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SPECIAL ISSUE RESEARCH ARTICLE

RGMa and *RGMb* Expression Pattern During Chicken Development Suggest Unexpected Roles for These Repulsive Guidance Molecules in Notochord Formation, Somitogenesis, and Myogenesis

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Background: Repulsive guidance molecules (RGM) are high-affinity ligands for the Netrin receptor Neogenin, and they are crucial for nervous system development including neural tube closure; neuronal and neural crest cell differentiation and axon guidance. Recent studies implicated RGM molecules in bone morphogenetic protein signaling, which regulates a variety of developmental processes. Moreover, a role for RGMc in iron metabolism has been established. This suggests that RGM molecules may play important roles in non-neural tissues. Results: To explore which tissues and processed may be regulated by RGM molecules, we systematically investigated the expression of *RGMa* and *RGMb*, the only RGM molecules currently known for avians, in the chicken embryo. Conclusions: Our study suggests so far unknown roles of RGM molecules in notochord, somite and skeletal muscle development. *Developmental Dynamics 241:1886–1900, 2012.* © 2012 Wiley Periodicals, Inc.

Key words: RGMa; DRAGON; RGMb; chicken; embryo; neural plate, neurogenesis; dorsal root ganglia; pharyngeal ectoderm; notochord; somite; dermomyotome; myotome

Key findings:

- *RGMa* and *RGMb* expression patterns in neural tissues are conserved in gnathostome vertebrates.
- *RGMa* might be associated with muscle stem cells capable of undertaking myogenesis, and *RGMb* with cells ready to enter myogenic differentiation.

ABBREVIATIONS **asp** anterior segmental plate **di** diencephalon **dm** dermomyotome **dml** dorsomedial lip of the dermomyotome **drg** dorsal root ganglion **ect** ectoderm **edl** Extensor Digitorum Longus **end** endoderm **f** fibula bone **fge** foregut endoderm **fp** floor plate of the neural tube **hm** head mesoderm **hy** hyoid (2nd) pharyngeal arch **m** myotome **ma** mandibular (1st) pharyngeal arch **mes** mesencephalon mx maxillary aspect of the 1st pharyngeal arch **myel** myelencephalon **node** Hensen's node (avian organizer) **not** notochord **np** neural plate **nt** neural tube **op** otic placode **os** optic stalk **ov** otic vesicle **pa** pharyngeal arch **pchpl** prechordal plate **p/not** posterior notochord **ps** primitive streak **r1** **r2** etc rhombomere 1 rhombomere 2 etc. **s0** epithelializing somite **s1** **s5** etc 1st somite 5th somite etc. **t** tibiotarsal bone **tel** telencephalon **V** trigeminal (5th cranial nerve) ganglion **VII** facial (7th cranial nerve) ganglion **vll** ventromedial lip of the dermomyotome.

Additional Supporting information may be found in the online version of this article.

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- *RGMa* and *RGMb* may promote myogenesis by modulating bone morphogenetic signaling and Shh signal transduction.
- RGM family members may act in maintaining notochord integrity and longitudinal axis formation.

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INTRODUCTION

Repulsive guidance molecules (RGM) are cysteine-rich proteins that can be found both as membrane-bound (by means of a GPI-anchor) or soluble forms. Invertebrate chordates have a single-copy RGM gene; owing to two rounds (teleosts three rounds) of genome duplication and subsequent gene loss (Holland et al., 1994; Taylor et al., 2001; Postlethwait, 2007), two RGM paralogs have been identified in the chicken (*RGMa*; *RGMb*, or *DRAGON*), mammals have an additional *RGMc* (*Hemojuvelin*, *HJV*, *HFE2*) gene and teleosts have a fourth *RGMd* gene (Camus and Lambert, 2007). RGM proteins show significant sequence homology to one another with 30–60% amino acid identity. Common structural features of RGM proteins include an N-terminal signal peptide, a partial von Willebrand factor type D domain which includes a catalytic cleavage site, and a C-terminal glycosylphosphatidylinositol (GPI) -anchor (Monnier et al., 2002; Samad et al., 2004). In addition, a putative cell–cell adhesion motif known as RGD (Arg-Gly-Asp) motif is conserved in RGMa and RGMc (Samad et al., 2004).

The first member of the RGM family to be found was chicken *RGMa*, initially identified as a repulsive extracellular guidance molecule, responsible for the precise projections of retinal ganglion cells to the superior colliculus of the embryonic tectum (Monnier et al., 2002). However, analysis of *RGMa* mutant mice revealed no defects of neuronal projections (Niederkofler et al., 2004). Instead, approximately 50% of mouse embryos lacking *RGMa* suffered from exencephaly, caused by a failure of cephalic neural tube closure and defective skull bone deployment (Niederkofler et al., 2004). RGMa was also identified as a neuronal cell survival factor, based on its ability to counteract the pro-apoptotic activity Neogenin (Matsunaga et al., 2004). Gain- and loss-of-function studies in chicken embryos reinforced the notion that, in addition to its role in axon guidance, *RGMa* also controls neuronal proliferation, differentiation and survival (Matsunaga et al., 2006). In mammals, *RGMa* was found to be up-regulated after injury of the adult central nervous system, and in vitro studies demonstrated that *RGMa* is a key inhibitor of neurite outgrowth of postnatal cerebellar neurons (Hata et al., 2006). The elevation of *RGMa* is due to microglia/macrophages that invade the lesion site and start producing RGMa to avoid novel axonal growth (Kitayama et al., 2011); in line with this, local administration of RGMa neutralizing antibodies significantly induced axon regeneration and locomotor improvement after spinal cord injury (Hata et al., 2006). Recently, *RGMa* has also been associated with multiple sclerosis and autoimmune encephalomyelitis (Nohra et al., 2010; Muramatsu et al., 2011), underlining the importance of *RGMa* for both the adult and embryonic nervous system.

The role of RGMa in delaying neurite outgrowth, growth cone repulsion, and growth cone retraction is mediated by the dependence receptor Neogenin, a homolog of DCC (Deleted in Colorectal Cancer) and known Netrin-1 receptor, and a cytoplasmatic signaling cascade that involves RhoA/Rho kinases, a family of small GTP-binding proteins that regulate cytoskeletal actin dynamics (Rajagopalan et al., 2004; Hata et al., 2006; Conrad et al., 2007; Itokazu et al., 2012). In brief, RGMa binds to Neogenin, but not to DCC, with high affinity to activate RhoA, Rho kinase and the PKC pathway, a common pathway to induce growth cone collapse (Rajagopalan et al., 2004; Conrad et al., 2007; Itokazu et al., 2012). The Neogenin-mediated activation of RhoA also dependents on Unc5B, a constitutively bound RGMa co-receptor, the RGS-RhoGEF family member Leukemia-associated guanine nucleotide exchange factor (LARG) and Focal adhesion kinase (FAK; Hata et al., 2009). In addition, the tumor necrosis factor-alpha converting enzyme (TACE) was recently included in the RGMa-Neogenin signaling pathway. TACE is a disintegrin and metalloprotease transmembrane protein involved in the shedding of the extracellular domain of Neogenin, thereby regulating the sensitivity of neurons for RGMa (Okamura et al., 2011).

RGMb or *DRAGON* was identified in a screen for genes whose promoters are regulated by DRG11, a homeobox transcription factor expressed in dorsal root ganglia (DRG) and embryonic dorsal horn neurons (Samad et al., 2004). However, rather than facilitating axonal repulsion, RGMb was shown to promote adhesion of mouse DRG neurons (Samad et al., 2004). Further biological roles for RGMb include mammalian reproduction (Xia et al., 2005); and, similar to RGMa, responses to nervous system injury (Liu et al., 2009). *RGMb* injection in *Xenopus* embryos induced endodermal, mesodermal, and in the ectoderm, neuronal markers while neural crest cell differentiation was inhibited (Samad et al., 2005). *RGMb* knockout mice died 3 weeks after birth, without evident defects in sensory and motor functions or nervous system development (Mueller et al., 2006).

RGMb was the first member of the RGM family to be identified as bone morphogenetic signaling (BMP) co-receptor, enhancing BMP signaling (Samad et al., 2005); subsequently the same activity was found for RGMa (Babitt et al., 2005) and RGMc (Babitt et al., 2006). However, in C2C12 myoblasts *RGMb* blocked BMP-induced osteoblastic differentiation by means of inhibition of Smad1 and Smad4 (Kanomata et al., 2009); a recent study linked Neogenin and BMP activity showing that BMP can use Neogenin as receptor, and the subsequent activation of RhoA inhibits Smad 1/5/8 phosphorylation, thereby inhibiting canonical BMP signal transduction (Zhou et al., 2010).

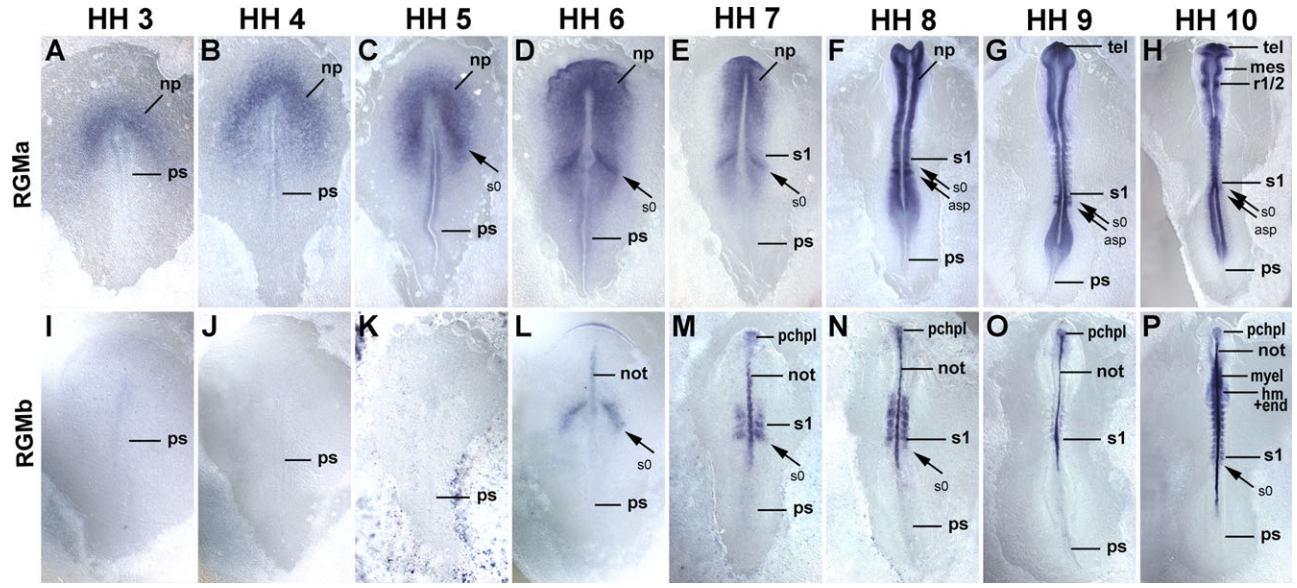

Fig. 1. *RGMa* and *RGMb* expression pattern during gastrulation and neurulation stages of chicken development. Dorsal views of whole embryos, anterior to the top. Chicken developmental stages are indicated at the top of the panel. For abbreviations, see list of abbreviations. *RGMa* is strongly expressed in the neural plate, anterior segmental plate, and epithelializing somites, *RGMb* in the notochord and somites.

RGMc was found based on its sequence similarity with RGMa and RGMb (Niederkofler et al., 2004; Schmidtmer and Engelkamp, 2004). *RGMc* is expressed in skeletal muscle, heart, liver, bone, and cartilage (Samad et al., 2004; Niederkofler et al., 2004; Papanikolaou et al., 2004; Rodriguez et al., 2007; Kanomata et al., 2009), but its function in these tissues remains largely unknown. Nevertheless, mutations of *RGMc* have been linked with Hereditary Hemochromatosis, a heterogeneous group of autosomal recessive diseases that cause increased intestine absorption and deposition of iron in heart, liver, endocrine glands, joints, and skin (revised by Pietrangelo, 2007). For this reason, *RGMc* is also known as *Hemojuvelin* (*HJV*) or *HFE2*. In the liver, RGMc primarily acts as membrane-bound BMP co-receptor, positively regulating the expression of the iron regulatory hormone Hepcidin (Babitt et al., 2006; Xia et al., 2008). However, it has been suggested that RGMc-Neogenin interaction also contributes to Hepcidin upregulation (Zhang et al., 2009). The soluble form of RGMc acts as a decoy and blocks BMP signaling and Hepcidin expression (Maxson et al., 2010). Studies regarding the production of soluble RGMc are controversial because on one hand, Neogenin has been shown to increase RGMc shedding (release of soluble RGMc) (Zhang et al., 2008); on the other hand, Neogenin has been shown to inhibit the secretion of soluble RGMc, thus increasing BMP signaling and Hepcidin production in a cell nonautonomous manner (Lee et al., 2010).

Given that new roles outside the nervous system are emerging for Neogenin, and given that BMP signaling controls the development of neural as well as non-neural tissues, we were wondering to which extent RGM molecules may control non-neural developmental processes. To begin elucidating the biological roles of *RGM* genes in these processes, we turned to the chicken embryo as this is the most accessible model for both early and late stages of embryonic development. The current edition of the chicken genome (build 3.1, NCBI) predicts the existence of only two *RGM* genes in this species: *RGMa*, localized on chromosome 10; and *RGMb*, on the sexual chromosome Z. We thus determined *RGMa* and *RGMb* expression patterns from gastrulation stages at Hamburger and Hamilton stage (HH) 3 to organogenesis stages at embryonic day (E) 5/HH27 of chicken embryonic development. Our analysis confirmed *RGMa* expression in the neural plate and neural tube, and *RGMb* expression in differentiating neurons and cranial and dorsal root ganglia. However, *RGMb* was also strongly expressed in the notochord. Moreover, both RGM molecules were expressed in the somite, with *RGMa* predominantly labeling muscle precursor and muscle stem cells and *RGMb* labeling differentiating muscle.

RESULTS

RGM Expression at Gastrulation and Neurulation Stages of Development

To investigate the expression of RGM molecules during early stages of development, chicken embryos from stage HH3 (early gastrulation stage) to HH10 (late neurulation stage) were isolated and subjected to whole-mount in situ hybridization using a **RGMa** antisense probe (Fig. 1A–H) or a *RGMb* antisense probe (Fig. 1I–P). For a small collection of embryos a *RGMb* sense probe was used as negative control; these embryos did not show any staining (not shown). Upon in situ hybridization, details of expression pattern were analyzed on serial vibratome cross sections (Fig. 2).

Early Chicken *RGMa* Expression

At HH3–HH8, the most prominent expression domain of *RGMa* was in the neural plate (Figs. 1A–F, 2A,C; np); however, weak, transient expression was also found in the primitive

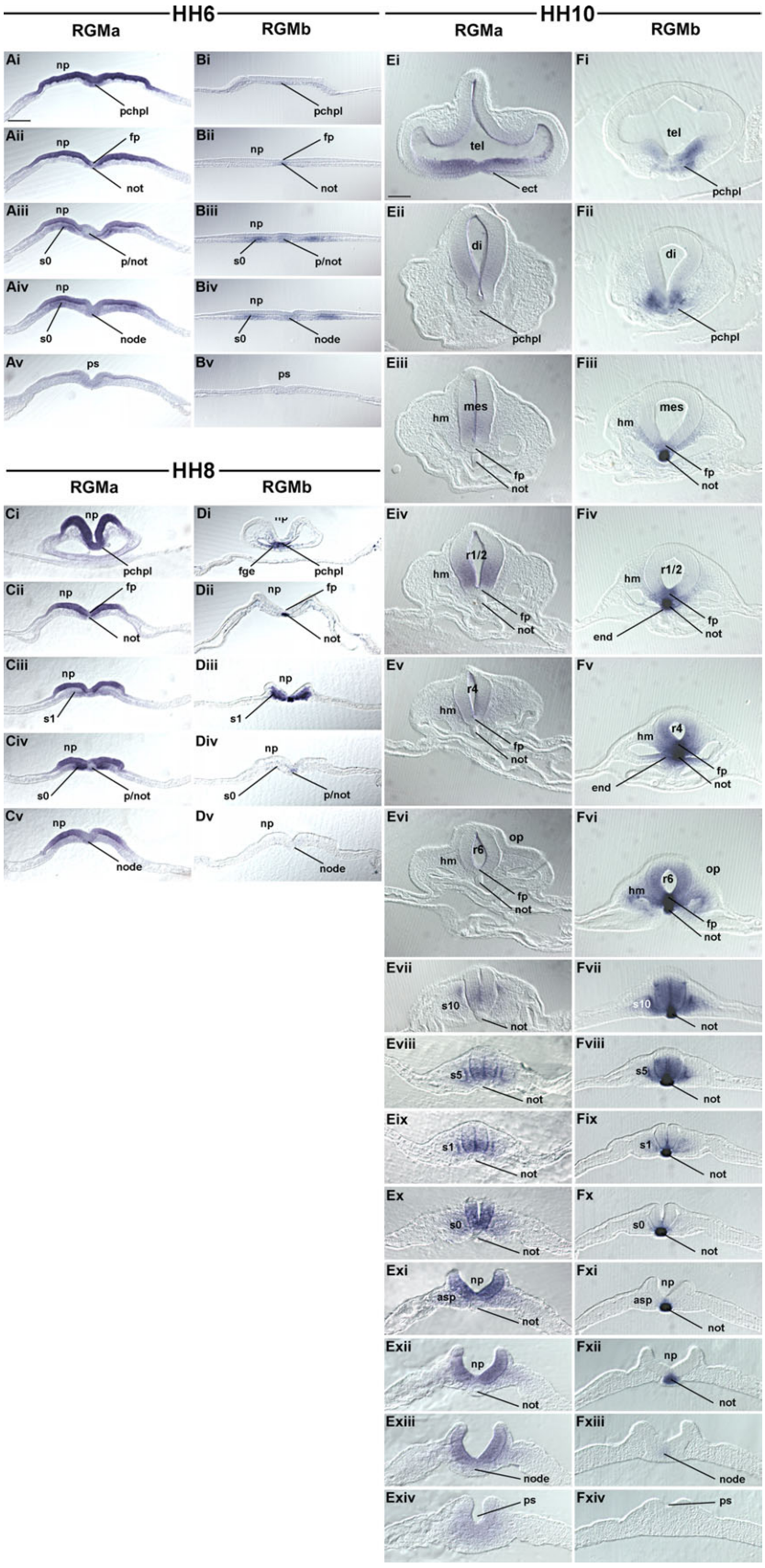

Fig. 2.

streak (ps). During neurulation, *RGMa* expression declined in the dorsal neural tube and floor plate, restricting *RGMa* expression to the dorsal part of the basal plate; moreover, *RGMa* expression declined in the posterior hindbrain (Figs. 1G,H, 2E). However, ventral expression was well maintained in the telencephalon, encompassing the underlying ectoderm (Fig. 2Ei). In the paraxial mesoderm, *RGMa* expression appeared as soon as the 1st somite began to condense (Fig. 1C,D; arrow). Subsequently, *RGMa* transcripts were always found in the anterior-most segmental plate and epithelializing somite (Fig. 1E–H, arrows; Fig. 2A,C,E, asp and s0). As the somites matured, diminished but detectable expression was found in the medial wall of the somite (Fig. 2Eix, Eviii) and the muscle pioneers that lay down the primary myotome (Kahane et al., 1998).

Early Chicken *RGMb* Expression

RGMb did not commence before HH6 when it labeled the notochord (Figs. 1L, 2B, not) and the somite about to epithelialize (Figs. 1L, arrow; 2Biii,iv, s0). At HH7–HH10, the developing as well as the recently formed somites expressed *RGMb*, with expression becoming restricted to the medial wall of the somite and the muscle pioneers (Figs. 1M–P, 2Fx-vii). Moreover, new expression domains appeared, encompassing the prechordal plate (Figs. 1M–P, 2Fi,ii, pchpl) and the ventromedial head mesoderm (Fig. 2Fiii-vi, hm). At the level of rhombomere 4 where the pharyngeal endoderm just had fused in the ventral midline, the

Fig. 2. *RGMa* and *RGMb* expression pattern during gastrulation and neurulation stages of chicken development. Cross-sections, dorsal to the top. For abbreviations, see list of abbreviations. *RGMa* is strongly expressed in the neural plate, anterior segmental plate and epithelializing somites; additional expression domains are in the primitive streak, the medial wall of the somites, subdomains of the developing fore-, mid-, and hindbrain and the ectoderm underlying the telencephalon. *RGMb* expression is found in the notochord and medial wall of the somites, and in addition in the prechordal plate and head mesoderm, the floor plate of the neural tube, and subdomains of the fore-, mid-, and hindbrain.

medial aspect of this tissue also expressed *RGMb* (Fig. 2Fv). At HH10, the floor plate of most of the neural tube was positive for *RGMb*; in the fore brain, expression expanded further dorsally (Fig. 2Fi,ii), and in the myelencephalic area and upper spinal cord expression encompassed also most of the alar plate (Fig. 2Fv-viii).

RGM Expression at Organogenesis Stages of Development

To establish RGM expression at organogenesis stages of development, we continued our analysis with embryos at HH14–HH27, i.e., half-day intervals. Due to the large size of embryos at HH24–HH27, we focused our analysis on the trunk. Embryos were subjected to whole-mount in situ hybridization and subsequent cross-sectioning as before.

RGMa Expression

RGMa remained strongly expressed in the neural tube, the most prominent staining being in the telencephalon and mesencephalon (Fig. 3A,C,E,G). In the immature spinal cord neighboring the HH14 segmental plate, expression encompassed most of the neural tissue excluding the floor plate and the dorsal-most aspect of the alar plate (Fig. 4Q). In more anterior and hence more mature regions, expression was cleared from most of the alar plate (Fig. 4O,M). At HH16–HH20, expression shifted toward the ventral aspect of the alar plate (Fig. 4K,I,G), and by HH21, the signal was confined to the intermediate layer that actively generates interneurons (Fig. 4E, Gross et al., 2002; Müller et al., 2002). Moreover, *RGMa* was observed in mesenchymal cells surrounding the dorsal root ganglia (Fig. 4A,C,E), and in cells demarcating the dorsal root entry zone (Fig. 4E, arrow). At HH24 (Fig. 4C), discrete domains in the spinal cord were labeled, suggesting that *RGMa* was expressed in specific neuronal subpopulations (Supp. Fig. S1, which is available online).

In addition to its expression associated with the nervous system, *RGMa* was expressed in the ectoderm overlying the pharyngeal arches (Figs. 3E,G, 5A–C). In the trunk, *RGMa* expression was associated with the somites (Fig. 3A,C,E,G,I,K,M, s). Similar to earlier stages, expression encompassed the anterior segmental plate ready to form a somite (Fig. 4S,Q, asp). In epithelial somites, expression became successively

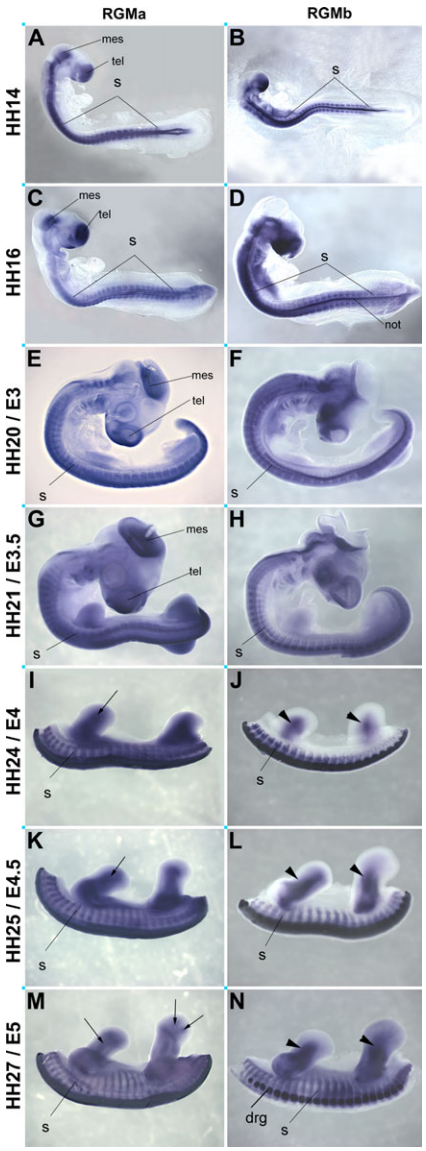

Fig. 3. *RGMa* and *RGMb* expression at early organogenesis stages of chicken development. Dorsal views of whole embryos, anterior to the top. Chicken developmental stages are indicated at the top of the panel. In Figure 3N, the neural tube was removed to reveal expression in the dorsal root ganglia. For abbreviations, see list of abbreviations. Note prominent RGMa expression in the telencephalon (tel), mesencephalon (mes), spinal cord, pharyngeal arches, somites (s) and developing limb bud cartilages (I,K,M, arrows). *RGMb* is expressed in the neural tube, cranial and dorsal root ganglia (N, drg), pharyngeal arches, notochord (D, not), somites (s) and developing limb muscles (J,L,N, arrowheads).

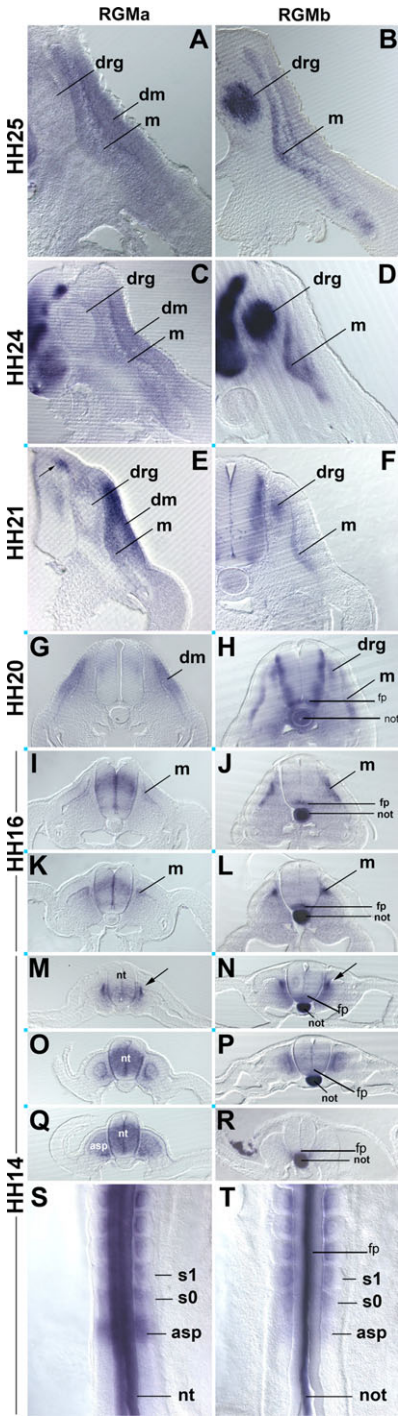

Fig. 4. *RGMa* and *RGMb* expression at early organogenesis stages of chicken development. Cross-sections at trunk levels (A–R), dorsal to the top. Dorsal views of HH14 whole trunk, anterior to the top (S,T). For abbreviations, see list of abbreviations. Note *RGMa* expression in the medial wall of young somites. In mature somites, *RGMa* is expressed in the central dermomyotome and in myotomal cells located between and dorsal and ventral to the differentiated muscle fibers. *RGMb* expression overlaps with that of *RGMa* in the medial wall of the somites and the myotome.

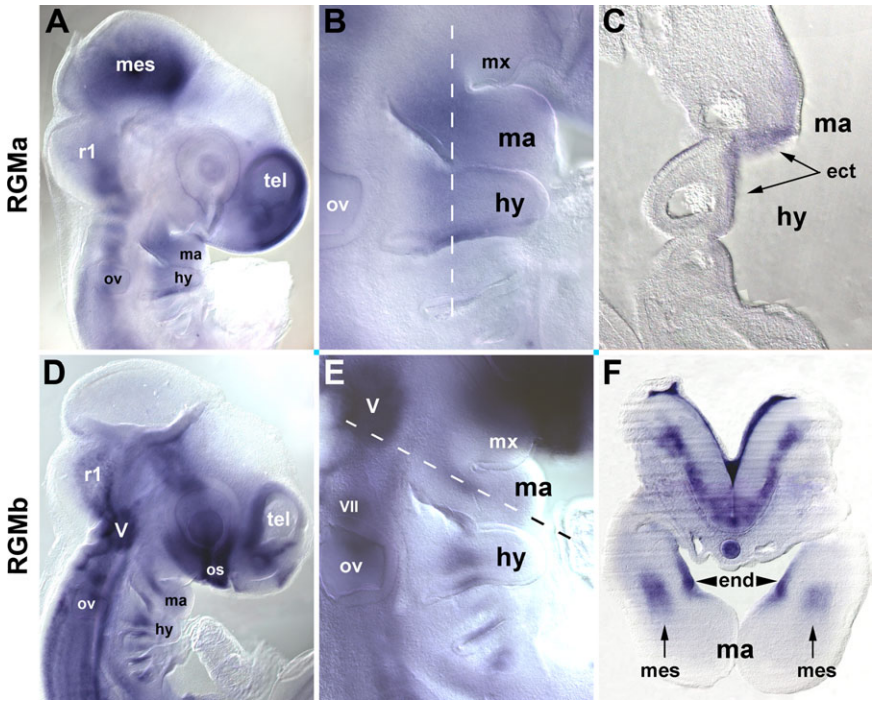

Fig. 5. Details of cranial *RGMa* and *RGMb* expression. Dorsolateral views of the head (A,D); details of the pharyngeal arches (B,E); frontal (C) and transverse (F) sections at the positions indicated in (B) and (E), respectively. For abbreviations, see list of abbreviations. *RGMa* is expressed in the telencephalon, mesencephalon, rhombencephalon, and the ectoderm lining the pharyngeal arches. *RGMb* is expressed in the telencephalon, ventral diencephalon, rhombencephalon, optic stalk, notochord, pharyngeal endoderm and the mesodermal core of the pharyngeal arches.

restricted to the medial wall (Fig. 4S,O,M). As the somites differentiated, weak expression was found in the myotome (Fig. 4K,I). Significantly, at HH20 *RGMa* was expressed in the medial and central aspect of the dermomyotome (Fig. 4G, dm) and from HH21 onward, expression was found in the dermomyotome and in cells that have entered the myotome directly from the dispersing central dermomyotome (Fig. 4E,C,A, m).

RGMb Expression

Similar to the earlier stages of development, *RGMb* was strongly expressed in the notochord (Figs. 3B,D,F, 4T,R,P,N,L,J,H, not), the floor plate of the neural tube (Figs. 3B,D,F, 4T,R,P,N,L,J,H, fp) and in the immediately adjacent cells that are known to deliver the ventral (V3) interneurons upon induction by Shh (Ericson et al., 1997, Briscoe et al., 2000). While the signals in the notochord and floor plate faded at HH21 (Figs. 3H, 4F), new expression domains emerged from HH20 onward in the mantle layer of the neural tube that contains the differentiated neurons (Figs. 3F,H,J,L; 4H,F,D, 5F; Lee and Pfaff, 2001), in the trigeminal (Fig. 5D,E; V), facial (Fig. 5D,E; VII), and dorsal root ganglia (Figs. 3N, 4H,F,D,B, drg), and in the optic stalk (Fig. 5D, os). Outside the nervous system, *RGMb* was expressed in the pharyngeal endoderm and pharyngeal pouches (Fig. 5F, end). The most prominent non-neural expression domain, however, was the developing skeletal musculature. Similar to earlier stages, *RGMb* expression labeled the medial wall of the epithelial somite (Fig. 4T,P), the muscle pioneers (Fig. 4N,L), and the myotome during all waves of myogenesis (Fig. 4J,H,F,D,B; m). In addition, *RGMb* was also expressed at the core of the pharyngeal arches that delivers the branchiomeric musculature (Figs. 3D,F,H, 5D,E,F; mes).

Comparative Analysis of RGM and Marker Gene Expression in the E4 Chicken Somite

To determine more precisely, which cells in the somitic dermomyotome and myotome may express the RGM genes, and which cells may express other players involved in RGM signaling, we comparatively analyzed the expression of *Pax7*, *FgfR4*, *Myf5*, sarcomeric Myosin, *Neogenin*, *Netrin-1*, *Netrin-2*, *RGMa* and *RGMb* on cross-sections at HH24 (Fig. 6). *Pax7* is an established marker for dermomyotomal, myotomal, and adult muscle stem cells, i.e., stem cells competent to undertake myogenesis (Seale et al., 2000; Fig. 6A). *FgfR4* is a marker for embryonic stem cells that populate the myotome when the dermomyotome deepithelializes, and hence, *FgfR4* and *Pax7* expression overlaps in the myotome (Ahmed et al., 2006; Fig. 6A,B). *Myf5* labels cells that have entered myogenic differentiation (Ott et al., 1991; Fig. 6C), while sarcomeric Myosin is expressed in cells that have reached terminal differentiation (Lyons et al., 1990; Fig. 6D). Neogenin is the receptor for both RGM and Netrin ligands, and Netrin-induced signaling has been associated with late myogenic differentiation (Kang et al., 2004; Bae et al., 2009). We found that *RGMa* expression closely matched the expression of *Pax7* (Fig. 6A,E), suggesting that it demarcates dermomyotomal and muscle stem cells. *RGMb* expression partially overlapped with that of *FgfR4* and *Myf5*, suggesting that it labels cells committed to and ready to begin myogenesis (Fig. 6B,C,F). Neogenin showed a widespread expression, suggesting that the receptor was available at RGM expression sites. *RGMa*, *Neogenin*, *Netrin-1*, and *Netrin-2* expressions overlapped exclusively in the dermomyotome (Fig. 6E,G,H,I, dm), suggesting that here, Netrin molecules and RGMa may compete for the receptor. In contrast, only *RGMa*, *RGMb* and *Neogenin* were found in the myotome (Fig. 6E,F,G, m), suggesting a specific role of the RGM/Neogenin system during myotome differentiation.

Comparative Analysis of *RGMa*, *RGMb*, and *Neogenin* Expression During Limb Development

Our expression analysis at stages HH24–HH27 suggested that both

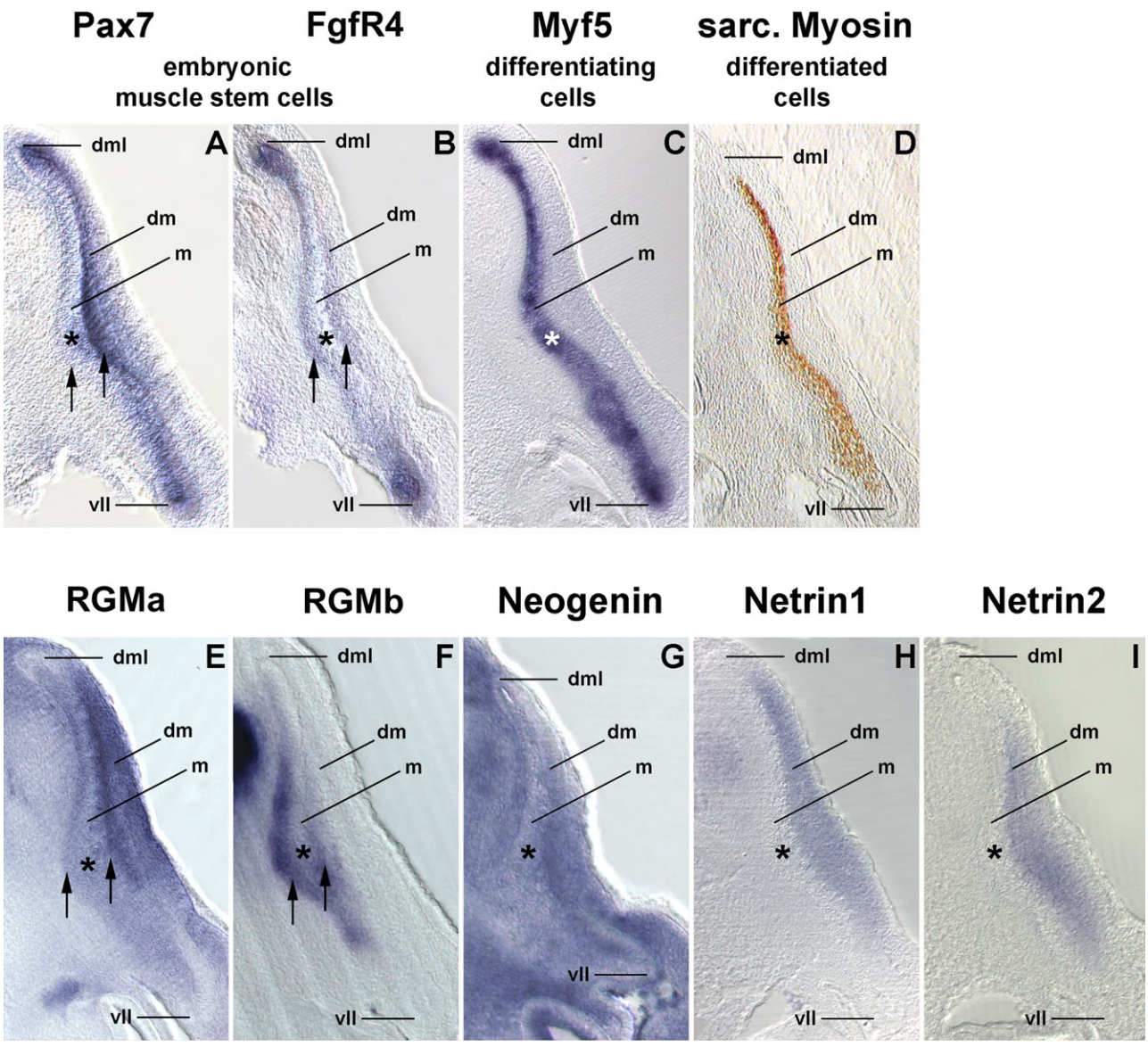

Fig. 6. Vibratome cross-sections of HH24 (E4) flank somites after whole-mount in situ hybridization using *Pax7*, *FgfR4*, *Myf5*, *Neogenin*, *Netrin-1*, *Netrin-2*, *RGMa*, *RGMb* probes, or after whole-mount antibody staining using the MF20 antibody that detects sarcomeric Myosin, as indicated on top of the panel. Dorsal is to the top, lateral to the right; for abbreviations see list of abbreviations. Embryonic muscle precursors that populate the myotome when the dermomyotome disperses are marked by arrows. Differentiated muscle fibers are marked by an asterisk. Note that *RGMa* expression overlaps with that of *Pax7*, *RGMb* expression partially overlaps with that of *FgfR4* and *Myf5*. *RGMa* expression also overlaps with that of *Neogenin*, *Netrin-1*, and *Netrin-2* in the dermomyotome; whereas only *RGMa* and *b* and *Neogenin* were found in the myotome. *Pax7*, *FgfR4*, *Myf5*, and Sarcomeric Myosin images were adapted from previous results (Ahmed et al., 2006).

RGM genes are expressed in the developing limbs (Fig. 3I,K,M,J,L,N, arrows, arrowheads). In addition, recent studies have demonstrated the association of *Neogenin* with digit patterning (Hong et al., 2012), and also with bone formation (Zhou et al., 2010). To better characterize RGM and Neogenin expression in the limbs, we investigated stages HH30–HH34 of development when endochondral bone formation is under way (Pechak et al., 1986). We found that in the shank (zeugopod), *RGMa* was expressed in the limb dermis, the undifferentiated mesenchyme surrounding the cartilages, and also in limb muscles (Figs. 3I,K,M, 7A,B,Bi-ii, arrows indicate the EDL muscle). In the foot (autopod), *RGMa* was detected in the mesenchyme surrounding tendon primordia with stronger expression at the future metatarsal-phalangeal and interphalangeal joints (Fig. 7B,C,C-inset, arrowheads). *RGMb* expression overlapped with that of *RGMa* in the undifferentiated mesenchyme between cartilages, and also in muscles (Figs. 3J,L,N, 7D,E,Ei-ii, arrows for EDL muscle). *Neogenin* expression overlapped with that of *RGMa* and *RGMb* in the undifferentiated mesenchyme, but it was weaker at skeletal muscle (Fig. 7G,H,Hi-ii). Instead, the most prominent *Neogenin* expression was found in the connective tissue surrounding these muscles (perimysium; Fig. 7Hi-ii).

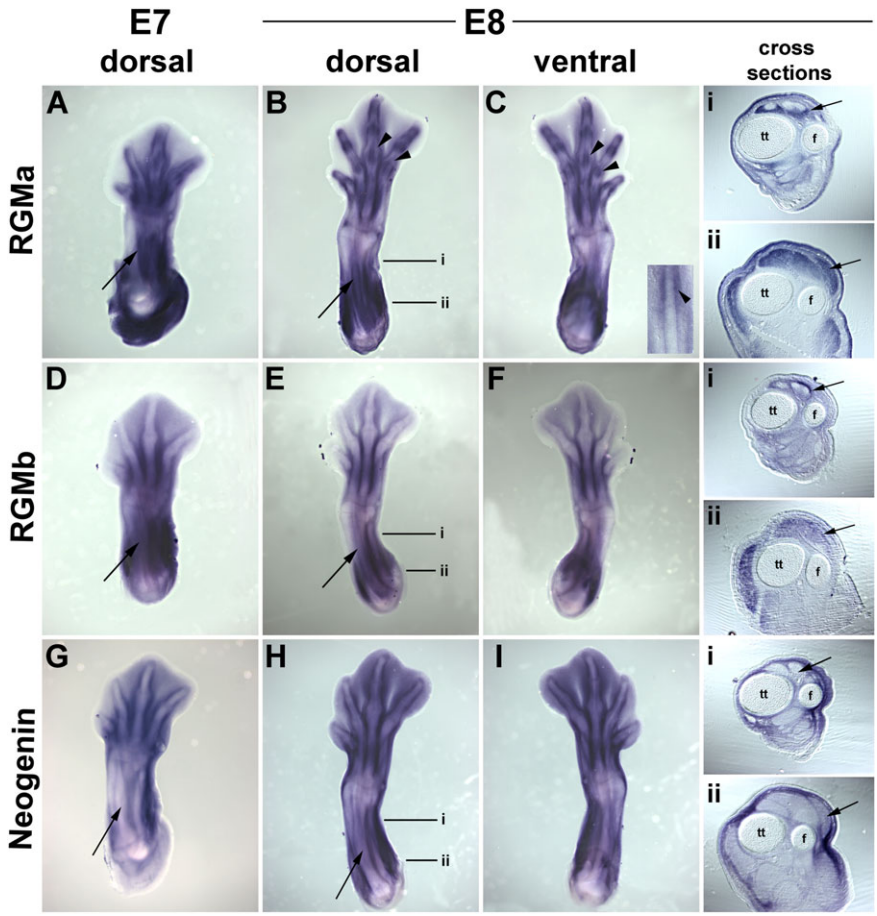

Fig. 7. Details of *RGMa*, *RGMb*, and *Neogenin* expressions in E7 and E8 chicken hindlimbs. Dorsal (A,B,D,E,G,H) and ventral (C,F,I) views of the whole hindlimbs at E7 and E8. A planar section of the ventral side of the RGMa-stained foot (autopod; inset in C); and cross-sections of the E8 shanks (zeugopod) at the distal (i) and proximal (ii) ends, as indicated in (B,E,H). Arrows indicate EDL muscle; and arrowheads indicate distal tendon primordia at the autopod future metatarsal-phalangeal joints. Distal is to the top, proximal to the bottom. (tt) for tibiotarsus and (f) for fibula. Note *RGMa* expression in the dermis, undifferentiated mesenchyme between cartilages, along tendons (with stronger expression over joints), and in muscle; *RGMb* expression in the undifferentiated mesenchyme between cartilages and muscles; and *Neogenin* expression in undifferentiated mesenchyme between cartilages, muscles (weaker), and connective tissue surrounding the muscles (perimysium). The deeper muscles are incompletely stained, due to limited penetration of the tissue by probe and antibody.

DISCUSSION

Repulsive guidance molecules (RGM) were originally identified as high-affinity Neogenin ligands that negatively regulate axonal outgrowth, promoting growth cone collapse of retinal ganglion cells in the chicken tectum (Monnier et al., 2002). Since then, RGM molecules have been associated with neural tube closure (Niederkofler et al., 2004); neuronal cell survival (Matsunaga et al., 2004); inhibition of axonal outgrowth after injury (Hata et al., 2006; Liu et al., 2009); control of neuronal proliferation and differentiation (Matsunaga et al., 2006) and inhibition of neural crest cell differentiation (Samad et al., 2005). Significantly, roles of RGM molecules outside the nervous system are now emerging, including reproduction (RGMb; Xia et al., 2005) and iron metabolism (RGMc; Papanikolaou et al., 2004). However, the expression, and hence function, of RGM members in non-neural tissues has not been systematically investigated.

Mammals have three, teleosts four, but chicken, according to the current edition of the chicken genome (build 3.1, NCBI), only two *RGM* family members (Camus and Lambert, 2007; Jorge and Dietrich, unpublished observations). We therefore reasoned that the most relevant roles of RGM molecules would have been preserved with these two family members, and we analyzed their spatiotemporal expression during chicken development. Our results (summarized in Table 1) indicate shared roles for RGMa and RGMb molecules in vertebrate nervous system development. Importantly, we identified expression domains of these molecules outside the nervous system, most prominently in the notochord and in the dermomyotome and myotome of somites, suggesting novel biological roles for RGM molecules in notochord and skeletal muscle development. Moreover, the avian *RGM* expression domains points toward an involvement of RGM not only in Neogenin, but also in BMP and Shh signaling.

RGM EXPRESSION IN NEURAL TISSUES IS CONSERVED IN GNATHOSTOME VERTEBRATES

Our expression analysis revealed chicken *RGMa* expression in the early neural plate, at neurulation stages of development followed by prominent expression in the ventral telencephalon and the basal plate of the neural tube. In the mature neural tube, *RGMa* was expressed in the telencephalon, mesencephalon, and hindbrain; in the spinal cord expression was highly dynamic, shifting from the basal plate to the ventral alar plate, then the intermediate layer of the neural tube containing differentiating neurons to subsets of differentiated neurons. Moreover, expression was observed in the mesenchyme surrounding the dorsal root ganglia and in the dorsal root entry zone. *RGMb* expression commenced later, at neurulation stages encompassing the ventral telencephalon, transiently the prospective myelencephalon and upper spinal cord, and the floor plate. The mature neural tube expressed *RGMb* in the telencephalon, hindbrain and spinal cord, specifically labeling the floor plate, differentiated neurons in the mantle layer, and finally subpopulations of differentiated

TABLE 1. Summary of Chicken *RGMa* and *RGMb* Expression Domains and Comparison to Known Expression Domains of Mouse, *Xenopus*, and Zebrafish *RGMa* and *RGMb*, and to Sites of Neogenin, Bmp, and Shh Signaling[a]

	RGMa	RGMb
Tissues with RGM expression		
Tissues involved in gastrulation	Primitive streak	–
Endodermal derivatives	–	Pharyngeal endoderm and pouches
Mesodermal derivatives	–	Prechordal plate
	–	Notochord
	–	Ventromedial head mesoderm
	–	Branchiomeric muscles
	Anterior segmental plate	–
	Medial wall of the condensing and epithelial somites	Medial wall of the condensing and epithelial somites
	Muscle pioneer cells	Muscle pioneer cells
	Medial and central dermomyotome, embryonic muscle stem cells	–
	Myotome (weak)	Myotome (strong; all waves of myogenesis)
	Limb muscle	Limb muscles
	Limb mesenchyme	Limb mesenchyme
	Cells lining the foot tendons	–
Ectodermal derivatives	Pharyngeal ectoderm	–
	Neural plate	–
	Early neural tube: ventral telencephalon, basal plate	Early neural tube: ventral telencephalon, transient in prospective myelencephalon and upper spinal cord, floor plate
	Mature neural tube: telencephalon, mesencephalon, hindbrain; dynamic expression in the spinal cord (basal plate-ventral alar plate-intermediate layer-subsets of neurons)	Mature neural tube: telencephalon, hindbrain and spinal cord, floor plate, mantle layer; subsets of neurons
	Mesenchyme surrounding DRG and at DREZ	DRG, trigeminal and facial ganglia
		Optic stalk
	Complementary domains	**Overlapping domains**
RGMa and RGMb complementary or overlapping expression domains	Pharyngeal arch endoderm – ectoderm	Medial wall of the somites
	Dermomyotome - myotome of mature somites	Muscle pioneer cells
	basal plate – notochord & floor plate of the early neural tube	Limb muscle
	Intermediate layer - mantle layer of the mature neural tube	Limb mesenchyme between cartilages
	mesenchyme around DRG and DREZ – DRG	Telencephalon
		Possibly neuronal subpopulations
	RGMa	**RGMb**
Overlapping or nearby expression of Neogenin	partially overlapping in the mature neural tube	–
	DRG	DRG
	Somites	Somites
	Undifferentiated mesenchyme between limb cartilages	Undifferentiated mesenchyme between limb cartilages

TABLE 1. (Continued)

	RGMa		RGMb	
Overlapping or nearby expression of BMP2/4/6	BMP2 – overlapping in the primitive streak; nearby in the lateral plate mesoderm, apical ectodermal ridge and posterior mesenchyme of the limb bud BMP4 – overlapping in the primitive streak; nearby along the neural plate border and dorsal neural tube, ventromedial area of epithelial somites, lateral plate mesoderm, anterior and posterior limb mesenchyme BMP6 – overlapping in anterior segmental plate; nearby limb bud cartilage and bone		BMP2 – nearby in the lateral plate mesoderm, apical ectodermal ridge and posterior mesenchyme of the limb bud BMP4 – overlapping in and pharyngeal pouches; nearby in the dorsal neural tube, ventromedial area of epithelial somites, lateral plate mesoderm, anterior and posterior limb mesenchyme BMP6 – nearby limb bud cartilage and bone	
Overlapping or nearby expression of Shh	Notochord and floor plate Posterior limb bud		Notochord and floor plate Posterior limb bud	
	Chicken	Mouse	*Xenopus*	Zebrafish
Conserved expression domains in bony vertebrates for RGMa	Dermomyotome Neural tube with high expression levels in tel- and mesencephalon	Dermomyotome Neural tube with high expression levels in tel- and mesencephalon	Somites Neural tube with high expression in tel- and mesencephalon	Somites (weak signal) Neural tube with high expression in tel- and mesencephalon
	Chicken	Mouse	*Xenopus*	Zebrafish
Conserved expression domains in bony vertebrates for RGMb	Myotome Mantle layer of neural tube Trigeminal ganglion and DRG Optic stalk	Myotome Mantle layer of neural tube Trigeminal ganglion and DRG Retinal ganglion cells and optic nerve	Not described	Not described (weak expression in somites) Not described Trigeminal ganglion and DRG Optic stalk

[a]Expression domains of the *Neogenin* receptor according to (Fitzgerald et al., 2006; Gad et al., 1997; Mawdsley et al., 2004, this study); *BMP2,4,6* (Andrée et al., 1998; Chapman et al., 2002; Danesh et al., 2009; Zhou et al., 2010), *Shh* (Roelink et al., 1995; Marti et al., 1995), mouse *RGMa* and *RGMb* (Schmidtmer and Engelkamp, 2004; Niederkofler et al., 2004; Oldekamp et al., 2004); *Xenopus RGMa* and *RGMb* (Samad et al., 2005; Wilson and Key, 2006; Gessert et al., 2008; Kee et al., 2008), and zebrafish *RGMa* and *RGMb* (Samad et al., 2004; Bian et al., 2011). Hyphens indicate absence of corresponding expression domains for the chicken paralogs.

neurons. Moreover, *RGMb* was expressed in the DRG, trigeminal and facial ganglia, and the optic stalk.

RGMa expression in the chicken neural plate matches *RGMa* expression and function for neural tube closure in the mouse (Niederkofler et al., 2004). Moreover, *RGMa* expression in the mes- and telencephalon is the same for chicken, mouse, *Xenopus*, and zebrafish (Matsunaga et al., 2004; Schmidtmer and Engelkamp, 2004; Samad et al., 2004; Oldekamp et al., 2004; Wilson and Key, 2006; Gessert et al., 2008; Kee et al., 2008). Likewise, *RGMb* expression in the mantle layer of the neural tube is known for mouse *RGMb*, and *RGMb* expression in the trigeminal ganglion, DRG, and optic stalk has been described for mouse and zebrafish (Samad et al., 2004, 2005; Conrad et al., 2010). This suggests that *RGMa* and *RGMb* have conserved roles in the development of the nervous system in all gnathostome vertebrates. Several chicken *RGMa* and *b* expression domains are complementary, with *RGMa* being expressed in the basal plate and *RGMb* in the floor plate of the early neural tube, *RGMa* in the intermediate and *RGMb* in the mantle layer of the mature neural tube, and *RGMa* in the mesenchyme surrounding the DRG and *RGMb* directly in the DRG. Thus, the two RGM molecules may have specialized roles in these tissues. In contrast, *RGMa* and *b* expression overlap in the telencephalon and possibly also in subpopulations of spinal cord neurons. Thus, in these tissues, the RGM molecules may have redundant roles, and these redundant roles may account for the absence of the expected axon guidance phenotypes in mice deficient for a single RGM gene (Niederkofler et al., 2004).

Newly Discovered Expression Domains of *RGMa* and *RGMb* Point Toward Role in Somite Development and Myogenesis

In addition to *RGM* expression domains associated with the development of the nervous system, we found prominent expression domains in non-neural tissues. *RGMa* was expressed in the primitive streak, pharyngeal ectoderm, and later, in the limb mesenchyme surrounding cartilage anlagen, and cells lining tendon primordia. However, the most prominent non-neural expression of *RGMa* was associated with the paraxial mesoderm. Expression commenced in the anterior segmental plate, becoming readily restricted to the medial wall of the epithelial somites that delivers the muscle pioneer cells (Kahane et al., 2007). Expression faded as the cells differentiated and formed the primary myotyome. However, as somites matured, expression re-emerged in the dorsal and central dermomytome. Upon dermomyotome deepithelialization, expression appeared in the myotome, and later, in the limb muscles. Non-neural expression of *RGMb* was found in the pharyngeal endoderm and pouches, the axial mesoderm (prechordal plate, notochord) and the paraxial mesoderm including the ventromedial head mesoderm and branchiomeric muscle anlagen, the medial wall of the epithelial somites, the myotome during all waves of myogenesis. In the late myotome and in limb muscle, expression was similar to the observed for *RGMa*. However, overall, *RGMb* expression was shifted toward differentiating cells.

Expression studies in the mouse, while focusing primarily on neural tissues, mentioned *RGMa* expression in the dermomyotome, and *RGMb* and *RGMc* expression in the myotome of developing somites (Schimdtmer and Engelkamp, 2004; Oldekamp et al., 2004); somitic expression has also been mentioned for *Xenopus* and zebrafish *RGMa*, zebrafish *RGMc* and (weakly) for zebrafish *RGMb* (Samad et al., 2004, 2005; Gessert et al., 2008; Bian et al., 2011). Netrin molecules are expressed in somites as well, and expression is present at stages when the somitic myotome and developing dermis are being innervated by the spinal nerves (this study and E. Jorge and S. Dietrich, unpublished observations). This innervation occurs in a stereotype pattern (Fetcho, 1987). Moreover, the Neogenin receptor is found on axonal growth cones (Wilson and Key, 2006). Thus, it is possible that RGMa and Netrins molecules are facilitating muscle and dermis innervation. However, *Neogenin* also showed widespread expression in the somite itself (Mawdsley et al., 2004, this study). Moreover, *Neogenin* expression overlapped specifically with that of *RGMa* and *b*, not with that of *Netrin-1* and *–2*, in the myotome. Furthermore, at earlier stages, we found *RGMa* and *b* expression in the segmental plate and epithelial somites, respectively, long before axonal outgrowth is initiated, with *RGMa* expression resembling that of the dermomyotomal and muscle stem cell marker *Pax7* (Seale et al., 2000; Ahmed et al., 2006), and *RGMb* expression overlapping with that of the muscle stem cell marker *FgfR4* and the muscle determination factor *Myf5* (Ott et al., 1991; Seale et al., 2000; Ahmed et al., 2006). This suggests that *RGMa* is associated with stem cells capable of undertaking myogenesis, and *RGMb* with cells ready to enter myogenic differentiation. In this vein, in vitro studies on mouse C2C12 myoblasts and on primary myoblasts obtained by means of adult muscle stem cell (satellite cell) activation suggested that RGMc (RGMa, b not tested) may have some ability to bind to Neogenin to promote myoblast differentiation and recruitment into myotubes. However, the effect of RGMc was less pronounced than the effect of the well established Neogenin ligand Netrin (Kang et al., 2004; Bae et al., 2009). Moreover, no myotome or muscle phenotypes have been reported for *RGM* mouse mutants (Niederkofler et al., 2004; Ma et al., 2011; Xia et al., 2011). However, similar to the nervous system, RGM molecules may have overlapping roles in myogenesis. Furthermore, specifically RGMa and b may act before terminal differentiation and fusion occurs. The fact that somitic expression domains of *RGMa* and *b* are rather conserved, and that RGMa continues to be expressed in limb muscles, and *RGMb* in craniofacial, body, and limb muscle anlagen as well reinforces the idea of an association of RGM molecules with myogenesis.

RGM Molecules May Maintain Notochord Integrity

In the chicken, *RGMb* was strongly expressed in the prechordal plate and

notochord. Interestingly, in the mouse, notochordal expression was shown for *RGMa* (Schmidtmer and Engelkamp, 2004; Niederkofler et al. 2004), whereas in the zebrafish, the notochord expressed *RGMc* (Gibert et al., 2011). In zebrafish morphants devoid of *RGMc*, the notochord was disrupted, and as a consequence, somites showed a U-shaped rather than chevron-shaped morphology (Gibert et al., 2011). Recent studies on the downstream effects of Neogenin suggested that Neogenin has prominent roles in controlling cell adhesion because in *Neogenin* mutants, tissue architecture of epithelial tissues such as the neural plate/neural tube and the mammary gland was disrupted (Srinivasan et al., 2003; Mawdsley et al., 2004). Likewise, in myoblast fusion Neogenin facilitates tight cell–cell contact (Kang et al., 2004). RGM molecules, when GPI-anchored, are known to control the cytoarchitecture and shape of cells in the neural plate and of the axonal growth cone (Kee et al., 2008). Thus, even though in different vertebrates RGM molecules may have swapped roles, RGM family members may act in maintaining notochord integrity and longitudinal axis formation.

In Endodermal and Mesodermal Tissues, RGM May Act by Means of Modulating BMP Signaling

RGM molecules have been established as high affinity ligands for Neogenin, and the roles of RGM molecules in the nervous system have been associated with Neogenin activation (Rajagopalan et al., 2004; Itokazu et al., 2012). However, RGM molecules also serve as BMP co-factors, with GPI anchored molecules supporting and the soluble molecules inhibiting BMP signaling (Babitt et al., 2005; Halbrooks et al., 2007). BMP molecules are expressed at various sites in the embryo, most notably overlapping with *RGM* expression domains in the primitive streak, and pharyngeal pouches, and neighboring *RGM* expression domains in the somitic dermomyotome and myotome, in the neural plate, in the dorsal neural tube, and in the limb (Andrée et al., 1998; Chapman et al., 2002; Danesh et al., 2009, this study). BMP molecules in the primitive streak and lateral mesoderm promote lateral mesoderm development and suppress somite and muscle development (Reshef et al., 1998). Given that RGM are expressed at sites of muscle formation, they may act by suppressing BMP signaling. Likewise, in the early embryo, BMP molecules suppress neural and promote surface ectoderm development, and *RGMa* expression in the neural plate may counterbalance this effect. In limbs, *BMP6* is expressed in the cartilages, and BMP-Neogenin signaling has been shown to promote cartilage development (Zhou et al., 2010). However, we found prominent *RGMa*, *b* (weaker), and *Neogenin* expression in the surrounding undifferentiated mesenchyme between cartilages, suggesting that also at this site RGM molecules may suppress BMP signaling and, in consequence, cartilage/endochondral bone formation. To solve this question, it will be important to establish using proteomics, whether RGM molecules are present as GPI-anchored or soluble forms.

RGM Molecules May Mediate Shh-Dependent Processes

Chicken *RGMa* and *b* are both expressed at sites and during processes subject to extensive Shh signaling. For example *RGMb* is expressed in the notochord and floor plate of the neural tube at the time when Shh signals from the notochord induce the also Shh expressing floor plate (Roelink et al., 1995; Marti et al., 1995). Likewise, *RGMa* and *b* are expressed in myogenic cells when Shh released from notochord and floor plate, together with Wnt signals from the dorsal neural tube, induce myogenesis (Münsterberg et al., 1995; Wagner et al., 2000; McDermott et al., 2005). *RGM* expression may merely be a result of earlier Shh signaling. However, Neogenin has been shown to bind to the Shh co-receptors Cdo and Boc to promote myoblast fusion (Kang et al., 2004). Thus, the Neogenin ligands Netrins and RGM may partially act by positively regulating Shh signal transduction in this process. However, in the limb, loss of Neogenin leads to polydactyly associated with gain of Shh signaling (Hong et al., 2012). Here, Neogenin, by means of binding of Cdo and Boc may diminish their availability for Shh signaling. Moreover, Neogenin also acts downstream of these receptors and at the level of the Shh signaling mediators, the Gli transcription factors (Hong et al., 2012). Thus, in this process Neogenin may negatively regulate Shh pathway. In the somite, high Shh levels promote sclerotome and suppress myotome formation (Pourquie et al., 1993; Brand-Saberi et al., 1993; Fan and Tessier-Lavigne, 1994). Therefore, it cannot be excluded that RGM functions to modulate Shh signal transduction, thereby promoting myogenesis.

In summary, our expression analysis of chicken *RGMa* and *b* genes confirmed the conserved roles of repulsive guidance molecules in nervous system development and canonical Neogenin signaling. However, our analysis revealed prominent non-neural sites of expression, consistent with so far unknown in myogenesis, notochord development, and BMP and Shh signaling.

EXPERIMENTAL PROCEDURES

Chicken RGM Clones and Riboprobes Synthesis

Chicken *RGMa* clone was obtained from an embryonic skeletal muscle cDNA library cloned between *Not*I-*Sal*I cloning sites of pSPORT1 vector (Invitrogen), deposited at GenBank under the accession number CO506306 (Jorge et al., 2004). Chicken *RGMb* clone was obtained from BBRSC collection (*RGMb*, ChEST380b4, GenBank entry CR354149.1). Chicken *Neogenin*, *Netrin-1*, and *Netrin-2* clones were kindly provided by Dr. Frank Schubert. All clones were confirmed by sequencing. For RGMs clones, M13 universal primers were used to amplify templates to produce RNA antisense and sense (control) probes by in vitro transcription, using T7 and SP6 RNA polymerases, respectively, and digoxigenin-labeled UTP (Roche). For *Neogenin* and *Netrin* probes, plasmids were linearized by *Eco*RI (*Neogenin*) and *Xho*I (*Netrins*)

enzymes and the antisense RNA probes were synthesized by T3 (*Neogenin*) and T7 (*Netrins*) RNA polymerases, also in the presence of digoxigenin-labeled UTP (Roche).

Chicken Embryos

Fertilized chicken eggs were obtained from Winter Farm (Royston). Eggs were incubated at 38.5°C in a moist atmosphere, developmental stages were defined according to Hamburger and Hamilton (1951).

Whole-Mount In Situ Hybridization and Antibody Staining

Whole-mount in situ hybridization and antibody staining was performed as previously described (Dietrich et al., 1997, 1998; Mootoosamy and Dietrich, 2002).

Sectioning

Embryos were embedded in 20% gelatin (Sigma) and sectioned to 40 μm in a Pelco 1000 Vibratome, as previously described (Dietrich et al., 1997).

Photomicroscopy

After in situ hybridization, embryos were cleared in 80% glycerol/PBS. Embryos and sections were photographed on a Zeiss Axiophot, using Nomarski optics. Images were assembled using Adobe Photoshop.

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