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ORIGINAL ARTICLE

Modification of secondary head-forming activity of microinjected Δβ-catenin mRNA by co-injected spermine and spermidine in *Xenopus* early embryos

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Abstract Polyamines are essential for cell growth and differentiation. In *Xenopus* early embryos, per embryo level of spermine is extremely low as compared with that of spermidine. To disclose the possible function of polyamines in *Xenopus* early embryos, we tested the effect of co-injection of spermine and spermidine on the functioning of exogenously microinjected in vitro-synthesized, Δβ-catenin mRNA which is known to induce a secondary head after being microinjected into a ventral vegetal blastomere in 8-celled *Xenopus* embryos. Microinjection of Δβ-catenin mRNA in fact induced a secondary axis with a secondary head, and co-injection of spermine or spermidine suppresses induction of the secondary head and secondary axis without drastic effects like induction of immediate cell death or execution of apoptosis at blastula stage. The inhibitory effects were dosage dependent, and at lower doses the inhibition was mainly on secondary head formation rather than on secondary axis formation. We performed similar experiments using GFP mRNA and confirmed that expression of GFP mRNA was also suppressed by co-injection of spermine. We mixed Δβ-catenin mRNA with different amounts of spermine and performed electrophoresis on agarose gels, with a finding that the prior mixing greatly suppressed mRNA migration. These results suggest that an excess amount of spermine as well as spermidine exerts inhibitory effects on mRNA translation, and that the inhibition may be due to direct binding of polyamines to mRNA and a reduction of negative charges on mRNA molecules that might also induce the formation of cross link-like networks among mRNAs.

Keywords Δβ-Catenin mRNA · Secondary head formation · Secondary axis formation · *Xenopus* 8-celled embryos · Microinjection · Polyamines · Spermine · Spermidine · GFP mRNA · Gel electrophoretic mobility

Abbreviations

MBT	Midblastula transition
SAMDC	*S*-adenosylmethionine decarboxylase
ODC	Ornithine decarboxylase
SAM	*S*-adenosylmethionine
GFP mRNA	Green fluorescent protein mRNA
FITC-dextran	Fluorescein isothiocyanate-dextran
PCR	Polymerase chain reaction
MBS	Modified Barth's solution

Introduction

Natural polyamines (putrescine, spermidine and spermine) which are essential for cellular proliferation occur ubiquitously in both prokaryotic and eukaryotic cells, and

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pathways of polyamine synthesis are strictly regulated not only by the physiological conditions of the cell but also by the cellular levels of polyamines themselves (Igarashi and Kashiwagi 2010). Prokaryotic *Escherichia coli* cells have a high content of putrescine and spermidine, with no detectable amount of spermine, for the lack of enzyme necessary for conversion of spermidine into spermine (Davis et al. 1992). Eukaryotic cells, by contrast, have a relatively high content of spermidine and spermine (Guirard and Snell 1964). In the regulation of these polyamines, two essential enzymes, spermidine synthase and spermine synthase, which synthesize spermidine and spermine, respectively, from putrescine and spermidine are constitutively synthesized within cells (Heby and Persson 1990), and two key enzymes in polyamine metabolism, ornithine decaroboxylase (ODC) and *S*-adenosylmethionine decarboxylase (SAMDC) are rate-limiting enzymes in polyamine biosynthesis (Igarashi and Kashiwagi 2010). ODC has a very short turnover time and its activity increases rapidly and sensitively, responding to a variety of growth stimuli, thereby increasing the level of putrescine and other polyamines (Davis et al. 1992). On the other hand, SAMDC provides an aminopropyl group to putrescine and spermidine to form spermidine and spermine, respectively, by the function of spermidine synthase and spermine synthase. SAMDC is synthesized as a single polypeptide precursor and undergoes an internal cleavage to give rise to active molecules, and efficiency of both these processes is also under the control of putrescine (Pajunen et al. 1988).

Polyamines are also essential for cellular differentiation in eukaryotic cells (Heby 1981). In *Xenopus laevis*, changes in ODC activity and amounts of polyamines have been investigated during oogenesis (Osborne et al. 1989), oocyte maturation (Sunkara et al. 1981; Younglai et al. 1980; Bassez et al. 1990), and early embryogenesis (Osborne et al. 1993; Rosander et al. 1995; Shinga et al. 1996). Using *Xenopus* ODC cDNA as a probe, it has also been shown that the level of ODC mRNA is relatively high at the beginning of oogenesis, decreases once in the later phases of oogenesis, and in embryogenesis increases from the early gastrula stage (Osborne et al. 1991). These results imply that zygotic expression of ODC gene starts shortly after the midblastula transition (MBT), which is accompanied by the general activation of zygotic gene activity (Newport and Kirschner 1982; Nakakura et al. 1987; Shiokawa et al. 1994). We focused our efforts on the other key enzyme, SAMDC, and cloned the cDNA of this enzyme, determining the developmental changes in its mRNA level during early embryonic stages (Shinga et al. 1996). Also, we determined the changing levels of putrescine, spermidine, and spermine during oogenesis, oocyte maturation, and embryogenesis to understand more about the function of polyamines in amphibian embryogenesis, and found that in *Xenopus* early embryos the level of spermine is extremely low: the approximate amount of putrescine, spermidine, or spermine in a *Xenopus* cleavage stage embryo is approximately 2,000–3,000 pmol, 500–1,000 pmol, or ca. 20 pmol or less, respectively (Shinga et al. 1996). We showed that the sequence of *Xenopus* SAMDC is evolutionally quite conserved and its mRNA is expressed following a unique and characteristic pattern during oogenesis, oocyte maturation, and embryogenesis, explaining at least partly the maintenance of the characteristic polyamine composition in *Xenopus* early embryogenesis (Shinga et al. 1996). In addition, we overexpressed the SAMDC enzyme in early embryos and found that the overexpression results in exhaustion of its substrate S-adenosylmethionine (SAM), thereby inducing execution of the maternal program of apoptosis at around MBT stage (Shibata et al. 1998; Kai et al. 2000; Kai et al. 2003; Shiokawa et al. 2008; Shiokawa et al. 2000; Shiokawa et al. 2005; Shiokawa et al. 2010).

On the other hand, in the research on early embryogenesis searching for mechanisms of body plan establishment, it has long been known that early embryonic development is driven by various maternal stockpiles, among which are mRNAs and proteins for growth factors and various transcriptional regulators (Heasman 2006). In *Xenopus laevis*, fertilized eggs undergo very characteristic cell cycles: during the 90 min of the first cell cycle, cortical cytoplasm moves towards the sperm entrance point and this determines both the future dorsal and ventral sides. The next eleven division cycles proceed at about 30 min-intervals with no apparent gap phases (G_1 and G_2), altogether after 12 rounds of cell cycles, the embryo forms a ball of ca. 4,000 cells, in which Spemann organizer appears and invaginates into the blastocoel to form ectodermal, mesodermal and endodermal cells. As a result of this so-called gastrulation movement, the future dorso-ventral and head-to-tail axes become visible due to the formation of blastopore, and then the embryo develops into tadpole with head, trunk, and tail structures (Heasman 2006). Such embryonic patterning is known to be controlled at the first step after the fertilization by the relocation of the vegetally localized dorsal determinants which moves along with the cortical rotation (Scharf and Gerhart 1980; Holwill et al. 1987). The key molecules here are a component of the canonical Wnt signaling pathway, Wnt11 mRNA (Kofron et al. 2001) and β-catenin, the transcriptional co-activator of the Wnt target genes whose activation triggers the pathway to form the embryo axis and head structure (Schneider et al. 1996; Yost et al. 1996). The maternal transcription factor, β-catenin, is originally located in the vegetal most region of the unfertilized egg, and during the

first cell cycle, this is moved to the opposite side of the sperm entrance point along with the cortical rotation, and the new position of this β-catenin localization becomes the center for the establishment of the future embryonic dorso-ventral axis (Vincent and Gerhart 1987). In doing so, β-catenin is transferred to the nucleus and modulates the Wnt signal pathway (Schneider et al. 1996; Brannon et al. 1997; Tao et al. 2005). Previous experiments have shown that microinjection of β-catenin mRNA into the future ventral vegetal blastomere induces a strong secondary axis and a complete secondary head (Yost et al. 1996; Schohl and Fagotto 2002).

In the present experiments, with an expectation that polyamines might be involved as essential modulators, also in these morphogenetic molecular interactions, we studied the possible effects of spermine and spermidine on the secondary head-forming activity, in these morphogenetic events in *Xenopus* embryos, making use of in vitro-synthesized, naked, mRNA for the β-catenin. In this experiment, since spermine is the least component of the three well-known polyamines (Shinga et al. 1996), we first performed experiments to microinject 25–250 pmol of spermine into 8-celled *Xenopus* embryos, but later experiments with spermidine were also performed. The results obtained showed that co-injection of polyamines almost completely suppresses the formation of secondary head as well as the secondary axis to be induced by β-catenin, and the reason for the inhibition was probably the inhibition of translation of the injected mRNA, probably due to the too much binding of polyamines to the injected mRNA, which resulted in the depletion of negative charges on the nucleotides and formation of cross link-like molecular aggregation among the injected mRNA molecules.

Materials and methods

Plasmids and in vitro translation

A Δβ-catenin cDNA, in which the first 47 amino acids at N-terminus were deleted and a new initiation codon was inserted, was isolated from *Xenopus* embryo mRNA by use of reverse transcriptase and polymerase chain reaction (PCR), using oligonucleotides to insert a new initiation codon as described by Yost et al. (1996). The amplified cDNA was cloned into the CS2 + vector and used as in Kai et al. (2003). The Δβ-catenin cDNA was sequenced using the Sequenase Kit (United States Biochemicals, Cleveland, OH, U.S.A.) or the BcaBEST dideoxy sequencing Kit (Takara Shuzo, Japan), and its identity as the Δβ-catenin was confirmed (Yost et al. 1996). In this cDNA, the ubiquitination site has been eliminated, and therefore, this deleted cDNA is expected to provide Δβ-catenin protein which escapes from ubiquitination followed by degradation by proteosomes, thereby surviving longer within the cell than the wild-type β-catenin (Yost et al. 1996). GFP mRNA was obtained from pbGFP/RN3P as described previously by Zernicka-Goetz et al. (1996) and by Kai et al. (2003). mRNAs for type IIA activin receptor and its truncated form, in which the intracellular serine-threonine domain was deleted, were produced according to Kondo et al. (1991) and used as references in mRNA gel electrophoresis performed, as described in our previous paper (Kajita et al. 2000).

All the DNA constructs were linearized at the PvuII or NotI site, and transcribed in vitro with SP6 RNA polymerase (Ambion, Austin, TX, USA) in the presence of a cap analog (New England Biolabs, Beverly, MA, USA). All RNA preparations were heated once at 60°C for 15 min for RNA denaturation. All the microinjected mRNAs were confirmed to have expected sizes before being subjected to gel electrophoresis, performed using 1.2% gels under denaturation conditions (Kajita et al. 2000).

Microinjection of mRNA

Unfertilized eggs of *Xenopus laevis* were manually ovulated from gravid females which had been injected with a human chorionic gonadotropic hormone (Gonatoropin; Aska Pharmaceutical Co., Japan). Eggs were artificially fertilized, and dejellied in 2% sodium deoxycholate, pH 7.6.

mRNA was dissolved in sterile distilled water together with 20 ng of FITC-dextran as a tracer, which was confirmed not to be toxic to embryos in our preliminary experiments (Mishina et al., unpublished observation). The mixture of mRNA and FITC-dextran was microinjected into 8-celled embryos together with or without polyamines as specified in each experiment. The amount of the whole mixture was ca. 30 nl or less throughout experiments. Microinjection was performed in 1× modified Barth's solution (MBS), containing 50 units/ml of penicillin and 50 μg/ml of streptomycin (Fu et al. 1989). Injected embryos were kept in 1× MBS until stage 7 (Nieuwkoop and Faber 1967), then transferred into either 0.1× or 1× Steinberg's solution as indicated in each experiment. Only sibling embryos were used in each experiment, and embryos were cultured at 21–23°C in the presence of 50 units/ml of penicillin and 50 μg/ml of streptomycin (Fu et al. 1989).

In all the experiments, successful injection of the intended amount of mRNA-containing mixture into the intended blastomere was confirmed by the transfer of fluorescence in each experiment after the injection.

Results

Induction of secondary head and secondary axis formation by microinjected Δβ-catenin mRNA and suppression of the effects of Δβ-catenin mRNA by co-injection of spermine

We microinjected 100 pg/egg of Δβ-catenin mRNA plus 20 ng of FITC-dextran as a tracer into a ventral vegetal blastomere of *Xenopus* 8-celled embryos. Control embryos which received only distilled water that was used for mRNA dissolution developed normally. As shown in Fig. 1, Δβ-catenin mRNA-injected embryos formed twin-headed and double-axed swimming tadpoles at 58 h (Fig. 1, top). At 120 h after mRNA microinjection, swimming tadpoles with well-developed secondary head with two eyes developed (Fig. 1, bottom). In this experiment, out of 11 embryos which were injected with the mRNA, 7 (64%) formed such typical two-headed tadpoles, 3 embryos (27%) produced secondary head-less double axis, and 1 embryo (9%) was an abnormal embryo of smaller size with single head and single axis.

Since Δβ-catenin mRNA injected here induced secondary head formation as expected from literature (McMahon and Moon 1989; Brannon et al. 1997), we used the Δβ-catenin mRNA to test the effect of spermine on mRNA functioning within *Xenopus* embryonic cells. Here, we tested a large amount of spermine (250 pmol) by co-injecting it with 80 pg of Δβ-catenin mRNA plus 20 ng of FITV-dextran. Under these conditions, co-injection of spermine completely suppressed secondary head formation. In the right panels in Fig. 2, a set of typical embryos with a secondary head (top) and the embryo whose secondary head formation due to Δβ-catenin mRNA injection was suppressed completely are shown (bottom). In the left graph of Fig. 2, it is apparent that while as much as 80% of embryos formed duplicated head in embryos that received Δβ-catenin mRNA only (experiment 2), no embryos produced secondary head in embryos co-injected with mRNA and 250 pmol of spermine (experiment 3), although as much as 40% of the spermine-co-injected embryos stopped development and died. We checked the pH of the mixture of 80 pg of Δβ-catenin mRNA and 250 pmol of spermidine, but the pH was not greatly deviated from its normal value (pH 6.2). Probably 40% of embryo death was due to the toxic effects of this high dosage of spermine, since Osborne et al. (1993) reported that 250 pmol of spermidine injected into *Xenopus* fertilized eggs immediately induced developmental arrest. Since the basicity of spermine is higher than that of spermidine, yet most of the 8-celled embryos that received 250 pmol of spermine did not stop development immediately and 60% of the total spermine-injected embryos survived (Fig. 2, experiment 3), we assume that co-injected Δβ-catenin mRNA might have reduced the toxicity of spermine by trapping spermine to the mRNA.

We then reduced the amount of spermine to 50 pmol and performed the same experiment as in Fig. 2. As shown in Fig. 3, at this lowered dose of spermine, the percentage of secondary head formation which was as high as 90% in

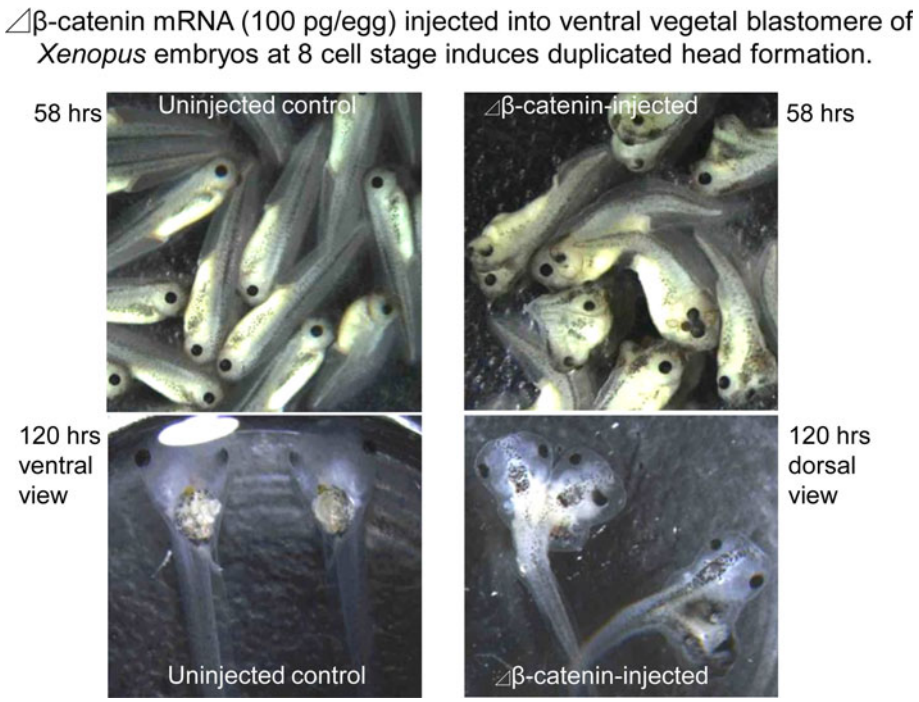

Fig. 1 Induction of the formation of secondary head and secondary axis in Δβ-catenin mRNA-microinjected embryos. One hundred pg/egg of Δβ-catenin mRNA was microinjected into a ventral vegetal blastomere in 8-celled embryos. Embryos were filmed at 58 h (**a**, **b**) and at 120 h (**c**, **d**) after injection. At 144 h after the injection, the number of embryos with a secondary head and a secondary axis was 64% of the total injected embryos (11 embryos) and the number of embryos with only secondary axis was 27%. In 52 control embryos neither secondary head nor secondary axis was formed

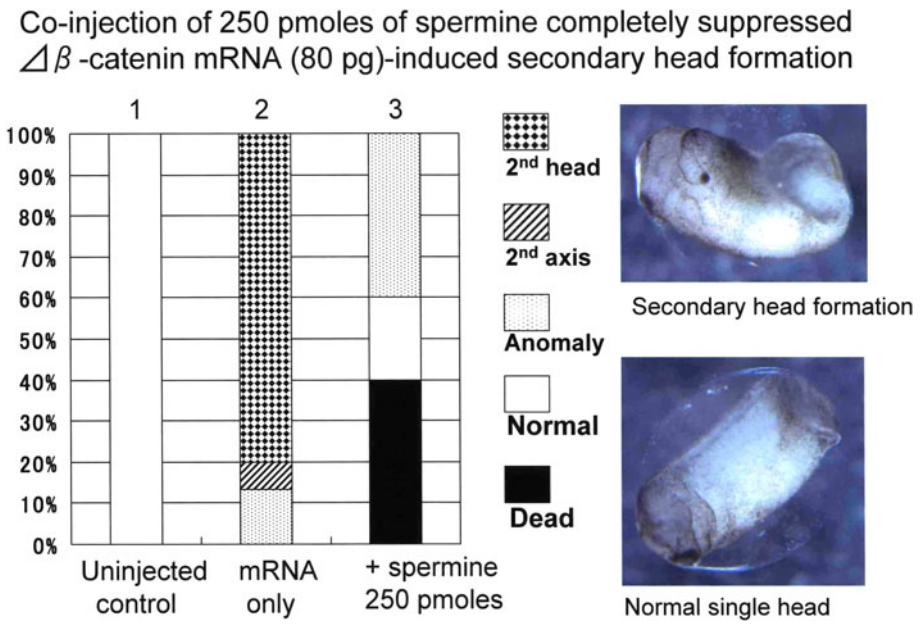

Fig. 2 Suppression of the induction of secondary head formation by co-injection of 250 pmol of spermine. Δ*β*-Catenin mRNA (80 pg) was co-injected with 250 pmol of spermine and 20 ng of FITC-dextran. Embryos were examined 144 h after the injection and percentage composition of different phenotypes of embryos is shown in the *left graph*. Number of embryos used for the experiment 1, 2, and 3 was 31, 15, and 10, respectively. Out of 15 embryos that were injected with Δ*β*-catenin mRNA (80 pg) alone, 80% formed duplicated head. However, such secondary head formation took place in none of the embryos injected with Δ*β*-catenin mRNA (80 pg) together with 250 pmol of spermine, and 20% of the embryos became normal tadpoles, although in this case 40% of the total injected embryos became abnormal tailbud embryos and eventually died. In the *right panel*, a typical double-headed embryo formed (*top*) after injection of Δ*β*-catenin mRNA (80 pg) alone, and a normal embryo filmed at 24 h after co-injection of Δ*β*-Catenin mRNA (80 pg), 250 pmol of spermine, and 20 ng of FITC-dextran are shown

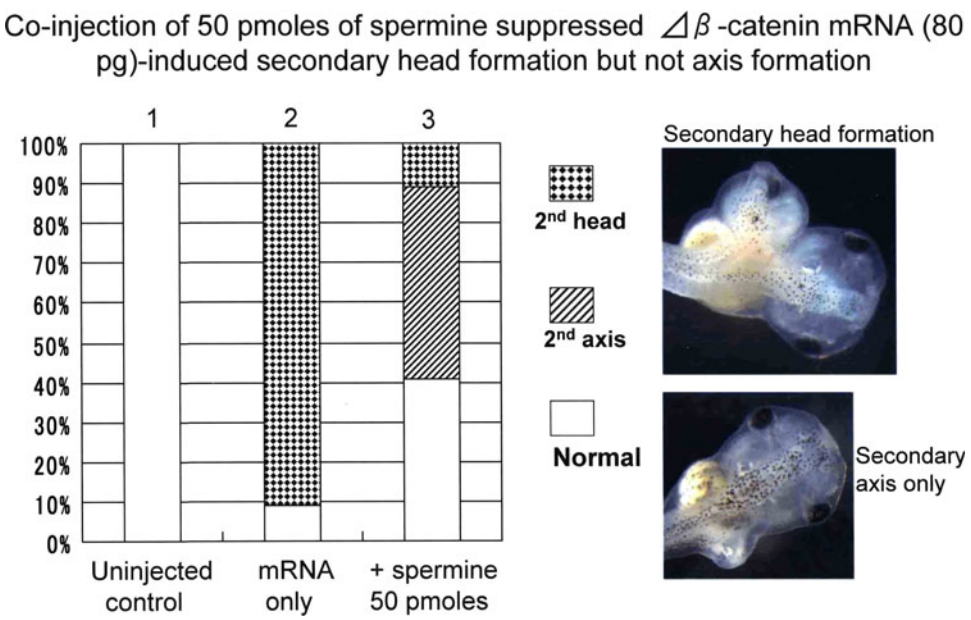

Fig. 3 Suppression of the induction of secondary head formation by co-injection of 50 pmol of spermine. Δ*β*-Catenin mRNA (80 pg) was co-injected with 50 pmol of spermine and 20 ng of FITC-dextran. Embryos were examined 144 h after the injection and percentage composition of different phenotypes of embryos is shown in the graph on the left side. Number of embryos used for the experiment 1, 2, and 3 was 29, 11, and 27, respectively. Out of 11 embryos that were injected with Δ*β*-Catenin mRNA (80 pg) alone, 90% formed duplicated head. In 27 embryos injected with Δ*β*-catenin mRNA (80 pg) together with 50 pmol of spermine only 10% formed secondary axis and 50% of embryos formed only duplicated axes but there was no head structure formation

the embryos that received only Δβ-catenin mRNA was reduced to a value as low as 10% in the embryos co-injected with Δβ-catenin mRNA (80 pg) and 50 pmol of spermine (experiment 3). Interestingly, in this case, as much as 40% of the spermine-co-injected embryos were normal and, furthermore, the rest 50% of the co-injected embryos only had a secondary axis without a secondary head. In the right panel of Fig. 3, a set of the embryos with a typical secondary head injected by Δβ-catenin mRNA alone (top) and a spermine-co-injected embryo with a typical secondary axis without a head (bottom) are shown. These results indicate that 50 pmol of spermine greatly suppressed the twin head-forming effect of Δβ-catenin mRNA (80 pg), without exerting appreciable toxic effects on the development (there were no dead embryos). The large increase in the percentage of embryos which formed only double axes may suggest that polyamines suppress the process of head formation more strongly than the process of secondary axis formation, and this seems to be reasonable because during development, head formation takes place later than the axis formation.

Throughout the experiments, FITC-dextran was injected together with Δβ-catenin mRNA and spermine, and this dye serves not only to confirm the site of the injection but also as a cell lineage tracer. In Fig. 4, we show tadpoles filmed in the UV light in the dark: one with a second head induced by the injection of Δβ-catenin mRNA alone (top), and two embryos which were co-injected with Δβ-catenin mRNA and spermine and formed only a secondary axis without a head (middle and bottom). It is apparent here that the secondary head and secondary axis are both marked by FITC-dextran, indicating that these structures were induced by the injected Δβ-catenin mRNA.

Since a *Xenopus* embryo at early stages contains only a low level of spermine (20 pmol or less) (Shinga et al. 1996), we then tested the effect of the low level of spermine under the same conditions as above. In this experiment, we used 150 pg of Δβ-catenin mRNA in the control embryos. As shown in Fig. 5, embryos that received only Δβ-catenin mRNA-induced secondary head in ca. 70% of injected embryos and the rest 30% of the embryos formed only a secondary axis (without a secondary head). In embryos co-injected with 25 pmol of spermine, ca. 25% of embryos became normal and percentage of both two-headed embryos and embryos with only double axis were reduced slightly but significantly (respectively to 50 and 25%). Thus, even this low dose of spermine did reduce the embryos, though slightly, from the twin-head forming effect of Δβ-catenin.

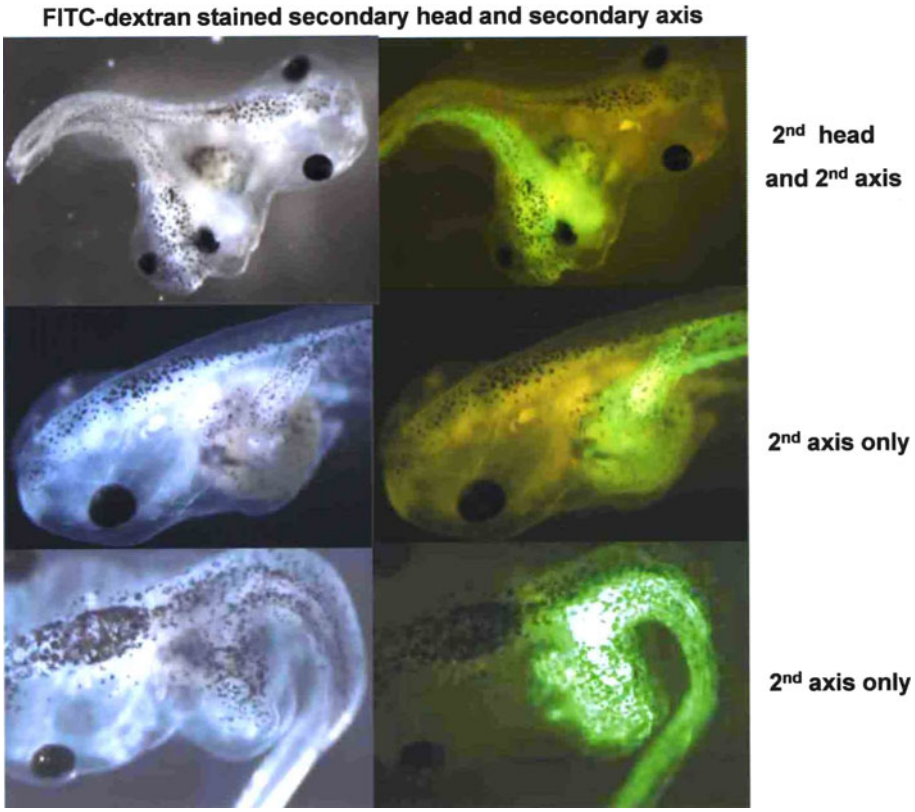

Fig. 4 A typical double-headed embryo and two embryos with a typical double axis but without a secondary head. The *top panel* indicates a tadpole with double axes and two heads, and the *middle* and *bottom panels* indicate two tadpoles with two axes but without a secondary head. *Left panels* are filmed in the day light, whereas the *right panels* indicate tadpoles filmed with a UV light. It is apparent here that only the induced structure has FITC-dextran, indicating that the induced structures are formed by the injected Δβ-catenin mRNA

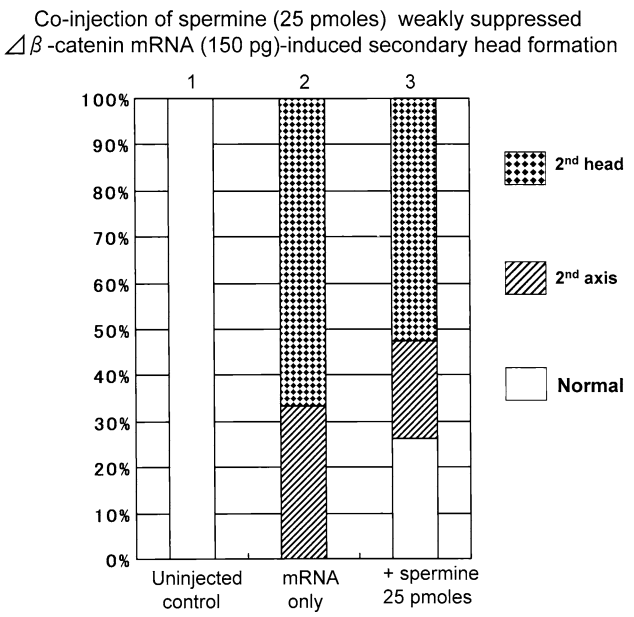

Fig. 5 Weak but significant suppression of the secondary head formation by co-injection of a low dose (25 pmol) of spermine. Δβ-Catenin mRNA (150 pg) was co-injected with 25 pmol of spermine and 20 ng of FITC-dextran. Embryos were examined 144 h after the injection and percentage composition of different phenotypes of embryos is shown in the graph. Number of embryos used for the experiment 1, 2, and 3 was 26, 9, and 19, respectively. Out of 9 embryos that were injected with Δβ-catenin mRNA (80 pg) alone, ca. 70% formed duplicated head and ca. 30% formed embryos with a secondary axis without a head. In 19 embryos injected with Δβ-catenin mRNA (80 pg) together with 25 pmol of spermine, 53% of the embryos formed a secondary head and 21% of embryos formed a secondary axis without a secondary head, and the remaining 26% of the embryos became normal. Thus, even under these conditions of low dosage of spermine, the double head-forming activity of Δβ-catenin mRNA was slightly but significantly suppressed

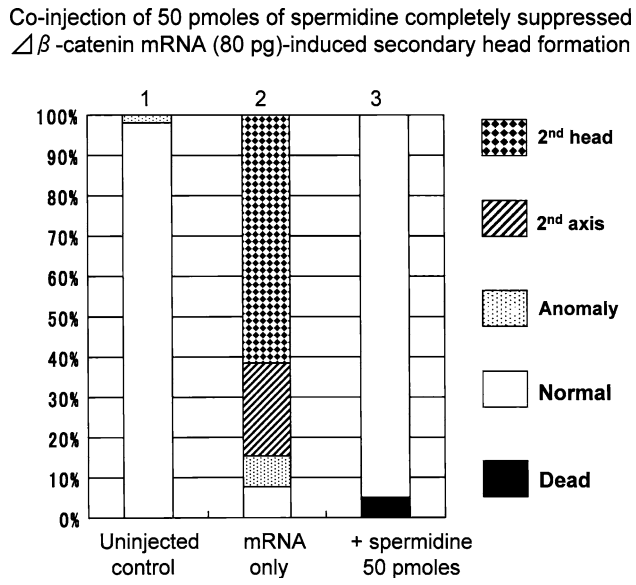

Fig. 6 Suppression of the induction of secondary head formation by co-injection of 50 pmol of spermidine. Δβ-Catenin mRNA (80 pg) was co-injected with 50 pmol of spermidine and 20 ng of FITC-dextran. Embryos were examined 144 h after the injection and percentage composition of different phenotypes of embryos is shown in the graph. Number of embryos used for the experiment 1, 2, and 3 was 38, 13, 19, respectively. Out of 13 embryos that were injected with Δβ-Catenin mRNA (80 pg) alone, ca. 60% formed a duplicated head and ca. 25% formed embryos with a double axis without a head. In 19 embryos injected with Δβ-catenin mRNA (80 pg) together with 50 pmol of spermidine, 95% developed as normal embryos and the remaining 5% died. Thus, not only spermine but also spermidine suppresses the activity of Δβ-catenin mRNA

Suppression of Δβ-catenin mRNA-induced secondary head and secondary axis formation by co-injected spermidine

We next tested if spermidine also has a similar suppressing effect on the secondary head forming activity of Δβ-catenin mRNA. In this experiment, we injected 80 pg of Δβ-catenin mRNA alone (experiment 2) or co-injected Δβ-catenin mRNA (80 pg) with 50 pmol of spermidine into a ventral vegetal blastomere of 8-celled embryo. In embryos into which only Δβ-catenin mRNA was injected, a significant number of injected embryos (60%) formed a secondary head and ca.25% of the injected embryos formed a secondary axis without a head (Fig. 6, experiment 2). In the embryos co-injected with 50 pmol of spermidine, as many as 95% of the embryos develop into normal embryos with a single head and a single axis. These results clearly show that spermidine just as spermine is effective in inhibiting the functioning of Δβ-catenin mRNA.

Suppression of expression of fluorescence from microinjected GFP mRNA by co-injected spermine

We tested if co-injected spermine inhibits the expression of mRNA other than Δβ-catenin mRNA. We selected GFP mRNA (800 pg) as the test mRNA and injected here GFP mRNA into uncleaved fertilized eggs instead of the 8-celled embryos. The GFP mRNA injected alone into uncleaved fertilized eggs was expressed extensively as can be seen by the appearance of fluorescence at blastula stage and at tailbud stage (Fig. 7). The injection of as much as 800 pg of GFP mRNA together with 400 pmol of spermine turned out to be considerably toxic in this experiment, but a significant number of embryos developed beyond tailbud stage as shown in Fig. 7. In such spermine-co-injected embryos, the extent of expression of GFP signal was very weak as compared with that in embryos injected with GFP mRNA alone both at late blastula and muscular-response stages (Fig. 7). These results indicate that the activity of spermine to suppress the function of co-injected mRNA also holds true in this different system, in which mRNA

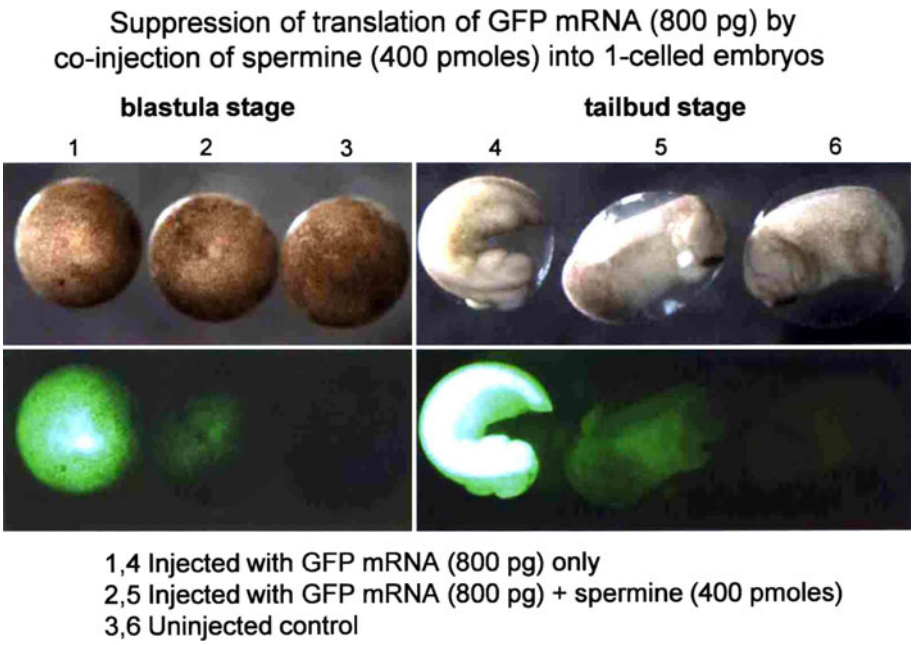

Fig. 7 Suppression of the induction of fluorescence from injected GFP mRNA by co-injection of spermine. GFP mRNA (800 pg) was either injected alone or injected with 400 pmol of spermine into the uncleaved fertilized eggs. Embryos were filmed at blastula (*left panel*) and tailbud (*right panel*) stages. *Upper panel* was filmed in the day light and the *lower panel* was filmed in a UV light. It is apparent at both stages that expression of GFP fluorescence in the co-injected embryos was very weak as compared with that in embryos injected with GFP mRNA alone

was replaced from Δ*β*-catenin mRNA to GFP mRNA and the stage of the injection was shifted to the 1-celled stage.

Changes in gel electrophoretic mobility of Δ*β*-catenin mRNA after being mixed with spermine

Since RNAs are acidic substances and polyamines are basic substances, and furthermore both Δ*β*-catenin mRNA and GFP mRNA are in vitro-transcribed, and therefore, naked as opposed to protein-coated RNA, we expected direct interactions between these two kinds of molecules when they became close to each other. To test this possibility, we first mixed Δ*β*-catenin mRNA (200 ng) and spermine (125 nmol) in a molar ratio which was comparable to that in the above microinjection experiments (e.g. Fig. 3), and electrophoresed the mixture on an agarose gel (Fig. 8). Type IIA activin receptor mRNA which was cloned in our laboratory (Kondo et al. 1991) was also electrophoresed as a size marker (coding for 514 amino acids and 2303 nucleotides long). It is clearly shown here that while Δ*β*-catenin mRNA alone migrated at the position slightly slower than activin receptor mRNA in the gel, Δ*β*-catenin mRNA mixed with spermine before or after RNA denaturation did not migrate and remained almost at the starting gel slot. This indicated that the mixing of mRNA with spermine inhibited mRNA migration.

We repeated the gel electrophoresis using a combination of Δ*β*-catenin mRNA (200 ng) with 1/10th amount of spermine (12.5 nmol), which has been shown to inhibit Δ*β*-catenin mRNA-induced secondary head formation only slightly (Fig. 5). In this experiment, Δ*β*-catenin mRNA migrated just as the Δ*β*-catenin mRNA which was not mixed with spermine (Fig. 9). Therefore, at the dose used, spermine did not stop migration of Δ*β*-catenin mRNA. This result is correlated to the weak effect of this dose of spermine in the suppression of the twin head-forming activity of Δ*β*-catenin mRNA as shown in the experiment in Fig. 5.

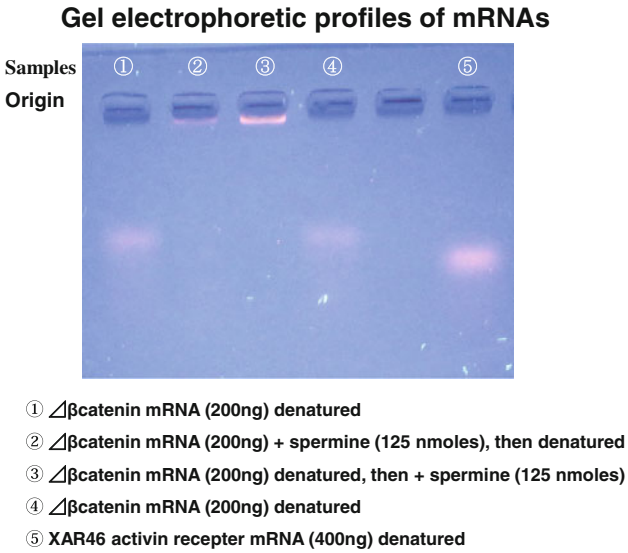

Fig. 8 Gel electrophoretic profile of Δ*β*-catenin mRNA mixed or not mixed with spermine. Δ*β*-Catenin mRNA was electrophoresed before and after the mixing with spermine. In *lanes 2* and *3*, Δ*β*-Catenin mRNA (200 ng) was mixed with spermine (125 nmol) after (*lane 2*) or before (*lane 3*) heat-denaturing of RNA. In *lane 5* XAR *Xenopus* activin receptor mRNA (400 ng) was electrophoresed as a reference

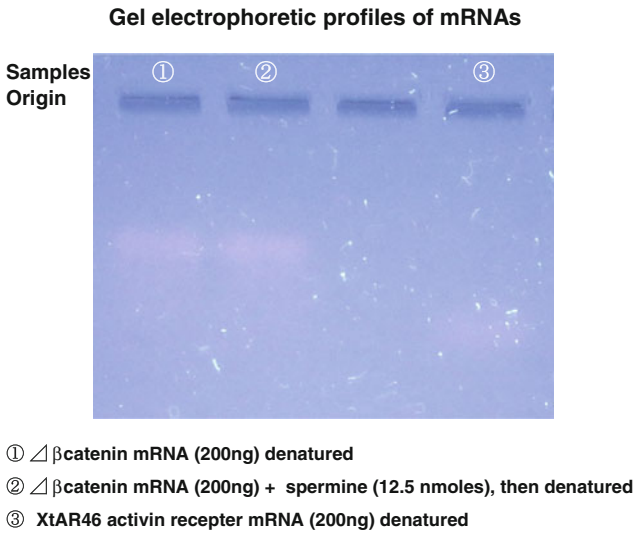

Fig. 9 Gel electrophoretic profile of Δβ-catenin mRNA mixed or not mixed with a lower dose spermine. Δβ-Catenin mRNA was electrophoresed before (*lane 1*) and after (*lane 2*) mixing with spermine (12.5 nmol). In *lane 3*, XAR *Xenopus* activin receptor mRNA (200 ng) was electrophoresed as a reference

Discussion

In this paper we tried to explore a new experimental system to study possible function of polyamines in developing embryos using *Xenopus* eggs as a model system. In *Xenopus* embryos, gene expression starts following cleavage-related unique sequence of events (Shiokawa and Yamana 1967; Shiokawa et al. 1989; Shiokawa et al. 1994; Shiokawa et al. 2008; Yang et al. 2002), and early phase of the development is characterized mainly by function of maternal mRNAs, or maternally stored proteins (Heasman 2006). While most maternal proteins express their functions after they reached the right place within the egg cytoplasm, maternal mRNAs express their functions later during development after being translated into proteins which are essential to drive the development. Among them are many families of growth factors or transcriptional factors as reviewed by Heasman (2006).

In the present experiments, we selected a well-studied transcriptional factor, β-catenin which plays the most essential part in dorso-ventral axis specification which leads to the formation of axial structure and head structure (Tao et al. 2005; Schneider et al. 1996; Schohl and Fagotto 2002; Yost et al. 1996). Using Δβ-catenin mRNA as a test RNA, we examined the effect of spermine, which is the least component in *Xenopus* early embryos, and spermidine by injecting them into 8-celled embryos together with Δβ-catenin mRNA.

As expected (Yost et al. 1996), microinjection of Δβ-catenin mRNA alone induced the formation of secondary head and secondary axis, spermine as well as spermidine inhibited dosage-dependently, the formation of such embryos with double axes with twin-head. Thus, while 25 pmol of spermine which is comparable to the amount of free spermine within the *Xenopus* embryo was only slightly effective, 250–50 pmol of spermine completely or greatly, respectively, suppressed the formation of double axes and double-headed embryos by Δβ-catenin mRNA. Interestingly, spermidine exerted similar effect as spermine at 50 pmol per embryo, which is approximately only 1/10 level of the per embryo amount of endogenous spermidine. These results indicate that both spermine and spermidine suppress the function of exogenously introduced mRNA. We performed the co-injection experiments replacing Δβ-catenin mRNA with GFP mRNA and found that also the expression of fluorescence from GFP mRNA was strongly suppressed by being co-injected with spermine. These results suggest that polyamines suppress translation of the co-injected mRNA. At physiological pH, putrescine, spermidine, and spermine possess two, three, and four positive charges, respectively, and these compounds have been reported to bind to negatively charged macromolecules such as DNA (Basu et al. 1997), RNA (Igarashi and Kashiwagi 2010) and proteins (Peng and Jackson 2000; Pollard et al. 1999) to influence the specific interactions among these molecules (Tabor and Tabor 1984; Guo et al. 2005). Therefore, our present results appear to be reasonable in the light of these previous studies.

To obtain some idea about direct molecular interactions between mRNA and polyamines, we mixed Δβ-catenin mRNA with spermine and subjected the mRNA-polyamine mixture to gel electrophoresis (Fig. 8). The results obtained here showed that spermine at the concentration used greatly (almost completely) inhibited the mRNA migration within the agarose. In this experiment, approximately 10 nmol of spermine was mixed with 200 ng of mRNA prior to microinjection. Since the mRNA used here consists of approximately 2,500 nucleotides and the average molecular weight of a nucleotide is ca. 300, the molecular weight of mRNA used here was approximately 7.5×10^5. Based on this rough calculation, the amount of mRNA used for the mixing was approximately 2.6×10^{-4} mol. The molar ratio of the mixing of spermine and mRNA was, therefore, $10{:}2.6 \times 10^{-4}$. Since the mRNA consists of ca. 2,500 nucleotides, the molar ratio of the mixing of spermine and one nucleotide of mRNA was $10{:}6.5 \times 10^{-1}$. Thus, the number of the spermine molecules per one molecule of nucleotide within the mRNA was ~15, indicating that our experiments were performed not at an extremely large molar excess of polyamine per mRNA. In this relation, in the gel electrophoretic experiment in Fig. 9, the amount of spermine per one nucleotide of the mRNA was reduced to 1.5, and in the condition of this reduced level of the added spermine the data showed that

the spermine does not reduce the mRNA migration at all within the gel. This data is consistent with the biological data that prior mixing of spermine of this reduced level does not greatly suppress the function of Δβ-catenin mRNA in the formation of double-headed embryos (Fig. 5). These results strongly suggest that free polyamines such as spermine and spermidine added only moderately (ca. tenfolds) in excess to mRNA nucleotides probably directly bind to the naked mRNA and inhibit translation of the mRNA, simply by neutralizing the negative charges of mRNA, although there might remain a possibility of formation of some cross-link-like structures among mRNAs that may also interfere with the mRNA translation. At present, we are examining changes in the levels of three polyamines (putrescine, spermidine, and spermine) in polyamine-injected embryos on one hand, and also looking for the different conditions or combination of Δβ-catenin mRNA and polyamines in which the secondary head-forming activity of the injected Δβ-catenin mRNA may be strengthened due to a possible structural stabilization of mRNAs by co-injected polyamines on the other.

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