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*Short Contribution*

# Overexpression of *S*-adenosylmethionine decarboxylase (SAMDC) activates the maternal program of apoptosis shortly after MBT in *Xenopus* embryos

MASATAKE KAI, TAKAYASU HIGO, JUN'ICHI YOKOSKA, CHIKARA KAITO, ERI KAJITA, HIROSHI FUKAMACHI, EIJI TAKAYAMA[1], KAZUEI IGARASHI[2], and KOICHIRO SHIOKAWA*

*Laboratory of Molecular Embryology, Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo, 113-0033, Japan; [1]Department of Parasitology, National Defense Medical College, Namiki 3-2, Tokorozawa, Saitama, 359-8513, Japan; [2]Faculty of Pharmaceutical Sciences, Chiba University, Yayoi-cho 1-33, Inage-ku, Chiba 263-8522, Japan*

**ABSTRACT Overexpression of *S*-adenosylmethionine decarboxylase (SAMDC) mRNA in 1- and 2-cell stage *Xenopus* embryos induces cell autonomous dissociation at the late blastula stage and developmental arrest at the early gastrula stage. The induction of cell dissociation took place "punctually" at the late blastula stage in the SAMDC-overexpressing cells, irrespective of the stage of the microinjection of SAMDC mRNA. When we examined the cells undergoing the dissociation, we found that they were TUNEL-positive and contained fragmented nuclei with condensed chromatin and fragmented DNA. Furthermore, by injecting *Xenopus* Bcl-2 mRNA together with SAMDC mRNA, we showed that SAMDC-overexpressing embryos are rescued completely by Bcl-2 and become tadpoles. These results indicate that cell dissociation induced by SAMDC overexpression is due to apoptotic cell death. Since the level of *S*-adenosylmethionine (SAM) is greatly reduced in SAMDC-overexpressing embryos and this induces inhibition of protein synthesis accompanied by the inhibition of DNA and RNA syntheses, we conclude that deficiency in SAM induced by SAMDC overexpression activates the maternal program of apoptosis in *Xenopus* embryos at the late blastula stage, but not before. We propose that this mechanism serves as a surveillance mechanism to check and eliminate cells physiologically damaged during the cleavage stage.**

KEY WORDS: *SAMDC overexpression, maternal apoptosis program, MBT, surveillance mechanism.*

In *Xenopus* embryogenesis, fertilized eggs cleave rapidly, and when embryos reach the late blastula stage, $G_1$ phase appears in the cell cycle (Graham and Morgan, 1966), and midblastula transition (MBT) takes place (Newport and Kirschner, 1982). Shortly after MBT (at the late blastula stage), cleavage becomes asynchronous (Signoret and Lefresne, 1971; Newport and Kirschner, 1982), and zygotic nuclei are activated (Newport and Kirschner, 1982; Shiokawa *et al.*, 1994; Almouzni *et al.*, 1994) to provide mRNAs necessary to support the development beyond the gastrula stage (Gurdon, 1988). Along with these changes, cells in the future dorsal region acquire motility (Minoura *et al.*, 1995), and transcription of ribosomal RNA genes (Shiokawa *et al.*, 1981) and formation of definitive nucleoli start (Nakahashi and Yamana, 1976).

In our previous experiments, we microinjected into *Xenopus* fertilized eggs the mRNA for *Xenopus S*-adenosylmethionine decarboxylase (SAMDC) (*XSD3*, Shinga *et al.*, 1996), a key enzyme in the metabolism of both polyamines and *S*-adenosylmethionine (SAM), and found that embryos overexpressed with SAMDC undergo cell-autonomous dissociation shortly after MBT and stop development at the gastrula stage. An interesting aspect of this phenomenon was that cleavage proceeded apparently normally and embryos became normal-looking blastulae at the correct time. In this experiment, we found that SAMDC-induced dissociated cells cultured in a hypotonic medium (0.1X Steinberg's solution) autolyze due to osmotic shock (Shibata *et al.*, 1998), but in isotonic media (1X Steinberg's solution and Stearn's complete medium) they do not autolyze and remain intact at least in their outer appearance for quite a long period, although they neither divide nor synthesize DNA, RNA and protein.

In the present experiments, we injected *Xenopus* SAMDC mRNA into *Xenopus* fertilized eggs, and examined the nature of SAMDC-induced dissociated cells which appeared during the late blastula to early gastrula stages. As in our previous experiments (Shibata *et al.*,

*Abbreviations used in this paper:* SAMDC, *S*-adenosylmethionine decarboxylase; SAM, *S*-adenosylmethionine; MBT, midblastula transition; TUNEL, TdT-mediated dUTP digoxygenin nick end labeling; GFP, green fluorescent protein.

***Address correspondence to:** Koichiro Shiokaswa. Laboratory of Molecular Embryology, Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo, Japan. FAX: 81 3 5841 4431. e-mail: xlshioka@biol.s.u-tokyo.ac.jp

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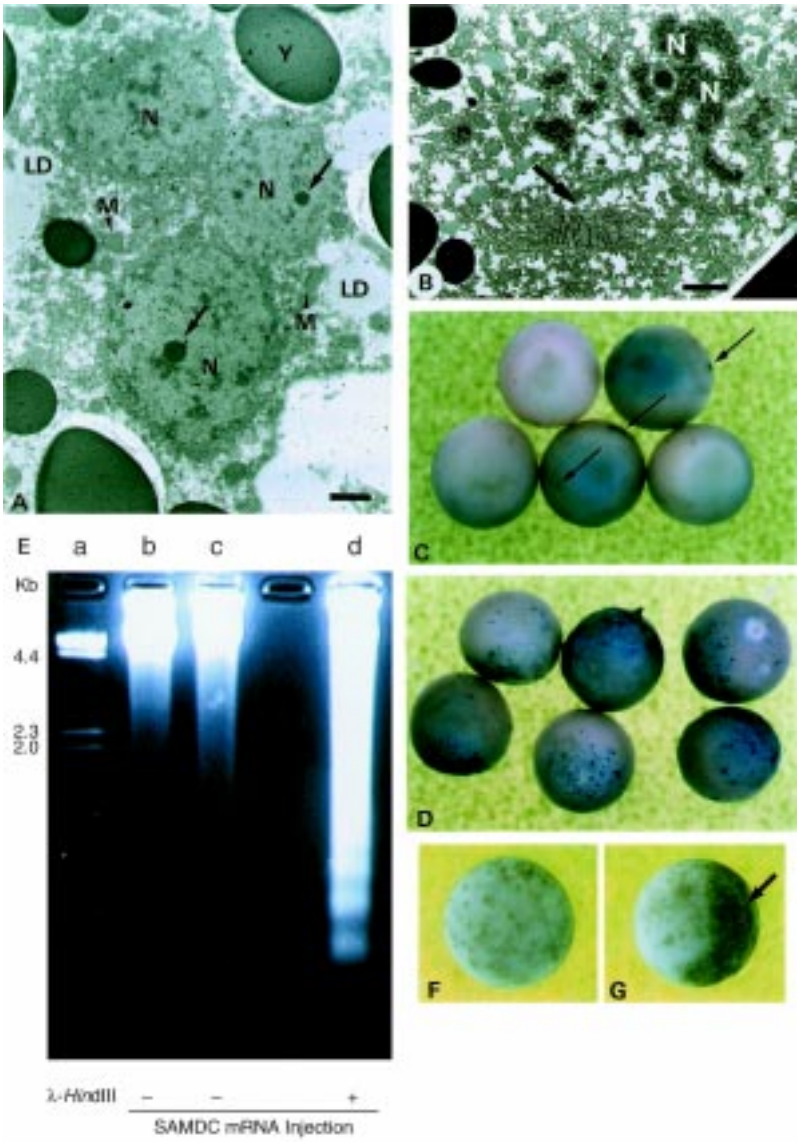


**Fig. 1. Results of cytological studies of SAMDC-induced dissociated cells. (A)** *An electron microscopic picture of a SAMDC mRNA-induced dissociated embryonic cell. Fertilized eggs were injected with 1 ng of SAMDC mRNA, and processed for electron microscopy at the early gastrula (stage 10.5). The nucleus is fragmented into three portions, two of which contained the "condensed chromatin" (arrows).* **(B)** *The embryo used in (A) was fixed 30 min later. Note that the chromatin is more condensed, and the nucleus appears to have been further fragmented, elongated and intermingled. Annulate lamellae (arrow) were often observed at this stage, although their physiological meaning is unclear. N, nucleus; M, mitochondria; Y, yolk platelet; LD, lipid droplet. Scale bars: 2 μm.* **(C, D)** *TUNEL staining of (C) control and (D) SAMDC mRNA-injected embryos. Arrows in (C) indicate random patches of TUNEL-positive cells, which represent presumably "spontaneous" apoptotic cells. Embryos in (D) were injected with 1 ng of SAMDC mRNA at the stage of uncleaved fertilized egg and examined at the early gastrula stage. Widespread TUNEL-positive cells (purple spots) are observed.* **(E)** *Internucleosomal fragmentation of DNA from SAMDC mRNA-injected embryos. SAMDC mRNA (1 ng/egg) was injected at the stage of uncleaved fertilized egg. Lane a (λ/HindIII). Total DNA was extracted from uninjected (lane b), β-globin mRNA-injected (lane c), and SAMDC mRNA-injected (lane d) embryos at the late gastrula stage (stage 12), and electrophoresed on a 2% agarose gel. In lane d, internucleosomal fragmentation of DNA ("ladder") is observed.* **(F)** *Fertilized eggs were injected with 2 ng of SAMDC mRNA and cultured in 0.1X Steinberg's solution after stage 6. The whole embryo was dissociated into cells and underwent autolysis due to osmotic shock at the early gastrula stage.* **(G)** *Fertilized eggs were injected with 2 ng of SAMDC mRNA as in (F) and at 2-cell stage further injected with 0.5 ng of Bcl-2 mRNA into one of the blastomeres. At the gastrula stage, one half portion of the embryo (arrow) was found to be rescued by Bcl-2 mRNA injection.*

1998), mRNA-injected embryos cleaved normally until the blastula stage and underwent cell dissociation at the early gastrula stage (stage 10.5). Injections of SAMDC RNA without a cap structure and mRNAs for other proteins such as β-galactosidase, type IIA activin receptor, nrp-1 mRNA and eIF4E did not induce such an effect (see also Shibata *et al.*, 1998). When we fixed the dissociated cells in SAMDC mRNA-injected embryos at the early gastrula stage and processed them for transmission electron microscopy, we found that their nuclei were fragmented into two or three portions, although their cell membrane, nuclear envelope and mitochondria appeared to be intact (Fig. 1A). In such nuclei circular electron-dense bodies were frequently observed (Fig. 1A, arrows). When such cells were examined at the midgastrula stage (30 min later), density of nuclei increased greatly, and nuclei appeared to be further fragmented, elongated and intermingled (Fig. 1B). In such SAMDC mRNA-injected embryos, a large number of cells became TUNEL-positive at the late blastula stage (stage 9) (Fig. 1D). In control embryos which were not injected with SAMDC mRNA, cells were mostly TUNEL-negative, but we occasionally observed TUNEL-positive cells (Fig. 1C, arrows), as reported previously by Hensey and Gautier (1998). When we electrophoresed the DNA from these SAMDC mRNA-injected embryos (at stage 12) on agarose gels, we found that it was fragmented (showed a "DNA ladder") (Fig. 1E, lane d). When we injected 2 ng of SAMDC mRNA into fertilized eggs, and after the first cleavage, further injected 0.5 ng of *Xenopus* Bcl-2 (*xR11*) mRNA (Cruz-Reyes and Tata, 1995) into one of the blastomeres, we found that a half portion of the embryo did not undergo cell dissociation (Fig. 1 F,G). Using GFP mRNA as a tracer, we confirmed that cells in the normal-looking half were from the blastomere injected with Bcl-2 mRNA (data not shown).

While we were performing these experiments, similar cell dissociation and embryo death were found to take place in *Xenopus* embryos when they were treated with γ–ray (Anderson *et al.*, 1997; Hensey and Gautier, 1997), hydroxyurea (Stack and Newport, 1997), cycloheximide (Stack and Newport, 1997; Hensey and Gautier, 1997) and α-amanitin (Sible *et al.*, 1997; Hensey and Gautier, 1997) during the cleavage stage. Furthermore, cells dissociated by these treatments have been shown to be TUNEL-positive, form fragmented nuclei, and contain fragmented DNA, and the cell dissociation was modified by Bcl-2. Thus, the changes observed here in

dissociated cells in SAMDC mRNA-injected embryos were quite similar to those observed in γ-ray-, α-amanitin- and cycloheximide-treated cells (Sible *et al.*, 1997; Hensey and Gautier, 1997). We, therefore, conclude that overexpression of SAMDC in *Xenopus* cleavage stage embryos induces apoptotic cell death shortly after MBT (at the late blastula to early gastrula stage), as in the case of the embryos treated with γ-ray, α-amanitin and cycloheximide (Sible *et al.*, 1997; Hensey and Gautier, 1997).

In the experiments reported by Sible *et al.* (1997) and Hensey and Gautier (1997), the injection of Bcl-2 mRNA modifies the onset of apoptosis at the late blastula stage, but the "rescued" embryos stop development completely after 2-3 h. To see to what extent the Bcl-2 mRNA injection "modifies" the apoptosis in SAMDC mRNA-injected embryos, we coinjected SAMDC and Bcl-2 mRNAs into uncleaved fertilized eggs at different dosage combinations, and examined the development of the coinjected embryos. We found here that in embryos coinjected with SAMDC and Bcl-2 mRNAs cell dissociation at the early gastrula stage was completely suppressed (Fig. 2 A,B,C). Furthermore, we found that a significantly large percentage of the injected embryos reached tailbud stage (Fig. 2D) and developed into tadpoles. As shown in Figure 2E, this effect was dosage-dependent: As the ratio of the injected amount of Bcl-2 mRNA to SAMDC mRNA was increased from 0.5/1 to 1/0.5 and to 2/0.5, the percentage of embryos which developed into tadpoles increased from 5% to 20% and to 40%. We followed here the level of SAMDC mRNA (1 ng/embryo) injected into fertilized eggs, and found that it decreases rapidly and disappears from the embryo by the end of the gastrula stage (data omitted). Furthermore, when we followed the level of SAMDC in the embryos coinjected with SAMDC mRNA (0.5 ng/embryo) and Bcl-2 mRNA (2 ng/embryo), we found that the level of SAMDC per embryo greatly increased as in our previous report (Shibata *et al.*, 1998), reached the maximum at the blastula stage, then decreased thereafter (data omitted). Therefore, the complete rescue of SAMDC mRNA-injected embryos by Bcl-2 mRNA injection was not due to the inhibition of translation of the injected SAMDC mRNA, but probably due to the decrease in the level of the overexpressed SAMDC in the post-gastrula stages.

Finally, we performed the experiment to inject different volumes of SAMDC mRNA solution (2.0, 1.0, 0.50, 0.25, 0.13 and 0.063 nl; all at 1 ng/nl) into one of the animal top blastomeres at different stages (1-, 2-, 4-, 8-, 16- and 32-cell stage, respectively). Here, the concentration of the injected SAMDC mRNA within the injected blastomere was expected to be approximately 2 ng/μl (an egg is roughly 1 μl), although in later stage embryos it must be slightly larger, since the volume of the cytoplasm of animal top blastomeres is slightly smaller than that of the vegetal blastomeres. In this experiment, all the embryos injected with SAMDC mRNA at later stages (16- and 32-cell stages) developed into tadpoles, without stopping development at the early gastrula stage. However, in the experiment to coinject SAMDC mRNA (ca. 2 ng/μl cytoplasm) and GFP mRNA (ca. 0.2 ng/μl cytoplasm) into one of the animal side blastomeres at 8- and 16-cell stages, we found that cells which received SAMDC mRNA were dissociated and confined into the blastocoel at the early gastrula stage and disappeared from the embryo (data omitted). Therefore, we concluded that SAMDC mRNA injection induces apoptosis constantly at the late blastula stage, irrespective of the stage of the mRNA injection.

In SAMDC mRNA-injected embryos, inhibition of protein synthesis takes place prior to cell dissociation, and this is preceded by a large decrease in the level of SAM (the substrate of SAMDC)

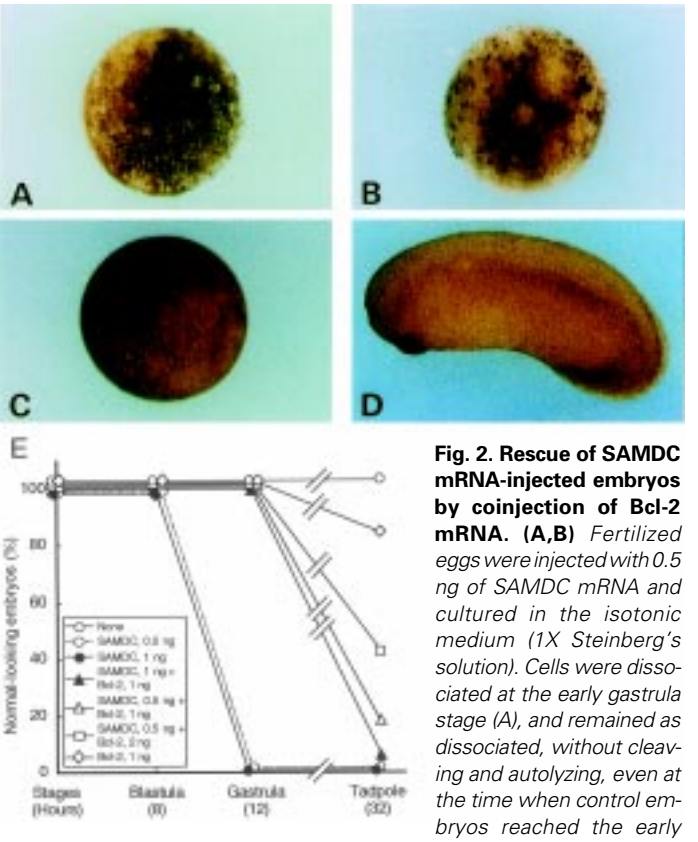


**Fig. 2. Rescue of SAMDC mRNA-injected embryos by coinjection of Bcl-2 mRNA. (A,B)** *Fertilized eggs were injected with 0.5 ng of SAMDC mRNA and cultured in the isotonic medium (1X Steinberg's solution). Cells were dissociated at the early gastrula stage (A), and remained as dissociated, without cleaving and autolyzing, even at the time when control embryos reached the early tailbud stage (B).* **(C,D)** *Fertilized eggs were injected with 0.5 ng of SAMDC mRNA together with 2 ng of Bcl-2 mRNA. All the injected embryos showed no sign of cell dissociation at the early gastrula stage (C), and approximately 40% of the injected embryos (the total number of embryos used here was 27) developed into tailbud embryos (D).* **(E)** *Dosage-dependent effects of Bcl-2 mRNA. Fertilized eggs were injected with SAMDC and Bcl-2 mRNAs together at the indicated dosage combinations. The percentage of normal-looking embryos determined at each developmental stage was plotted against the ordinate. Open and closed symbols indicate the results obtained with different batches of embryos. The number of embryos used for each experimental group was ca. 30.*

(Shibata *et al.*, 1998) and is followed by the inhibition of DNA and RNA syntheses (Shibata *et al.*, 1998). Therefore, we assume that the sequence of events which take place in SAMDC mRNA-injected embryos are 1) over-function of SAMDC due to its overexpression; 2) induction of a SAM-deficient state; 3) inhibition of protein synthesis, probably due to interference with methylation of either mRNA cap or DNA; 4) inhibition of DNA and RNA syntheses; 5) activation of the maternal program of apoptosis, which results in the induction of cell dissociation. Thus, SAMDC overexpression is a new condition to execute the maternal program of apoptosis shortly after MBT in *Xenopus* embryogenesis.

We, however, propose here that there is a potentially important difference between SAMDC-induced apoptosis and apoptosis induced by other agents such as γ–irradiation, α-amanitin or cycloheximide. In the experiments by Sible *et al.* (1997) and Hensey and Gautier (1997), Bcl-2 only shifted the onset of apoptotic cell death from stage 10.5 to stage 12 (by 2-3 h). In our system, by contrast, considerably large percentages of SAMDC-overexpressed embryos were "rescued completely" by Bcl-2 mRNA injection and developed into tadpoles. We assume that the reason for the difference may be

that, while the agents used by Sible *et al.* (1997) and Hensey and Gautier (1997) are not the normal metabolites which probably remain as toxic substances within the cells, the agent used here is a naturally-occurring enzyme of a high turn-over nature (cf. Heby and Persson, 1990).

## Experimental Procedures

### In vitro *transcription of mRNAs*

The plasmid harboring cDNA for wild-type *Xenopus* SAMDC (pBluescript SK(-)-XSD3) was maintained and used as described by Shibata *et al.* (1998). The ORF was subcloned into *Nco*I and *Xba*I sites in pSP36T (gift from Drs. Amaya and Kirschner), a derivative of pSP64T (Krieg and Melton, 1984), that carried 5' and 3'-UTRs (untranslated regions) of *Xenopus* β-globin mRNA. To obtain GFP mRNA, pbGFP/RN3P (Zernicka-Goetz *et al.*, 1996) was used. Both constructs were linearized respectively at the *Pvu*II or *Sfi*I site and transcribed *in vitro* with SP6 RNA polymerase in the presence of a cap analog, $m^7G(5')ppp(5')G$ (New England Biolabs). RNAs were dissolved in distilled water, and kept at -80°C until used.

### *mRNA injection*

Unfertilized eggs of *Xenopus laevis* were manually ovulated from gravid females which had been injected with a human chorionic gonadotropic hormone, Gonatropin (Teikoku Zoki Co.). Eggs were artificially fertilized, and dejellied in 2% cysteine-HCl (pH 8.0) (Koga *et al.*, 1999). mRNAs (0.01 to 10 ng in 10 nl of distilled water) were microinjected into the region specified in each experiment in 1X modified Barth's solution (MBS), which contained 3% Ficoll 400 and 50 mg/ml gentamycin. Microinjection was performed using a programmable microinjector (IM 300; Narishige). Injected embryos were kept in 1X MBS until stage 7 (Nieuwkoop and Faber, 1967), when they were transferred into either 0.1X Steinberg's solution or fresh 1X Steinberg's solution as specified in each experiment. All the embryos were cultured at 21°C.

### *Transmission electron microscopy*

Embryos were fixed overnight in 2.5% glutaraldehyde in 0.1 M Cacodylate buffer (pH 7.4) at 4°C. They were post-fixed with 1% $OsO_4$ for 2 h, dehydrated in a graded series of ethanol, embedded in Embedding Resin (TAAB), and sectioned at ca. 90 nm in thickness. Sections were stained with uranyl acetate and lead citrate, and examined in an electron microscope (100CX; JEOL).

### *TUNEL methods*

Embryos microinjected with SAMDC mRNA at the 1- or 2-cell stage were fixed at stages 8 to 10 (Nieuwkoop and Faber, 1967). TUNEL staining of embryos was carried out according to Hensey and Gautier (1997). Briefly, fixed embryos were labeled with fluorescein-labeled TdT for 24 h at 37°C, and signals were detected by substrate reaction with alkaline phosphatase conjugated to anti-fluorescein antibody.

### *DNA gel electrophoresis*

Embryos were homogenized in DNA extraction buffer (10 mM Tris [pH 8.0], 0.1 mM EDTA, 50 mg/ml RNase A, 0.5% SDS) (Sible *et al.*, 1997) and incubated at 37°C for 1 h. To the homogenate, proteinase K was added to a final concentration of 100 μg/ml, and incubation performed at 50°C for 2 h. Samples were treated with phenol:chloroform (1:1), and DNA was precipitated with ethanol. Electrophoresis was performed on a 2% agarose gel and DNA stained with ethidium bromide.

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