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From Cell Biology to Tissue Engineering

Review

Role of skeletal muscle in motor neuron development

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Summary. The current paper is a continuation of our work most recently described in Kablar, 2011. Here, we show lists of up- and down-regulated genes obtained by a cDNA microarray analysis that compared developing mouse *MyoD*-/- limb musculature (MyoD-dependent, innervated by Lateral Motor Column motor neurons) and *Myf5*-/- back (epaxial) musculature (Myf5-dependent, innervated by Medial Motor Column motor neurons) to the control and to each other, at embryonic day 13.5 which coincides with the robust programmed cell death of motor neurons and the inability of myogenesis to undergo its normal progression in the absence of *Myf5* and *MyoD* that at this embryonic day cannot substitute for each other. We wanted to see if/how the myogenic program couples with the neurotrophic one, and also to separate Lateral from Medial column trophic requirements, potentially relevant to Motor Neuron Diseases with the predilection for the Lateral column. Several follow-up steps revealed that *Kif5c, Stxbp1* and *Polb*, differentially expressed in the *MyoD*-/- limb muscle, and *Ppargc1a, Glrb* and *Hoxd10*, differentially expressed in the *Myf5*-/- back muscle, are actually regulators of motor neuron numbers. We propose a series of follow-up experiments and various ways to consider our current data.

Key words: *MyoD*, *Myf5*, Myogenesis, Neurotrophins, Motor neuron, Microarray

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General introduction

Most of the research in our laboratory has focused on the epigenetic (N.B., this term is employed in the Waddingtonian sense) influence of skeletal muscle on the development of various tissues and organs, with much of the work arising from the existence of a mouse embryo and fetus that completely lacks skeletal muscle, due to the knock-out of two myogenic regulatory factors (MRFs), *Myf5* and *MyoD* (Rudnicki et al., 1993). (N.B., in fact, it has been shown that MRF4 is also a primary MRF, and therefore had been eliminated in these mutants; Kassar-Duchossoy et al., 2004) We have thus far used this *Myf5*-/-:*MyoD*-/- double mutant to examine the influence of developing skeletal muscle activity on the development of, among others, the motor neurons in general (Kablar and Belliveau, 2005; Stephens et al., 2005; Geddes et al., 2006; Angka and Kablar, 2007, 2009; Angka et al., 2008), and this particular review considers the action of muscle-derived neurotrophic factors on the central nervous system (CNS) during development and in conditions that involve neuronal degeneration and/or loss. Keeping in mind that the definition of motor neuron diseases (MNDs), such as amyotrophic lateral sclerosis (ALS), or Lou Gehrig's Disease, includes the death of both the upper and lower motor neurons, we proposed that the muscles in the motor neurons' microenvironment (N.B., motor neuron axons and skeletal muscle are physically connected via synapses), which may act as a provider of trophic factors for the survival of those neurons, could be vital to the pathogenesis of the related MNDs. For instance, whereas the intrinsic properties of the neurons themselves can lead to the death of motor neurons, as in the superoxide

dismutase 1 o*r SOD1*-/- mouse (Ripps et al., 1995), the microenvironment around the motor neurons, such as the surrounding CNS, Schwann cells along the axonal path, neuromuscular junction and the muscle, could contribute some paracrine signals that may trigger the motor neurons' programmed cell death. While endocrine signaling may happen in all our examples of Waddingtonian genetics, therefore not representing a distinguishing feature between them, in case of the muscle-motor neuron developmental relationship (especially at earlier stages), paracrine signaling and direct contact between cells eventually evolving into synaptic signaling, may be the feature of the muscle-motor neuron relationship. In the context of the current and previous publications from our laboratory this relationship has been described as "paracrine." Some signals from skeletal muscle, such as insulin-like growth factor 1 (IGF-1), which has been implicated in the regulation of muscle and nerve tissue anabolism, inducing muscle hypertrophy and promoting neuronal survival (Musaro and Rosenthal, 2006), may influence neuronal survival, axonal growth, and maintenance of synaptic connections, and it has also been shown that localised expression of the co-inherited MLC/mIGF-1 transgene in the muscles of SOD1G93A mice counteracts the symptoms of ALS and activates regeneration (Dobrowolny et al., 2005, 2008).

In addition, different motor neurons may depend on different muscle-associated factors for their survival. Several investigations in our laboratory (reviewed in Angka and Kablar, 2009) have indeed established that the ability of a particular neurotrophin to support the survival of motor neurons in general does depend on the presence of skeletal muscle. There is a differential requirement of the neurotrophic factors: brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) by motor neurons of the lateral motor column (LMC), innervating MyoD-dependent limb muscles, *versus* the medial motor column (MMC) motor neurons innervating Myf5-dependent back musculature (Kablar and Belliveau, 2005; Geddes et al., 2006; Angka and Kablar, 2007; Angka et al., 2008; Alaynick et al., 2011). The present report therefore summarises our more recent findings regarding the interaction of the limb and back muscle groups and their associated motor neurons.

Neurotrophic factors and muscle-associated neurons

Neurotrophic factors are small versatile proteins that promote the survival, development and function of specific neuron populations (Lewin and Barde, 1996). Their list includes, but is not limited to: (a) members of the neurotrophin family, nerve growth factor (NGF), BDNF, NT-3 and NT-4/5; (b) the cytokines, cardiotrophin-1, ciliary neurotrophic factor (CNTF) and leukocyte inhibitory factor (LIF); (c) the transforming growth factor-beta family members, GDNF, neurturin and persepin; and (d) various growth factors, such as hepatocyte growth factor/scatter factor (HGF/SF), IGF-1, IGF-2, as well as the fibroblast growth factors FGF-1, 2 and 5. They are secreted by the target tissues and other tissues in the physical proximity of the neurons, and act as survival factors by preventing the associated neurons from initiating programmed cell death, thus allowing the neurons to survive. For instance, Zhao et al. (2004), have shown that overexpression of GDNF in the astrocytes of *GFAP-GDNF* transgenic mouse embryos increases the survival of their brachial, lumbar and thoracic motor neurons by about 30%, by rescuing them during the period of naturally occurring cell death. Neurotrophins also induce the differentiation of progenitor cells to form neurons, and different factors may act in synergy and in different subpopulations of motor neurons (Henderson et al., 1998). Defective neurotrophic factor production and/or function may therefore be a key factor in MNDs, and it is possible that skeletal muscles, as major effectors of neural impulses and contributory providers of neurotrophic factors, play a considerable active role in the etiology and pathogenesis of MNDs.

Our continuing research project includes experiments to test the neurotrophic hypothesis (that developing motor neurons require limited trophic support from their target tissues for their survival and maintenance), by comparing the intramuscular expression of molecules that may be important in the support of motor neuron survival, in embryos lacking either of the MRFs *Myf5* and *MyoD*, and therefore having their muscles at different levels of specification (back muscle in Myf5 nulls) and commitment (limb muscle in MyoD nulls). This work was largely stimulated by the finding that (a) the major phase of mammalian myogenesis, giving rise to approximately 80% of the muscle in the newborn, happens to coincide with the peak of naturally occurring motor neuron cell death in the spinal cord, starting at embryonic day (E)13.5 and continuing to E18.5 in the mouse (Harris et al., 1989) and that (b) during that same period, mouse embryos lacking striated muscle due to the *Myf5*-/-*:MyoD*-/- double mutation completely lose all somatic motor neurons from the spinal cord to the brain (Kablar and Rudnicki, 1999).

The purpose of the project is to find out if there is a muscle-provided trigger of motor neuron death ultimately relevant to such motor neuron disorders as ALS, and thereby attempt to clarify the precise *in vivo* role of muscle-associated neurotrophic factors in the integration of nerve and muscle development. We therefore used two methods (morphometric and microarray analyses) to evaluate: (a) motor neuron survival in the spinal cord's LMC and MMC, where motor neuron development, survival, and maintenance appear to depend, to a large extent, on factors from the limb and back musculature, respectively (Ordahl and Williams, 1998; Kablar and Belliveau, 2005), and (b) gene expression in those specific muscle groups (limb and back) innervated by motor neurons from the LMC and the MMC, respectively, and whose development

depends on the MRFs *MyoD* (limb muscle) and *Myf5* (back muscle).

Therefore, in our approach, we combine the events leading to muscle differentiation with the events that make that muscle capable of producing (or just containing) factors essential for motor neuron survival and maintenance, and attempt to identify the muscle-associated regulators of motor neuron numbers in the CNS.

Morphometric analysis of muscle-associated neurons in the spinal cord of the *MyoD*-deficient *mdx* mouse

In order to study the effect of partial versus total ablation of the *MyoD* gene on motor neural survival in a known model of a human muscle dystrophy disease, *mdx* mice (which carry a loss-of-function point mutation in the X-linked *dystrophin* gene and are a model for human Duchenne and Becker muscular dystrophy) (Stedman et al., 1991; Anderson et al., 1998) were bred with *MyoD*-/- mice to generate *mdx:MyoD*+/- and *mdx:MyoD*-/- mice. These mice were subsequently backcrossed (the tenth generation of *mdx:MyoD*+/- mice was bred with the first generation of *mdx:MyoD*-/- mice) until no viable *mdx:MyoD*-/- newborn was detectable. The embryos generated in the described manner were thus designated *mdx:MyoD*-/-^{9th} to indicate that they were double-mutant products after nine generations of backcrossing (Inanlou et al., 2003).

Morphometric analyses of all neuron types in the LMC and MMC of the spinal cord were performed at E18.5 as in Kablar and Rudnicki, 1999; Kablar and Belliveau, 2005 and Geddes et al., 2006. Briefly, two 4-μm serial coronal sections taken at 40 μm intervals were collected on each slide, and every fourth slide was counted. Cells with a large nucleus (diameter >10 μm), and/or positive for Islet-1/2 and/or HB9 were counted as motor neurons, and the number of motor neurons per section was calculated from the average obtained in the two sections on the counted slides. The total number of motor neurons per motor column per slide was then determined for both the MMC and the LMC.

Our results show that whereas the MMC neurons (in the thoracic spinal cord, T4-T6) appear similar in *mdx:MyoD*+/- and *mdx:MyoD*-/- mice, the number of LMC neurons (in the lumbar spinal cord, L3-L4) is greatly reduced in the double-mutant (*mdx:MyoD*-/-) mice compared to their *mdx:MyoD*+/- littermates (Fig. 1), indicating that some muscle-expressed factor(s), possibly related to *MyoD* (but not to *Myf5*), may be involved in the causation of this particular phenotype.

Microarray analysis of gene expression in the muscle of *MyoD-/-* and *Myf5-/-* mouse embryos during early myogenesis

The next part of the project called for the use of a cDNA microarray analysis protocol to examine gene expression in the muscle of mouse embryos individually lacking either of the primary MRFs *Myf5* and *MyoD*, at the critical period of major embryonic myogenesis when *Myf5* and *MyoD* cannot substitute for each other (Kablar et al., 1997), and coinciding with the onset of neuronal naturally occurring programmed cell death (E13.5). The muscles examined were the limb and back muscle groups, which, respectively, are associated with the survival of neurons in the LMC and MMC of the spinal cord (Ordahl and Williams, 1998; Kablar and Belliveau, 2005).

Gene expression profiles are best measured on the basis of the transcribed mRNA, rather than protein. Molecular form of RNA and DNA is more uniform, lending itself more conveniently to laboratory procedures than proteins do. There is a trade-off however: mRNA and protein expression/distribution levels do not always correlate well. Post-translational protein modifications with an essential effect on its function cannot be measured here. Proteomics approach based on mass spectrometry would be an asset. Meanwhile, we plan to use Gene Ontology Consortium for protein function ontology, such as the Center for Biological Sequence Analysis at the Technical University of Denmark Lyngby, for predicting protein function, or ProtFun, for comprehensive classification of proteins.

Homozygous *MyoD*-deficient (*MyoD*-/-) or *Myf5*-deficient (*Myf5*-/-) embryos were obtained by the interbreeding of homozygous *MyoD*-/- or heterozygous

Table 1. Primers for transcripts used in PCR verification of gene expression differences.

Target	5' to 3' primer sequence; forward (f) and reverse (r)
Slc4a4	f: TCA CAA ACC TTT CAG CAA AAG AGT GC r: CAA AGA GCA ACA GTC AGA CAG C
Polb	f: GGC TCC GCA GGA GAC CCT CAA CGG C r: CCC GAT GCC AGT AAC TCG AGT CAG GAA
Fgf2	f: AGA GCG ACC CAC ACG TCA AAC r: CCA ACT GGA GTA TTT CCG TGA CC
Stxbp1	f: ACT CCG CTG ACT CTT TCC AA r: TGG ATC GTC GGC TTT ATA GG
Kif5c	f: CGG ATT CTT CAG GAC TCT TT r: TTG TCT ATG ATG GGG GTG TT
Afp	f: AAA GCT GCG CTC TCT ACC AG r: GAG TTC ACA GGG CTT GCT TC
Uox	f: ACC TCC CGT CAT TCA CTC T r: ACT GTC CCT GTT ATT TTG CC
Hoxd10	f: GAA GAG GTG CCC TTA CAC CA r: TCG ATT CTC TCG GCT CAT CT
Bdnf	f: ATG GGA CTC TGG AGA GCG TGA A r: CGC CAG CCA ATT CTC TTT TTG C
Glrb	f: GCA ACT TGA GAG CTG TAT GT r: ACT TGG CTG GGC TTA CAT AT
Ppargc1a	f: GTC AAC AGC AAA AGC CAC AA r: TCT GGG GTC AGA GGA AGA GA

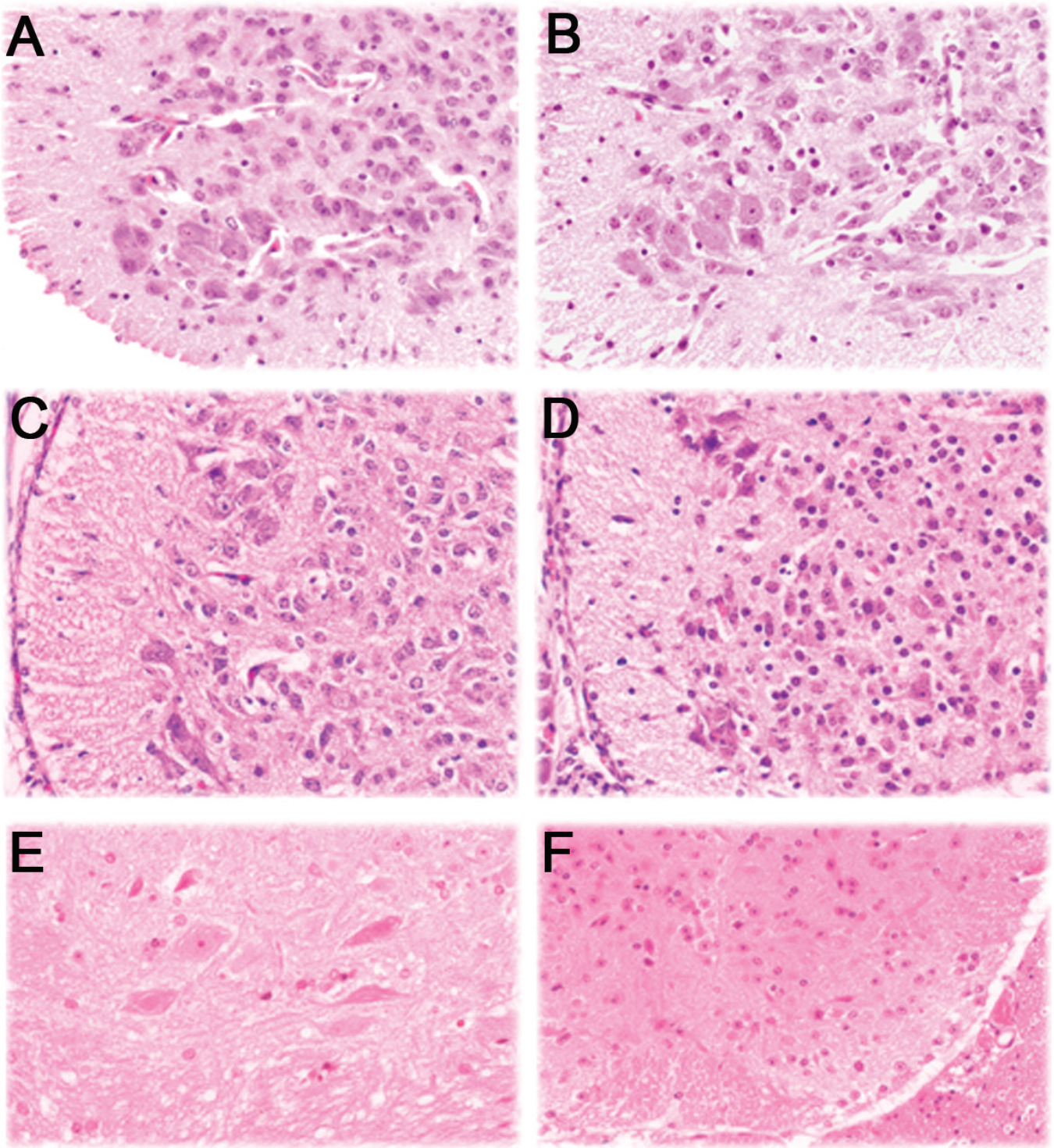

Fig. 1. Comparison between MMC and LMC in mdx:*MyoD*-/- 9th fetuses. Representative micrographs (H&E) of the thoracic spinal cord (T4-T6) **(A, B)** MMC of mdx:MyoD+/- 9th **(A)** and Mdx:MyoD-/- 9th **(B)** E18.5 fetuses show no difference in neuronal appearance and numbers (7±2 vs. 8±2, P>0.05) between the genotypes (three fetuses of the each genotype were employed; n=3). By contrast, representative micrographs of the lumbar spinal cord (L3-L4) **(C, D)** LMC of mdx:MyoD+/- 9th **(C)** and mdx:MyoD-/- 9th **(D)** E18.5 fetuses show a drastic 53% decrease in neuronal number in the double mutant (17±2 vs. 8±3, P<0.05). In addition, the mdx:MyoD-/- 9th **(D)** spinal cord shows gliosis, probably as a reaction to the motor neuron death, as evidenced by the presence of numerous darkly stained small nuclei of glia cells (also GFAP positive; data not shown). **E and F.** Show morphology of LMC motor neurons in mdx:MyoD+/- 9th **(E)** and the complete lack of LMC motor neurons (in this particular section) of mdx:MyoD-/- 9th **(F)** spinal cord. Original magnification, A-D, x 400, E, F, x 630

Table 2. Primers used for comparing neurotrophic factor mRNA levels.

Target	5' to 3' primer sequence; forward (f) and reverse (r)
Bdnf	f: ATG GGA CTC TGG AGA GCG TGA A r: CGC CAG CCA ATT CTC TTT TTG C
Gdnf	f: CGC TGA CCA GTG ACT CCA ATA TGC r: GTT AGC CTT CTA CTC CGA GAC AGG
Ntf3	f: CTT ATC TCC GTG GCA TCC AA r: TCT GAA GTC AGT GCT CGG ACG T
Ntf5	f: CAC TGG CTC TCA GAA TGC AA r: TCC TCC GGG AGA ACT CCT AT

Myf5+/- parents, respectively, as described in Rudnicki et al., 1993. All embryos were collected by Cesarean section at E13.5 and genotyped by RT-PCR as previously described (Inanlou and Kablar, 2005).

Total limb muscle RNA was isolated using the RNeasy™ kit from Qiagen, Mississauga, Ont., Canada, according to the manufacturer's instructions. For each group (wild-type or *MyoD*-/-), RNA from the limb muscle of six embryos was pooled. Similarly, back muscle RNA was pooled from five wild-type and *Myf5*-/- embryos. Fluorescent labeling of cRNA

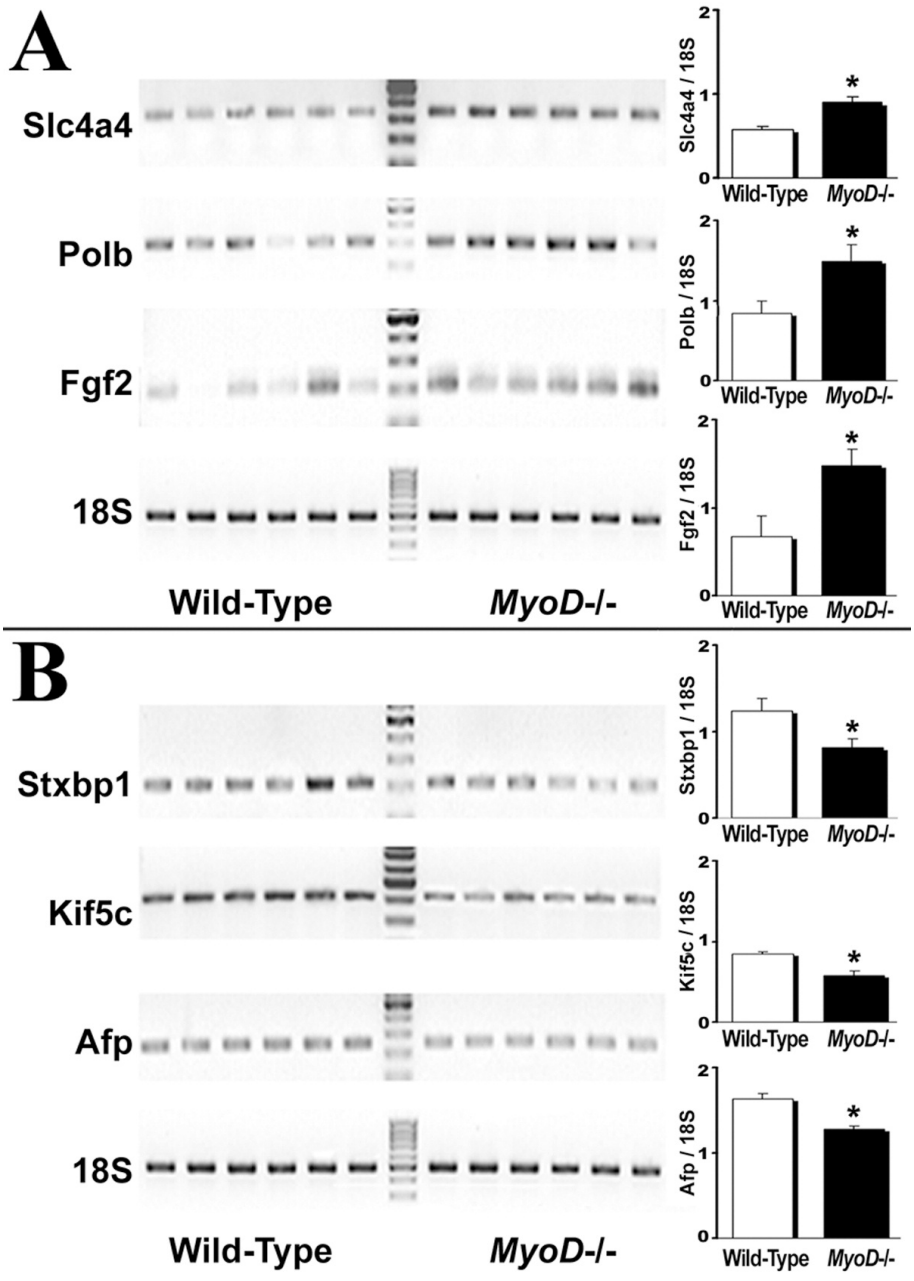

Fig. 2. RT-PCR confirmation of differential gene expression in the limb muscle of MyoD-/- mutant embryos. Up-regulated (**A**), down-regulated (**B**). Graphs plot expression relative to 18S rRNA, mean ± SEM, n=6; *, significantly different from expression in wild-type embryos, $P<0.05$. Note: the microarray analysis showed that, of the well-known neurotrophic factors, only Fgf2 was differentially expressed, and it was included here for that reason. However, its up-regulation was only 1.91-fold, so it is not listed in Table 3.

fragments obtained from the pooled samples and their simultaneous hybridization to MOE340 GeneChip mouse genome arrays was performed at the Ottawa Genome Centre according to standard Affymetrix (Santa Clara, CA) protocols as in Seale et al., 2004. The hybridized chips were then scanned and the results

Table 3. Genes up-regulated ≥ 3.5-fold in E13.5 *MyoD-/-* limb muscle, sorted by function and log2 (ratio) expression value.

Gene	$\log_2$ (ratio)	Myf5[a]	Gene title	SME[b]	SCE[b]	Molecular function
Zfp64	4.78	0.04	zinc finger protein 64	250	185	
Eaf2	4.46	-	ELL associated factor 2	210	180	
Esco1	4.42	-0.23	establishment of cohesion 1 homolog 1 (S. cerevisiae)	400	500	Transcription
2410018L13Rik	3.75	-0.01	RIKEN cDNA 2410018L13 gene	10	53	Factor Activity
Runx1	3.71	-1.32	runt related transcription factor 1	35	15	
Zfp444	3.67	-1.54	zinc finger protein 444	100	60	
Pelp1	3.56	0.45	proline, glutamic acid and leucine rich protein 1	340	275	
Ednra	5.07	-0.71	endothelin receptor type A	185	100	
Tac1	4.52	0.22	tachykinin 1	100	400	
Plcg1	4.12	1.31	phospholipase C, gamma 1	40	100	
Cntnap2	4.03	1.29	contactin associated protein-like 2	125	260	Receptor and Signal
Cd180	4.01	-0.15	CD180 antigen	200	170	Transduction Activity
Ppp2r2c	3.96	-1.49	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), gamma isoform	50	1300	
Nomo1	3.91	0.57	nodal modulator 1	1250	800	
Gpc5	3.62	-	glypican 5	40	325	
Slc4a4	5.15	-1.81	solute carrier family 4 (anion exchanger), member 4	320	380	
Bpifc	4.94	-	BPI fold containing family C	140	160	Transport/
Mob1a	4.93	0.16	MOB kinase activator 1A	NA[c]	NA	Carrier Activity
Anxa4	4.44	-0.28	annexin A4	200	200	
P4ha3	5.96	-	oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide III	200	220	
Prcp	4.51	-	prolylcarboxypeptidase (angiotensinase C)	100	140	
Ube1y1	4.47	-	ubiquitin-activating enzyme E1, Chr Y 1	37	13	
Cwf19l2	4.05	-0.64	CWF19-like 2, cell cycle control (S. pombe)	425	200	Catalytic Activity
Ggact	3.96	-	gamma-glutamylamine cyclotransferase	120	260	
Wee2	3.79	-	WEE1 homolog 2 (S. pombe)	NA	NA	
Psma1	3.54	-0.06	proteasome (prosome, macropain) subunit, alpha type 1	1700	750	
Rbpms	4.84	0.41	RNA binding protein gene with multiple splicing	100	80	
Snhg14	4.79	0.22	small nucleolar RNA host gene 14	25	250	
Birc6	4.36	3.35	baculoviral IAP repeat-containing 6	38	23	Metabolic and
Polb	4.1	-0.53	polymerase (DNA directed), beta	480	240	Housekeeping
Tpp2	3.78	-1.05	tripeptidyl peptidase II	390	225	
Pdxdc1	3.67	0.79	pyridoxal-dependent decarboxylase domain containing 1	NA	NA	
Mfap1a	7.55	0.35	microfibrillar-associated protein 1A	NA	NA	
3110007F17Rik	4.56	-	RIKEN cDNA 3110007F17 gene	235	185	
Dsg2	4.17	-0.18	desmoglein 2	50	50	Structural and
Cdhr4	4.1	0.63	cadherin-related family member 4	135	135	Cytoskeletal
Spaca1	4.01	0.95	sperm acrosome associated 1	10	15	
Spp2	3.59	-	secreted phosphoprotein 2	50	125	
Cep162	4.4	0.67	centrosomal protein 162	20	35	
Invs	4.02	-0.04	inversin	250	230	Cell Cycle Regulation
Wac	3.9	0.52	WW domain containing adaptor with coiled-coil	60	80	
3110002H16Rik	5.68	-0.59	RIKEN cDNA 3110002H16 gene	210	260	
1700097N02Rik	4.95	-	RIKEN cDNA 1700097N02 gene	175	180	
4930513N10Rik	4.89	-	RIKEN cDNA 4930513N10 gene	235	190	
5830407P18Rik	4.68	-	RIKEN cDNA 5830407P18 gene	NA	NA	
2310076G05Rik	4.53	-0.35	RIKEN cDNA 2310076G05 gene	NA	NA	
C80998	4.31	-	expressed sequence C80998	NA	NA	
2900046F13Rik	4.2	-	RIKEN cDNA 2900046F13 gene	NA	NA	Not yet specified
2810002D19Rik	3.99	-0.29	RIKEN cDNA 2810002D19 gene	35	25	
4833411C07Rik	3.94	-	RIKEN cDNA 4833411C07 gene	6.5	5.5	
B130021B11Rik	3.71	0.64	RIKEN cDNA B130021B11 gene	NA	NA	
5830444B04Rik	3.71	-	RIKEN cDNA 5830444B04 gene	NA	NA	
4930588G05Rik	3.67	-0.15	RIKEN cDNA 4930588G05 gene	NA	NA	
C920006O11Rik	3.6	-	RIKEN cDNA C920006O11 gene	NA	NA	
4833413E03Rik	3.5	-	RIKEN cDNA 4833413E03 gene	NA	NA	

[a]: up- or down-regulation in *Myf5-/-* mutant. [b]: expression (arbitrary units) in the adult mouse skeletal muscle (SME) and spinal cord (SCE), (Su et al., 2002). [c]: NA: data not available.

analyzed using the Affymetrix statistical expression algorithms to obtain the expression ratios and fold changes between the wild-type and mutant embryo muscle. As is common (Iida and Nishimura, 2002), we used a more traditional gene expression assay as a way of validating the microarray data. Thus, to confirm the differential mRNA expression and direction of change in expression (up- or down-regulation in *MyoD-/-* or *Myf5-/-* muscle versus their controls), total RNA from the muscle of six embryos in "MyoD group" or from the back muscle of five fetuses in the "Myf5 group" was individually reverse-transcribed with Omniscript™ reverse transcriptase (Qiagen) and amplified using 35 cycles of 45 sec at 95°C, 60 sec at 58°C and 60 sec at 72°C with the primers listed in Table 1 (*Slc4a4, Polb, Fgf2, Stxbp1, Kif5c* and *Afp* for *MyoD-/-* limb muscle, and *Uox, Hoxd10, Bdnf, Glrb, Ppargc1a* and *Afp* for *Myf5-/-* back muscle). DNA levels were subsequently

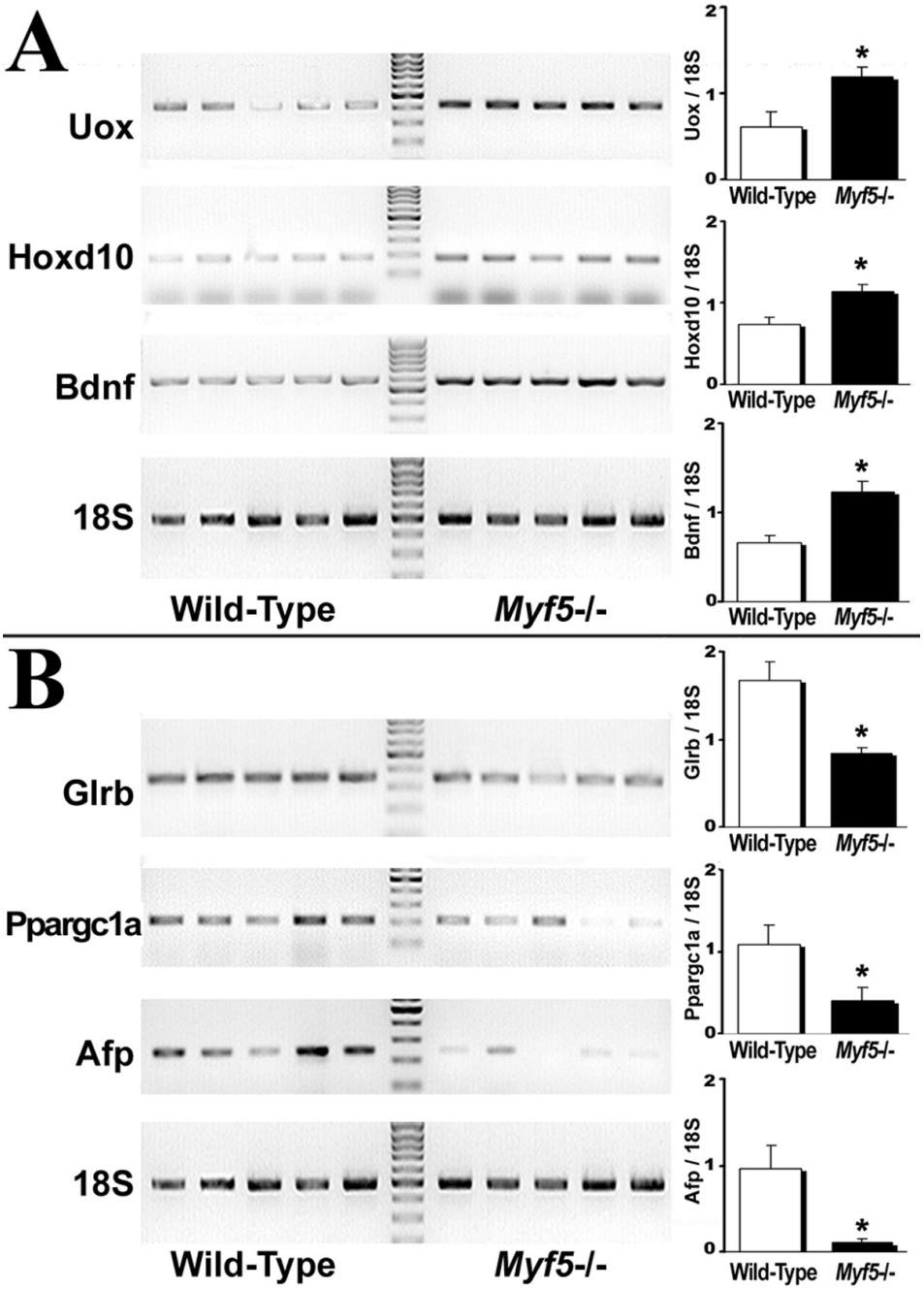

Fig. 3. RT-PCR validation of differential gene expression in the back muscle of Myf5-/- mutant embryos. Up-regulated **(A)**, down-regulated **(B)**. Graphs plot expression relative to 18S rRNA, mean ± SEM, n=5; *, significantly different from expression in wild-type embryos, P<0.05. Note: the microarray analysis showed that, of the well-known neurotrophic factors, Bdnf was the most differentially expressed, and it was included here for that reason. However, its up-regulation was only 2.98-fold, so it is not listed in Table 5.

normalized against the QuantumRNA™ 488 bp 18S ribosomal PCR product (Ambion, Austin, TX) amplified from the same RT reaction, as previously described (Baguma-Nibasheka et al., 2007). These expression data were then compared using a *t*-test (wild-type *versus* mutant) with differences of $P<0.05$ considered significant.

In addition, using the same cycling conditions and the primers listed in Table 2, the mRNA expression of four well-known neurotrophic factors: BDNF, GDNF, NT-3 and NT-4/5 was also compared in the limb muscle of wild-type and *MyoD*-/- embryos.

Microarray analysis identified a large number of genes that were differentially expressed between the

Table 4. Genes down-regulated ≥3.5-fold in E13.5 *MyoD-/-* limb muscle, sorted by function and log2 (ratio) expression value.

Gene	$\log_2$ (ratio)	Myf5[a]	Gene title	SME[b]	SCE[b]	Molecular function
Lztfl1	-5.59	0.35	leucine zipper transcription factor-like 1	350	530	
Gatad2b	-4.73	0.34	GATA zinc finger domain containing 2B	525	675	Transcription Factors
Hoxc10	-4.38	-	homeo box C10	535	550	
Polr1a	-3.65	-0.08	polymerase (RNA) I polypeptide A	220	125	
Cd247	-4.5	-	CD247 antigen	60	40	
Opn1sw	-4.43	-	opsin 1 (cone pigments), short-wave-sensitive (color blindness, tritan)	50	40	
Ms4a1	-4.21	-	membrane-spanning 4-domains, subfamily A, member 1	20	12	
Cox16	-4.16	-1.52	COX16 cytochrome c oxidase assembly homolog (S. cerevisiae)	650	475	Receptor and Signal
Bmx	-4.06	-	BMX non-receptor tyrosine kinase	50	35	Transduction Activity
Unc5cl	-4.05	-	unc-5 homolog C (C. elegans)-like	100	150	
Fcer1a	-3.92	-	Fc receptor, IgE, high affinity I, alpha polypeptide	40	45	
Ifngr2	-3.81	-	interferon gamma receptor 2	NA[c]	NA	
Nfam1	-3.75	-	Nfat activating molecule with ITAM motif 1	500	500	
Afp	-5.59	-8.69	alpha fetoprotein	1000	500	
Aqp7	-5.49	-	aquaporin 7	460	350	
Ran	-4.59	-	RAN, member RAS oncogene family	1000	850	
Clic1	-4.23	0.76	chloride intracellular channel 1	550	250	
Ipo7	-3.84	-0.46	importin 7	120	75	
Abcc2	-3.83	-	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	100	110	Transport/
Stxbp1	-3.82	-0.12	syntaxin binding protein 1	200	4200	Carrier Activity
Slc25a19	-3.76	-0.7	solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19	320	340	
Pfdn5	-3.77	-0.71	prefoldin 5	80	75	
Vamp5	-3.56	-	vesicle-associated membrane protein 5	1050	200	
Actr1a	-3.55	-	ARP1 actin-related protein 1 homolog A (yeast)	700	1100	
Acaa1b	-5.03	0.73	acetyl-Coenzyme A acyltransferase 1B	100	100	
Scd2	-4.62	0.51	stearoyl-Coenzyme A desaturase 2	300	2000	Lipid Metabolism
Pisd	-4.25	0.48	phosphatidylserine decarboxylase	275	600	Activity
Mtmr7	-3.94	3.98	myotubularin related protein 7	50	250	
Acot2	-3.54	-0.34	acyl-CoA thioesterase 2	210	90	
Psmd6	-4.18	-0.47	proteasome (prosome, macropain) 26S subunit, non-ATPase, 6	2300	800	
Alpi	-3.91	1.53	alkaline phosphatase, intestinal	NA	NA	Other Metabolic and
Fam20b	-3.55	0.75	family with sequence similarity 20, member B	600	750	Housekeeping Activity
Ctps2	-3.53	-0.38	cytidine 5'-triphosphate synthase 2	400	600	
Kif5c	-4.6	-0.35	kinesin family member 5C	30	300	Structural and
Tectb	-3.76	-	tectorin beta	23	18	Cytoskeletal
Ccnb1	-5.18	-0.1	cyclin B1, related sequence 1	100	100	Cell Cycle
B230216G23Rik	-4.56	-	RIKEN cDNA B230216G23 gene	100	85	Regulation
AU019752	-4.89	0.73	expressed sequence AU019752	NA	NA	
1700034I23Rik	-4.54	-	RIKEN cDNA 1700034I23 gene	20	20	
Zak	-4.43	0.37	sterile alpha motif and leucine zipper containing kinase AZK	3500	600	
Fam117b	-4.33	-0.48	family with sequence similarity 117, member B	300	500	
AU017263	-4.26	-	expressed sequence AU017263	600	450	
4933400F21Rik	-4.25	-	RIKEN cDNA 4933400F21 gene	NA	NA	Not yet specified
2210411G17Rik	-3.97	-	RIKEN cDNA 2210411G17 gene	NA	NA	
E330010L02Rik	-3.93	-	RIKEN cDNA E330010L02 gene	NA	NA	
4933430H15Rik	-3.86	-	RIKEN cDNA 4933430H15 gene	120	70	
A930011E06Rik	-3.84	-	RIKEN cDNA A930011E06 gene	NA	NA	
Ccdc83	-3.63	-	coiled-coil domain containing 83	310	340	

[a]: up- or down-regulation in *Myf5-/-* mutant. [b]: expression (arbitrary units) in the adult mouse skeletal muscle (SME) and spinal cord (SCE), (Su et al., 2002). [c]: NA: data not available.

Table 5. Genes up-regulated ≥ 3.5-fold in E13.5 *Myf5-/-* back muscle, sorted by function and log2 (ratio) expression value.

Gene	log_2 (ratio)	MyoD[a]	Gene title	SME[b]	SCE[b]	Molecular function
Prox1	4.97	0.75	prospero-related homeobox 1	50	80	
Eomes	4.27	-0.26	eomesodermin homolog (Xenopus laevis)	95	65	Transcription Factors
Hoxd10	3.94	-0.14	homeo box D10	50	600	
Dmtf1	3.71	0.09	cyclin D binding myb-like transcription factor 1	395	395	
Cbx3	4.76	0.27	chromobox homolog 3 (Drosophila HP1 gamma)	180	270	
Rpain	3.98	-0.07	RPA interacting protein	NA[c]	NA	
Slx	3.89	-	Sycp3 like X-linked	50	50	Chromosome Organization
Rmi1	3.83	-0.71	RMI1, RecQ mediated genome instability 1, homolog (S. cerevisiae)	NA	NA	and Biogenesis; DNA Repair
Sycp3	3.82	-	synaptonemal complex protein 3	100	100	
Caskin1	3.76	-	CASK interacting protein 1	123	123	
Crtc2	3.98	-1.46	CREB regulated transcription coactivator 2	100	85	
Epha5	3.76	-1.31	Eph receptor A5	20	55	Receptor and Signal
Cd300lf	3.68	-	CD300 antigen like family member F	30	27	Transduction Activity
Olfr15	3.5	-	olfactory receptor 15	200	250	
Kcnip4	4.54	-	Kv channel interacting protein 4	60	135	
Letm1	4.35	-0.39	leucine zipper-EF-hand containing transmembrane protein 1	200	175	
Cacng5	4.23	-	calcium channel, voltage-dependent, gamma subunit 5	250	280	Transport/Carrier Activity
Yipf6	3.89	0.5	Yip1 domain family, member 6	175	125	
Glyat	3.79	-	glycine-N-acyltransferase	60	20	
Uox	5.62	0.29	urate oxidase	100	100	
Pon2	5.14	-1.8	paraoxonase 2	115	85	
Cela3b	5.13	-	chymotrypsin-like elastase family, member 3B	100	100	
Ppp1r3g	4.36	0.38	protein phosphatase 1, regulatory (inhibitor) subunit 3G	60	225	
Arsj	3.98	-1.93	arylsulfatase J	128	112	
Mtmr7	3.98	-3.94	myotubularin related protein 7	NA	NA	Catalytic Activity
Ggct	3.97	0.12	gamma-glutamyl cyclotransferase	75	130	
Mpp5	3.64	1.09	membrane protein, palmitoylated 5 (MAGUK p55 subfamily member 5)	315	450	
Farsa	3.57	0.02	phenylalanyl-tRNA synthetase, alpha subunit	750	500	
Egln3	3.61	1.14	EGL nine homolog 3 (C. elegans)	270	200	
Pdik1l	3.55	-0.28	PDLIM1 interacting kinase 1 like	130	200	
Ccdc38	4.3	-	coiled-coil domain containing 38	50	125	
Clps	3.98	-	colipase, pancreatic	100	100	Other Metabolic and
Ttc14	3.83	-1.03	tetratricopeptide repeat domain 14	NA	NA	Housekeeping Activity
Adig	3.54	-	adipogenin	100	100	
Oog3	3.71	-	oogenesin 3	NA	NA	
Cnbd2	3.66	-	cyclic nucleotide binding domain containing 2	245	245	Gametogenesis
Elavl2	3.59	2.26	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)	285	235	
Mcc	3.61	-	mutated in colorectal cancers	12.5	23	Cell Cycle Regulation
4930578I06Rik	5.88	-	RIKEN cDNA 4930578I06 gene	180	100	
DXBay18	5.35	-	DNA segment, Chr X, Baylor 18	90	50	
Igsf23	4.81	-	immunoglobulin superfamily, member 23	300	275	
D1Ertd507e	4.7	-	DNA segment, Chr 1, ERATO Doi 507, expressed	NA	NA	
3021401N23Rik	4.7	-	RIKEN cDNA 3021401N23 gene	320	350	
4930404A10Rik	4.55	-	RIKEN cDNA 4930404A10 gene	350	260	
4921508D12Rik	4.47	-0.45	RIKEN cDNA 4921508D12 gene	90	125	
Gm9796	4.45	-0.1	predicted gene 9796	NA	NA	
4833406M21Rik	4.41	-	RIKEN cDNA 4833406M21 gene	NA	NA	
4921521C08Rik	4.38	-	RIKEN cDNA 4921521C08 gene	NA	NA	Not yet specified
A330008L17Rik	4.29	-	RIKEN cDNA A330008L17 gene	55	85	
4930540M05Rik	4.23	-	RIKEN cDNA 4930518C04 gene	NA	NA	
AU022320	4.05	-	expressed sequence AU022320	NA	NA	
4930518C04Rik	4.03	-0.48	RIKEN cDNA 4930518C04 gene	NA	NA	
5430434G16Rik	4.01	-	RIKEN cDNA 5430434G16 gene	NA	NA	
2810414N06Rik	3.91	3.41	RIKEN cDNA 2810414N06 gene	NA	NA	
8030497I03Rik	3.88	-	RIKEN cDNA 8030497I03 gene	NA	NA	
9430091N11Rik	3.76	-	RIKEN cDNA 9430091N11 gene	NA	NA	
4930533K18Rik	3.51	-	RIKEN cDNA 4930533K18 gene	610	390	

[a]: up- or down-regulation in *MyoD-/-* mutant. [b]: expression (arbitrary units) in the adult mouse skeletal muscle (SME) and spinal cord (SCE), (Su et al., 2002). [c]: NA: data not available.

control and the *MyoD*-/- mutant limb muscles, and an arbitrary cut-off value of log2(3.5-fold) (i.e. ≥3.5-fold or ≤-3.5-fold), which translates to 11.5-fold, was chosen as a means of determining the up- and down-regulated probesets, respectively. A total of 103 named genes met this criterion. Fifty five genes were up-regulated more than 3.5-fold in the limb muscle of mutant embryos (Table 3), whereas a total of 48 were down-regulated (Table 4).

Similarly, a large number of genes were found to be

Table 6. Genes down-regulated ≥3.5-fold in E13.5 *Myf5-/-* back muscle, sorted by function and $\log_2$ (ratio) expression value.

Gene	$\log_2$ (ratio)	MyoD[a]	Gene title	SME[b]	SCE[b]	Molecular function
Hsf2	-3.99	-	heat shock factor 2	230	175	Transcription Factors
Polr3c	-3.88	0.35	polymerase (RNA) III (DNA directed) polypeptide C	190	90	
Cenpp	-4.87	0.32	centromere protein P	NA	NA	
Wrnip1	-4.31	-0.05	Werner helicase interacting protein 1	200	375	
Smchd1	-3.97	1.03	SMC hinge domain containing 1	200	260	Chromosome Organization
Parp3	-3.94	-	poly (ADP-ribose) polymerase family, member 3	NA[c]	NA	and Biogenesis; DNA Repair
Ncor1	-3.63	1.03	nuclear receptor co-repressor 1	305	140	
Hmgn1	-3.62	-0.17	high mobility group nucleosomal binding domain 1	130	200	
Dgke	-5.42	1.12	diacylglycerol kinase, epsilon	140	310	
Ptprc	-4.8	2.85	protein tyrosine phosphatase, receptor type, C	750	300	
Fcgr3	-4.38	-	Fc receptor, IgG, low affinity III	220	275	
Ppargc1a	-4.22	-1.23	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	200	270	
Ly75	-3.92	-	lymphocyte antigen 75	22	25	Receptor and Signal
Gpr22	-3.91	-	G protein-coupled receptor 22	15	65	Transduction Activity
Pthlh	-3.87	0.05	parathyroid hormone-like peptide	75	25	
Glrb	-3.86	0.77	glycine receptor, beta subunit	200	2350	
Rgs7bp	-3.68	-	regulator of G-protein signalling 7 binding protein	NA	NA	
Asb5	-3.67	-	ankyrin repeat and SOCs box-containing protein 5	4750	300	
Afp	-8.69	-5.59	alpha fetoprotein	1000	500	
Rbp4	-4.7	-0.1	retinol binding protein 4, plasma	10	50	
Slc16a12	-4.48	-	solute carrier family 16 (monocarboxylic acid transporters), member 12	50	50	Transport/Carrier Activity
Ndufab1	-4.13	-0.48	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1	135	50	
Dock9	-3.76	0.67	dedicator of cytokinesis 9	650	900	
Ston1	-3.74	0.33	stonin 1	NA	NA	
Col9a2	-5.13	-	collagen, type IX, alpha 2	250	250	
Myl2	-4.49	-	myosin, light polypeptide 2, regulatory, cardiac, slow	5500	850	Structural, Cytoskeletal,
Prelp	-4.11	1.73	proline arginine-rich end leucine-rich repeat	600	260	and Motor Activity
Rshl2a	-3.66	0.08	radial spokehead-like 2A	NA	NA	
Myo19	-3.64	-1.13	myosin XIX	NA	NA	
Mgll	-4.48	0.73	monoglyceride lipase	450	450	Catalytic Activity
Pign	-3.72	1.09	phosphatidylinositol glycan anchor biosynthesis, class N	180	140	
Abi3bp	-5.29	0.29	ABI gene family, member 3 (NESH) binding protein	450	40	
Ankrd13c	-4.84	0.61	ankyrin repeat domain 13c	250	320	
Mrps14	-4.25	-0.48	mitochondrial ribosomal protein S14	12.5	12.5	
Pcolce2	-4.25	0.83	procollagen C-endopeptidase enhancer 2	250	250	
Pum2	-4.21	-0.62	pumilio 2 (Drosophila)	850	750	Other Metabolic and
Toporsl	-4.07	-	topoisomerase I binding, arginine/serine-rich like	175	130	Housekeeping Activity
Zfp748	-3.76	-	zinc finger protein 748	120	150	
Otud1	-3.72	-	OTU domain containing 1	180	100	
Cep295	-3.66	-	centrosomal protein 295	50	85	
Pigx	-3.63	0.85	phosphatidylinositol glycan anchor biosynthesis, class X	175	260	
Defb8	-4.26	-	defensin beta 8	50	50	Defense Response
Asb7	-4.79	0.73	ankyrin repeat and SOCS box-containing protein 7	320	270	
4933403J19Rik	-4.6	-0.42	RIKEN cDNA 4933403J19 gene	NA	NA	
5430434I15Rik	-4.4	-	RIKEN cDNA 5430434I15 gene	165	165	
Fam136a	-4.2	0.1	family with sequence similarity 136, member A	150	90	Not yet specified
AA408650	-4.18	0.49	expressed sequence AA408650	27	25	
4933424C09Rik	-3.69	-	RIKEN cDNA 4933424C09 gene	NA	NA	
2500004C02Rik	-3.65	0.79	RIKEN cDNA 2500004C02 gene	NA	NA	
D730035F11Rik	-3.51	-	RIKEN cDNA D730035F11 gene	NA	NA	

[a]: up- or down-regulation in *MyoD-/-* mutant. [b]: expression (arbitrary units) in the adult mouse skeletal muscle (SME) and spinal cord (SCE), (Su et al., 2002). [c]: NA: data not available.

differentially expressed between the control and the *Myf5*-/- mutant back muscle. A total of 107 named genes met this criterion. Fifty-seven named genes were up-regulated more than log2(3.5-fold) in the muscle of the mutants (Table 5), whereas a total of 50 were down-regulated (Table 6).

Tables 3 to 6 also show that most of the named genes are measurably expressed in skeletal muscle and the spinal cord of adult mice (Su et al., 2002).

Our own RT-PCR gene expression assays confirmed the direction of change for the individual genes we tested. These were, for *MyoD*-/- limb muscle: *Slc4a4*, *Polb* and *Fgf2*, up-regulated, and *Stxbp1, Kif5c* and *Afp*, down-regulated (Fig. 2), and for *Myf5*-/- back muscle: *Uox, Hoxd10* and *Bdnf*, up-regulated, and *Glrb, Ppargc1a* and *Afp* down-regulated (Fig. 3).

Finally, an assessment of mRNA levels for the neurotrophic factors BDNF, GDNF, NT-3 and NT-4/5 by RT-PCR revealed a lack of difference in their expression between the muscle of the *MyoD*-/- mutants and that of their control wild-type littermates (P>0.05, n=6, Fig. 4), confirming the noted lack of significant differential expression in the microarray analyses (Table 7). Previously, we found that the limb muscle lacking *MyoD* at E13.5 did not contain BDNF and NT-4/5, while having GDNF and NT-3 normally distributed (the distribution of all four molecules in the *Myf5*-/- back muscle was normal; Kablar and Belliveau, 2005). Here, therefore, we suggest a post-transcriptional regulation of BDNF and NT-4/5 distribution in the absence of *MyoD*. Combining the findings from our previous work with the current findings, at least two conclusions could be made: (a) that a MRF could be connected with the regulation of

Table 7. Results of cDNA microarray analysis, showing the $\log_2$ (ratio) expression value, in E13.5 *MyoD*-/- limb muscle, of factors known to affect motor neuron survival. -, expression not measurably different from normal/control littermates.

Gene Family	Factor	Log_2 Ratio
Neurotrophins	neurotrophin-3	-
	neurotrophin-4/5	-
	brain derived neurotrophic factor (BDNF)	-
Cytokines	cardiotrophin-1	0.11
	ciliary neurotrophic factor (CNTF)	0.04
	leukocyte inhibitory factor (LIF)	-
Transforming Growth Factor-beta (TGF-β)	Glial cell line-derived neurotrophic factor (GDNF)	-
	neurturin	-
	persepin	-
Hepatocyte Growth Factor/Scatter Factor (HGF/SF)	HGF/SF	-
Other Growth Factors	insulin-like growth factor-I (IGF-I)	0.22
	IGF-II	-0.69
	fibroblast growth factor-1 (FGF-1)	-0.71
	FGF-2	1.91
	FGF-5	-

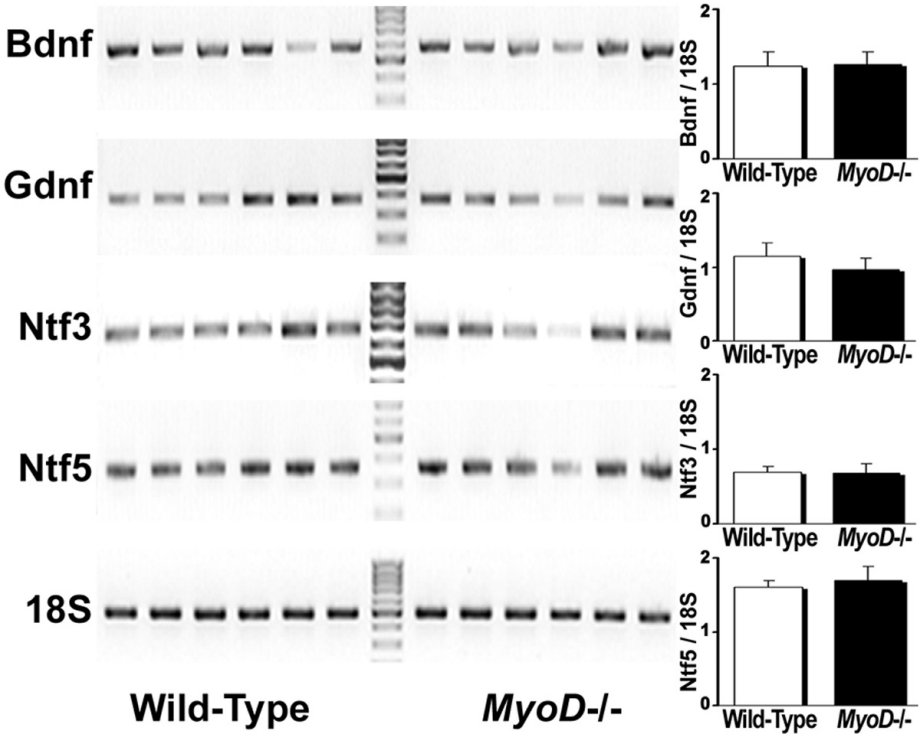

Fig. 4. MyoD-/- mutant limb muscle exhibits normal neurotrophin mRNA levels. RT-PCR assessment of mRNA levels for the genes encoding the neurotrophic factors BDNF, GDNF, NT-3 and NT-4/5 (Bdnf, Gdnf, Ntf3 and Ntf5, respectively). Graphs plot expression relative to 18S rRNA, mean ± SEM, n=6. There was no significant difference in neurotrophin expression between the MyoD-/- mutants and their control wild-type littermates (P<0.05, n=6).

neurotrophic factor expression and distribution, and (b) that there are neurotrophic factors not (so) essential for the survival of certain motor neurons (the number of LMC and MMC motor neurons in the spinal cord of *MyoD*-/- embryos was unaffected in the absence of BDNF and NT-4/5; Kablar and Belliveau, 2005).

A subsequent survey of the available literature indicates that the specific individual loss of many of the genes confirmed to be differentially expressed in the limb muscle of *MyoD*-/- mutants, such as *Kif5c, Stxbp1* and *Polb*, is also known (from their own knock-out models), to be associated with severe neurological abnormalities (Tables 8 and 9). The first two genes were found to be down-regulated in *MyoD*-/- mutants, whereas the last was up-regulated.

Similarly, we found that the individual ablation of a good number of the genes found by microarray analysis to be differentially expressed in the back muscle of the *Myf5*-/- mouse (and with already existing knock-out mouse models), such as *Ppargc1a, Glrb* and *Hoxd10*, also leads to major motor neural pathology (Tables 10 and 11). *Ppargc1a* and *Glrb* are down-regulated in *Myf5*-

Table 8. Genes up-regulated ≥3.5-fold in E13.5 *MyoD-/-* limb muscle and with knock-out (null mutant) mouse models.

Gene	Comments on deletion mutants	Reference
Slc4a4	Growth retardation, bowel obstruction, acidosis and death in three weeks	Gawenis et al., 2007
Ednra	Neural crest defects; death at birth due to blocked airway	Clouthier et al., 1998
Snhg14	Postnatal mortality; hypotonia, growth retardation, and neurological defects	Meng et al, 2013
Tac1	Healthy and fertile, but with significantly reduced pain response	Cao et al., 1998; Zimmer et al., 1998
Prcp	Decreased body length, weight, and fat pads, with resistance to diet-induced obesity	Wallingford et al., 2009
Eaf2	Heart and prostate hypertrophy, increased incidence of tumors, and premature death	Xiao et al., 2008
Anxa4	Normal phenotype except poor mothering behavior	Li et al., 2003
Birc6	Placental defects and complete perinatal lethality	Lotz et al., 2004
Dsg2	Embryonic lethality before somite formation	Eshkind et al., 2002
Plcg1	Abnormal hematopoiesis; lethality during organogenesis, before E10	Ji et al., 1997
Polb	Excessive neuronal apoptosis; respiratory failure and death at birth	Sugo et al., 2000
Cntnap2	Normal phenotype despite molecular abnormalities within the CNS	Poliak et al., 2003
Invs	Reversed left-right polarity	Yokoyama et al., 1993
Cd180	Impaired B cell function	Ogata et al., 2000
Spaca1	Male infertility, with globozoospermia and asthenozoospermia	Fujihara et al, 2012
Tpp2	Impaired T cell function; premature aging and death	Huai et al., 2008
Runx1	Massive CNS hemorrhage and death before E13	Wang et al., 1996

Table 9. Genes down-regulated ≥ 3.5-fold in E13.5 MyoD-/- limb muscle and with knock-out (null mutant) mouse models.

Gene	Comments on deletion mutants	Reference
Afp	Females masculinized and anovulatory due to impairment of the hypothalamic/pituitary system	De Mees et al, 2006
Aqp7	Obese and glyceroluric, with fasting hypoglycemia	Hibuse et al., 2005; Sohara et al., 2005
Ccnb1	Embryonic mortality by day 10	Brandeis et al., 1998
Acaa1b	Healthy and fertile with no detectable phenotypic defects	Chevillard et al., 2004
Scd2	70% neonatal lethality; leaky skin and abnormal liver lipid homeostasis in survivors	Miyazaki et al., 2005
Kif5c	Decreased motor neuron number and brain size	Kanai et al., 2000
Cd247	T-cell deficient, with neurosynaptic dysfunction	Huh et al., 2000
Hoxc10	Vertebral, pelvic and limb deformaties, decreased lumbar motor neuron number, impaired coordination, muscle wasting, and obesity	Wellik and Capecchi, 2003; Hostikka et al., 2009
Pisd	Death by E10, with mitochondrial fragmentation	Steenbergen et al., 2005
Clic1	Abnormal platelet numbers, activation and aggregation defects, and prolonged bleeding time	Qiu et al., 2010
Ms4a1	Healthy and fertile, but with abnormal B-cell physiology	Uchida et al., 2004
Bmx	Healthy and fertile with a normal phenotype	Rajantie et al., 2001
Fcer1a	Defective hematopoietic and immune system activity	Mayr et al., 2002
Abcc2	Healthy and fertile, but with hepatomegaly and hyperbilirubinemia	Vlaming et al., 2006
Stxbp1	Massive motoneural apoptosis causes death at birth due to respiratory failure	Verhage et al., 2000
Ifngr2	Hematopoietic and immune system deficiencies	Savinov et al., 2001
Pfdn5	Photoreceptor degeneration, CNS abnormalities, and male infertility	Lee et al., 2011
Slc25a19	Death by E12; numerous CNS malformations and other developmental defects	Lindhurst et al., 2006
Tectb	Low-frequency hearing loss	Russell et al., 2007

null mice, whereas *Hoxd10* was up-regulated.

Candidate molecules

With regard to neuropathology, the specific genes identified here that would appear to be of particular interest as candidates linking molecular events in the developing muscle to the survival of neurons include:

Kif5c (*kinesin family member 5C*), a structural and cytoskeletal gene, whose product is highly distributed in motor neurons of cranial nerves and spinal cord, and whose knock-outs exhibit decreased motor neuron numbers, particularly in some motor nuclei of the brainstem (Kanai et al., 2000). *Kif5c* belongs to the kinesin superfamily of genes, which regulate the transport of organelles and vesicles along microtubules within nerve cells and across synapses, and therefore play major roles in brain development and function. Mutations in these genes have thus been associated with familial ALS, intellectual disability, and epilepsy (Sepulveda-Falla et al., 2014; Willemsen et al., 2014).

Stxbp1 (*syntaxin binding protein 1*), a gene with transport and carrier molecular function, whose knock-outs have massive motor neuronal apoptosis in the lower brainstem and upper spinal cord (i.e., phrenic nerve motor neurons of the hypaxial motor column) leading to death at birth due to respiratory failure (Verhage et al., 2000). Dysfunction of this gene causes a variety of epileptic encephalopathies and parkinsonisms (Dravet syndrome, Ohtahara syndrome, etc.) in young children (Barcia et al., 2014; Carvill et al., 2014; Tso et al., 2014).

Polb (*polymerase (DNA directed), beta*), a metabolic and housekeeping gene, whose knock-outs also have excessive motor neuronal apoptosis (mostly in the hindbrain and the motor neurons of the sciatic nerve, i.e., the LMC) and die at birth due to respiratory failure (Sugo et al., 2000). Since the protein is involved in the repair of damaged DNA, defects in the gene are more commonly associated with tumorigenesis (Li et al., 2009; Donigan et al., 2012), but the defective neurogenesis noted in the mutant mouse ought to be a cause of concern in human medicine too.

Ppargc1a (*peroxisome proliferative activated receptor, gamma, coactivator 1 alpha*), one of the genes involved in the response to oxidative stress, and whose knock-outs exhibit among other phenotypes neuron degeneration (Cui et al., 2006) and muscle dysfunction with microvacuolization of the cortical pyramidal neurons (Leone et al., 2005). Because the protein is involved in mitochondrial biogenesis that is vital for cell survival, it has been implicated in neuronal death following acute brain injuries such as stroke from cerebral ischemia (Chen et al., 2011). In fact, our analysis of *Ppargc1a*-/- E17.5 fetuses (kindly provided by Dr. D. P. Kelly, Burnham Institute for Medical Research, Orlando, FL, USA, and Dr. D. Krainc, Harvard Medical School, Boston, MA, USA) revealed that, while the head and the thorax regions seem to contain normal number of motor neurons (facial motor nucleus in the head and MMC in the thoracic spinal cord), the lumbar spinal cord (LMC) seem to have a reduced number of LMC motor neurons in the mutant. At E17.5, fetuses show 44% decrease in neuronal number at the LMC level: 16±3 vs. 9±2, $P<0.05$ (the counts were performed as described previously in the current report for *mdx:MyoD* mutants). In addition, as

Table 10. Genes up-regulated ≥3.5-fold in E13.5 *Myf5-/-* back muscle and with knock-out (null mutant) mouse models.

Gene	Comments on deletion mutants	Reference
Uox	Severe nephropathy and 65% mortality within a month	Wu et al., 1994
Pon2	Increased susceptibility to atherosclerosis and bacterial infection	Ng et al., 2006
Prox1	Death at E14.5; impaired development of visceral organs and the lens	Wigle et al., 1999
Cbx3	Infertility	Naruse et al., 2007
Eomes	Defective optic nerve and retinal development	Mao et al., 2008
Crtc2	Decreased circulating corticosterone levels, impaired glucose homeostasis	Le Lay et al., 2009
Clps	60% preweaning mortality; postnatal growth retardation with impaired fat absorption, elevated cholesterol, and reduced triglycerides	D'Agostino et al., 2002
Hoxd10	Abnormal skeletal and neural morphogenesis	Carpenter et al., 1997
Yipf6	Colitis with decreased Paneth and goblet cells	Brandl et al., 2012
Rmi1	Decreased body weight and increased resistance to diet-induced obesity	Suwa et al., 2010
Sycp3	Infertility, aneuploidy, embryonic death	Yuan et al., 2000
Epha5	Abnormal sensory neuron projections in the retina	Feldheim et al., 2004
Dmtf1	Generalized tumorigenesis; premature death	Inoue et al., 2000
Cd300lf	Increased severity of experimental autoimmune encephalomyelitis with increased demyelination	Xi et al., 2010
Cnbd2	Male infertility associated with flagellar dysfunction and impaired spermiogenesis	Krahling et al., 2013
Egln3	Hepatomegaly, abnormal hematopoiesis, decreased litter size; abnormal adrenal, pupillary and neuronal morphology and function; reduced blood pressure	Takeda et al., 2008; Bishop et al., 2008
Mpp5	Viable and fertile, with behavioral defects; loss of cortex neurons due to premature differentiation and increased apoptosis	Kim et al., 2010
Mcc	Viable and fertile with no gross abnormalities	Young et al., 2011

expected, we observed down-regulation in the immunoreactivity pattern of proteins whose expression is regulated by *Ppargc1a* (St-Pierre et al., 2006), in *Ppargc1a*-/- E17.5 vs. control fetuses. These proteins, involved in reactive oxygen species (ROS) metabolism (namely, superoxide dismutases 1 and 2, glutathione peroxidase 1/2, catalase), failed however to show any differences between the limb and the back musculature (data not shown).

Glrb (*glycine receptor, beta subunit*), a gene involved in chloride channel function, and whose dysfunction results in a neurological disorder and premature death, together with extensive motor neuron loss (Molon et al., 2006). Severe phenotypic group of mutant mice showed a great reduction in both, the abundance and the size of motor neurons, and almost complete loss of motor neurons (Molon et al., 2006). JAX mice database search revealed sciatic nerve and cauda equine motor neuron loss (i.e., mostly LMC). The associated disease in human medicine, hyperekplexia, exhibits as neonatal hypertonia with an exaggerated startle reflex and frequent falls (Zhou et al., 2002).

Hoxd10 (*homeo box D10*), a transcription factor regulating columnar, divisional and motor pool identity of lumbar LMC neurons (Wu et al., 2008), and associated with abnormal neural morphogenesis when knocked out (Carpenter et al., 1997). This gene has been implicated in the proliferation, migration, and invasiveness of squamous cell carcinoma cells (Hakami et al., 2014).

Afp (*alpha fetoprotein*) was the only gene found to be greatly down-regulated in the muscle of both the *MyoD*-/- and the *Myf5*-/- embryos, and female mice homozygous for an alpha-fetoprotein (AFP) null allele are known to be sterile as a result of anovulation (Áet al, 2006), probably due to some defect in the hypothalamic-pituitary axis. Interestingly though, AFP-deficient human individuals appear fully healthy. Elevated serum AFP, on the other hand, has now been found to be a useful biomarker in the diagnosis of several heritable human ataxic neuropathies, including the one with abnormal ocular movements (Schieving et al., 2014). Data on the role of this gene in the regulation of motor neuron numbers are currently unavailable, but its participation in it is likely, considering the *Afp* down-regulation in our experiments and its connection with the movement disorders.

However, of the neurotrophic factors known to affect motor neuron survival, only *Fgf2* (*fibroblast growth factor 2*) was found to be differentially expressed to a significant extent (Fig. 2 and Table 7). Mice with the *Fgf2*-/- mutation exhibit reduced neuronal density in most layers of the motor cortex (Dono et al., 1998; Ortega et al., 1998), and FGF-2 is known to be neuroprotective in such conditions as multiple sclerosis (Mizuno, 2014). Previous work in this lab has already shown that *MyoD*-/- embryos, though exhibiting immunohistochemically detectable alterations in the distribution of particular neurotrophins, do have normal numbers of spinal cord motor neurons (Kablar and Belliveau, 2005). It is therefore conceivable that the *Fgf2* over-expression noted in this case is part of some compensatory response mechanism that assists in rescuing the motor neurons in *MyoD*-/- embryos from excessive programmed cell death, indicating that further examination of FGF-2's role in muscle-associated

Table 11. Genes down-regulated ≥ 3.5-fold in E13.5 Myf5-/- back muscle and with knock-out (null mutant) mouse models.

Gene	Comments on deletion mutants	Reference
Afp	Females masculinized and anovulatory due to impairment of the hypothalamic/pituitary system	De Mees et al, 2006
Dgke	Healthy and fertile; defective neuronal response during seizures	Rodriguez de Turco et al., 2001
Ptprc	Defective T cell function; premature death	Zhu et al., 2008
Rbp4	Viable and fertile but with impaired vision	Quadro et al., 1999
Mgll	Hypoalgesia, with increased body temperature, decreased fatty acid levels, and impaired lipolysis; improved glucose homeostasis on a high-fat diet	Schlosburg et al., 2010; Taschler et al., 2011
Fcgr3	Abnormal hematopoietis and immune-anaphylactic responses	Coxon et al., 2001
Pcolce2	Healthy and fertile with a normal phenotype	Heinzel and Bleul, 2007
Ppargc1a	Dysfunctional energy metabolism; neuron degeneration	Cui et al., 2006
Pum2	Exhibit seminiferous tubule degeneration and smaller testes, but healthy and fertile	Xu et al., 2007
Hsf2	High lethality; neuronal defects and intracranial hemorrhage; infertility	Wang et al., 2003
Smchd1	Females embryos die at midgestation; males remain healthy	Blewitt et al., 2008
Parp3	Viable and fertile, with no overall phenotypic abnormalities	Boehler et al., 2011
Ly75	Defective hematopoietic and immune system activity	Kronin et al., 2000
Gpr22	Excessive cardiac response to induced stress	Adams et al., 2008
Pthlh	Postnatal lethality from dischondroplasia-related asphyxia	Karaplis et al., 1994
Glrb	Spasticity and hyperreflexia due to motoneural pathology	Molon et al., 2006
Rgs7bp	Normal behavior and brain morphology	Anderson et al., 2007
Ncor1	Growth retardation; abnormal neurogenesis; death by E16	Jepsen et al., 2000, 2007
Hmgn1	Increased prenatal lethality and sensitivity to irradiation	Birger et al., 2003

neurogenesis may be warranted. However, data on the role of this gene in the regulation of motor neuron numbers are currently unavailable.

In conclusion, with the exception of *Afp* and *Fgf2*, whose role in motor neuron development remains uncertain, *Kif5c, Stxbp1* and *Polb*, affected in MyoD-dependent muscle (innervated by LMC), and *Ppargc1a, Glrb* and *Hoxd10*, affected in Myf5-dependent muscle (innervated by MMC), seem to regulate the number of motor neurons according to the described findings so far (i.e., each molecule's mouse mutant has major disturbances in motor neuron survival). Their expression and distribution pattern is consistent with their role in regulation of motor neuron numbers from the skeletal muscle, because they are present in both locations, but their presence is more robust in the spinal cord, except for *Polb* (Su et al., 2002). Therefore, this analysis increases the number of molecules known to regulate motor neuron numbers in the CNS from approximately 15 (Table 7) to 21 (an approximate 30% increase), and it contributes to the literature on the specific differences between MMC and LMC trophic requirements (ALS almost exclusively affects LMC neurons, and therefore the neurons that innervate the MyoD-dependent musculature).

Functional relationship of the molecules of interest

An examination of gene relationship (using the Reactome FI plugin in Cytoscape 2.7.0, which collects and aggregates pathway information from a variety of public sources) revealed that most of the identified genes are known to interact with one another, suggesting a coordinated regulation of gene expression and function (Fig. 5).

Future directions

As mentioned in the General Introduction section above, muscle-derived neurotrophic factors evidently affect the proliferation, differentiation and survival of neural precursor cells and regulate the physiological characteristics of mature neurons, including the motor neurons, and thus have great potential in biotherapy. In this study, we have used cDNA microarray analysis to detect a number of genes affected by the deletion of either of two MRFs, *Myf5* and *MyoD*, and shown that investigators working with those genes' knock-out

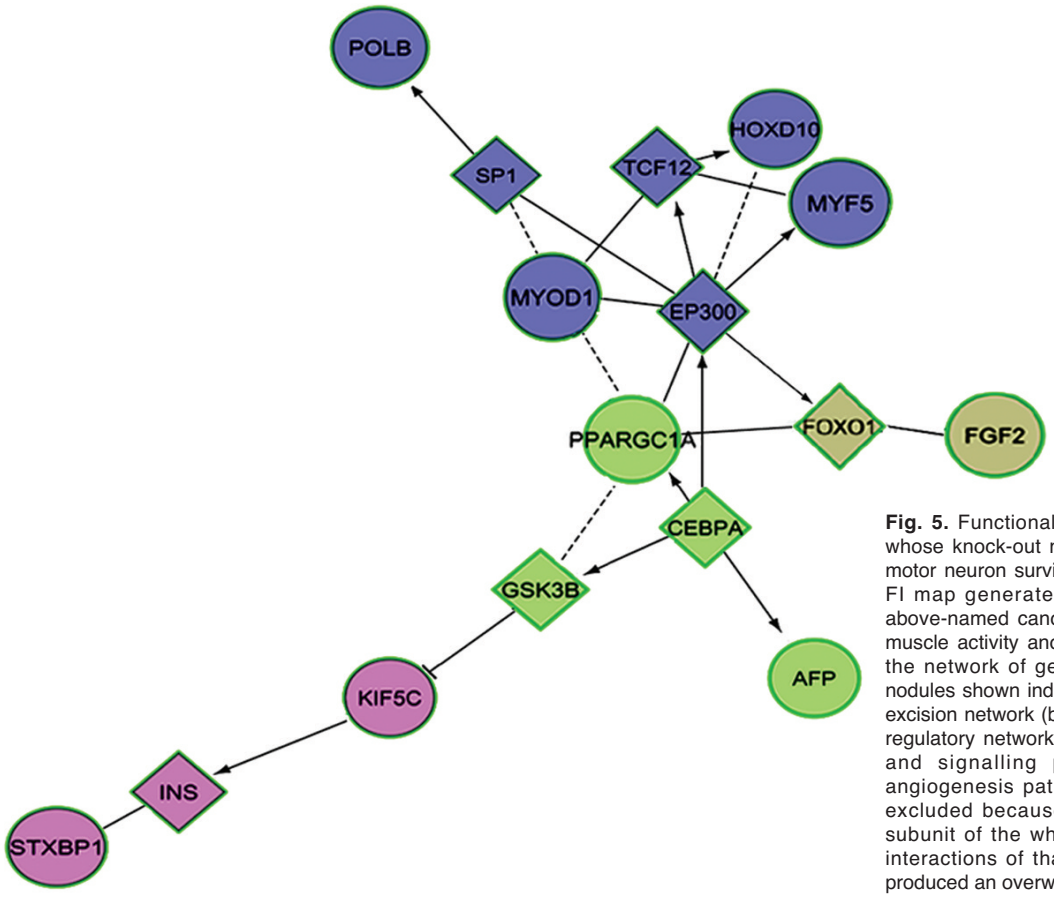

Fig. 5. Functional relationship of the molecules whose knock-out mouse mutants exhibit reduced motor neuron survival. Cytoscape 2.7.0 Reactome FI map generated from MyoD, Myf5, and the above-named candidates possibly linking skeletal muscle activity and motor neuron survival reveals the network of gene interactions pictured. The nodules shown indicate: the myogenesis and base excision network (blue), the glucocorticoid receptor regulatory network (green), the insulin processing and signalling pathway (purple), and the angiogenesis pathway (grey). (Note: Glrb was excluded because the gene encodes just one subunit of the whole glycine receptor, and the interactions of that receptor with other entities produced an overwhelming body of information.)

models have found neuromuscular pathology in the mutants.

The immediate step at this point would therefore be to assess precisely the neuron losses (or simply their numbers, which may be unaffected in some locations) in relevant anatomical locations (e.g., MMC, LMC, facial motor nucleus, motor cortex) in the eight above mentioned mouse mutants. This analysis would give us an idea of how exactly each of the identified molecules is implicated in the regulation of motor neuron (or giant pyramidal cell) numbers. At the moment we know that six of them regulate the numbers of motor neurons, but we do not know the details, except for *Ppargc1a* nulls. The next step would be to generate conditional mutants and together with other experiments (e.g., retrograde labelling, physiology, etc.) show if any of the identified molecules regulate the number of motor neurons from the muscle. At the moment we provide indirect evidence for this, but direct evidence would be essential. Another step would be to use the existing mouse mutants (Tables 8-11) and generate double- or triple-knockouts to see if a neuromuscular phenotype could be revealed behind the redundancy, unknown modifier genes, or other phenotype-masking factors. For example, synthetic lethality and genetic associations, where individual knock-outs produce viable offspring, while double knock-outs lethal, could benefit from STRING. STRING is a database of known and predicted protein interactions, including direct (physical) and indirect (functional) associations derived from four sources: Genomic Context, High-throughput Experiments, (Conserved) Co-expression and Previous Knowledge (e.g., PubMed). STRING quantitatively integrates interaction data from these sources for a large number of organisms, and transfers information between these organisms where applicable. Our hope is that individual laboratories with a specific interest in this topic, together with the efforts from the International Mouse Phenotyping Consortium (IMPC), Mouse Genome Informatics (MGI) and Gene Expression Database for Mouse Development (GDX), already extensively consulted for the current paper, will keep generating more data along these lines providing us with a possibility of precisely identifying the muscle-associated regulators of motor neuron numbers in the CNS. Our interest is in defining anatomically and molecularly the interface between the skeletal muscle and other tissues, organs and cells, such as: retina, ear, lung, bones (e.g., palate and mandible) and motor neurons (Kablar, 2011). In fact, our next immediate step is to deploy a systems biology approach (e.g., StarNet, BioBricks, Registry Biological Parts, Database of Interacting Proteins or DIP, Protein Data Bank or PDB, ProtFun, STRING, EmbryoNet, BioCreAtIvE, Phenomenet, Human Protein Atlas, ENCODE) in order to see if there are similarities in the interfaces described between the muscle and all other tissues.

Finally, all this is especially relevant if a mouse-to-human translation is possible. Parallel and analogous analysis of so called "lower vertebrates" would also be very informative and in some cases could provide answers faster (e.g., FRONTERA), but a move toward human translation is essential. (It could be started by employing Phenomenet, Human Protein Atlas and ENCODE.) To that end, we would like to stress that our approach is just a "model approach." It benefits from the fact that *Myf5* and *MyoD* cannot substitute for each other at E13.5, which coincides with the peak of programmed cell death of motor neurons. Later in life, there is no possibility to combine muscle differentiation with its ability to support motor neurons, because *Myf5* and *MyoD* soon become redundant in their functions. It is clear that most MNDs occur later in life, with the exception of Type I Spinal Muscular Atrophy (Werdnig-Hoffmann Disease), which starts in utero and becomes symptomatic within the first six months of life, and, maybe Type III Spinal Muscular Atrophy (Wohlfart-Kugelberg-Welander Disease), which also starts very early, around first or second year of life. Therefore, our approach is far from ideal. Nonetheless, it did produce results that could be further studied in order to determine if voluntary muscle plays an active role in the etiology and pathogenesis of ALS. Because of various details, we think that an (initially) asymptomatic muscle either sends a trigger(s) or/and does not send a trophic factor(s) to the motor neuron. This initiates a cascade of events, such as: peripheral axonopathy and peripheral inflammatory response, followed by the motor neuron death, central inflammatory response and subsequent muscle atrophy. Within the field, we are not the only ones with "interests in muscle as one of the earliest sites for the initiation of ALS-mediated degeneration" (from personal communication with Dr. Stanley Appel, Houston Methodist Neurological Institute, Houston, TX and Weill Cornell Medical College, New York, NY, USA). In addition to the "Musaro Laboratory," whose papers we already cited earlier in this manuscript, we found that others have studied the triggering events for motor neuron degeneration in ALS and tried to answer why motor neurons are selectively vulnerable. For example, it was revealed that skeletal muscle-restricted expression of human SOD1 actually causes motor neuron degeneration (Wong and Martin, 2010). Another group found that muscle mitochondrial uncoupling dismantles neuromuscular junction and triggers degeneration of motor neurons (Dupuis et al., 2009). All of us cited here agree that therapeutic strategies targeting muscle could be very valuable for the treatment and cure of ALS, in addition to the following groups: Suzuki et al., 2008; Hayhurst et al., 2012. In conclusion, we trust this is one possible explanation of the etiology and pathogenesis of some, but not necessarily all, cases of a MND.

Finally, our data can simply be used to compare the intricacies of Myf5- and MyoD-dependent muscle development, and therefore without the reference to the muscle's ability to provide the neurotrophic support to motor neurons. With this in mind, another search of

databases would be needed, and different but consequent follow-ups would lead to new discoveries.

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