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Knockdown of a galectin-1-like protein in zebrafish (*Danio rerio*) causes defects in skeletal muscle development

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Abstract We previously identified and characterized four galectin-1-like proteins in zebrafish, Drgal1-L1, Drgal1-L2, Drgal1-L3, and one splice variant of Drgal1-L2, of distinct ontogenic expression. Drgal1-L1 is maternal; Drgal1-L2 is zygotic and strongly expressed in the notochord, while Drgal1-L3 is both maternal and zygotic. Knockdown experiments in zebrafish embryos using a morpholino-modified antisense oligo targeted to the 5’-UTR sequence of Drgal1-L2 resulted in a phenotype with a bent tail and disorganized muscle fibers. This effect was dose-dependent as follows: 62–66% at 17 ng, 29–35% at 5.7 ng, 21–28% at 1.9 ng, and 14–17% at 0.6 ng. However, no (or a negligible number of) Drgal1-L1 knockdown embryos showed similar morphological defects, indicating that the observed effects are sequence-specific, and not due to the toxicity of the morpholino-modified oligos. Further, ectopic expression of native Drgal1-L2 specifically rescued the phenotype, as co-injection of the full-length sense Drgal1-L2 mRNA with Drgal1-L2-MO yielded 60–62% normal embryos. As the notochord serves as the primary source of signaling molecules required for proper patterning of adjacent tissues, such as neural tube, somites, and heart, these results suggest that galectins produced by the notochord play a key role in somitic cell differentiation and development.

Keywords Galectin · Drgal1-L2 · Zebrafish · Knockdown · Muscle defect

Complex carbohydrate structures encode information that modulates interactions between cells, or cells and the extracellular matrix (ECM), by specifically binding to carbohydrate-binding proteins, such as galectins [1, 2]. Galectins (previously known as S-type lectins) are a family of ß-galactoside-binding proteins, which are evolutionary conserved and have been identified in most organisms [3]. Based on structural features, galectins have been classified in three types: “proto”, “chimera”, and “tandem-repeat” [4]. Proto type galectins contain one carbohydrate recognition domain (CRD) per subunit, and are usually homodimers of non-covalently-linked subunits. The chimera type galectins have a C-terminal similar to the proto type and a non-CRD N-terminal domain rich in proline and glycine. Tandem-repeat galectins, in which two CRDs are joined by a linker peptide, are monomeric. Recently, a novel tandem-repeat type galectin with four CRDs has been described [5]. The dimerization of proto type galectins is critical for their function in mediating cell–cell or cell–ECM interactions [6, 7], and similar interactions via the N-terminus domain have been proposed for the chimera type galectins [8, 9].

Galectins have been proposed to participate in diverse biological functions related to development [10], apoptosis [11], and tumor metastasis [12]. The biological roles of galectins, and their therapeutic potential for pathological processes have been recently reviewed in detail [13]. Rigorous demonstration of their detailed mechanisms in mammalian experimental models, however, has remained elusive for most galectin types. The use of zebrafish as a model for addressing developmental questions in higher vertebrates, including mammals, has expanded dramatically in recent years [14–17]. The popularity of this animal model can be explained by the advantages it offers over mammalian systems [14, 16]. First, fertilization is external, the embryos develop rapidly *in vitro* and are transparent,

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making it possible to visualize the effects of genes involved in developmental processes, such as organogenesis, etc. Second, the early expression of these gene(s) can easily be manipulated in zebrafish embryos by various approaches such as dominant negative and antisense knockdowns, enabling visualization of effects of their normal or experimentally-modified expression. Third, a growing collection of mutations that affects early embryonic development has been characterized and mapped, providing a powerful resource for genetic studies on the function and mechanisms of action of developmentally-regulated genes. More importantly, many orthologous genes are shared among zebrafish, mouse, and man. This feature represents a significant advantage of zebrafish over other developmental models such as *C. elegans* and *Drosophila*, which lack genes mediating adaptive immunity, some aspects of neurological processing, and other functions typical of vertebrates.

We previously identified four galectin-1-like proteins in zebrafish, Drgal1-L1, Drgal1-L2, Drgal1-L3, and one splice variant of Drgal1-L2, and examined their ontogenic expression. Drgal1-L1 is maternal; Drgal1-L2 is zygotic, while Drgal1-L3 is both maternal and zygotic [18]. In this study, we identified a phenotype with a short/bent tail and disorganized muscle fibers resulting from disrupting its expression by a morpholino-modified antisense oligo targeted to the 5'-UTR sequence of Drgal1-L2. As the notochord serves as the primary source of signaling molecules required for proper patterning of adjacent tissues, such as neural tube, somites, and heart, galectins produced by the notochord may also play a key role in somitic cell differentiation and development.

Materials and methods

Reagents The monoclonal antibody F59 (specific for isoforms of myosin heavy chain associated with slow contracting muscle) was obtained from the Developmental Studies Hybridoma Bank, The University of Iowa (Iowa City, IA). Morpholino antisense oligos for translation blockers were based on the sequence near the ATG start site and were custom-synthesized by Gene Tools (Carvalis, OR). The morpholino antisense oligos were as follows: Drgal1-L1-MO: 5'-TGTATAAGCACAGTCTCATGCA-3'; Drgal1-L2-MO: 5'-ATAAGCACACCGGCCATTTT-GACGT-3'; standard control morpholino: 5'-CCTCTT-ACCTCAGTT-ACAATTTATA-3'. All other reagents were of the highest grade commercially available.

Maintenance of zebrafish and collection of embryos Zebrafish (*Danio rerio*, Cyprinidae) were raised according to the standard method previously described [19]. At the onset of light, one female and one male fish were placed in an embryo collection tank at 28.5 °C and fertilized embryos were collected within an hour after mating and used for microinjection.

Whole mount in situ hybridization and immunostaining In situ hybridization and immunostaining were carried out following the protocols previously described [18].

Validation of the morpholino-modified antisense oligonucleotide (Drgal1-L2-MO) by in vitro blocking of Drgal1-L2 protein expression In vitro direct translation of Drgal1-L2 was performed from pCS-Drgal1-L2 plasmid DNA (0.5 μg) using TNT SP6 Coupled Rabbit Reticulocyte Lysate System (Promega, Madison, WI) according to the manufacturer's instructions. For this purpose, Drgal1-L2 with 5'-UTR sequences was cloned into a pCS2$^+$ vector (a gift from D. Turner, R. Rupp, J. Lee, and H. Weintraub, Fred Hutchinson Cancer Research Center, Seattle, WA) to obtain the pCS-Drgal1-L2 construct. In this construct, a 27-nucleotide untranslated 5' leader (derived from the *Xenopus* β-globin mRNA 5'-end) is introduced between the SP6 promoter and the Drgal1-L2 insert. The pCS-Drgal1-L2 construct is expected to generate protein, when added to a cell-free protein synthesis system that is initiated by SP6 RNA polymerase. In the presence of the Drgal1-L2-MO, however, synthesis of protein should be blocked. To detect the translated product, [^{35}S]methionine was used with the methionine free amino acid mixture. For the blocking Drgal1-L2 expression, the same amount of plasmid DNA was mixed with Drgal1-L2-MO (170 ng and 340 ng). After completion of the reaction, the translated product was analyzed on 15% SDS-PAGE followed by autoradiography.

Microinjection of morpholino-modified antisense oligonucleotides into zebrafish embryos Morpholino antisense oligos were dissolved in Danieau buffer [20] to a final concentration of 0.5 mM or 1 mM and 1–2 nl was injected into the yolk of zebrafish embryos (1 to 4-cell stage) as described [21]. Five hundred embryos were injected for each antisense oligonucleotide, and raised to various stages for phenotypic analysis.

Plasmid construction and ectopic expression of native Drgal1-L2 on antisense oligo injected embryos To determine if the ectopic expression of native Drgal1-L2 rescues the phenotypes observed, Drgal1-L2 mRNAs were synthesized from a pCS-Drgal1-L2 construct using an *in vitro* transcription kit (mMESSAGE mMACHINE SP6, Ambion, Austin, Texas). In this construct, 27 nucleotides derived from the *Xenopus* β-globin 5'-UTR were used to replace the Drgal1-L2 5'-UTR as the Drgal1-L2-MO was specifically targeted to the Drgal1-L2 5'UTR. Thus, the Drgal1-L2-MO would only inhibit expression of the endogenous Drgal1-L2, but not of the injected Drgal1-L2 mRNA. The integrity of the transcribed RNA was examined on formaldehyde 1.5% agarose gels. For microinjection, mRNA was dissolved in distilled water to a final concentration of 100 µg/ml. The transcribed RNA solution (approximately 2 nl) was microinjected into the cytoplasm of zebrafish embryos at the one- or two-cell stage, and subsequently, the morpholino-modified galectin antisense oligonucleotides (17 ng) were microinjected into the yolk sac [21].

Results and discussion

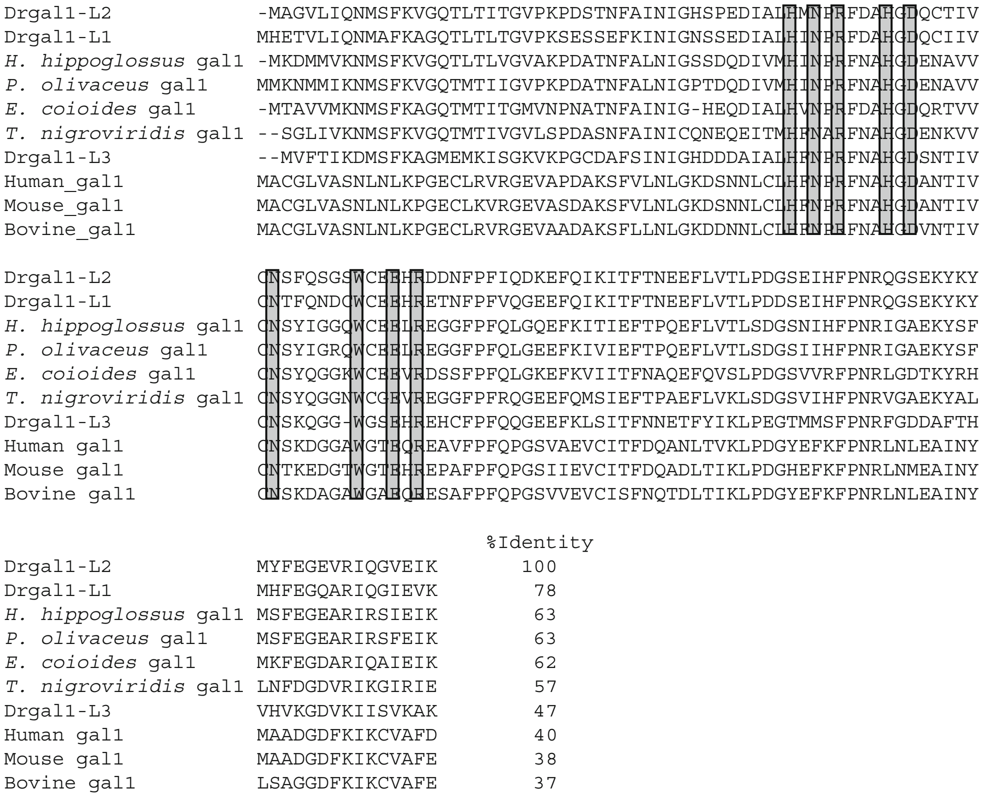
Molecular characterization of the Drgal1-L2

Sequence analysis of Drgal1-L2

The full-length cloning and sequencing of the Drgal1-L2 was previously reported [18]. Like mammalian galectin-1, the Drgal1-L2 contains all nine amino acid residues (H^{44}, N^{46}, R^{48}, H^{52}, D^{54}, N^{61}, W^{68}, E^{71}, R^{73}, the numbering is based on the bovine galectin-1 sequence, [22]) that are responsible for the carbohydrate-binding [22] [Fig. 1]. A blast search (TBLASTN) of Drgal1-L2 protein resulted highest hit with the galectin-1 or galectin-1 like proteins from higher and lower vertebrates. Besides other galectin-1-like proteins in zebrafish such as Drgal1-L1 (78%), the highest identity was observed with galectin-1-like proteins from the Atlantic halibut (*Hippoglossus hippoglossus*) and the Japanese flounder *Paralichthys olivaceus* (63%), followed by the orange-spotted grouper (*Epinephelus coioides*) (62%), the spotted green pufferfish (*Tetraodon nigroviridis*) (57%), and the mammalian *bona fide* galectins, such as those from bovine, murine and human (37–40%).

Gene organization of Drgal1-L2

A blast search (TBLASTN) against the nearly completed zebrafish genome, revealed that the gene encoding Drgal1-L2 is approximately 7 kb-long, and is located in chromosome 3, ranging from 1548913 bp to 1555901 bp. Like other galectin-1 genes, the Drgal1-L2 gene contains four exons, of which exon 3 houses all the amino acid residues that bind the carbohydrate ligand. Coding sequence lengths of the four exons are as follows: exon 1, 6 bp; exon 2, 80 bp; exon 3, 171 bp; and exon 4, 147 bp) [Fig. 2]. Overall, the genomic organization of the Drgal1-L2 gene showed similarity to the mammalian galectin-1 genes [23, 24], including the exon–intron boundaries.



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Drgal1-L2              -MAGVLIQNMSFKVGQTLTITGVPKPDSTNFAINIGHSPEDIALHMNPRFDAHGDQCTIV
Drgal1-L1              MHETVLIQNMAFKAGQTLTLTGVPKSESSEFKINIGNSSEDIALHINPRFDAHGDQCIIV
H. hippoglossus gal1   -MKDMMVKNMSFKVGQTLTLVGVAKPDATNFALNIGSSDQDIVMHINPRFNAHGDENAVV
P. olivaceus gal1      MMKNMMIKNMSFKVGQTMTIIGVPKPDATNFALNIGPTDQDIVMHINPRFNAHGDENAVV
E. coioides gal1       -MTAVVMKNMSFKAGQTMTITGMVNPNATNFAINIG-HEQDIALHVNPRFDAHGDQRTVV
T. nigroviridis gal1   --SGLIVKNMSFKVGQTMTIVGVLSPDASNFAINICQNEQEITMHFNARFNAHGDENKVV
Drgal1-L3              --MVFTIKDMSFKAGMEMKISGKVKPGCDAFSINIGHDDDAIALHFNPRFNAHGDSNTIV
Human_gal1             MACGLVASNLNLKPGECLRVRGEVAPDAKSFVLNLGKDSNNLCLHFNPRFNAHGDANTIV
Mouse_gal1             MACGLVASNLNLKPGECLKVRGEVASDAKSFVLNLGKDSNNLCLHFNPRFNAHGDANTIV
Bovine_gal1            MACGLVASNLNLKPGECLRVRGEVAADAKSFLLNLGKDDNNLCLHFNPRFNAHGDVNTIV

Drgal1-L2              CNSFQSGSWCEEHRDDNFPFIQDKEFQIKITFTNEEFLVTLPDGSEIHFPNRQGSEKYKY
Drgal1-L1              CNTFQNDCWCEEHRETNFPFVQGEEFQIKITFTNEEFLVTLPDDSEIHFPNRQGSEKYKY
H. hippoglossus gal1   CNSYIGGQWCEELREGGFPFQLGQEFKITIEFTPQEFLVTLSDGSNIHFPNRIGAEKYSF
P. olivaceus gal1      CNSYIGRQWCEELREGGFPFQLGEEFKIVIEFTPQEFLVTLSDGSIIHFPNRIGAEKYSF
E. coioides gal1       CNSYQGGKWCEEVRDSSFPFQLGKEFKVIITFNAQEFQVSLPDGSVVRFPNRLGDTKYRH
T. nigroviridis gal1   CNSYQGGNWCGEVREGGFPFRQGEEFQMSIEFTPAEFLVKLSDGSVIHFPNRVGAEKYAL
Drgal1-L3              CNSKQGG-WGSEHREHCFPFQQGEEFKLSITFNNETFYIKLPEGTMMSFPNRFGDDAFTH
Human gal1             CNSKDGGAWGTEQREAVFPFQPGSVAEVCITFDQANLTVKLPDGYEFKFPNRLNLEAINY
Mouse gal1             CNTKEDGTWGTEHREPAFPFQPGSIIEVCITFDQADLTIKLPDGHEFKFPNRLNMEAINY
Bovine gal1            CNSKDAGAWGAEQRESAFPFQPGSVVEVCISFNQTDLTIKLPDGYEFKFPNRLNLEAINY

                                                %Identity
Drgal1-L2              MYFEGEVRIQGVEIK            100
Drgal1-L1              MHFEGQARIQGIEVK             78
H. hippoglossus gal1   MSFEGEARIRSIEIK             63
P. olivaceus gal1      MSFEGEARIRSFEIK             63
E. coioides gal1       MKFEGDARIQAIEIK             62
T. nigroviridis gal1   LNFDGDVRIKGIRIE             57
Drgal1-L3              VHVKGDVKIISVKAK             47
Human gal1             MAADGDFKIKCVAFD             40
Mouse gal1             MAADGDFKIKCVAFE             38
Bovine gal1            LSAGGDFKIKCVAFE             37
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Fig. 1 Amino acid sequence comparison of the Drgal1-L2 with the mammalian galectin-1 or galectin-1-like proteins from lower vertebrates. Amino acid sequence of the galectins are aligned by ClustalW program (clustalw.genome.ad.jp/). The amino acids shown in *shaded boxes* interact with *N*-acetyllactosamine as determined from 3-D structure of the bovine galectin-1 [22]

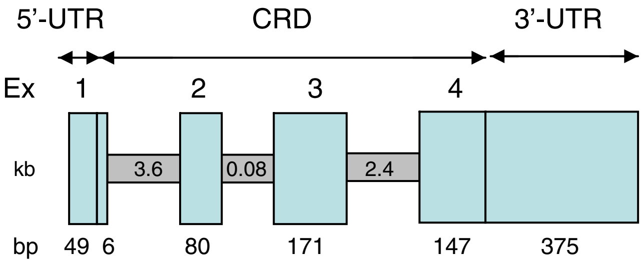

Fig. 2 Gene organization of Drgal1-L2. The *vertical boxes* represent exons, which are numbered at the top. The size of each exon (in bp) is indicated at the bottom. The *horizontal boxes* represent introns, whose sizes are indicated in kb

Drgal1-L2 expression in developing zebrafish

Drgal1-L2 was intensely expressed in the notochord of the developing embryos tested up to 24 hpf (hour post fertilization) embryos [18]. In this study, older embryos up to 3 dpf (days post fertilization) were examined for Drgal1-L2 expression. In these embryos, Drgal1-L2 expression remained intense in the notochord (not shown).

Functional characterization of Drgal1-L2

Validation of the morpholino-modified antisense oligonucleotide (Drgal1-L2-MO) by in vitro blocking of Drgal1-L2 protein expression

To test if Drgal1-L2-MO can block Drgal1-L2 expression, *in vitro* direct translation of Drgal1-L2 was performed from the pCS-Drgal1-L2 plasmid DNA (0.5 μg) using the TNT SP6 Coupled Rabbit Reticulocyte Lysate System in the presence or absence of Drgal1-L2-MO. Drgal1-L2-MO blocked the *in vitro* expression of Drgal1-L2 as little or no product was seen, when the translation reaction was performed in the presence of Drgal1-L2-MO [Fig. 3A, lanes 2 and 3]. Results from pCS-Drgal1-L2 plasmid DNA without Drgal1-L2-MO showed a single band corresponding to expected size of Drgal1-L2 (approximately 14 kDa) [Fig. 3A, lane 1].

Validation of the Drgal1-L2-MO by in vivo blocking of Drgal1-L2 protein expression

To determine if the injection of antisense oligos effectively blocked Drgal1-L2 protein expression *in vivo*, whole mount antibody staining was performed on injected embryos.

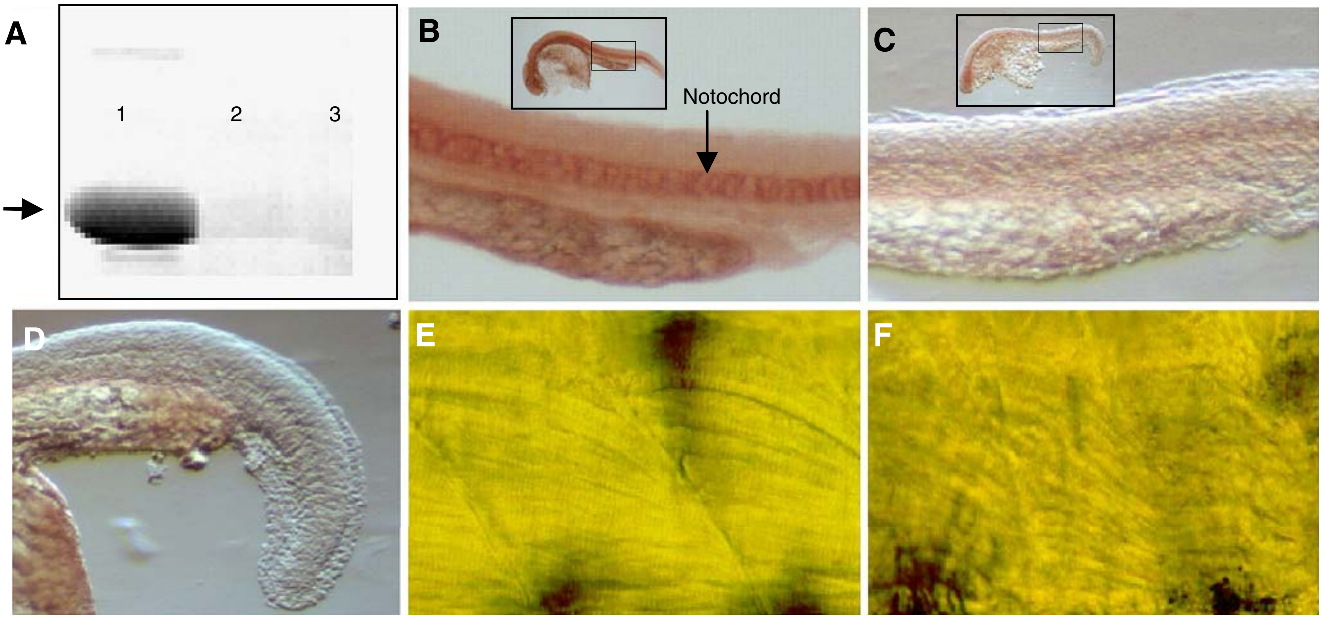

Fig. 3 Blocking of Drgal1-L2 protein expression by Drgal1-L2-MO. **A** *In vitro* blocking of Drgal1-L2 protein in a rabbit reticulocyte system. SDS-PAGE/ autoradiography analysis of the *in vitro* labeled Drgal1-L2 translation products. *Lane 1*: translation reaction in the absence of Drgal1-L2-MO (positive control); *lanes 2* and *3*: reactions in the presence of 170 ng and 340 ng of Drgal1-L2-MO, respectively. **B** Whole mount immunostaining of an uninjected embryo probed with anti-Drgal1-L2 antibodies (positive control). Lateral and dorsal view of a 27 hpf embryo. **C** In vivo blocking of Drgal1-L2 protein as shown by whole mount immunostaining of an embryo injected with Drgal1-L2-MO, and probed with anti-Drgal1-L2 antibodies. Lateral and dorsal view of a 27 hpf embryo. **D** Dorsal and lateral view of the embryo showing the short/bent tail macroscopic phenotype. **E**, **F** Dorsal and lateral view of the trunk showing disorganized muscle fibers in the Drgal1-L2-MO-injected embryo (**F**), as compared to the wild type control embryo (**E**)

Table 1 Dose response of phenotypes caused by Drgal1-L2-MO injection

Drgal1-L2-MO injected*	% Animals expressing the "short and bent tail" phenotype (animals expressing phenotype/total injected embryos)		
	Exp. 1	Exp. 2	Exp. 3
0.6 ng	17 (12/70)	17 (20/119)	14 (5/35)
1.9 ng	28 (17/60)	22 (8/36)	21 (8/38)
5.7 ng	29 (18/62)	35 (17/49)	34 (27/79)
17.0 ng	62 (40/64)	66 (25/38)	64 (55/86)

*2–8 Cell stage embryos were injected with increasing doses of Drgal1-L2-MO and examined at 24 hpf

Compared with the control (uninjected) embryos [Fig. 3B], injected embryos showed dramatically reduced or no Drgal1-L2 expression [Fig. 3C].

Identification of a phenotype in the Drgal1-L2-MO-knockdown

Knockdown experiments using Drgal1-L2-MO resulted in embryos with a bent tail [Fig. 3D, see also Fig. 3C inset] and disrupted organization of the muscle fibers [Fig. 3F]. The phenotype was dose-dependent as follows: 62–66% at 17 ng, 29–35% at 5.7 ng, 21–28% at 1.9 ng, and 14–17% at 0.6 ng (Table 1). However, a negligible number of embryos showed morphological defects when injected with the Drgal1-L1-MO (not shown), indicating that the observed effect is sequence-specific and not due to the toxicity of the morpholino-modified oligos themselves. The disorganized muscle was prominent as judged by whole mount immunostaining with the slow muscle marker F59 antibody (monoclonal anti-myosin antibody) (Fig. 4A,B) and *in situ* hybridization with *myod* (specific for both fast and slow muscle) (Fig. 4C,D).

The specificity of the phenotype was validated by rescue experiments. To determine if ectopic expression of Drgal1-L2 could rescue the phenotype, full-length sense Drgal1-L2 mRNA was co-injected with Drgal1-L2-MO. Because the 5'-UTR sequence of Drgal1-L2 mRNA was replaced by a β-globin gene 5'-UTR, it cannot be recognized by Drgal1-L2-MO. This ectopic expression of native Drgal1-L2 specifically rescued the phenotype, as co-injection yielded 60–62% of normal embryos.

The results from this study suggest that the Drgal1-L2 produced/secreted by the notochord plays a key role in somitic cell differentiation and development, since blocking

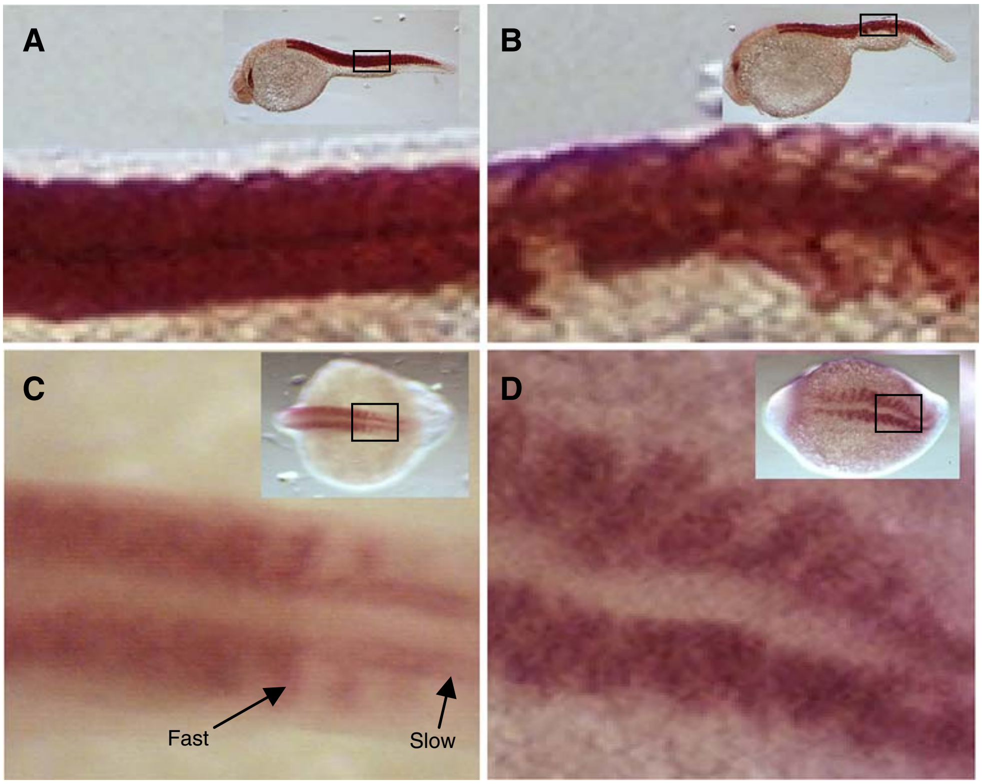

Fig. 4 Dorsal and lateral view of embryos showing the effect(s) of Drgal1-L2 gene expression knockdown. **A**, **B** Whole mount immunostaining of (**A**) wild type (uninjected) control and (**B**) Drgal1-L2-MO injected embryos (24 hpf) with F59 antibody. **C**, **D** Whole mount in situ hybridization of (**C**) uninjected and (**D**) Drgal1-L2-MO injected embryos (19 hpf) probed with myod antisense oligos. Compared to the wild type control embryos (**A**, **C**), the myofibers in Drgal1-L2-MO injected embryos (**B**, **D**) appeared less organized

of Drgal1-L2 expression appears to affect skeletal muscle formation resulting in disrupted myofibril organization.

Previous reports strongly suggest the role of galectin-1 in muscle differentiation by direct interactions with the myoblast or its precursors in the local environment [25, 26]. Recent *in vitro* and *in vivo* studies on myoblasts from a galectin-1 null mouse have rigorously demonstrated the direct role of galectin-1 in skeletal muscle development and regeneration [27]. Myoblasts derived from the galectin-1 null mouse exhibit decreased fusion *in vitro*, whereas a delay in muscle fiber development takes place in the null mouse neonatal stage, and a reduced muscle fiber diameter is observed in the adult. Similarly, a regeneration delay and reduced fiber size is observed during muscle recovery from experimental injury [27]. Further, galectin-1 has been implicated in the differentiation of stem cells into muscle fibers. When human fetal mesenchymal stem cells are exposed *in vitro* to galectin-1, about two thirds will differentiate into a muscle phenotype, consisting of multinucleated fibers that express both desmin and myosin. If the galectin-1-exposed cells are transplanted into regenerating murine muscle they form 4-fold more human muscle fibers than the unexposed cells. These and similar results obtained in a *scid/mdx* dystrophic mouse model, underscore the considerable myogenic potential of the galectin-1-exposed stem cells for intervention in degenerative muscle diseases, such as muscular dystrophies [28].

Based on the studies described herein, however, an alternative and potentially earlier mechanism for galectin-1 function in muscle development can be proposed. During early embryogenesis, the notochord serves as the primary source of signal molecules required for proper patterning of adjacent tissues, such as neural tube, somites, heart [29–31]. The bone morphogenic protein expressed in lateral mesoderm, plays a major role in promoting blood and heart development, activities that are modulated by its antagonist, Noggin, expressed in notochord [29]. The Wnt signaling molecules, including Wnt11, expressed in the notochord [30], and Wnt3a and Wnt8, produced by the neural tube, also influence strongly the formation of heart tissue and blood [31]. Furthermore, hedgehog signals secreted from the notochord play an important role in slow muscle induction [32–34]. The unique spatial expression and tissue localization of Drgal1-L2 in the notochord (but not in the myotome) of the early embryos at the time the knockdown experiments are carried out, and the distant tissue affected by disruption of Drgal1-L2 gene expression strongly suggest that at this developmental stage its role(s) in somitic cell differentiation and development may be indirect, that is by initiating or modulating signaling pathways originated in the notochord, rather than direct interactions between cells or cells and the ECM in the local environment of the somites that would take place at later developmental stages.

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