplasia and neonatal lethality typically accompanying CDH. Furthermore, although we cannot exclude that in some cases CDH-associated genes have cell-autonomous effects on lung development, we show here that impedance of lung growth by herniated tissue is sufficient to induce lung hypoplasia.

Development of Amuscular Regions That Cause CDH

Our experiments demonstrate that hernias form when localized amuscular regions develop within the muscular diaphragm. To determine when hernias and amuscular regions arise, we examined a developmental time series of mutant *Prx1Cre*$^{Tg/+}$*;Gata4*$^{\Delta/fl}$ embryos. Overt herniation of liver through the diaphragm

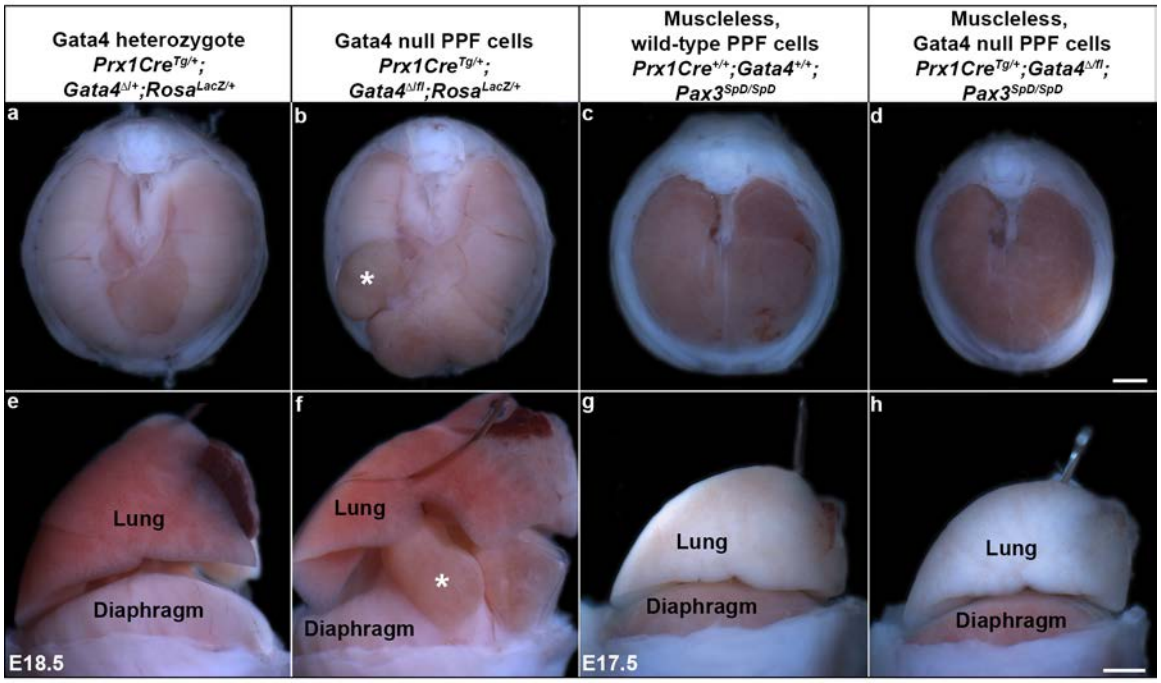

Fig. 3.4: In CDH, physical impedance by herniated tissue causes lung hypoplasia. a-b, e-f, As compared with lungs in mice with a normal diaphragm (a, e), development of lung lobar structure is impeded by liver herniated through a defective diaphragm (b, f). c-d, g-h, Lungs develop normally in mice with a muscleless diaphragm (c, g) or with a muscleless diaphragm with Gata4 null PPF cells (d, h). Hernia indicated with an asterisk. Scale bar = 1mm (a-d), 1 mm (e-h).

first occurs at E16.5 (Fig. 3.5a, e). However, defects in muscle are present by E14.5 (Fig. 3.5b, f), and neither differentiated myofibers nor muscle progenitors are present in amuscular regions (Fig. 3.5c-d, g-h). Even at E12.5, there is a profound defect in the number and localization of muscle progenitors (Fig. 3.5i-p). Thus, hernias are preceded by defects in the muscle progenitors at the earliest stages of diaphragm development.

To determine why fewer muscle progenitors are present in mutant embryos, we examined apoptosis and cell proliferation. At E12.5 in the developing diaphragm of control embryos, few TUNEL+ apoptotic cells are present (Fig. 3.5 i, k-s), while nearly all muscle progenitors are actively proliferating (Fig. 3.6 a, c-k). In contrast, in mutant embryos, there is a marked increase in apoptotic cells, many of which are present in regions abnormally devoid of muscle (Fig. 3.5j, n-v, Supplemental Videos 3.4-3.5). In addition, there is a profound decrease in EdU+ proliferative cells (Fig. 3.6b, f-n, Supplemental Videos 6-7). In the heart, Gata4 directly activates the transcription of cell cycle regulators Cyclin D2 and Cdk4 (Rojas et al., 2008; Yamak et al., 2012). Similarly, we find that loss of *Gata4* leads to decreased levels of Cyclin D2 and Cdk4 in PPF cells (Fig. 3.7a-l). In culture, isolated *Gata4* null PPF fibroblasts proliferate at less than half the level of wild-type fibroblasts (Fig. 3.6i). Moreover, when diaphragm myogenic cells are cultured with PPF cells, *Gata4* null fibroblasts (as compared with wild-type fibroblasts) fail to support the growth of myogenic cells (Fig. 3.6j-k). Thus, in mutant embryos, *Gata4* null fibroblasts cause both an increase in apoptosis and a decrease in proliferation of muscle progenitors.

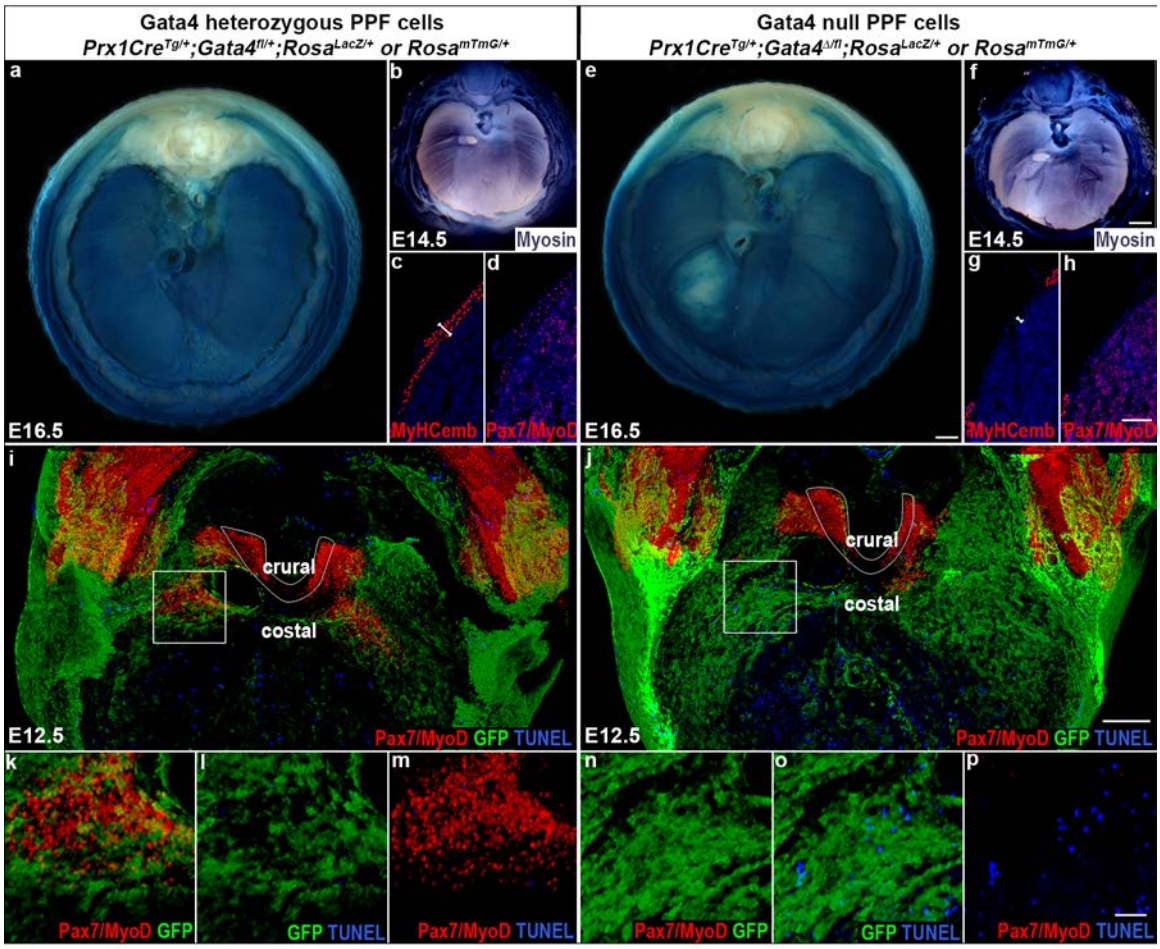

Fig. 3.5: CDH results from early defects in the localization of muscle progenitors and increased apoptosis. a, e, Overt liver herniation through diaphragms with Gata4 null PPF cells is first apparent at E16.5. b-d, f-h, At E14.5 differentiating myofibers are aberrant (b, f) and myofibers (c, g) and Pax7+/MyoD+ muscle progenitors (d, h) are absent in localized regions. i-p, At E12.5 there is a marked increase in apoptotic cells (i-j, l-m, o-p), particularly in the region (box j, magnified in n-p) destined to always give rise to hernias (n=33/33). Also, costal muscle progenitors are absent from regions with Gata4 null PPF cells in mutant diaphragms (i-j, k, n). a, b, Whole-mount β-galactosidase staining. b, f. Whole-mount myosin-alkaline phosphatase staining. c-d, g-h, Section immunofluorescence. i-p, Whole-mount immunofluorescence. Scale bar = 500 um (a, e), 500 um (b, f), 100um (c-d, g-h), 200 um (i-j), 50 um (k-p). White bars in show thickness of muscle in control (c) or amuscular tissue in mutant (g) diaphragms.

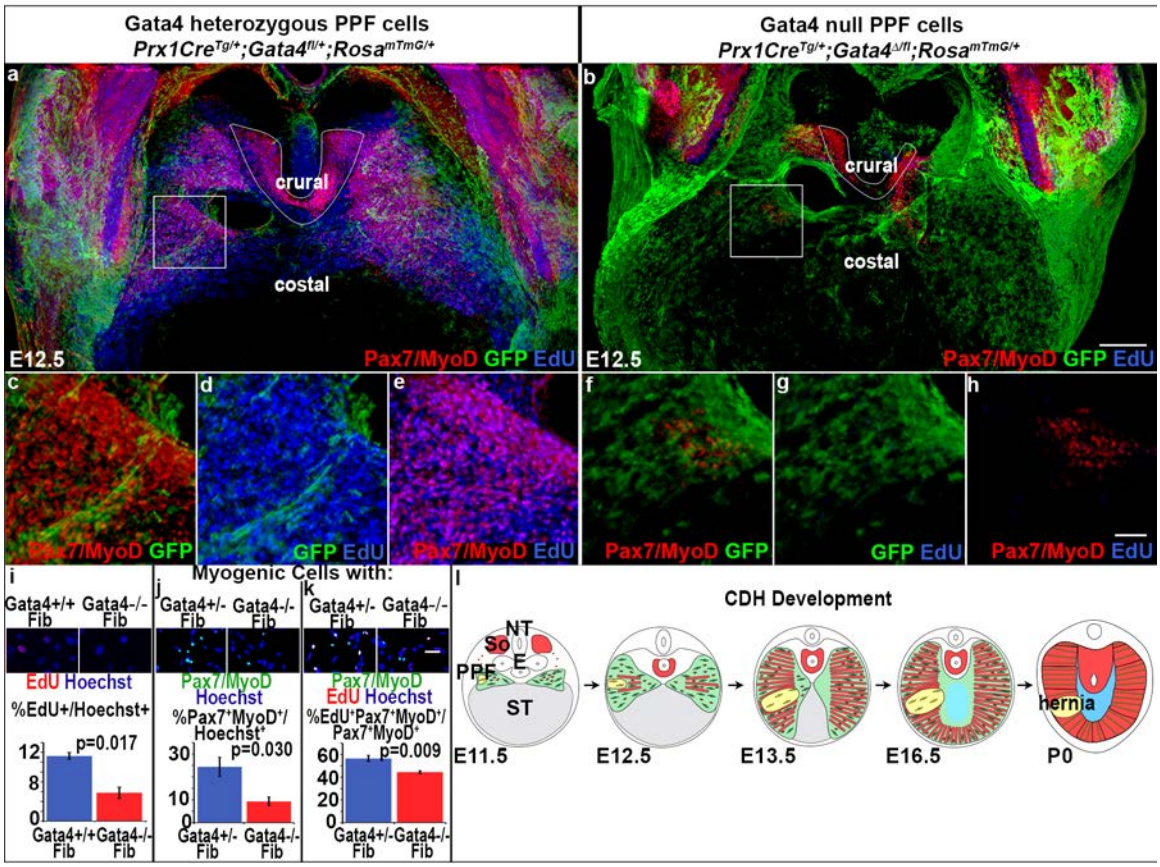

Fig. 3.6: Early defects in proliferation and localization of muscle progenitors lead to CDH. a-h, At E12.5 there is a marked decrease in proliferating cells, a decrease in costal muscle progenitors, and muscle progenitors are largely absent from regions with Gata4 null PPF cells in mutant diaphragms. In the boxed region (b and magnified in f-h) destined to give rise to hernias (n=33/33), the few myogenic cells are strikingly EdU-. i, When cultured Gata4 null, as compared to wild-type, PPF fibroblasts proliferate less. j-k, When cultured for 48 hours with Gata4 null PPF fibroblasts, as compared with Gata4 heterozygous fibroblasts, there are decreased numbers (j) and proliferation (k) of Pax7+/MyoD+ diaphragmatic myogenic cells. l, Model of CDH development. a-h, Whole-mount immunofluorescence. i-k, Immunofluorescence of cultured cells. Scale bar = 200 um (a,b), 50um (c-h), 50 um (i-k). PPF, pleuroperitoneal fold; NT, neural tube; E, esophagus; So, somite; ST, septum transversum.

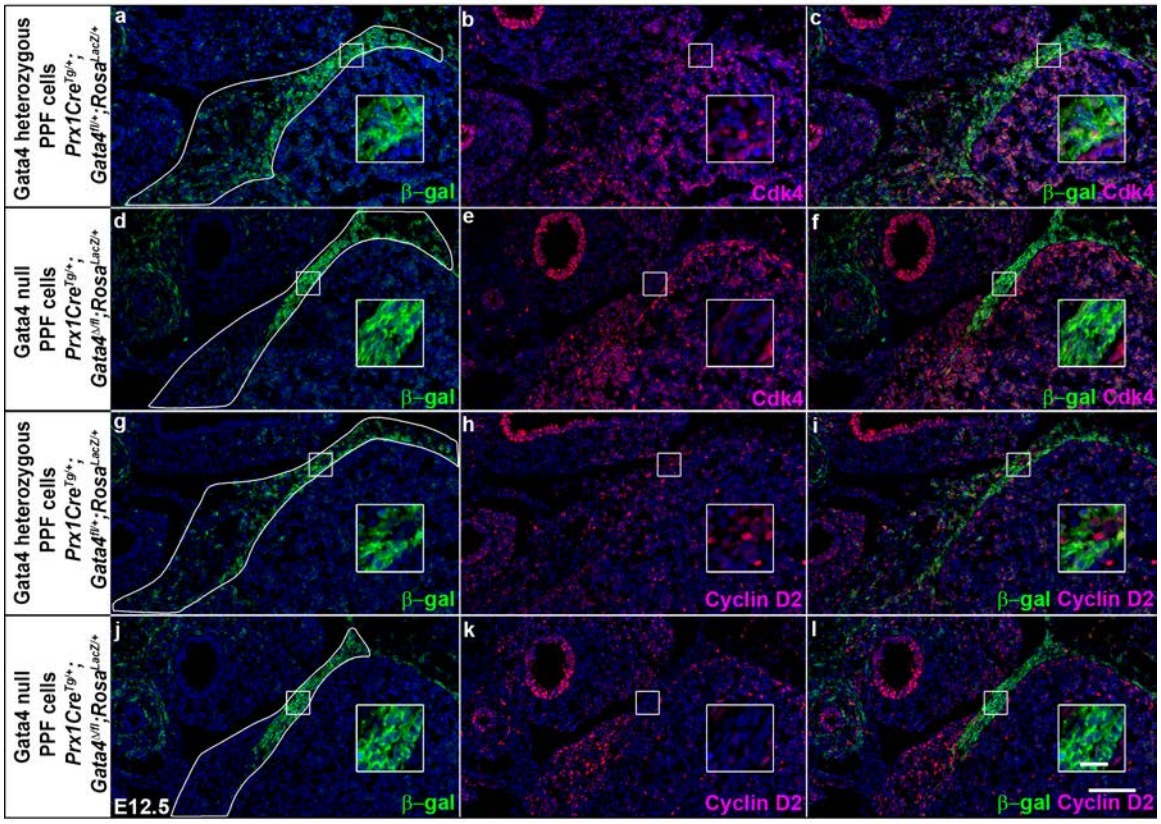

Fig. 3.7: Cell cycle proteins are down-regulated in PPF cells with deletion of Gata4. a-l, Cdk4 (a-f) and Cyclin D2 (g-l) are down-regulated in Gata4 null PPF cells. E12.5 Section immunofluorescence. Scale bar = 100um (a-l), 20um (insets a-l).

In mutant diaphragms, the number of muscle progenitors is greatly reduced by increased cell death and decreased proliferation, but how do localized amuscular regions develop? In control *Prx1Cre*$^{Tg/+}$;*Gata*$^{fl/+}$;*Rosa*$^{mTmG/+}$ embryos, muscle progenitors migrate into and develop completely surrounded by the GFP+ PPF cells (Fig. 3.4i, 3.5a). As the PPF cells expand, they carry with them the proliferating and differentiating myogenic cells. In contrast, in *Prx1Cre*$^{Tg/+}$;*Gata4*$^{\Delta/fl}$;*Rosa*$^{mTmG/+}$ mutants, at E12.5, myogenic cells are largely excluded from GFP+Gata4- regions (Fig. 3.4 j, 3.5 b). These localized amuscular regions result from the mosaic deletion of one *Gata4* allele by the *Prx1Cre* transgene, which incompletely recombines in PPF cells at E11.5-12.5 (Fig. 3.2d-i; the other *Gata4* allele is deleted in the germline); muscle is excluded from the PPF regions where *Gata4* has been deleted during this early time window. Only during this early time do myogenic cells appear to be sensitive to *Gata4*, as deletion of *Gata4* via *Tcf4*^{Cre} (Mathew et al., 2011), which causes recombination in PPF fibroblasts primarily after E12.5, does not result in hernias (n=16/16, data not shown). Altogether these data indicate that early mosaic deletion of *Gata4* in PPF cells leads to increased apoptosis and decreased proliferation of muscle progenitors and the development of localized amuscular regions, which ultimately allow herniation.

Discussion

Our study establishes that the PPFs and MCT fibroblasts, although previously unappreciated, are critical for the development of the diaphragm and CDH. PPF cells not only give rise to the diaphragm's MCT and the central

tendon, but the PPFs' MCT fibroblasts control the morphogenesis of the diaphragm's muscle. Our finding that the MCT regulates muscle development has precedence, as MCT fibroblasts regulate the pattern of limb muscles (Kardon et al., 2003) and the fiber-type of limb and diaphragm muscles (Mathew et al., 2011). Completely novel is our finding that the active, apparently collective, cell migration of MCT fibroblasts across the liver's surface controls the expansion of muscle progenitors and overall diaphragm morphogenesis.

We also definitively demonstrate for the first time that defects in the MCT fibroblast component of the PPFs are the cellular source of CDH and that *Gata4* null mutations in these cells cause CDH. Surprisingly, we show that hernias do not result from defects in PPF cell migration and the formation of holes in the diaphragm. Instead, PPF deletion of *Gata4* leads to the development of localized amuscular regions through which the growing liver and intestines herniate. In humans, such hernias with connective tissue surrounding the herniated tissue are classified as "sac hernias" (Pober, 2007), and we hypothesize that most hernias are covered by connective tissue early in development. Mechanistically, we show that CDH arises when weaker (thinner and more compliant) amuscular regions develop within the stronger (thicker and stiffer) muscularized diaphragm and allow herniation. Development of the amuscular regions in mutants results from an overall marked decrease in cell proliferation and increase in cell death and, most notably, the localized exclusion of myogenic cells from regions with early PPF deletion of *Gata4*. The early mosaic deletion of *Gata4* in PPF cells, in our mice via the *Prx1Cre* transgene, is essential for development of hernias and

strongly suggests that in humans, somatic mosaic mutations are critical for the etiology of CDH.

The role of mosaicism in human disease has received increasing attention in recent years (Biesecker and Spinner, 2013). A potential role for somatic mosaicism in CDH has previously been suggested by the discordant occurrence of CDH in monozygotic twins (Veenma et al., 2012a) and the finding of genetic mosaicism in CDH patients (Kantarci et al., 2010; Veenma et al., 2010). However, it was unknown when, in which tissue, and how somatic mosaicism contributes to the etiology of CDH. Here we show that early embryonic somatic mosaicism in Gata4 expression in the diaphragm's MCT causes locally weak amuscular regions and leads to CDH. Our finding that CDH arises from somatic mosaic mutations explains the notable incomplete penetrance and variable expressivity of many CDH-associated CNVs and genetic mutations, particularly *Gata4* (Arrington et al., 2012; Longoni et al., 2012; Wat et al., 2009; Yu et al., 2013). Our data suggest that loss of one *Gata4* allele confers susceptibility, but is not sufficient to cause CDH. Development of CDH requires somatic loss of the second allele in a subset of the MCT fibroblasts early during diaphragm morphogenesis. When and where this second allele is deleted determines the size and location of the amuscular region and hernia. In humans, somatic loss of the 8p23.1 region (which contains *Gata4*) is relatively common, as this region is highly susceptible to deletions (Bosch et al., 2009; Giglio et al., 2001; Longoni et al., 2012). Alternatively, the second somatic mosaic, CDH-causative "hit" may not be loss of the second *Gata4* allele, but another CDH-associated gene; multiple

CDH-associated genes are strongly expressed in the PPF cells, including *Zfpm2* (Ackerman et al., 2005) and *Nr2f2* (You et al., 2005), which are known to genetically interact with *Gata4*. Thus, the genetic architecture underlying CDH is likely to be complex. By combining developmental, genetic, and bioengineering studies, we elucidate for the first time that early somatic mosaic mutations in connective tissue have profound biomechanical consequences and lead to CDH. Our finding that early somatic mosaic mutations are critical will have an important impact into future research on CDH genetic susceptibility and approaches to therapeutic interventions.

Methods Summary

Embryos of various genotypes (detailed in text) at different days of development were harvested from timed pregnant dams. Pregnant females were given 40 µl of 1mM EdU by intraperitoneal injection 1 h before harvest. Section and whole-mount immunofluorescence and β-galactosidase staining were carried out as described in the Supplementary Methods and imaged via confocal microscopy. Primary PPF fibroblasts and myogenic cells were isolated from E15.5 diaphragms of various genotypes and fibroblasts or myoblasts with fibroblasts were cultured, EdU labeled, and processed for immunofluorescence (see Supplemental Methods). For diaphragm explants, E12.5 *Prx1Cre*$^{Tg/+}$*;Rosa*$^{mTmG/+}$ embryos were harvested, and diaphragms isolated, cultured 2-24 h, and imaged via 2 photon microscopy (see Supplementary Methods). All confocal 3-dimensional and 2 photon 4-dimensional data sets were rendered and visualized using FluoRender (Wan et al., 2009). Finite Element

Modeling was based on an E16.5 *Prx1Cre*$^{Tg/+}$;*Gata4*$^{\Delta/fl}$ herniated mouse diaphragm using FEBio (Maas et al., 2012) (see Supplemental Methods).

Supplemental Methods

Mice

All mice have been previously published. We used *Prx1*^{Cre} (Logan et al., 2002), *Pax3*^{Cre} (Engleka et al., 2005), and *HPRT*^{Cre} (Tang et al., 2002) Cre alleles; *Rosa*^{LacZ} (Soriano, 1999) and *Rosa*^{mTmG} (Muzumdar et al., 2007) Cre-responsive reporter alleles; and *Pax3*^{SpD} (Vogan et al., 1993) and *Gata4*^{fl} (Watt et al., 2004) mutant alleles. *Gata4*$^{del/+}$ mice were generated by breeding *Gata4*^{fl} mice to *HPRT*^{Cre} mice.

Immunofluorescence, β-Galactosidase Staining, and Microscopy

For section immunofluorescence, embryos were fixed, embedded, cryosectioned, and immunostained as described previously (Mathew et al., 2011). EdU (Life Technologies) was detected per manufacturer's directions. For whole-mount immunofluorescence, embryos were fixed 24 h in 4% PFA, dissected, incubated 24 h in Dent's bleach (1:2 30% H_2O_2:Dent's fix), and stored in Dent's fix (1:4 DMSO:MeOH) for at least 5 days. Embryos were washed in PBS, blocked 1 h in 5% serum + 20% DMSO, incubated in primary antibody at room temperature 48 h, washed in PBS, incubated in secondary antibody 48 h, washed in PBS, and subjected to EdU reaction 1 h. Diaphragms were cleared in BABB (33% Benzyl Alcohol, 66% Benzyl Benzoate). AP-conjugated mouse IgG1

α My32 was incubated for 48 h and detected with 250 µg/ml NBT and 125 µg/ml BCIP (Sigma) in NTMT.

Cultured cells were fixed for 20 minutes in 4% PFA, blocked in 5% serum in PBS with 0.1% Triton X-100 for 1 h, incubated in primary antibody at 4^{o}C overnight, washed with PBS, and incubated for 2 h in secondary antibody. Following antibody staining, EdU was detected as above. Cells were incubated in 2 mg/ml Hoechst for 5 minutes and mounted with Fluoromount-G.

Antibodies are listed in Table 3.1.

For whole-mount β-galactosidase staining, embryos were fixed 1.5 h in 4% PFA + 2mM $MgCl_2$. Diaphragms were dissected, washed in PBS and rinse buffer (100mM Sodium Phosphate, 2mM $MgCl_2$, 0.01% Na Deoxycholate, 0.02% Ipegal), and stained 16 h at 37^{o}C in X-gal staining solution (5mM potassium ferricyanide, 5mM potassium ferrocyanide, 1mg/ml Xgal).

Fluorescent images were taken on a Nikon A1 confocal. Optical stacks of whole-mounts were rendered using FluoRender (Wan et al., 2009). β-galactosidase stained embryos were imaged with a Qimaging camera.

Cell Culture

Fibroblasts were isolated from E15.5 *Gata4*$^{fl/fl}$*;Rosa*$^{mTmG/+}$ or *Rosa*$^{mTmG/+}$ diaphragms, cultured, and expanded 3 weeks. 4 uM TAT-Cre (M Hockin, MR Capecchi) was added to induce recombination. EdU was given 4 h prior to fixation and immunofluorescence. For co-cultures, fibroblasts and myoblasts were isolated from E15.5 *Prx1Cre*$^{Tg/+}$*;Gata4*$^{del/fl}$*;Rosa*$^{mTmG/+}$ or

Prx1Cre$^{+/+}$*;Gata4*$^{del/fl}$*;Rosa*$^{mTmG/+}$ diaphragms, cultured 2 days, and given EdU 1 h prior to fixation and immunofluorescence.

Diaphragm Explants

E12.5 *Prx1Cre*$^{Tg/+}$*;Rosa*$^{mTmG/+}$ mice were harvested and trimmed to only include the diaphragm with attached ribs, hind limbs, and liver. Explanted diaphragms were cultured 2-24 h in 100% horse serum, at 37^0C, in the presence of 5% CO_2. Explants were imaged on a Bruker (Prairie) 2 Photon microscope, and 4-dimensional datasets were rendered and visualized using FluoRender (Wan et al., 2009).

Modeling

The finite element model of hernia development was based on an E16.5 *Prx1Cre*$^{Tg/+}$*;Gata4*$^{\Delta/fl}$ herniated mouse diaphragm. The geometry of the diaphragm was based on surfaces created from segmenting microscopy images. Surfaces were discretized with quadratic-tetrahedral elements, and adequacy of the spatial discretization was confirmed with a mesh convergence study. Meshes were created with ANSA software (BETA CAE Systems USA) and analyzed with FEBio (Maas et al., 2012). For the simulations, the rib cage surrounding the diaphragm was rigidly constrained, while a uniform pressure of 380 Pa was applied to simulate the pressure applied by the liver. The pressure was chosen so that the bulge height of the muscle region predicted by the model matched the height measured in the experiment. The muscle was represented

with an isotropic hyperelastic Veronda-Westmann constitutive equation (Veronda and Westmann, 1970) with coefficients (C_1 = 2.1 MPa, C_2 = 0.1, K = 10) based on published data (Maas et al., 2012). The connective tissue of the hernia was represented with an isotropic hyperelastic Mooney-Rivlin constitutive equation (Mooney, 1940) using either the coefficients C_1 = 0.003 MPa, K = 1, or C_1 = 0.01 MPa, K = 10.

Table 3.1
Antibodies

Antibody	Type	Source	Product Number	Working Concentration	Antigen Retrieval	Secondary and Amplification
Pax7	Mouse IgG1	DSHB	PAX7	2.4µg/ml	Yes ≥ E14.5 No ≤E14.5	Dylight 594 or 649 goat α mouse IgG1
MyoD	Mouse IgG1	Santa Cruz Biotechnology	Sc-32758 (5.8A)	4µg/ml	Yes ≥ E14.5 No ≤E14.5	Dylight 594 or 649 goat α mouse IgG1
MyHCemb	Mouse IgG1	DSHB	F1.652	3µg/ml	Yes	Dylight 594 or 649 goat α mouse IgG1
MyHC (fast, neonatal) My32	Mouse IgG1	Sigma	M4276	10 µg/ml	No	Dylight 594 goat α mouse IgG1
Tcf4	Rabbit monoclonal	Cell Signaling	2569	0.7 µg/ml	No	Biotin goat or donkey α rabbit, Dylight 488 Conjugated Streptavidin
Gata4	Goat polyclonal	Santa Cruz	Sc-1237	2 µg/ml	No	Dylight 594 Donkey α Goat
β-gal	Chick polyclonal	Immunology Consultants Laboratory	CGAL-45A-Z	2 µg/ml	No	Dylight 488 goat or donkey α chick
GFP	Chick polyclonal	Aves Labs	GFP-1020	20µg/ml	no	Dylight 488 goat or donkey α chick
MyHC (fast, neonatal)-Alkaline Phosphatase My32-AP	Mouse IgG1	Sigma	A4335	12 µg/ml	No	-
Cdk4	Rabbit polyclonal	Santa Cruz	Sc-260	2 µg/ml	No	Dylight 594 goat α Rabbit
Cyclin D2	Rabbit polyclonal	Santa Cruz	Sc-593	2 µg/ml	No	Dylight 594 goat α Rabbit

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CHAPTER 4

DELETION OF *PORCN* IN THE PLEUROPERITONEAL FOLDS PRODUCES VENTRAL CONGENITAL DIAPHRAGMATIC HERNIAS

Abstract

Congenital diaphragmatic hernias (CDH) are frequently described in patients with focal dermal hypoplasia (FDH). FDH is caused by mutations in the *PORCN* gene, which is required for secretion of both canonical and non-canonical Wnt signals. This suggests that Wnt signaling may be critical during diaphragm development. To test the role of Wnt signaling in mouse diaphragm development, *Porcn* was specifically deleted in the pleuroperitoneal folds (PPF). Inhibition of Wnt signals from the PPF induced ventral midline hernias with 100% penetrance. Wnt signals are required specifically from the PPF, as deletion of *Porcn* in myogenic cells did not generate hernias. This indicates that Wnt signaling from the PPF is critical for diaphragm development. Inhibition of the canonical Wnt/β-catenin pathway in myogenic cells and the PPF did not recapitulate the phenotypes observed when *Porcn* is deleted in the PPF, indicating that the Wnt signals secreted from the folds are likely noncanonical.

This demonstrates that *Porcn* is specifically required in the PPF and that Wnt signals, likely noncanonical, are critical for diaphragm development.

Introduction

Patients with Focal Dermal Hypoplasia (FDH, also known as Goltz syndrome) present with a wide spectrum of phenotypes that can include congenital diaphragmatic hernias (CDH). FDH is a congenital disorder with variable severity characterized by regions with a thin or absent dermis, herniation of adipose tissue through these regions, limb defects such as syndactyly, and sternal defects. Patients also commonly present with congenital diaphragmatic hernias, which can be located anteriorly (ventrally) (Dias et al., 2010; Han et al., 2000; Kunze et al., 1979; Maas et al., 2009; Smigiel et al., 2011). FDH is inherited in an X-linked dominant pattern, and recent studies of patients have identified mutations in *PORCN* on the X chromosome as the cause of FDH (Grzeschik et al., 2007; Maas et al., 2009; Wang et al., 2007). *PORCN* is a key mediator of the Wnt pathway and nearly all patients (90%) are heterozygous females. Male patients are predicted to have mosaic somatic mutations in *PORCN,* since hemizygous mutations would be embryonically lethal (Goltz, 1992). The severity of phenotypes in females likely varies due to the size of mutant patches formed by stochastic X chromosome inactivation of the mutated allele, creating functional mosaicism (Temple et al., 1990). This random inactivation may also explain why hernias are not seen in all patients, as herniation would require large mutant patches within the diaphragm.

Recently, a conditional mouse allele of *Porcn* has provided further insight into the developmental functions of *Porcn* (Barrott et al., 2011). Breeding this conditional allele, which contains flanking loxP sites around exons 2-3, to *Sox2-Cre* mice generates heterozygous *Porcn*$^{\Delta/+}$ females. Random X-inactivation produces mosaic patches of mutant cells. These heterozygous females recapitulate the phenotypes observed in human FDH patients, with abnormal limb development and missing digits, regions of thin dermis, and sternal or body wall closure defects. Using the conditional allele, *Porcn*^{fl}*,* the *Porcn* gene can be deleted in specific tissues to investigate the role of *Porcn* in diaphragm development.

Mutations in *Porcn* cause disruption of Wnt signaling. Porcn is an endoplasmic reticulum-localized acyltransferase enzyme required for secretion of all Wnts. Posttranslational modification by Porcn is required for secretion of both canonical (β-catenin dependent) and noncanonical (β-catenin independent) Wnts (Barrott et al., 2011). In the canonical Wnt/β-catenin pathway, Wnt signaling prevents phosphorylation and degradation of β-catenin, allowing accumulation of β-catenin protein. β-catenin can then translocate to the nucleus, bind to Tcf/Lef proteins, and activate transcription of downstream targets (Niehrs, 2012). In addition to its function in Wnt signaling, β-catenin is also involved in cell-cell adhesion through interactions with cadherins (Hierholzer and Kemler, 2010; Valenta et al., 2011). To manipulate and investigate the role of Wnt signaling in development, *Porcn* can be deleted to prevent sending of Wnt signals from cells and β-*catenin* can be deleted to prevent activation of canonical Wnt signals.

Using these tools, it is possible to determine the role of *Porcn* and Wnt signaling in the development of the diaphragm and development of CDH.

As described in Chapter 3, diaphragm development requires the coordinated morphogenesis of somitically-derived muscle and pleuroperitoneal fold (PPF)-derived connective tissue. Muscle precursors migrate from the somite to the PPF. The PPF spread ventrally and dorsally, giving rise to muscle connective tissue fibroblasts and to the cells in the central tendon. Muscle precursors proliferate and spread both ventrally and dorsally from the folds. By embryonic day (E)16.5, the muscle from the left and right halves of the diaphragm has finished spreading and connected ventrally. In Chapter 3, I describe how deletion of *Gata4* in the PPF causes non-cell autonomous defects in the muscle. This suggests that signals from the PPF-derived connective tissue fibroblasts to the muscle are critical for diaphragm development.

Evidence of CDH in FDH patients indicates that *PORCN* and Wnt signaling are critical for proper diaphragm development, although it is unclear what tissues are required to secrete Wnt signals, whether these signals are canonical or noncanonical, and what tissues need to receive these signals. In this Chapter, I demonstrate that Wnt signals are required from the PPF, likely the PPF-derived connective tissue, and not from muscle cells during development. Surprisingly, although muscle precursors receive canonical Wnt signals during diaphragm development, inhibition of canonical Wnt/β-catenin signaling in muscle precursors does not reproduce the defects seen when *Porcn* is deleted in

the folds. This suggests that *Porcn* is required to secrete noncanonical Wnt signals during diaphragm development.

Results

Because studies of FDH patients with *PORCN* mutations indicate a role for Wnt signaling in diaphragm development, I first tested what cell type secretes the Wnt signals for diaphragm development. To do this, I generated *Prx1Cre*$^{Tg/+}$*;Porcn*$^{fl/Y}$ male mice, which delete *Porcn* specifically in the PPF cells. *Porcn*$^{fl/Y}$ mice contain loxP sites flanking exons 2 and 3, and recombination in hemizygous males deletes *Porcn* from the PPF cells, preventing sending of Wnt signals from these cells. Diaphragms of postnatal day zero (P0) *Prx1Cre*$^{Tg/+}$*;Porcn*$^{fl/Y}$ mice all had ventral hernias (n=13) (Fig. 4.1b), similar to reported human cases. These hernias were all located at the ventral midline where myogenic cells migrating from the left and right halves of the diaphragm connect. In all mice examined, the muscle failed to join appropriately at the midline and liver tissue had herniated through this defect. This indicates a critical role for secretion of Wnt signals from the PPF-derived connective tissue in the completion of diaphragm muscle migration.

Because it is still formally possible that Wnt signals are also required from the muscle during diaphragm development, I generated *Pax7*$^{iCre/+}$*;Porcn*$^{fl/Y}$ mice, in which *Porcn* is deleted specifically in muscle progenitors and all derived myogenic cells using *Pax7*^{iCre}. The diaphragms of these mice appear completely normal (n=6), with muscle correctly joined ventrally and no herniation (Fig.

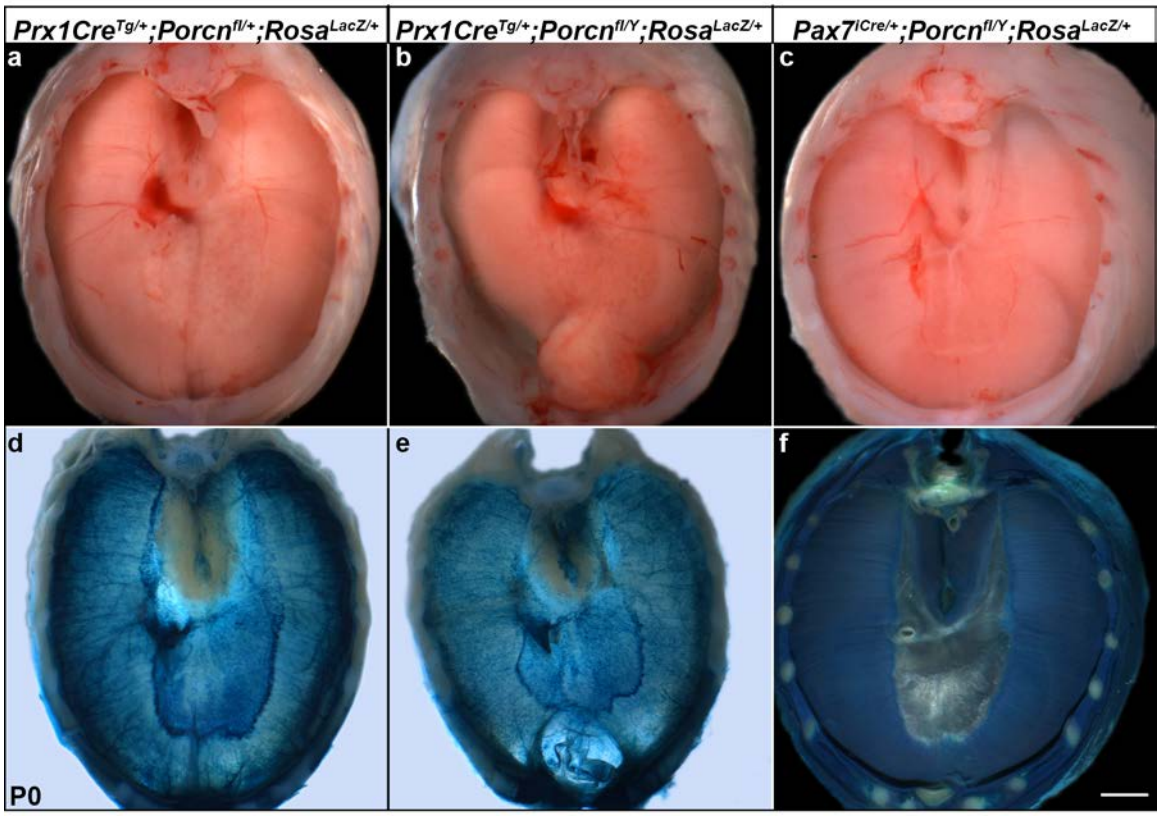

Fig. 4.1. Deletion of Porcn specifically in the pleuroperitoneal folds induces ventral congenital diaphragmatic hernias. a-b, *Prx1Cre*$^{Tg/+}$*;Porcn*$^{fl/Y}$ mice (b), but not *Prx1Cre*$^{Tg/+}$*;Porcn*$^{fl/+}$ mice (a) develop anterior diaphragmatic hernias. c and f, *Pax7*$^{iCre/+}$*;Porcn*$^{fl/Y}$ mice do not develop hernias. d-e, In control mice (corresponding to a) PPF-derived, β-gal+ cells expand throughout the developed diaphragm (d) and in mutant mice (corresponding to b) βgal+ cells expand throughout the diaphragm and over herniated regions (e). Scale bar = 1 mm.

4.1c,f) Thus, Wnt signals from muscle are not required for diaphragm development.

There are two possible explanations for the midline defects when *Porcn* is deleted in the PPF. It is possible that the midline defect in *Prx1Cre*$^{Tg/+}$*;Porcn*$^{fl/Y}$ mice occurs as a result of either the PPF failing to expand completely, thus causing defective muscle spreading, or that the PPF-derived connective tissue still spreads across the diaphragm, but fails to signal appropriately to the muscle to spread as well. To determine if the PPF-derived connective tissue still spreads across the diaphragm in these mice, *Prx1Cre*$^{Tg/+}$*;Porcn*$^{fl/Y}$ mice were bred to the *Rosa*^{LacZ} reporter line to visualize the recombined connective tissue fibroblasts. In *Prx1Cre*$^{Tg/+}$*;Porcn*$^{fl/Y}$*;Rosa*$^{LacZ/+}$ mice, PPF-derived cells delete *Porcn* and permanently express *LacZ*. When examined, labeled cells still spread across the herniated region (Fig. 4.1e). Thus, the loss of Wnt signals from the PPF-derived tissue does not prevent the PPF from spreading completely to form the diaphragm. Instead, this indicates that signals are required from the PPF-derived connective tissue to the muscle for complete expansion of muscle across the diaphragm.

Deletion of *Porcn* indicated that the sending of Wnt signals was critical during diaphragm development, but which cells received Wnt signals was still unknown. To address this question, I used mice that contained a reporter of canonical Wnt/β-catenin signaling, *BATgal*, to determine what cell types early in development receive canonical Wnt signals. *BATgal* mice contain seven Tcf/Lef binding sites driving expression of a nuclear *LacZ* reporter. Following active

canonical Wnt signaling, β-catenin binds to the Tcf/Lef sites and initiates expression of *LacZ*. When examined in whole-mount for β-galactosidase expression at E12.5, canonical signals were received in a subset of cells that matched the location of myogenic precursors (Fig. 4.2a). By E14.5, β-gal+ nuclei appeared at the leading edge of muscle in the developing diaphragm in a linear manner, suggesting these cells are myonuclei of muscle fibers. To determine what cell type received Wnt/β-catenin signaling, I examined the PPF in E12.5 *BATgal* mice in section. In *BATgal*$^{Tg/+}$ mice, none of the β-gal+ cells overlapped with Gata4+ PPF cells (Fig. 4.2c-e). However, β-gal+ cells clearly overlapped with Pax7+/MyoD+ myogenic precursors (Fig. 4.2 f-h), suggesting that muscle precursors receive Wnt/β-catenin signaling.

Because Wnt/β-catenin signaling is active in diaphragm muscle precursors, I next tested the requirement for reception of Wnt/βcatenin signaling by muscle in the developing diaphragm. To do this, β-catenin was deleted specifically in the muscle cells by generating *Pax7*$^{iCre/+}$*;β-catenin*$^{\Delta 2\text{-}6/fl2\text{-}6}$*;Rosa*$^{LacZ/+}$ mice. Recombination of this β-catenin allele deletes exons 2-6 and creates a functional null in the background of an animal heterozygous for β-catenin, thus inhibiting canonical Wnt signaling through β-catenin (Brault et al., 2001). If canonical Wnt signals from the PPF-derived connective tissue to the muscle were required for diaphragm development, hernias should form in *Pax7*$^{iCre/+}$*;β-catenin*$^{\Delta 2\text{-}6/fl2\text{-}6}$ mice similar to those seen in *Prx1Cre*$^{Tg/+}$*;Porcn*$^{fl/Y}$ mice. However, deleting β-catenin in the myogenic progenitors does not produce similar hernias

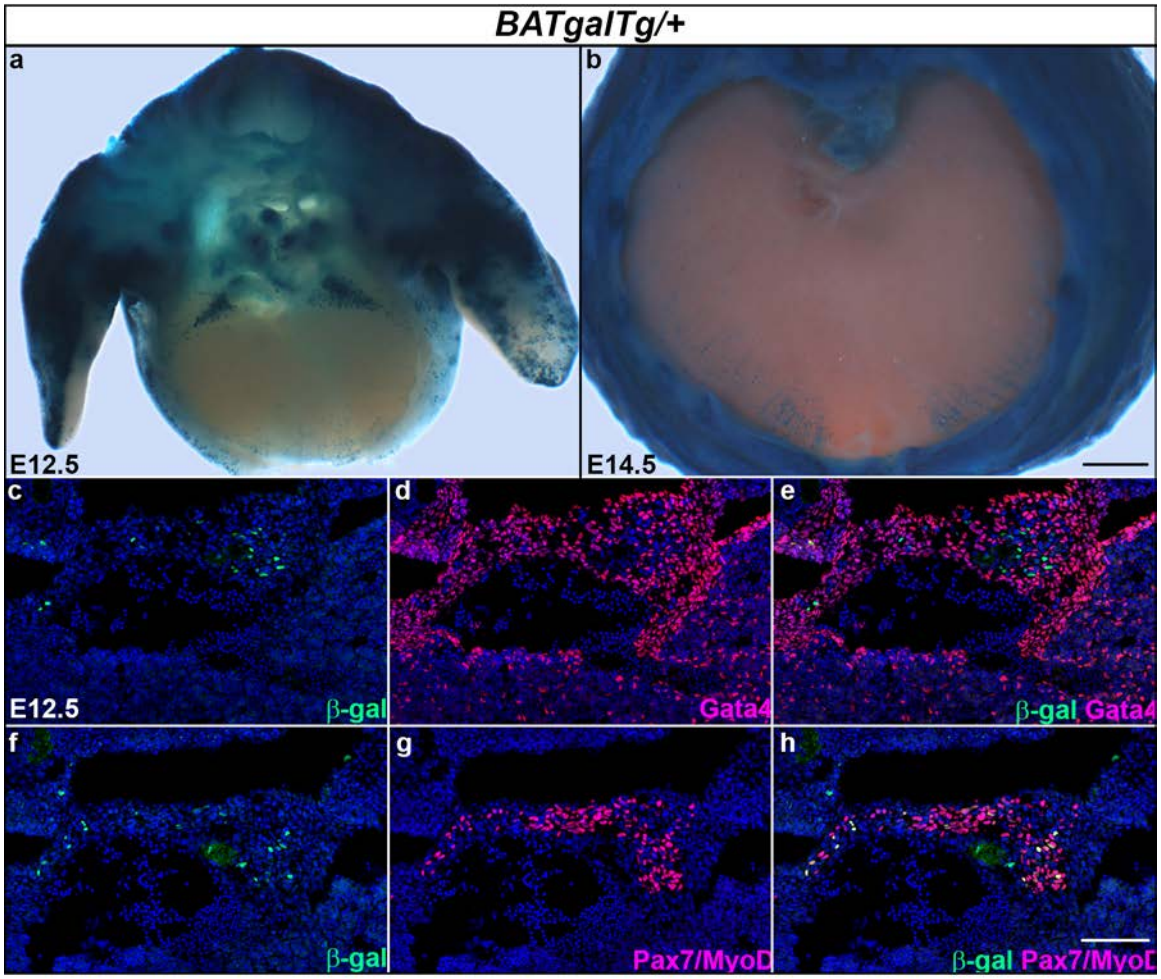

Fig. 4.2. Canonical Wnt/β-catenin signaling is actively received by myogenic cells. a, β-gal+ nuclei in BATgal$^{Tg/+}$ reporter mice indicate active Wnt/β-catenin signaling in cells at E12.5. b, β-gal+ nuclei appear to be myonuclei of the myofibers at E14.5. c-e, At E12.5, β-gal+ nuclei do not overlap with Gata4+ PPF cells. f-h All β-gal+ nuclei are Pax7/MyoD+. Scale bar a-b = 500 μm, c-h = 100 μm.

(n=15) (Fig. 4.3b). This indicates that the phenotype observed when *Porcn* is deleted in the PPF is not attributable to the loss of canonical Wnt signals from PPF-derived connective tissue to muscle. However, *Pax7$^{iCre/+}$;β-catenin$^{Δ2\text{-}6/fl2\text{-}6}$* mice appeared to have thinner diaphragm muscle than littermate controls, suggesting β-catenin does play a role in regulating myogenesis of the diaphragm. This could indicate that myogenic precursors receive canonical Wnt signals from another source beside Pax7 and Prx1-derived cells. Alternatively, because β-catenin is involved in cell-cell adhesion, β-catenin could be required in myogenic cells for cell adhesion instead of its signaling role. Interestingly, constitutive β-catenin stabilization in muscle precursors produced the opposite phenotype as β-catenin deletion. In β-*catenin$^{fl3/+}$* mice, Cre-mediated recombination produces stable β-catenin that cannot be targeted for degradation (Harada et al., 1999). *Pax7$^{iCre/+}$;β-catenin$^{fl3/+}$* mice have a thicker diaphragm muscle than control animals, as examined in whole-mount, and even have some myogenic cells present in the central tendon (Fig. 4.3c). Although canonical Wnt/β-catenin signaling to muscle precursors is not required to prevent herniation, these results indicate that altering β-catenin levels in myogenic cells does produce defects in myogenesis of the diaphragm muscle.

Subsequently, I tested whether the Wnt/β-catenin signaling in muscle precursors was activated by Wnt signals from the PPF. To do this, I generated *Prx1Cre$^{Tg/+}$;Porcn$^{fl/Y}$;BATgal$^{Tg/+}$* mice which contain the Wnt/β-catenin reporter, but have Wnt signals from the PPF deleted. If the muscle received Wnt signals from the PPF, the Wnt/β-catenin reporter should not be expressed when this

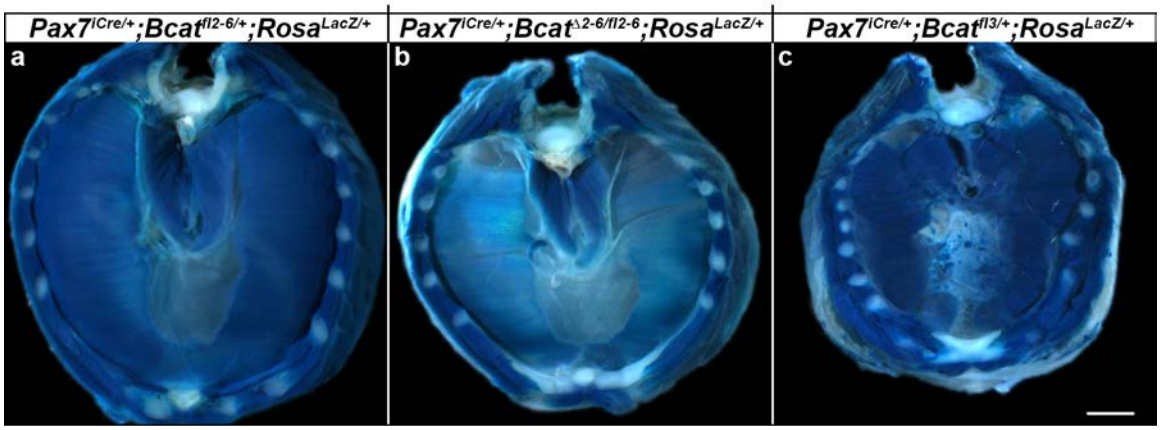

Fig. 4.3. Inhibiting reception of canonical Wnt signaling in muscle does not induce ventral diaphragmatic hernias. a-b, Diaphragms of *Pax7$^{iCre/+}$;β-catenin$^{Δ2-6/fl2-6}$;Rosa$^{LacZ/+}$* mice develop completely (b), although the muscle appears thinner than in *Pax7$^{iCre/+}$;β-catenin$^{fl2-6/+}$;Rosa$^{LacZ/+}$* control mice (a). c, Diaphragm muscle of *Pax7$^{iCre/+}$;β-catenin$^{fl3/+}$;Rosa$^{LacZ/+}$* mice (c) appears thicker than in control mice (a) and has ectopic muscle patches in the central tendon. Scale bar = 1 mm.

signal is deleted. At E12.5, when the β-catenin reporter is active in muscle precursors, inhibition of Wnt secretion from the PPF does not appear to change the activity of Wnt/β-catenin signaling in the muscle precursors (Fig. 4.4). This indicates that Wnt signaling to muscle precursors at E12.5 does not originate from the PPF, although it is possible that *Prx1Cre* may not delete *Porcn* early enough to see the loss of BATgal reporter at this stage.

Another possible target of the Wnt signals secreted by the PPF-derived connective tissue is the connective tissue itself. To test if reception of Wnt/β-catenin signaling in the PPF is required for diaphragm development, reception of canonical Wnt signaling in the connective tissue was ablated by deleting β-catenin specifically in the PPF-derived connective tissue. I examined diaphragms of $Prx1Cre^{Tg/+};\beta\text{-}catenin^{\Delta 2\text{-}6/fl2\text{-}6}$ mice and determined that some of the diaphragms did have ventral diaphragm defects (Fig.4. 5c). However, *Prx1Cre* also induces recombination in the body wall, and these defects all appeared to be due to body wall defects observed in these mice. $Prx1Cre^{Tg/+};\beta\text{-}catenin^{\Delta 2\text{-}6/fl2\text{-}6}$ body walls did not always close completely (Fig. 4.5 b-c), and as a consequence, intestine and liver could sometimes herniate between the ventral edge of the diaphragm and the tissue covering the body wall defect. It is unlikely that this corresponds with the phenotype observed in $Prx1Cre^{Tg/+};Porcn^{fl/Y}$ mice, as the deletion of β-catenin did not reproduce the muscle defects in ventral diaphragmatic hernias. This indicates that the connective tissue does not need to receive canonical Wnt signals for diaphragm development.

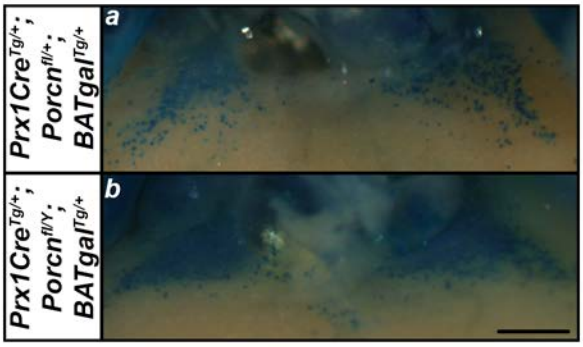

Fig. 4.4. Muscle progenitors do not receive canonical Wnt/β-catenin signals from the pleuroperitoneal fold cells. a-b, The number of β-gal+ myogenic cells receiving Wnt signaling is not reduced in mutant *Prx1Cre*$^{Tg/+}$*;Porcn*$^{fl/Y}$*;BATgal*$^{Tg/+}$ mice (b) compared to control *Prx1Cre*$^{Tg/+}$*;Porcn*$^{fl/+}$*;BATgal*$^{Tg/+}$ mice. Scale bar = 250 µm.

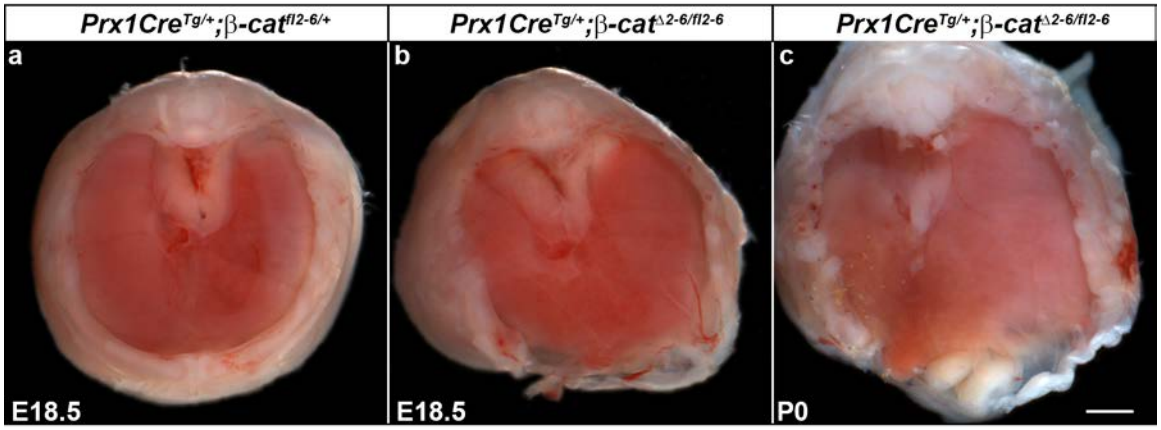

Fig. 4.5. Deletion of β-catenin with *Prx1Cre* causes body wall defects, but not muscle defects causing ventral herniation of liver. a, Compared to control *Prx1Cre*$^{Tg/+}$*;β-catenin*$^{fl2\text{-}6/+}$ mice (a), *Prx1Cre*$^{Tg/+}$*;β-catenin*$^{\Delta 2\text{-}6/fl2\text{-}6}$ mice have body wall closure defects (b-c). More severe defects can allow herniation between the body wall and diaphragm (c), but do not match the hernias and muscle defects present in *Prx1Cre*$^{Tg/+}$*;Porcn*$^{fl/Y}$ mice. Scale bar = 1 mm.

Discussion

My results demonstrate that Porcn is required within PPF-derived cells of the developing diaphragm for correct migration of the muscle to the midline of the diaphragm, and thus Wnt signals from the PPF are critical for complete formation of the diaphragm. Without direction from the PPF-derived cells, muscle does not form completely in the ventral region of the diaphragm. Furthermore, this suggests that these Wnt signals are required only at the final stages of diaphragm development, as the diaphragm muscle connects ventrally. This could indicate that the signals required to finalize diaphragm formation are different from earlier signals in diaphragm development, and suggests that alternative mechanisms may cause herniation in *Prx1Cre*$^{Tg/+}$*;Porcn*$^{fl/Y}$ mice compared to hernias induced by other genes, such as Gata4 (see Chapter 3).

Furthermore, my results also suggest that these Wnt signals are most likely noncanonical, although altering β-catenin levels did produce diaphragm defects (Fig. 4.6). Deleting β-catenin in cells likely to be targets of Wnt signaling, the muscle and fold cells, failed to reproduce the defects seen with conditional deletion of *Porcn* in the folds. Although this does not rule out an alternative cell type required to receive canonical Wnt signaling, this seems unlikely to be the case because the majority of diaphragm cells are derived from these two populations. Interestingly, though, deletion and stabilization of βcatenin in the muscle produced defects in diaphragm myogenesis, as the muscle appeared either thinner or thicker, respectively. This defect could arise from either altered reception of Wnt/βcatenin signaling from an alternative Wnt source, or from

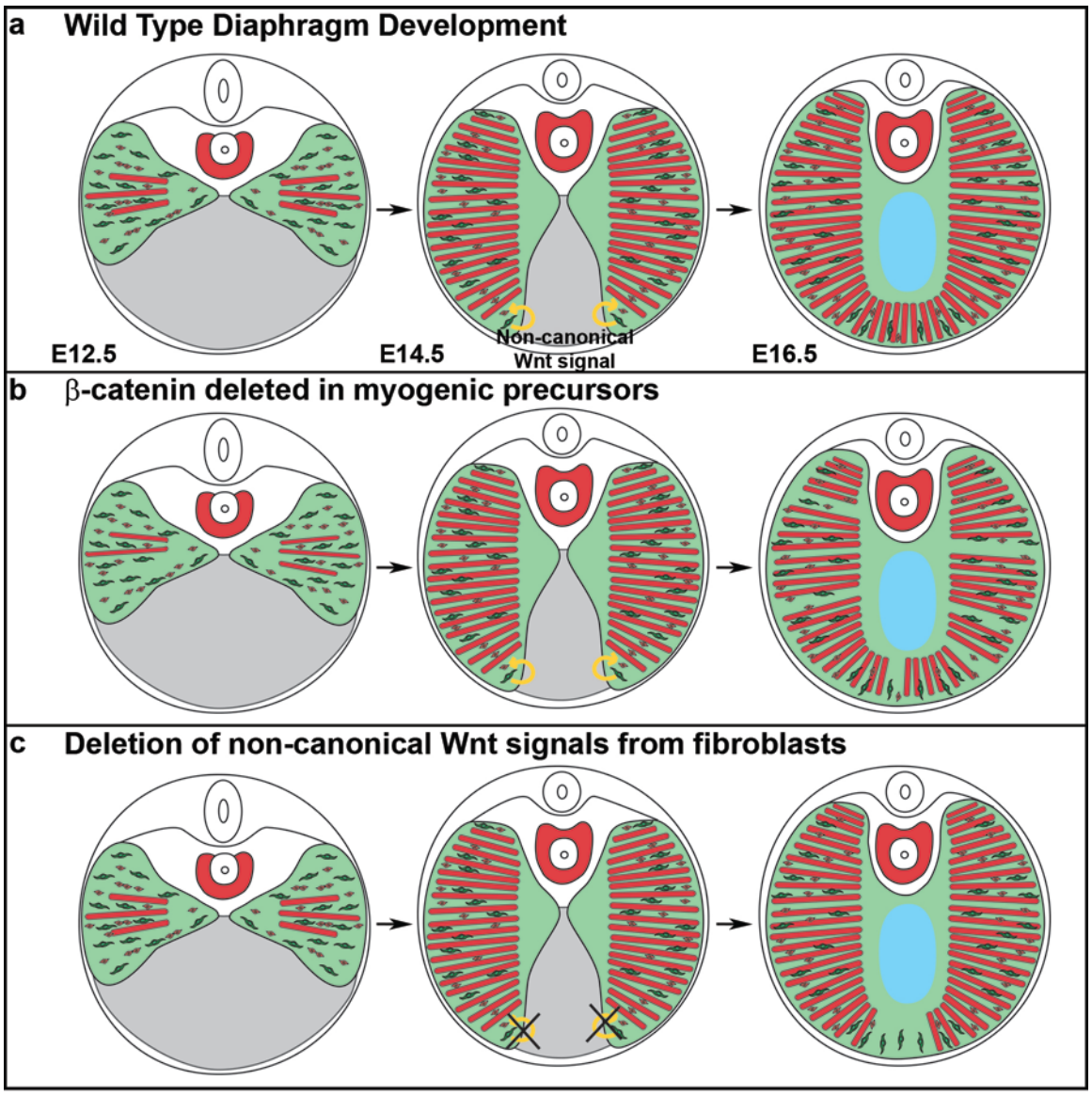

Fig. 4.6. β-catenin is likely required for myoblast fusion early in diaphragm development while noncanonical Wnt signals are required late in diaphragm development to direct muscle migration ventrally. a, Wild-type myoblasts migrate ventrally and fuse into muscle fibers during development. b, Deletion of β-catenin in muscle precursors produces thinner diaphragm muscle, likely because of adhesion defects in myoblast fusion. c, Wnt signals from fibroblasts, likely noncanonical, are required for muscle to spread ventrally in the last stages of diaphragm development.

changes in the adhesive properties of myogenic cells following reduced or stabilized expression of β-catenin.

Materials and Methods

Mice

All mice have been previously published. We used *Prx1Cre* (Logan et al., 2002) and *Pax7*^{iCre} (Keller et al., 2004) Cre alleles, the *Rosa*^{LacZ} (Soriano, 1999) Cre-responsive reporter allele, the *BATgal* (Maretto et al., 2003) Wnt/βcatenin reporter allele, and *Porcn*^{fl} (Barrott et al., 2011), βcatenin$^{fl2\text{-}6}$ (Brault et al., 2001), and βcatenin^{fl3} (Harada et al., 1999) mutant alleles.

Immunofluorescence, β-Galactosidase Staining, and Microscopy

For section immunofluorescence, embryos were fixed at 4^{o}C in either 1% PFA/PBS overnight or in 4% PFA/PBS for 1.5 h. Embryos were then put through 10% and 20% sucrose and embedded in OCT. Embryos were cryosectioned in 8 µm thick sections. Slides were fixed in 4% PFA/PBS for 5 minutes, washed in PBS, then blocked for 1 h in 5% goat serum/PBS. Sections were incubated in primary antibody at 4^{o}C overnight. Following primary antibody, slides were washed in PBS and incubated in secondary antibody for two hours. Slides were then washed, postfixed, and incubated in Hoechst for 5 minutes. Fluoromount mounting media (Southern Biotech) was used to coverslip slides. Antibodies are listed in Table 4.1.

Table 4.1

Antibodies

Antibody	Type	Source	Product Number	Working Concentration	Antigen Retrieval	Secondary and Amplification
Pax7	Mouse IgG1	DSHB	PAX7	2.4µg/ml	Yes ≥ E14.5 No ≤E14.5	Dylight 594 goat α mouse IgG1
MyoD	Mouse IgG1	Santa Cruz Biotechnology	Sc-32758 (5.8A)	4µg/ml	Yes ≥ E14.5 No ≤E14.5	Dylight 594 goat α mouse IgG1
Gata4	Goat polyclonal	Santa Cruz	Sc-1237	2 µg/ml	No	Dylight 594 Donkey α Goat
β-gal	Chick polyclonal	Immunology Consultants Laboratory	CGAL-45A-Z	2 µg/ml	No	Dylight 488 goat or donkey α chick

For whole-mount β-gal staining, embryos were fixed 1.5 h in 4% PFA + 2mM $MgCl_2$. Diaphragms were dissected, washed in PBS and rinse buffer (100mM Sodium Phosphate, 2mM MgCl2, 0.01% Na Deoxycholate, 0.02% Ipegal), and stained 16 h at 37^{o}C in X-gal staining solution (5mM potassium ferricyanide, 5mM potassium ferrocyanide, 1mg/ml Xgal (Inalco)).

Fluorescent images were taken on a Nikon A1 confocal and displayed as a maximum projection. β-gal stained embryos were imaged with a Qimaging camera.

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CHAPTER 5

EVOLUTION OF THE MAMMALIAN DIAPRHAGM

Abstract

A muscularized diaphragm is a unique and characteristic skeletal muscle in all mammals, but how the diaphragm evolved is unclear. Evolution of a muscularized diaphragm likely required developmental innovations that allowed muscle precursors to delaminate from the cervical somites and migrate to the pleuroperitoneal folds (PPFs) of the developing diaphragm. To test this, I compared development of a mammal (the mouse) to development of a mammalian outgroup (the chick), to determine what developmental diaphragm structures or signals are conserved or differ between these groups. The presence of conserved structures would indicate that these structures and signals were present prior to the final evolutionary steps that generated a muscularized diaphragm, while the presence of unique structures or signals in mammals would indicate potential developmental innovations important for diaphragm evolution. I determined that Tcf4+ PPF-like structures are present in chick, suggesting that the PPFs are not unique to mammals. Importantly, in chick, these structures do not express hepatocyte growth factor (HGF), a ligand highly expressed in mouse PPFs and a critical signal required for promoting

migration of muscle precursors into the developing diaphragm and muscularization of the diaphragm. However, in chick, somitic muscle precursors do express Met, the receptor for HGF, and these precursors are capable of delaminating from the somite when presented with HGF-soaked beads, indicating that muscle precursors are competent to respond to signals to migrate to the diaphragm. Together, these data argue that mammalian expression of HGF in the PPFs may be the key developmental innovation that allowed for evolution of a muscularized diaphragm.

Introduction

A muscularized diaphragm is a unique and defining characteristic of all mammals (Buchholtz et al., 2012), but the developmental innovations that triggered its evolution are unknown. Evolution of a muscularized diaphragm was likely critical for the evolution of the constant high metabolic rate characteristic of mammals (Perry et al., 2010). Contraction of the diaphragm creates a negative thoracic pressure and is critical for respiratory inspiration. This ability to generate a negative thoracic pressure is thought to be critical for evolution of low-compliance lungs. In turn, the evolution of low-compliance lungs with alveoli, reduced lung volume, but increased lung surface area, was critical for the evolution of a high, constant metabolic rate in mammals. In addition, a muscularized diaphragm may also have been important for generation of the high abdominal pressure required live births of placental mammals (Perry et al., 2010). Despite these critical roles of a muscularized diaphragm in mammals, it is unclear how the diaphragm evolved in mammalian ancestors. This evolution is

likely to have occurred through the acquisition of developmental innovations. In this Chapter, I will compare the development of a mammal, the mouse, and a mammalian outgroup, the bird, to determine what developmental steps are unique in mammals and have allowed the evolution of a muscularized diaphragm.

The presence of a muscularized diaphragm is a defining mammalian characteristic, but it is unclear when developmental components of the muscularized diaphragm are uniquely mammalian. The septum transversum is a developmental structure proposed to be important in diaphragm development, although its role is unclear. However, previous studies have determined that the septum is ancient, first appearing in reptiles, including birds, but not present in fish and amphibians (Fig. 5.1) (Goodrich, 1930; Perry et al., 2010). The septum separates the heart and liver (Perry et al., 2010), and is present very early in development (Dunwoodie et al., 1998). Because the septum is present in reptiles and birds, mammalian outgroups, innovations in septum development are unlikely to account for the evolution of the mammalian muscularized diaphragm.

My work on diaphragm development (Chapter 3) indicates that the pleuroperitoneal folds (PPFs) and muscle are critical in diaphragm development, and evolution of these tissues was likely a critical step for the evolution of a muscularized diaphragm. During development, I have shown that the PPFs are critical for diaphragm morphogenesis (Chapter 3). The PPFs may also be a source of signals and a target for the muscle progenitors migrating to the

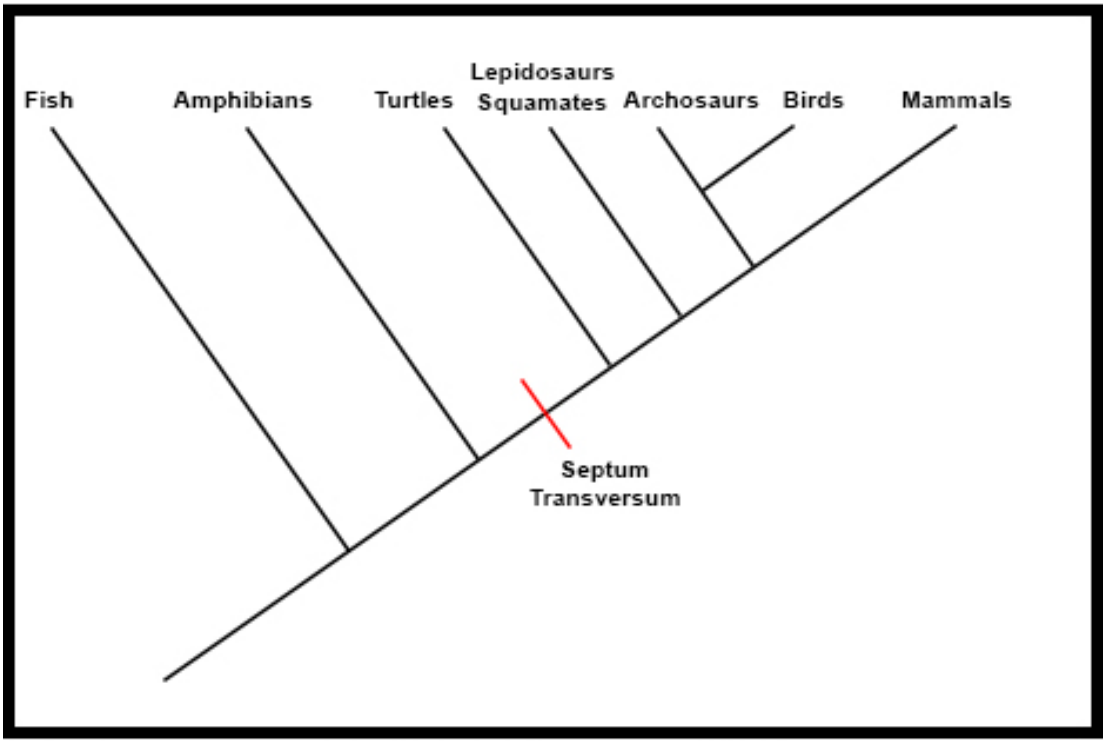

Fig. 5.1. The septum transversum is an ancient structure. While a muscularized diaphragm is unique to mammals, the septum is present in mammals, birds, and reptiles.

developing diaphragm. Thus, acquisition of PPFs or signals from PPFs may have been a critical developmental innovation that allowed for muscularization of the diaphragm. In addition, muscle precursors must be competent to respond to the signals directing migration to the diaphragm. This suggests that acquisition of competency to respond to signals may be another key developmental innovation. To determine what developmental innovations allowed muscularization of the diaphragm, I compared mouse, *Mus musculus,* diaphragm development to the development of a bird (a mammalian outgroup), the chicken G*allus gallus.*

For muscle to reach the PPFs, muscle precursors must first delaminate from the somites. Migrating muscle precursors from the somite, marked by expression of Lbx1 (Dietrich et al., 1998), give rise to the muscle of the tongue, limbs, and diaphragm. Delaminated muscle precursors from somites 1-6 give rise to the tongue musculature (Huang et al., 1999), while cervical and thoracic somites give rise to the migrating muscle precursors of the limb and diaphragm. In the mouse, the muscle of the diaphragm likely originates from somites 5-7 (Dietrich et al., 1999), while somites 8-14 give rise to the forelimb muscle (Burke et al., 1995). In nonmammalian species, the number of cervical somites and vertebrae vary dramatically, likely because these somites do not need to contribute to a muscularized diaphragm (Buchholtz et al., 2012). The chicken has 14 cervical somites (Burke et al., 1995), and somites 16-21give rise to the muscle of the chick forelimb (Beresford, 1983). Delamination of muscle progenitors from the somites requires HGF/Met signaling. In particular, delamination requires that somitic Met+ muscle progenitors receive an

hepatocyte growth factor (HGF) signal. Mutations in either HGF or Met prevent delamination and produce amuscularized limbs and diaphragm (Bladt et al., 1995; Maina et al., 1996).

In addition to being required for muscle precursor delamination, HGF may have other important functions in the formation of a muscularized diaphragm. Previous work has shown that muscle precursors migrate to the diaphragm along a track of HGF expression (Dietrich et al., 1999). This suggests that HGF signaling may play a role in directing migration of muscle to the PPFs. Additionally, experiments have demonstrated that HGF signaling promotes motility and muscle proliferation while inhibiting differentiation (Leshem et al., 2002; Maina et al., 1996; Scaal et al., 1999). HGF may therefore be critical as muscle precursors migrate to the diaphragm, possibly in guiding the muscle precursors and keeping the cells motile, while also expanding the pool of muscle precursors to ultimately increase the muscularization of the diaphragm.

To gain insight into the evolution of the mammalian diaphragm, I compared mouse and chick embryos to determine if developmental structures and signals critical for diaphragm development were present or absent in non-mammals. I discovered that chick embryos contained Tcf4+ cells in regions potentially similar to the mouse Tcf4+ PPFs. Additionally, I determined that HGF signaling, present in mouse PPFs, was absent in the Tcf4+ region in chick embryos. Through bead implantation studies, I determined that muscle precursors in the chick can respond to HGF signaling, suggesting that muscle precursors are competent to respond to HGF. Thus, acquisition of HGF

expression in the PPFs may have been a critical step in the evolution of the mammalian diaphragm.

Results

Because of the critical importance of the PPFs in diaphragm development, I determined in section if chick embryos had a structure similar to the PPFs. Using an antibody to Tcf4, which marks PPF cells in the mouse (Fig. 5.2a), I examined chick embryos to see if Tcf4+ PPF-like tissue was present. In chick, the septum transversum separates the heart and liver. At the junction of the septum and body wall in Hamburger-Hamilton stage 25 chicks, a thickening of tissue contains Tcf4+ cells (Fig. 5.2b). Although further characterization is needed to determine if these cells resemble PPF cells in other aspects, such as testing whether other markers of the PPF are expressed in this tissue, this suggests that Tcf4+ PPFs may not be unique to mammals.

In addition to the presence of PPFs, diaphragm development also likely requires signals from the PPFs, possibly HGF, to support muscle migration to the PPFs. HGF is strongly expressed at E12.5 in the mouse PPF, as detected by *in situ* hybridization (Fig. 5.2c). Furthermore, HGF is strongly expressed in the PPFs of *Pax3*$^{SpD/SpD}$ mice (Fig. 5.3), in which muscle precursors do not migrate from the somite (Underhill et al., 1995). The strong expression of HGF in these mice indicates that the PPF are the source of HGF signals. However, no HGF expression is detected in the potential chick PPF region (Fig. 5.2d) at stage 25. This suggests that, although Tcf4+ PPF cells could be present in chick, they do not secrete the same signals as the mouse PPFs.

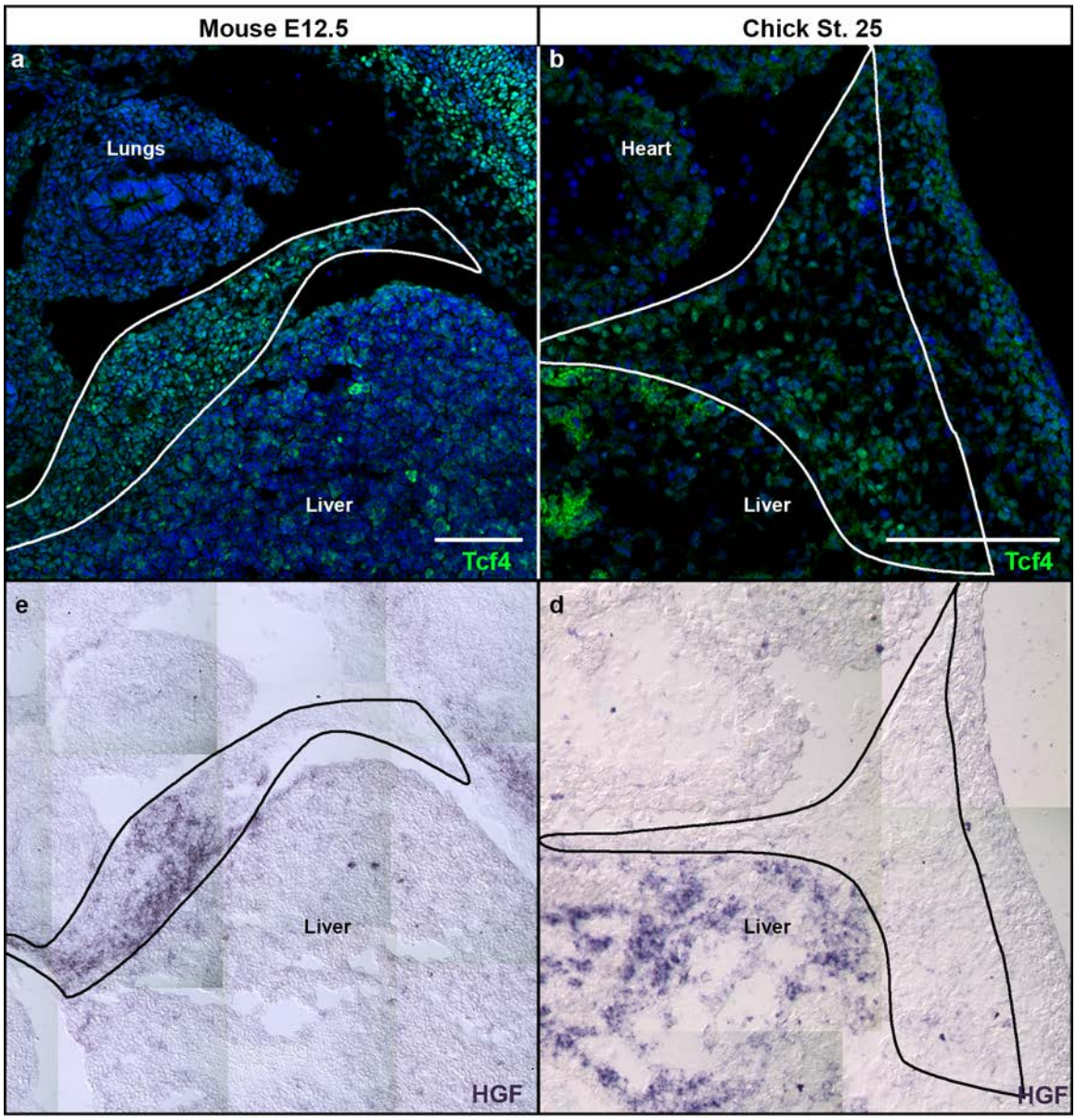

Fig. 5.2. PPF-like structures in the chick express Tcf4, like mouse PPFs, but do not express HGF signals. a, Mouse PPFs express Tcf4. b, PPF-like structures near the chick septum also express Tcf4. c-d, Mouse PPFs strongly express HGF (c), but PPF-like structures in chick do not (d). Scale bars = 100 μm.

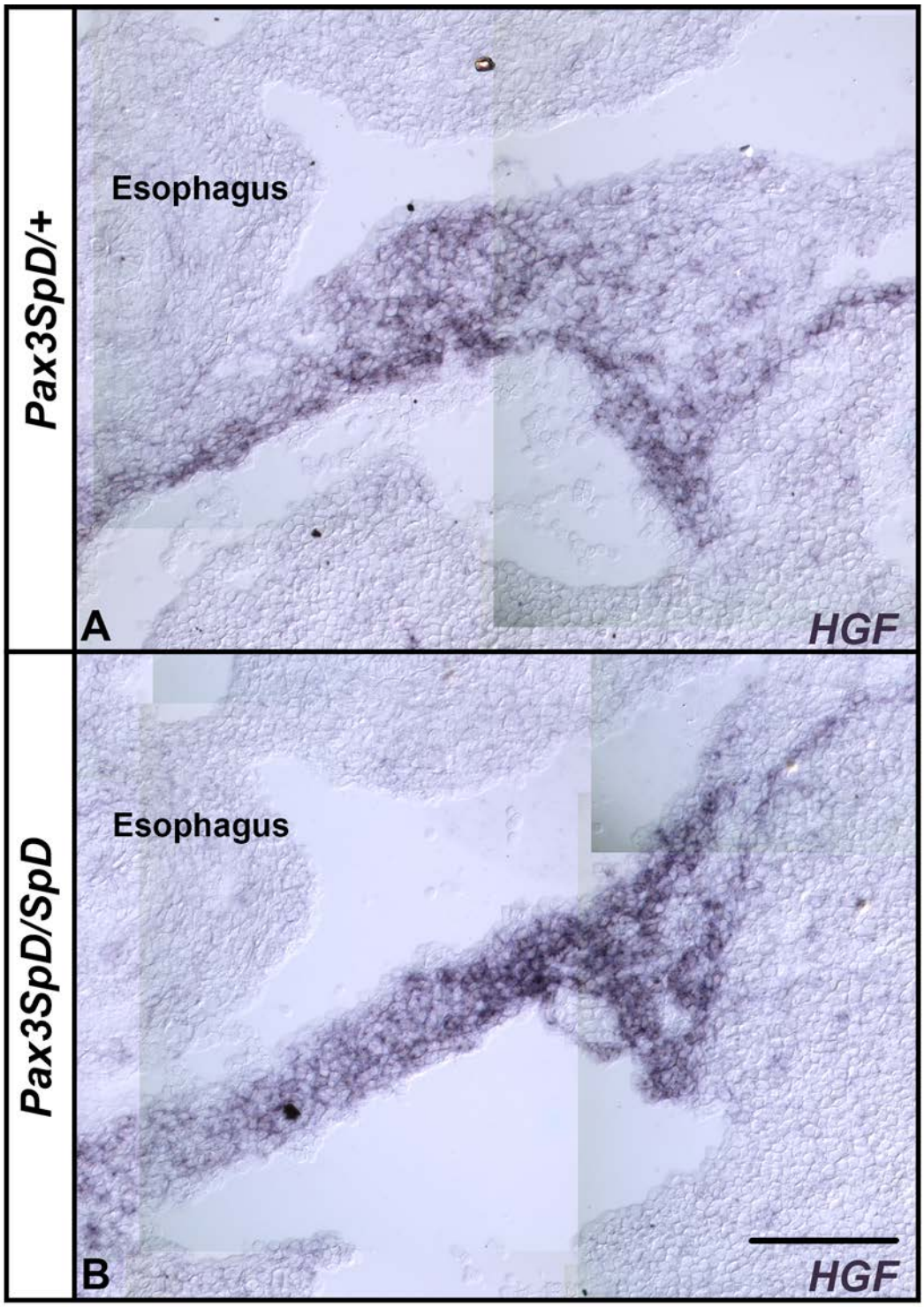

Fig. 5.3. HGF is strongly expressed by non-muscle cells in the PPFs. a-b, HGF is strongly expressed by cells in the PPF (a), even in the absence of muscle (b).

I next examined whether chick muscle precursors were competent to respond to HGF signals by implanting HGF-soaked beads near cervical somites. Heparin-coated acrylic beads soaked in 200µg/ml recombinant HGF (R&D) or 0.1% BSA/PBS were inserted on the lateral side of the somites in chick embryos. To avoid confusion with muscle precursors migrating to the tongue (somites 1-6) and the limb (somites 16-21), beads were implanted next to somites 10 or 11. Myogenic precursors were visualized by Pax3 and Pax7 expression through whole-mount immunohistochemistry to determine the effect of HGF on muscle delamination. In no cases (0/7, Fig. 5.4a,c) did control BSA beads promote delamination and migration of muscle from the cervical somites. HGF beads, in contrast, induced delamination of muscle precursors in 12/13 embryos analyzed (Fig. 5.4b,d). Thus, HGF signals are sufficient to induce muscle delamination in chick cervical somites. Gain of HGF expression in mammals to induce delamination may have been a critical step in diaphragm evolution (Fig. 5.5).

Conclusions

My results suggest that pleuroperitoneal fold-like structures may have been present prior to the divergence of the mammalian and avian lineages. Chick embryos contain Tcf4+ cells, which may be similar to the PPFs. This suggests that the PPFs may have been present prior to the development of the diaphragm and are not the developmental innovation that allowed acquisition of a muscularized diaphragm. However, further work will be required to determine if this tissue is indeed analogous to the PPF. It would be useful to look near other septa, such as the postpulmonary septum, to determine if other PPF-like tissues

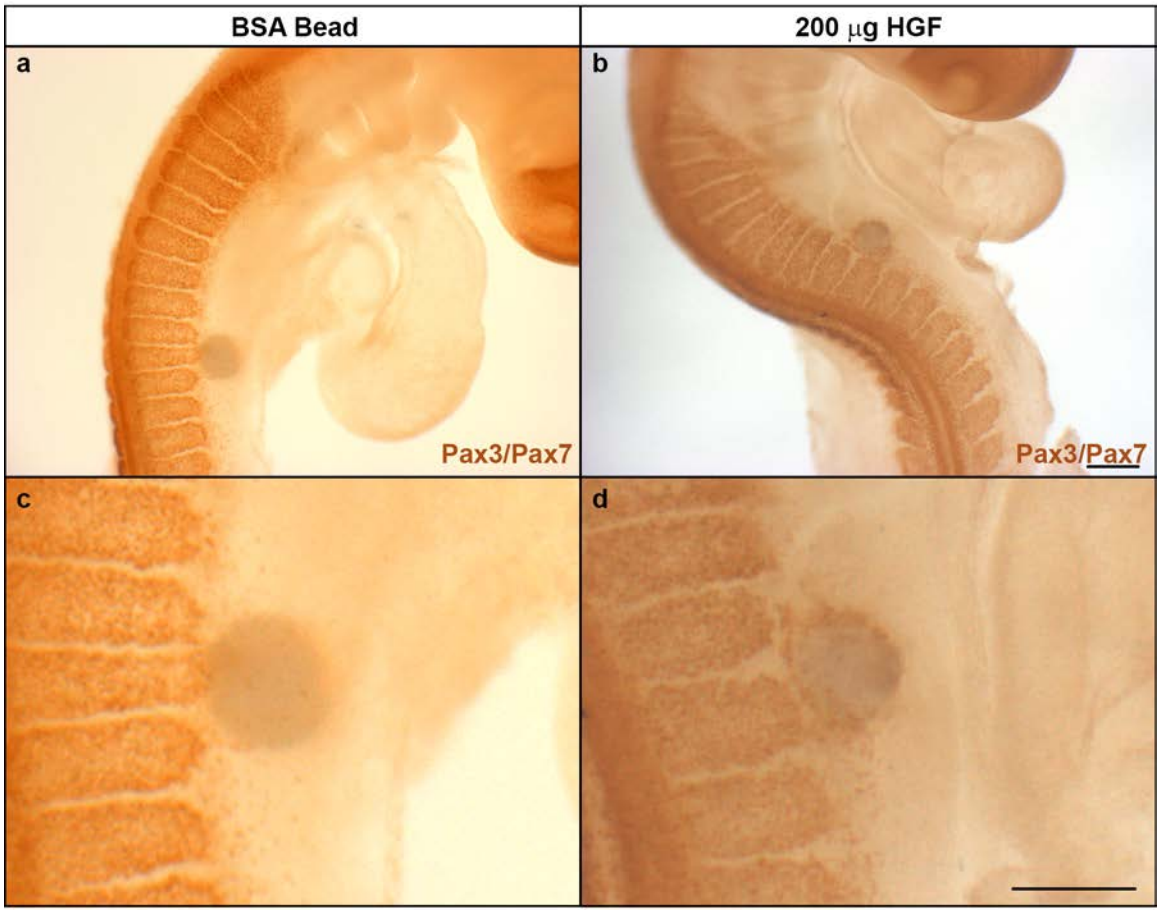

Fig. 5.4. HGF signaling is sufficient to induce migration of chick cervical somite muscle precursors. a, BSA beads do not induce migration of muscle precursors from cervical somites (enlarged in c). b, HGF-soaked beads induced delamination and migration of muscle precursors in chick embryos (enlarged in d). Scale bar = 250 μm (a-b) or 200 μm (c-d).

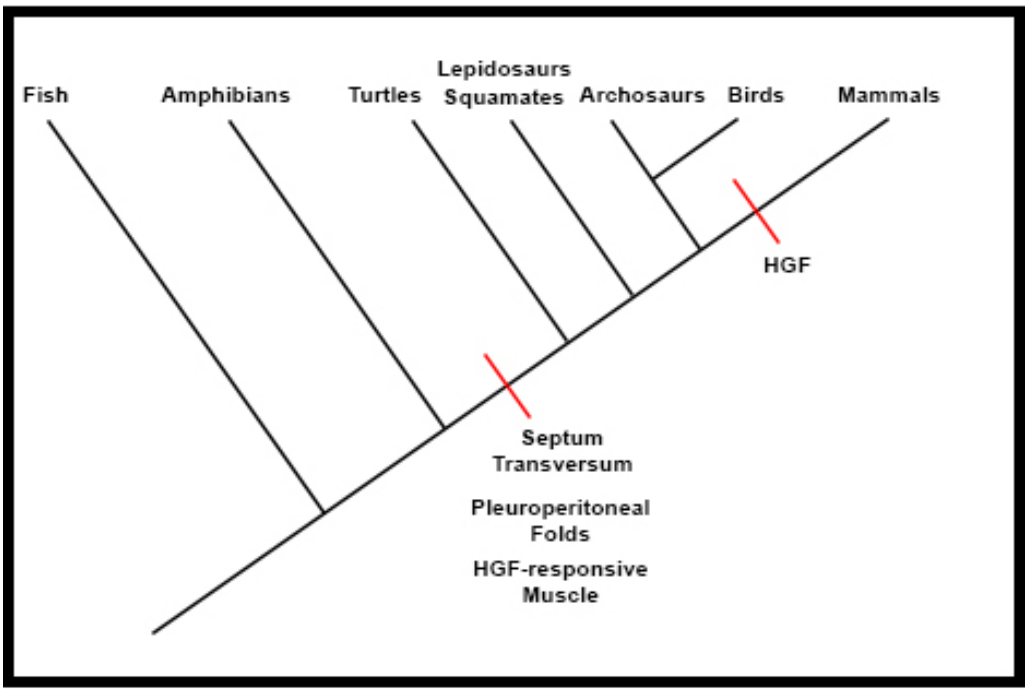

Fig. 5.5. Development of the PPF and HGF-responsive muscle may have evolved anciently, but HGF signaling in the PPFs could be a mammalian innovation. Migrating muscle precursors and pleuroperitoneal folds appear to be present in birds, suggesting they evolved before the mammal and bird lineages split. HGF expression in the folds may be critical for muscularization of the diaphragm.

may be present. To further characterize PPF-like tissues, it will be important to perform whole-mount immunofluorescence in chick embryos to detect markers of the PPFs. Likely candidates to examine are Tcf4, Gata4, and other transcription factors shown to be expressed in non-muscle cells of the folds such as Wt1 and COUP-TFII (Clugston et al., 2008).

My experiments suggest that mammalian, but not avian, PPFs express HGF, a signal that is critical to support muscle migration to the diaphragm. HGF is strongly expressed in mouse PPFs, but the presumptive chick PPFs do not express this signal. Because previous work suggests HGF signaling increases muscle precursor motility and proliferation, acquisition of HGF expression in the PPFs may have been a critical evolutionary step in directing muscle to the diaphragm and ensuring a large enough pool of muscle precursors. Interestingly, Met+ muscle precursors in chick cervical somites are capable of responding to HGF ligand and migrate from the somite when presented with HGF signals. This indicates that muscle precursors in chick embryos are competent to respond to signals involved in diaphragm development, and acquisition of the HGF signal may have been a critical step to allow migration of muscle to the diaphragm and the formation of a fully muscularized diaphragm. HGF expression in the PPFs likely occurred as the mammalian ancestor either gained an HGF enhancer or lost repressors, promoting expression in the PPFs and expanding the expression pattern of HGF to the diaphragm. Enhancers required to express HGF and allow diaphragm development are likely critical in all mammals, so comparing

conservation of genomic regions of HGF should indicate critical regions for further analysis.

Methods

Chick Surgeries

Heparin-coated acrylic beads were washed in PBS and soaked in 200µg/ml HGF or 0.1% BSA/PBS for 1.5 h at room temperature. Beads were inserted between the somite and intermediate mesoderm in 15-20 somite chick embryos. Beads were embedded next to somite 10 or 11. Embryos were then incubated at 37^{o}C for 24 h, harvested, and fixed in 4% PFA/PBS for 24 h.

Immunofluorescence and Immunohistochemistry

Section immunofluorescence was performed as described in Chapter 3. For whole-mount immunohistochemistry, fixed embryos were bleached using Dent's Bleach (1:2 30% H_2O_2:Dent's fix) at 4^{o}C for 24 h, then incubated in Dent's Fix (1:4 DMSO:MeOH) at 4^{o}C for 24 h. Embryos were washed in PBS, blocked in 5% goat serum at room temperature for 1 h, and incubated with mouse IgG_{2a} α Pax3 and mouse IgG_1 α Pax7 antibodies (DSHB, 1:100 and 1:10, respectively) for 24 h at room temperature. Embryos were washed in PBS and incubated in biotinylated goat α mouse (Jackson ImmunoResearch, 1:500) for 24 h at room temperature. DAB (3,3′-Diaminobenzidine, Sigma) stain was used to visualize antibody staining. Reaction solution was made with 10mg DAB, 7.5µL H_2O_2, and 50ml PBS and developed in the dark.

Section In Situ Hybridization

Full length mouse HGF cDNA was cloned into a pCR4-TOPO vector. Full length chick HGF probe was received from Claudio Stern. Sections were fixed in 4% PFA, washed, incubated in 1µ/ml proteinase K for 10 minutes, and post-fixed in 4% PFA. Sections were acetylated (0.25% acetic anhydride in 0.1M triethanolamine) for 15 minutes, then washed and prehybridized in hybridization solution at 65^{o}C for 1 h prior to incubation in 2µg/ml probe overnight at 65^{o}C. Sections were washed with increasingly stringent SSC washes and treated with RNAse A (20µg/ml) to remove unhybridized probe. Hybridized DIG-labeled probe was incubated with sheep α DIG-AP (1:2000, Roche) and detected with 250 µg/ml NBT and 125 µg/ml BCIP (Sigma) in NTMT.

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Understanding the processes of diaphragm development and the mechanisms by which development goes awry are critical for human health. A functional diaphragm requires that muscle and muscle connective tissue must be integrated to form the characteristic radial array of muscle fibers surrounding a central tendon that make up an adult diaphragm (Pearce, 2009). This occurs developmentally as muscle precursors migrate from the somites to the pleuroperitoneal folds (PPFs), proliferate, and spread and differentiate to form the radial array of muscle fibers (Babiuk et al., 2003). Defects in diaphragm development give rise to congenital diaphragmatic hernias (CDH), which cause severe health consequences. These hernias develop as the diaphragm fails to form completely, allowing abdominal contents to herniate into the thoracic cavity (Pober, 2007).

The developmental processes required for diaphragm development were previously unknown, largely due to a lack of genetic tools. From previous studies, it was unclear what mechanisms regulate muscle progenitors as they spread throughout the diaphragm, what is the fate of the PPF, and what function the

PPF play in diaphragm development. This dissertation discusses the integral role of the PPF-derived muscle connective tissue in all stages of diaphragm development and morphogenesis. Furthermore, defects in the PPFs non-cell autonomously regulate muscle to cause congenital diaphragmatic hernias. This demonstrates that the PPFs are not simply transient structures, but are actively and crucially involved in diaphragm development.

Diaphragm Development

This dissertation demonstrates that the pleuroperitoneal folds (PPFs) give rise to the connective tissue fibroblasts of the diaphragm and are the driving force behind diaphragm morphogenesis. The fate of the PPF during diaphragm development had not been determined because of a previous lack of genetic tools. We permanently labeled the PPFs by crossing *Prx1Cre* mice (Logan et al., 2002) to the *Rosa*^{LacZ} reporter mouse (Soriano, 1999). By examining a developmental time series, we determined that the PPF form independently of muscle by embryonic day (E)11.5 and spread ventrally and dorsally to contribute to cells of the central tendon and muscle connective tissue fibroblasts throughout the diaphragm by E14.5. This demonstrates for the first time that the PPFs are not just transient, developmental structures, but actively contribute to the cells of the adult diaphragm. Migration of the PPFs occurs completely independently of muscle, as PPF-derived cells still spread in the absence of muscle. *Ex vivo* imaging of live diaphragm development has demonstrated that fibroblasts are actively moving and migrating as they spread to form the diaphragm. Furthermore, it appears that PPF cells may spread through collective cell

migration, although further studies are needed to definitively determine this. Future studies should also examine how the PPF cells spread to give rise to cells throughout the diaphragm. It is unclear whether these cells are directed to specific locations, or are simply proliferating and filling all available space. Using a tamoxifen-inducible *Prx1Cre*^{ER} mouse, it may be possible to label subsets of the PPF and determine where these cells migrate within the diaphragm.

An interesting future area of study will be investigating how PPF cells are specified into muscle connective tissue fibroblasts, which likely promote muscle development, and tendon cells, which likely inhibit the formation of muscle (Kardon, 1998; Kardon et al., 2003). The central tendon is completely devoid of muscle fibers, suggesting that tendon cells secrete signals that either repel myogenic cells or that inhibit the process of myogenesis. It is unclear at what point PPF cells become specified to become a particular cell type, but it would be interesting to examine the PPFs for early markers of the tendon fate. Further study of the fibroblasts and tendon cells could provide insight into the mechanisms determining which fate these cells adopt. These studies may also provide insight into the mechanisms by which fibroblasts promote and tendon cells inhibit myogenesis.

Although it has been theorized that the septum transversum gives rise to the central tendon (Iritani, 1984) as muscle precursors migrate around the septum to form the costal muscle, there is no evidence to suggest this is the case. The surprising contribution of the PPF to the central tendon suggests the septum may not actually contribute to the central tendon or to the adult

diaphragm. Although the presence of PPF-derived cells in the central tendon does not rule out the idea that the septum may also contribute cells, we have seen no evidence of septum contribution. Without a genetic tool to label the septum and track the contribution of septum cells to the adult diaphragm, there is no way to test whether the septum gives rise to anything beside a layer of cells lining the cranial surface of the liver. However, even if the septum does not contribute to the diaphragm, it may still be important as a surface that PPF cells migrate across to form the diaphragm. If a septum transversum-specific Cre driver were developed, it would be possible to do lineage studies to determine if the septum contributes to the diaphragm. It would also be possible to ablate the septum and determine whether it plays a functional role in diaphragm development.

Our investigation of how the diaphragm develops in the absence of the PPFs has been hampered by incomplete recombination of PPF cells using the *Prx1Cre* allele, but is worthy of further investigation. In our study of $Prx1Cre^{Tg/+};Rosa^{DTA/+}$ mice, the majority of mice (22/25) developed complete diaphragms. This is likely due to incomplete recombination and compensatory proliferation of PPF cells that have escaped ablation, as these diaphragms still contained a large number of Tcf4+ PPF-derived cells. Interestingly, 3/25 diaphragms had minor defects, manifesting as small regions either containing ectopic muscle or missing tissue. These defects may have occurred as a result of more efficient recombination and ablation of PPF cells. It would be useful to identify or develop a Cre driver that induces recombination in the PPF with 100%

efficiency. Should such a tool be identified, complete ablation of the PPF will determine if any part of the diaphragm can form in the absence of this tissue.

Comparisons of the development of PPFs and muscle suggest that PPFs are critical in regulating the expansion and pattern of the diaphragm muscle. Prior to the spread of muscle precursors, the PPFs expand ventrally to give rise to connective tissue fibroblasts throughout the diaphragm. The mechanism by which the PPF-derived connective tissue influences the spread of muscle is unknown. One possibility is that muscle is passively carried along as the PPF cells spread. Alternatively, the connective tissue could secrete signals inducing or directing the migration of muscle. Future experiments using 2-photon imaging of live, *ex vivo* diaphragms with labeled muscle precursors can determine whether muscle spreads passively or by active migration. In addition, experiments which label both the fibroblasts and the myogenic cells to visualize how these two cell populations interact during morphogenesis will provide interesting insights into diaphragm formation. Costal diaphragm muscle fibers have a very specific, radial orientation. Although the mechanism by which myofibers are aligned is unclear, myofibers are radially aligned at the earliest stages of muscle fusion. It is likely the PPF-derived connective tissue may regulate the fusion and orientation of myofibers. *Ex vivo* cultures of the diaphragm offer an excellent system to study the process of muscle fusion and myofibers orientation. Since the diaphragm muscle is conducive to imaging, it should be possible to visualize the fusion of myogenic cells into muscle fibers live.

Connective Tissue Defects Induce CDH

Previous studies have proposed that defects within the PPF give rise to CDH (Babiuk et al., 2003; Clugston and Greer, 2007), but these experiments did not specifically test which tissues are critical for diaphragm development. Using multiple Cre drivers, we systematically deleted *Gata4,* a candidate CDH-causing gene, in tissues that give rise to the diaphragm to determine when, where, and how defects leading to CDH arise. Using *Prx1Cre*, which specifically recombines in PPF cells, we deleted *Gata4* by generating $Prx1Cre^{Tg/+};Gata4^{\Delta/fl}$ mice. $Prx1Cre^{Tg/+};Gata4^{\Delta/fl}$ mice develop diaphragmatic hernias with 100% penetrance. Using *Pax3Cre*, which specifically drives recombination in myogenic cells, we deleted *Gata4* by generating $Pax3^{Cre/+};Gata4^{\Delta/fl}$ mice. Deletion of *Gata4* in myogenic cells does not produce diaphragmatic hernias. Therefore, the formation of CDH is specific to deletion of *Gata4* within the PPF. This directly demonstrates for the first time that deletion of a CDH gene within the connective tissue is sufficient to produce CDH. Many other CDH-candidate genes are expressed in the PPFs (Clugston et al., 2008), suggesting that PPF-derived connective tissue may be defective in most cases of CDH.

A common mechanism proposed for CDH is that hernias form from the failure of the PPFs to spread completely, although no studies have tracked the movement of PPF cells in CDH models. Based on studies using teratogens as a model of CDH, regions of the PPFs were missing when examined in section. It was proposed that these regions would ultimately cause hernias because PPF cells would be missing in these regions, leading to a loss of associated muscle

and a hole in the diaphragm (Clugston and Greer, 2007; Greer et al., 2000). To directly test this hypothesis, I generated *Prx1Cre*$^{Tg/+}$;*Gata4*$^{\Delta/fl}$;*Rosa*$^{LacZ/+}$ mice, in which mutant cells are β-galactosidase+ (β-gal+). Surprisingly, β-gal+ PPF-derived cells still spread across the herniated region, demonstrating that hernias are not holes that develop from the failure of the PPF to spread completely across the diaphragm. Instead, deletion of *Gata4* specifically within the folds causes non-cell autonomous defects in the muscle, as muscle fails to spread to these regions. While this experiment demonstrates that defects in the spreading of the PPF throughout the diaphragm do not cause CDH, β-gal+ cells within the region of the hernia do not appear as evenly dispersed as control animals. This could indicate that, although the PPF-derived tissue spreads throughout the diaphragm, there may still be some subtle defects in the migration of fibroblasts. This can be tested by imaging the migration of *Gata4* mutant fibroblasts using *ex vivo* culture methods and 2-photon imaging.

As hernias induced by deletion of *Gata4* in the PPF are not holes arising from a failure of the PPF to spread, we examined the mechanism through which regions of the diaphragm give rise to diaphragmatic hernias. Herniated regions do not contain muscle, but instead are composed of connective tissue. One possibility is that connective tissue alone, without the additional strength of muscle fibers, may be too weak to prevent herniation of the liver. However, *Pax3*$^{SpD/SpD}$ mice (Underhill et al., 1995), which have amuscularized diaphragms, do not develop CDH. Therefore, connective tissue alone is sufficiently strong to prevent herniation. This indicates two possible mechanisms why hernias form

when *Gata4* is deleted. First, connective tissue in those herniated regions was generated by *Gata4*- fibroblasts and is inherently and structurally weaker than regular connective tissue. Alternatively, the herniated regions could have connective tissue of regular strength, but because this region is adjacent to strong, muscularized tissue it creates a relative weak spot through which liver will herniate. These hypotheses were tested by deleting *Gata4* in the folds in the background of $Pax3^{SpD/SpD}$ mice with completely amuscularized diaphragms. If the connective tissue is weaker in *Gata4*- regions, it would be expected that hernias still form in these weak spots in the absence of muscle. However, if herniation requires juxtaposition of connective tissue and stronger, muscularized regions, the absence of muscle should rescue the herniation phenotype. Interestingly, hernias are absent when *Gata4* has been deleted in the PPFs in the background of an amuscularized diaphragm. This clearly shows that herniation through the diaphragm cannot occur without adjacent, muscularized tissue and indicates that the important step for herniation is the juxtaposition of strong, muscularized tissue next to relatively weaker, amuscularized tissue. This imbalance creates a relative weak spot through which abdominal contents will herniate. In the complete absence of muscle, the strength of the connective tissue within the diaphragm is uniform, preventing herniation despite the deletion of *Gata4.* These experiments definitively show that the critical step in the development of CDH is the juxtaposition of amuscularized and muscularized regions, which are generated by the formation of amuscularized regions within a muscularized diaphragm.

Our experiments demonstrate that hernias form in amuscularized regions, so we looked earlier in development at muscle progenitors to see when defects in the muscle first arise. When *Gata4* is deleted in the PPFs, defects in muscularization occur early in development and are present by E12.5. At this time point, *Prx1Cre*$^{Tg/+}$*;Gata4*$^{\Delta/fl}$ mice show a defect in the number and localization of muscle precursors within the PPFs. Compared to control animals, mice with *Gata4* deleted specifically in the PPFs have a marked increase in apoptosis and a dramatic decrease in the number of proliferating myogenic cells. This demonstrates that PPF cells non-cell autonomously regulate myogenic cells. The decreased number of myogenic cells when *Gata4* is deleted in the PPFs demonstrates the critical role for PPF-derived connective tissue fibroblasts in myogenesis. It would be extremely informative to do an RNA sequencing screen to determine what signals are altered in PPF fibroblasts when *Gata4* is deleted. Determining what signals are secreted by the fibroblasts will advance our understanding of how fibroblasts normally promote myogenesis and could implicate additional signaling pathways in the formation of CDH.

The deletion of *Gata4* in the PPFs also produces mosaic defects in the localization of muscle precursors. In control animals, myogenic cells are interspersed with PPF cells. However, in mutant embryos, the muscle precursors appear to be excluded from regions in which *Gata4* has been deleted in connective tissue fibroblasts. Experiments testing deletion of *Gata4* with the *Tcf4*^{Cre}, which causes recombination largely after E12.5 (Mathew et al., 2011), indicate that muscle cells are only sensitive to *Gata4* loss when *Gata4* is deleted

early. This suggests that local amuscularized regions occur at the sites of the earliest recombination and deletion of *Gata4.* Neighboring muscularized regions could be produced from cells that either recombine later or do not recombine. Thus, early, mosaic deletion of *Gata4* in the PPF cells is critical for development of hernias. This requirement for mosaic genetic defects in the mouse to produce CDH suggests that humans may develop CDH from somatic mosaic mutations in the diaphragm. Mosaicism in human patients is supported by discordant occurrence of CDH in monozygotic twins and mosaic genetic mutations in CDH patients (Kantarci et al., 2010; Veenma et al., 2012). I predict that development of CDH in patients heterozygous for a CDH-gene would require mosaic loss of the second allele of this gene in PPF cells early in development. Mosiacism in patients would explain the variable expressivity and incomplete penetrance frequently observed in families of CDH patients, as not all family members would lose the second allele of the gene in the right time and place. Future studies of human patients should sequence both the amuscularized region that herniates as well as the muscularized regions to determine if somatic genetic mutations underlie CDH development in patients.

Now that we have a more complete understanding of the mechanisms that induce herniation when *Gata4* is deleted, it will be useful to determine if deletion of other CDH genes cause hernias by the same mechanism. Disruption of the retinoic acid (RA) pathway has been strongly implicated in causing CDH, so a useful gene to test would be *Aldh1a2* (Raldh2), which is required for the production of RA and is expressed in the PPFs (Clugston et al., 2010). It will be

informative to test whether Raldh2 is required within the PPFs, and whether herniation in this model would also be induced through aberrant muscle patterning. Testing other genes will help clarify the general mechanisms by which hernias form.

The Requirement for Wnt Signaling in Diaphragm Development

Human studies of patients with CDH associated with focal dermal hypoplasia (FDH) have suggested a role for Wnt signaling in diaphragm development. FDH is caused by mutations in Porcupine (*PORCN*), which is required for the secretion of Wnt ligands. To determine whether diaphragm development requires the secretion of Wnt ligands from PPF cells, we generated *Prx1Cre*$^{Tg/+}$*; Porcn*$^{fl/Y}$ mice to delete *Porcn* specifically within the PPFs. Ventral midline CDHs were present in all *Prx1Cre*$^{Tg/+}$*; Porcn*$^{fl/Y}$ mice, demonstrating that Wnt ligands secreted from PPF cells are required for diaphragm development. In these mice, PPF-derived cells still spread completely across the diaphragm, but the muscle failed to finish spreading ventrally and did not meet at the ventral midline. Surprisingly, reception of canonical Wnt signaling was not required in either the myogenic cells or connective tissue fibroblasts, as deletion of β-catenin, a central mediator of canonical Wnt signaling, in either tissue did not induce hernias. This suggests that noncanonical Wnt signaling is required from the PPFs. However, it is unclear what cells (muscle or PPF cells) need to receive noncanonical signals. The hernias generated by PPF-specific deletion of *Porcn*

demonstrate a role for Wnt signaling in the final stages of diaphragm development, and likely occur by a different mechanism than those generated by early deletion of *Gata4*. Further analysis of the *Prx1Cre*$^{Tg/+}$*; Porcn*$^{fl/Y}$ mice may provide insights into the normal process that regulates the complete migration of muscle to the midline and the final steps of diaphragm development.

Diaphragm Evolution

Despite the critical importance of the mammalian diaphragm muscle, the mechanism by which this muscularization evolved is unclear. To determine which developmental structures and processes required for development of a muscularized diaphragm were unique to mammals, we analyzed the chick, a nonmammal. In the developing chick, PPF-like structures are present and express the marker Tcf4, which is expressed in mammalian PPF cells. Although we have not definitively determined that these structures are homologous, this suggests that PPFs may be conserved. HGF, the ligand for the Met receptor (expressed in somitic muscle precursor cells), has been implicated in motility and proliferation of myogenic cells. HGF is expressed in the developing mammalian limb and PPF cells, and previous studies have shown that muscle fails to migrate into the developing limb and diaphragm in Met- animals (Bladt et al., 1995; Maina et al., 1996). The analogous chick structures do not express HGF; however, we have shown that muscle precursors from the chick somite express the HGF receptor Met, and are competent to respond to migratory signals. These data suggest that mammals acquired signals to direct muscle precursor migration to

the diaphragm. It is possible that gaining expression of HGF in the PPF was a critical step in the evolution of a muscularized diaphragm.

Future studies into the role of the PPFs and HGF expression in diaphragm development should focus on determining why HGF is expressed in the mammalian PPFs, further characterizing the chick PPF-like structures, and determining the role of HGF in diaphragm development. Comparing conservation of HGF enhancers between mammals and nonmammals may provide insight into the mechanism driving *HGF* expression in the mammalian PPFs. It would also be useful to determine if the PPF-analogous structures in the chick express other genes expressed in the mammalian PPFs, such as Gata4, Wt1, and COUP-TFII. Furthermore, whole-mount images of chick embryos stained by immunofluorescence for such markers will aid in the visualization of the 3-D structure of the chick PPF-like tissue and determining if it is really analogous to mammalian PPFs. The role of HGF in diaphragm development can be tested by conditionally deleting HGF in various tissues and at different time points in development, determining if HGF is required for targeting muscle to the diaphragm or maintaining an undifferentiated, proliferating pool of muscle progenitors.

Conclusion

Work in this dissertation demonstrates that the pleuroperitoneal fold-derived connective tissue fibroblasts are critical for all stages of diaphragm development. These cells may be instrumental in directing muscle precursors to the PPFs and diaphragm. During normal development, the PPFs signal to

regulate the proliferation and spreading of myogenic cells throughout the muscularized regions of the diaphragm. Genetic defects in the PPFs cause non-cell autonomous defects in the patterning of muscle, producing regions without muscle that give rise to congenital diaphragmatic hernias. Finally, Wnt signals from the PPF are also critical for muscle to migrate completely around the diaphragm and meet at the ventral midline. These results demonstrate that PPF-derived connective tissue fibroblasts are critical in all stages of diaphragm development and are crucial for human health, as defects in the PPFs generate congenital diaphragmatic hernias.

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