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Tissue-specific regulation of the number of cell division rounds by inductive cell interaction and transcription factors during ascidian embryogenesis

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ABSTRACT

Mechanisms that regulate the number of cells constituting the body have remained largely elusive. We approached this issue in the ascidian, *Halocynthia roretzi*, which develops into a tadpole larva with a small number of cells. The embryonic cells divide 11 times on average from fertilization to hatching. The number of cell division rounds varies among tissue types. For example, notochord cells divide 9 times and give rise to large postmitotic cells in the tadpole. The number of cell division rounds in partial embryos derived from tissue-precursor blastomeres isolated at the 64-cell stage also varied between tissues and coincided with their counterparts in the intact whole embryos to some extent, suggesting tissue-autonomous regulation of cell division. Manipulation of cell fates in notochord, nerve cord, muscle, and mesenchyme lineage cells by inhibition or ectopic activation of the inductive FGF signal changed the number of cell divisions according to the altered fate. Knockdown and missexpression of Brachyury (Bra), an FGF-induced notochord-specific key transcription factor for notochord differentiation, indicated that Bra is also responsible for regulation of the number of cell division rounds, suggesting that Bra activates a putative mechanism to halt cell division at a specific stage. The outcome of precocious expression of Bra suggests that the mechanism involves a putative developmental clock that is likely shared in blastomeres other than those of notochord and functions to terminate cell division at three rounds after the 64-cell stage. Precocious expression of Bra has no effect on progression of the developmental clock itself.

Introduction

The mechanisms by which embryos regulate the number of cells constituting the body are a key issue in developmental biology (Slack, 1996). Although the mechanisms that control the number of cell division rounds in specific tissues or organs have been considered important for proper embryonic development, their nature has remained unclear. In *Drosophila* embryos, it has been reported that Dacapo, a Cdk inhibitor, is involved in the exit of embryonic cells from the cell cycle (de Nooij et al., 1996; Lane et al., 1996). Similarly, the Cdk inhibitor cki-1 facilitates transient arrest of cell division in vulval precursor cells of *C. elegans* (Clayton et al., 2008), but the mechanism that determines the timing of expression or activation of Cdk inhibitors is not fully understood.

Several hypotheses have been proposed for the mechanism that terminates cell division at specific stages of embryogenesis. Embryos may have a developmental clock that measures the time elapsed from fertilization or counts the number of cell divisions and determines when the cells stop dividing. Totally distinct mechanisms are also possible. The timing of final mitosis may depend on terminal cell conditions and not on developmental history. For example, cells may stop dividing when the cell volume falls below a definitive threshold, or sense the nucleo-cytoplasmic ratio (N/C ratio), which was first proposed for the control of timing of the mid blastula transition in *Xenopus* embryos (Newport and Kirschner, 1982a,b). It has been shown that when 2-cell embryos are separated into two blastomeres, each half-embryo develops into a larva with half the normal number of cells, in both regulative eggs of echinoderms (Hörstadius, 1973; Dan-Sohkawa and Satoh, 1978) and mosaic eggs of ascidians (Yamada and Nishida, 1999). However, the results of these experiments do not distinguish between the possibilities mentioned above.

Ascidian embryos provide a good system for approaching this issue. The eggs develop into simple tadpole larvae with a relatively small number of cells: approximately 3000. This means that embryonic cells divide 11 times on average after fertilization. The number of rounds of cell division has been investigated for several major embryonic tissues. For example, muscle in the tail comprises 42 large postmitotic cells, which are the result of 9 divisions after fertilization (Nishida, 1987). Similarly, the 40 large and postmitotic cells that constitute the notochord also result from 9 divisions occurring from fertilization to

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the tadpole stage. The numbers of cell division rounds in muscle and notochord are conserved among several distantly related ascidian species with various egg sizes (Nishida and Satoh, 1983). On the other hand, there are a large number of small mesenchyme cells in the hatched larvae. Each of the tissues of an ascidian embryo seems to have a distinct mechanism that determines the number of division rounds.

In our previous study, we demonstrated that at least three kinds of mechanisms regulate the number of cell division rounds during ascidian embryogenesis. We analyzed the total numbers of cells in larvae that had developed from various kinds of egg fragments, in which the egg volume had been reduced by half or the egg pronucleus was removed (Yamada and Nishida, 1999). These mechanisms include one that is regulated by cell volume, one by the N/C ratio, and one by neither of the first two. When each tissue was analyzed individually, the cell division rounds in mesenchyme and epidermis cells appeared to be regulated by a cell volume factor. As mesenchyme cells in particular become very small after many cell divisions, it is likely that they divide until they reach a minimum cell size limit. Cell division rounds in notochord and muscle are not affected by either cell volume or N/C ratio, implying the presence of a developmental clock. These observations suggest that the mechanisms controlling cell division are tissue-specific.

In the present study, we demonstrate that the number of future cell divisions is determined at the fate restriction stage and thereafter regulated cell-autonomously. When cell fates were converted through manipulation of inductive events, the tissue-specific number of cell division rounds was also changed in accordance with the altered cell fate. A transcription factor, Bra, whose expression is promoted in the notochord by inductive FGF signaling, is involved in both cell differentiation processes and control of cell division rounds. However, precocious initiation of *Bra* expression did not affect the timing of terminal cell division in the notochord lineage.

Materials and methods

Animals and embryos

Adults of the ascidian *Halocynthia roretzi* were collected near the Asamushi Research Center for Marine Biology, Aomori, Japan, the Otsuchi International Coastal Research Center, Iwate, Japan, and the Marine Biology Center for Research and Education, Gangneung, Korea. Naturally spawned eggs were fertilized with a suspension of non-self sperm and raised in Millipore-filtered seawater containing 50 μg/ml streptomycin sulfate and 50 μg/ml kanamycin sulfate at 13 °C. The embryos develop into swimming tadpoles and hatch at 36 hours after fertilization.

Isolation of blastomeres and cell count of partial embryos

Embryos were manually devitellinated with tungsten needles prior to blastomere isolation and reared in 1% agar-coated plastic dishes filled with seawater. Blastomeres were identified and isolated from embryos at the 32- or 64-cell stage with a fine glass needle under a stereomicroscope (SZX-12; Olympus). Isolated blastomeres were cultured separately as partial embryos in agar-coated dishes, and then the partial embryos were fixed at the hatching stage (Kobayashi et al., 2003). For cell counts of the resulting partial embryos, samples were fixed with 70% ethanol for 2 minutes at room temperature. After washes with PBS containing 0.1% Tween 20, they were transferred into VECTASHIELD mounting medium for fluorescence with DAPI (Vector Laboratory) to stain nuclear DNA. The specimens were then transferred to 80% glycerol and mounted. They were gently squashed on glass slides by compressing them with cover slips until the constituent cells spread into a monolayer (modified from Yamada and Nishida, 1999).

Treatment with FGF and the signaling inhibitor

Blastomeres isolated at the beginning of the 32-cell stage were transferred to seawater containing 0.1% bovine serum albumin (BSA; Sigma) and 10 ng/ml recombinant human bFGF protein (Sigma) (Nakatani et al., 1996; Kim et al., 2000). We reconfirmed that this concentration of FGF was sufficiently effective for inducing notochord and suppressing muscle formation. As controls, blastomeres were treated with BSA in seawater. To inhibit the FGF signaling pathway, embryos were treated with 2 μM U0126 (Promega) after the 16-cell stage until hatching. U0126 is an MEK inhibitor that inhibits both the activation of MEK by Raf and the activation of ERK by MEK (Favata et al., 1998). U0126 was dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mM and stored at −20 °C. Stock solutions were diluted with seawater to the final concentration just before use (Kim and Nishida, 2001). We reconfirmed the effects of this drug on notochord and mesenchyme induction. As controls, blastomeres were treated with DMSO in seawater.

Morpholino antisense oligos and mRNA

To suppress Hr-Bra function, we injected a 25-mer morpholino oligonucleotide (MO; Gene Tools), covering the first methionine, into fertilized eggs. The nucleotide sequence was 5′-TTGTAATTGACA-TAATTCCTTGTAC-3′ (Matsumoto et al., 2007). As a control, a standard MO, 5′-CCTCTTACCTCAGTTACAATTTATA-3′ was injected. About 500–1000 pg of each MO was injected into the fertilized eggs. mRNAs were transcribed from each of the plasmids with a mMessage mMachine kit (Ambion). About 10–25 pg of mRNA was injected. Differentiation of notochord cells was monitored by immunostaining with the Not-1 monoclonal antibody (Nishikata and Satoh, 1990; Nakatani and Nishida, 1994). Indirect immunofluorescence staining was carried out by standard methods using a TSA fluorescein system (Perkin-Elmer Life Sciences). To inhibit cell division, cleavage was permanently arrested with 2.5 μg/ml cytochalasin B (Sigma) at the 64-cell stage (Kobayashi et al., 2003). Whole-mount in situ hybridization was basically performed according to Nakatani et al. (1996). Specimens were hybridized with the digoxigenin (DIG)-labeled *Hr-Bra* probe.

Plasmid construction for mRNA synthesis

To synthesize mRNA used for the over/mis-expression experiment, the *Hr-Bra* open reading frame was cloned into pBluescript-RN3, and the mRNA was transcribed from the plasmid. To synthesize mRNA used for the rescue experiments, we cloned into pBluescript-RN3 a *Hr-Bra* cDNA with synonymous nucleotide changes in the MO target site to prevent hybridization with Bra MO. The following upstream PCR primer was used to generate the altered cDNA: 5′-GAGAATTCaatag-tacgtagATGTCgATcACgAATAATATGGAGTCGCCATCTGACAGC-3′, the underlined bases corresponding to the translation initiation site, and the lower case bases representing those altered from the endogenous Bra sequence. To visualize Hr-Bra protein translation and distribution, cDNA of mCherry was inserted in-frame to the 5′ end of the cDNA of the Hr-Bra protein-coding region in the pBluescript-RN3 vector, and it was used for mRNA synthesis.

mRNA encoding eGFP fused to a nuclear localization signal (NLS-eGFP) was transcribed from the pBluescript-RN3:NLS-eGFP in which a NLS sequence was inserted between the *Eco*RI and *Bgl*II sites. To visualize the descendant cells of the tissue precursors, mRNA encoding histone H2B protein (from the appendicularian *Oikopleura dioica*) fused with mCherry fluorescent protein was synthesized from the plasmid pSD64TF carrying the ORF of the fusion protein (kindly provided by Dr. A. Nishino, Osaka University) and injected into tissue precursors at the 32- and 64-cell stages.