Characterization of *Danio rerio* Nanog and Functional Comparison to *Xenopus* Vents

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Nanog is a homeodomain transcription factor associated with the acquisition of pluripotency. Genome analyses of lower and higher vertebrates revealed that the existence of *Nanog* is restricted to *gnathostomata* but absent from *agnatha* and invertebrates. To elucidate the function of Nanog in nonmammalia, we identified the *Danio rerio* ortholog of *Nanog* and characterized its role in gain and loss of function experiments. We found Nanog to be crucial for survival of early zebrafish embryos, because depletion of Nanog led to gastrulation defects with subsequent lethality. Mouse Nanog overexpression could rescue these defects. Vice versa, zebrafish Nanog was found to promote proliferation and to inhibit differentiation of mouse embryonic stem cells in the absence of leukemia inhibitory factor. These findings indicate functional conservation of Nanog from teleost fishes to mammals. However, *Nanog* was lost in the genome of the anurans *Xenopus laevis* and *Xenopus tropicalis*. Phylogenetic analysis revealed that deletion probably occurred in a common anuran ancestor along with chromosomal translocations. The closest homologs of Nanog in *Xenopus* are the Vent proteins. We, therefore, investigated whether the *Xvent* genes might substitute for Nanog function in *Xenopus*. Although we found some similarities in phenotypes after overexpression and in the regulation of several marker genes, *Xvent1/2* and Nanog cannot substitute each other. Depletion of Nanog in zebrafish cannot be rescued by ectopic expression of *Xvent*, and *Xvent* depletion in *Xenopus* cannot be overcome by ectopic expression of zebrafish Nanog.

Introduction

THE UNDIFFERENTIATED CELL state in vertebrate embryogenesis and the maintenance of pluripotency in embryonic stem (ES) cells are characterized by the expression of factors, such as Oct4, Nanog, Dax1, Tert, or Dppa1-5. Expression of Oct4 appears to be a prerequisite for the maintenance of the undifferentiated state and self-renewal in mammalian ES cells [1–3], but despite an increasing knowledge about target gene regulations and molecular interactions in different species, the current data do not allow us to get a general view of signaling in maintaining pluripotency. Comparing the genes involved in pluripotency from an evolutionary point of view, the situation becomes even more complicated. In placental mammals (eutheria), only one Oct4 encoding gene (known as *Pou5f1*) exists, whereas monotremes (protheria) and marsupials (metatheria) additionally possess a *Pou2* gene. In the amphibian *Xenopus*, the *Pou2* gene has even been triplicated (*Oct60*, *Oct25*, *Oct91*) [4,5], whereas the *Pou5f1* gene is lacking [6]. In some other vertebrates, such as chicken, zebrafish, or axolotl, either a *Pou2* or the *Pou5f1* gene exists [7]. In addition, several Oct4 regulation cascades are modified

between mammals and nonmammals [6,8]. The pluripotency-associated factor, Dppa5, is completely absent in lower vertebrates [9]. Dppa2 and Dppa4 exist in lower vertebrates, such as *Anolis* (reptile) or *Xenopus* (amphibian), in form of only one homolog [10]. For a handful of other factors, which are associated with the maintenance of stem cell character, that is, Zfp206, Rex1, Dax1, Klf4, Dppa1, Dppa3, and even for Nanog, the data obtained from lower species are insufficient. Since there is also a stemness-like or a pluripotent state described for nonmammalian organisms [11,12], it is still not clear how this status is maintained in these species. Do these factors exist in lower vertebrates, and are they embedded in the same signaling cascades as in higher vertebrates?

In the current study, we focus on the homeobox transcription factor Nanog, another master regulator of pluripotency [13–15]. Nanog was identified by screening mouse cDNA libraries for ES cell specific transcripts [16,17] and by expression cloning from an ES cell cDNA library as a factor maintaining cytokine-independent self-renewal of murine ES cells [even in the absence of leukemia inhibitory factor (LIF)] [13]. Although Nanog is unable to re-induce stem cell character without Oct4 [18], Nanog is re-expressed in fully

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reprogrammed induced pluripotent stem cells and promotes transfer of pluripotency after cell fusions [19,20]. This indicates that Nanog is a central player in the core transcriptional regulatory circuitry of pluripotency [14,21,22] and is a gateway to the pluripotent ground state [15]. *Nanog*^{-/-} mice embryos are lethal, and ES cells form extraembryonic endoderm at the expense of epiblast. Since *Nanog* is still expressed in *Oct4*^{-/-} mouse embryos, it was suggested that these 2 transcription factors act in parallel pathways [13]. Although Oct4 cannot substitute Nanog in self-renewal of murine ES cells in the absence of LIF [23], these 2 factors share a high overlap of target genes [14,21]. In addition, a mutual protein binding between Oct4 and Nanog was found, recruiting HDAC1/2 and NuRD, Sin3A, Pml, and Mta1/2 to form repression complexes called NODE (*N*anog and *O*ct4 associated deacetylase) [24].

A vertebrate homolog of *Nanog* in nonmammalian organisms was first reported for chicken [25,26]. It was postulated that *Nanog* is an amniote-specific gene and is absent from anamniotes and invertebrates [25]. However, *Nanog* homologs have recently been reported for the teleost fish (medaka) [27,28] and for the urodele amphibian axolotl [29]. It was suggested that the mechanisms governing pluripotency might be conserved from urodele amphibians to mammals [29]. However, this does not consider the situation in fish, where *Nanog* is present, or in the anuran amphibian *Xenopus tropicalis*, where a *Nanog* homolog is missing [30]. Therefore, it would be interesting to know which parts of *Nanog* function are still conserved between different species and which genes may account for *Nanog* function in *Xenopus*.

Here, we characterize the *Nanog* gene of *Danio rerio* and demonstrate by gain and loss of function (LOF) experiments that *Nanog* function is essential for zebrafish embryogenesis. We show that *Nanog* knockdown in zebrafish can be rescued by murine *Nanog*, whereas the closest sequence homologs to *Nanog* proteins in *Xenopus*, the homeobox transcription factors *Xvent1* and *Xvent2* [31,32], fail to substitute for *Nanog* function in *D. rerio*. Additionally, we demonstrate that zebrafish *Nanog* in contrast to *Xvent2* promotes proliferation and inhibits differentiation of mouse ES cells even in the absence of LIF. These findings indicate that *Nanog* is functionally conserved from bony fishes (teleosts) to mammals.

Materials and Methods

Database analyses

Sequence analyses, EST, and genomic database search and sequence alignments were done by using bioinformatic programs: www.ncbi.nlm.nih.gov/blast; www.ensembl.org/index.html; <http://www.ebi.ac.uk/clustalw/>; <http://genome.wustl.edu/tools/blast>.

Antisense morpholino oligonucleotides

D. rerio NanogMO (5'-ATCTTCCAGTCCGCCATTTCGCCCGT-3') (GeneTools) is directed against the translation start site (reverse complementary is underlined). *Xvent1* and *Xvent2* morpholino oligonucleotides (MOs) were previously reported [33]. For control injections in *Xenopus*, a control MO (ctrlMO) derived from the human β -globin gene (5'-CCTCTTACCTCAGTTACAATTATATA-3') was used. For controls in zebrafish,

a ctrlMO (5'-ATGCAAACAATACATACACCTCGTG-3') was used. In vitro test of the morpholino specificity of NanogMO was done as previously described [10].

Cloning of genes and in vitro transcription

A *Nanog* clone of *D. rerio* was obtained from imaGenes (accession no. **BC162318**). The open reading frame was cloned into pCS2 expression vector. For rescue experiments, silent mutations were introduced in the MO-binding site of *D. rerio* *Nanog* (*mut-Nanog*: 5'-cgGaattcATGTcACgGcAcGAgGG-3') via polymerase chain reaction (PCR) using mutated primers. The translation start is underlined; silent mutations are shown in lowercase. For GST fusion proteins, *Nanog* open reading frames (murine and *D. rerio*) were cloned into pGEX-4T-1 vector. *Nanog* mutants (HD+: 164–179 aa; Δ N: 194–384 aa; Δ C: 1–261 aa) and *Nanog* Δ HD, lacking aa 195–261, were cloned into pCS2 vector. For RNA injection, plasmids were transcribed with SP6. Digoxigenin labeled antisense RNA for in situ hybridization was obtained with T7 polymerase.

Protein expression and GST-pulldown assay

GST-fusion proteins were expressed in *E. coli* BL21 (DE3) Plus (Stratagene) and purified in batch with Glutathione-Sepharose™ 4B (Amersham Biosciences) under native conditions according to the manufacturer's protocol. Purified proteins were dialyzed overnight at 4°C (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 20% glycerol). ³⁵S-methionine labeled proteins were translated with TNT®T7/SP6 Coupled Reticulocyte Lysate System (Promega). Pull-down analyses were done as previously described [34].

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) were performed with double-stranded oligonucleotides of the murine *Tcf3* promoter (5'-AAACCTGTTAATGGGAGCGCAT-3' [35]). Oligonucleotides were 5'-end-labeled with γ -ATP using T4 polynucleotide kinase and subsequently annealed. Gel-purified probes were incubated with *Xvent2* or *Nanog* proteins (murine or *Danio*) for 25 min on ice in 30 μ L binding buffer [36] containing the unspecific competitor poly(dIC). For specific competition, unlabeled probes were used. Samples were separated on 8% nondenaturing polyacrylamide gels and analyzed by autoradiography (BAS 1500–Fuji).

ES cell culture

In general, mouse ES cells (E14.1; 129/Ola-derived) were cultured with (DMEM+GlutaMax, 10% FCS, 1.5 g/L Na₂CO₃, 10 μ g/L LIF (SIGMA), and 0.1% penicillin/streptomycin) on irradiated neomycin resistant mouse embryonic feeder cells (MEFs). *M. m.* *Nanog*, *D. rerio* *Nanog*, and *Xvent2* sequences were integrated into the eukaryotic expression vector pFIEN, and ES cells were electroporated (Biorad Gene Pulser, 240V) with recombinant constructs or pFIEN vector as control (40 μ g). To obtain stably transfected clones, the ES cells were selected with G418 (250 μ g/mL; Gibco) for 8 days and then sorted for GFP expression (BD, FACS Aria).

For differentiation assays, ES cells were trypsinized, separated from MEFs and 2 $\times$ 10³ cells/40 μ L differentiation media [IMDM (Gibco), 1.5 g/L Na₂CO₃, 10% FCS, 0.1%

penicillin/streptomycin, 20 μ L/L monothioglycerol, 1.1 $\times$ nonessential amino acids] were incubated as hanging drops to form embryoid bodies. For controls, 10 μ g/L LIF was added to the differentiation media to inhibit differentiation. At day 2 of incubation, embryoid bodies were transferred on gelatinized 24-well plates with differentiation media. Differentiation of embryoid bodies was monitored at day 3 by morphology and at day 4 by AP staining (Alkaline Phosphatase Substrate Kit III; Vector). Images were taken with a Keyence microscope.

BrdU and cell tracking assays

Stably transfected ES cells were dissociated with trypsin and re-seeded at a density of 5×10^5 cells. Cells were incubated in 10 μ M BrdU solution (APC BrdU Flow Kit; BD Biosciences) in phosphate-buffered saline (PBS), harvested at different time points, re-trypsinized, and fixed in Cytofix/Cytoperm (BD Sciences) buffer. Washing, re-fixation, and BrdU staining were performed according to the manufacturer's manual. For counterstaining, cells were incubated in 7-amino-actinomycin (BD Biosciences) and analyzed on FACSCalibur.

For cell tracking, the same amounts of cells were incubated for 45 min in 15 μ M Cell Tracker Red CMPTX (Promega). Dye working solution was replaced, and cells were washed with PBS. Cells were trypsinized. Washing, incubation, and fixation were performed according to the manufacturer's protocol (Promega). Cells were analyzed on FACSCalibur.

Real-time reverse transcriptase-PCR

Total RNA extraction, purification, first-strand cDNA synthesis, and real-time reverse transcriptase (RT)-PCR were done as previously described [10]. Data are presented in relative units. Primers for RT-PCR are listed in Supplementary Table S1A, B; Supplementary Data are available online at www.liebertonline.com/scd.

Embryonic staging and in situ hybridization

Embryonic stages were determined for *Xenopus* [35] and for zebrafish [37]. Whole mount in situ hybridizations were performed according to standard procedures [38,39].

Results

Nanog is present in gnathostomata except for *Xenopus*

To identify Nanog orthologs in lower vertebrates, we performed BLAST analyses with genomic, EST, and protein databases from different species. We detected Nanog orthologs not only in pro- and metatheria (platypus and opossum), but also in several bony fishes (*Cyprinidae*: *D. rerio*; *Gasterosteidae*: stickleback; *Gadidae*: Atlantic cod; *Salmonidae*: salmon; *tetradontidae*: fugu and tetraodon) (Fig. 1A). The EST [CO049347](#) of the little skate (*Leucoraja erinace*) gives rise to the assumption that Nanog already exists in cartilaginous fishes. However, we were unable to detect Nanog in the genome of the lamprey (*Petromyzon marinus*), a representative of jaw-less vertebrates (*agnatha*), in acranial vertebrates

(*Branchiostoma*), and tunicates [*urochordata* (ie, *Ciona*)]. In addition, Nanog-like genes do not exist in non-vertebrates, for example, insects (*D. melanogaster*) or nematodes (*Caenorhabditis elegans*). Thus, Nanog appeared in chordates with the evolution of jaw (*gnathostomata*).

Interestingly, Nanog is lacking in the genome of *X. tropicalis* [30]. Since we also failed to detect corresponding ESTs for Nanog in *Xenopus laevis*, we suppose that Nanog is absent from the entire genus *Xenopus* and perhaps from the complete pipidian subfamily *Xenopodinae*. To understand how Nanog was deleted from the *Xenopus* genome, we performed an extensive synteny analysis of the Nanog loci (Fig. 1B). The human Nanog is located at chromosome 12:7. The genomic surroundings of Nanog are almost identical from human to chicken. Nevertheless, we found in pro- and eutheria some chromosomal inversions and in chicken, the absence of distinct genes, such as *GDF3* and *Dppa3*. Even in the genome of the reptile *Anolis carolinensis*, Nanog can be located with partially existing synteny in scaffold (sc) 86. Upstream of the *Anolis* Nanog gene, there are orthologous neighboring genes, such as *AM2* or *APOBEC1*. Downstream of Nanog are 7 genes, whose human orthologs can be found on human chromosome 19. The ortholog genes in *X. tropicalis* are located within sc_1120. Another block of genes neighboring Nanog in *A. carolinensis* can be found in *X. tropicalis* on sc_735 and sc_1151, but no Nanog gene was found within these scaffolds. In teleost fishes, the synteny is completely lost. We checked the surroundings of the Nanog gene in *D. rerio* and found them to be identical to fugu, tetraodon, and medaka Nanog genes, but there is no conservation of the Nanog locus in comparison to non-fishes. The Nanog gene of *D. rerio* is located at chromosome 24:11. By tracking the opposite way, we then checked the genomic surroundings of the Nanog in teleost fishes (medaka and zebrafish) and analyzed the location of orthologous genes in other vertebrates (Fig. 1C). The genes surrounding the fish Nanog genes are found in higher vertebrates as orthologous on different chromosomes indicating various chromosomal translocations and rearrangements within the Nanog locus. Such chromosomal translocations are even found in *X. tropicalis*, but again there is no evidence for a Nanog gene coupled to any of these neighboring genes in this species.

Divergence of Nanog proteins in different species and relationship to Xvents

Murine and human Nanog were described as 3-domain proteins containing a homeodomain (HD), a serine enriched N-terminal, and a C-terminal transactivator domain containing several tryptophan repeats [40,41]. The tryptophan repeats were shown to be crucial for dimerization, proliferation, the maintenance of Nanog-dependent cell self-renewal, and for binding of Nac1 [42–44]. However, when comparing the mammalian Nanog proteins to those of medaka or chicken, they are highly divergent. Therefore, we asked for common characteristics of all Nanog proteins and aligned the orthologs from different species (Supplementary Fig. S1). In addition to rather high rates of identity inside the HD, we found conserved aspartate/glutamate stretches and serine/threonine residues in both the C- and the N-terminus. The C-terminally located tryptophan repeats are mainly found in eutherians, but 2 conserved residues are still found

A

Phylogenetic tree illustrating the relationships between various vertebrate groups and specific species. The tree is rooted on the left and branches out to the right. The groups and species are listed on the right side of the tree, with species names in red text.

- Gnathostoma**
 - Agnatha**
 - Petromyzontidae** *Petromyzon marinus*
 - Branchiostomidae** *Branchiostoma floridae*
 - Cionidae** *Ciona intestinalis*
 - Osteichthyes**
 - Chondrichthyes** **Rajidae** *Leucoraja erinacea*
 - Teleostei**
 - Salmonidae** *Salmo salar*
 - Gadidae** *Gadus morhua*
 - Adrianichthyidae** *Oryzias latipes*
 - Tetraodontidae** *Takifugu rubripes*
 - Gasterosteidae** *Gasterosteus aculeatus*
 - Cyprinidae** *Danio rerio*
- Tetrapoda**
 - Amphibia**
 - Caudata**
 - Ambystomatidae** *Ambystoma mexicanum*
 - Salamandridae** *Notophthalmus viridescens*
 - Anura** **Xenopodinae** *Xenopus tropicalis*
 - Amniota**
 - Sauropsidia**
 - Reptilia** *Anolis carolinensis*
 - Aves** *Gallus gallus*
 - Mammalia**
 - Eutheria** *Homo sapiens*
 - Metatheria** *Monodelphis domestica*
 - Protheria** *Ornithorhynchus anatinus*

in pro- and metatheria (pos. 198 and 203 of the mouse protein).

Inspection of the Nanog HD reveals a striking aspect being characteristic for the BarH subfamily of HD proteins [45], that is a specific amino acid exchange from an aliphatic nonpolar residue, such as valine or isoleucine, to the polar residue threonine (pos. 241 of the zebrafish protein) (Fig. 1D). This exchange can only be detected in a few *Xenopus* HD proteins, such as the Vent proteins and Hox11, but not in Nkx or distal-less (Dll) proteins, which also share substantial identities with the HD of Nanog. The highest rates of identity for Nanog proteins exist with *Xenopus* Vent proteins. Xvent2.1 from *X. tropicalis* (we used this name for gene1 of *Xvent2*, because all *Xvent1/2/3* genes were found to be duplicated in *X. tropicalis*) is not a Nanog protein, but belongs to the BarH class [45] and is closely related to Nanog (Fig. 1D; Supplementary Fig. S1). However, a crucial attribute of the Nanog HD is a highly conserved aliphatic, nonpolar residue in helix $\alpha 2$ (M at pos. 225 in zebrafish Nanog), which contributes to DNA binding through minor groove contacts [36]. All other HD proteins, including the Vent proteins, have a basic amino acid (mostly lysine or arginine) at this position. Further, we found 3 invariant positions in the Nanog HD (zebrafish Q218, Y237, and K238) that were exchanged in Xvent2.1. Further, a triplet of amino acids (tp ϕ), which is invariant in all Nanog proteins, is partially conserved in Xvent2.1 (S at pos. 71 in murine Nanog) (Supplementary Fig. S1), but not in other BarH proteins or in the related Dll proteins (data not shown). We also found several S/T residues in the N-terminus of Xvent2.1 as described for Nanog. However, the 2 invariant amino acids flanking a polar residue adjacent to the HD domain of Nanog at the C-terminus, a basic and a tryptophan residue (b ρ -W), are not detected in Xvent2.1.

Expression pattern and functional characterization of *D.r. Nanog*

To investigate the role of Nanog in lower vertebrates, we analyzed its function in zebrafish. The full coding sequence of *D.r. Nanog* was obtained from the image clone **BC162318**. The corresponding protein contains 384 amino acids. We determine the temporal expression pattern of *Nanog* by real-time RT-PCR. *Nanog* RNA can already be detected in unfertilized eggs (Fig. 2A). The amount of transcript is strongly increased at the beginning of gastrulation, peaks at germ ring stage, and is then decreasing. When segmentation starts at 9 h postfertilization, *Nanog* transcripts are greatly diminished. From somite stage onward, *Nanog* cannot be detected anymore. We next examined the spatial expression pattern by whole mount in situ hybridization (Fig. 2B). *Nanog* can already be detected in 4-, 16-, and 128-cell stages. High levels of *Nanog* are observed during gastrulation, but at the end of gastrulation (75% epiboly), transcripts are already strongly diminished. From segmentation onward (90% epiboly), *Nanog* cannot be detected anymore.

Next, we performed gain of function studies by Nanog overexpression in zebrafish embryos. Injection of *D.r. Nanog* RNA did not prevent gastrulation, and embryos did not display obvious abnormalities during gastrulation (data not shown). To obtain more precise information on genetic dysregulation, several marker genes were analyzed by in situ

hybridization (Fig. 3A; Supplementary Table S2A, S2B). We tested *Sox17* (endodermal marker), *sonic hedgehog* (*Shh*), a marker for neural ectoderm, and its target *Ptc-1*. We found in all cases disturbed expression patterns with a loss of the boundaries of their expression domains. *Southpaw* (*Spaw*), a nodal-related gene, which is normally expressed at the left side at somite stages [46], is bilaterally expressed in *D.r. Nanog* RNA injected embryos. Marker gene analysis via real-time RT-PCR (Fig. 3B, for details see Supplementary Table S3A) revealed that *Shh* was repressed in embryos injected with higher amounts of *Nanog* RNA. *Pou2* was upregulated, even at a low dose of *Nanog* RNA. This result is consistent with the situation in mammals, where Nanog stimulates Oct4 expression and vice versa [14,44]. We observed no change of ventral markers (*BMP4*, *Vent*, *Vox*, or *Ved*) at low amounts of *Nanog* RNA, but an upregulation of *Ved* (ventrally expressed *dharma/bozozok* antagonist) and *BMP4* at higher amounts of *Nanog* RNA. Interestingly, *gooseoid* (*Gsc*), a marker of dorsal mesoderm, is also upregulated. This finding indicates that Nanog signaling does not only shift the cells toward a ventral fate, but also acts in a more complex manner. Moreover, we observed a strong decrease of the neuroectodermal marker and key regulator of midbrain-hindbrain boundary, *Pax2a* [47]. Surprisingly, the chemokine receptor *Cxcr4b*, known as a guidance molecule of primordial germ cells in zebrafish and medaka [48,49], was repressed. This was unexpected, as *Cxcr4b* was shown to be a direct target of Nanog in medaka and was downregulated after Nanog depletion [50]. *Gata4* and *Gata6*, which were shown to be regulators of endodermal cell fate in *Xenopus* [51], are not significantly changed, although they are repressed by Nanog in human ES cells [52,53].

LOF studies were performed by using the morpholino (MO) based knockdown strategy. Specificity of NanogMO was demonstrated in vitro by coupled transcription/translation assays with *D.r. Nanog* RNA and mutated *Nanog* (*mut-Nanog*) RNA, containing 11 silent mutations in the putative MO binding site, in the presence of NanogMO or an unrelated ctrlMO (Supplementary Fig. S2). Translation of *D.r. Nanog* RNA is blocked by NanogMO but not by ctrlMO. In addition, NanogMO cannot suppress translation of *mut-Nanog* RNA. NanogMO or ctrlMO were then injected in zebrafish embryos at one cell stage. Although NanogMO injection does not result in disturbed embryogenesis before gastrulation (data not shown), we found the epiboly progression to be strongly impaired in 99% of injected embryos (Fig. 3C, Supplementary Table S2C). Although the blastoderm continues to become thin and has almost covered the yolk cells at 90% epiboly in uninjected and ctrlMO injected embryos, the epiblast in the NanogMO injected embryos is strongly thickened and covered only approximately half of the yolk. Later on, NanogMO injected embryos die, whereas control siblings develop normally. To ensure that the phenotypes yielded by NanogMO injections are specific for Nanog depletion and not a toxic side effect of the NanogMO, we performed rescue experiments. In contrast to NanogMO injection alone, we found that NanogMO and *mut-Nanog* RNA co-injected embryos overcome critical stages and develop further (Supplementary Table S2C; see also Fig. 6A).

We also analyzed Nanog depleted embryos by in situ hybridization and RT-PCR for the expression of ventral marker genes, such as *BMP4*, *Ved*, and *Vent* (Fig. 3C, D; Supplementary Tables S2D and S3B). Although *BMP4* expression,

FIG. 2. *Nanog* expression during zebrafish embryogenesis. **(A)** Temporal expression of *D.r. Nanog* analyzed by real-time RT-PCR. *Histone H4* is used as internal control, and expression is given in relative units. **(B)** Expression analysis of *D.r. Nanog* by whole mount in situ hybridization. Four-cell to 75% epiboly are viewed from top, the insert shows a lateral view of the shield (red arrowhead). About 90% epiboly to long pec stage are shown laterally. RT-PCR, reverse transcriptase-polymerase chain reaction.

responsible for the specification of ventro-posterior cell fate in the late phase of BMP signaling [54], is almost unaffected, the maternally expressed *Ved* [55] is strongly repressed. In addition, *Vent* and *Vox*, which are transcribed at midblastula transition (MBT), are decreased in *Nanog* depleted embryos. *Gata4* and *Gata6* expression is also decreased (Fig. 3D). We further observe in *Nanog* depleted embryos a repression of *Gsc* and *Pou2*, confirming the results that *Nanog* overexpression can activate *Gsc* and *Pou2* expression (Fig. 3B, D). This is in contrast to medaka, where *Nanog* depletion has no influence on *Oct4* expression level [27]. In addition, *Pax2a* and *Cxcr4b*, which were repressed by overexpression, are strongly upregulated in *Nanog* depleted embryos (Fig. 3B, D).

Since the *Vent* genes display a close sequence homology to zebrafish *Nanog* and a similar temporal expression profile with transcriptional activation around MBT [31,32], we

asked whether overexpression of zebrafish *Nanog* and *Xvent* in *Xenopus* results in the same or a similar phenotype. Bilateral injections of *D.r. Nanog* or *Xvent2* RNA at 2-cell stage delay blastopore formation (Fig. 4A, Supplementary Table S2E). Dorsal injections of *D.r. Nanog* or *Xvent2* RNA in 4-cell stage embryos lead to lacking head structures (Fig. 4A). We also performed real-time RT-PCR for selected marker genes at different developmental stages after injection of mouse and *D.r. Nanog* as well as *Xvent1/2* RNA (Fig. 4B, Supplementary Table S3C). We focused on markers for the undifferentiated state (*Oct60*, *Tert*, and *Klf4*), germ layer formation (such as *Xbra*, *Gsc*, and *Siamese*), cell cycle (*Wee1b*), and metabolism (*PepCK*, *Sod2*), including genes that by microarray analyses were reported to be changed by *Nanog* in ES cells (marked by asterisks) [56]. Importantly, mouse and *D.r. Nanog* RNA injections mainly lead to identical regulations. Although many genes are similarly affected by *Xvent* RNA

FIG. 3. Gain of function (GOF) and loss of function (LOF) analyses of *D.r. Nanog*. (A) Whole mount in situ hybridization of wild-type, control (unrelated nucleotide sequence), and *Nanog* RNA (400 pg) injected embryos hybridized with *Sox17* (80% epiboly), *Shh* (90% epiboly), *Ptc-1* (90% epiboly), or *Spaw* (12 somite stage). (B) Real-time RT-PCR of *D.r. Nanog* RNA injected embryos at 6 somite stage for indicated marker genes relative to wild-type embryos. (C) Uninjected control, ctrlMO, and *Nanog*MO (1.5 ng) injected embryos at 90% epiboly (lateral view) and in situ hybridization (animal view, the ventral side is left orientated) with *BMP4*, *Ved*, and *Vent*. (D) Real-time RT-PCR of *Nanog*MO injected embryos in relation to ctrlMO injected embryos for indicated marker genes at 80% epiboly. *Histone H4* is used as internal control. Green: upregulation (≥ 1.5), red: decrease (≤ 0.75), gray: no change in transcript level ($0.75 < x < 1.5$). MO, morpholino oligonucleotide; ctrl MO, control MO.

injections, that is, *Sod2*, *Wee1b*, *XSox3*, or *Oct60*, we found an inverse regulation of *Gsc* and *Sox2* (Fig. 4B).

Murine and *D.r. Nanog* show similar protein interactions and substitute for each other in vivo

Mammalian *Nanog* acts as a dimer in the maintenance of self-renewal in the absence of LIF, and dimerization of *Nanog* was shown to be dependent on C-terminally located tryptophan repeats [42,43]. Axolotl *Nanog* was reported to be unable for dimerization, because it lacks the tryptophan repeats [29]. Since these repeats are obviously specific for mammals (Supplementary Fig. S1), they are also absent from zebrafish. However, by GST-pulldown analyses, we found that *D.r. Nanog* can self-associate (Fig. 5A). Size exclusion chromatography of radiolabeled *D.r. Nanog* on Sephadex G-100 revealed that 70% of labeled protein eluted as dimers and 30% as monomers (data not shown). We prepared deletion constructs of *Nanog* to delineate the putative dimerization domain and found that the N-terminal region as well as the HD are required for the dimerization process (Fig. 5A). Noteworthy, in contrast to mammalian and axolotl *Nanog*, the N-terminal region of zebrafish *Nanog* displays an enrichment of tryptophan residues (Supplementary Fig. S1). We next investigated whether *D.r. Nanog* also physically interacts with the Pou2 homologous *Xenopus* protein Oct25. Mammalian and axolotl *Nanog* bind to Oct4 [22,24,29]. GST-pulldown analyses with Oct25 and murine or zebrafish *Nanog* demonstrated that both HD transcription factors interact with Oct25 (Fig. 5B).

Next, we tested by EMSA a 22mer of the murine *Tcf3* promoter, which was shown to be sufficient for murine *Nanog* binding [36], for binding of *D.r. Nanog* and *Xvent2*. The reported consensus binding sequences of murine *Nanog* (TAATGG, [36]) and *Xvent2* (CTAATT, [57,58]) show a certain overlap. However, we found that the murine as well as the zebrafish *Nanog* can bind the 22mer, whereas *Xvent2* does not interact with this binding site (Fig. 5C). This difference in DNA binding between *Xvent* and *Nanog* might be due to the already mentioned amino acid exchange within the $\alpha 2$ helix of the HD (basic residue $\rightarrow$ aliphatic, nonpolar residue).

We then asked whether *D.r. Nanog*, mouse *Nanog*, and *Xvent* can substitute each other after LOF in zebrafish or *Xenopus* embryos, respectively. First, we tried to overcome *Nanog*MO dependent gastrulation defects and subsequent death by co-injection with mutated *D.r. Nanog*, mouse *Nanog*, or *Xvent2* RNA (Fig. 6A). Although the blastoderm in *Nanog* depleted embryos remains thick and does not cover the yolk, co-injection of mouse *Nanog* RNA successfully substituted for zebrafish *Nanog*, as the gastrulation defects were reverted, and the embryos survived (Fig. 6A; Supplementary Table S2C). However, co-injection of *Xvent2* RNA at different amounts does neither inhibit nor attenuate the *Nanog*MO based gastrulation defects. Vice versa, we depleted *Xvent1* and *Xvent2* in *Xenopus* embryos by MO injections and tried to rescue the wild-type phenotype by co-injection of *Danio Nanog* RNA (Fig. 6B). Co-injections of *Xvent1*MO/*Xvent2*MO result in hyper-dorsalized embryos with strongly reduced body axis [33]. Co-injections of murine (data not shown) or zebrafish *Nanog* RNA at various concentrations did never restore dorso-ventral body axis or attenuate the *Xvent1/2* depletion phenotype. Therefore, we conclude that *Nanog* and *Xvent* cannot

FIG. 4. Comparison of *Xvent* and *D.r. Nanog* overexpression in *Xenopus laevis*. **(A)** Overexpression of *Nanog* or *Xvent2* in *X. laevis* embryos results in similar phenotypes. Embryos are shown at stage 11 (vegetal view), and at stage 29 (lateral view). RNAs were bilaterally injected at indicated amounts into 2-cell stage embryos or into the 2 dorsal cells at 4-cell stage, respectively. **(B)** Schematic presentation of real-time RT-PCR data derived from overexpression of *Nanog* and *Xvent1/2* in *X. laevis* at stages 10, 11, and 12. *M.m. Nanog* (400 pg), *D.r. Nanog* (400 pg), *Xvent1* (350 pg), or *Xvent2* RNA (350 pg) were bilaterally injected into 2-cell stage embryos. *Histone H4* was used as internal control. Green: upregulation (≥ 1.5), red: decrease (≤ 0.75), gray: no change in transcript level ($0.75 < x < 1.5$). Marker genes that were shown to be regulated by *Nanog* in ES cells [56] are indicated by asterisks. ES, embryonic stem.

FIG. 5. Protein and DNA binding properties of Nanog. **(A)** Scheme of Nanog deletion mutants used for pulldown analysis of GST-Nanog. White box: HD; Δ HD: deletion of the HD; HD+: HD of Nanog and surrounding 5 amino acids. Note that dimerization of *D.r.* Nanog does not require the C-terminus. **(B)** Oct25 interacts with mouse as well as with *D.r.* Nanog. 35 S methionine-labeled mouse and zebrafish Nanog proteins were incubated with the GST-fusion protein of Oct25. **(C)** Electrophoretic mobility shift assay of a 22mer of the murine *Tcf-3* promoter. *D.r.* and murine Nanog but not Xvent2 interact with the Nanog binding site.

substitute each other in gastrulation processes of zebrafish and in dorso-ventral patterning of *X. laevis*.

D.r. Nanog but not Xvent promotes proliferation and LIF-independent self-renewal of mouse ES cells

To test whether *M.m.* Nanog, *D.r.* Nanog, or Xvent2 have the same effects regarding cell differentiation and cell proliferation in ES cells, we generated stably transfected mouse ES cell lines of these constructs (Supplementary Fig. S3). The effects on cell differentiation were monitored by embryoid body assays, where the morphology and the loss of pluripotency were analyzed. With LIF, that is inhibiting differentiation of ES cells, embryoid bodies of all tested cell lines kept a round, undifferentiated shape, and mainly consisted of pluripotent cells. Without LIF, control cell lines (E14 and pFIEN vector control) and Xvent2 overexpressing cells started differentiation and lost their pluripotent state, which was indicated by the outgrowing and expanding morphology and the decreased blue AP staining of the embryoid bodies after 4 days

of culturing (Fig. 7A, B). In contrast, *M.m.* Nanog as well as *D.r.* Nanog overexpression were able to inhibit differentiation, visible by the still round shape and blue AP staining of the cultured embryoid bodies. This could be further substantiated by an expression analysis of some selected marker genes in the absence of LIF. Although the pluripotency genes *Pou5f1* and *Sox2* are highly expressed in *D.r.* Nanog and *M.m.* Nanog transfected cells, the differentiation markers *T (brachyury)* and *Sox17* exhibited a strong increase in Xvent2 and pFIEN vector transfected cells (Supplementary Fig. S4).

Previous studies implicate an important role of Nanog in cell proliferation not only for the mammalian [42] but also for the axolotl and medaka protein [27,29]. To test whether *D.r.* Nanog and Xvent2 also enhance cell proliferation, we performed BrdU and cell tracking assays with the transfected ES cell lines. We observed a much higher level of BrdU incorporation in mouse Nanog and *D.r.* Nanog transfected cells in contrast to Xvent2 or vector transfected ES cells (Fig. 7C). To verify these results, we also performed a cell tracking assay. ES cells were incubated with Red CMPTX as cell tracker. A high proliferation rate results in a higher basic value of fluorescence intensity coupled

FIG. 6. Mouse *Nanog* rescues zebrafish embryos after *Nanog* knockdown. **(A)** Co-injection of 400 pg mouse *Nanog* RNA and 1.5 ng *Nanog*MO in zebrafish embryos prevents the knockdown dependent gastrulation defect and subsequent death, whereas co-injection of 400 pg *Xvent2* RNA cannot do so. Upper: uninjected control (90% epiboly), *Nanog*MO injected and knockdown embryos co-injected with *mut-Nanog* RNA. Lower: knockdown embryos co-injected with *Xvent2* RNA or co-injected with mouse *Nanog* RNA at 90% epiboly and at somitogenesis. **(B)** Co-injection of *D.r. Nanog* RNA cannot rescue the *Xvent1/2*MOs (45 ng) caused phenotype in *X. laevis* embryos. Left: uninjected *X. laevis* embryos, stage 29, middle: *Xvent1*MO and *Xvent2*MO injected embryos. Right: *Xvent1*MO and *Xvent2*MO injected embryos co-injected with 100 pg *D.r. Nanog* RNA.

with an enhanced decrease of fluorescence intensity, when the cell tracker is removed. We here observed that fluorescence intensities after 45 min incubation were higher and decreased much faster in *D.r.* and mouse *Nanog* transfected ES cells than in pFIEN or *Xvent2* transfected cells (Fig. 7D). Accordingly, the ratio of mean fluorescence intensity of 0 h/1 day and, especially 0 h/2 day after removal of cell tracker, was higher in the mouse and *D.r. Nanog* than in the control and *Xvent2* transfected cells (Fig. 7D). Both experimental approaches, BrdU assay and cell tracking, suggest that murine as well as *D.r. Nanog* enhance proliferation.

Discussion

The *Nanog* gene is present in amphibia but lacking from the anuran *Xenopus*

EST data and genomic linkage analyses suggest a model according to which *Nanog* arose when *gnathostomata* evolved. In amphibians, *Nanog* was already identified in the newt *Notophthalmus viridescens* [59] and in the axolotl *Ambystoma mexicanum* [29], but *Nanog* was reported to be absent from the *X. tropicalis* genome [30]. Since we were also unable to identify ESTs for *Nanog* in *X. laevis*, we assume that *Nanog* is missing from the whole genus *Xenopus*. The genomic linkage analysis indicates that the *Nanog* gene has been deleted

during some chromosomal translocations in *Xenopodinae*. During evolution, *Nanog* passed at least 2 chromosomal rearrangements, first from *Osteichthyes* to *Tetrapodae*, and second within amphibians, where *Nanog* was depleted from anurans or, more specifically, from the *Xenopus* genome.

Characterization of *Nanog* in *D. rerio*

The *Nanog* gene in zebrafish is maternally expressed and strongly upregulated after the blastula stage of development. Transcripts are detected in the shield, are most abundant during gastrula stage, but are rapidly degraded by 75% epiboly. The murine *Nanog* expression was detected during embryogenesis from the morula stage onward, is later restricted to the epiblast, and excluded from primitive endoderm [16,17]. Mouse *Nanog* is detectable in the primitive streak, but is downregulated in cells ingressing through the streak to form mesoderm and definitive endoderm [60]. It is enriched in pluripotent cell lines, such as ES cells, embryonic germ cells, and embryonic carcinoma cells, but absent from somatic tissues [13,17]. Overexpression of zebrafish *Nanog* revealed an upregulation of the pluripotency factor *Pou2* and of ventral (*BMP4*, *Ved*) and dorsal-mesodermal (*Gsc*) markers, whereas the *Cxcr4b* gene was downregulated. Vice versa, knockdown of *Nanog* led to an increase of *Cxcr4b* and *Pax2a*,

FIG. 7. Mouse and zebrafish Nanog promote self-renewal and cell proliferation. **(A)** Morphology of embryoid bodies after 3 days with (+) and without (–) LIF (leukemia inhibitory factor). **(B)** Alkaline phosphatase (AP) staining of embryoid bodies with and without LIF after 4 days of culturing. **(C)** Nanog promotes cell proliferation. Percentages of BrdU positive ES cells detected with FACSCalibur in correlation to the incubation time in hours. **(D)** Histograms of fluorescence intensity of transfected ES cells treated with Cell Tracker Red CMTPX, in correlation to the percentages of cell numbers analyzed on FACSCalibur, and diagram presenting the ratio of mean fluorescence intensity (MFI) of 0 h to 1 or 2 days after removal of the cell tracker.

whereas *Pou2*, *Gsc*, *Vent*, *Vox*, *Ved*, *Gata4*, and *Gata6* were downregulated. Nanog overexpression does not affect the phenotype during gastrulation, but in situ hybridizations revealed a delocalized expression of *Sox17*, *Shh*, *Ptc-1*, and *Spaw*. Similar to that previously reported for medaka [27], Nanog knockdown in zebrafish prevents epibolic movements during gastrulation and is lethal.

The proliferative effect of Nanog is evolutionarily conserved

D.r. Nanog forms homodimers and maintains self-renewal of mouse ES cells in the absence of LIF. This finding correlates to the effects of murine Nanog, whereas axolotl Nanog was shown to maintain self-renewal, when tryptophan residues in the C-terminus were introduced [29]. Moreover, we found that murine Nanog is able to rescue depletion of zebrafish Nanog during gastrulation. These findings indicate that both *D.r.* Nanog and mouse Nanog behave as functional homologs.

An important aspect of Nanog function is the proliferative effect that was already described for mammalian, medaka, and axolotl Nanog [27,29,42]. Here, we demonstrate by BrdU incorporation and cell tracking that *D.r.* Nanog transfected mouse ES cells show a significant increase of proliferation. We, therefore, postulate that the regulation of proliferation is an important common role for all Nanog proteins which has been evolutionarily conserved.

Is Nanog in frogs replaced by Xvents?

How can an organism, such as *Xenopus*, survive without an important gene for vitality? Either some basic developmental processes in anurans/*Xenopodinae* have evolutionarily been changed, which made the *Nanog* gene dispensable, or the function(s) of *Nanog* gene might have been replaced by functionally related genes. Therefore, we have asked whether *Xenopus Vent* genes could have compensated for the missing *Nanog*. Sequence homologies, especially regarding the BarH specific threonine residue and the conserved serine residue outside the HD, which cannot be found in any other BarH proteins, suggest that *Vent* and *Nanog* arose from a common ancestor. In addition, the temporal expression profiles of zebrafish *Nanog* and *Xenopus Vents* are very similar. Overexpression of zebrafish *Nanog* and *Xenopus Vents* in frogs revealed similar phenotypes, and many developmentally important genes were regulated in the same manner. Moreover, zebrafish *Nanog* as well as *Xvent2* can dimerize and bind to Oct25. However, there are other important genes, such as the dorsalizing genes *Gsc* and *Chordin*, which are regulated by *Nanog* and *Xvent* in an opposite way. Moreover, *Nanog* stimulates cell proliferation, whereas *Xvent* has no stimulating effect. Finally, we could show that neither *Nanog* LOF in zebrafish can be overcome by *Xvent* overexpression nor LOF of *Xvents* in frogs can be overcome by zebrafish *Nanog* overexpression. In contrast, murine *Nanog* is still able to rescue the depletion of *Nanog* in zebrafish embryos during gastrulation. These experiments clearly demonstrate that *Nanog* function in *Xenopus* cannot simply be substituted by *Xvent* genes. Either there are other genes involved or *Nanog* function is *per se* dispensable in *Xenopus*. The identification of *Nanog* from additional amphibians, in particular of other anurans, would help in un-

derstanding the role of *Nanog* in embryogenesis and why it is dispensable in *Xenopus*.

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Author Disclosure Statement

There are no conflicts of interest relating to this work for any of the authors.

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