Effects of reproductive stage, GH, and 11-ketotestosterone on expression of growth differentiation factor-9 in the ovary of the eel, *Anguilla australis*

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Abstract

In order to study the regulation of the growth differentiation factor-9 (*gdf9*) gene in a primitive teleost with semelparous life history, we cloned a cDNA encoding shortfinned eel Gdf9, expressed a partial peptide in *Escherichia coli*, and raised an antiserum to evaluate changes in Gdf9 expression during its pituitary homogenate-induced reproductive cycle. The effects of *in vivo* and *in vitro* exposure to the androgen 11-ketotestosterone (11-KT), known to affect previtellogenic (PV) oocyte growth, were also determined. Furthermore, we investigated whether Gdf9 expression was metabolically gated by treating PV fish with recombinant GH *in vivo*. Immunoreactive proteins of ca. 52 and 55 kDa were identified by western blot analysis. Gdf9 message and protein were most abundant in PV oocytes, and peaked slightly earlier for mRNA than for protein. Captivity resulted in reduced *gdf9* mRNA levels, which were restored following pituitary homogenate treatment. As oocytes progressed through induced oogenesis, Gdf9 expression decreased. Neither 11-KT nor GH treatment affected *gdf9* mRNA levels in PV fish, although GH could partially restore handling- or captivity-induced decreases in *gdf9* mRNA levels. Semelparous eels thus show an expression pattern of Gdf9 during oogenesis that is similar to that seen in other vertebrates, that appears responsive to handling or captivity stress, and whose control remains to be elucidated.

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Introduction

Female teleosts support the early ontogeny of their offspring by provisioning eggs with a variety of nutrients, such as yolk and lipids, and developmental instructions via maternal mRNAs. During oocyte development, growth is achieved primarily by yolk protein accumulation; however, substantial growth occurs well ahead of this period, and is characterized, among others, by intense RNA synthesis, increased abundance of ribosomes and mitochondria, and evidence of crosstalk through microvilli between oocyte and granulosa cells (reviewed in Le Menn *et al.* (2007)). The role played by the endocrine system in previtellogenic (PV) growth has only recently become topical (e.g. Campbell *et al.* 2006, Lokman *et al.* 2007, Luckenbach *et al.* 2008). Moreover, information on up- or down-regulated genes during early stages of oogenesis is only beginning to emerge (e.g. Wen *et al.* 2003, Luckenbach *et al.* 2008).

In mammals, the early growth of ovarian follicles, an event arguably analogous to PV oocyte growth, is likely to be mostly regulated by paracrine and autocrine factors

(Matzuk 2000, McGee & Hsueh 2000). Furthermore, this growth phase appears to be gonadotropin independent, a conclusion supported by the phenotype (development of multi-layered primary follicles up to the preantral stage only) presented by FSH (Kumar *et al.* 1997) or FSH receptor (FSHR; Danilovich *et al.* 2001) knockout mice. In contrast, preantral follicular development in mice was dramatically impaired when the gene growth differentiation factor-9 (*Gdf9*, encoding a member of the transforming growth factor- β (TGF β), superfamily) was knocked out; this was reflected by the absence of normal follicles with more than one layer of granulosa cells in *Gdf9* knockout mice (Matzuk 2000). Indeed, notwithstanding some species-specific observations, the generic function of GDF9 appears to be its involvement in regulating granulosa cell proliferation during early folliculogenesis in mammals (e.g. Vitt *et al.* 2000, Gilchrist *et al.* 2006) and chicken (Johnson *et al.* 2005). Moreover, *GDF9* has been dubbed the 'fecundity gene' due to the increased ovulation rates that are seen when its bioavailability is reduced, for instance

following immunization to GDF9 (McNatty *et al.* 2003, Juengel *et al.* 2004) or in response to a mutation of the *GDF9* gene (c.f. Hanrahan *et al.* 2004, Moore *et al.* 2004). A phenotype of increased fecundity is probably realized via reduced suppression of *fshr* gene transcription, resulting in increased sensitivity to FSH (Moore *et al.* 2004). Similar high-fecundity phenotypes have also been described in some mammals with reduced bioavailability of bone morphogenetic protein-15 (BMP15), the closest sister peptide to GDF9 (Dube *et al.* 1998), while in mice lacking *Bmp15*, a subfertile phenotype was seen (—/— by knockout; Yan *et al.* 2001). Interestingly, both these genes are repressed by germ cell nuclear factor, an orphan nuclear receptor (Lan *et al.* 2003). Other local signaling molecules that have been implicated in progression of early follicular development were recently reviewed by Skinner (2005) and include the likes of kit ligand/stem cell factor, several members of the TGF β superfamily, and fibroblast growth factor-like peptides.

Although an involvement of several growth factors in regulating aspects of previtellogenesis has been shown or suspected on the basis of expression profile, functional evidence appears to be limited to insulin-like growth factor 1 (Igf1; Lokman *et al.* 2007). A role for Gdf9 is expected, given that mRNA levels are highest during the PV phase in both zebrafish (Danio rerio; Liu & Ge 2007) and seabass (*Dicentrarchus labrax*; Halm *et al.* 2008). However, functional studies on these peptides have not as yet been conducted, while regulation of *gdf9* gene expression is limited to a demonstrated inhibitory effect of LH in zebrafish, a model fish species with asynchronous ovaries. It is unclear whether or not the gene expression profile seen in zebrafish is species specific or reproductive strategy specific.

We aimed to determine whether the expression pattern of the *gdf9* gene in the fish ovary is related to reproductive strategy and whether its expression is affected by two hormones with demonstrated effects on PV ovaries (Lokman *et al.* 2007), i.e. 11-ketotestosterone (11-KT) and IGF1. We therefore analyzed *gdf9* mRNA levels in wild and artificially maturing eels, *Anguilla* spp., fish believed to exhibit semelparity. Furthermore, we investigated the *in vivo* and *in vitro* effects of 11-KT on *gdf9* gene expression. Finally, we indirectly tested the hypothesis that metabolically gated interaction between the nutritional status and reproduction is mediated by Igf1, by subjecting eels to treatment with GH, an Igf1 secretagogue.

Results

Isolation of shortfinned eel *gdf9* cDNA, *gdf9* gene structure and tissue distribution of *gdf9* mRNA

The shortfinned eel *gdf9* cDNA comprises 2163 bp, encoded by two exons (Fig. 1). A 253 bp intron with consensus splicing sites (exon 1-AG|GU....AG|AG-exon 2) separated these exons at base 400. The cDNA

Figure 1 Gene and protein structure of eel (*Anguilla australis*) growth differentiation factor-9 (Gdf9). A cDNA sequence of 2163 bp was obtained, encoded by two exons. The single intron was 230 bp in length and had consensus nucleotide sequences at the deduced splicing site. The cDNA encoded a prohormone of 430 amino acids, and a putative signal peptide (SiP) of 24 residues was identified using protein analysis software (ExPASy Tools). A basic cleavage site (RXXR) was found, enabling the release of mature Gdf9 of 131 amino acids.

could be translated into a protein of 430 amino acids (predicted 49 kDa) when using the second ATG as the start codon; accordingly, a signal peptide of 24 or 27 amino acids was predicted by PROSITE and SOSUI respectively (Fig. 1). Typical BMP family characteristics were retained, given the recognized TGF β domain (BLAST searching) and the basic furin RXXR cleavage site (Benoit *et al.* 1987). Cleavage after RWRR, between residues 299 and 300, would yield a mature C-terminal peptide of 131 amino acids and a predicted MW of 14.8 kDa; a second possible site with cleavage after RSRR (between residues 192 and 193) was also identified. In accord with other GDF9 orthologs, sfGdf9 was found to contain six cysteine residues in conserved positions.

When submitting the mature peptide sequence for analysis by ExPASy Tools, the N-terminal serine residue (Ser⁵) was identified as a possible O-glycosylation site; N-glycosylation sites were not predicted. By contrast, the prohormone yielded two possible N-glycosylation sites, i.e. NLTC (residues 220–223) and NDTS (254–257), while the threonine at position 33 was predicted to be a possible site for O-glycosylation. Alignment of the predicted mature peptide with GDF9 and BMP15 sequences from human, zebrafish, and chicken, using eel anti-Müllerian hormone as the outgroup, saw sfGdf9 cluster with other GDF9, rather than with other TGF β -like, peptides (Fig. 2).

Tissue distribution

gdf9 mRNA could be detected by quantitative PCR (qPCR) in a variety of tissues (Table 1), but was primarily expressed in the ovary. Indeed, relative transcript levels were estimated to be at least 100-fold higher in ovary than in most other tissues examined, with the notable exception of the thyroid gland (5% of levels in the ovary)

Figure 2 Phylogenetic relationships between the mature peptides for growth differentiation factor-9 (GDF9) and bone morphogenetic protein-15 (BMP15) homologs from eel (*Anguilla australis*), zebrafish (*Danio rerio*), seabass (*Dicentrarchus labrax*), chicken, and human. The tree was constructed on the basis of the unweighted pair group method with arithmetic mean, using Japanese eel (*A. japonica*; AB074569) anti-Müllerian hormone (Amh) as outgroup. Peptide sequences were deduced from translated nucleotide sequences, and the C-terminus after the RXXR basic cleavage site (~120–130 amino acid residues in length) was assumed to represent the mature, biologically active hormone. Accession numbers were as follows: NM_001012383 (zebrafish Gdf9); AM933667 (seabass Gdf9); AY566700 (chicken GDF9); NM_005260 (human GDF9); NM_001020484 (zebrafish Bmp15); AM933668 (seabass Bmp15); AY725199 (chicken BMP15); NM_005448 (human BMP15).

and skeletal muscle (10% of ovary). Small amounts (50–500-fold lower than ovary) of mRNA encoding Gdf9 were also observed in a number of classical endocrine glands (brain, pituitary, interrenal, and testis).

Production of recombinant partial eel proGdf9 in *Escherichia coli* and production of polyclonal antiserum

Affinity chromatography resulted in the effective removal of most proteins other than immunoglobulins (Fig. 3a and b). These purified IgGs, when used for western blot analysis, detected two ovarian proteins with approximate molecular weights of 52 and 55 kDa (Fig. 3b). Glycosidase treatment did not result in a shift in molecular weight of either peptide (data not shown).

Localization of gdf9 mRNA

The signal for gdf9 mRNA was strongest in oocytes in the oil droplet stage (Fig. 4a and b), regardless of whether fish were treated with salmon pituitary homogenate

(SPH) or NaCl (data not shown). In early vitellogenic (EV) oocytes, message was detectable, but at low signal intensity (Fig. 4b). In contrast, sense cRNA did not yield any signal (Fig. 4c).

Experimental findings

Experiment I: ovarian gdf9 mRNA levels in wild shortfinned eels

Gonadosomatic indices (GSI) in EV ($GSI_{2005} = 3.11 \pm 0.11\%$; $n=8$; $GSI_{2006} = 3.07 \pm 0.23\%$; $n=6$) eels were over tenfold higher than those in PV eels ($GSI_{2005} = 0.20 \pm 0.35\%$; $n=8$; $GSI_{2006} = 0.24 \pm 0.06\%$; $n=6$). gdf9 mRNA levels (expressed per microgram RNA) were comparable in eels of either life-history stage ($F_{1,24} = 0.855$; $P=0.364$), irrespective of year of collection ($F_{1,24} = 1.263$; $P=0.272$). Immunohistochemical analysis revealed that Gdf9 was most strongly expressed in oocytes at the end of the primary growth phase, just preceding entry into vitellogenesis (Fig. 5a–d).

Experiment II: ovarian gdf9 gene expression in artificially maturing shortfinned and Japanese eels

Pretreatment and saline-injected controls were grouped for the statistical analysis, as no differences between the different controls could be detected ($F_{3,7} = 0.49$; $P=0.72$). Subsequent analysis of relative gdf9 transcript means revealed that gdf9 mRNA levels in shortfinned eel ovaries varied with reproductive stage ($F_{3,19} = 4.04$; $P<0.05$), peaking in the EV stage (Fig. 6). However, statistically significant differences in gdf9 mRNA levels could not be detected between stages

Table 1 Tissue distribution of mRNA encoding growth differentiation factor-9 (Gdf9) in previtellogenic shortfinned eel, *Anguilla australis*.

Tissue	GDF9 mRNA (fg/100 pg ELF)
Anterior brain	1.09
Midbrain	0.57
Posterior brain	1.25
Pituitary	1.87
Eye	0.28
Gill	0.37
Heart	0.48
Liver	0.26
Spleen	0.09
Interrenal	8.50
Mesonephros	0.74
Posterior gut	0.91
Intestine	0.47
Muscle	47.85
Ovary	476.28
Thyroid	24.16
Testis (immature)*	2.12
Testis (matured)*	1.48

For comparison, testicular tissue from an immature and fully artificially matured male (*) is included. cDNA encoding Gdf9 (50 ng) was amplified, quantified using real-time quantitative PCR, and standardized over transcript abundance for elongation factor-1 α (Elf).

Figure 3 Affinity purification of immunoglobulins (Ig) by protein G and western blot analysis for growth differentiation factor-9 (Gdf9). Gels were stained with Coomassie Brilliant Blue (a) or transferred to PVDF membrane and probed with anti-Gdf9 Ig and alkaline phosphatase-conjugated goat anti-rabbit IgG (b). Samples: MW, Novex Sharp Protein Standards (Invitrogen), expressed in kDa. Panel a: L1, whole antiserum; L2, affinity-purified Ig (~50 kDa); L3, homogenate of eel (*Anguilla australis*) previtellogenic ovarian tissue (~80 µg). Panel b: L1, affinity-purified rabbit Ig; L2, eel ovarian homogenate.

($P_{EV \text{ versus } MV} = 0.054$) when conducting Scheffé's conservative *post hoc* test due to the wide variation within the EV group and the reduced expression of elongation factor-1 α (*elf*) at more advanced stages of development. Indeed, a clear separation was noticeable within the EV group between eels treated for 2 weeks compared with those treated for 4 weeks ($F_{1,4} = 10.48$; $P < 0.05$); when separating these EV groups and repeating the ANOVA, a highly significant result was obtained, and could be attributed to differences between EV_{2 weeks} and both mid vitellogenesis (MV) and migratory nucleus stage (MN).

Unexpectedly, relative *gdf9* mRNA levels did not change significantly between ovarian follicles isolated and sieved into size classes ($P > 0.05$; data not shown).

In vivo qPCR trends were reinforced by immunohistochemical analyses; thus, oocytes in early stages of development (i.e. ovaries from pretreatment and saline-injected controls) stained more intensely than did those in later stages of oogenesis (Fig. 7a), while signals were essentially undetectable in migratory nucleus stage oocytes (Fig. 7b). Furthermore, staining appeared to be confined to oocytes.

Experiment III: *in vivo* and *in vitro* effects of 11-KT on ovarian *gdf9* mRNA levels in shortfinned eel

Notwithstanding the death of one 11-KT treated fish, eels were vigorous and in good condition by the end of the

experiment. Treatment of eels with 11-KT for 2 weeks resulted in notable changes in morphology; indeed, by as little as 5 days of treatment, pectoral fins had turned pitch black (c.f. Lokman *et al.* 2003), as opposed to the brownish translucent pectoral fins of controls. Effects were also manifested internally, as seen by a significantly higher GSI ($F_{1,11} = 9.493$; $P < 0.01$) for eels treated with 11-KT ($0.42 \pm 0.033\%$; $n = 6$) than that for controls ($0.26 \pm 0.037\%$; $n = 7$). However, oocytes from androgen-treated PV female eels were not markedly different from those of control fish in terms of general cytology or in oocyte diameter. Androgen-treated fish averaged around 25.4 copies *gdf9* mRNA/ 10^3 copies *elf* mRNA, not significantly lower ($P = 0.253$) than the average value of around 29.6 copies *gdf9* mRNA/ 10^3 copies *elf* mRNA in control fish (data not shown). *In vitro* incubation of ovarian fragments with or without 11-KT similarly yielded slightly lower mean *gdf9* mRNA values for androgen-treated fish, but differences were not statistically significant ($F_{1,5} = 5.375$; $P = 0.068$; data not shown).

Experiment IV: *in vivo* effects of GH on ovarian *gdf9* mRNA levels in Japanese eel

Treatment of juvenile eels at 3-day intervals for 12 days resulted in reductions in body weight (BW) of, on average, around 10% in all groups. Despite the size of the animals, gonad tissue, albeit small, could be successfully found in all fish. Histology revealed that oocytes were in the early oil droplet stage and measured around 50 µm in diameter, regardless of treatment. Bioactivity of rjeGH was confirmed by significant effects on liver *igf1* mRNA levels, which were significantly reduced to around half in both groups of fish treated with GH compared with pretreatment and saline-injected controls ($F_{3,25} = 8.194$; $P < 0.05$; data not shown). In the ovary, *gdf9* mRNA levels did not differ between treated fish (Fig. 8); however, treatment itself (handling and manipulation) appeared to affect *gdf9* mRNA levels as

Figure 4 *In situ* hybridization analysis for growth differentiation factor-9 (Gdf9) in the early vitellogenic ovary of the eel, *Anguilla australis*. Adjacent sections were stained with hematoxylin and eosin (a), or probed with anti-sense (b) or sense cRNA encoding Gdf9 (c). Signal intensity is strongest for small oocytes in the oil droplet stage (b). Bar: 100 µm.

Figure 5 Micrographs of previtellogenic (a and c; gonadosomatic index 0.35%) and early vitellogenic (b and d; gonadosomatic index 2.46%) ovarian tissue from wild-caught shortfinned eel, *Anguilla australis*. Sections were either stained with hematoxylin and eosin (a and b) or immunostained for growth differentiation factor-9 (c and d). Note that although the sections are adjacent, corresponding parts of the adjacent sections could not be found. F, fat; PV, previtellogenic oocyte; EV, early vitellogenic oocyte. Bar: 100 μ m.

reflected in lower relative abundance in control-injected fish compared with non-injected, pretreatment controls ($F_{3,25}=3.622$; $P<0.05$). Three samples were lost due to escape or due to an accident with snap-freezing in liquid nitrogen.

Discussion

Control of PV oocyte growth in oviparous vertebrates has recently become of interest, yet very few key players have been identified to date. In mammals, an important role has been identified for the BMPs GDF9 and BMP15. We therefore aimed to investigate the pattern and control of *Gdf9* expression in a primitive teleost fish, the eel.

A cDNA was isolated whose deduced amino acid sequence had high homology to mature GDF9 of other vertebrate species. Moreover, a number of key conservations, notably the alkaline cleavage site RWRR, the number of cysteine residues, the TGFB domain and the gene structure (two exons, 1 intron), provided further indication that the cloned cDNA corresponded to that

encoding *Gdf9*. Following the induction of recombinant *Gdf9* synthesis by *Escherichia coli*, an antiserum was obtained that recognized proteins with molecular weights of 52 and 55 kDa. These weights are very comparable with that of the deduced prohormone (49 kDa). In contrast, the mature monomer, predicted to weigh 14.8 kDa, was not detected; it is plausible that the 35 amino acids from the mature monomer sequence, which were included into the recombinant peptide (antigen for antibody generation), were insufficient for antibody generation. Indeed, the use of an epitope predictor (BepiPred; Larsen *et al.* 2006) reinforced the notion that the N-terminus of the mature monomer is only weakly immunogenic, and that this immunogenicity spans the RWRR furin cleavage site, i.e. the weak epitope is quite possibly lost following furin cleavage.

Glycosylation of GDF9 has been proposed (McGrath *et al.* 1995, Hayashi *et al.* 1999). Elvin *et al.* (1999) considered it likely that a 21 kDa GDF9 immunoreactive band corresponded to *N*-glycosylated mouse GDF9, as compared with the unprocessed GDF9 peptide that was predicted to weigh 15.6 kDa. A second mouse peptide of 60 kDa was predicted to correspond to glycosylated

Figure 6 Transcript abundance for growth differentiation factor-9 (*gdf9*) in the ovary of the shortfinned eel (*Anguilla australis*) during artificial induction of maturation by salmon pituitary homogenate (SPH). *gdf9* mRNA levels were determined by real-time quantitative PCR, and normalized over mRNA levels encoding elongation factor-1 α (*elf*). Controls (CTRL) sampled at different time points (pretreatment and following different number of injections with Ringer) were not statistically different and therefore grouped together. Data are presented as means $\pm$ S.E.M., and sample sizes are given at the base of each bar. Significant differences between means do not share the same lettering above bars ($P<0.05$). PV, previtellogenic; EV₂, early vitellogenic eels treated for 2 weeks following injections with SPH; EV₄, early vitellogenic eels treated for 4 weeks following injections with SPH; MV, midvitellogenic; MN, migratory nucleus stage.

Figure 7 Micrographs of ovarian tissue from cultivated, artificially maturing Japanese eel, *Anguilla japonica*. Fish were sampled at different developmental stages, e.g. saline-injected controls in previtellogenesis (a and d; gonadosomatic index, GSI = 1.44%), or salmon pituitary extract-injected eels in early (b and e; GSI = 4.14%) or late vitellogenesis (c and f; GSI = 19.28%). Sections were either stained with hematoxylin and eosin (a–c) or immunostained for growth differentiation factor-9 (d–f). Bar: 100 μ m.

GDF9 prohormone. Similarly, mature porcine GDF9 was estimated at ~ 20 kDa (Li *et al.* 2008), while that of rhesus monkey was ~ 22 kDa (Duffy 2003). The Asn–Leu–Ser motif, a conserved putative *N*-glycosylation site in mature tetrapod GDFs, was absent in both zebrafish (Liu & Ge 2007) and eel, but two putative *N*-glycosylation sites (NLTC^{220–223}; NDTS^{254–257}) and a possible *O*-glycosylation site were predicted for the eel at Ser³⁰⁴. *O*-glycosylation was recently reported for recombinant human BMP15 (Saito *et al.* 2008). However, exposure to variety of glycosidases did not result in a shift in molecular weight, suggesting that eel Gdf9 is not glycosylated.

Relative mRNA levels encoding Gdf9 were highest in the ovary, but notable message was, unexpectedly, also obtained in skeletal muscle. Within the ovary, strong *in situ* hybridization and immunohistochemical signals were obtained in the oocyte, while granulosa and other somatic cell layers appeared unstained. The latter finding remains tentative, however, as it is impossible to separate the oocyte from surrounding follicle cells, and absence of staining in the squamous granulosa cells is difficult to ascertain conclusively. Oocyte-specific localization patterns largely similar to those observed for the eel have been obtained for GDF9 in other vertebrates, both oviparous (e.g. zebrafish, Liu & Ge 2007; chicken, Johnson *et al.* 2005; seabass, Halm *et al.* 2008) and mammalian, and our findings in that regard are therefore merely confirmatory in nature. Our immunohistochemical data further suggest that staining of the oocyte was most intense in the PV stage, just prior to puberty, and

that expression was marginally detectable in fully-grown oocytes. Using *in situ* hybridization, a comparable signal was seen, but abundance peaked in the oil droplet stage, rather than the stage just preceding puberty. Our qPCR data broadly agree with this picture, as *gdf9* gene expression was comparable in PV and EV eels; however, it should be noted that PV fish from the wild were in a very immature stage, and it is plausible that *gdf9* mRNA levels could increase with advancing PV oocyte growth stage. These findings are somewhat different from those reported for zebrafish, in which mRNA levels were highest in the primary growth phase (Liu & Ge 2007), and from seabass, in which *gdf9* gene expression remains roughly constant throughout PV oocyte growth (Halm *et al.* 2008).

Determination of the function(s) of GDF9 in the ovary of oviparous vertebrates remains. We transfected the *Drosophila* S2 embryonic macrophage cell line with a construct encoding mature eel Gdf9, but recombinant protein could not be obtained. Likewise, attempts to generate recombinant zebrafish Gdf9 have, to date, been unsuccessful (Wei Ge, University of Hong Kong, personal communication, June 2008). Furthermore, very little is known about the regulation of *gdf9* gene expression. To address this issue, fish were exposed for 2 weeks to 11-KT, an androgen with notable effects on PV eel oocytes in terms of lipid accumulation and growth (Rohr *et al.* 2001, Lokman *et al.* 2007). No effects of the androgen on *gdf9* gene expression in the eel were noted. While comparable studies on possible effects of androgen on *GDF9* gene expression do not

Figure 8 Growth differentiation factor-9 (*Gdf9*) mRNA levels in ovarian tissue of juvenile eel, *Anguilla japonica*, after treatment with saline (ctrl) or 500 ng/g high (hi GH) or low molecular weight recombinant eel GH (lo GH) for 12 days. Injections were made every third day. An untreated group of fish (pre) was sampled on day 0. *Gdf9* transcript levels were normalized over those of elongation factor-1 α (Elf). Data are expressed as means $\pm$ S.E.M. Significant differences between groups are indicated by an asterisk ($P < 0.05$).

appear to have been conducted previously, a notable stimulatory effect of androgen on GDF9-mediated proliferation of porcine granulosa cells has been reported (Hickey *et al.* 2005).

To investigate whether *gdf9* gene expression was metabolically gated, we treated juvenile eels with oocytes in the early oil droplet stage with the Igf1 secretagogue rjeGH as a proxy for changed metabolic status. To ensure that this treatment was effective, hepatic *igf1* mRNA levels were measured 3 days after the fourth injection of rjeGH; surprisingly, a decrease in *igf1* mRNA levels was observed, which is clearly not in keeping with the well-recognized role of GH as an inducer of Igf1 transcription and secretion. However, in many of the studies aimed at assessing the effects of GH, hepatic *igf1* mRNA measurements were made within hours of GH administration. A recent study by Moriyama *et al.* (2006) on effects of recombinant GH in glass eel of *Anguilla japonica* and by Ponce *et al.* (2008) on redbanded seabream, *Pagrus auriga*, has revealed that increased hepatic mRNA levels of *igf1* were maintained for only up to 1 day post GH treatment. A similar trend was obtained in perch, *Perca fluviatilis*, in which GH failed to elicit any changes at all with regard to hepatic *igf1* mRNA (Jentoft *et al.* 2004); when measurements were made at later time points, GH effects were typically not significant, e.g. in rainbow trout (*Oncorhynchus mykiss*; Shablott *et al.* 1995) and catfish, *Ictalurus punctatus* (Peterson *et al.* 2005). Why a reduction in *igf1*

mRNA levels was seen in the eel is not clear, but it is tempting to speculate that negative feedback by rjeGH-induced Igf1 on endogenous GH stores within 1 day of injection may have led to decreased *igf1* mRNA levels by day 3 after injection when measurements were made.

Regardless of the mechanisms responsible for reduced liver *igf1* mRNA levels following rjeGH administration, differences in GH/Igf1 status between treatment groups did not result in changed *gdf9* mRNA levels in the ovary. Interestingly, however, a significant reduction in *gdf9* mRNA levels was observed in the saline-injected controls compared with pretreatment controls (day 0), suggesting that handling stress (injections every third day for 12 days) or time as a factor affected *gdf9* gene expression. The interaction between stress and ovarian *Gdf9* remain essentially unexplored, although a recent study has implicated low doses of environmental pollutants as responsible for reduced GDF9 protein levels in fetal sheep ovaries (Fowler *et al.* 2008). Our present data further indicate that the decrease in *gdf9* mRNA levels in saline-injected controls could be prevented, at least in part, by GH treatment. In this context, it is of interest to emphasize that *gdf9* mRNA levels also decreased in EV shortfinned eels when fish were held captive prior to induction of vitellogenesis, and that mRNA levels encoding *Gdf9* rapidly increased following SPH treatment (Experiment II). It is tempting to speculate that the observed decrease in *gdf9* mRNA levels in both experiments and their subsequent (partial) restoration following rjeGH and SPH treatment respectively hinge on similar mechanisms. Further studies are warranted to explore the effects of nutrition and stress on *gdf9* gene expression in more detail.

In conclusion, *Gdf9* has been demonstrated at the protein and mRNA level in oocytes of an oviparous vertebrate, the eel, although function could not be ascertained due to the unavailability of isolated native or recombinant *Gdf9*. Expression appears highest preceding the onset of puberty, and then declines. Regulation of *gdf9* gene expression is independent of 11-KT, but GH, or its mediator Igf1, appears to interact with a stress-mediated reduction in *gdf9* mRNA levels in juvenile eels.

Materials and Methods

Animals

Shortfinned eels (*Anguilla australis*) were either caught by fyke nets set overnight ('wild eels') or purchased from Gould Aquafarms (Leeston, New Zealand), a commercial eel processor. Japanese eels (*A. japonica*) were obtained from an eel farm, feminized in the glass eel stage (Ijiri *et al.* 1998), and reared on commercial dry feeds.

Experiments and design

Isolation of shortfinned eel *gdf9* cDNA, *gdf9* gene structure and tissue distribution of *gdf9* mRNA

Ovarian tissue was collected from an EV ('silver') shortfinned eel and total RNA isolated with Invitrogen's Trizol Reagent. One microgram of total RNA was reverse transcribed into cDNA using Superscript II (Invitrogen). Primers were designed on the basis of regions of GDF9 that were conserved (GSPVHTM and EDMVAST) among mouse (accession number AAA53035), rat (AF099912), sheep (O77681), goat (AAU09020), bovine (AF307092), pig (AAT67460), human (CA488820), chicken (NP_996871), and fugu (CAF99068), resulting in the following degenerate primers: forward (FW): 5'-GGNWSNCCNGTNCAYACNATGGT-3'; reverse (RV): 5'-CANCKNGTNGCNACCATRTCYTC-3'. DNA amplification was done (Roche) by gradient polymerase chain reaction (PCR; 45–58 °C) on an MJ Research (Watertown, MA, USA) thermal cycler and the amplicon (expected size 170 bp) ligated into pGEM T-Easy vector (Promega Corp.) before amplification of constructs in transformed *E. coli* strain XL-1. Plasmid vectors were isolated using QIAGEN Plasmid Mini Kit (Qiagen GmbH) and prepared for automated dideoxy sequencing (Allan Wilson Centre, Palmerston North, New Zealand). The entire mRNA sequence was obtained using RACE, following construction of SMART RACE or Marathon cDNA libraries (Clontech) according to the manufacturer's instructions. Libraries were constructed using ovarian total RNA from a shortfinned eel in the PV (SMART 3'- and 5'-RACE) or the EV stage (SMART 5'-RACE). As difficulties in amplifying 5'-RACE amplicons were encountered, an additional library (Marathon cDNA Amplification Kit, Clontech) was constructed using poly-A+ RNA from testis from a male shortfinned eel (BW 129 g) in early spermatogenesis; spermatogenesis was induced by single treatment with 1000 U/kg human chorionic gonadotropin and testicular tissue retrieved following death 9 days later.

3'-RACE PCR. The 3'-end of the *gdf9* transcript was amplified using the gene-specific primer (GSP-1) 5'-GCTGGACTCCT-CAGTACCTAGGCCCT-3' (1.5 µl; 10 µM; see Fig. 9) and the SMART Kit universal primer mix (UPM; 1 µl). The reaction mixture also contained 1 µl dNTPs (10 mM), 3 µl 10× reaction buffer and 0.6 µl 50× 'Advantage 2' Taq polymerase in a final volume of 30 µl. Conditions for 3'-RACE were as follows: 94 °C

Figure 9 Strategy used to amplify the cDNA encoding eel (*Anguilla australis*) growth differentiation factor-9 (Gdf9). Using ovarian cDNA, a product was amplified by degenerate PCR and sequenced (1). Successive 3'- or 5'-RACE PCRs were used (2–5) to construct the full cDNA sequence. A near-complete sequence was then amplified using primers at the 5'- and 3'-ends (6).

for 1 s, 40 cycles of 94 °C for 5 s, (68–0.5×cycle number)°C for 10 s and 72 °C for 3 min, followed by a final extension step of 68 °C for 15 min. PCR products were electrophoresed, excised from the gel, and prepared for sequencing as described above.

5'-RACE PCR. Upstream sequence data were obtained using the same reagents, but using GSP-2, 5'-GGCAATGGAGC-CATCCTTTTCAATGGT-3' (Fig. 9), on a SMART 5'-RACE cDNA library (PV eel). Temperature parameters were identical to the manufacturer's program 1, using an extension time of 2 min. The same protocol, but with a 4 min extension, was then run on the Marathon cDNA library with arbitrary primer AP2 and GSP-3, 5'-TGAATCCCATGGCCCTTGGACAAAT-3' (Fig. 9), to yield a further 813 bp. A third 5'-RACE PCR yielded the 5'-UTR when using the SMART 5'-RACE library constructed from EV ovarian tissue and primed with UPM and GSP-4, 5'-CGCGCGTCACGAGTTTCAGAAAGTAT-3' (Fig. 9), for 2 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min, and a final extension step of 72 °C for 3 min. To confirm that the RACE PCR products overlapped and represented a single transcript, sfGDF9-FULL FW, 5'-CGAGGGCTGATCTTTGAGAC-3', and sfGDF9-FULL RV, 5'-TGCTTGCTTTTATTCTTTCCA-3' (Fig. 9) were used, and six clones sequenced. It is noted that the PCR product obtained from this reaction misses 3 bp of the 5'-UTR and 45 bp of the 3'-UTR due to difficulties in obtaining a PCR product when using primers further upstream or downstream respectively.

***gdf9* gene structure.** Genomic DNA was extracted from frozen ovarian tissue from EV shortfinned eel using QIAGEN's Blood & Cell Culture DNA Mini Kit according to the manufacturer's instructions. The *gdf9* gene structure was revealed using sfGDF9-FULL FW and RV.

Tissue distribution of *gdf9* mRNA. A variety of tissues were collected from a PV shortfinned female, and testes from an immature and a maturing male and subjected to RT and qPCR analyses according to the methodology outlined in the section 'RNA isolation, cDNA synthesis, and quantitative real-time PCR analysis of *gdf9* mRNA'.

DNA sequence analyses. DNA sequence information was evaluated using freely downloaded software of CLC Sequence Viewer 4. Exon–intron boundaries were identified after aligning cDNA and genomic DNA sequences and after confirming consensus splicing sites. The deduced amino acid sequence was subjected to SOSUI (classification and secondary structure prediction system for membrane proteins; <http://bp.nuap.nagoya-u.ac.jp/sosui/>) and PROSITE (<http://www.expasy.ch/prosite/>) for characterization of the peptide, while Clustal was used to evaluate phylogenetic relationships between different GDF9 orthologs.

Production of recombinant partial eel proGdf9 in *E. coli* and production of polyclonal antiserum

We aimed to generate recombinant protein for use in antiserum production in such a way that i) specificity of the antiserum for Gdf9 could be ensured and that ii) the antiserum could be used for immunohistochemical analyses. A target sequence of 132

amino acid residues in the mid-part of the prohormone was chosen, representing 93 residues upstream of the RXXR TGFB consensus cleavage site and the N-terminal 35 amino acids of the mature peptide downstream of the cleavage site. The highly conserved C-terminus seen among TGFB superfamily members could thus be avoided. Accordingly, a cDNA encoding a partial proGdf9 was amplified by PCR (FW 5'-**GGATCCTTCTC-CACCCCTTATT**-3'; RV 5'-**AAGCTTGAAGTCATACAAGC-CACA**-3'; restriction enzyme overhangs represented by bold italic lettering) and cloned in frame into the BamHI and HindIII sites of the pQE30 vector (Qiagen) containing instructions for addition of the HHHHHHGS octapeptide at the N-terminus, and the KLN sequence at the C-terminus. The construct was transfected into *E. coli* strain M15, and, following identification of transformed colonies, protein expression induced using IPTG, according to the manufacturer's instructions (QIAexpressionist, Qiagen). Recombinant protein (reGdf9) was recovered from inclusion bodies using BugBuster (Novagen) before affinity purification for histidine repeats by Ni-NTA agarose column chromatography (Qiagen). Fractions were eluted with 8 M urea in phosphate buffer (10 mM Tris-HCl in 0.1 M sodium phosphate of varying pH), and aliquots displayed on a Coomassie Brilliant Blue (CBB) were stained with 15% acrylamide gel containing 10% SDS-PAGE; fractions eluted by mobile phase at pH 3.6 were retained and concentrated by ultrafiltration (Amicon, Millipore, Bedford, MA, USA).

A New Zealand white rabbit was immunized s.c. with antigen in Freund's adjuvant (1:1; 0.25 mg) on days 0, 14, 28, and 35. Blood was collected on days 0, 18, 25, 32, and 42, allowed to clot, and serum aspirated and stored frozen. Serum from the third bleed was used in western blot analysis.

Western blot analysis. Ovarian tissue from a PV shortfinned eel was homogenized (IKA Labortechnik, Staufen, Germany) in lysis buffer (10 mM PBS, pH 7.4, containing Roche's Protease Inhibitor Cocktail in accordance with product instructions) and subjected to SDS-PAGE on 10% custom-made gels (~80 µg/lane). Proteins were stained with CBB or transferred to methanol-activated PVDF membrane by electroblotting at ~5 mA/cm² for 60 min. The membrane was subsequently incubated at room temperature for 3 h in Tris-buffered saline (TBS) containing 5% skim milk to reduce nonspecific binding. Immunoglobulins were affinity purified using Protein G (Protein G HP SpinTrap, GE Healthcare, Little Chalfont, UK) and diluted 1:150 (equivalent to neat antiserum diluted at least 1:1000) in blocking solution prior to incubation at 4 °C overnight. After two washes in TBS-Tween (0.025% Tween-20 in TBS) and two washes in TBS (15 min each), the membrane was exposed to alkaline phosphatase-conjugated goat anti-rabbit IgG (Vector AP1000, Vector Laboratories, Burlingame, CA, USA) in blocking solution for 1 h. A further two washes in TBS-Tween and two washes in TBS (15 min each) were followed by a rinse in staining buffer (100 mM Tris, 100 mM NaCl, and 50 mM MgCl₂ (pH 9.5)). Colour development was done by the addition of 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) substrate in staining buffer, followed by a rinse in distilled water to terminate alkaline phosphatase activity.

Glycosidase treatment. Aliquots of ovarian homogenate were incubated with N-glycosidase F or O-glycosidase + neuraminidase (from *Vibrio cholerae*; all Roche Diagnostics), or a combination of the three glycosidase enzymes. Incubations were carried out at 37 °C for 5 h in a final volume of 40 µl containing 7 µl ovarian homogenate and 2 mU N-glycosidase F in buffer 1 (100 mM phosphate buffer, pH 7.0, containing 10 mM EDTA, 1% CHAPS, 0.1% SDS, and 1% 2-mercaptoethanol) or 2 mU neuraminidase + 0.5 mU O-glycosidase in buffer 2 (10 mM NaCl and 1 mM CaCl₂ in 0.1 M NaOAc, pH 5.5). Digestions with a combination of all three glycosidases were done in a 1:1 mixture of both buffers.

Localization of *gdf9* mRNA in the eel ovary

Two captive female EV shortfinned eels were injected twice (days 0 and 7) with either 0.9% NaCl or with 10 mg/kg acetone-dried SPH, administered i.p. Fish were euthanized in 0.3 g/l benzocaine on day 14, and ovarian tissue was collected and preserved in Bouin's solution overnight prior to *in situ* hybridization analysis (section 'In situ hybridization').

Experiment I: ovarian *gdf9* mRNA levels in wild shortfinned eels

Eight PV and eight EV shortfinned eels were wild caught during the downstream migratory season of 2005. An additional six fish in each stage were sampled a year later to ensure that effects were not attributable to interannual variation. Fish were transported alive to the field laboratory and placed in flow-through spring water at 12 °C until sampling the same day. Fish were killed in 0.3 g/l benzocaine after which BW and gonad weight (GW) were recorded, GSI (Lokman *et al.* 1998) were calculated, and ovarian tissue was quickly preserved by snap freezing in liquid nitrogen or fixing in buffered 4% paraformaldehyde (PFA) or Bouin's solution. Tissues were analyzed for *Gdf9* mRNA and protein levels by qPCR and immunohistochemical analysis, as outlined in the section 'Analyses' below.

Experiment II: ovarian *gdf9* gene expression in artificially maturing shortfinned and Japanese eels

Twenty-three silver shortfinned eels were acclimated for 2 weeks to artificial seawater (30 ppt) at 20 °C in 1 m³ recirculating tanks during the late austral autumn season of 2005. Three fish were killed in 0.3 g/l benzocaine at the start of experimentation (day 0) to serve as pretreatment controls, and processed as described in Section 'Localization of *gdf9* mRNA in the eel ovary' above. Remaining animals were tagged i.p. with a passive integrated transponder (Trovan, Keysborough, Victoria, Australia) while under anesthesia in 0.15 g/l benzocaine and either received fortnightly i.m. injections of Ringer's solution (Miura *et al.* 1991; *n*=8) or 10 mg/kg SPH suspended in Ringer (*n*=12). Final maturation and ovulation were induced with a priming injection of SPH followed by treatment with 2 mg/kg 17-hydroxyprogesterone (Lokman & Young 2000). Groups of SPH-treated fish were killed periodically (*n*=3 after 3, 5, 7–9 and 13 weeks) in such a way that a range of reproductive states could be sampled. These samplings

were complemented by samplings of control fish after 3, 5 (both $n=3$) and 13 weeks ($n=2$). Tissues were analyzed for *gdf9* mRNA levels and for routine histology (section 'Analyses').

In order to determine which oocyte size classes express Gdf9, 43 female cultivated Japanese eels were artificially matured by weekly treatment with 30 mg/kg SPH suspended in eel Ringer, injected i.p. Fish were terminally sampled at varying time points in order to ensure that samples in all stages of development could be collected. BW, GW, and GSI were determined after which ovarian tissue was preserved in 4% buffered PFA and processed for embedding in paraffin blocks. Following staging of hematoxylin–eosin-stained sections from ovaries, blocks from three fish in each of the following developmental stages were randomly chosen for immunohistochemical analysis (section 'Analyses'): pretreatment control; Ringer control; EV; MV; MN.

Finally, to confirm whether signal intensity by immunohistochemistry coincided with that of message, we isolated ovarian follicles from four artificially matured, ovulated Japanese eels (BW 349–650 g at ovulation) used for artificial propagation trials. Follicle isolation was done by gently stripping eggs from suspended ovarian tissue in Ringer solution using forceps. Follicles were sieved (750, 600, 425, and 220 μ m) into size classes corresponding to migratory nucleus stage, late vitellogenic, MV, EV, and PV respectively, and snap frozen in liquid nitrogen to estimate *gdf9* mRNA levels. All eel ovarian tissue was kindly donated by Mr Keisuke Sago, Faculty of Fisheries Sciences, Hokkaido University. Details on maturation methodology are essentially as above. Final maturation and ovulation were induced by an i.p. injection of 2 mg/kg 17,20 β -dihydroxy-4-pregnen-3-one, either alone or in combination with thyroxine (T_4). Maternal T_4 administration was attempted as thyroid hormones can be transferred to the eggs, and have been linked, albeit inconclusively, to egg quality and larval survival rates (e.g. review by Blanton & Specker (2007)). Fertilization rates ranged between 1.3 and 50.4%, and hatching rates between 0.63 and 19.8%; such variability is common in artificially propagated eels (e.g. Ohta *et al.* 1996, Furuita *et al.* 2006) and is most likely attributable to the extensive hormonal manipulations that are required to induce full sexual maturity (see the recent study by Okamura *et al.* (2007) for further discussion).

Experiment III: in vivo and in vitro effects of 11-KT on ovarian gdf9 mRNA levels in shortfinned eel

PV eels (522 ± 8.3 g; mean $\pm$ S.E.M.) were acclimated to a 1 m³ tank containing recirculating fresh water for 1 week prior to treatment with either a placebo implant (95% cholesterol and 5% cellulose) or with an implant containing 2.5 mg 11-KT. Once anesthetized (0.15 g/l benzocaine), a small incision was made on the ventral side of the abdomen, a few cm anterior of the anus. Implants and a PIT tag were subsequently placed in the body cavity and the wound was closed with a single stitch. Control- and androgen-treated groups (seven fish each) were held in separate 1 m³ tanks at 15–17 °C for 2 weeks for the treatment to take effect. They were then killed in benzocaine, morphometric measurements were made, and

ovarian tissue was retrieved and stored frozen until analysis for *gdf9* mRNA levels or preserved in PFA for routine histological analysis (section 'Analyses').

Experiment IV: in vivo effects of GH on ovarian gdf9 mRNA levels in Japanese eel

Thirty-two 0+ juvenile Japanese eels (24–32 g), feminized while in the glass eel stage, were retrieved from a small indoor concrete stock pond (ca. 8 m²; 23–26 °C) and randomly assigned to one of the following four treatment regimes: pretreatment control; saline control (0.9% NaCl); 500 ng/g recombinant Japanese eel GH long form (rjeGH-I); 500 ng/g GH short form (rjeGH-II). Injections were made i.p. on days 0, 3, 6, and 9 under anesthesia in 0.1% phenoxyethanol. Following injection, fish were placed in a 20 l plastic basket (one basket per treatment group) and the basket was placed in the concrete pond. Fish were fed commercial eel feed throughout the experimental period, except on days of injection or sampling. Terminal sampling took place on day 12 during which BW was recorded and gonad tissue recovered and snap frozen until analysis for routine histology and *gdf9* mRNA levels. Recombinant jeGH-I and -II (Kishida *et al.* 1987) were produced using the *Drosophila* S2 expression system established for production of piscine gonadotropins (Zmora *et al.* 2007, Kazeto *et al.* 2008). Fine details of the GH expression and isolation procedures are to follow (Y Kazeto, S Akiyama, Y Ozaki, T Todo, S Adachi & K Yamauchi, unpublished observations).

Analyses

RNA isolation, cDNA synthesis, and quantitative real-time PCR analysis of gdf9 mRNA

Total RNA from ovarian tissues was extracted using TriZol Reagent (Invitrogen), and oligo-dT_{15–20}-primed cDNA synthesized from 1 μ g isolate (Superscript III, Invitrogen). cDNA was subjected to qPCR using an intron-spanning *gdf9* qPCR FW primer 5'-CAACAAAGCAAAGAGATTTTATGC-3' and the reverse primer *gdf9* qPCR RV 5'-GGACGTCCAGGTAGCA-GACT-3' to yield a 148 bp product (397–544 bp). QPCR was conducted on Stratagene MX3000P Real Time PCR machine, programed at 50 °C for 2 min, 95 °C for 2 min, and 40 cycles at 95 °C for 15 s and 62 °C for 15 s, followed by melting curve analysis. Reactions were run in final volumes of 20 μ l, containing the following reagents: 15–25 ng total RNA equivalent; 0.8 μ l FW primer (10 μ M); 0.2 μ l RV primer (10 μ M); 10 μ l 2 $\times$ Platinum SYBR Green qPCR Supermix-UDG (Invitrogen). To ensure that these primers could be used to amplify DNA from both shortfinned eel and Japanese eel, a partial *gdf9* cDNA was cloned from the latter using FW 5'-AGCTTTATCGGAGCAGGCTA-3' and RV 5'-TTACGGC-GACTCCTTCTGT-3'.

Standard curves were constructed for quantitation of *gdf9* mRNA levels by successive tenfold dilutions of pGEM T-Easy harboring inserts generated by PCR with *sfgdf9*-FULL FW and RV. Constructs were linearized by restriction enzyme digestion just downstream of the insert, and then quantified by spectrophotometry and converted to copy number ($MW_{ssDNA} = 1.58$ MDa; 1 fg = 380 copies *gdf9* mRNA). *gdf9* mRNA

copy numbers were normalized over those for *elf* ($MW_{ssDNA} = 1.25$ MDa; $1 \text{ fg} = 506$ copies *elf* mRNA), as published previously (Lokman *et al.* 2007). Variability in *elf* mRNA levels was seen, but treatment means in the sections 'Experiment III: *in vivo* and *in vitro* effects of 11-KT on ovarian *gdf9* mRNA levels in shortfinned eel' and 'Experiment IV: *in vivo* effects of GH on ovarian *gdf9* mRNA levels in Japanese eel' were comparable, suggesting that for these experiments, *elf* is a suitable normalizer. However, when comparing between different reproductive stages (wild fish: PV and EV), notable differences in levels of *elf* mRNA were observed; thus, *elf* mRNA levels per microgram RNA were significantly higher in PV fish than in EV fish and data for this comparison are therefore presented in nonnormalized form. During artificial maturation, differences in *elf* mRNA were also seen, but these did not affect the statistical outcome, and normalization of *gdf9* mRNA levels over *elf* mRNA levels was therefore applied.

Histology and immunohistochemistry for *Gdf9*

Ovarian tissue was dehydrated, embedded in paraffin, and sectioned at 5–6 μm . Sections from several eels representing different treatment groups were mounted on the same slide to avoid slide-related variability. Sections were stained with hematoxylin and eosin for routine histological analysis, e.g. staging of oocytes. Where considered relevant (i.e. when detecting differences in *gdf9* mRNA levels between treatments), sections were mounted onto aminopropylsilane-coated glass slides (Matsunami, Tokyo, Japan) and immunostained with the *Gdf9* antiserum as follows: slides were deparaffinized and transferred to PBS (0.15 M NaCl in 20 mM phosphate buffer, pH 7.4) before antigen retrieval in 10 mM sodium citrate (pH 6.0) for 5 min at 105 °C in an autoclave. Slides were rinsed in PBS, incubated with 10% goat serum in PBS in a humidified chamber (30 min, room temperature), again rinsed in PBS (twice, 5 min each), and incubated overnight at 4 °C with *Gdf9* antiserum (1:1000 in PBS). A rinse in PBS was followed by exposure of slides to alkaline phosphatase-conjugated goat anti-rabbit-IgG (Section 'Western blot analysis') for 30 min at room temperature in a humidified chamber. Following rinses in PBS and TBS, 0.2 M Tris (pH 8.0) containing Vector Red Reagent (Vector Laboratories) and 0.5 mM levamisole was added for colour development according to the manufacturer's instructions. Slides were finally rinsed with Tris, dehydrated through a graded series of ethanol and xylene, and mounted.

In situ hybridization

A cDNA fragment of 875 bp encoding shortfinned eel *Gdf9* was amplified by RT-PCR using the following primers: 5'-ACGTCCAAGAGCAGGAACAT-3' and 5'-CCAAATATGC-GAGGTGCAAA-3'. The cDNA fragments were subcloned into pGEM-T Easy vector and used to synthesize digoxigenin (DIG)-labeled sense and anti-sense cRNA probes with Roche's DIG RNA Labeling Mix (Roche).

To perform *in situ* hybridization, simplified methods described previously (Braissant & Wahli 1998, Kusakabe *et al.* 2002) were employed; thus, Bouin-preserved ovarian tissue was dehydrated through an ethanol series and embedded in paraffin. After cutting at 5 μm thickness, sections were

deparaffinized in xylene and, subsequently, ethanol. Sections were air dried, refixed with 2% PFA in PBS, and treated with 1 $\mu\text{g}/\text{ml}$ proteinase K (Roche) in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) at 37 °C for 30 min. Sections were refixed again in 2% PFA–PBS, and then processed as described in the previous report (Braissant & Wahli 1998) with minor modifications. Hybridized probes were detected immunohistochemically with an anti-DIG antibody conjugated with alkali phosphatase (Roche) and visualized by adding NBT/BCIP as substrates.

Statistical analysis

Data were analysed for normality by Q–Q plots and for homoscedasticity using Levene's test and log-transformed if these assumptions were not met. Treatment means were compared using Univariate one-way ANOVA and Scheffé's *post hoc* multiple comparisons. Treatment means from *in vitro* experiments were evaluated using two-way ANOVA and defining FISH as a random variable in the analysis. Statistical tests were conducted using SPSS 14.0 (SPSS Inc., Chicago, IL, USA), and means were considered different for probabilities <5%.

Declaration of interest

The authors declare that there is no conflict of interest that would impact on the impartiality of this work.

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