

## Overexpression of S-adenosylmethionine decarboxylase (SAMDC) in *Xenopus* embryos activates maternal program of apoptosis as a “fail-safe” mechanism of early embryogenesis

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### ABSTRACT

In *Xenopus*, injection of S-adenosylmethionine decarboxylase (SAMDC) mRNA into fertilized eggs or 2-cell stage embryos induces massive cell dissociation and embryo-lysis at the early gastrula stage due to activation of the maternal program of apoptosis. We injected SAMDC mRNA into only one of the animal side blastomeres of embryos at different stages of cleavage, and examined the timing of the onset of the

apoptotic reaction. In the injection at 4- and 8-cell stages, a considerable number of embryos developed into tadpoles and in the injection at 16- and 32-cell stages, all the embryos became tadpoles, although tadpoles obtained were sometimes abnormal. However, using GFP as a lineage tracer, we found that descendant cells of the blastomere injected with SAMDC mRNA at 8- to 32-cell stages are confined within the blastocoel at the early gastrula stage and undergo apoptotic cell death within the blastocoel, in spite of the continued development of the injected embryos. These results indicate that cells overexpressed with SAMDC undergo apoptotic cell death consistently at the early gastrula stage, irrespective of the timing of the mRNA injection. We assume that apoptosis is executed in *Xenopus* early gastrulae as a “fail-safe” mechanism to eliminate physiologically-severely damaged cells to save the rest of the embryo.

**Key words:** SAMDC overexpression, maternal apoptosis program, processing-defective SAMDC mutant, MBT, fail-safe mechanism.

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Received April-4-2003 Revised May-1-2003 Accepted May-13-2003

## INTRODUCTION

In *Xenopus* embryogenesis, cleavage proceeds rapidly without G<sub>1</sub> phase in the cell cycle, and during the cleavage stage cells do not synthesize much RNA[1], since informational molecules necessary for this early phase of development are provided mainly as maternal mRNAs and preformed proteins[2, 3]. When embryos reach the late blastula stage, G<sub>1</sub> phase appears in the cell cycle[4], and important changes in cellular activities, which are collectively called MBT (midblastula transition), take place before embryos start gastrulation[5]. The main changes which take place here are transcriptional activation[1, 5, 6], shift from the synchronous to asynchronous cell division, and commencement of cell movement[5, 7, 8]. Along with these changes, transcription of ribosomal RNA genes is also initiated[9], and definitive nucleoli start to appear as the cytological manifestation of the rRNA synthesis[10].

Recently, we performed experiment to inject S-adenosylmethionine decarboxylase (SAMDC) mRNA into *Xenopus* fertilized eggs, and found that the overexpression of SAMDC in *Xenopus* cleavage stage embryos induces cell dissociation shortly after MBT[11], due to activation of the maternal program of apoptosis[12]: The dissociated cells were TUNEL positive, contained fragmented nuclei and the fragmented DNA, and the effect of SAMDC mRNA injection was abolished by Bcl-2 mRNA injection[12]. Since the whole effect of SAMDC mRNA injection was canceled by coinjection of SAM (S-adenosylmethionine), the apoptosis induced was concluded to be due to the deficiency of SAM.

While we were performing these experiments, similar apoptotic reaction was found to take place in *Xenopus* embryos when they were treated with  $\alpha$ -ray [13, 14], hydroxyurea[15], cycloheximide[14, 15] and  $\alpha$ -amanitin [14, 16] during cleavage stage. The apoptotic cell death was also found to occur in normally-developing *Xenopus* embryos[17], although the site of the appearance of apoptotic cells or the number of the apoptotic cells differed from embryo to embryo. More recent studies report that the apoptotic reaction can be observed in embryos altered in DNA methylation[18, 19], defective in endogenous function of XChkl kinase[20] or

blocked DNA replication[20]. A similar apoptotic reaction has been found to take place also in zebrafish embryos when they were treated with hydroxyurea, aphidicolin or camptothecin during the cleavage stage [21].

An interesting aspect of the programmed apoptotic reaction found in *Xenopus* embryos is that it takes place constantly during the late blastula to early gastrula stages and never before [11-16, 18-20, 22]. In the previous study, we have tested a wide dosage range of SAMDC mRNA (0.1 pg to 10 pg) and found that when the “apoptosis-induced” developmental arrest took place it always took place at the early gastrula stage[11]. Furthermore, it has been suggested that DNA damage response is an element of MBT, and *Xenopus* embryos have multiple mechanisms to block the apoptotic pathway after the MBT[22]. These results strongly suggest that the apoptotic program may be regulated by a clock mechanism.

In this study, we intended to know how and when the apoptosis takes place when SAMDC mRNA was injected at different stages of cleavage. To address this issue, we injected here SAMDC mRNA into only one blastomere at cleavage stages later than 2-cell stage. By doing this experiment, we also expected to know how embryos behave when the apoptosis was induced in a relatively small population of cells within the embryo. We found here that almost all the embryos injected with SAMDC mRNA at later stages (8- to 32-cell stages) develop into tadpoles without showing any sign of cell dissociation at the early gastrula stage at least in their outer appearance. This was at first not easy to understand, since we expected that the apoptosis should take place shortly after the MBT irrespective of the stage of the mRNA injection. In such apparently normally developing embryos, however, we found that cells which received SAMDC mRNA overexpress SAMDC and are dissociated at the early gastrula stage to disappear from the embryo after being confined within the blastocoel. Based on these results, we suggest here that in *Xenopus* embryos the maternal program of apoptosis constitutes a failsafe mechanism of early embryogenesis, which eliminates physiologically-severely damaged cells to save the rest of the embryo.

## MATERIALS AND METHODS

### *Plasmids and in vitro transcription of mRNAs*

pSP36T-XSD3, which contained the wild-type *Xenopus* SAMDC cDNA (XSD3)[23], was maintained and used as described[11, 12].

A processing-defective *Xenopus* SAMDC mutant cDNA was newly constructed from XSD3 by replacing the serine residue with alanine at the putative reaction center (at residue 70)[23, 24]. Primer pairs used in PCR to prepare the 5'-half of the mutant ORF were SDF1 (5'-CGA TCG ATA TCA TGA AGA TGG AGG AGA GC-3') and SDR2 (5'-CAA ACG AGA CCA GAT CTG GTC TCT GAG TAC ATA ACG TTC C-3') underlined are *Bsa*I sites), and those used to prepare the 3'-half of the mutant ORF were SDF2 (5'-CTC AGA GAC CAG ATC TGG TCT CGT TTG TCT CCA AGA GAC G-3'; underlined are *Bsa*I sites) and SDR1 (5'-TCC CCG CGG TTG CTA GCT CTG CTG TGG CTG GAT C-3')[11]. To ligate the 5'-half and the 3'-half of the mutated ORF, the above PCR products were subjected to second PCR for five cycles, then to further cycles using SDF1 and SDR1 as new primer pairs. The PCR product finally obtained was inserted into pTD315 at *Bsp*HI and *Nhe*I sites (pTD315-XSD3-R). A double-stranded DNA fragment (with 5'-protruded ends) was prepared by annealing two single-stranded oligo nucleotides, 5'-ACT CAG TGA GGC TAG CAT G-3' and 5'-CAA ACA TGC TAG CCT CAC T-3' (boldfaces are the mutated bases), and this was inserted into pTD315-XSD3-R at *Bsa*I sites. The sequence of the DNA obtained was confirmed, subcloned into pSP36T at *Nco*I and *Xba*I sites {pSP36T-XSD3(S70A)} and used as the template for the mutated SAMDC.

Plasmids carrying the wild-type human SAMDC (pCM9) and the processing-defective human SAMDC (S68A) cDNAs (in which serine at the reaction center was replaced by alanine) were used as described by[25]. GFP mRNA was obtained from pbGFP/RN3P as described[26].

All the DNA constructs were linearized at the *Pvu*II or *Not*I site, and transcribed *in vitro* with SP6 RNA polymerase (Ambion, Austin, TX, USA) in the presence of a cap analog, m<sup>7</sup>G(5')ppp(5')G (New England Biolabs, Beverly, MA, USA). Sizes of all the *in vitro* transcribed RNAs were confirmed by gel electrophoresis.

### *Microinjection of mRNA*

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., Tokyo, Japan). Eggs were artificially fertilized, and dejellied in 2% cysteine-HC1 (pH 8.0)[27].

mRNAs dissolved in distilled water were microinjected into embryos as specified in each experiment in I × modified Barth's solution (MBS), containing 3% Ficoll 400 and 50 µg/ml gentamycin. Injected embryos were kept in I × MBS until stage 7[28], then transferred into either 0.1 × or 1 × Steinberg's solution as indicated in each experiment. Embryos were cultured at 21-23°C throughout experiments.

### *Light and electron microscopic observations*

For light microscopic observations, embryos were fixed for 2 h with MEMFA (0.1 M [pH 7.4], 2 mM EGTA, 1 mM MgSO<sub>4</sub>, 3.7%

formaldehyde), dehydrated in a graded series of ethanol, and embedded in paraffin. Samples were sectioned serially at 5 µm, stained with hematoxylin and eosin, and examined under a light microscope [11].

For 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<sub>4</sub> for 2 h, dehydrated in a graded series of ethanol, embedded in Embedding Resin (TAAB, Aldermaston, UK), and sectioned at 90 nm. Sections were stained with uranyl acetate and lead citrate, and examined in an electron microscope (100CX; JEOL, Tokyo, Japan)[12].

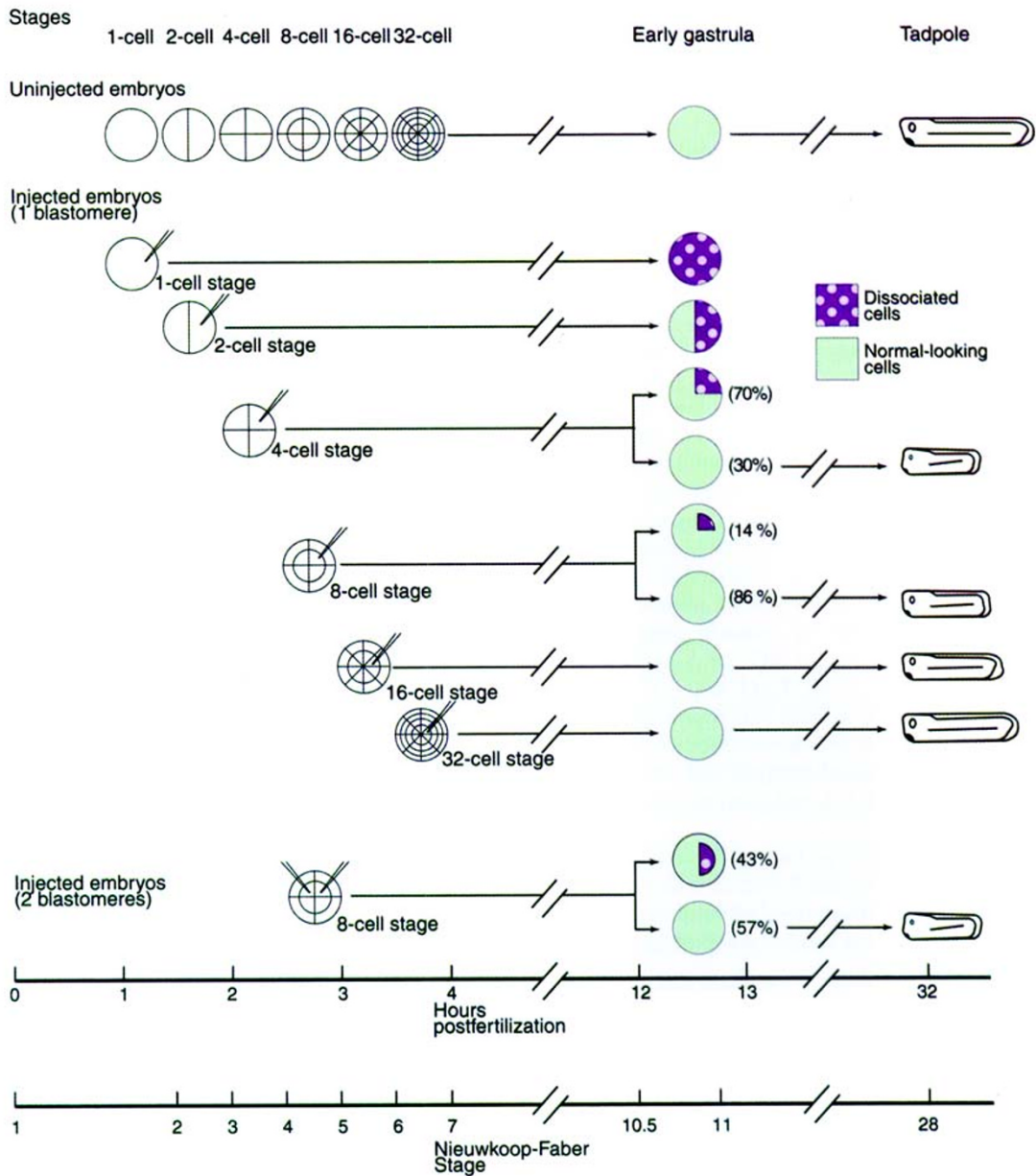
### *Assays for SAMDC activity*

Embryos were homogenized in 50 mM HEPES-KOH (pH 7.4), containing 50 mM KCl, 0.1 mM EDTA, 2.5 mM DTT, 10% glycerol and 20 mM Futhan (Torii Pharmaceutical, Tokyo, Japan). Soluble fractions, obtained by centrifugation at 16,000 rpm for 10 min, were dialyzed twice for 2 h at 4°C against the homogenizing buffer. Samples were incubated with 2mM putrescine and 0.2mM of (carboxyl-14C)SAM (2.6 kBq/mmol) (Perkin Elmer, Wellesley, MA, USA) at 37 °C for 1 h. Reaction was stopped by adding an equal volume of 2 M citric acid at 37 °C, and release of <sup>14</sup>CO<sub>2</sub> was measured as described previously[23].

## RESULTS

We previously showed that SAMDC mRNA injection into 1- or 2-cell stage *Xenopus* embryos induces deleterious cell dissociation followed by developmental arrest due to apoptosis shortly after MBT[12]. We injected here 20, 10 and 5.0 nl of SAMDC mRNA solution (all at 0.1 ng/nl) into the animal top portion of one of the blastomeres of 1-, 2- and 4-cell stage embryos, respectively. Using the same batch of embryos, we also injected 2.5, 1.3 and 0.63 nl of the same SAMDC mRNA solution into one of the animal top blastomeres of 8-, 16- and 32-cell stage embryos, respectively. These experimental designs are shown diagrammatically in Fig 1 (left half). Since the volume of an egg is roughly 1 µl, the amount of SAMDC mRNA injected here was approximately 2 ng per u1 of the cytoplasm within the injected blastomere at 1-, 2- and 4-cell stages. We assume that the concentration of SAMDC mRNA in the blastomeres of embryos injected at stages later than 4-cell stage was slightly larger than 2 ng/nl, since animal top blastomeres are usually smaller than vegetal side blastomeres in these stages.

When we cultured these SAMDC mRNA-injected embryos in 0.1 × Steinberg's solution, all the embryos injected at 1- and 2-cell stages underwent cell dissociation at the early gastrula stage (stage 10.5; 12.5 h postfertilization) and autolyzed (Fig 1) due to osmotic



**Fig 1.** Experimental designs and results of the experiments to inject SAMDC mRNA at different stages of cleavage. We injected different volumes of SAMDC mRNA solution (20, 10, 5.0, 2.5, 1.3 and 0.63 nl; all at 1 ng/nl) into only one of the animal top blastomeres at different stages (respectively, 1-, 2-, 4-, 8-, 16- and 32-cell stage, or 1.25, 1.75, 2.25, 2.75, 3.15 and 3.75 h post-fertilization) or into two animal top blastomeres (2.5 nl of 1 ng/nl solution to each blastomere) at 8-cell stage (2.75 h post-fertilization) (bottom). In these experiments, we did not distinguish the presumptive dorsal or ventral blastomeres. All embryos were kept cultured in  $0.1\times$  Steinberg's solution after stage 6. All embryos injected at 1- or 2-cell stages underwent cell dissociation and autolyzed due to osmotic shock at the early gastrula stage (stage 10.5). Embryos injected with SAMDC mRNA into one of the blastomeres at 4- and 8-cell stages either arrested development at the early gastrula stage (70% and 14%, respectively) or developed into tadpoles (30% and 86%, respectively). All the embryos injected at 16- and 32-cell stages developed into tadpoles. In embryos injected with SAMDC mRNA into two animal side blastomeres at 8-cell stage, 43% of the embryos underwent cell dissociation and arrested development, but the rest 57% developed into tadpoles. Some of the tadpoles developed from SAMDC mRNA-injected embryos were normal, but some were abnormal. To the right of the figure, only abnormal tadpoles are drawn schematically, reflecting the extent of the anomaly in their outer appearance. In all cases, one experimental group consisted of at least 20 embryos.

shock (see [11]). The dissociated cells which appeared here were TUNEL positive, contained the “ladder”-forming DNA, and the cell dissociation was suppressed by Bcl-2 (data not shown, but see [12]), confirming that the cell dissociation is due to the execution of the maternal program of apoptosis. In embryos injected with SAMDC mRNA at 4- or 8-cell stage, however, cell dissociation accompanied with embryo death took place only in 70% or 14% of the embryos, respectively. The rest of the embryos developed into tadpoles without showing any sign of cell dissociation at least in their outer appearance (Fig 1), although they showed some abnormality in their morphology. In embryos injected at 16- or 32-cell stage, cell dissociation was totally not observed at least in their outer appearance, and all the injected embryos developed into tadpoles, although here again they were more or less abnormal. Roughly speaking, the earlier the stage of the injection, the larger the extent of the anomaly, and the anomaly included small head (sometimes acephaly), small trunk and tail, bending of body axis, and shortening of the body length (see also Fig 4).

We injected 2.5 nl each of the above-used SAMDC mRNA solution (0.1 ng/nl) into two animal-side blastomeres at the 8-cell stage (the concentration was ca. 0.25 ng/blastomere or slightly larger) (Fig 1, bottom). In this case, as much as 43% of the injected embryos underwent cell dissociation and died at the early gastrula stage: The rest 57% of the embryos developed into tadpoles, although again their morphology was sometimes abnormal. Thus, embryos injected with SAMDC mRNA into two animal blastomeres at the 8-

cell stage developed like the embryos injected with the mRNA into only one blastomere at the 4-cell stage, suggesting that it is the total volume of SAMDC mRNA-injected cytoplasm per embryo that determines whether embryos undergo massive cell dissociation or continue development.

SAMDC activity increases in SAMDC mRNA-injected embryos irrespective of the timing of the mRNA injection

We repeated the experiment in Fig 1 using embryos at 2-, 8- and 32-cell stages. In this experiment, all the embryos injected with SAMDC mRNA at the 2-cell stage stopped development at the early gastrula stage, whereas all the embryos injected with SAMDC mRNA at the 32-cell stage and 80% of embryos injected at the 8-cell stage developed into tadpoles. At the blastula stage, however, we detected substantial increases in SAMDC activity in all the three groups of these embryos (Tab 1). We then normalized the SAMDC activities according to the estimated volume of the injected blastomere, and obtained SAMDC activities which were large enough to induce cell dissociation for all the three injection groups (144 to 320 units/ml of the cytoplasm) (Tab 1) (see [11]). Thus, the absence of developmental arrest in embryos injected with SAMDC mRNA at stages later than 4-cell stage (Fig 1) could not be explained by the failure of the overexpression of SAMDC.

*Developmental fate of descendant cells of the blastomere injected with SAMDC mRNA at 8-, 16- or 32-cell*

**Tab 1.** SAMDC activities in embryos injected with SAMDC mRNA into only one of the blastomeres at different stages of development

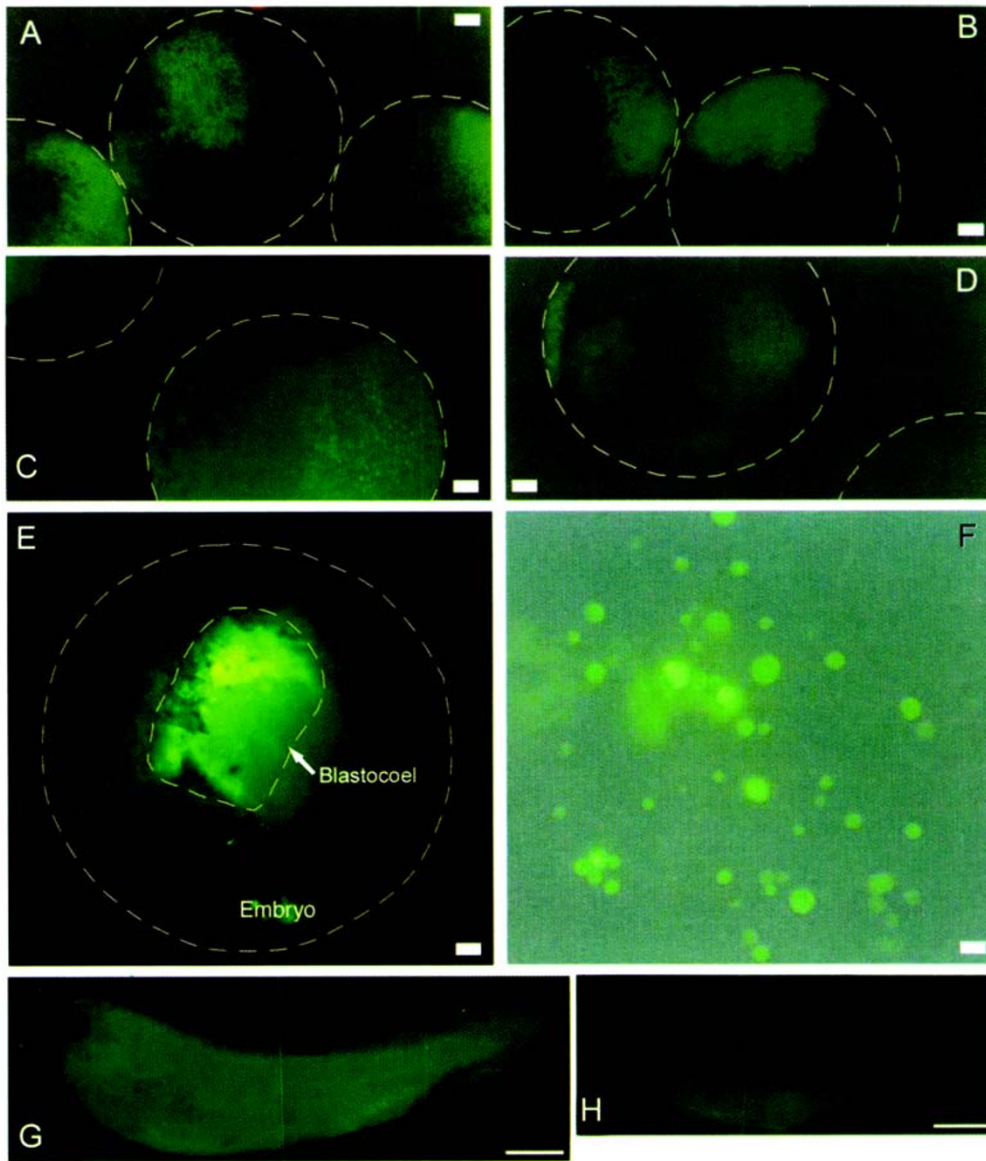
mRNA injection*	Stage of injection (hours post-fertilization)	Estimated volumes of the cytoplasm injected with mRNA ( $\mu$ l)	SAMDC Activity** (pmoles <sup>14</sup> CO <sub>2</sub> /h/embryo)	SAMDC activity calculated for 1 embryo-equivalent volume of the mRNA-injected cytoplasm (1 $\mu$ l)
None		1	0.82	0.82
1 ng	2-Cell (1.75)	1/2	160	320
0.25 ng	8-Cell (2.75)	1/8 <sup>#</sup>	27	216 <sup>##</sup>
0.063 ng	32-Cell (3.75)	1/32 <sup>#</sup>	4.5	144 <sup>##</sup>

\*Embryos were injected with *Xenopus* SAMDC mRNA at ca. 2 ng/ml cytoplasm into one animal side blastomere as explained in the text (the volume of an egg being estimated to be ca. 1  $\mu$ l).

\*\*SAMDC activity measured at the stage 9 using 50 embryos per sample before cell dissociation became apparent.

<sup>#</sup>These values are the possible maximal estimates, since animal side blastomeres are usually smaller than vegetal side blastomeres.

<sup>##</sup>These values are the possible minimal estimates as explained in<sup>#</sup>.



**Fig2.** GFP tracing of SAMDC mRNA-injected cells at the midblastula, early gastrula and tadpole stages. Embryos were injected with 0.25 ng of processing-defective or wild-type SAMDC mRNA together with 0.025 ng of GFP mRNA into one animal side blastomere at the 8-cell stage, and GFP luminescence was examined at the midblastula (8 h post-fertilization) (**A** and **B**), early gastrula (12 h post-fertilization) (**C-F**) and tadpole (32 h post-fertilization) (**G** and **H**) stages. (**A** and **B**) Luminescent cells were observed from the outside equally in embryos injected with the processing-defective (**A**) and wild-type (**B**) SAMDC mRNAs. Note that luminescent cells are not distributed in the whole area, since only one blastomere was injected at the 8-cell stage. (**C** and **D**) At the early gastrula stage, embryos injected with processing-defective SAMDC mRNA (**C**) showed luminescent cells spread widely on the surface of the embryo, whereas embryos injected with wild-type SAMDC mRNA (**D**) showed little such luminescent cells. (**E**) When the embryo in (**D**) was stripped off the vitelline membrane and its animal cap portion was removed surgically, round luminescent cells were found abundantly in the blastocoel. The embryo looks here larger than those in (**D**), due to flattening caused by the absence of the vitelline membrane. (**F**) When the embryo in (**E**) was further dissected manually, luminescent dissociated cells which had been kept in the blastocoel were scattered into the medium. Scale bars indicate 0.1 mm. (**G** and **H**) At the early tadpole stage, the embryo injected with mRNA for processing-defective SAMDC (**G**) shows strong luminescence throughout the embryo axis, the embryo injected with wild-type SAMDC mRNA (**H**) shows little luminescence. Note that the embryo in (**H**) is shorter in body length than that in (**G**) due to the apoptotic loss of a certain number of cells. Scale bars=5 mm



## stage

We injected SAMDC mRNA (ca.2ng/ml cytoplasm) and GFP mRNA (ca.0.2 ng/ml cytoplasm) together into one of the animal top blastomeres at 8-, 16- or 32-cell stage, and followed the fate of cells derived from the injected blastomere by tracing the GFP signal. In this experiment, control embryos were injected with the mRNA for processing-defective *Xenopus* SAMDC (S70A), which like mRNA for the human processing-defective mutant SAMDC (S68A) neither increased the SAMDC activity within the embryo nor induced cell dissociation (Tab 2).

At the midblastula stage (Fig 2A, B) we found no apparent difference in the distribution of luminescent cells on the surface of embryos between the control and wild-type SAMDC mRNA-injected groups. At the early gastrula stage, widely-spread luminescent cells occurred on the surface of the control embryos (Fig 2C), but not on the surface of the embryos injected with wild-type SAMDC mRNA (Fig 2D). We then removed the animal cap from wild-type SAMDC mRNA-injected embryos, and found many round luminescent cells within their blastocoel (Fig 2E). The round luminescent cells were scattered into the medium when the embryos were manually further disrupted (Fig 2F).

When we examined the stage 9 embryos under a light microscope, many round dissociated cells were in fact found within the blastocoel (Fig 3A). Roughly, the number of the dissociated cells in the

blastocoel was again dependent on the timing of injection; it decreased when the injections had been performed at later stages (data not shown). At stage 10, the number of dissociated cells appeared to have been increased within the blastocoel (Fig 3B), and some of them seemed to have been already autolyzed (Fig 3B, arrows). Under an electron microscope, these cells were found to have fragmented nuclei (Fig 3E), and in some cells nuclear envelope was not seen. Furthermore, chromatin of such cells was extremely condensed (Fig 3E, F) as compared with that in the control (Fig 3D). At the neurulastage (stage 15), however, the number of abnormal round cells were not so large, and embryos appeared to be recovering from the effect of cell dissociation at the gastrula stage (Fig 3C, arrow). Finally, at the swimming tadpole stage, we found many luminescent cells throughout the embryo axis in control embryos (Fig 2G). However, we found few luminescent cells in wild-type SAMDC-injected embryos (Fig 2H).

Based on these data, we concluded that cells derived from the SAMDC mRNA-injected blastomere are confined within the blastocoel shortly after MBT and disappear from the embryo due to apoptotic cell death, irrespective of the stage of the mRNA injection.

*Embryos injected with SAMDC mRNA into a future dorsal or ventral blastomere at later stages of cleavage give rise to tadpoles with different types of anomaly*

Presumptive fates of blastomeres at early cleav-

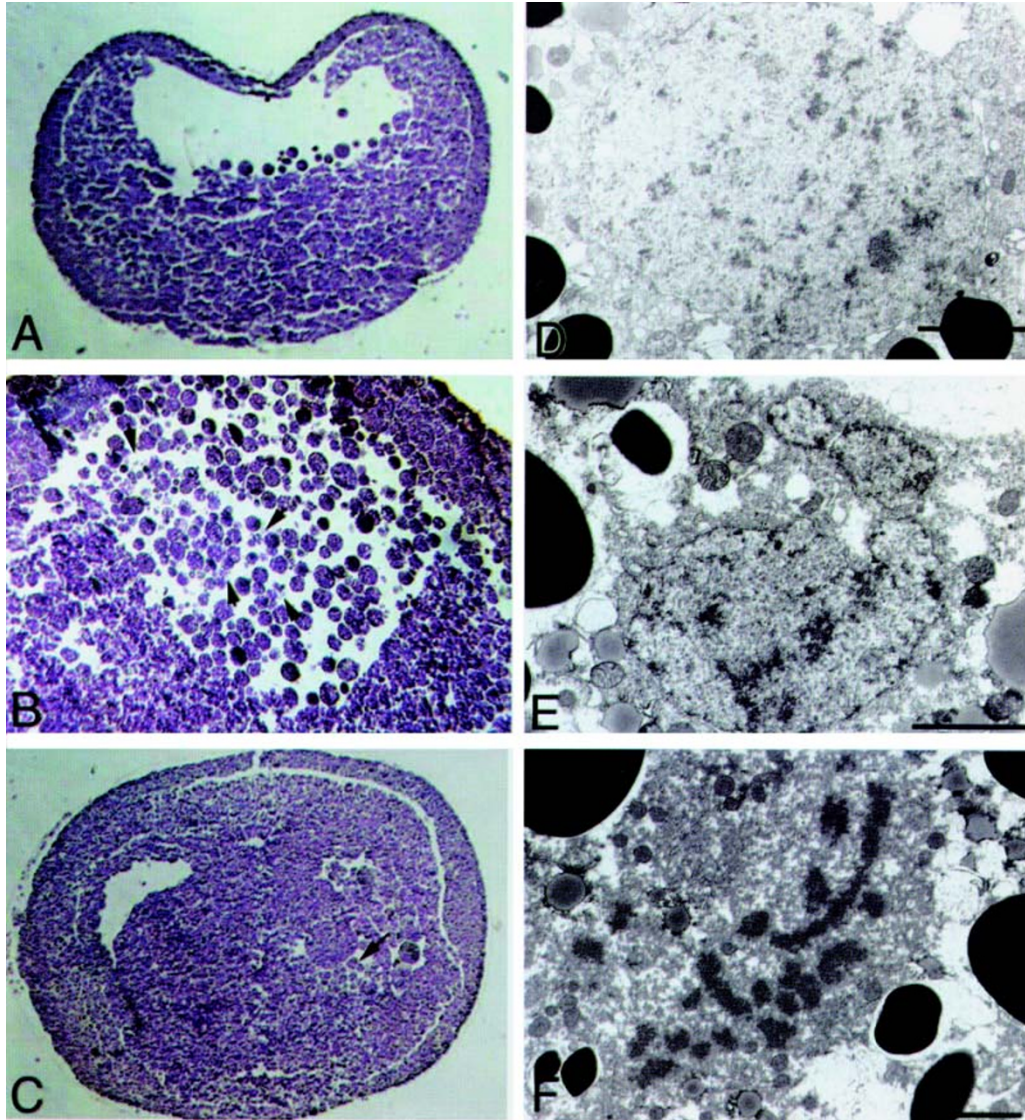
Tab 2. Development and the level of SAMDC activity in *Xenopus* embryos injected with mRNAs for wild-type or processing-defective mutants of *Xenopus* and human SAMDC

Injection*	Source of the injected SAMDC mRNA	Number of embryos used for cell dissociation test	Number of embryos which underwent cell dissociation (%)**	SAMDC activity (pmoles <sup>14</sup> CO <sub>2</sub> /h/embryo)***
None		30	0 (0)	0.73
Wild type SAMDC	<i>Xenopus</i>	20	20 (100)	220
Processing-defective SAMDC (S70A)	<i>Xenopus</i>	25	1 (4)	0.85
None		29	0 (0)	0.95
Wild type SAMDC	Human	25	22 (88)	88
Processing-defective SAMDC (S68A)	Human	23	0 (0)	0.73

\*Uncleaved fertilized eggs were injected with 1 ng of either wild type or processing-defective SAMDC mRNA.

\*\*Cell dissociation was examined at stage 11.

\*\*\*Determined at the stage 9 (late blastula stage) using 50 embryos for each series. This experiment was performed using the same lot of SAMDC mRNA as in (\*\*) but using a different batch of embryos.

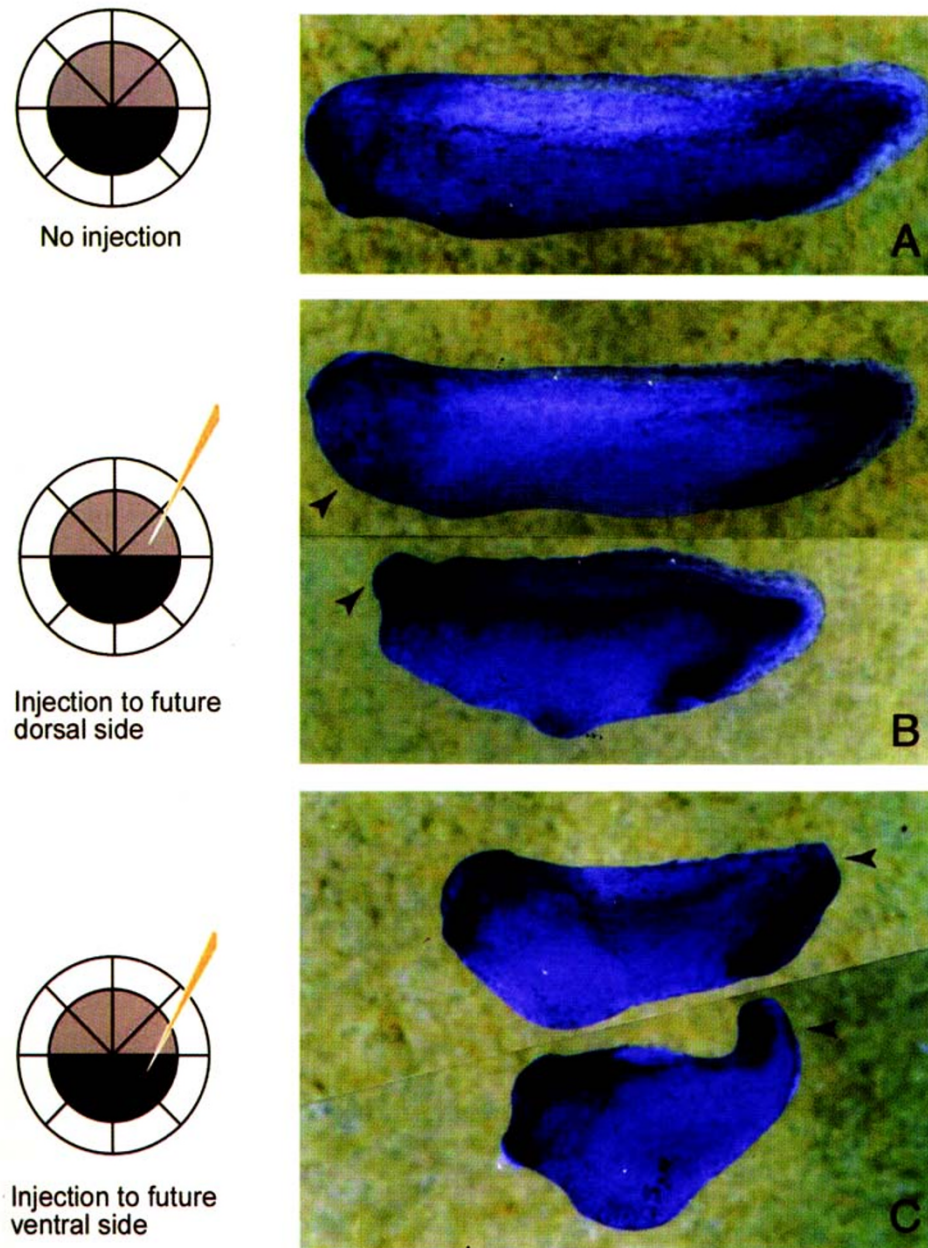


**Fig 3.** Light and electron microscopic observations of SAMDC mRNA-injected embryos. **(A-C)** Embryos were injected with 0.013 ng of SAMDC mRNA into an animal side blastomere at 16-cell stage, fixed at the late blastula stage (stage 9) **(A)**, early gastrula stage (stage 10) **(B)** or neurula stage (stage 15) **(C)** and examined under light microscope. **(A)** Round dissociated cell were observed to be confined within the blastocoel. Note that their membrane structures appeared to be intact. **(B)** Many dissociated cells were found in the blastocoel, and some of them appeared to have been broken (arrows) at the early gastrula stage. **(C)** Not many dissociated cells were seen at the neurula stage, although there were some abnormal cells remaining (arrow). **(D-F)** Electron microscopic observations. **(D)** A control cell at the midgastrula stage. **(E, F)** Fertilized eggs were injected with 1 ng of SAMDC mRNA, and fixed for the electron microscopy at the midgastrula stage (1 h after the cell dissociation was observed). In the cell in **(E)**, the nucleus is fragmented into three portions, and furthermore, chromatin is condensed. Nuclear envelope is not seen in the cell in **(F)**. Bars=2  $\mu$ l

age stage could be identified by the pigmentation pattern [29, 30]. We, therefore, compared the developmental fate of embryos injected with SAMDC mRNA (0.13 ng/blastomere) into one of the future dorsal-an-

mal or ventral-animal blastomeres at the 16-cell stage. Here again, cell dissociation accompanied with developmental arrest took place in none of the injected embryos. However, when SAMDC mRNA was injected





**Fig 4.** Effects on development of SAMDC mRNA injection into one animal side blastomere of either future dorsal or ventral side at the 16-cell stage. Embryos were injected with 0.13 ng of SAMDC mRNA into one blastomere of either future dorsal or ventral side at 16-cell stage as schematically shown in the diagram, and cultured until the tailbud stage. (A) A control uninjected embryo. (B) Embryos injected into the dorsal side. The cement gland is missing (upper arrowhead), and furthermore head part is absent (acephaly) (lower arrowhead) or not normal. (C) Embryos injected into the ventral side. Posterior and ventral structures such as trunk and tail are poorly developed (arrowheads).

into the future dorsal animal blastomere, 38% of the injected embryos became abnormal in dorsoanterior structures (Tab 3): They formed no cement gland and/or had defects in the head part (Fig 4B). Similarly, 27% of the embryos injected with SAMDC mRNA into the future ventral animal blastomere developed into em-

bryos abnormal in ventroposterior structures (Tab 3): They had poorly developed trunk and tail structures, although they formed normal-looking head and well developed cement gland (Fig 4C). Therefore, we concluded that the elimination of cells at the gastrula stage by SAMDC overexpression significantly affects subse-

quent morphogenesis, reflecting the developmental fate of the eliminated cells.

## DISCUSSION

Previous experiments to inject SAMDC mRNA into *Xenopus* embryos have been performed only at 1- or

2-cell stage, and the outcome of the experiments was constantly deleterious and massive cell dissociation followed by whole embryo dissolution at the early gastrula stage [11, 12]. In the present experiment, we injected SAMDC mRNA into only one of the blastomeres

**Tab 3.** Development of the embryos injected with SAMDC mRNA into one of either future dorsal or ventral blastomere at 16-cell stage

Injection*	Number of embryos injected	Normal (%)	Abnormal**		
			Defect in head part (%)	Defect in trunk length and tail (%)	Others (%)
None	18	18 (100)	0 (0)	0 (0)	0 (0)
Dorsal	13	7 (54)	5 (38)	0 (0)	1 (8)
Ventral	15	10 (67)	1 (7)	4 (27)	0 (0)

\*Injection was performed at 0.13 ng/blastomere at 16-cell stage into one of either dorsal or ventral animal blastomeres.

\*\*Anomaly of embryo was examined at stage 28.

of *Xenopus* embryos at different stages of cleavage ranging from 2-cell to 32-cell stages, and examined the development of the injected embryos. At first, we found that the response of embryos to the SAMDC mRNA injection differs apparently “greatly” depending on the stage of the mRNA injection: While embryos injected with SAMDC mRNA at 1- and 2-cell stages constantly underwent massive cell dissociation and stopped development at the early gastrula stage as before [11, 12], those injected with the mRNA at and after the 8-cell stage did not arrest development and often became normal-looking tadpoles. This was a puzzling result to us, since in embryos which developed into tadpoles, SAMDC was shown to be overexpressed as expected when some of the sibling embryos were sacrificed and measured for SAMDC activity at the late blastula stage. Here, the extent of the overexpression corrected for the size of the injected blastomere was just comparable to that obtained with embryos which were injected with SAMDC mRNA at 1- or 2-cell stage and whose development was arrested completely at the early gastrula stage.

We then followed the fate of the cells which received SAMDC mRNA using GFP as a lineage tracer. We found here that even in embryos which were not arrested at the early gastrula stage, descendant cells of the SAMDC mRNA-injected blastomere undergo apoptotic cell death “punctually” at the early gastrula stage after being confined in the blastocoel. Based on these results, we concluded that in SAMDC mRNA-injected cells, the apoptotic program is executed “consistently” at the early gastrula stage, irrespective of

the stage of the mRNA injection.

An interesting point of the present results is that such dissociated cells were first confined within the blastocoel. This is probably due to the loss of cell adhesion in such apoptosis-induced cells. It is then tempting to consider that the blastocoel provides a space for apoptotic cells to be collected and disrupted when the maternal program of apoptosis is first executed at the late blastula to early gastrula stage. Another interesting point is that when a relatively small number of cells were eliminated by the execution of the apoptotic program, embryos remain apparently intact and continue development, although such embryos often become abnormal in morphology. It appears here that the maternally-inherited program of apoptosis in *Xenopus* embryos eliminates damaged cells from the embryo by “altruistic cell death”. In this sense the maternal program of apoptosis appears to serve as a “fail-safe” mechanism of *Xenopus* embryogenesis, since it eliminates damaged cells without stopping the development as a whole.

As Hensey and Gautier [14] pointed out, *Xenopus* embryonic cells seem to check themselves shortly after MBT to see if they are physiologically valid, when G<sub>1</sub> phase first appears in the cell cycle: If they find themselves physiologically aberrant, they execute the apoptotic program and disappear at cellular level but not as the embryo as a whole. The points to be checked here seem to be diverse, including intactness of DNA structure, occurrence of normal DNA replication, normal RNA transcription, and normal translation, as can be expected from the results ob-

tained with g-lray[13, 14], hydroxyurea[15], a-lamanitin [14, 15], and cycloheximide[14, 16]. Also, *Xenopus* embryonic cells seem to check if DNA methylation is normal or not, and if it is not normal they execute the same apoptotic program[18, 19]. These situations could be summarized as in Fig 5, in which the apoptotic program is expressed as a “fail-safe” mechanism of normal *Xenopus* embryonic development.

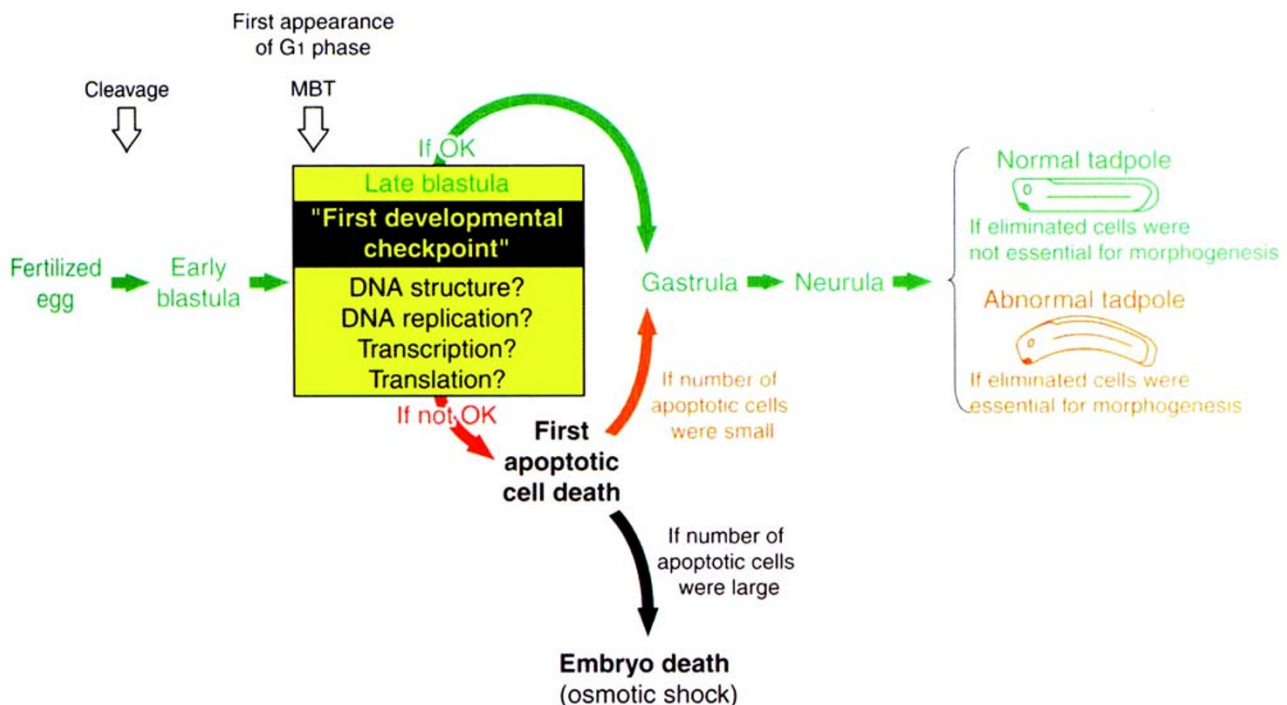
#### ACKNOWLEDGMENTS

We thank Professor A. E. Pegg (Pennsylvania

State University) for providing us with plasmids for human SAMDC and its processing-defective mutant S68A. We also thank Professor J. B. Gurdon (Cambridge University) for providing us with the plasmid for GFP. Special thanks are due to Dr. Yoshio Yaoita for valuable discussions especially on the experiment Fig 1.

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**Fig 5.** A model which shows how early development proceeds. This model includes apoptotic check point which seems to function as a part of the surveillance and “fail-safe” mechanism in *Xenopus* early embryonic development. The fertilized egg cleaves rapidly until it reaches the early blastula stage. Shortly after MBT, the “first developmental checkpoint” comes when G<sub>1</sub> phase first appears and this check mechanism cell-autonomously determines if the cell should continue on development or be eliminated by executing the maternal apoptosis program. However, even when the apoptotic program was set on operation, embryos could follow two different courses. If the number of apoptotic cells was large, the whole embryo stops development and dies, since apoptotic cells are dissociated and autolyzed due to osmotic shock (eggs are laid in the water). However, if the number of apoptotic cells is small, they are confined within the blastocoel and the embryo itself continues on development. Tadpoles which come out from such survived embryos may be normal. However, when the eliminated cells are too many or indispensable for normal morphogenesis, embryos develop into abnormal tadpoles.

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