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Tissue regulation of somitic *colloid-like1* gene expression

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ABSTRACT

Body skeletal muscles formation starts with somite differentiation, due to signals from surrounding tissues. Somite ventral portion forms the sclerotome while its dorsal fraction constitutes the dermamyotome, and later the dermatome and myotome. Relative levels of BMP activity have been proposed to control several aspects of somite development, namely the time and location of myogenesis within the somite. The fine-tuning of BMP activity is primarily achieved via negative regulation by diffusible BMP inhibitors, such as Noggin and Chordin, and on a secondary level by proteins cleaving these inhibitors, such as BMP1/Tolloid metalloprotease family members. Herein, we carefully described the somitic expression of colloid-like1, one of the chick BMP1/Tolloid homologues, and found that this gene is specifically expressed in the 10 most anterior somites, suggesting that it may be involved in neck muscle formation. By using *in ovo* microsurgery and tridimensional embryo tissue culture techniques we assessed the function of surrounding structures, neural tube, notochord, surface ectoderm and lateral plate mesoderm, on the maintenance of somitic colloid-like1 gene expression. We unveil that a signal coming from the neural tube is responsible for this expression and rule out the main candidate pathway, Wnt. By comparing the somitic colloid-like1 gene expression with that of related signaling partners, such as BMP4, Noggin and Chordin, we propose that colloid-like1 plays a role in the reinforcement of BMP4 activity in the medial portion of the 10 most anterior dermomyotomes, thus belonging to the molecular machinery controlling neck muscle development in the chick.

1. Introduction

Somites are on the basis of the segmental pattern of the adult vertebrate body and give rise to vertebrae, intervertebral discs, ribs, the dermis of the back and all skeletal muscles of the adult body. Somites form along the antero-posterior embryonic axis, bilaterally to the neural tube and notochord, budding off from the cranial end of the presomitic mesoderm (PSM) [1] while new cells are caudally added as a consequence of gastrulation [2]. Initially, the somite is formed by a sphere of epithelial cells but, as development proceeds, its ventral part undergoes an epithelial-to-mesenchymal transition, forming the sclerotome. Meanwhile, its dorsal part remains epithelial and constitutes the dermomyotome, which later originates dermatome and myotome. In the latter, two domains can be defined from which the two sets of the body skeletal muscles arise; a dorsomedial/epaxial and a ventrolateral/hypaxial. Signals from surface ectoderm (SE), neural tube, notochord and lateral plate mesoderm (LPM) have been identified as crucial for somitic axes specification [3]. Bone Morphogenetic Protein 4 (BMP4) is produced by both dorsal neural tube and LPM and was shown to be crucial in several aspects of somite differentiation [4]. Several studies in *Xenopus*, chick and mouse revealed that secreted proteins like Noggin and Chordin create a gradient of BMP4 by directly binding to it, thus preventing an interaction with its receptor [5]. A further level of regulation is introduced by the secreted zinc metalloprotease, Tolloid, which belongs to the conserved family BMP1/Tolloid-like [6]. In early embryo, these proteins have been described as positive regulators of BMP4 activity by proteolytically cleaving Chordin, generating small fragments and thus reducing its affinity to BMP4 [7]. Contrarily to other species, very little is known about the function of these metalloproteases in the chick. Two BMP1/Tolloid family members have already been cloned, chicken BMP1/Tolloid and colloid-like1, and their expression patterns described (*bmp1/Tolloid* [8,9]; *colloid-like1* [10]). In this work, we characterise somitic colloid-like1 gene expression in embryos up to stage 15^{+}HH. We assess the *in vivo* regulation of this expression by the different surrounding embryonic tissues such as the axial structures, neural tube and notochord, SE and flanking LPM. The data obtained allows us to

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propose a function of somitic colloid-like1 gene expression in neck muscle formation.

2. Materials and methods

2.1. Chicken embryos

Fertilised chicken (*Gallus gallus domesticus*) eggs were obtained from commercial sources, stored at 16 °C and incubated in a 45% humidified atmosphere at 37 °C. Incubated embryos were harvested, washed in PBS without Ca^{2+}/Mg^{2+} and staged according to Hamburger and Hamilton classification system [11]. Embryos of stages 7–15^{+}HH were fixed overnight at 4 °C in a 4% formaldehyde solution and then progressively dehydrated in methanol series and stored in 100% methanol at −20 °C.

2.2. RNA probes

Colloid-like1, BMP4, chordin, noggin and myoD anti-sense RNA probes were provided by Fabienne Pituelo, Paul Brickell, Tom Jessel and Richard Harland, respectively [10,12–14].

2.3. Whole-mount in situ hybridization

Whole-mount in situ hybridisation was carried out according to [15]. Stained embryos were photographed using a Leica D200 camera or a Zeiss SteREOLumarV12 Stereomicroscope coupled with a Zeiss Axiocam MRc camera and Zeiss Axiovision software. All images were adjusted for brightness and contrast using Adobe PhotoShop CS3.

2.4. Embryo sectioning procedure

Selected hybridised embryos were progressively dehydrated with ethanol, embedded in resin (Technovit 8100) and sectioned at 20 μm thickness using an ultramicrotome. The slides were then mounted in Neomount (Merck) and photographed using a Zeiss Axio Imager Z2 Fluorescence microscope coupled with a Zeiss ICc3 Axiocam camera. All images were adjusted for brightness and contrast using Adobe PhotoShop CS3.

3. Microsurgical experiments

3.1. In ovo surgical slit

A small window was made in the eggshell and an Indian ink:PBS (1:1) solution was injected into the sub-blastodermic cavity. In embryos ranging from 10HH to 12HH, the right row of somites was separated from axial organs by a slit made through the three germ layers (Fig. 2A), along four to six somites posteriorly to the second to fourth most cranial somites. Operated embryos were reincubated for 12–16 h, harvested, fixed, dehydrated as described above and kept in methanol at −20 °C for in situ hybridisation.

3.2. In vitro Notochord ablation

10HH–12HH staged embryo were removed from the egg yolk as described for the Early Chick (EC) culture technique [16] and placed into resin-coated Petri dishes in PBS without Ca^{2+}/Mg^{2+}, ventral side up to facilitate access to the notochord. A portion of the notochord spanning six somites posteriorly to the first to second most cranial somites was mechanically removed from the neural tube and flanking somites (Fig. 2B) and the embryos were cultured for 12–16 h. Embryos were harvested, fixed and processed for in situ hybridization as described above.

3.3. In ovo neural tube ablation

Embryos were accessed as described above. In 10HH–12HH staged embryos, SE incisions were made bilaterally to the neural tube. After a brief treatment with pancreatin (Sigma), a piece of neural tube with a four to six somite length posterior to the second to fifth most cranial somites (Fig. 2C) was dissected out and removed using a micropipette. The operated area was rinsed with heat-inactivated foetal calf serum and the embryos were reincubated for 12–16 h, then harvested, fixed and processed for in situ hybridization as described above.

3.4. In vitro SE removal

10HH–12HH embryos were surgically removed from the egg yolk into resin-coated Petri dishes in PBS without Ca^{2+}/Mg^{2+} where the microsurgery was performed (Fig. 2D). On the right side of the embryo, a longitudinal incision on the ectoderm overlying the middle of the neural tube was made starting from the second to fourth most cranial somites and spanning along a six somite length. An equivalent incision was made on the ectoderm overlying LPM, and the delimited area was carefully peeled off. By cutting the neural tube down the midline two type of explants were generated: on the right side of the embryo, the exposed area was cultured as an explant deprived of all SE and on the left side of the embryo an equivalent explant (with SE) was cultured as a control.

Explants were cultured for 7.5–9 h on polycarbonate filters (0.8 mm pore size; Millipore) as described in [17], fixed and processed for in situ hybridisation.

3.5. In vitro LPM removal

10HH–12HH embryos were surgically removed from the egg yolk into resin-coated Petri dishes as described above. On the right side of the embryo, a longitudinal incision was made between somites and LPM starting from the second to third most cranial somites and spanning along a six somite length (Fig. 2E). Two explants were generated by cutting the neural tube down the midline: the right portion of the embryo was cultured as an explant deprived of LPM and a left equivalent explant (with SE) was cultured as a control.

Both explants were cultured as described above for 7.5–9 h, harvested, fixed and processed for in situ hybridisation.

3.6. In vitro Wnt inhibition

10HH–12HH embryos were surgically removed from the egg yolk into resin-coated Petri dishes as described above. A six somite length explant containing all embryonic tissues was delimited from the first to third somites (Fig. 3) and cultured as described above in Medium 199 supplemented with two concentrations (2 μl/ml; 3.5 μl/ml) of the commercial available Wnt Pathway Inhibitor VII, Cardamonin (Calbiochem). Control explants were cultured in normal medium as described above for 7.5–9 h, harvested, fixed and processed for in situ hybridisation.

4. Results and discussion

4.1. Somitic colloid-like1 gene expression is restricted to the 10 most rostral somites

We evaluated the somitic expression of *colloid-like1* gene by whole-mount in situ hybridisation and section analysis of embryos ranging from stages 6HH [11] to 15HH (Fig. 1). In embryos from stages 7^{+}HH to 15^{+}HH, *colloid-like1* transcripts are broadly detected