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Identification of novel genes including *Dermo-1*, a marker of dermal differentiation, expressed in trout somitic external cells

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SUMMARY

The external cell layer that surrounds the fish primary myotome provides the myogenic precursors necessary for muscle growth, suggesting that this epithelium is equivalent to the amniote dermomyotome. In this study we report the identification of a trout orthologue of the dermal marker *Dermo-1*, and show that trout somitic external cells, which are all potentially myogenic as indicated by the transcription of *Pax7* gene, express Dermo-1. This finding and our previous observation that external cells express collagen I show that these cells have dermis-related characteristics in addition to exhibiting myogenic features. In an effort to identify novel genes expressed in the external cell epithelium we performed an *in situ* hybridisation screen and found both collectin sub-family member 12, a transmembrane C-type lectin, and Seraf, an EGF-like repeat autocrine factor. *In situ* hybridisation of staged trout embryos revealed that the expression of Dermo-1, collectin sub-family member 12 and Seraf within the external cell layer epithelium was preceded by a complex temporal and spatial expression pattern in the early somite.

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Key words: myogenesis, somite, dermomyotome, myotome, dermatome, *Dermo-1*, *Pax7*, Seraf, collectin, teleost.

INTRODUCTION

The fish somite gives rise to two major compartments: the myotome and the sclerotome (Scaal and Wiegreffe, 2006). The development of the myotome begins before somite formation with the expression of myogenic regulatory factors in the adaxial cells located in the most medial presomitic mesoderm adjacent to the notochord (Weinberg et al., 1996). Shortly after somite formation, the adaxial cells differentiate and migrate radially to reach the lateral surface of the myotome, where they form peripheral slow-twitch muscle fibres (Devoto et al., 1996). As adaxial cells begin to differentiate deep in the somite, cells in the posterior of the somite start to express myogenic regulatory factors. Later on, behind the wave of adaxial cell migration, posterior somite cells differentiate into medial fast muscle fibres. Cells of the sclerotome, for their part, express Pax9 (Nornes et al., 1996), twist (Stickney et al., 2000), bapx1 (Yasutake et al., 2004) and collagen I (Rescan et al., 2005), and are initially present at the ventromedial surface of the somite. As development proceeds, they migrate dorsally around the notochord where, at a later stage, the vertebral column forms (Morin-Kensicky and Eisen, 1997).

For many years, several authors have reported the presence of flattened cells that are lateral to the developing fish myotome (Waterman, 1969; Vegetti et al., 1990; Johnston, 1993; Lopez-Albors et al., 1998). These cells do not exhibit any characteristics of immature muscle fibres, such as the presence of contractile filaments (Waterman, 1969; Johnston, 1993; Lopez-Albors, 1998; Rescan et al., 2005; Devoto et al., 2006), but express the paired box transcription factors Pax3 and Pax7 suggesting that they form a myogenic epithelium homologous to the amniote dermomyotome (Devoto et al., 2006; Groves et al., 2005; Hammond et al., 2007; Feng et al., 2006; Steinbacher et al., 2006; Steinbacher et al., 2007). The myogenic capacity of the external cells has recently been demonstrated in zebrafish by vital dye staining and lineage tracking techniques, which revealed that some external cells are incorporated into the myotome, generating new myofibres contributing to the burst of growth taking place in larval myotome (Hollway et al., 2007; Stellabotte et al., 2007). Furthermore vital dye staining and lineage tracking techniques showed that external cells are initially present at the anterior border of epithelial somites before migrating to the external surface of the somite. Expression of collagen genes in somitic external cells of teleost (Le Guellec et al., 2004; Rescan et al., 2005) substantiated the view that the external cell epithelium contributes to dermis formation. The only dermal marker available to date is Dermo-1 (twist-2) a basic helix–loop–helix (bHLH) transcription factor. In mouse, *Dermo-1* gene is expressed in the newly formed dermis and subsequently restricted to the epidermal–dermal junction (Li et al., 1995). In chick, Dermo-1 transcript is detected in the mediodorsal subectodermal mesenchyme of the integument (Scaal et al., 2001). Dermo-1 is not an exclusive marker of the differentiating dermis since it is also expressed in sclerotome and limb mesenchyme (Li et al., 1995; Scaal et al., 2001). However, its crucial role in dermis formation has been demonstrated by the generation of *Dermo-1*(–/–) mice that exhibit a thin, loose dermis (Sosic et al., 2003). We report in this study the cloning of a trout orthologue of *Dermo-1* and show that this gene is expressed in Pax7-positive somitic external cells, indicating that these cells have dermis-related characteristics in addition to exhibiting myogenic features. We also report here the identification of two new genes expressed in external cells, a gene related to *Seraf*, which encodes an EGF-like repeat autocrine factor involved in developmental processes of Schwann cell lineage in chicken (Wakamatsu et al., 2004), and a gene encoding collectin sub-family

member 12 (also called collectin 1 precursor CL-3), a transmembrane lectin potentially involved in innate immunity and cell–cell interactions (Drickamer and Taylor, 1993; Hogenkamp et al., 2006). The somitic expression of a collectin and a *Seraf* gene is reported for the first time in vertebrates.

MATERIALS AND METHODS

Animals

All the experiments were carried out on the rainbow trout *Oncorhynchus mykiss* (Walbaum). Eggs were collected at the experimental facilities of the INRA Drennec fish farm (Finistère, France). After artificial insemination, eggs were incubated at 10°C in recirculating dechlorinated water. Chemical water parameters were regularly monitored. Oxygen levels were always above 98% saturation. Samples were taken every day within the developmental period from the beginning of somitogenesis to the eyed stage (7–15 days post fertilisation).

Whole-mount *in situ* hybridisation

Dermo-1, collectin subfamily member 12, Seraf and slow myosin light chain 1 (GenBank accession no. 076946) cDNAs were derived from a large-scale, rainbow trout 3′ and 5′ sequencing project (Govoroun et al., 2006). *Pax7* exon 1 was obtained from the screening of a rainbow trout bacterial artificial chromosome (BAC) library (Palti et al., 2004) using primers designed from salmon *Pax7* cDNA sequence (Gottenspare et al., 2006). Myogenin was previously characterised (Rescan et al., 1995). All digoxigenin-labelled antisense RNA probes were synthesised from a PCR-amplified template using appropriate RNA polymerases. The embryos were dechorionated with fine forceps and fixed overnight at 4°C in paraformaldehyde in phosphate buffered saline (PBS). Specimens were dehydrated and stored in methanol at –20°C. After rehydration in graded methanol–PBS baths, embryos were processed according to established automated procedures (Quiring et al., 2004) with minor modifications. Double whole-mount *in situ* hybridisations were performed as previously described (Rescan et al., 2001).

Histological methods

For histological examinations, embryos were embedded in 30% ovalbumin, 0.5% gelatine and 1% gluteraldehyde in PBS. Blocks were sectioned at 30 μm on a Leica vibratome. Alternatively, embryos were dehydrated and mounted in paraffin, and 10 μm sections were then prepared. Sections were counterstained with nuclear Fast Red and mounted in Mowiol (Calbiochem, La Jolla, CA, USA).

RESULTS

Trout somitic external cells express both Pax7 and the dermal marker Dermo-1

To show that trout external cells have myogenic potential as reported in other teleosts (Devoto et al., 2006; Groves et al., 2005; Hammond et al., 2007; Feng et al., 2006; Steinbacher et al., 2006; Macqueen et al., 2007), we first analysed the expression of Pax7, a paired homeodomain transcription factor that is involved in the emergence and survival of muscle progenitors (Buckingham and Relaix, 2007). Using an antisense riboprobe corresponding to the first exon, we observed that the somitic expression of Pax7 was first detected around the 20 somite stage and was restricted to the anterior part of the 12–15 more rostral somites. Frontal sections in various staged embryos confirmed the initial localisation of Pax7 transcript in the anterior compartment of the somite and indicated

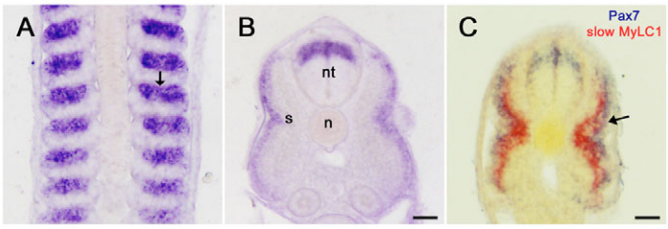

Fig. 1. Expression of Pax7 in trout embryo. (A) Stage 16 embryo (approximately 40 somites). Frontal section through anterior tail: labelling is present in the anterior domain of the somite (arrow indicates the boundary between two somites). (B) Eyed-stage embryo. Transverse section through middle trunk: labelling is present in the dorsal domain of the neural tube and at the external surface of the somite. (C) Dual-colour *in situ* hybridisation for Pax7 (dark blue) and slow myosin light chain 1 (MyLC1; red) transcripts. Eyed-stage embryo. Transverse section through the middle trunk: Pax7-positive somitic external cells (arrow) are lateral to the slow muscle fibres. n, notochord; nt, neural tube; s, somite. Scale bar, 25 μm in A, 30 μm in B and 35 μm in C.

that Pax7 transcript was excluded from the adaxial cells (Fig. 1A). As the somite matured rostro-caudally, Pax7 labelling progressively moved from the anterior to the lateral part of the somite (not shown) to become, at the end of the segmentation period, visible in the somitic external cells (Fig. 1B) lateral to slow fibres (Fig. 1C). We have previously reported that external cells express collagen I (α1) suggesting that they form an epithelium sharing many characteristics with amniote dermatome (Rescan et al., 2005). To further examine the relationship between the trout external cell epithelium and amniote dermis we analysed the gene expression pattern of *Dermo-1* a reliable marker for the onset of dermis differentiation (Li et al., 1995). A trout *Dermo-1* cDNA (GenBank accession no. EU004088) was identified from a large-scale rainbow trout 3′ and 5′ sequencing project (Govoroun et al., 2006). The trout Dermo-1 putative protein, which contains a bHLH domain, showed 82% identity to mouse twist-related protein 2 (Dermo-1) and only 65% identity to twist-related protein 1 (Fig. S1 in supplementary material). Whole-mount *in situ* hybridisation indicated that the expression of Dermo-1 started around the 10-somite stage [stage 10B of Ballard (Ballard, 1973)] in the head mesenchyme and in somites (Fig. 2A). No labelling was observed in the segmental plate from which somites form. As somitogenesis proceeded rostro-caudally, the labelling progressively appeared in more caudal somites (Fig. 2B,C) and did not exhibit any antero-posterior variation within individual somites. Transverse sections indicated that Dermo-1 expression was initially present in the dorsal domain of the somite (Fig. 2D), immediately above the myogenin-positive adaxial cells (Fig. 2H). As the somite matured, Dermo-1 expression extended to the dorso-lateral domain of the somite (Fig. 2E,F) partially overlapping the myogenin-positive embryonic myotome (Fig. 2I,J). At around the 20-somite stage, sclerotome cells located in the ventromedial region of the somite started to express Dermo-1 (Fig. 2E). Later on, Dermo-1-expressing sclerotome cells exhibited an apparent migration towards the dorsal side of the notochord (Fig. 2F), as was expected for migrating sclerotome cells contributing to vertebral cartilage and connective tissue. At the eyed stage, Dermo-1 expression was observed within the external cell layer epithelium (Fig. 2G) surrounding the myogenin-positive embryonic myotome (Fig. 2K) and in postmigratory sclerotome cells (Fig. 2G,K). Taken together, our observations show that all the external cells in late trout embryos express both the paired

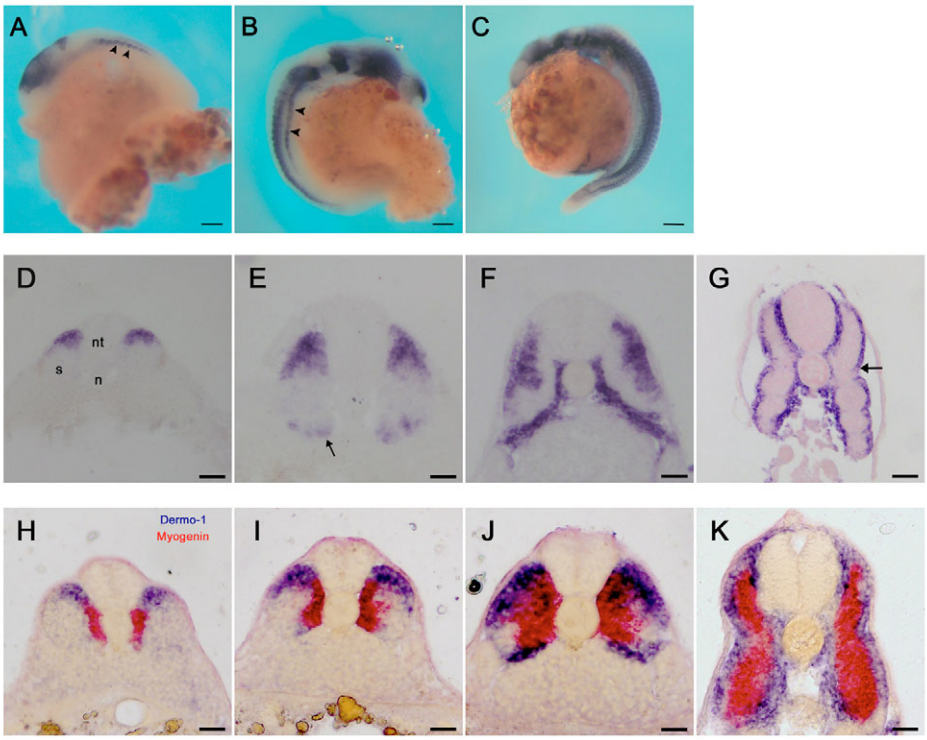

Fig. 2. Expression of Dermo-1 in trout embryo. (A) Stage 10B embryo (approximately 10 somites). Lateral view: labelling is observed in the dorsal domain of the somites (arrowheads) and in cephalic mesenchyme. (B) Stage 13 embryo (approximately 30 somites). Lateral view: Dermo-1 transcript appears in the ventral domain of rostral somites (arrowheads). (C) Stage 18 embryo (segmentation is nearly complete). Lateral view: labelling has progressed up to post-anal somites. (D,E) Stage 12 (20 somite) embryo. Transverse sections through posterior (D) and anterior (E) trunk: labelling is initially present in the dorsal domain of the somite and then appears in the ventral domain of the somite (E: arrow). (F) Stage 40 somite embryo. Transverse section through middle trunk: labelling narrows to the outermost domain of the somite and is observed in sclerotome cells. (G) Stage 20 (somitogenesis is complete). Transverse section through posterior trunk: Dermo-1 transcript is present in somitic external cells (arrow), sclerotome cells and cells intercalated between neural tube and myotome. (H–K) Dual-colour *in situ* hybridisation for Dermo-1 (dark blue) and myogenin (red). (H–J) Serial transverse sections through posterior (H), middle (I) and anterior trunk (J) of a stage 12 embryo (approximately 20 somites). Dermo-1 and myogenin expression progressively intermingle in the maturing somite. (K) Transverse section through posterior trunk of an eyed-stage embryo. Dermo-1 labelling is external to the myogenin-expressing primary myotome. n, notochord; nt, neural tube; s, somite. Scale bar, 200 μm in A and B, 250 μm in C, 40 μm in D, 30 μm in E, 35 μm in F, 45 μm in G and 35 μm in H–K.

homeodomain transcription factor Pax7 and the dermal marker Dermo-1.

Identification of two novel genes expressed in trout somitic external cells: collectin sub-family member 12 and Seraf

We screened eyed-stage embryos for genes expressed at the surface of the myotome by random *in situ* hybridisation using riboprobes generated from a normalised library prepared from trout multitissue cDNAs (Govoroun et al., 2006). We thus isolated two cDNA clones from this screening, one related to collectin sub-family member 12 and the other to Seraf. The collectin cDNA (GenBank accession no. EU008733) encoded an incomplete protein that exhibited approximately 50% identity with chicken collectin sub-family member 12 and very limited identity with other proteins. The deduced amino acid sequence of the trout collectin cDNA included a carbohydrate recognition domain (CRD) containing the conserved Gln–Pro–Asp motif involved in sugar binding, as well as a collagen-like region consisting of Gly–X–Y triplets (Fig. S2 in supplementary material). Whole-mount *in situ* hybridisation showed that the expression of collectin sub-family member 12 started around the 10-somite stage (Fig. 3A). At this stage, collectin sub-family member 12 transcript was detected in somites, lateral plate and cephalic mesenchyme (Fig. 3A). As somitogenesis proceeded along an antero-posterior axis, the labelling progressively appeared in more caudal somites (Fig. 3B,C). Transverse sections showed that collectin transcript initially accumulated in the dorsal domain of the somite (Fig. 3D). As the somite matured, collectin sub-family member 12 expression extended to a large domain of the somite including the sclerotome at the ventromedial edge of the somite (Fig. 3E). Later on, collectin sub-family member 12 expression was observed in external cells surrounding the primary myotome and sclerotome cells adjacent to the axial notochord (Fig. 3F). At the eyed stage, collectin sub-family member 12 expression was observed in branchial arches (Fig. 3C), external cells, sclerotome cells and epidermis (Fig. 3G).

The trout Seraf cDNA (GenBank accession no. EU004089) that we isolated encoded a nearly full-length protein possessing a hydrophobic, potential signal sequence as well as five EGF-like repeats (Fig. S3 in supplementary material). This protein showed the strongest identity with avian Seraf (60%) and a significant conservation with other EGF-repeat-containing proteins, such as Wnt inhibitory factor 1 and Notch ligands. Whole-mount *in situ* hybridisation showed that the somitic expression of Seraf started around the 10-somite stage (Fig. 4A). At this stage, labelling was also observed in the developing notochord. As somitogenesis proceeded, Seraf expression progressively appeared in more caudal

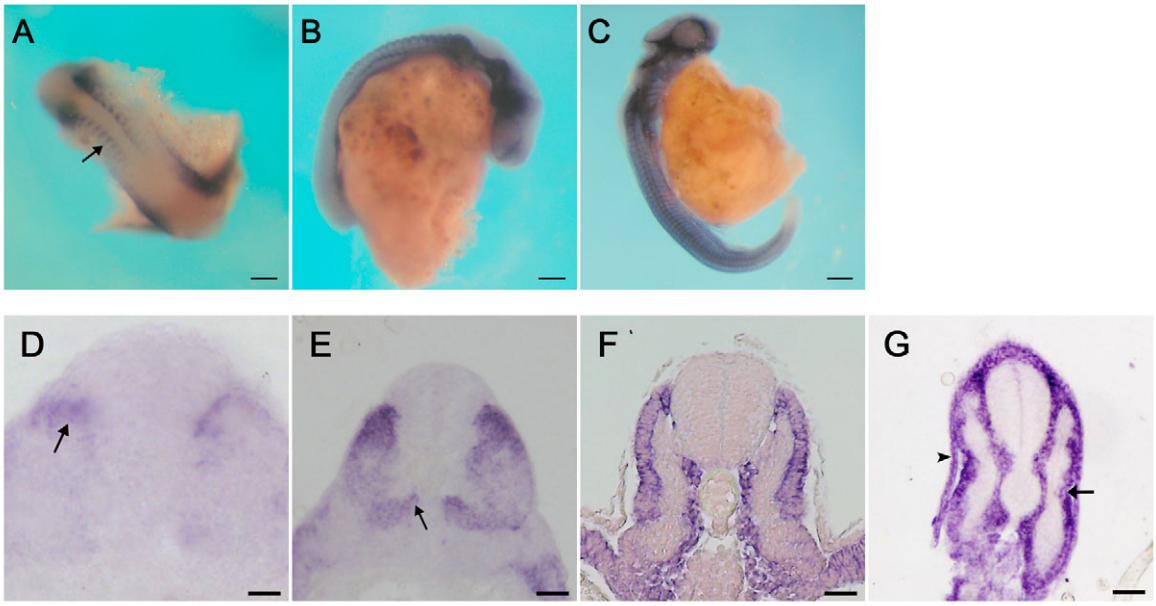

Fig. 3. Expression of collectin subfamily member 12 in trout embryo. (A) Stage 10B embryo (approximately 10 somites). Dorsal view (anterior to the top): labelling is present in somites (arrow), lateral mesoderm and cephalic mesenchyme. (B) Stage 12 embryo (approximately 25 somites). Lateral view: labelling progresses caudally as somites form. (C) Eyed-stage embryo. Lateral view: labelling has progressed up to the most caudal somites. (D) Stage 10B embryo. Transverse section through the middle trunk: labelling is confined to a narrow dorsal domain of the somite (arrow). (E) Stage 20 somite. Transverse section through the anterior trunk: labelling extends to a large domain of the somite and is detectable in sclerotome cells (arrow). (F) Stage 35 somite embryo. Transverse section through the anterior trunk: labelling has narrowed to the external cells and is present in sclerotome cells surrounding the notochord. (G) Stage 20 embryo (segmentation is complete). Transverse section through the middle trunk: labelling is evident in the external cells (arrow), the facing epidermis (arrowhead) and in sclerotome cells. Scale bar, 150 μm in A, 200 μm in B, 300 μm in C, 20 μm in D, 40 μm in E and F, and 50 μm in G.

somites (Fig. 4B), but diminished rostro-caudally in the notochord coinciding with notochord differentiation. Transverse sections through the somites showed that Seraf labelling initially covered most parts of the somite excluding the domain close to the notochord (Fig. 4C). At this stage Seraf expression partially overlapped the myogenin-positive domain of the primary myotome (not shown). Later on, expression of Seraf narrowed to the lateral domain of the somites (Fig. 4D). At the eyed stage, Seraf expression was observed in external cells, in ventral and dorsal edges of the myotome, in sclerotome cells surrounding the notochord and at the dorsal midline (Fig. 4E). Frontal sections in different-staged embryos never revealed antero-posterior variation of the labelling in individual somites.

DISCUSSION

The external cell layer that covers the entire lateral surface of the superficial slow muscle fibres and separates the myotome from the

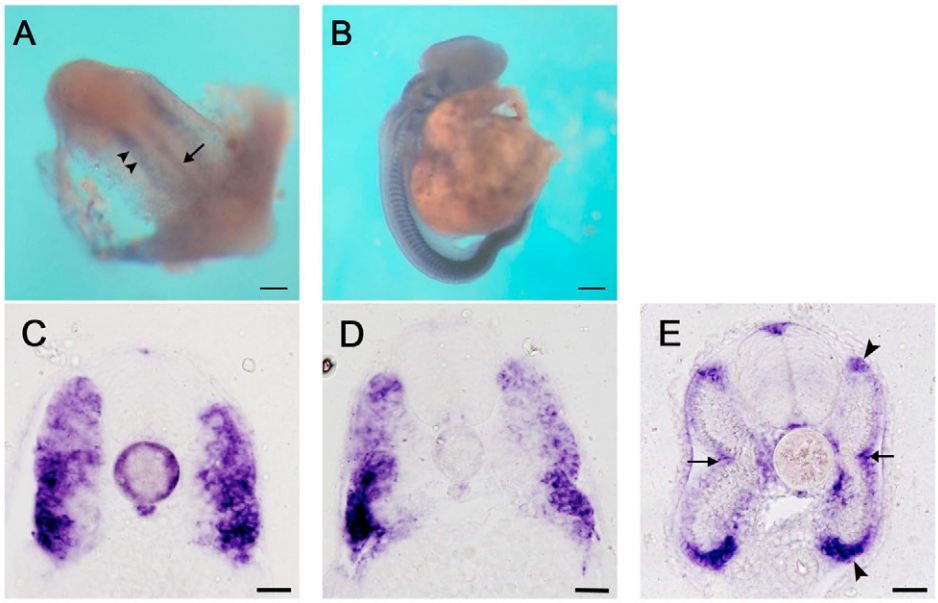

Fig. 4. Expression of Seraf in trout embryo. (A) Stage 10B embryo (approximately 10 somites). Dorsal view (anterior to the top): labelling is observed in somites (arrowheads) as well as in differentiating notochord (arrow). (B) Stage 20 embryo (segmentation is complete). Lateral view: labelling is observed in somites and cephalic mesenchyme. (C,D) Stage 13 embryo (approximately 30 somites). Transverse sections through posterior (C) and anterior trunk (D) showing the progressive disappearance of Seraf transcript in the notochord and the medial part of the somite. (E) Eyed-stage embryo. Transverse section through the middle trunk. Seraf transcript is observed in the ventral and dorsal tips of the myotome (arrowheads), in external cells (arrows) and at the dorsal midline. Scale bar, 100 μm in A, 200 μm in B, 25 μm in C, and 30 μm in D and E.

overlaying epidermis has been reported in many fish species by different authors for many years (Waterman, 1969; Johnston, 1993; Lopez-Albors et al., 1998). The recent observation that this epithelium expresses Pax3 and/or Pax7 (Devoto et al., 2006; Feng et al., 2006; Hammond et al., 2007; Steinbacher et al., 2006; Steinbacher et al., 2007; Macqueen et al., 2007), and the experimental demonstration that external cells in zebrafish give rise to new muscle fibres (Hollway et al., 2007; Stellabote et al., 2007) have led to the suggestion that the external cell epithelium is the evolutionary homologue of amniote dermomyotome generating myogenic cells for myotome growth (Devoto et al., 2006; Stellabote and Devoto, 2007). In line with these findings we show here that all of the cells forming the external cell epithelium surrounding the trout embryonic myotome are potentially myogenic as indicated by Pax7 expression. The homology between teleost external cells and amniote dermomyotome implies that some dermis-related characteristics might be associated in addition to myogenic features. It has been shown that external cells of zebrafish (Le Guellec et al., 2004) and trout (Rescan et al., 2005) express type I collagen, a major marker gene for dermis (Epstein and Munderloh, 1978; Niederreither et al., 1992). Expanding these data, we further show in this study that all somitic external cells in trout embryo also express Dermo-1, a twist-like bHLH transcription factor that marks in amniotes the onset of dermis differentiation. Taken together these observations indicate that trout external cells exhibit features of bipotency and raise the important issue of the contribution of external cells to dermis formation. One possibility is that trout somitic external cells colonize the subectodermal space and give rise to dermis as demonstrated for the (at least) bipotent cells present in the chick dermomyotome sheet (Ben-Yair and Kalcheim, 2005). The other possibility is that external cells only contribute to the production of the early collagenous stroma of the dermis. Further long-term fate mapping studies will be necessary to clarify the exact role of the trout external cells in forming the integument with the epidermis. In zebrafish, lineage-tracking experiments led to the observation that some anterior somitic cells migrate dorsally and ventrally to a position consistent with a dermal layer (Hollway et al., 2007) but the relationship between zebrafish external cells and the dermis still remains unclear. Regarding Dermo-1 function, it has been shown that it acts as a repressor for MyoD activation (Gong and Li, 2001) and, as such, Dermo-1 might play a role in preventing the entry of somitic external cells into the myogenic programme. Dermo-1 also inhibits myogenin transactiviting activity through competition for E-proteins (Li et al., 1995). It is therefore possible that Dermo-1, which is transiently expressed in some domains of the trout embryonic myotome, a feature not observed in amniotes (Li et al., 1995; Scaal et al., 2001), participates in the regulation of the early myogenic differentiation in this fish species. The mechanisms by which Dermo-1 may regulate differentiation of dermal cells are unknown. However, its crucial role in dermis formation has been demonstrated by analysis of *Dermo-1* null mutants that show an atrophic dermis (Sosic et al., 2003). The observation that in vertebrate embryos Dermo-1 transcript most often co-localizes with that of collagen I (Li et al., 1995; Niederreither et al., 1992) (this study) is suggestive of a role for Dermo-1 in regulating collagen I transcription in the external cells.

In addition to Pax7 and Dermo-1, trout external cells express collectin sub-family member 12 and Seraf. Collectins are collagenous calcium-dependent defence lectins involved in innate immunity through binding to oligosaccharide structures and/or lipid moieties on the surface of micro-organisms. Collectin sub-family member 12, which is a transmembrane domain-containing lectin, has been shown to be expressed in most human adult tissues and mainly in vascular cells (Hogenkamp et al., 2006). Its developmental expression pattern, however, has not been reported until now. In this study we show that collectin sub-family member 12 is notably expressed in epidermis and branchial arches. The localisation of collectin transcript in epidermis and branchial arches is in agreement with the role of these tissues as a first line of defence against pathogens. The precise function of collectin sub-family member 12 in developing somite/myotome as well as in sclerotome cells remains enigmatic. Given that the C-type lectin CRD motif, which is present in collectins, mediates a diversity of functions in cell recognition, cell–cell adhesion and embryonic development (Drickamer and Taylor, 1993), it is possible that somitic expression of this lectin contributes to myotome and sclerotome morphogenesis.

The dynamic expression of a *Seraf* gene in trout developing somites was unexpected because *Seraf* gene transcription in chicken embryo is restricted to a subpopulation of migrating neural crest cells committed to form Schwann cell precursors (Wakamatsu et al., 2004). It is possible, given the ancient whole-genome duplication that occurred in the teleost fish lineage, subsequent to its divergence from mammals (Jaillon et al., 2004) and the recent additional genome duplication event specific to salmonids, that multiple *Seraf* orthologues with distinct expression patterns co-exist in the trout genome. The presence in trout databases of several EST cDNAs highly related but distinct from our cDNA clone is in line with this interpretation. The function of Seraf protein remains largely unknown. In chicken, Seraf acts in an autocrine/paracrine fashion on Schwann cell precursors and regulates their distribution in embryos (Wakamatsu et al., 2004). Since Seraf protein shows significant sequence homology with Wnt inhibitory factor 1, it is possible that Seraf produced by external cells controls morphogenetic gradients of Wnt signalling activity in regions subjacent to the ectodermal epidermis. It is interesting to note that Seraf transcript also accumulates in dorsal and ventral tips of the myotome where new myofibres form during the stratified hyperplasia (Rowlerson and Veggetti, 2001; Steinbacher et al., 2007), suggesting that this gene may play a role in regulating stratified growth of the second phase of myogenesis in fish.

The expression of Dermo-1, collectin sub-family member 12 and Seraf within the external cell layer epithelium was preceded by a complex transcription pattern in the early somite, notably affecting the primary myogenin-positive myotome. This contrasts with the expression of Pax7, which mirrors the migration of external cell progenitors from the anterior to the external surface of the somite. The mechanisms that govern the dynamic expression of Dermo-1, collectin sub-family member 12 and Seraf in somitic cells remain to be determined.

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