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Teashirt 3 expression in the chick embryo reveals a remarkable association with tendon development

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Abstract

Drosophila teashirt (*tsh*) is involved in the patterning of the trunk identity together with the *Hox* genes. In addition, it is also a player in the Wingless and the Hedgehog pathways. In birds and mammals, three *Tshz* genes are identified and the expression patterns for mouse *Tshz1* and *Tshz2* have been reported during embryogenesis. Recently, we showed that all three mouse *Tshz* genes can rescue the *Drosophila tsh* loss-of-function phenotype, indicating that the function of the *teashirt* genes has been conserved during evolution. Here we describe the expression pattern of chick *TSHZ3* during embryogenesis. Chick *TSHZ3* is expressed in several tissues including mesodermal derivatives, the central and peripheral nervous systems. Emphasis is laid on the dynamic expression occurring in regions of the somites and limbs where tendons develop. We show that *TSHZ3* is activated in the somites by FGF8, a known inducer of the tendon marker *SCX*.

Keywords: *Teashirt*; Chick; Mouse; *Drosophila*; *Scleraxis*; Tendon; Neurons

1. Results and discussion

Drosophila teashirt (*tsh*) encodes for a zinc finger transcription factor that is crucial for the patterning of the trunk identity in collaboration with the *Hox* genes (Fasano et al., 1991; Röder et al., 1992). Tsh acts also in the Wingless and the Hedgehog pathways (Angelats et al., 2002; Gallet et al., 1998, 1999). In addition, *tsh* function is required for the midgut morphogenesis (Mathies et al., 1994) and for the development of adult appendages (Bessa et al., 2002; Erkner et al., 1999; Pan and Rubin, 1998; Soanes et al., 2001; Wu and Cohen, 2000). In vertebrates, three *teashirt (Tshz)* genes have been identified in mouse and human. Expression patterns during embryogenesis were reported for mouse *Tshz1* and *Tshz2* and are consistent with a role in trunk specification in vertebrates (Caubit et al., 2000). Recently, we tested whether *Tshz1*, *Tshz2*, or *Tshz3* could rescue *tsh* loss-of-function in flies. We showed that all three mouse *Tshz* rescued with high efficiency homeotic transformation and abnormal trunk morphogenesis, two defects observed in *tsh* null mutant *Drosophila* embryos. Rescue of *Drosophila tsh* null mutant by the mouse orthologs demonstrates that the function of *Tshz* genes is phylogenetically conserved (Manfroid et al., 2004). Here we describe the expression of the third member of the *Tshz* genes family, chick *TSHZ3*, during chick embryogenesis and show a remarkable expression in tendons.

1.1. Identification of chick Tsh genes

In a BLAST search with the amino acid sequences of mouse *Tshz* genes against the chick draft genome database (Ensembl Genome Browser (currently v.36-Dec2005),

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http://www.ensembl.org/Gallus_gallus/index.html), we found three genes. The sequences show high similarity to the *tsh*/*tio* family. Alignment of these sequences revealed characteristic amino acids thereby unequivocally identifying the three *Tshz* genes of vertebrates (data not shown). Phylogenetic analyses based on the protein sequences using Neighbor-joining method clearly groups chick *TSHZ3* with other vertebrate *Tshz3* sequences (Fig. 1). Based on these results, we named the new genes (Chick)*TSHZ1*, (Chick)*TSHZ2* and (Chick)*TSHZ3*. Chromosomal locations of (Chick)*TSHZ* genes have been identified. *TSHZ1*, *TSHZ2* and *TSHZ3* are located on chromosome 2, 20 and 11, respectively.

1.2. Overall TSHZ3 expression during early chick embryogenesis

We could not detect *TSHZ3* mRNAs by in situ hybridization prior to HH stage 10 when a faint expression takes place in the neural plate (not shown). Between HH stage 10 and 15, additional sites of expression are observed. *TSHZ3* demarcates the neural tube, the lateral mesoderm (Fig. 2A) and the region of the foregut (Fig. 2B). Rostrally, rhombomere r4, anterior to the otic vesicle, constitutes the anterior limit of expression in the neural tube (Fig. 2B). At HH stage 21 (Figs. 2C and D), *TSHZ3* is found in the mesenchyme of the posterior aspect of the limb buds, in branchial arches (BA) posterior to BA I, at the level of the foregut and in the lateral mesoderm between the fore- and the hindlimb buds. The most striking expression is observed in the somites. The expression becomes detectable in the posterior part by HH stage 18, and intensifies as development proceeds (Figs. 2C and D). No or very weak *TSHZ3* expression is observed in the four most anterior somites (occipital somites, Fig. 2D). The staining in the head, not reproducible, is likely due to the trapping of the probe/substrate. Around HH stage 24 (Fig. 2E), *TSHZ3* appears in the anterior part of the somites in addition to the posterior domain of expression. We focused our analysis on this interesting expression.

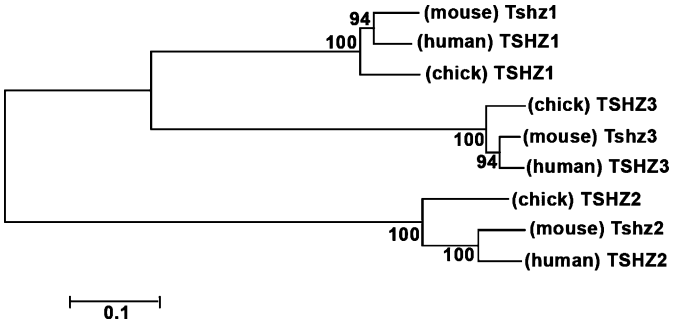

Fig. 1. Phylogenetic tree of Tshz sequences. Tshz proteins fall into three classes. The numbers of the interior branches refer to the bootstrap values with 100 replicates.

1.3. Expression of TSHZ3 in the forming tendons

A dorsal view of the trunk at HH stage 27 uncovers the similarity between *TSHZ3* and *Scleraxis* (*SCX*) expression in the somites (Figs. 2F and G). *SCX* marks the syndetome, a somitic compartment formed by the tendon progenitors localized at the anterior and posterior margins of the somites (Brent et al., 2003; Schweitzer et al., 2001). Transverse sections (Figs. 2H and I) show that *TSHZ3* and *SCX* both delineate the same region – a narrow stripe of mesenchyme underlying the myotome. However, frontal sections reveal that the *TSHZ3* domain is broader than the thin, V-shaped *SCX* expression domain (Figs. 2J and K). *TSHZ3* and *SCX* do not overlap with the myofibers immunostained with the anti-myosin heavy chain MF-20 antibody. Surprisingly, in slightly younger embryos (HH stage 24), *TSHZ3* and *SCX* match more remarkably (Figs. 2L and M). Thus, *TSHZ3* expression in the somites follows that of *SCX* (Brent et al., 2003; Brent and Tabin, 2004), first in the same domain as *SCX* (HH stage 24), and subsequently in a broader domain (HH stage 27).

We also examined the expression in the limbs. At HH stage 23, *TSHZ3* labels the posterior and anterior part of the hindlimb bud (Fig. 2N). *TSHZ3* transcripts are similarly distributed in the forelimb bud (not shown). These expression domains are distinct from the area defined by the tendons progenitors and the forming muscles, since, at this stage, both cell types occupy the central region of the limb bud (Schweitzer et al., 2001). On sections, *TSHZ3* is separate from *TCF4*, which is intimately associated with forming limb muscles and tendons (Figs. 2O and P, Kardon et al., 2003). By HH stage 27, *TSHZ3* expression pattern becomes more complex (Fig. 2Q) and partial overlapping appears between *TSHZ3* and *SCX* (Figs. 2R and S). In older embryos, *TSHZ3* displays the most pronounced staining in the myotendinous junctions, which are also strongly marked by *SCX* (HH stage 34, Figs. 2T and U). Astonishingly, while early *TSHZ3* expression is excluded from the myotome and from the limb muscles, subsequent *TSHZ3* transcription is visible in muscles and in surrounding connective tissues at HH stage 34. Thus, *TSHZ3* marks broader domains than *SCX* at later stages.

It has been shown that *SCX* expression is induced by myotomal FGFs (Brent et al., 2003). Here we show that, as demonstrated for *SCX*, insertion of FGF8-coated beads in the trunk somites results in a faint but reproducible ectopic *TSHZ3* expression after 24 h. This upregulation occurs in cells surrounding the beads that normally do not express *TSHZ3* nor *SCX* (HH stage 26, Figs. 2V and W). Enhanced *TSHZ3* transcription is also detected upon shorter treatments with FGF8 (after 12 h, not shown).

1.4. Other places of TSHZ3 expression in the chick embryo

In the neural tube, we noticed dissimilarity between *TSHZ3* expression at brachial and lumbar positions. At

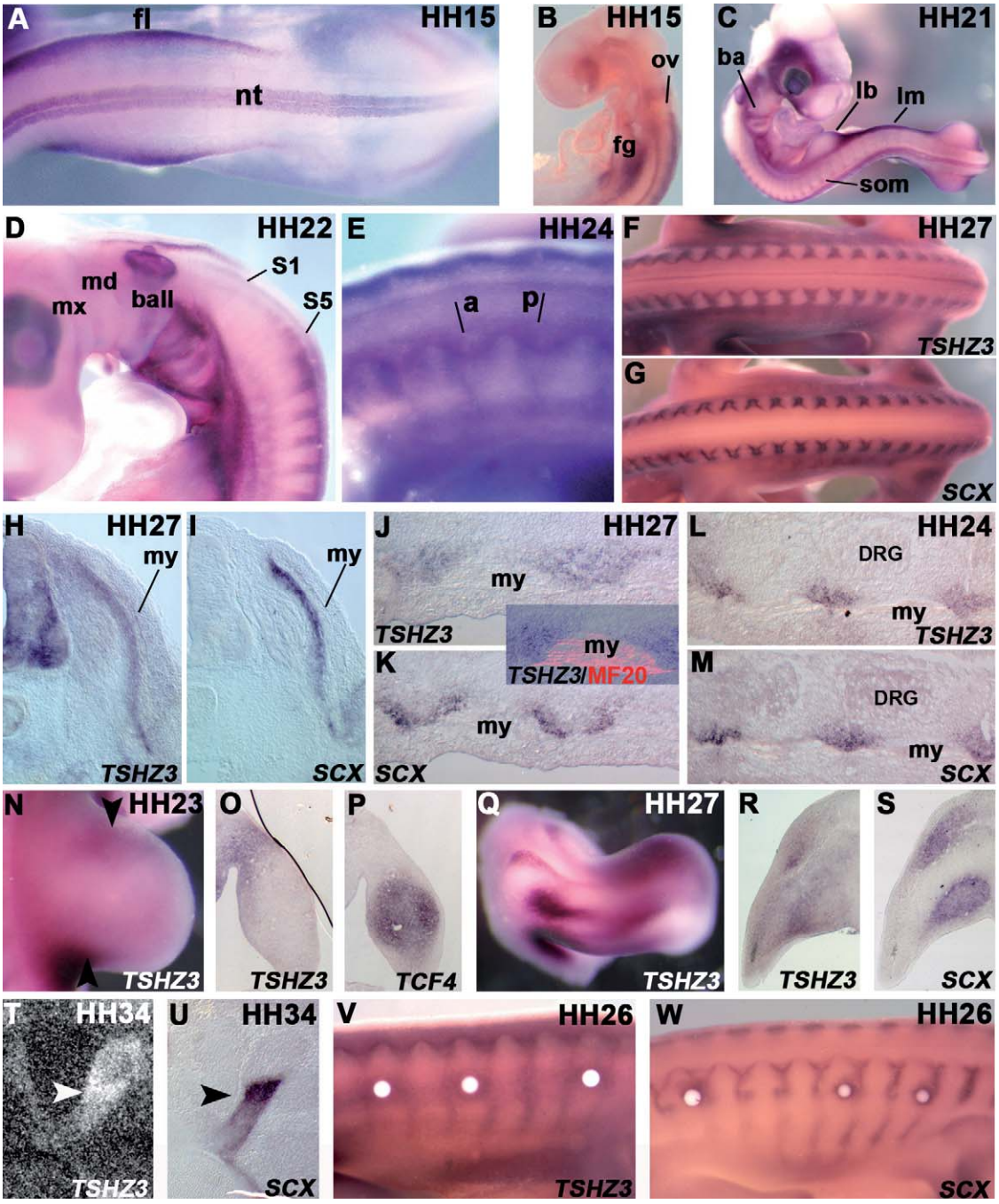

Fig. 2. Expression of *TSHZ3* in the chick embryo. (A and B) Whole mount in situ hybridizations at HH stage 15. (C) Additional sites of expression at HH stage 21. (D) Close-up of the branchial arches and the anterior somites at HH stage 22. (E) Close-up, at HH stage 24, of the brachial somites (a: anterior, p: posterior parts of the somites). (F and G) *TSHZ3* (F) and *SCX* (G) expression in the trunk at HH stage 27 (dorsal view). (H and I) Transverse adjacent sections at the brachial level hybridized with *TSHZ3* (H) and *SCX* (I) probes at HH stage 27. (J and K) Frontal sections at the same stage and same A/P axis level hybridized with *TSHZ3* (J) and *SCX* (K) probes. The inset shows *TSHZ3* and MF20. (L and M) Transverse sections at the brachial level through a HH stage 24 embryo. (N and Q) Whole mount *TSHZ3* expression in a hindlimb bud at HH stage 23 (N), and in a HH stage 27 forelimb (Q). The arrowheads point out the *TSHZ3*-expressing posterior and anterior regions. (O, P–R, and S) Parasagittal transverse sections through a HH stage 23 hindlimb (O and P) and HH stage 27 forelimb (R and S) stained for *TSHZ3* (O and R) and *TCF4* (P and S). (T and U) Section through the thigh at HH stage 34 hybridized with ^{35}S-labelled *TSHZ3* (T) probe or DIG-*SCX* (U). Arrowheads designate the robust expression in the myotendinous junction. (V and W) Up-regulated *TSHZ3* (V) and *SCX* (W) expression around FGF8-coated beads. DRG: dorsal root ganglia, fg: foregut, fl: forelimb bud, lb: limb bud, lm: lateral mesoderm, my: myotome, ov: otic vesicle, som: somite, nt: neural tube. Whole mount and frontal sections, except in (B): anterior to the left. Transverse and sagittal sections: dorsal to the top.

the brachial level of a HH stage 21 embryo, *TSHZ3* demarcates a ventral region located dorsal to the floor plate comprising motor neurons progenitors. *TSHZ3* expression is also detected in lateral regions of the spinal cord in the marginal zone (Fig. 3A, arrowhead). Later, at HH stage 34, the dorsal half of the spinal cord displays robust expres-

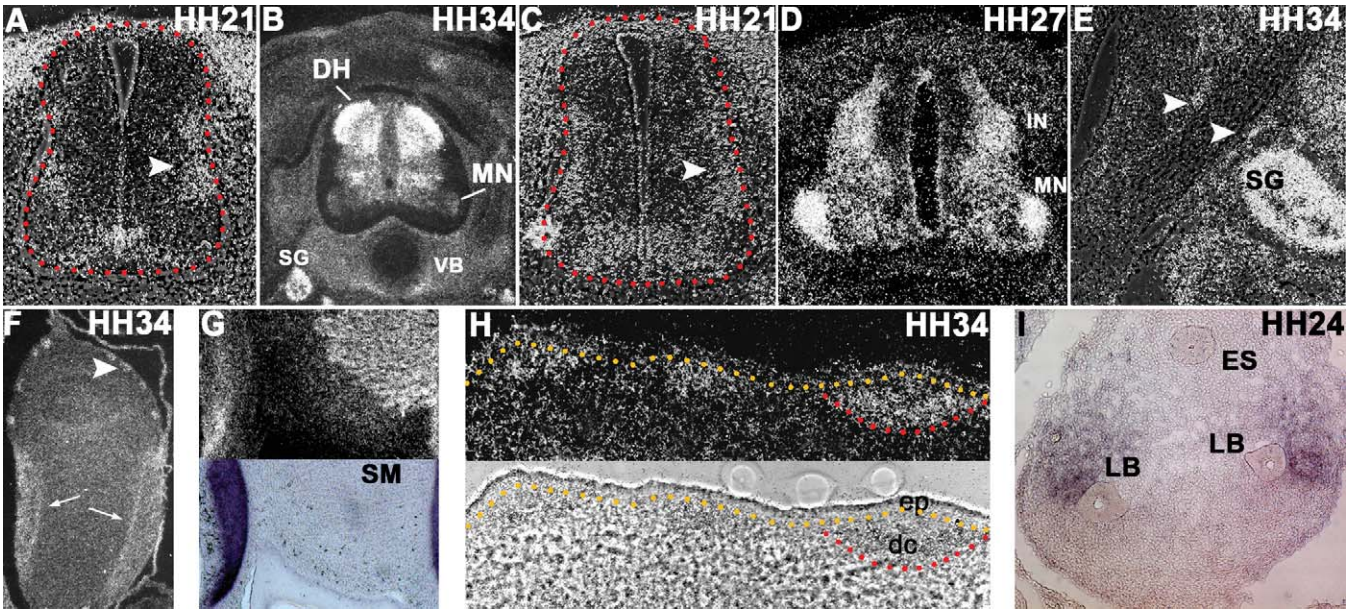

Fig. 3. Other places of *TSHZ3* expression. (A and C) ^{35}S-labelled *TSHZ3* transverse sections through the neural tube at HH stage 21 at brachial (A) and lumbar (C) level. Arrowheads point to the lateral marginal zone. Red dotted lines delineate the spinal cord. (B and D) Same as (A and C) at HH stage 34 (B) and 27 (D). (E) Axonal tract arising from the spinal cord at HH stage 34. The arrowheads indicate *TSHZ3*-expressing cells at the border. (F and G) Two sections through the HH stage 34 gizzard labelled with *TSHZ3* (F and G, top) and *SCX* (G, bottom) probes. The arrows indicate the laminar tendon and the arrowhead point to one of the forming enteric nervous systems elements (eight ganglia are observed on this section). (H) Transverse section through the skin. The ectoderm is apparent in bright field (demarcated by a yellow dotted line). The red line underlines the condensing mesenchyme (dermal condensates) beneath the ectodermal placode of the feather bud. (I) Zone of the esophagus (dorsal) and of the two lung buds (ventral). Photographs (A, C, E) are bright field/black field composite images. DH: dorsal horn, dc: dermal condensate, ep: ectodermal placode, ES: esophagus, IN: interneurons, LB: lung bud, MN: motoneurons, SG: sympathetic ganglia, SM: smooth muscle, VB: vertebral body.

sion; the *TSHZ3* positive domain covers the subventricular zone and the dorsal horn (Fig. 3B). A very weak staining is also observed in a lateral population of motor neurons. At the lumbar level of HH stage 21 embryos, *TSHZ3* exhibits expanded ventral expression compared to the brachial level (Fig. 3C). *TSHZ3* is found in the marginal zone as well (arrowhead). Later, in contrast to the brachial level, sustained expression is encountered in motor neurons (Fig. 3D). Dorsally, *TSHZ3* is prominently expressed in the mantle layer indicating expression in the alar plate interneurons. In addition to the central nervous system, *TSHZ3* is also expressed in the peripheral nervous system. Notable examples are the sympathetic ganglia (Figs. 3B and E), cells along the axonal tracts where the Schwann cells develop (Fig. 3E) and the enteric nervous system. In the latter, strong *TSHZ3* expression is observed in aggregates of cells within the outer gut mesenchyme of the gizzard constituting the ganglia of the myenteric plexus (Fig. 3F, arrowhead). In addition, consistent with *TSHZ3* expression in tendons, *TSHZ3* is found in the laminar tendon of the gizzard (compare with *SCX* in Figs. 3F and G). Smooth muscles also express *TSHZ3* (Fig. 3G, and in the intestine, not shown).

In addition, *TSHZ3* is detected in zones of condensing mesenchyme forming the cartilage (see the vertebral body encircling the notochord in Fig. 3B). *TSHZ3* expression is also detected in several tissues where epithelio-mesenchymal interactions take place, such as the feather anlagens (HH stage 34, Fig. 3H, also evident in Fig. 3B) and in the mesenchyme in the area of the lung buds (HH stage 24, Fig. 3I).

TSHZ3, like *Tshz1* and *Tshz2* in mouse, is expressed in the nervous system and in mesodermal derivatives. A noteworthy feature of *TSHZ3* is its expression in developing tendons and exclusion from the forming muscles at early stages. Later, *TSHZ3* displays enlarged expression in the myotendinous junctions, the muscles (skeletal and smooth muscles) and connective tissues. We hope this study will improve our knowledge of the *Tshz* genes expression patterns for understanding their specific and redundant functions.

2. Experimental procedures

2.1. Identification of TSHZ3 clones and phylogenetic reconstruction

In a BLAST search with the amino acid sequences of mouse *Tshz* genes we identified a chick EST clone presenting a high degree of homology with the mouse and human *Tshz3* genes (C482, kind gift of Dr. Nat Bumstead). C482 was used to identify a longest chick EST clone, ChEST257k10 (ID BU471594). This clone was used to generate *TSHZ3* riboprobe. Sequence alignments were analyzed by Neighbor-joining (NJ) (Gamma model of distances and sites pairwise deletion) with MEGA version 3.0 (Kumar et al., 2004). Confidence estimates included bootstrap analysis with 100 replicates.

2.2. Processing of the tissues

Fertilized chicken eggs were purchased from a commercial source. Eggs were routinely incubated, opened and staged according to Hamburger and Hamilton (1951). The specimens were fixed in 4% paraformaldehyde and processed for whole-mount in situ hybridization or cryopreserved in 30% sucrose and embedded in OCT (Tissue-Tek) for freezing and sectioned at 10–15 μm on the cryostat for tissue section in situ hybridization and immunodetection.

2.3. Chick TSHZ3 and SCX probes and in situ hybridizations

Whole mount and on sections in situ hybridizations were performed using digoxigenin (Boehringer)-labelled chick *TSHZ3* (DNA linearization: *Not*I, antisense RNA synthesis: T3), *Scleraxis* (*SCX*) (Schweitzer et al., 2001; DNA linearization: *Eco*RI, antisense RNA synthesis: T3), and *TCF4* (Kardon et al., 2003) riboprobes according to Henrique et al. (1995). Embryos were photographed using a Leica MZ8 dissecting microscope with a Canon D30 colour digital camera. Zeiss Axiophot2 microscope equipped with a Nikon DXM1200 Digital Camera. The automatic camera tamer software (ACT-1 Version 2.10, Nikon Corporation) was used to allow operation of the Digital Camera Control unit from a networked high-performance PC. The *SCX* cDNA is a kind gift of D. Duprez.

Radioactive in situ hybridizations with ^{35}S-labelled *TSHZ3* riboprobe on sections were performed as described in Caubit et al. (2005).

2.4. Immunohistochemistry staining procedure

Immunodetection of the myosin heavy chain was performed on cryosections of embryos previously processed for *TSHZ3* whole mount in situ hybridization. 1:20 dilution of an MF-20 hybridoma supernatant directed against the embryonicmyosin heavy chain (Developmental Studies Hybridoma Bank) and was detected by Alexa 546 fluorophor-labelled secondary antibodies (Jackson).

2.5. FGF8-soaked beads procedure

Heparin-immobilized acrylic beads (Sigma) were saturated overnight at 4 °C in a solution of 1 μg/μl of FGF8 (R&D systems) diluted in PBS 0.2% BSA. Beads were then implanted in the interlimb somites of a HH stage 23 embryos 24 h prior to dissection.

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