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Review

Striated-for-smooth muscle replacement in the developing mouse esophagus

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Summary. The esophagus is a muscular tube which transports swallowed content from the oral cavity and the pharynx to the stomach. Early in mouse development, an entire layer of the esophagus, the muscularis externa, consists of differentiated smooth muscle cells. Starting shortly after mid-gestation till about two weeks after birth, the muscularis externa almost entirely consists of striated muscle. This proximal-to-distal replacement of smooth muscle by the striated muscle depends on a number of factors. To identify the nature of the hypothetical "proximal" (mainly striated muscle originating) and "distal" (mainly smooth muscle originating) signals that govern the striated-for-smooth muscle replacement, we compared the esophagus of *Myf5:MyoD* null fetuses completely lacking striated muscle to the normal control using cDNA microarray analysis, followed by a comprehensive database search. Here we provide an insight into the nature of "proximal" and "distal" signals that govern the striated-for-smooth muscle replacement in the esophagus.

Key words: Esophagus, Myogenesis, Myogenic regulatory factors, cDNA microarrays

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Introduction

The esophagus is a segment of the alimentary (digestive) system which transports swallowed content from the oral cavity and the pharynx to the stomach. The wall of the mouse esophagus consists of mucosa, submucosa, muscularis externa (ME) and adventitia (connective tissue). The esophageal mucosa consists of nonkeratinized stratified squamous epithelium, connective tissue of the lamina propria and smooth muscle cells of the muscularis mucosae. The esophageal submucosa consists of connective tissue. The esophageal ME consists of two layers of muscle separated by connective tissue and the myenteric (Auerbach's) plexus. Early in mouse development, the entire ME consists of differentiated smooth muscle cells, but starting at embryonic day (E) 12.5 till about postnatal day (P) 14, the ME almost entirely consists of striated muscle and this proximal-to-distal replacement of smooth muscle by the striated muscle depends on a number of factors, including the myogenic regulatory factors such as *Myf5* and *MyoD* (Kablar et al., 2000; Reddy and Kablar, 2004 and the references therein).

The striated-for-smooth muscle replacement is a fascinating phenomenon whose orchestration, if disturbed, may have a number of clinically relevant implications collectively known as esophageal motility disorders. It is therefore the focus of our manuscript to contribute in revealing the molecular players that potentially have a role in: recruiting the striated muscle precursor cells, replacing differentiated smooth muscle cells by the striated muscle, and instructing other cellular and morphogenetic mechanisms underlying the striated-

for-smooth muscle replacement process.

It is a fundamental fact in histology that the body consists of only four basic tissue types: epithelium, connective tissue, muscle and nervous tissue. There is another example of tissue replacement during development, where the embryonic skeleton made of hyaline cartilage is replaced by the bone. While both the cartilage and the bone are connective tissues, skeletal-for-smooth muscle replacement is therefore the only example of a naturally occurring tissue replacement within the category of muscle basic tissue types (includes smooth and cardiac muscle).

Currently, the striated-for-smooth muscle replacement in the esophagus is described in the following manner (Krauss et al., 2016 and the references therein): *Mesp1* expressing cranial mesoderm progenitor cells, also expressing *Tbx1*, give rise to migratory *Isl1* expressing esophageal striated muscle cell progenitors. These cells arrive to the upper tip of the developing esophagus, within the developing ME, and express first *Pax7* (proliferating striated muscle-like progenitors) and then additionally *Myf5* and *MyoD* (committing to the striated muscle-like lineage muscle progenitors). This area, known as "transition zone", also contains differentiating and differentiated myoblasts (also *myogenin* expressing). The transition zone moves in proximal-to-distal direction, while smooth muscle cells undergo fascicular reorientation and are mostly located distal to the descending transition zone. The identity of "proximal" (promote movements distally) and "distal" (promote fascicular reorientation) signals is unknown, but the striated muscle cells in the transition zone may be the source of these signals.

It is important to state that there are other views on this process. For example, the presence of numerous apoptotic smooth muscle cells, described in the literature (Wörl and Neuhuber, 2005; Wörl et al., 2009), has not been entirely accounted for by the current view on the striated-for-smooth muscle replacement in the esophagus, and therefore there is a possibility that apoptosis is the main reason for the disappearance of smooth muscle cells during esophageal development. Another example is the hypothesis of smooth-to-striated muscle transdifferentiation, connected to the lack of observable apoptotic smooth muscle cells in the developing esophagus (Patapoutian et al., 1995; Kablar et al., 2000).

In fact, approximately two decades ago, we studied striated muscle development in the esophagus of *Myf5:MyoD* null embryos and fetuses and found that striated muscle in the esophagus cannot be found in the absence of these two myogenic regulatory factors (Kablar et al., 2000). In order to identify "proximal" and "distal" signals that govern the striated-for-smooth muscle replacement in the esophagus we compared the esophagus of *Myf5:MyoD* null fetuses completely lacking striated muscle to the normal control using cDNA microarray analysis and performed a comprehensive literature and database search and communicated with a number of scientists. Here we provide some insights into the nature of hypothetical "proximal" (striated muscle originating or "striated") and distal (smooth muscle originating or "smooth") signals that govern the striated-for-smooth muscle replacement in the esophagus.

Materials and methods

Animal breeding and fetal collection

Double-mutant (*Myf5-/-:MyoD-/-*) fetuses were obtained by the interbreeding of heterozygous (*Myf5+/-:MyoD+/-*) parents, as previously described (Rudnicki et al., 1993). All fetuses were collected by Cesarean section at E18.5 and genotyped by PCR using *Myf5* and *MyoD* primers (Inanlou and Kablar, 2005). In addition, the presence or absence of skeletal muscle was confirmed by myosin-fast immunostaining (data not shown). Animal use and care was in accordance with all institutional guidelines.

RNA isolation and analysis

Total esophagus RNA was isolated from two wild-type and two *Myf5-/-:MyoD-/-* fetuses using the RNeasy™ kit from Qiagen, Mississauga, Ont., Canada, according to the manufacturer's instructions. For each group of fetal esophagi (wild-type or mutant), RNA was pooled to minimize the effect of individual differences. Fluorescent labeling of cRNA fragments obtained from the pooled samples and their simultaneous hybridization to MOE430 2.0 GeneChip mouse genome arrays was performed at the Ottawa Genome Centre according to standard Affymetrix (Santa Clara, CA) protocols as described in Seale et al. (2004). The hybridized chips were then scanned and the results analyzed using the Affymetrix statistical expression algorithms to obtain the expression ratios and fold changes between the wild-type and double-mutant fetuses' esophagus.

Results

Microarray analysis identified a large number of genes that were differentially expressed between the control and the double-mutant esophagus, and an arbitrary cut-off value of 4-fold was chosen as a means of determining the up- and down-regulated probesets, respectively. A total of 133 named genes met this criterion. Thirty-five genes were up-regulated more than 4-fold (Table 1), whereas a total of 98 were down-regulated (Table 2). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE122017 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE122017).

Tables 1 and 2 also show that a great majority of the named genes are measurably expressed in the skeletal

muscle and small intestine (smooth muscle) of the normal adult mouse (Su et al., 2002).

Our next step was to identify for each of the 133 named genes a genetically engineered (mostly knockout) mouse and their phenotypes. We searched the MGI (www.informatics.jax.org) and PubMed (https://www.ncbi.nlm.nih.gov/pubmed) to gather this information. Seven up-regulated genes had a knockout and several were displaying the potentially relevant phenotype (Table 3), whereas 43 down-regulated genes had a knockout and several were displaying the potentially relevant phenotype (Table 4). However, no direct evidence was found regarding the "esophageal" phenotype.

Considering that, after detailed analysis and reading of the available literature, it was still not possible to detect any esophagus-specific phenotype in the knockout mice identified, we decided to contact the authors of the relevant papers (as listed in Tables 3 and 4). It was not clear from the papers if the topic was not studied or simply not described, since in many cases describing an esophageal phenotype would not be relevant to the topic of the original publication. In other words, the papers listed in Tables 3 and 4 contained only the aspects of the knockout mouse phenotype relevant to the original scope of the paper, and that scope was not the esophagus. This is why we assumed that the esophagus-related data may be known, but have remained unreported due to the lack of their relevance to the topic of the original publication. With that in mind, we communicated with all the principal authors of the knockout mice publications from Tables 3 and 4, and asked specific, esophagus-related phenotype questions. The esophagus-related phenotype questions pertained to any morphological or/and functional alterations in the esophagus: dysphagia (difficulty swallowing), achalasia (impaired esophageal

Table 1. Genes up-regulated ≥4-fold in E18.5 *Myf5-/-:MyoD-/-* mutant mouse esophagus, sorted by function and log2 (ratio) expression value.

Gene	log_2 (ratio)	Gene Title	SM[a]	SI	Molecular Function
Mcm6	4.15	minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)	568	3366	Transcription Factor Activity
2810047C21Rik	4.02	RIKEN cDNA 2810047C21 gene	195	162	
Eif2s3y	7.38	eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	2523	1006	Catalytic Activity
Ttc41	5.48	tetratricopeptide repeat domain 41	39	31	
Ddx3y	5.26	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	107	90	
Trim12a	4.56	tripartite motif-containing 12A	96	88	
Arg2	4	arginase type II	64	51530	
Ibsp	5.34	integrin binding sialoprotein	101	95	Structural or Cytoskeletal and Cell Adhesion Activity
Cadm2	5.33	cell adhesion molecule 2	243	219	
Col4a6	4.31	collagen, type IV, alpha 6	52	43	
Cplane1	4.03	ciliogenesis and planar polarity effector 1	119	116	
LOC665506	4.83	(T-cell receptor beta-2 chain C region-like)	NA[c]	NA	Receptor and Signal Transduction Activity
Wnt9b	4	wingless-type MMTV integration site 9B	56	53	
Lman1	5.18	lectin, mannose-binding, 1	1841	3920	
March4	4.05	membrane-associated ring finger (C3HC4) 4	NA	NA	
Mroh7	5.37	maestro heat-like repeat family member 7	121	157	Immune Response
H2-T24	4.34	histocompatibility 2, T region locus 24	142	128	
Spesp1	4.8	sperm equatorial segment protein 1	115	99	Other Processes (fertilization, blood clotting, cell cycle)
Glcci1	4.67	glucocorticoid induced transcript 1	972	1302	
F9	4.41	coagulation factor IX	44	41	
Slxl1	4.22	Slx-like 1	605	329	
Astx	5.39	amplified spermatogenic transcripts X encoded	NA	NA	Not yet specified
4921504E06Rik	5.01	RIKEN cDNA 4921504E06 gene	56	52	
8030497O21Rik	4.87	RIKEN cDNA 8030497O21 gene	55	53	
Urm1	4.61	ubiquitin related modifier 1 homolog (S. cerevisiae)	184	259	
Cnnm1	4.36	cyclin M1	62	59	
9330175H22Rik	4.43	RIKEN cDNA 9330175H22 gene	138	368	
A730041O05Rik	4.34	RIKEN cDNA A730041O05 gene	NA	NA	
8430437N05Rik	4.29	RIKEN cDNA 8430437N05 gene	101	92	
5430437J10Rik	4.28	RIKEN cDNA 5430437J10 gene	85	82	
D16Ertd778e	4.19	DNA segment, Chr 16, ERATO Doi 778, expressed	NA	NA	
6330571C24Rik	4.18	RIKEN cDNA 6330571C24 gene	89	79	
4930563E18Rik	4.14	RIKEN cDNA 4930563E18 gene	94	88	
4930549O18Rik	4.03	RIKEN cDNA 4930549O18 gene	208	160	
4930523O13Rik	4	RIKEN cDNA 4930523O13 gene	129	117	

[a]: expression (arbitrary units) in the skeletal muscle (SM). [b]: small intestine (SI), of the normal adult mouse (Su et al., 2002). [c]: NA: data not available.

peristalsis and a lack of lower esophageal sphincter relaxation during swallowing), gastroesophageal reflux, and the presence of striated muscle, muscular dystrophies and myopathies. Some of these symptoms could also result in failure to thrive, pneumonias and an early (e.g., neonatal) death, which were in some cases

Table 2. Genes down-regulated ≥4-fold in E18.5 *Myf5-/-:MyoD-/-* mutant mouse esophagus, sorted by function and log_2 (ratio) expression value.

Gene	log_2 (ratio)	Gene Title	SM[a]	SI[b]	Molecular Function
Tceal7	-8.7	transcription elongation factor A (SII)-like 7	NA[c]	NA	
Lmcd1	-6.26	LIM and cysteine-rich domains 1	6433	54	
Pax7	-5.69	paired box gene 7	56	53	
Smyd1	-5.13	SET and MYND domain containing 1	1312	117	
Vgll2	-4.78	vestigial like 2 homolog (Drosophila)	541	102	
Nolc1	-4.71	nucleolar and coiled-body phosphoprotein 1	1011	1135	
Egr2	-4.44	early growth response 2	129	107	Transcription Factor Activity
Prox1	-4.42	prospero-related homeobox 1	148	137	
Jrk	-4.4	jerky	59	55	
Shox2	-4.21	short stature homeobox 2	83	75	
Myog	-4.12	myogenin	233	391	
Hbp1	-4.11	high mobility group box transcription factor 1	6079	1771	
Isl1	-4.03	ISL1 transcription factor, LIM/homeodomain	50	48	
Onecut2	-4.01	one cut domain, family member 2	49	47	
Myh1	-10.1	myosin, heavy polypeptide 1, skeletal muscle, adult	26932	78	
Tnnc2	-9.83	troponin C2, fast	472085	59	
Ttn	-8.3	titin	15190	1446	
Neb	-7.92	nebulin	NA	NA	
Myh3	-7.71	myosin, heavy polypeptide 3, skeletal muscle, embryonic	NA	NA	
Mybph	-7.57	myosin binding protein H	24299	65	
Myl3	-7.24	myosin, light polypeptide 3	34937	120	
Tnni1	-7.21	troponin I, skeletal, slow 1	1125	42	
Tpm3	-7.2	tropomyosin 3, gamma	7002	41892	
Myom2	-7.12	myomesin 2	25233	47	
Myh8	-7	myosin, heavy polypeptide 8, skeletal muscle, perinatal	389	145	
Acta1	-6.51	actin, alpha 1, skeletal muscle	418196	460	
Myl1	-6.22	myosin, light polypeptide 1	473430	397	Structural, Cytoskeletal, and Motor Activity
Mylpf	-6.19	myosin light chain, phosphorylatable, fast skeletal muscle	315081	35	
Myoz2	-6.13	myozenin 2	8363	43	
Myh6	-5.79	myosin, heavy polypeptide 6, cardiac muscle, alpha	109	103	
Tnnc1	-5.25	troponin C, cardiac/slow skeletal	21025	62	
Lmod2	-5.19	leiomodin 2 (cardiac)	1280	135	
Myot	-4.92	myotilin	132172	57	
Myom3	-4.81	myomesin family, member 3	87	86	
Cobl	-4.78	cordon-bleu	212	15803	
Synpo2l	-4.69	synaptopodin 2-like	4140	222	
Ldb3	-4.66	LIM domain binding 3	1435	67	
Nefl	-4.46	neurofilament, light polypeptide	NA	NA	
Smpx	-4.4	small muscle protein, X-linked	86156	199	
Sgcg	-4.03	sarcoglycan, gamma (dystrophin-associated glycoprotein)	1021	112	
Wif1	-7.16	Wnt inhibitory factor 1	141	119	
Cox6a2	-7.15	cytochrome c oxidase, subunit VI a, polypeptide 2	108673	70	
Art1	-6.44	ADP-ribosyltransferase 1	75	71	
Akr1c14	-6.25	aldo-keto reductase family 1, member C14	310	352	
Dhrs7c	-6.05	dehydrogenase/reductase (SDR family) member 7C	6131	44	
Pgam2	-5.08	phosphoglycerate mutase 2	133394	45	
Capn1	-4.97	calpain 1	50	142	
Trim63	-4.68	tripartite motif-containing 63	185	127	Catalytic Activity
Mark1	-4.43	MAP/microtubule affinity-regulating kinase 1	38	36	
Srl	-4.39	sarcalumenin	133087	147	
Acsl4	-4.37	acyl-CoA synthetase long-chain family member 4	184	158	
Padi3	-4.33	peptidyl arginine deiminase, type III	44	44	
Mettl22	-4.17	methyltransferase like 22	NA	NA	
Psmd6	-4.15	proteasome (prosome, macropain) 26S subunit, non-ATPase, 6	18861	4256	
Tecrl	-4.11	trans-2,3-enoyl-CoA reductase-like	242	182	

indicated by the authors in Tables 3 and 4.

Here are the data obtained via "personal communications." The most striking esophageal phenotypes were considered those with the "complete lack of striated muscle" as observed in *Mylpf* nulls (communicated by Yingcai Wang, ywang@med.miami.edu), or with "almost complete lack of striated muscle" in *Acta1* nulls (James L. Lessard, james.lessard@cchmc.org; Kristin Nowak, kristen.nowak@perkins.uwa.edu.au) and in *Myog* nulls (Frank W. Booth, boothf@missouri.edu) and, finally, with "significantly reduced striated muscle" in *Pax7* nulls (Peter Gruss, peter.gruss@gv.mpg.de). In fact, it has been recently shown that PAX7 is required for patterning of the esophageal musculature (Chihara et al., 2015). Several years earlier, it was shown that the deletion of *Pax7* changes the mouse esophageal ME from a striated to a mixed smooth and striated muscle phenotype (Wörl et al., 2009). Finally, the "striated muscle damage" was communicated for *Ryr1* nulls (Hiroshi Takeshima,

Table 2. (Continued).

Gene	$\log_2$ (ratio)	Gene Title	SM[a]	SI[b]	Molecular Function
Actn2	-7.74	actinin alpha 2	44643	62	
Trdn	-7.64	triadin	75964	232	
Cavin4	-6.68	caveolae associated 4	6475	72	
Sln	-6.4	sarcolipin	NA	106	
Arhgap36	-5.26	Rho GTPase activating protein 36	198	155	Signal Transduction Activity
Prex2	-5.1	phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2	NA	NA	
Hbs1l	-4.76	Hbs1-like (S. cerevisiae)	6494	3783	
Csrp3	-4.48	cysteine and glycine-rich protein 3	4106	152	
LOC100047138	-4.24	(similar to tescalcin)	NA	NA	
Khdrbs3	-4.15	KH domain containing, RNA binding, signal transduction associated 3	2024	542	
Ryr1	-7.12	ryanodine receptor 1, skeletal muscle	98446	62	
Atp1b4	-6.9	ATPase, (Na+)/K+ transporting, beta 4 polypeptide	69	65	
Hfe2	-6.52	hemochromatosis type 2 (juvenile) (human homolog)	74564	141	
Hbb-y	-5.67	hemoglobin Y, beta-like embryonic chain	NA	NA	Receptor or Channel and Transport or Carrier Activity
Mmgt1	-5.32	membrane magnesium transporter 1	4883	1438	
Chrna1	-5.31	cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle)	51	49	
Abcb7	-4.53	ATP-binding cassette, sub-family B (MDR/TAP), member 7	1293	448	
Atp2a1	-4.34	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	694632	170	
Ehd1	-4.29	EH-domain containing 1	105	108	
Nkap	-4.95	NFKB activating protein	112	114	
Adig	-4.77	adipogenin	55	74	Regulation of Cell Differentiation
Unc45b	-4.7	unc-45 homolog B (C. elegans)	7698	92	
Klhl41	-9.47	kelch-like 41	NA	NA	
Mymx	-6.69	myomixer, myoblast fusion factor	NA	NA	
Txlnb	-6.65	taxilin beta	36743	87	
Iffo1	-6.43	intermediate filament family orphan 1	84	57	
Fndc5	-5.25	fibronectin type III domain containing 5	60	37	
5730526G10Rik	-5.04	RIKEN cDNA 5730526G10 gene	NA	NA	
AA672641	-5.04	expressed sequence AA672641	NA	NA	
Lmod3	-5	leiomodin 3 (fetal)	11763	107	
Fndc3c1	-5	fibronectin type III domain containing protein 3C1	77	71	
Lrrn1	-4.96	leucine rich repeat protein 1, neuronal	206	191	
LOC100044533	-4.94	(similar to Zic protein zinc finger protein of the cerebellum 1)	NA	NA	Not yet specified
Ccdc186	-4.8	coiled-coil domain containing 186	678	2965	
Lrrc41	-4.6	leucine rich repeat containing 41	103	196	
Thsd7b	-4.53	thrombospondin, type I, domain containing 7B	258	167	
Mon1b	-4.4	MON1 homolog b (yeast)	147	143	
Cenpl	-4.4	centromere protein L	NA	NA	
AI131651	-4.37	expressed sequence AI131651	NA	NA	
Fam25c	-4.29	family with sequence similarity 25, member c	268	226	
Map3k7cl	-4.16	Map3k7 C-terminal like	69	65	
1110002E22Rik	-4.14	RIKEN cDNA 1110002E22 gene	NA	NA	
4930522H14Rik	-4	RIKEN cDNA 4930522H14 gene	49	45	

[a]: expression (arbitrary units) in the skeletal muscle (SM). [b]: small intestine (SI), of the normal adult mouse (Su et al., 2002). [c]: NA: data not available.

takeshim@m.u.tokyo.ac.jp) and an "aberrant diaphragm" was communicated for *Atp2a1* nulls (David H. MacLennan, david.maclennan@utoronto.ca).

The next group of communicated data relate to the disturbances in muscle function. For example, "progressive muscle weakness" was observed in Neb nulls (communicated by Siegfried Labeit, Labeit@embl.de), whereas "impaired muscle function" was communicated for *Trdn* nulls (Isabelle Marty, isabelle.marty@ujf-grenoble.fr) and for *Srl* nulls (Hiroshi Takeshima, takeshim@mail.tains.tohoku.ac.jp).

"Myopathy" and "dystrophy" were communicated for *Ehd1* nulls (Hamid Band, hband@unmc.edu) and *Sgcg* nulls (Elizabeth M. McNally, elizabeth.mcnally@northwestern.edu), respectively. "Myopathy with dysphagia" was communicated for *Ldb3* nulls (Ju Chen, juchen@ucsd.edu). Potentially dystrophy-related "motor coordination" problems were observed in *Prex2* nulls (Heidi C.E. Welch, heidi.welch@babraham.ac.uk). In fact, we performed routine, hematoxylin and eosin stained, histological analysis of P0 and P7 *Prex2*-/- esophagi, including wildtype and heterozygote controls (kindly provided by Dr. Heidi C.E. Welch), and did not find any obvious esophageal phenotype. (N.B., we observed some signs of muscular dystrophy in several muscles of the *Prex2* nulls head, but these findings are outside of the scope of this manuscript.)

Furthermore, several phenotypes were related to problems with innervation. For example, a "neuromuscular" phenotype was communicated for *Chrna1* nulls (Kuo-Fen Lee, klee@salk.edu), while "motor innervation" problems were communicated for *Nefl* nulls (Jean-Pierre Julien, jean-pierre.julien@fmed.ulaval.ca).

Surprisingly, "excess of striated muscle fibers" was communicated for *Myoz2* nulls (Frank W. Booth, boothf@missouri.edu), whereas "disturbed smooth muscle function" was communicated for *Myh1* nulls (Leslie A Leinwand, Leslie.Leinwand@colorado.edu).

Several mutant mice were "neonatal lethal" or had "impaired growth" or/and a "failure to thrive," with consequent early lethality. These are: *Lman1* nulls (Bin Zhang, zhangb@ccf.org), *Wnt9b* nulls (Andrew P. McMahon, mcmahona@usc.edu), *Klhl41* (Ramirez-Martinez et al., 2017), *Mymx* (Quinn et al., 2017), Erg2 nulls (Thomas Gridley, gridlt@mmc.org; Patrick Charnay, patrick.charnay@ens.fr), *Jrk* nulls (Miklos Toth, mtoth@med.cornell.edu), and *Onecut2* nulls (Frederic Clotman, frederic.clotman@uclouvain.be; Frederic P. Lemaigre, lemaigre@horm.ucl.ac.be). However, we performed routine histological analyses (hematoxylin and eosin staining) of E16.5, E17.5 and P0 *Lman1*-/- esophagi, including wildtype and heterozygote controls (kindly provided by Dr. Bin Zhang), and did not identify any obvious esophageal phenotype.

There were a number of mutant mice that were "embryonic lethal" at various stages: E8.5 (*Mcm6* nulls: John C. Schimenti, jcs92@cornell.edu; *Myl1* nulls: Nadia Rosenthal, nadia.rosenthal@jax.org; *Nkap* nulls: Virginia Smith-Shapiro, shapiro.virginia1@mayo.edu; *Abcb7* nulls: Mark D Fleming, mark.fleming@childrens.harvard.edu), E8.5-12.5 (*Tpm3* nulls: Peter Gunning, p.gunning@unsw.edu.au; David Wieczorek, david.wieczorek@uc.edu), E9.5 (*Unc45b* nulls: www.informatics.jax.org(a)ref), E10.5 (*Smyd1* nulls: Paul D. Gottlieb, gottlieb@uts.cc.utexas.edu), E11.5 (*Isl1* nulls: Thomas M Jessell, tmj1@columbia.edu), E13.5 (*Myh6* nulls: Jeffrey Robbins, Jeff.Robbins@chmcc.org), E14.5 (*Shox2* nulls: Adriana C. Gittenberger-de Groot, acgitten@lumc.nl), and E15.5 (*Prox1* nulls: Guillermo Oliver, guillermo.oliver@stjude.org).

Lastly, there were a number of mutants whose authors communicated that an esophageal phenotype would be "unlikely" (based on other features of the phenotype, such as growth, feeding behavior, etc.) in their mice even if examined: *Ibsp* (Malaval et al., 2008), *Spesp1* nulls (Masaru Okabe, okabe@gen-info.osaka-u.ac.jp), *F9* nulls (Darrel W. Stafford, dws@email.unc.edu), *Col4a6* nulls (Yoshi Ninomaya, yoshinin@cc.okayama-u.ac.jp), *Arg2* nulls (Jaye P.F. Chin-Dusting, jaye.chin-dusting@bakeridi.edu.au), *Ttn* nulls (Michael Gotthardt, gotthardt@mdc-berlin.de), *Wif1* nulls (David M. Thomas, david.thomas@petermac.org), *Cox6a2* nulls

Table 3. Genes up-regulated ≥4-fold in E18.5 *Myf5-/-:MyoD-/-* mutant mouse esophagus with knockout (null mutant) mouse models (in order of $\log_2$ ratio).

Gene	Comments on Deletion Mutants	Reference
Ibsp	Bone is undermineralized in fetuses and young adults	Malaval et al., 2008
Lman1	Preweaning lethality, with dilated endoplasmic reticulum in hepatocytes	Zhang et al., 2011
Spesp1	Males show decreased fertilization frequency and delayed fertilization	Fujihara et al., 2010
F9	Premature death due to abnormal blood coagulation; reduced levels of factor IX	Lin et al., 1997
Col4a6	Viable, fertile and healthy with no apparent phenotypic defects	Fox et al., 2007
Mcm6	Prenatal or premature lethality, chromosomal instability, abnormal erythrocyte morphology, increased tumor incidence	Chuang et al., 2010; Pruitt et al., 2007
Wnt9b	Neonatal lethality, disrupted ureteric bud branching, impaired Mullerian duct formation, and incompletely penetrant cleft lip and palate	Carroll et al., 2005
Arg2	Viable, but with hypertension and elevated plasma arginine levels	Shi et al., 2001; Huynh et al., 2009

Table 4. Genes down-regulated ≥ 4-fold in E18.5 *Myf5-/-:MyoD-/-* mutant mouse esophagus with knockout (null mutant) mouse models (in order of log2 ratio).

Gene	Comments on Deletion Mutants	Reference
Myh1	Kyphosis, reduced growth, muscular weakness, and abnormal kinetics of muscle contraction and relaxation	Acakpo-Satchivi et al, 1997
Klhl41	Defective sarcomeric formation; neonatal death	Ramirez-Martinez et al., 2017
Ttn	Embryogenesis defects; vascular, cardiac and skeletal muscle defects causing growth retardation, muscle weakness, abnormal posture, and death between embryonic day 11.5 and 8 weeks of age	Lane, 1985; Weinert et al., 2006
Neb	Growth retardation, kyphosis, abnormal gait, progressive muscle weakness, and death within 3 weeks	Witt et al., 2006
Trdn	Viable and fertile but with impaired skeletal muscle function	Oddoux et al., 2009
Tpm3	Early embryonic death, prior to blastocyst formation	Hook et al., 2004
Wif1	Viable and fertile but highly susceptible to osteosarcomas	Kansara et al., 2009
Cox6a2	Cardiac dysfunction due to abnormal ventricular filling or diastolic dysfunction under maximal cardiac load	Radford et al., 2002
Ryr1	Skeletal abnormalities, fragmented muscle fibers, and perinatal death from respiratory failure	Takeshima et al., 1994
Mymx	Abnormal skeletal muscle morphology; neonatal death	Quinn et al., 2017
Hfe2	Decreased hepcidin expression, severe iron overload, and male sterility	Niederkofler et al., 2005
Acta1	Scoliosis, reduced body weight/size, atrophy of brown adipose tissue, depleted glycogen stores, muscle weakness, and death by postnatal day 10	Crawford et al., 2002
Sln	Increased cardiac contractility, with no apparent change in cardiac muscle morphology	Babu et al., 2007
Myl1	Developmental delay, failure to form mesoderm, and death by embryonic day 8.5	Jiang et al., 2002
Mylpf	Completely lacking skeletal muscle, all die immediately after birth, presumably due to respiratory failure	Wang et al., 2007
Myoz2	Excess of skeletal muscle fibers; cardiac hypertrophy when chronically stressed	Frey et al., 2004
Myh6	Embryonic death by day 13, associated with gross heart defects	Jones et al., 1996
Pax7	Retarded growth, muscle weakness, craniofacial malformations, abnormal gland morphology, and death within three weeks	Mansouri et al., 1996
Hbb-y	Viable, with a normal phenotype through adulthood	Hu et al., 2007
Chrna1	Neonatal lethality, kyphosis, carpoptosis, abnormal endplate potential, increased motor neuron number, and abnormal neuromuscular synapse morphology	An et al., 2010
Lmod2	Contractile dysfunction and dilated cardiomyopathy; death within a month	Pappas et al., 2015
Smyd1	Enlarged heart, and developmental abnormalities of the right ventricle; embryonic death at day 10.5	Gottlieb et al., 2002
Prex2	Motor coordination defects, more pronounced in females, progressively worsening with age	Donald et al., 2008
Capn1	Decreased platelet aggregation and defective clot retraction	Azam et al., 2001
Nkap	Thymus hypoplasia and impaired T cell differentiation with decreased total thymocytes	Pajerowski et al., 2009
Myot	Normal lifespan and fertility, and no abnormal phenotype detected	Moza et al., 2007
Cobl	Exencephaly due to defects in neural tube closure	Carroll et al., 2003
Unc45b	Embryonic lethality at day 9 without placental abnormalities	www.informatics.jax.org(a)
Trim63	Cardiac hypertrophy, and most die in two weeks with heart failure	Witt et al., 2008
Ldb3	Myopathy, dysphagia, heart vascular congestion, dilated heart ventricles, cyanosis, respiratory distress, and death within a few days after birth	Zhou et al., 2001
Abcb7	Hemizygous male and heterozygous female mice carrying a maternally inherited null allele display prenatal lethality	Pondarre et al., 2006
Csrp3	Heart ventricle dilation, hypertrophy and fibrosis, decreased contractility, and premature death	Arber et al., 1997
Nefl	Lack of neurofilaments in the axons, and motor axons are reduced in both size and number	Zhu et al., 1997
Egr2	Defective axonal migration, disrupted myelination of Schwann cells, slow respiratory and jaw opening rhythms, skeletal abnormalities, and perinatal lethality	Swiatek and Gridley, 1993
Padi3	Morphological alterations in the hair coat	Basmanav et al., 2016
Prox1	Death by embryonic day 15 with impaired development of the lens, lymphatic system, liver and pancreas	Wigle and Oliver, 1999
Smpx	No apparent defects in heart or skeletal muscle morphology or development	Palmer et al., 2001
Jrk	Elevated seizure susceptibility, impaired postnatal growth, reduced life span, male sterility and impaired female fertility	Toth et al., 1995
Srl	Impaired calcium store functions in skeletal and cardiac muscle cells, resulting in slow contraction and relaxation phases	Yoshida et al., 2005
Acsl4	Female heterozygotes exhibit accumulation of prostaglandins in the uterus, reduced fertility with few and small litters, and very low transmission of the mutant allele	Cho et al., 2001
Atp2a1	Respiratory distress, progressive cyanosis, and death within 2 hours after birth, the lung tissues and diaphragm muscle showing aberrant morphology	Pan et al., 2003
Ehd1	Perinatal and postnatal lethality, decreased body weight, and male infertility due to defective spermatogenesis	Rainey et al., 2010
Shox2	Abnormal heart development and pericardial edema, death by embryonic day 14	Blaschke et al., 2007
Myog	Kyphosis, muscle hypoplasia, no spontaneous movement, and death within minutes due to respiratory failure	Hasty et al., 1993; Tseng et al., 2000
Sgcg	Abnormalities in muscles and heart similar to muscular dystrophy	Hack et al., 1998
Isl1	Abnormal heart and pancreas development, failure to develop motor neurons, and death by embryonic day 11.5	Pfaff et al., 1996
Onecut2	Abnormal liver and pancreas development, failure to thrive	Clotman et al., 2005; Dusing et al., 2010

(Daniel J. Garry, garry@umn.edu), *Hfe2* nulls (Silvia Arber, silvia.arber@unibas.ch), *Sln* nulls (Muthu Periasamy, periasamy.1@osu.edu), *Hbb-y* nulls (Steven Fiering, fiering@dartmouth.edu), *Lmod2* (Pappas et al., 2015), *Capn1* nulls (Athar H. Chishti, Athar_Chishti@cchcs.org), *Myot* nulls (Monia Moza, monica.moza@helsinki.fi), *Cobl* nulls (John Klingensmith, kling@cellbio.duke.edu), *Trim63* nulls (Siegfried Labeit, Labeit@embl.de), *Csrp3* nulls (Silvia Arber, silvia.arber@unibas.ch), *Padi3* (U Basmanav et al., 2016), *Smpx* nulls (Richard P. Harvey, r.harvey@victorchang.unsw.edu.au), and *Acsl4* nulls (Tokuo T. Yamamoto, tomoko-y@faculty.chiba-u.jp).

Discussion

In order to reveal molecular players with a potential role in esophageal muscle development, we performed cDNA microarray analysis comparing *Myf5:MyoD* null esophagi with no striated muscle (Kablar et al., 2000) to the wild-type control esophagi at E18.5. We obtained a profile of genes potentially relevant to the developing esophageal striated muscle (Tables 1, 2). The differential expression patterns obtained by the microarray analysis resulted in a large number of genes, so we added another criterion, the transcriptome expression pattern (Su et al., 2002), as shown in Tables 1, 2. Lastly, we added the third criterion, the esophageal (and esophagus-related) phenotype, as published on PubMed (Tables 3, 4) or personally communicated, to gain insight into the knockout mouse phenotypes relevant to the esophageal development for each of the differentially expressed genes, when available. It is important to realize that some histopathologic and other phenotypic changes reported here as "personal communication" are not necessarily specific to the esophagus alone. For example, the dystrophy-related "motor coordination" change in the *Prex2* nulls does not relate to any esophageal phenotype.

Considering that the gene up- or down-regulation and the transcriptome expression level are criteria that are not as reliable as the knockout mouse phenotype, we decided to form conclusions primarily based on the phenotype and only secondarily (as additional, supporting information) on the level of up- or down-regulation and transcriptome expression.

Using these criteria, we propose that the potential candidates for the "proximal" or striated muscle originating signals ("striated") are: *Mylpf* (complete lack of striated muscle in the esophagus, down-regulated 6.19 times, and more than 300,000 transcriptome expression level in the striated muscle and almost none in smooth muscle), *Acta1* (almost complete absence of striated muscle in the esophagus, down-regulated 6.51 times, and more than 400,000 transcriptome expression level in striated muscle and very low in smooth muscle), *Myog* (almost complete lack of striated muscle in the esophagus, down-regulated 4.12 times), *Pax7* (significantly reduced striated muscle in the esophagus, down-regulated 5.69 times) and *Ryr1* (striated muscle damage in the esophagus, down-regulated 7.12 times, and almost 100,000 transcriptome expression level in striated muscle and very low in smooth muscle). *Myf5, MyoD* and *Mrf4* should be included in this group, considering the amount of strong evidence previously reported that supports their involvement in skeletal and esophageal striated muscle development (Rudnicki et al., 1993; Kablar et al., 2000; Reddy and Kablar, 2004; Kassar-Duchossoy et al., 2004).

Several other candidates for the "proximal" or "striated" signals could be included, based on striated muscle function, disease (e.g., myopathy and/or dystrophy) or innervation pathologies (phenotypes): *Neb* (progressive muscle weakness, down-regulated 7.92 times), *Trdn* (impaired muscle function, down-regulated 7.64 times, and almost 100,000 transcriptome expression level in the striated muscle and very low in smooth muscle), *Srl* (impaired muscle function, down-regulated 4.39 times, and more than 100,000 transcriptome expression level in striated muscle and very low in smooth muscle), *Ehd1* (myopathy and dystrophy, down-regulated 4.29 times), *Sgcg* (myopathy and dystrophy, down-regulated 4.03 times, and more than 1,000 transcriptome expression level in striated muscle and very low in smooth muscle), *Ldb3* (myopathy with dysphagia, down-regulated 4.66 times, and more than 1,000 transcriptome expression level in striated muscle and very low in smooth muscle). *Chrna1* (neuromuscular pathology, down-regulated 5.31 times), and *Nefl* (motor innervation pathology, down-regulated 4.46 times). The last two genes were included in this list because motor innervation (e.g., the neuromuscular junction development) and striated-for-smooth muscle replacement are interconnected developmental events in the esophagus (Reddy and Kablar, 2004).

The potential candidates for the "distal" or smooth muscle originating signals ("smooth") are: *Myoz2* (excess of striated muscle, down-regulated 6.13 times) and *Myh1* (smooth muscle pathology, down-regulated 10.1 times). However, contrary to our logic behind the criteria employed here, *Myh1* has almost 30,000 transcriptome expression level in striated muscle and very low (and should have been very high) in smooth muscle (Table 2), however it was included here based on phenotype, the strongest of the three groups of criteria.

Genes listed in conjunction with the neonatal lethality, impaired growth or failure to thrive and embryonic lethality are not considered further. This phenotypic information is not sufficiently specific at this point in time, because it is difficult to envision a mechanism connecting the genes in question to esophageal muscle development in the absence of further esophagus-specific information. Therefore, additional studies, involving a large number of participants (e.g., via the IMPC, International Mouse Phenotyping Consortium), are required to obtain more detailed information on the potential involvement of each of the identified genes in processes that are specific

to esophageal muscle development. In fact, in our recent publication we discussed future directions for an approach of study analogous to the current one (Baguma-Nibasheka et al., 2016).

Lastly, the involvement of neurotrophic factors, as previously reported (Reddy and Kablar, 2004), was not confirmed in the current study, in spite of the fact that NT-3 appeared to be involved in esophageal striated muscle development (Reddy and Kablar, 2004; Angka and Kablar, 2007, 2009 and data not shown).

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