



Title	Visualization and motility of primordial germ cells using green fluorescent protein fused to 3'UTR of common carp nanos-related gene
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1 **Visualization and motility of primordial germ cells using green fluorescent protein**  
2 **fused to 3'UTR of common carp *nanos*-related gene**

3

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30 **Abstract**

31 Primordial germ cells (PGCs) are the only cells in developing embryos with the  
32 potential to transmit genetic information to the next generation. We previously  
33 visualized the PGCs of several teleostean embryos by injecting RNA synthesized from  
34 constructs encoding green fluorescent protein (GFP) fused to the 3'UTR of the  
35 zebrafish (*Danio rerio*) *nanos1* gene (*nos1*). However, this technique was not always  
36 suitable for visualizing PGCs in embryos from all teleost species. In this study, we  
37 compared the visualization of PGCs in common carp (*Cyprinus carpio*) embryos using  
38 two artificial constructs containing GFP fused to the 3'UTR of *nanos* from either  
39 common carp or zebrafish. Visualization was better using GFP fused to the 3'UTR of  
40 the *nanos* gene from common carp, compared with that from zebrafish. The visualized  
41 PGCs successfully migrated toward the gonadal ridge after transplantation into goldfish  
42 host embryos, suggesting that they maintained normal migratory motility. These  
43 techniques could be useful for the production of inter-specific germline chimeras using  
44 common carp donor PGCs.

45

46 *Keywords:* *nanos*, primordial germ cell, common carp, goldfish, germline chimera

47

## 48 **1. Introduction**

49 Germ cells are the only cells with the potential to transmit genetic information  
50 to the next generation. In most organisms, primordial germ cells (PGCs) are set aside  
51 from somatic cells in early development. Germplasm is defined as a substance present  
52 in the cytoplasm of the eggs and early embryos of many species that is inherited by only  
53 some cells of the embryo, and determines the fate of the PGCs (Eddy, 1975). The  
54 germplasm includes electron-dense masses of granules and fibrils, rich in RNA and  
55 protein (Wylie, 2000), which are known as polar granules in *Drosophila* (Mahowald,  
56 1962). Many genes are required for the localization and formation of these polar  
57 granules in *Drosophila* (Nusslein-Volhard et al., 1987). The *nanos* gene, which encodes  
58 an RNA-binding zinc-finger protein, was first identified as a polar granule component  
59 in *Drosophila* (Wang and Lehmann, 1991). Although the formation of PGCs does not  
60 require *nanos* activity, the absence of maternal *nanos* results in abnormal PGC  
61 morphology and their failure to migrate to and be incorporated in the gonad (Kobayashi  
62 et al., 1996; Forbes and Lehmann, 1998; Deshpande et al., 1999).

63 *nanos*-related genes have been reported in vertebrates: *Xcat2* in *Xenopus laevis*  
64 (Mosquera et al., 1993), *nos1* and *nos2*, (Köprunner et al., 2001) in zebrafish (*Danio*  
65 *rerio*) and *nanos1* (Haraguchi et al., 2003), *nanos2* and *nanos3* (Tsuda et al., 2003) in

66 mouse (*Mus musculus*). It is known that *Xcat2* RNA is transported with the germplasm  
67 to the vegetal cortex during oogenesis, where it is associated with germinal granules in a  
68 process that depends on cis-acting elements in the 3'UTR (Kloc et al., 2000). Although  
69 mouse *nanos1* is not detected in PGCs (Haraguchi et al., 2003), *nanos2* and *nanos3* are  
70 differentially expressed in PGCs. *nanos2* is predominantly expressed in male germ cells,  
71 and the elimination of this gene results in a complete loss of spermatogonia. *nanos3* is  
72 found in migrating PGCs, and the elimination of this factor results in the complete loss  
73 of germ cells in both sexes (Tsuda et al., 2003).

74         Expression of *nos2* mRNA in zebrafish is first detected in the forming somites  
75 and in specific domains in the nervous system, but it is not detected in the PGCs during  
76 the first 2 days post-fertilization (dpf), while *nos1* is expressed in the germplasm and in  
77 the PGCs. Knock-down experiments have revealed that this gene may play roles in the  
78 migration and survival of PGCs during embryogenesis (Köprunner et al., 2001). Young  
79 female zebrafish *nos1* mutants contain oocytes, but fail to maintain oocyte production  
80 (Draper et al., 2007). Moreover, *nos1* 3'UTR-containing RNAs were specifically  
81 stabilized in the PGCs, but exhibited rapid degradation in somatic tissues (Köprunner et  
82 al., 2001). Using this phenomenon, PGCs in embryos can be visualized by injecting  
83 artificial RNA encoding a construct that includes green fluorescent protein (GFP) and/or

84 DsRed, fused to the 3'UTR of *nos1* (Köprunner et al., 2001; Weidinger et al., 2002;  
85 Slanchev et al., 2005).

86 We have successfully produced intra- and/or inter-specific germline chimeras in  
87 teleosts by transplanting GFP-visualized PGCs from embryos injected with GFP-*nos1*  
88 3'UTR strand-capped mRNA (Saito et al., 2008; Higaki et al., 2010; Kawakami et al.,  
89 2010). Thus visualization of PGCs using GFP-*nos1* 3'UTR derived from the zebrafish  
90 gene could provide a powerful tool for producing inter-specific germline chimeras in  
91 teleosts using the single PGC transplantation method (Saito et al., 2008; Kawakami et  
92 al., 2010). However, the efficiency of PGC visualization varies among teleost species,  
93 and few studies have compared the efficiency of PGC visualization using GFP  
94 constructs with zebrafish *nos1*-3'UTR with that using the *nanos*-3'UTR from the  
95 species to be visualized.

96 Common carp (Cypriniformes, *Cyprinus carpio*) is one of the most important  
97 commercial species of fish because of its high market value. However, carp take a  
98 relatively long time to reach maturity (2 or 3 years), and it may be possible to reduce  
99 this period by using host fish with shorter pubertal periods, such as zebrafish and/or  
100 goldfish. It is therefore important to establish a suitable technique for visualizing  
101 common carp PGCs. In this study, we therefore cloned the common carp *nanos*-related

102 gene (*ccnanos*) and established a construct including GFP fused to the 3'UTR of  
103 *ccnanos*. We then confirmed the GFP-visualized PGCs of embryos injected with mRNA  
104 synthesized from the construct. We also analyzed the motility of the visualized PGCs  
105 after transplantation into goldfish hosts (*Carassius auratus*).

106

## 107 **2. Materials and methods**

### 108 *2.1. Fishes*

109

110 Immature 1-year-old female common carp, mature common carp, goldfish, and  
111 zebrafish were maintained in the Nanae Fresh Water Laboratory, Hokkaido University.  
112 Ovary samples from immature common carp were frozen in liquid nitrogen and kept at  
113 -80°C prior to the preparation of total RNA or poly(A) + RNA.

114 Fertilized common carp eggs and sperm were obtained in June–July 2010.

115 Stock mature fish maintained at 13–16°C in a plastic tank were moved to a 1,000-l  
116 spawning tank, containing water at a depth of 30–50 cm and at a temperature of  
117 20–24°C with a spawning bed, to induce ovulation by thermal treatment (males: 5–10  
118 fish per tank, females: 2–3 fish per tank). Mature eggs and sperm were artificially  
119 stripped from the parent fish during the early morning, after checking spawning

120 behavior.

121           Mature goldfish eggs were obtained after artificial induction by hormonal  
122 injection of 10 IU/g body weight of human chorionic gonadotropin (Asuka Seiyaku,  
123 Tokyo, Japan). Sperm was collected from anesthetized male goldfish using  
124 microcapillary tubes.

125           Artificial insemination was performed by the dry method. The eggs were  
126 stripped on polyvinylidenechloride film (Saran Wrap; Asahi Chemical Industry, Tokyo,  
127 Japan and/or Kure Wrap; Kureha Chemical Industry, Tokyo, Japan), inseminated with  
128 the sperm, and then fertilized in fertilization solution (tap water containing 0.2% urea  
129 and 0.25% NaCl). The combinations of eggs and sperm are described below.

130 Dechoriation of the eggs was carried out before blastodisc formation by a slight  
131 modification of the method described by Yamaha and Yamazaki (1993). Fertilized eggs  
132 were dechorionated with Ringer's solution (128 mM NaCl, 2.8 mM KCl and 1.8 mM  
133 CaCl<sub>2</sub>) containing 0.1% trypsin (Difco, Detroit, MI, USA) and 0.4% urea for about 10  
134 min, and then washed with culture Ringer's solution containing 1.6% albumen.

135 Operated and control denuded embryos without chorion were cultured in separate wells  
136 filled with the culture Ringer's solution for 1 day, and thereafter moved to separate  
137 wells filled with second culture solution (1.8 mM CaCl<sub>2</sub> and 1.8 mM MgCl<sub>2</sub>) until 4

138 dpf.

139           The developmental schedule for goldfish was staged from fertilization to the  
140 end of epiboly at 20°C based on Yamaha et al. (1999). Hatching occurred at 4 dpf. After  
141 hatching, fry were fed with *Artemia nauplii* for about 1 month, and thereafter with a  
142 commercial fish feed.

143           We used zebrafish from a golden-strain that had been maintained in our  
144 laboratory in a closed colony since 2006. Parental brood stock was maintained at  
145 26–28°C under a 16-h light:8-h dark photoperiod. Embryos were dechorionated with  
146 0.1% trypsin (Difco) in Ringer’s solution. Dechorionated embryos were then cultured at  
147 26°C in 96-well plates individually filled with Ringer’s solution for 24 h, followed by  
148 culture in different wells filled with a second culture solution.

149

## 150 2.2. Reverse transcription-polymerase chain reaction (RT-PCR) and cDNA cloning

151

152           Total RNA was extracted from common carp ovaries using Trizol reagent  
153 (Invitrogen, Carlsbad, CA, USA). Poly(A)<sup>+</sup> RNAs were subsequently isolated from  
154 total RNA using Oligotex-dT-30 (Takara, Otsu, Japan). Isolated RNA was denatured at  
155 70°C for 10 min, placed on ice, and reverse transcribed with M-MLV. Second-strand

156 cDNA was synthesized and single-stranded overhangs were removed, using Takara's  
157 cDNA cloning system (Takara).

158 *ccnanos* cDNA fragments were amplified using sense and antisense degenerate  
159 primers designed based on a consensus sequence from the aligned deduced amino acid  
160 sequences of *nos1* from several vertebrates species, using DNASIS version 3.5 (Hitachi  
161 Software Engineering, Yokohama, Japan) (Table 1). PCR was carried out in a final  
162 volume of 50  $\mu$ l containing 0.5–1 pg cDNA, 400 nM of each primer, 800  $\mu$ M of each  
163 dNTR, and 2.5 U Ex Taq (Takara). PCR was carried out for 35 cycles using a Thermal  
164 Cycler Dice Gradient (Takara) under the following conditions: denaturing at 94°C for  
165 30 s annealing at 51°C for 30 s, and extension at 72°C for 20 s. PCR products were  
166 separated by 1% agarose gel electrophoresis, and selected bands were cut out and  
167 purified using QIAprep Spin Miniprep Kit (Qiagen, Venlo, Netherlands). Purified DNA  
168 fragments were subcloned into the plasmid vector pGEM-T Easy (Promega, Madison,  
169 WI, USA), using a Ligation-Convenience Kit (Nippon Gene, Tokyo, Japan), and  
170 positive clones were sequenced using Big Dye Terminator v3.1 Cycle Sequencing Kit  
171 (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 3100 Genetic Analyzer  
172 (Applied Biosystems).

173

174 2.3. 5' and 3' rapid amplification of cDNA ends (RACE)-PCR

175

176 Common carp ovary was used for the construction of cDNA for RACE-PCR  
177 using a SMART-RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). For  
178 both the 3'-RACE and the 5'-RACE, nested primers (NP1, NP2 and NP3, NP4,  
179 respectively) were designed from *ccnanos* cDNA fragments (Table 1). Based on the  
180 *nanos* cDNA fragments amplified by 5'- and 3'-RACE-PCR, sense (*ccNanos*-UTR-S)  
181 and antisense (*ccNanos*-UTR-A) primers were designed for the untranslated regions  
182 (Table 1). PCR was carried out as described above (denaturation at 94°C for 30 s,  
183 annealing at 56°C for 30 s, and extension at 72°C for 30 s).

184 These PCR products were sequenced following the method described above,  
185 and *ccnanos* cDNA sequences containing the entire open reading frame were obtained.  
186 *ccnanos* cDNA products representing independent, full-length PCR clones were each  
187 sequenced five times to detect PCR errors.

188

189 2.4. Sequence analysis

190 The GenBank accession numbers of the sequences compared with our common  
191 carp *nanos* sequence were: *ccnanos* (AB576134), zebrafish *nos1* and *nos2* (AY052376

192 and AI585000), Japanese medaka (*Oryzias latipes*) *nanos1* and *nanos2* (BJ074258 and  
193 EU074259), Atlantic salmon (*Salmo salar*) *nanos1* (BT050235), *Xenopus laevis* *Xcat2*  
194 (X72340), mouse (*M. musculus*) *nanos1*, *nanos2* and *nanos3* (AB095029, AB095972  
195 and AB095973).

196 A phylogenetic tree was constructed using the neighbor-joining method (Saitoh  
197 and Nei, 1987). For this analysis, 1,000 bootstrap replicates were carried out using  
198 ClustalW version 1.83 (DNA Data Bank of Japan,  
199 <http://www.ddbj.nig.ac.jp/index-j.html>).

200

#### 201 2.5. Construction of GFP-ccnanos-3'UTR vector

202 A GFP-containing vector specifically-expressed in PGCs, designated  
203 pCS-GFP-ccnanos-3'UTR, was constructed as follows: GFP with the SP6 promoter at a  
204 5' upstream region, introducing a *Hind* III site at the 5' end (*Hind*III-SP6-GFP-S) and a  
205 *Xho*I site at the 3' end (*Xho*I-GFP-A), and a 343-bp fragment of the *nanos* 3'UTR with  
206 a reduced poly(A) tail introducing a *Xho*I site at the 5' end (*Xho*I-ccNanos-3'UTR-S)  
207 and an *Xba*I site at the 3' end (*Xba*I-ccNanos-3'UTR-A), were inserted at the *Hind*III  
208 and *Xba*I sites of the pCS2+ vector (Table 1).

209

210 2.6. *Microinjection of mRNA*

211 Common carp, goldfish, and zebrafish embryos were injected at the 1–4-cell  
212 stage with 80 pg of GFP-*ccnanos*-3'UTR or GFP-zebrafish *nos1*-3'UTR strand-capped  
213 mRNA, synthesized by SP6 transcription from a *NotI*-linearized plasmid using the  
214 mMESAGE mMACHINE system (Ambion, Austin, TX). Common carp and goldfish  
215 embryos injected with GFP-*ccnanos* 3'UTR were cultured at 15, 18 or 20°C in 96-well  
216 plates, homogenized at the appropriate stage corresponding to 10–15 or 15–20 somites,  
217 and GFP-labeled cells were then obtained. Zebrafish embryos were then cultured at  
218 26°C in 96-well plates, and somatic embryos with GFP-labeled PGCs were counted  
219 after 24 h.

220

221 2.7. *Whole-mount in situ hybridization (WISH) using vasa probe*

222 Dechorionated embryos were fixed with 4% paraformaldehyde in  
223 phosphate-buffered saline overnight at 4°C at each stage during development. Fixed  
224 embryos were stored in 100% methanol at -20°C. The identity of the GFP-labeled cells  
225 in common carp embryos as PGCs was confirmed by comparing the locations of the  
226 GFP-labeled cells with *vasa* (GenBank accession number: AF479820)  
227 mRNA-expressing cells, using WISH, according to a modification of the method

228 described by Otani et al. (2002) and Saito et al. (2006). GFP-labeled embryos were  
229 fixed overnight in 4% paraformaldehyde at 4°C. Common carp *vasa* mRNA in embryos  
230 was analyzed by amplifying a 0.5-kbp cDNA fragment including the 3'-UTR by PCR,  
231 using primers that introduced an SP6-promoter site at the 3' end (*Vasa-Probe-S* and  
232 *SP6-Vasa-Probe-A* in Table 1). The cDNA fragment was purified by  
233 phenol/chloroform/isoamyl alcohol and ethanol precipitation. Antisense digoxigenin  
234 (DIG)-labeled RNA *vasa* probes were applied using 100–200 ng *vasa* cDNA fragment  
235 and a DIG RNA labeling kit (Roche, Mannheim, Germany).

236

## 237 2.8. PGC transplantation

238 Donor embryos injected with GFP-*ccnanos*-3'UTR or GFP-zebrafish  
239 *nos1*-3'UTR strand-capped mRNA were incubated at 15, 18 or 20°C. Donors with  
240 visualizable PGCs (Fig. 1A) were dissociated into single cells using 0.25% sodium  
241 citrate in Ringer's solution. PGCs from dissociated cells were transplanted into host  
242 goldfish blastulae, according to the method of Kawakami et al. (2010). The  
243 transplantation procedure took around 2 h, during which one isolated PGC was picked  
244 up with a glass micro-needle under a stereomicroscope (Fig. 1B) and transplanted into  
245 the marginal region of the blastodisc of each goldfish blastula (Fig. 1C). Chimeric

246 embryos were observed and photographed using a fluorescence stereomicroscope,  
247 model MZ16F (Leica Microsystems, Inc., Bannockburn, IL, USA).

248

### 249 **3. Results and discussion**

250 The nucleotide sequence of the cloned cDNA in this study is shown in Fig. 2A.

251 The common carp cDNA contains an open reading frame of 462 bp, encoding 154  
252 amino acid residues. All the cysteines in both proteins are found within the homology  
253 region and in the same positions. The C-terminal region of this gene contains two  
254 unusual Cys-Cys-His-Cys (CCHC) motifs (underlined in Fig. 2A), which each bind one  
255 equivalent of zinc with high affinity in *Drosophila* (Curtis et al., 1997). The CCHC  
256 motifs characteristic of the *nanos* (*nos*) gene can be represented as:

257 Cys-X2-Cys-X12-His-X10-Cys-X7-Cys-X2-Cys-X7-His-X4-Cys (Fig. 2B) (Köprunner  
258 et al., 2001; Mosquera et al., 1993). When the amino acid sequence corresponding to the  
259 common carp gene was compared with that of other known *nanos* (*nos*) genes, the  
260 protein showed the highest homology with zebrafish *nos1* (Fig. 3). Partial sequences for  
261 the zebrafish *nos2* gene have been reported, and include a zinc-finger domain with high  
262 homologies to medaka *nanos1* and Atlantic salmon *nanos1* (see Fig. 2B). Thus the  
263 common carp gene cloned in this study appeared to be a *nanos* gene, most closely

264 related to zebrafish *nos1*.

265 Köprunner et al. (2001) developed a PGC-labeling technique using RNA  
266 synthesized from a construct including GFP fused to the 3'UTR of zebrafish *nos1*. This  
267 technique was able to produce germline chimeras using donor GFP-visualized PGCs as  
268 follows: 50–100 GFP-expressing cells from the marginal region of a mid-blastula stage  
269 zebrafish injected with GFP-zebrafish *nos1*-3'UTR mRNA were picked up and  
270 transplanted into host embryos. The GFP-zebrafish *nos1*-3UTR mRNA was rapidly  
271 degraded in the somatic cells, but was stably maintained in PGCs, leading to GFP  
272 protein expression specifically in PGCs. Moreover, the GFP-visualized PGCs migrated  
273 to the gonadal ridge of the host, and donor PGC-derived gametes were induced. This  
274 technique has been utilized for labeling zebrafish PGCs (Weidinger et al., 2002;  
275 Slanchev et al., 2005; Higaki et al., 2010; Kawakami et al., 2010). In the current study,  
276 GFP-visualized cells were confirmed at the somite stage in common carp embryos  
277 injected with mRNA synthesized from a GFP-*ccnanos*-3'UTR construct (Fig. 1A). *vasa*  
278 RNA has been demonstrated to be a germ cell marker in teleosts, including zebrafish  
279 (Raz, 2003), gibel carp (*Carassius auratus gibelio*) (Xu et al., 2005), Nile tilapia  
280 (*Oreochromis niloticus*) (Kobayashi et al., 2000), and medaka (Shinomiya et al., 2002).  
281 *vasa* protein expression has been analyzed during zebrafish (Braat et al., 2000) and

282 gibel carp (Xu et al., 2005) embryogenesis. WISH demonstrated that common carp cells  
283 labeled with GFP-*ccnanos*-3'UTR mRNA colocalized with cells expressing *vasa*  
284 mRNA (Fig. 1D and 1E), confirming the identity of the GFP-labeled cells as PGCs.

285           In zebrafish, the mechanisms underlying germline-specific *nos1* expression  
286 have been reported to involve the interaction of the microRNA, miR-430 and the  
287 *nos1*-3'UTR. Although the miR-430 target mRNAs are equally susceptible to repression  
288 in somatic cells and PGCs (Giraldez et al., 2005, 2006), the deadenylation element in  
289 the *nos1*-3'UTR compensates for miR-430-mediated repression in PGCs (Mishima et al.,  
290 2006). Mechanisms such as this are likely to be highly conserved among teleost fish,  
291 and PGCs in several teleost embryos have been visualized by injection of  
292 GFP-zebrafish *nos1*-3'UTR, e.g., in Clupeiformes (herring, *Clupea pallasii*),  
293 Cypriniformes (pearl danio, *Danio albolineatus*; goldfish, *C. auratus*; loach, *Misgurnus*  
294 *anguillicaudatus*), Beloniformes (medaka) and Perciformes (ice goby, *Leucopsarion*  
295 *petersii*) (Saito et al., 2006; Yamaha et al., 2010). The results of this study demonstrated  
296 that PGCs in common carp embryos could also be visualized using  
297 GFP-*ccnanos*-3'UTR, suggesting that this 3'UTR has the same function as the zebrafish  
298 *nos1*-3'UTR. However, injection of the artificial GFP mRNA fused with the  
299 *ccnanos*-3'UTR visualized both common carp and goldfish PGCs more effectively than

300 injection of the zebrafish *nos1*-3'UTR (see Table 2). The reason for this difference is  
301 currently unclear, though the new method represents a powerful tool, with potential  
302 applications related to the visualization of common carp and/or goldfish PGCs.

303         When these GFP-visualized cells were isolated and transplanted into goldfish  
304 blastulae, they migrated to the gonadal ridge at 4 dpf (Fig. 1F and 1G). Migration to the  
305 gonadal ridge is one the characteristics of PGCs (Saito et al., 2008; Kawakami et al.,  
306 2010), thus supporting the identification of the GFP-visualized cells as PGCs. PGCs  
307 from earlier-stage embryos were more likely to migrate to the host gonadal ridge than  
308 those from more advanced developmental stages (Table 3). Thus PGCs isolated from  
309 earlier developmental stages are likely to be more successful in producing germline  
310 chimeras, as in zebrafish (Kawakami et al. 2010; Saito et al., 2010). The replacement of  
311 germ cells requires removal of the host endogenous PGCs. PGC differentiation was  
312 blocked by injection of a *dead end* (*dnd*) antisense morpholino oligonucleotide (MO)  
313 (Ciruna et al., 2002; Weidinger et al., 2003), and germline chimeras using zebrafish  
314 hosts have successfully been achieved by injection of a *dnd* antisense (Saito et al., 2008;  
315 Higaki et al., 2010; Kawakami et al. 2010). Further studies are needed to develop  
316 techniques for asexualizing goldfish to use as hosts.

317

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321

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323

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425 **Figure Legends**

426 **Fig. 1.** The visualization and migration of green fluorescent protein (GFP)-labeled cells  
427 (primordial germ cells: PGCs) in common carp embryos using GFP-*ccnanos*-3'UTR  
428 mRNA. (A) Common carp 10–15-somite embryo. Arrows indicate GFP-labeled cells.  
429 (B) GFP-labeled cells isolated from common carp 15–20-somite embryo. (C)  
430 Transplantation of GFP-labeled cells from common carp 15–20-somite embryo to  
431 goldfish host. (D) GFP-labeled PGCs. (E) *vasa in situ* hybridization. Note that the same  
432 cells are identified by GFP-labeling and *vasa in situ* hybridization. (F, G) Migration of  
433 donor-derived PGCs in host. Host at 4 days post-fertilization. a, b, c, d and e were  
434 estimated to represent the same signals, respectively. Bars, 500  $\mu$ m.

435

436 **Fig. 2.** The common carp *nanos* gene encodes a *nanos*-like zinc-finger protein. (A)  
437 Nucleotide sequence of common carp *nanos* gene, with the predicted amino acid  
438 sequence indicated in single-letter code below the open reading frame. The  
439 characteristic C-terminal CCHC CCHC zinc-finger domain with its conserved residues  
440 is underlined. (B) Comparison of the amino acid sequence of the zinc-finger domain  
441 among *nanos*-like proteins from common carp (*nanos*), zebrafish (1: *nos1*, 2: *nos2*), and  
442 medaka (1: *nanos1*, 2: *nanos2*). The amino acid residues comprising the conserved

443 CCHC zinc finger domain are labeled in gray. 3'UTR sequences of 504–846 bps were  
444 used for constructs in this study.

445

446 **Fig. 3.** Phylogenetic tree of eight vertebrate *nanos* (*nos*). Branch lengths are  
447 proportional to genetic distance. One thousand boot-strap replicates were performed,  
448 and values supporting nodes are shown next to inner branches. Carp; common carp  
449 *nanos* (AB576134), Zebrafish; zebrafish *nos1* (AY052376), Medaka1; medaka *nanos1*  
450 (BJ074258), Medaka2; medaka *nanos2* (EU074259), Atl.salmon; Atlantic salmon  
451 *nanos1* (BT050235), *Xenopus*; *Xenopus laevis* *Xcat2* (X72340), Mouse1; mouse *nanos1*  
452 (AB095029), Mouse2; mouse *nanos2* (AB095972) and Mouse3; mouse *nanos3*  
453 (AB095973).

454

455

**Table 1** Primers used for cloning, PCR, vector construction and whole mount *in situ* hybridization analysis of common carp *nanos* (*ccnanos*).

Name	Primer sequence	Nucleotide numbers corresponding to the annealing site (Fig. 2)
<i>ccNanos</i> -DP-S	5'-TGGAVSGACTACHTGGGTCT-3'	
<i>ccNanos</i> -DP-A	5'-CCNGTGGCNCRCAYAGNGGACA -3'	
NP1	5'-GGACCCTGACGACGGAGAAAAGAGA-3'	223–247 bp
NP2	5'-TTCTGCAGCTTCTGCAAACACAACG-3'	294–318 bp
NP3	5'-GCGCGGTCTTTTAAGTAGTGCGAGGT-3'	338–364 bp
NP4	5'-TGCAGAACTTCTTCTCGGATGGGTG-3'	275–300 bp
<i>ccNanos</i> -UTR-S	5'-TCAGCGCGTGCTTCACGGTTC-3'	1–21 bp
<i>ccNanos</i> -UTR-A	5'-AGCATTAAAAGCGTTTATATATTTCCACCAC-3'	817–846 bp
<i>Hind</i> III-SP6-GFP-S	5'-AAGCTTATGGTGAGCAAGGGCGAGGA -3'	
<i>Xho</i> I-GFP-A	5'-CTCGAGTTACTTGTACAGCTCGTCCA-3'	
<i>Xho</i> I- <i>ccNanos</i> -3' UTR-S	5'-CTCGAGACCGGACGTTTCTGAACCG-3'	
<i>Xba</i> I- <i>ccNanos</i> -3' UTR-A	5'-TCTAGAAGCATTAAAAGCGTTTATATATTT-3'	
<i>Vasa</i> -Probe-S	5'-AGTGTCCAGTGCTGGTGGCCACG -3'	
SP6- <i>Vasa</i> -Probe-A	5'-ATTTAGGTGACACTATAGAACATTTTAAACAAACATGCTG -3'	

DPs (degenerate primers), primers for amplification of *ccnos1* fragments; *ccnanos*-UTRs (untranslated regions), sense and antisense primers for the sequencing of *ccnanos* containing the open reading frame.

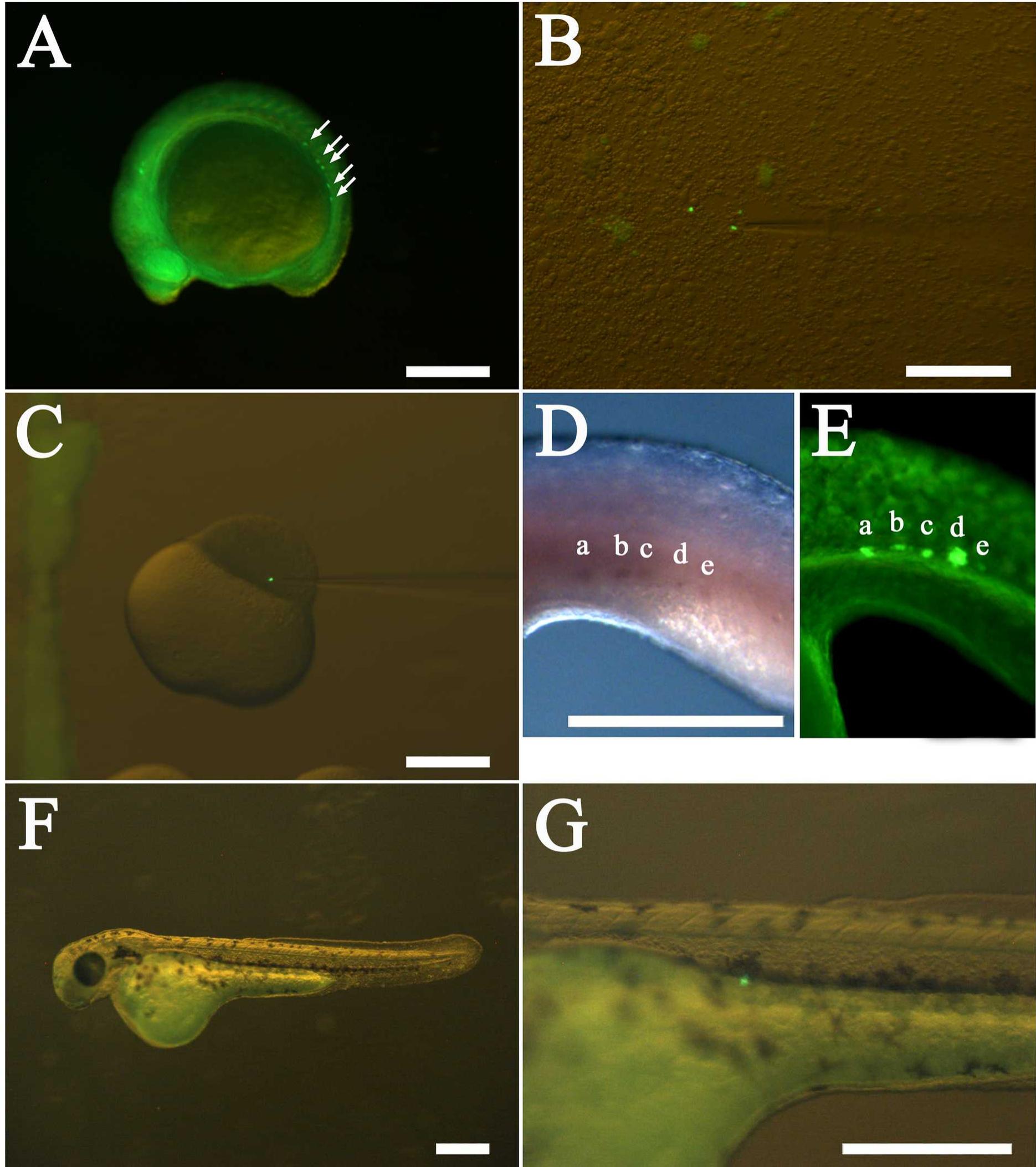
**Table 2** Appearance of somatic embryos with GFP-labeled PGCs injected with GFP-*ccnanos*-3'UTR or GFP-zebrafish *nos1*-3'UTR mRNA at 1–4 cells.

Construct	Injected embryo	Experiments	No. of injected embryos	No. of normal embryos	No. of embryos with GFP-labeled PGCs (%)
GFP- <i>ccnanos</i> -3'UTR	Common carp	1	101	72	53 (73.6)
		2	95	80	46 (57.5)
		3	91	78	38 (50.7)
	Zebrafish	1	40	36	33 (91.7)
		2	35	31	31 (100.0)
		3	61	58	56 (96.6)
	Goldfish	1	38	37	23 (62.2)
		2	42	37	25 (67.6)
		3	54	53	31 (58.5)
GFP-zebrafish <i>nos1</i> -3'UTR	Common carp	1	48	37	2 (5.4)
		2	50	40	4 (10.0)
	Goldfish	1	44	43	10 (23.3)
		2	47	46	11 (23.9)
		3	53	50	8 (16.0)

**Table 3** Localization of transplanted PGCs from common carp and/or goldfish embryos in host goldfish at 4 days post-transplantation at 20°C

Trial no.	Donor	Stage (donor)	No. of manipulated embryos	No. of normal embryos (%)	No. of embryos with PGCs at		
					Total (%)	Gonadal ridge (%)	Ectopic (%)
1	Common Carp	10–15 somite	158	148 (93.7)	140 (88.6)	44 (27.8)	96 (60.8)
2	Common Carp	15–20 somite	33	21 (63.6)	17 (51.5)	3 (9.1)	14 (42.4)
3	Common Carp	15–20 somite	75	34 (45.3)	19 (25.3)	8 (10.7)	11 (14.7)
4	Common Carp	15–20 somite	56	48 (85.7)	46 (82.1)	13 (23.2)	33 (58.9)
5	Common Carp	15–20 somite	50	22 (44.0)	22 (44.0)	5 (10.0)	17 (34.0)
Control							
6	Goldfish	10-15 somite	24	19 (79.2)	15 (62.5)	7 (29.2)	8 (33.3)
7	Goldfish	10-15 somite	10	10 (100.0)	10 (100.0)	4 (40.0)	6 (60.0)
8	Goldfish	15-20 somite	20	9 (45.0)	8 (40.0)	3 (15.0)	5 (25.0)

PGCs of donor embryos were labeled by injecting GFP-*ccnanos*-3'UTR mRNA



**Fig.1**

**A**

TCAGCGCGTGCTTCACGGTTCTGTTCGCACCTGGCGGATGTC 41

ATG GCA TTT TCT CTT CTC CAT TAC ATC TTG TCG GCT CAT GGA TCT ATG GAG TCT ACA 98  
M A F S L L H Y I L S A H G S M E S T 19

AAT CAG TAC TTT CAG CCC TGG AAG GAT TAC ATG GGT CTG GCA GAC ATG ATC AGA GGC 155  
N Q Y F Q P W K D Y M G L A D M I R G 38

ATG CAG CGG CCA GCA GAG CAG CCA GAC GCG CTC CTG GAG TCT CCA AGC GGC CCG ACG 212  
M Q R P A E Q P D A L L E S P S G P T 57

CGA GCG CAC GGG ACC CTG ACG ACG GAG AAA AGA GAC CCG GAA CGC GGC AAG AGC ACC 269  
R A H G T L T T E K R D P E R G K S T 76

CGC AGC AAC CCA TCC GAG AAG AAG TTC TGC AGC TTC TGC AAA CAC AAC GGA GAG ACC 326  
R S N P S E K K F C S F C K H N G E T 95

GAA GCC GTG TTC ACC TCG CAC TAC TTA AAA GAC CGC GCT GGA GAC GTG ACG TGC CCG 383  
E A V F T S H Y L K D R A G D V T C P 114

TAC TTG AGC CAG TAC GTG TGT CCC CTG TGC GGA GCC ACC GGG GCC AAA GCG CAC ACC 440  
Y L S Q Y V C P L C G A T G A K A H T 133

AAG AGA TTC TGC CCG CTC GTG GAC AAA ACC TAC AGC TCC GTG TAC GCC AAA TCA ACA 497  
K R F C P L V D K T Y S S V Y A K S T 152

TGG TGA ACCGGACGTTTCTGAACCGAAGAAGATCTGGCCACGGTATAACAAGAAGGACGTTTTTACGAGTAGTT 570  
W \* 153

TTAATATTCCAGTTTTAATTGTTCAATCCATAATGGCTTGTGTGTAAGTTTGCATGCATGTGTGCTTTTTTTTTTT 645

TTGGTGTGTTGATTTTTGCACGGTTTTTTGTCTTCCTCTTGTGTGCAGTGGTGTGTTTTTCACTCTAACAACTT 720

GTACACAAGCCAGTTGGCTTGCTACAGGTGCAACCACGTGTGAACTAGCGCTTCTTGTTAATTTTACTAAAAAA 795

AAAGTATCTTGTGATTAATCTGTGGTGAAATATATAAACGCTTTTAAATGCT 846

**B**

Common Carp	C	S	F	C	K	H	N	G	E	T	E	A	V	F	T	S	H	Y	L	K	D	R	A	G	D	V	T	C	P	Y	L	S	Q	Y	V	C	P	L	C	G	A	T	G	A	K	A	H	T	K	R	F	C
Zebrafish 1	C	S	F	C	K	H	N	G	E	T	E	A	V	Y	T	S	H	Y	L	K	N	R	D	G	D	V	M	C	P	Y	L	R	Q	Y	K	C	P	L	C	G	A	T	G	A	K	A	H	T	K	R	F	C
Zebrafish 2	C	V	F	C	R	N	N	G	A	P	E	E	V	Y	G	S	H	V	L	K	T	P	D	G	R	V	V	C	P	I	L	R	A	Y	T	C	X	L	C	S	A	N	G	D	N	A	H	T	I	X	Y	C
Medaka 1	C	V	F	C	R	N	N	G	A	P	E	E	V	Y	G	T	H	I	L	K	T	G	E	G	R	V	L	C	P	I	L	R	A	Y	T	C	P	L	C	S	A	N	G	D	N	A	H	T	I	K	Y	C
Medaka 2	C	S	F	C	R	H	N	G	E	S	E	M	V	Y	R	S	H	W	L	K	N	Q	K	G	D	V	L	C	P	Y	L	R	Q	Y	V	C	P	L	C	G	A	T	G	A	K	A	H	T	K	R	F	C
Atl.Salmon1	C	V	F	C	R	N	N	G	A	P	E	E	V	Y	G	S	H	V	L	K	T	P	D	G	G	V	V	C	P	I	L	R	A	Y	T	C	P	L	C	S	A	N	G	D	N	A	H	T	I	K	Y	C

Fig.2

