***Tsukushi* expression is dependent on Notch signaling and oscillated in the presomitic mesoderm during chick somitogenesis**

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Abstract

During somitogenesis, segmentation of the body axis occurs by epithelial somites budding off from the rostral end of the unsegmented presomitic mesoderm (PSM), and its molecular regulation is achieved by a molecular oscillator and signaling molecules. Tsukushi (TSK) is a unique secreted protein and involved in diverse biological cascades in vertebrate embryos by modulating several signaling pathways at the extracellular region. However, the involvement of *TSK* in somitogenesis remains unknown. In this study, we investigated the detailed expression patterns of *TSK* at different developmental stages of a chick embryo. Chick-*TSK* (*C-TSK*) is expressed in the PSM and shows an oscillation pattern with three phases. The oscillation pattern of *C-TSK* in the PSM is similar to that of *c-Notch1* and *c-hairy1*, but not to *c-Delta1*. Our in vitro data showed that Notch signaling is necessary for the normal expression of *C-TSK* and that expression of *C-TSK* is an intrinsic property of the anterior PSM. These data suggest that *TSK* plays a role in chick somitogenesis.

Key words: Tsukushi, Notch signaling, Somitogenesis, Chick, Oscillation

1. Introduction

Somitogenesis is one of the most fascinating and fundamental processes of vertebrate early development by which the blueprint of the vertebral column, the “somites,” form from both sides of the developing neural tube [1]. These somites are transient bilateral repeated segments of the paraxial mesoderm that differentiate into the axial skeleton, body skeletal musculature, and dermis. Segmentation of the vertebrate body axis is initiated through somitogenesis, whereby epithelial somites bud off in pairs periodically from the rostral end of the unsegmented presomitic mesoderm (PSM) [2].

Somite formation involves extensive cellular readjustments, namely, cell packing and polarization, when preparing for the required mesenchymal-to-epithelial transition [3]. It is widely accepted that this process is controlled by a molecular oscillator that drives periodic waves of gene expression caudo-rostrally through the PSM with the same periodicity as somite formation [4]. Several clock genes belonging to the Wnt, Notch, *shh*, or FGF pathways play roles in the process of somite formation and oscillation [5]. This periodic mechanism is repeated a specific number of times until the embryo acquires a defined species-specific final number of somites at the end of the process of axis elongation [4].

Previously, we reported that Tsukushi (TSK) is a member of the Small Leucine-Rich Proteoglycan (SLRP) family belonging to the subclass IV [6]. *TSK* binds nodal/VG1 [7], BMP4/Chordin [6], FGF8 [8], Frizzled4 [9], and Delta [10] and modulates their downstream intracellular signaling pathways, indicating multiple regulatory functions of *TSK* during early development. *TSK* is also involved in anterior commissure formation in the mouse brain [11]. *TSK*, therefore, play multiple

roles as a signaling mediator at the extracellular region during many key biological events [12].

In this study, we investigated the detailed expression pattern of *C-TSK* at different developmental stages of chick somitogenesis. Using an *in situ* hybridization technique, we found that *C-TSK* is expressed in the PSM and shows an oscillation similar to that of *c-hairy1* and *c-Notch1*, but not to *c-Delta1*. Further, an *in vitro* culture assay showed that Notch signaling is necessary for the normal expression of *C-TSK* and that the expression of *C-TSK* is an intrinsic property of the PSM. Our results suggest that *TSK* plays a role in chick somitogenesis with oscillation by interacting Notch signaling.

2. Materials and methods

2.1. Chick

Chick eggs were incubated at 38°C in a humidified incubator. The embryos were staged according to the developmental table of Hamburger and Hamilton [13]. The stage specific (stage 7, stage 8, stage 10, stage 15, stage 18, stage 23) embryos were used for *in situ* hybridization. The newly formed somites were designated S1, S2, S3, the forming somite was designated S0, and the one somite length unsegmented PSM was designated S-1. All experiments on chick were conducted in accordance with the guidelines of the Center for Animal Resources and Development (CARD), Kumamoto University, Japan.

2.2. In situ hybridization

In situ hybridization was performed as previously described [6] to assess *C-TSK*, *c-hairy1*, *c-Notch1* and *c-Delta1* expression patterns in the presomitic mesoderm. For

experiment on oscillation pattern, stage 15 (somites 25) embryos were selected by counting the somite numbers, cut the embryos into two equal halves, and complementary pairs were used for *in situ* hybridization. Sense and antisense riboprobes labeled with Digoxigenin (DIG) were synthesized from the corresponding DNA constructs [14]. A color reaction was initiated with 337.5 µg/ml 4-Nitro blue tetrazolium (NBT, Roche) and 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP, Roche) in freshly prepared Alkaline Phosphatase (AP) buffer (0.1 M Tris-HCL, pH 9.5, 0.1M NaCl, 50 mM MgCl₂, 1% Tween-20, and 0.48 mg/ml levamisole hydrochloride).

2.3. Chick embryo and explants *in vitro* culture

Chick embryos ranging from 15 to 20 somites were cultured in L15 medium (L-15 Leibovitz medium, Gibco) supplemented with 15% fetal calf serum (FCS) and 0.1% gentamycin (Sigma Aldrich) in a roller incubator at 37°C. For pharmacological inhibition of signaling pathways, embryos were cultured for 9 hours in 10–100 µM N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, (DAPT, Calbiochem). The DAPT was diluted in culture medium from stock solutions in dimethylsulphoxide (DMSO) to maintain a final concentration of 1%. For *in vitro* culture of the explants (stage 14) containing PSM, embryos were isolated from surrounding tissues and the cultured (0-6 hours) embryos were hybridized with C-*TSK* probes.

2.4. Image Analysis

Embryos processed for whole mount *in situ* hybridization were photographed, using an Olympus DP70 digital camera coupled with a Leica MZFLIII stereomicroscope equipped with a DP Controller image manager (Olympus

Corporation, Japan). Cryosectioned samples were photographed using a Keyence BZ-9000 fluorescence microscope (Keyence Corporation, Japan).

3. Results

3.1. C-TSK is expressed in PSM during chick somitogenesis

During chick embryogenesis, *C-TSK* is expressed in various tissues, including the limb bud, nasal pit, and PSM [7,8]. To determine *TSK* mRNA expression in detail in the PSM and newly forming somites, we examined the expression pattern of *C-TSK* in the PSM of different developmental stages of chick embryos (Fig. 1). *C-TSK* was detected beginning at stage 7, where the first somite pair originated from both sides of the neural tube (Fig. 1A). As development proceeded, the number of somites increased and the most mature somites reached near the neck of the embryo, while the newly formed somites were located near the posterior part of the embryo (Fig. 1B and C). At stage 15, the expression of *C-TSK* was detected in the anterior PSM (Fig. 1D). During stage 18, *C-TSK* is also expressed in the newly forming somites and the PSM that reached beyond the leg bud (Fig. 1E). At stage 23, *C-TSK* was expressed in the somites and PSM near the tip of the tail and also in the wing/leg buds (Fig. 1F). In a sagittal section of the last formed somite and the PSM at stage 15, *C-TSK* was expressed as two bands, a narrow band in the caudal half of the newly formed somite 'S1', a thick band in the caudal half of the forming somite 'S0' and in the unsegmented posterior PSM (Fig. 1G). These results suggest that, during embryonic development, *C-TSK* is expressed at different stages in the chick PSM.

3.2. The expression of C-TSK oscillates in the chick anterior PSM

To determine whether *C-TSK* expression oscillates, we compared its expression with *c-hairy1*, *c-Notch1*, and *c-Delta1* mRNA in the chick anterior PSM (Fig. 2). The expression of *c-hairy1* mRNA in the PSM oscillates with the same periodicity as the somite forms and one complete cycle of *c-hairy1* oscillation represents the formation of one somite [15]. Importantly, *c-hairy1* is a target of the Notch signaling cascade [15]. In our study, the expression patterns were divided into three phases (phases I–III) as per somite formation periodicity in a one cycle and a new somite is formed from the rostral-most part of the PSM. At phase I, *c-hairy1* was visible as two bands, one narrow band in the caudal part of the forming somite S0, and another thick band that extended caudally from the S-1 to the tail area (Fig. 2A). In contrast, *C-TSK* expression started from the caudal part of the newly forming somite S0 as a single and extended band along the PSM (Fig. 2A). At phase II, with the progression of somite formation, *C-TSK* expression was detected as two bands: the very narrow band was detected in the caudal half of the forming somite S0, while the thick band was detected in the caudal part of the S-1. In the complementary band, *c-hairy1* expanded anteriorly and the caudal-most part vanished (Fig. 2B). At phase III, this wave front like expression of both *c-hairy1* and *C-TSK* eventually narrowed and reduced towards the S0 (Fig. 2C). In this phase, the formation of somite S0 was nearly completed.

Next, we compared *C-TSK* and *c-Notch1* expression in the anterior PSM (Fig. 2D–F). At phase I, *c-Notch1* was expressed weakly at the caudal region of newly formed somite S1, in the PSM it expresses weakly from the forming somite S0, and extended strongly from the unsegmented PSM S-1 (Fig. 2D). At phase II, *c-Notch1* was expressed at the caudal half of S1 and extended to S0, S-1, and posterior PSM (Fig. 2E). At phase III, *c-Notch1* expression moved more anterior and posterior part of the PSM. Here, *c-Notch1* occupied the entire last formed somite S1, then it expressed

posteriorly beyond the S-1 (Fig. 2F). In our observations, we found upregulated expression of *c-Notch1* in the anterior part of the PSM in compare to posterior part. As development proceeded, the expression pattern of *c-Notch1* was gradually downregulated towards the posterior part of the PSM. In contrast, *C-TSK* expression was observed as a distinct band along the A-P axis. S0 and S-1 at phase II became S1 and S0, respectively, at phase III. *C-TSK* expression is not visible at the last formed somite S1 at phase III (Fig. 2C, F, I). In our experiment the expression pattern of *c-hairy1* and *c-Notch1* in chick somitogenesis has similarity with the previous study [16].

Finally, we compared *C-TSK* and *c-Delta1* in the anterior PSM (Fig. 2G-I). Expressions of both *C-TSK* and *c-Delta1* were robust in the anterior PSM during somitogenesis. We found that *c-Delta1* was expressed in a very distinct domain in chick PSM, which is similar with the previous study [16]. But, *c-Delta1* expression was stable at all phases in the anterior PSM, and did not show the dynamic oscillation pattern of *C-TSK*, *c-hairy1*, and *c-Notch1*. This comparative expression pattern suggests that *C-TSK* exhibits a dynamic oscillation in the PSM.

3.3. Notch signaling is necessary for the normal expression of C-TSK and the expression of C-TSK is an intrinsic property of the PSM

To examine how Notch signaling interacts with *C-TSK* in chick somite development, we used a pharmacological inhibition approach by the Notch inhibitor DAPT (Fig. 3A-D). We isolated unsegmented mesoderm at embryonic stage 14, cultured in the presence or absence of DAPT, and analyzed target genes either with DAPT (Fig. 3B and D) or without (Fig. 3A and C). To confirm that DAPT-mediated inhibition was not due to a usual disruption of the PSM identity patterning, we

checked the expression of the PSM marker T-box transcription factor 6 (*Tbx6*). After 9 hours of cultivation, *C-TSK* (Fig. 3A and B) and *Tbx6* (Fig. 3C and D) expression patterns were analyzed. *C-TSK* expression at the PSM in medium containing DAPT was reduced in comparison to the control (DMSO).

To determine whether the expression of *C-TSK* is intrinsically controlled by the PSM, we performed an explant culture assay and an *in situ* hybridization experiment (Fig. 3E-G). In our study, 14-20 somites stage chick PSM was detached from the surrounding tissues, cultured for 0 (Fig. 3E), 3 (Fig. 3F), or 6 (Fig. 3G) hours, and then hybridized with a *C-TSK* probe. In this experiment, the ectoderm and endoderm were not associated with the PSM. Under these conditions, we did not see any segments, but the expression of *C-TSK* was sustained. The expression of *C-TSK* was progressively downregulated and shifted to the more caudal parts of the explants (Fig. 3G). These results suggest that Notch signaling is necessary for the normal expression of *C-TSK* in the chick PSM and expression of *C-TSK* is autonomously regulated by the unsegmented mesoderm or PSM. Therefore, the expression of *C-TSK* is an intrinsic property in PSM development.

4. Discussion

Early patterning of the body during animal development is a fundamental process to subsequent events including cell differentiation, tissue and organ formation, and correct function of the adult body [17]. To trigger this rhythmic production of somitic segments, three major signaling pathways, Notch, Wnt, and FGF integrate into a molecular network that generates a traveling wave of gene expression along the embryonic axis, called the “segmentation clock” [18]. In addition to these signaling pathways, *TSK*, a member of the extracellular proteoglycan gene family [19] also

plays a role in somitogenesis. As *TSK* is expressed in the PSM and is involved in the above signaling pathways [12], it is plausible to expect the involvement of *TSK* in somitogenesis. In this study, the *in situ* hybridization data provide evidences for the involvement of *C-TSK* in different stages of somitogenesis. This detailed expression pattern of chick embryogenesis denotes the timing, location, and pattern of *C-TSK* expression that is required for somite formation and progression.

Figure 4 compared and summarized the expression pattern of *C-TSK* and Notch signaling molecules during somitogenesis. During early phase, *c-hairy1* was first expressed in the posterior PSM, and then gradually, it is expressed in the more anterior domains. *C-TSK* also showed caudal-to-rostral shifting of expression and refined in later phases to retain the wave-like expression. Though the *c-hairy1* and *C-TSK* expression domains and band size is not similar, both of them showed a dynamic expression pattern in the anterior PSM. The involvement of the Notch signaling pathway in somitogenesis was first revealed by the finding that Notch1 and its ligand Delta-like 1 (Dll1), which are highly expressed in the PSM of mouse embryos, play important roles in inter- and intra-somitic boundary formation [20-22]. In our observation, *c-Notch1* expression was upregulated in anterior domains of the PSM. This oscillation of *c-Notch1* is consistent with the pattern of *C-TSK* expression during early development. Both *C-TSK* and *c-Delta1* expression was seen robustly in the anterior PSM during somitogenesis. Though *c-Delta1* was expressed in a very distinct domain in chick PSM, expression was stable in the anterior PSM, and did not show a pattern of oscillation similar to *C-TSK*, *c-hairy1*, and *c-Notch1*. Therefore, *C-TSK* expression seems unlikely to be related to *c-Delta1* expression and this comparison supports the oscillatory behavior of *C-TSK* in the anterior PSM.

Our *in vitro* culture experiment and pharmacological inhibition data showed that Notch signaling is essential for the normal expression of *C-TSK*. The regulation of clock gene oscillations by the Notch signaling pathway plays a key role in the molecular mechanism of the segmentation clock in a number of different vertebrate species [23]. *C-TSK* expression was downregulated in the presence of DAPT, which pharmacologically inhibited the Notch signaling pathway. The PSM still possesses mesenchymal congruity by expressing the PSM marker *Tbx6* that has previously been shown to be a Wnt target in the mouse PSM [24]. Inhibition of Notch may cause residual Wnt activity to upregulate *Tbx6* expression. As Notch is an integral part of the oscillator and segmentation critically depends on its signaling, *C-TSK* expression also relies on Notch signaling in the anterior PSM. Therefore, *C-TSK* expression was reduced in embryos incubated in DAPT to inhibit the corresponding pathways. It is well known that somitogenesis can proceed *in vitro*, provided that the ectoderm and endoderm remain associated with the PSM [25]. Moreover, in complete isolation, the PSM does not form any somites but the cyclic expression of *c-hairy1* is maintained [26]. In our study, no somites or segments were formed, but that the expression of *C-TSK* was sustained. The expression of *C-TSK* was autonomously regulated by the unsegmented mesoderm and the expression of *C-TSK* intrinsic to the PSM. However, it is not yet clear whether the periodic gene expression of *C-TSK* is associated with the segmentation clock due to the cyclic gene network. TSK directly binds several signaling molecules in the extracellular space through protein-protein interactions, and it functions as an extracellular modulator of signaling cascades by enhancing or inhibiting their activity during early embryonic development [12]. Considering this, we hypothesize that *TSK* also maintains its own gene network and acts as an

extracellular signaling mediator for somite formation and oscillations in gene expression.

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Figure legends

Fig. 1. Expression of *C-TSK* in the PSM at different stages of chick embryos. (A-F) Whole mount *in situ* hybridization. *C-TSK* is detected at stage 7 (A), stage 8 (B), stage 10(C), stage 15 (D), stage 18 (E), and stage 23 (F) (violet color in all stages). The black arrowheads show the PSM expressing *C-TSK*. The boxed areas are magnified views of the PSM. (G) Sagittal section of the PSM at stage 15, where *C-TSK* is expressed weakly in the caudal half of the newly formed somite ‘S1’, strongly from the caudal half of the forming somite ‘S0’ and in the unsegmented posterior PSM. ps, primitive streak; tb, tail bud; wb, wing bud; lg, leg bud. Scale bars: 150 μ m in A; 300 μ m in B; 400 μ m in C-E; 2 mm in F; 50 μ m in G.

Fig. 2. Comparison of *C-TSK* with *c-hairy1* (A-C), *c-Notch1* (D-F), and *c-Delta1* (G-I) in the chick anterior PSM at stage 15. (A) At phase I, *C-TSK* is expressed as a single band and *c-hairy1* expressed as two bands in S0, S-1. (B) At phase II, *C-TSK* and *c-hairy1* expressed as two bands at S0 and S-1 (C) At phase III, expression of both *C-TSK* and *c-hairy1* are narrowed. (D) At phase I, *C-TSK* express along the S-1, while *c-Notch1* is weakly expressed at the caudal half of S1, S0, but express strongly from the of S-1. (E) At phase II, *C-TSK* express as two bands, S0 and S-1, *c-Notch1* is expressed in the caudal half of S1, and entire S0, S-1 (F) At phase III, *C-TSK* narrowed but *c-Notch1* expression covers the S1, S0, S-1. (G-I) *C-TSK* oscillates at S0 and S-1, while *c-Delta1* expression does not show oscillations at the anterior PSM. Scale bars: 150 μ m in A-C; 100 μ m in D-F; 100 μ m in G-I.

Fig. 3. *C-TSK* expression is dependent on Notch signaling and intrinsically regulated by the unsegmented mesoderm. Embryos at stage 14 were cultured at 37°C with

DAPT (B, n = 11; D, n = 9) or control (A, n = 10; C, n = 13). After 9 hours of culture, *C-TSK* (A, B) and *Tbx6* (C, D) expression patterns were analyzed by *in situ* hybridization. *C-TSK* expression in medium containing DAPT (B) was diminished in comparison with the control (A). (E-G) PSM at the 14-20 somites stage was isolated from the surrounding tissues and was cultured for 0 (A), 3 (B), or 6 (C) hours. Note that the expression of *C-TSK* was maintained and progressively shifted to the more caudal parts of the explants. r, rostral; c, caudal. Scale bar: 300 μ m in A-D; 200 μ m in E-G.

Fig. 4. Schematic comparison of the oscillatory expression patterns in the anterior PSM. At phases I to III, *C-TSK* (red) expression is observed as dynamic oscillation. At phases I to II, *c-hairy1* (violet) was expressed as two extended bands, but at phase III as a single narrow band. At phase I, *c-Notch1* (green) is weakly expressed at S0, but dispersed from the caudal half of the anterior PSM. Later, it extends anteriorly up to S1. At phase III, *c-Notch1* is extended beyond the S-1. In all three phases, *c-Delta1* expression (blue) is characterized as stable and does not show oscillations at the anterior PSM. In phase III, somite S0 is nearly ready to bud off as a new somite S1. The end of this phase starts again as phase I.

Figure -1

Figure -2

Figure -3

Figure -4