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MECHANISMS OF DEVELOPMENT

Vestigial-like 2 acts downstream of *MyoD* activation and is associated with skeletal muscle differentiation in chick myogenesis

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ABSTRACT

The co-factor *Vestigial-like 2* (*Vgl-2*), in association with the Scalloped/Tef/Tead transcription factors, has been identified as a component of the myogenic program in the C2C12 cell line. In order to understand *Vgl-2* function in embryonic muscle formation, we analysed *Vgl-2* expression and regulation during chick embryonic development. *Vgl-2* expression was associated with all known sites of skeletal muscle formation, including those in the head, trunk and limb. *Vgl-2* was expressed after the myogenic factor *MyoD*, regardless of the site of myogenesis. Analysis of *Vgl-2* regulation by Notch signalling showed that *Vgl-2* expression was down-regulated by Delta1-activated Notch, similarly to the muscle differentiation genes *MyoD*, *Myogenin*, *Desmin*, and *Mef2c*, while the expression of the muscle progenitor markers such as *Myf5*, *Six1* and *FgfR4* was not modified. Moreover, we established that the Myogenic Regulatory Factors (MRFs) associated with skeletal muscle differentiation (*MyoD, Myogenin* and *Mrf4*) were sufficient to activate *Vgl-2* expression, while *Myf5* was not able to do so. The *Vgl-2* endogenous expression, the similar regulation of *Vgl-2* and that of *MyoD* and *Myogenin* by Notch signalling, and the positive regulation of *Vgl-2* by these MRFs suggest that *Vgl-2* acts downstream of *MyoD* activation and is associated with the differentiation step in embryonic skeletal myogenesis.

1. Introduction

During development, head, trunk and limb muscle progenitors are specified by different genetic programs (reviewed in Grifone and Kelly, 2007; Mootoosamy and Dietrich, 2002; Duprez, 2002). Once specified, myogenic cells use a common differentiation program at various places in the body. Transcriptional control of skeletal muscle gene expression is dependent on four basic-helix-loop-helix transcription factors: *Myf5*, *MyoD*, *MRF4* and *Myogenin*, which are named the Myogenic Regulatory Factors (MRFs) (reviewed in Berkes and Tapscott, 2005). MRFs have the ability to trigger skeletal muscle differentiation in non-muscle cells in vitro (Weintraub et al., 1991) and in vivo (Delfini and Duprez, 2004). Maturation

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of skeletal muscle fibres into distinct phenotypes (slow and fast) involves transcriptional activation of transcription factor, cytoskeletal, calcium handling, metabolic and contractile proteins (Zierath and Hawley, 2004).

Searching for genes regulating vertebrate embryonic skeletal muscle identity, we investigated the vertebrate equivalents of the Drosophila *Vestigial* gene. In Drosophila, *Vestigial* is involved in the specification and subsequent differentiation of embryonic somatic muscles and one type of adult muscles, the indirect flight muscles (Sudarsan et al., 2001; Bernard et al., 2003, 2006, 2009; Deng et al., 2009). *Drosophila Vestigial* and the Vertebrate equivalent *Vestigial-like* (*Vgl*) genes code for co-transcription factors that do not contain any DNA binding sequence (Vaudin et al., 1999; Halder and Carroll, 2001; Maeda et al., 2002; Gunther et al., 2004). They are characterised by a Vestigial domain, which interacts with members of the Scalloped/Tef/Tead transcription factor family. Tead family members control the transcription of muscle-specific genes through binding to MCAT elements, in cardiac, smooth and skeletal muscle lineage (reviewed in Yoshida, 2008). TeaD family members share a highly-conserved DNA binding domain called the Tea domain and comprise four members described in human and mice, including *Tead1,2,3* and 4 (reviewed in Yoshida, 2008). *Tead1,3* and 4 members have been described as widely expressed in multiple adult tissues, while *Tead2* is selectively expressed in embryonic tissues (reviewed in Yoshida, 2008). Given the ubiquitous expression of the *Tead* genes, it is generally assumed that the specificity of action of these Tead family members results from the specific expression of their cofactors.

The human and mouse *Vestigial-like 2* (*Vgl-2*) genes were identified following screens for orthologues of Drosophila *Vestigial* and for genes specifically expressed in skeletal muscles (Mielcarek et al., 2002; Maeda et al., 2002). Two zebrafish *Vgl-2* orthologues have also been identified as being expressed in terminally differentiated muscle fibres (Mann et al., 2007). Three other genes belonging to the Vestigial-like family have been identified in Human: *Vgl-1* and *Vgl-3*, which are mainly expressed in placenta (Maeda et al., 2002), and *Vgl-4*, which has been described as being specific to cardiac muscles (Chen et al., 2004a). Recently, the mouse *Vgl-3* gene has been isolated and associated with the myogenic lineage during early mouse embryonic development (Mielcarek et al., 2009).

In vitro studies have shown that *Vgl-2* transcripts are up-regulated following muscle differentiation in C2C12 cells (Maeda et al., 2002; Mielcarek et al., 2002). Although *Vgl-2* alone is not sufficient to initiate myogenic differentiation in fibroblast cell lines, *Vgl-2* enhances the *MyoD*-mediated myogenic conversion of 10T1/2 (Maeda et al., 2002; Gunther et al., 2004). Conversely, down-regulation of *Vgl-2* expression using siRNA or morpholino in C2C12 cells prevents muscle cells from forming myotubes (Gunther et al., 2004; Chen et al., 2004b). These experiments suggest a role for *Vgl-2* in promoting *MyoD*-induced myogenesis. Mutant mice for *Vgl-2* have not been produced to date. However, *Vgl-2* expression has been observed in myotomes and limb muscles of mouse embryos and in adult skeletal muscle tissues (Maeda et al., 2002; Mielcarek et al., 2002). The *Vgl-2* expression and regulation in chick embryos is not known.

In the present study, we have identified and isolated chick *Vgl-2* mRNA and analysed its expression during embryonic chick development. Comparing *Vgl-2* expression with that of known muscle markers allowed us to establish that *Vgl-2* was expressed after *MyoD* in all sites of skeletal muscle formation, head, trunk and limb. We also analysed the regulation of *Vgl-2* expression by the Notch/Delta pathway and by the four MRFs (*Myf5*, *MyoD*, *Myogenin* and *Mrf4*). Our results suggest that in the chick embryo, *Vgl-2* is associated with skeletal muscle differentiation after the *MyoD* step.

2. Results

2.1. *Identification of chicken Vestigial-like 2 cDNA*

Chicken *Vgl-2* cDNA was isolated by RT-PCR using mRNAs extracted from chick embryos of various stages. Sequence analyses revealed that chicken Vgl-2 protein is coded by 4 exons and is constituted of 303 amino acids with a predicted molecular mass of 32 kDa (data not shown). The comparison of the deduced amino acid sequences of Vgl-2 proteins between different species showed that cVgl-2 is more homologous with human Vgl-2 (67%) than with mouse Vgl-2 (63%). In zebrafish, two *Vgl-2* genes, *Vgl-2a* and *Vgl-2b* have been identified; *Vgl-2a* has been shown to be more similar to mammalian *Vgl-2* than *Vgl-2b* (Mann et al., 2007). Chick Vgl-2 protein is more similar to Vgl-2a than to Vgl-2b zebrafish proteins (58% versus 34%). The region homologous to the *Drosophila* SID (Scalloped Interaction Domain) of the different species is the most conserved region between Vgl-2 proteins. The SID of cVgl-2 is, respectively, 79% and 73% identical to the SID of human Vgl-2 and mouse Vgl-2 (data not shown).

2.2. *Vgl-2 expression is associated with skeletal muscle formation in chick embryos*

We analysed *Vgl-2* expression in the different sites of skeletal myogenesis (head, trunk and limb) during chick development by *in situ* hybridisation. In order to correlate *Vgl-2* expression with the different steps of myogenesis, we systematically compared *Vgl-2* expression with that of *MyoD*, whose expression reflects the commitment of myoblasts into the muscle differentiation program (Amthor et al., 1998; Delfini et al., 2000; Hirsinger et al., 2001; Bergstrom et al., 2002).

2.2.1. *Head myogenesis*

In chick embryos, *Vgl-2* transcripts were first detected at 12-somite stage in anterior regions in the future branchial arches (shown for 15-somite stage, Fig. 1A and B, arrows). Transverse sections of 17-somite stage embryos at the future branchial arch level showed that *Vgl-2* expression was restricted to the surface ectoderm and to the pharyngeal endoderm (Fig. 1C). No *Vgl-2* expression was observed in the mesenchymal cells of the future branchial arches until HH22 (Fig. 1C and D), while at stage HH26, *Vgl-2* transcripts were now detected in the myogenic core of the first branchial arch (Fig. 1E, arrow). In order to define *Vgl-2* expression in head muscles, we compared its expression with that of *MyoD* on adjacent sections. In the first branchial arch, at HH21, *Vgl-2* expression was not observed in the myogenic core, while