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Review

Fishing Pluripotency Mechanisms *In Vivo*

Ana V. Sánchez-Sánchez[1], Esther Camp[2], and José L. Mullor[1] ✉

1. Fundación para la Investigación Hospital La Fe, Valencia 46009, Spain.
2. School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, UK.

✉ Corresponding author: jlmullor@fundacionlafe.org; Tel: +34 961 973 400; Fax: +34 963 494 416.

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Abstract

To understand the molecular mechanisms that regulate the biology of embryonic stem cells (ESCs) it is necessary to study how they behave *in vivo* in their natural environment. It is particularly important to study the roles and interactions of the different proteins involved in pluripotency and to use this knowledge for therapeutic purposes. The recent description of key pluripotency factors like Oct4 and Nanog in non-mammalian species has introduced other animal models, such as chicken, Xenopus, zebrafish and medaka, to the study of pluripotency *in vivo*. These animal models complement the mouse model and have provided new insights into the evolution of Oct4 and Nanog and their different functions during embryonic development. Furthermore, other pluripotency factors previously identified in teleost fish such as Klf4, STAT3, Sox2, telomerase and Tcf3 can now be studied in the context of a functional pluripotency network. The many experimental advantages of fish will fuel rapid analysis of the roles of pluripotency factors in fish embryonic development and the identification of new molecules and mechanisms governing pluripotency.

Key words: Nanog, Oct4, teleost fish, Medaka, pluripotency.

Introduction

ESCs have the potential to be induced to differentiate into almost any cell type of an embryo or adult tissue [1] presenting great therapeutic promise in regenerative medicine [2, 3]. To derive appropriate cell types for clinical use it is necessary to understand how pluripotency gene networks function *in vivo*. The regulation of pluripotency has been described mainly in mammals and *in vivo* functional analysis of *Nanog*, *Oct4 (Pou5f1)* and *Sox2*, which form the gene core of pluripotency, has been performed using the mouse model [4-7]. This was partly due to the lack of *Nanog* orthologs identified in other non-mammalian species in the past. However, *Nanog* and *Oct4* sequences have recently been published in non-mammalian vertebrate species, such as chicken [8, 9], *Xenopus* (only *Oct4* homologs) [10], zebrafish (*Danio rerio)* [11, 12] and medaka fish (*Oryzias latipes*) [12, 13], demonstrating that these key pluripotency factors are not exclusive to mammals. The third key pluripotency factor of the triad is Sox2, which acts as a cofactor with Oct4 to maintain pluripotency [14, 15]. Sox2 was initially described as an early neural marker and an important factor for eye development [16, 17], however its putative interaction with Oct4 in fish has not been studied in detail. Additionally, other important pluripotency factors in mammals have been studied in fish such as Klf4, Tcf3, STAT3 and telomerase (Table 1). Although their embryonic roles have been studied in different developmental contexts, little is known about their function in fish pluripotency.

Here, we will review the recent introduction and use of fish models to study early pluripotency *in vivo* comparing the results to those obtained in mammals. The many experimental advantages that offer teleost

animal models [18, 19] allow researchers to combine embryological, molecular and genetic analyses required in the study of pluripotency. This makes them an excellent choice to study pluripotency *in vivo*, thus complementing the mouse model.

The introduction of non-mammalian animal models to study pluripotency.

Oct4 and Nanog proteins are central to the maintenance of ESC pluripotency [4, 6, 7]. The roles of Oct4 and Nanog in pluripotency were first described in mice. Both proteins mOct4 and mNanog are expressed in the inner cell mass (ICM) and epiblast of the early mouse embryo and in mouse germ cells (Figure 1). *mNanog* null embryos fail to form epiblast and the ICM differentiates into parietal endoderm-like cells [7], while ICM from *mOct4* null embryos is not pluripotent and differentiates into trophoblast [4].

Table 1. Pluripotency genes present in human *(Homo sapiens)*, mouse *(Mus musculus)*, medaka *(Oryzias latipes)* and zebrafish *(Danio rerio)*. Numbers identify the Entrez Gene ID for each gene except for zebrafish Nanog that represents the NCBI reference sequence.

	Homo sapiens	*Mus musculus*	*Oryzias latipes*	*Danio rerio*
Oct4	5460	18999	100049520	30333
Nanog	79923	71950	100301580	NP_001091862.1
Sox2	6657	20674	100049368	378723
Klf4	9314	16600	-	65238
Stat3	6774	20848	100049477	30767
Tcf3	6929	21423	-	30310

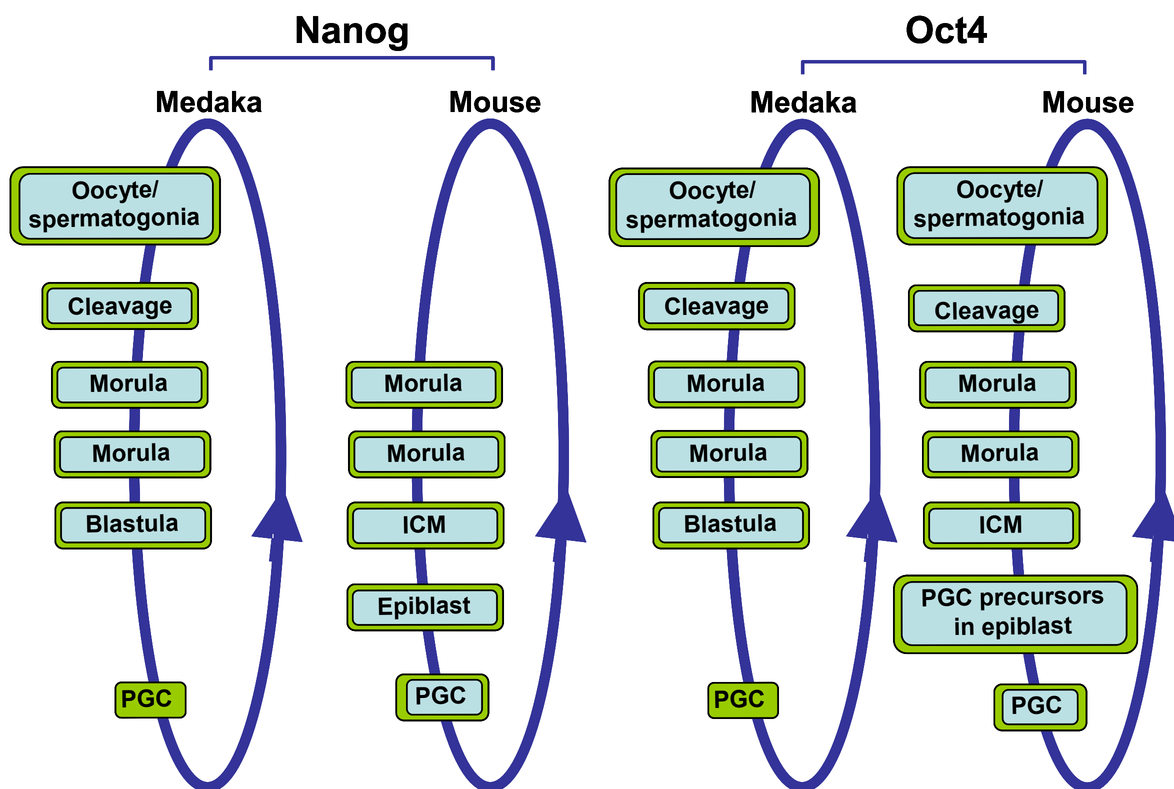

Figure 1. Nanog and Oct4 expression during medaka and mouse development. The circular blue arrow indicates the totipotent cycle. Cells and tissues types that express Oct4 or Nanog are boxed in blue when their mRNA expression are detected and/or boxed in green when their protein expressions are detected. Both Nanog and Oct4 have similar expression patterns in medaka and mouse, although mouse Nanog is first detected at the morula stage.

In *Xenopus*, *Nanog* homologs have not been detected. On the other hand, Morrison and Brickman [10] described three *Oct4* homolog genes in *Xenopus laevis*, *XlPou25*, *XlPou60* and *XlPou91*, grouped under the name *XlPouV*. Depleting the levels of these three *XlPouV* genes using morpholino oligos caused embryonic lethality in injected *Xenopus* embryos. In order to further evaluate their functional conservation, all three genes were transformed into *Oct4*-null mouse ESCs. Only *XlPou91* was able to rescue the self-renewal capability of the cells almost to the same extent as *mOct4*, suggesting that in *Xenopus*, *XlPou91* may be the gene that during evolution has retained the same role in regulating pluripotency as the mammalian *Oct4* gene [10]. *Xenopus* embryos do not form trophectoderm, therefore *XlPouV* genes depletion can not produce differentiation to this tissue, as observed after *mOct4* depletion in the mouse embryo. However, *XlPouV* genes depletion induces an expansion of *Xcad3* expression, which is the *Xenopus* homologue of mouse *Cdx2*, a marker of trophectoderm. *XlPouV* genes depletion also induces an early progression to a committed cell type, provoking an increase in the expression of early commitment markers such as *Goosecoid*, *Chordin* and *Cerberus*, and a decrease in genes associated with the inhibition of differentiation such as *Bmp4* [10]. In view of the results obtained in *Xenopus*, the role of Oct4 is associated with preventing the premature commitment of pluripotent cells before and during gastrulation.

In chicken, *cNanog* and *cOct4* sequences have been described with only one homolog of each identified to date [8, 9]. The functional conservation of these genes was analyzed by performing overexpression experiments of *cNanog* and *cOct4* in mouse and chicken ESC cultures and comparing their effects with those of *mNanog* and *mOct4*. Both *cNanog* and *cOct4* share some functional roles with their mouse homologous genes. *mNanog* or *cNanog* overexpression maintains proliferation of mouse ESCs in the absence of Leukaemia Inhibition Factor (LIF), while overexpression of *cOct4* induces differentiation in chicken ESCs and mouse ESCs. On the other hand, inactivation of *cNanog* or *cOct4* induces differentiation of chicken ESCs and inhibits their proliferation. Moreover, *cNanog* and *cOct4* expression is detected in chicken primordial germ cells (PGCs) just as observed in the mouse [6, 8, 9].

In zebrafish and medaka, embryonic stem-like cells have been isolated and characterised. These ESCs from fish share the *in vitro* properties with murine ESCs and exhibit self-renewal capacity [20-22]. *Oct4* orthologous sequences have been identified in medaka *OlOct4 (Olpou5f1)* [13] and zebrafish *pou2/spg* [11, 23]. Furthermore, *in vitro* analysis has demonstrated that *mOct4* promoter can be activated in medaka ESCs, suggesting that the pluripotency regulatory network in mammals is conserved in fish [24]. In addition, a single *Nanog* orthologous gene has been found in medaka and zebrafish genomes and the medaka Nanog (*OlNanog*) gene has been functionally characterized [12].

Other genes associated with pluripotency in mammals (*Sox2, Tcf3, Klf4, STAT3*) have been described and analyzed in other species such as *Xenopus*, fish or chicken. However, the initial absence of *Nanog* or *Oct4* orthologs in these species suggested that pluripotency might be an exclusive mammalian property and focused the study of these genes to their roles in other aspects of development. The recent description and characterization of *Nanog* and *Oct4* in non-mammal animals paves the way for a thorough comparative analysis of the genetic networks regulating embryonic pluripotency. Furthermore, the experimental advantages provided by these species, particularly zebrafish and medaka embryos, such as the number of embryos and their transparency, *ex utero* development, easy gene function manipulation and transgenic generation, will further expand the field of *in vivo* pluripotency. For example, new *in vivo* genetic screens can be designed to reveal other proteins involved in pluripotency, generation of inducible transgenes or the roles of pluripotency genetic networks in adult stem cells and regenerating tissues.

Comparing the roles of Oct4 between mammals and fish.

In mouse ESCs, repression of mOct4 expression causes differentiation to trophectoderm and loss of pluripotency, while overexpression produces the differentiation to primitive mesoderm and endoderm [25]. Therefore, precise levels of Oct4 control the maintenance of pluripotency. However Oct4 alone is necessary but not sufficient to support stem cell renewal in these cells [25]. On the other hand, Oct4 is one of the transcription factors needed for the reprogramming of human and mouse fibroblasts into induced pluripotent stem cells (iPSC) [26-28]. In fact, Oct4 is the only transcription factor that appears to be irreplaceable for reprogramming to occur [29], making Oct4 a potential key factor to be used in regenerative medicine.

During evolution, the ancestral *Oct4* gene seems to have been duplicated once, resulting in *pou5* and *pou2*. However *pou5* was subsequently lost in teleost fish and *pou2* was lost in mammals, thus, the remaining copy of the ancestral *Oct4* gene in each class of vertebrate would most likely have retained *Oct4*

functions and/or acquired new ones [30]. Expression and functional analysis shows that *Oct4* is expressed in the ICM of mouse embryos and in all cells from zygote to gastrula embryos in zebrafish and medaka (Figure 1) [31-33]. During later stages of development, medaka and zebrafish *Oct4* also share an expression domain in the posterior part of the embryo. Additionally, mouse, chicken and medaka *Oct4* are expressed in the PGCs [9, 31, 33] and, in mouse, *mOct4* is necessary for PGC survival [34]. This suggests that *Oct4* in PGCs may be maintained during PGC migration without *de novo* synthesis. In sum, the duplicated *Oct4* gene copy which remains in teleost fish and mammals has maintained similar expression patterns before gastrulation and in PGCs throughout evolution in the two classes of vertebrates, although in zebrafish *pou2/spg* is not expressed in PGCs. This is a particular interesting evolutionary difference of the *Oct4* homolog between medaka and zebrafish. The *pou2/spg* gene in zebrafish is not duplicated and the question remains of how the roles of *Oct4* in PGC and gonad development in zebrafish may have been substituted. It will be interesting to analyze the expression patterns of the *Oct4* homolog in fugu (*Takifugu rubripres)* and *Tetraodon* to determine how these functions may have evolved in different teleost species.

In this evolutionary context, it is interesting to note that after gastrulation, the zebrafish *Oct4* homolog, *spg/pou2*, has a different spatial pattern of expression compared to Oct4 homologues in other vertebrates, and it has acquired other functions. One specific difference is that *spg/pou2* is differentially expressed in the developing zebrafish brain where it plays an important role in regionalization [32]. Furthermore maternal and early zygotic *spg/pou2* expression has been found to play an important role in endoderm development [35]. Moreover, although the presence of Spg/pou2 protein has not been analyzed, *spg/pou2* expression has not been detected in the PGCs, and it has also been found not to be necessary for the correct development of PGCs in zebrafish [35] in contrast to medaka and mice. Thus, it appears that in zebrafish, Oct4 has acquired a new role during brain development and lost its function in PGC biology. Additionally, initial cross-species complementation experiments suggest that *spg/pou2* cannot rescue *mOct4* function and maintain ES cell renewal when transfected into *mOct4* mutant mouse ESCs [10]. This indicates that at least some interactions necessary for pluripotency maintenance in mice have been lost in zebrafish, although in fish *pou2* may still regulate pluripotency. On the other hand, the function of the *OlOct4* homolog during medaka development and its capabilities to rescue ES cell renewal and cross-species pluripotency characteristics have not yet been examined. These studies will help to understand the evolution of *Oct4* and determine the extent to which functional homology has been conserved between fish and mammals.

Comparing the roles of Nanog between mammals and fish.

Nanog is a homeodomain (HD) transcription factor expressed in early embryo cells and PGCs in mouse [6, 7], chicken [9] and medaka fish [12]. mNanog expression is initially detected at the morula stage of the mouse embryo [6, 7], while in medaka it is maternally inherited (Figure 1) and its expression is detected as early as the unfertilized egg [12]. In other teleost species the expression pattern of *Nanog* has not been described and thus we will focus on the medaka *OlNanog* for comparison with the mouse *mNanog*. Lack of *mNanog* in mouse embryos results in early embryonic lethality [7]. Similarly, in medaka, OlNanog inhibition using morpholino oligos causes embryos to die without completing epiboly [12]. Thus, in both mouse and medaka, Nanog plays a central role in early embryo survival.

Additionally, in mouse ESC cultures, *mNanog* is a necessary factor for ESC to maintain their ability to differentiate into multiple cell lineages acting as a gatekeeper of the gateway to pluripotency [36, 37]. However, mNanog protein is expressed in mouse ESC in a mosaic pattern and cells which do not express mNanog in these cell cultures still retain expression of pluripotency markers and possess the ability to self-renew [38]. Moreover, mouse chimeras formed by implanting *mNanog* knock-out cells into wild type embryos develop normally and demonstrate that *mNanog* depleted cells can differentiate into all tissues except the gonads [38]. Thus, in the mouse, mNanog seems to be necessary to maintain pluripotency only during a short period of time since loss of mNanog does not irremediably provoke cell differentiation.

A similar finding is also observed in medaka, where OlNanog depleted embryos maintain the expression levels of pluripotency markers such as *Oct4*, *Tert*, and *Tcf3* [12]. Moreover, in these medaka OlNanog morphant embryos, in which OlNanog may not have been removed completely, the expression levels of differentiation markers associated with early lineage commitment such as *Bra*, *Sox17, Gata3* or *Sox2* were not significantly changed [12]. These results suggest that OlNanog function in OlNanog-depleted embryonic cells can be rescued by neighbouring OlNanog positive cells, indicating a non-cell autonomous OlNanog-mediated effect on undifferentiated cells. Thus, mouse and medaka *in vivo* studies suggest

that the crucial role of Nanog in pluripotency may affect proliferation and survival. However, cross-species complementation experiments are necessary between medaka and mouse or human Nanog to determine the extent of functional conservation among species.

Functional characterization of pluripotency genes in teleost fish can provide new clues to understand the roles and evolution of pluripotency in mammals, particularly in humans. For example, the study of Nanog function using medaka embryos revealed that Nanog controls cellular proliferation during the S phase transition by regulating *CyclinA* expression [12]. These results are consistent with findings in human ESCs, where overexpression of human NANOG results in an increase in proliferation. Simultaneously to the medaka studies, Zhang and colleagues described the role of human NANOG in regulating the transition from G1 to S phase of the cell cycle in human ESCs by direct regulation of *CDC25A* and *CDK6* expression [39]. In view of these results, the function of medaka Nanog is similar to that observed in human cells, hence validating the use of teleost fish as models to study pluripotency.

Later in development, Nanog expression is restricted to PGCs in human, mouse, chicken and medaka (Figure 1) [6, 8, 9, 12, 40, 41]. In mice, Nanog deleted ESCs were implanted in wild-type morulae to form chimeras that developed normally. In these chimeric mice, *Nanog*$^{-/-}$cells can be detected in all tissues, but PGCs lacking *Nanog* do not mature [38]. In fact, Nanog depletion using shRNA induced cell death in the migratory PGCs [42]. In medaka embryos, Ol-Nanog loss-of-function experiments using morpholinos provoked an altered migration and abnormal distribution of PGCs. In fish and mice, the signaling chemokine *Sdf1* and its receptor *Cxcr4* are necessary for PGC migration [43-47]. In medaka, *Ol-Nanog* binds to the regulatory region of *Cxcr4b* and regulates its expression. Thus *Ol-Nanog* mediates correct PGC migration by directly regulating the expression of *Cxcr4b* [41]. The role of Nanog in controlling PGC migration and apoptosis could explain the mouse chimera phenotype where Nanog-deficient ESCs do not generate mature PGCs [38]. It would be beneficial to investigate the relationship between the apoptotic and migratory processes in that Nanog could be acting in the mouse PGCs.

Other pluripotency genes in fish embryonic development

Several genes involved in mammalian pluripotency have been described in fish. Their analysis in fish embryos may help to resolve questions about the evolution of pluripotency in the vertebrate lineage and clarify some discrepancies that have raised in the gene functions in mice and humans. A clear example is the *STAT3* gene which is essential to maintain mouse ESC in an undifferentiated state, however, in human and monkey ESC *STAT3* seems to be dispensable. In medaka, analysis of *STAT3* showed that *STAT3* was also inactive in medaka ESC and blastula embryos, as in primates. These results suggest that the requirement of *STAT3* in mouse pluripotency may be species specific, whereas in medaka, monkey and human *STAT3* seems to be inactive [48]. Thus, from an evolutionary point of view, further studies in fish are providing clues about the evolution of pluripotency and which mechanisms are conserved among vertebrates.

Other important genes for mammalian pluripotency have been described in medaka, although their roles in early fish pluripotency have not been studied in detail. For example, *Klf4* belongs to the Krüppel-like factor (KLF) family of transcriptional regulators and is necessary for maintaining mouse pluripotency. In zebrafish, many *Klf* orthologs have been identified and functional studies suggest that at least *Klf1* and *Klf4* play important roles in zebrafish hematopoiesis. However, other *Klf* orthologs, such as *Klf2a* and *Klf2b* which are expressed from 70% epiboly, may have a role in early fish pluripotency [49-52]. Finally, telomerase is necessary to maintain telomere length and is active in all stem cells. Telomerase RNA template and its catalytic subunit, Telomerase Reverse Transcriptase (TERT), have been described in medaka and zebrafish [53-55] which provide further circumstancial evidence that pluripotency mechanisms have been conserved in the vertebrate lineage. It is interesting to note that telomerase activity can not be detected in most human somatic tissues, but it is ubiquitously detected in teleost fish somatic tissues. This telomerase expression pattern and its similarity to its human homolog substantiate the use of teleost fish as a model to easily study telomerase function and molecular mechanisms *in vivo* [56].

Nanog and Oct4 in the gonads

Nanog and Oct4 are expressed in gonads of human, mouse, chicken and medaka suggesting that both proteins may play a role in gamete differentiation and/or gonadal stem cell maintenance [6-9, 12, 33, 40, 57-59]. Also, telomerase activity is detected in the gonads of both mammals and teleost fish [53, 54, 56]. However, experimentation in these adult tissues is difficult and it remains an important task to characterize the *in vivo* roles of pluripotency genes in the gonads. Nevertheless, the expression patterns of these

genes provide some clues that point to a putative role in maintaining pluripotency in the gonad germ cells.

In medaka female gonads, OlNanog and OlOct4 transcript and proteins are detected in the small pre-vitellogenic oocytes, however the signal diminishes and becomes undetectable in medium to large pre-vitellogenic oocytes, which are arrested in meiosis. In medaka male gonads, OlNanog and OlOct4 transcript and protein are detected in the periphery of the testis, where undifferentiated spermatogonia, which constitutes the germ stem cell population of the testis, are located [12, 33].

The expression of Nanog is similar in medaka and mouse developing gonads. Mouse Nanog is expressed in germ cells and its expression is down-regulated in cells undergoing meiosis in female gonads, and at the onset of mitotic arrest in male gonads (Figure 1) [12, 42]. Thus, the initial role of Nanog may be to provide gonad stem cells with pluripotency characteristics and, therefore, its expression would be lost during differentiation. However, it is interesting to note that in mice mNanog is detected in type A spermatogonia and in pachytene spermatocytes, during haploid germ cell maturation, suggesting that it may play a role as an epigenetic modifier [59]. This putative role of mNanog as an epigenetic modifier during gamete maturation would introduce a new twist into Nanog function that may extend to its roles in pluripotency maintenance.

In the case of Oct4, medaka and mouse adult testis also share a similar Oct4 expression pattern. Both Oct4 mRNA and protein are detected in the most undifferentiated type A spermatogonia population in mice and medaka [33, 57]. Experiments in mice have shown that mOct4 is required for spermatogonia stem cell self-renewal [60]. On the other hand, mOct4 expression in mice ovaries has not been described and, in medaka, OlOct4 is detected in the most undifferentiated germ cells and in the germ plasm of oocytes, which will form the embryo germ cells [33]. Thus, the Oct4 expression pattern suggests that this transcription factor plays a role during gamete maturation. This hypothesis is supported by the results described in mice, where conditional inactivation of Oct4 in PGCs generates a sterility phenotype in the adult [34].

Conclusion

The different embryological and genetic manipulations to which zebrafish and medaka are amenable will expand the possibilities to study the roles of the different pluripotency factors that operate during vertebrate embryonic development (Table 2).

Table 2. **Expression and function of Nanog and Oct4 in different species.** The sites of expression and roles of these genes described in the text are summarized. Empty boxes represent not determined characteristics.

Oct4	Early expression	Early embryo function	PGC expression	PGC function	Gonad expression	Gonad function	Brain expression	Brain function	mOct4-/- rescue
Mouse	yes	yes	yes	yes	yes	yes	no	no	yes
Chicken	yes		yes		yes				yes
Xenopus	yes	yes	no	no					yes
Zebrafish	yes	yes	no	no	no	no	yes	yes	no
Medaka	yes		yes		yes		no		

Nanog	Early expression	Early embryo function	PGC expression	PGC function	Gonad expression	Gonad function
Mouse	yes	yes	yes	yes	male	
Chicken	yes		yes			
Xenopus						
Zebrafish						
Medaka	yes	yes	yes	yes	yes	

The studies in medaka during early embryo development have already contributed important information on Nanog function. Furthermore, the use of medaka or zebrafish will allow researchers to design new screening strategies to identify proteins involved in pluripotency *in vivo*. Experiments in fish may also provide valuable information on new specific molecular interactions *in vivo* between Nanog, Oct4, Klf4, Sox2, TERT, STAT3 or Tcf3 which may be differentially conserved in mice or humans. The generation of transgenic animals for promoter analysis and conditional gene manipulation will further contribute to the understanding of the roles of these genes during embryonic development. Thus, the introduction of medaka and zebrafish to the study of embryonic pluripotency raises new and exciting questions and opportunities for the field.

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Conflict of Interests

The authors have declared that no conflict of interest exists.

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