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The Oct4 homologue PouV and Nanog regulate pluripotency in chicken embryonic stem cells

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Embryonic stem cells (ESC) have been isolated from pregastrulation mammalian embryos. The maintenance of their pluripotency and ability to self-renew has been shown to be governed by the transcription factors Oct4 (Pou5f1) and Nanog. *Oct4* appears to control cell-fate decisions of ESC in vitro and the choice between embryonic and trophectoderm cell fates in vivo. In non-mammalian vertebrates, the existence and functions of these factors are still under debate, although the identification of the zebrafish *pou2* (*spg*; *pou5f1*) and *Xenopus Pou91* (*XlPou91*) genes, which have important roles in maintaining uncommitted putative stem cell populations during early development, has suggested that these factors have common functions in all vertebrates. Using chicken ESC (cESC), which display similar properties of pluripotency and long-term self-renewal to mammalian ESC, we demonstrated the existence of an avian homologue of *Oct4* that we call chicken *PouV* (*cPouV*). We established that *cPouV* and the chicken *Nanog* gene are required for the maintenance of pluripotency and self-renewal of cESC. These findings show that the mechanisms by which *Oct4* and *Nanog* regulate pluripotency and self-renewal are not exclusive to mammals.

KEY WORDS: Nanog, Oct4, Avian homologue, cPouV, Stem cells

INTRODUCTION

Embryonic stem cells (ESC) are self-renewing pluripotent cells that can be maintained in culture for an indefinite period. In mammals, pluripotency is under the control of key transcription factors, including Oct4 (also known as Pou5f1 – Mouse Genome Informatics) (Nichols et al., 1998), Nanog (Mitsui et al., 2003; Chambers et al., 2003), Sox2 (Avilion et al., 2003) and FoxD3 (Hanna et al., 2002). Oct4 is found in oocytes and is expressed in cleavage stage cells up to the morula stage (Kirchhof et al., 2000), and subsequently in the epiblast of the pre-primitive streak stage embryos. *Oct4* expression is downregulated in trophectodermal cells but maintained in the inner cell mass, becoming restricted to primordial germ cells and oocytes (Kehler et al., 2004; Boiani et al., 2002). In vitro, *Oct4* is expressed in proliferating murine and primate (including human) ESC, as well as in tumourigenic cells such as embryonal carcinoma (EC) (Ben-Shushan et al., 1995) and germ cell tumour (GCT) cells (Looijenga et al., 2003).

Oct4 appears to control cell-fate decisions of ESC in vitro. Inhibition of *Oct4* expression in mouse ESC (mESC) causes a loss of proliferation and the induction of trophectodermal and endodermal markers (Velkey and O'Shea, 2003; Hay et al., 2004). By contrast, overexpression of *Oct4* leads to primitive endoderm differentiation (Niwa et al., 2000) and it appears that a fine balance between *Oct4* and *Cdx2* expression controls the choice between embryonic and trophectoderm cell fates (Niwa et al., 2005; Tolkunova et al., 2006).

Oct4 contains a POU-specific domain and a POU homeodomain and belongs to the class V POU homeodomain family of transcription factors. A complex of proteins including Oct4 and Sox2 has been found to regulate expression of the growth factor Fgf4 (Dailey et al., 1994; Ambrosetti et al., 1997) and of the transcription factors Utf1 (Nishimoto et al., 1999), Zfp42 (Rex1) (Ben-Shushan et al., 1998), Fbx15 (also known as Fbxo15 – Mouse Genome Informatics) (Tokuzawa et al., 2003), Nanog (Kuroda et al., 2005; Rodda et al., 2005) and Sox2 itself (Tomioka et al., 2002). Different nuclear receptors participate in the regulation of *Oct4* expression including Sf1 (Barnea and Bergman, 2000), Lrh-1 (Nr5a2) (Gu et al., 2005a), Gcnf (Nr6a1) (Fuhrmann et al., 2001; Gu et al., 2005b), CoupTF (Nr2f2) (Ben-Shushan et al., 1995) and Rar/Rxr heterodimers, the latter being responsible for the downregulation of *Oct4* expression by retinoic acid (Schoorlemmer et al., 1994; Pikarsky et al., 1994). *Oct4* expression is under the control of its own protein (Okumura-Nakanishi et al., 2005; Chew et al., 2005) through specific response elements located in its own promoter (Yeom et al., 1996; Nordhoff et al., 2001; Gu et al., 2005a; Gu et al., 2005b).

Nanog expression is also confined to pluripotent tissues and cell lines and its overexpression is able to maintain mESC in an undifferentiated state, even in the absence of Lifr/gp130 stimulation. Inhibition of *Nanog* expression in mESC results in their differentiation into primitive endoderm (Chambers et al., 2003; Mitsui et al., 2003).

To date, this relationship between *Oct4* and/or *Nanog* and stem cell pluripotency has only been demonstrated in mammals. Indeed, in zebrafish, it was reported that the *pou2* gene (also known as *spg* and *pou5f1* – ZFIN), initially identified by a mutation that caused neural and endoderm defects, is the fish homologue of the mammalian *Oct4* gene based on protein similarities, chromosomal syntenic relationship and developmental expression pattern, but not in terms of function (Burgess et al., 2002). No evaluation of a

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putative role in fish ESC pluripotency was described in an assessment of murine *Oct4* activity in medaka ESC (Hong et al., 1998). The *X. laevis Pou91* (*XlPou91*) gene product, encoded by one of three *X. laevis PouV* genes, has been demonstrated to have a similar activity to the mouse *Oct4* gene in mESC and to participate in the maintenance of putative stem cell populations during early development (Morrison and Brickman, 2006).

Given that *Oct4* appears to be so important in the maintenance of pluripotency, a report suggesting that the chicken genome lacks a homologue of *Oct4* (Soodeen-Karamath and Gibbins, 2001) was very surprising. Indeed, no corresponding sequence was identified in the chicken genome annotation, even in the latest release (Ensembl 42, December 2006).

Here we report the isolation of chicken *PouV* (*cPouV*) and *Nanog* (*cNanog*), homologues of mammalian *Oct4* and *Nanog*. Both genes are expressed in early embryos before gastrulation and thereafter in germ cells. Taking advantage of chick ESC (cESC) (Pain et al., 1996; Petitte et al., 2004), we demonstrate that chicken *PouV* and *Nanog* are required for the maintenance of cESC pluripotency and for continued proliferation. Together, these findings show that the mechanisms by which these two genes regulate pluripotency and self-renewal are not exclusive to mammals.

MATERIALS AND METHODS

Oligonucleotides and cDNA sequences

Oligonucleotides (Proligo) were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are listed in Table 1. The coding sequences of the different genes were identified using the chicken genome assembly (http://www.ensembl.org/Gallus_gallus/), GenBank (http://www.ncbi.nlm.nih.gov) or sequenced directly from newly isolated clones.

Subtractive liquid hybridisation

Total RNA from cESC and from 2-day-old chicken embryoid bodies (cEB) obtained as previously described (Pain et al., 1996) were reverse transcribed. The cDNAs were subject to a subtractive liquid hybridisation procedure (http://www.genome-express.com/). The transcripts enriched in cESC were subcloned, sequenced and filtered sequences assembled using PHRAP software. Target sequences delivered by the assembly process were subject to BLAST analysis.

Library screening and cloning of *cPouV*

The cDNA library from chicken embryonic stem cell mRNA (Acloque et al., 2001) was screened using T7 or T3 vector primers and internal sequences P06(381)S (5′-GTTGTCCGGGTCTGGTTCT-3′) or P06(382)AS (5′-GTGGAAAGGTGGCATGTAGAC-3′) derived from the 1P06g01 initial clone. A 5′-RACE strategy was developed with the P06RAAS2 (5′-TGAGTGAAGCCCAGCATGAT-3′) primer followed by a second amplification with P06RAAS1 (5′-AACATCTTCCCATA-GAGCGTGC-3′) and AnchPS (5′-GACCACGCGTATCGATGTC-GACTTTTTTTTTTTTTTT-3′) primers. A second round of amplification using P06(pL7-2)AS (5′-TGCTTGAGGTCCTTGGCAAA-3′) and PCRprimseq primers led to the isolation of 300 bp upstream of the 1P06g01 clone, including an in-frame ATG. A full-length cDNA was cloned into pGEM-T-easy (Promega) using primers P06EcoRIS (5′-AT-GAATTCATGCATGTAAAAGCCAAA-3′) and P06EcoRIAS (5′-AT-GAATTCTCAGTGGCTGCTGTTGTT-3′).

RNA extraction and RT-PCR

Real-time RT-PCR was performed using the MXP-3000P PCR-system (Stratagene) using Mix-Quantitect SYBR Green (Qiagen). Samples were run in duplicate and gene expression levels were calculated using the ΔΔCt method (http://www.gene-quantification.info) with the chicken ribosomal gene *RS17* (X07257) as reference. The number of independent experiments performed is indicated in each figure legend.

Table 1. Oligonucleotides used for gene expression analysis

		Oligonucleotide (5′ to 3′)	
Gene	Gene identification	Sense	Antisense
Chicken			
AP	U19108	CCTGACATCGAGGTGATCCT	CAAAGAGACCCAGCAGGAAG
Cdx2	NM_204311	TCAAAACCAGGACGAAGGAC	CCAGATTTTCACCTGCCTCT
Gata4	XM_420041	TGAGAAAAGAGGGCATTCAGA	GCAGGATGAATTGAAGATCCA
Gata6	NM_205420	CCGACCACTTGCTATGAAAAA	CAGCCCATCTTGACCTGAATA
Gcnf	ENSGALT00000001607	GTTTGCCAGGACTTCACAGAG	CGGGACATTCACCATCTTTC
Nanog	DQ867025	CAGCAGACCTCTCCTTGACC	TTCCTTGTCCCACTCTCACC
PouV	DQ867024	GTTGTCCGGGTCTGGTTCT	GTGGAAAGGTGGCATGTAGAC
Rarg	X73973	TCTACAAACCGTGCTTCGTCT	TCCTCCTTCACCTCCTTCTTC
RS17	X07257	ACACCCGTCTGGGCAACGACT	CCCGCTGGATGCGCTTCATCA
Sox2	U12532	GCAGAGAAAAGGGAAAAAGGA	TTTCCTAGGGAGGGGTATGAA
Sox3	U12467	TGTTCGCTTCCGAGTCTTAAA	CCTTTCCGTAGGAACAAAACC
Tert	AY502592	CCCAATAGAAGGGGCATAGAG	CTTGGTAACTGCGGGAATACA
Mouse			
brachyury	NM_009309	CCGGTGCTGAAGGTAAATGT	CCTCCATTGAGCTTGTTGGT
Cdx2	NM_007673	TCTCCGAGAGGCAGGTTAAA	GCAAGGAGGTCACAGGACTC
Fgf4	NM_010202	CGAGGGACAGTCTTCTGGAG	GTACGCGTAGGCTTCGTAGG
Gata4	AF179424	GCAGCAGCAGTGAAGAGATG	GCGATGTCTGAGTGACAGGA
Gata6	AF179425	GCCAACTGTCACACCACAAC	TGTTACCGGAGCAAGCTTTT
Hnf1 (*Tcf1*)	M57966	GATGTCAGGAGTGCGCTACA	CTGAGATTGCTGGGGATTGT
laminin B1	M15525	GTTCGAGGGAACTGCTTCTG	GTTCAGGCCTTTGGTGTTGT
Nanog	AY278951	AAGTACCTCAGCCTCCAGCA	GTGCTGAGCCCTTCTGAATC
Oct4 (*Pou5f1*)	NM_013633	CACGAGTGGAAAGCAACTCA	AGATGGTGGTCTGGCTGAAC
Rex1 (*Zfp42*)	NM_009556	GGCCAGTCCAGAATACCAGA	GAACTCGCTTCCAGAACCTG
Rs17 (*Rsp17*)	BC086901	ATGACTTCCACACCAACAAGC	GCCAACTGTAGGCTGAGTGAC
Sox17	NM_011441	CTCGGGGATGTAAAGGTGAA	GCTTCTCTGCCAAGGTCAAC
Sox2	U31967	CACAACTCGGAGATCAGCAA	CTCCGGGAAGCGTGTACTTA
Tert	AF051911	ACTCAGCAACCTCCAGCCTA	CATATTGGCACTCTGCATGG
Utf1	D31647	TTACGAGCACCGACACTCTG	GGCCCAGAACTGTTGAGATG

In situ hybridisation

Hens' eggs were incubated for 0-36 hours and embryos staged according to Eyal-Giladi and Kochav (Eyal-Giladi and Kochav, 1976) for pre-primitive streak stages and according to Hamburger and Hamilton (Hamburger and Hamilton, 1951) for later stages. Embryos were subjected to whole-mount in situ hybridisation (Streit and Stern, 2001). Fluorescent *Vasa* and *cPouV* probes were labelled with digoxigenin and fluorescein, respectively, and successively revealed using an HRP-coupled anti-digoxigenin and an HRP-coupled anti-fluorescein antibody and the TSA-Plus Cyanine3/Fluorescein system (Perkin Elmer). SSEA-1 labelling (DSHB, Iowa) was performed on frozen sections (15 μm) and revealed with an anti-mouse IgM conjugated to Texas Red (Abcam). Adjacent sections were processed for in situ hybridisation as previously described (Strähle et al., 1994).

Expression constructs

Reverse-transcribed chicken embryonic stem cell mRNA was used with P06GFPEcoRIS (5′-GGGAATTCGCATGTAAAAGCCAAA-3′) and P06GFPKpnIAS (5′-ATGGTACCTCAGTGGCTGCTGTTGT-3′) primers to amplify the *cPouV* coding region. The product was subcloned into pEGFP-C1 (Clontech) to produce the pGFP-cPouV expression vector. *cPouV* cDNA was amplified with P06EcoRIS (5′-ATGAATTCATG-CATGTAAAAGCCAAA-3′) and P06EcoRIAS (5′-ATGAATTCTCAG-TGGCTGCTGTTGTT-3′) primers and cloned into pCAGIP (Niwa et al., 2000). The 1.8 kb *pou2* zebrafish coding sequence was amplified from a pCSL2-Pou2 template using Pou2EcoRIS (5′-ATAGAATTCTATGAC-GGAGAGAGCGCAG-3′) and Pou2EcoRIAS (5′-GTAGAATTCTTAG-CTGGTGAGATGACCC-3′) primers and cloned into pCAGIP. Murine *Oct4* and *Nanog* coding sequences were reverse transcribed from mESC total RNA with primer pairs mOct4EcoRIS (5′-ATGAATTCTGCTGGAC-ACCTGGCTTC-3′) with mOct4EcorIAS (5′-ATGAATTCTTAACCC-CAAAGCTCCAG-3′) and mNanogXhoIS (5′-GTCTCGAGATGAGT-GTGGGTCTTCC-3′) with mNanogNotIAS (5′-ATGCGGCCGCTCAT-ATTTCACCTGGT-3′), respectively, then inserted into pCAGIP. The *cNanog* coding sequence was obtained from reverse-transcribed cESC total RNA using cNanogEcoRIS (5′-ATGAATTCATGAGCGCTCACCTG-GCC-3′) and cNanogEcoRIAS (5′-ATGAATTCCTAAGTCTCATAAC-CATT-3′) primers and cloned into pCAGIP.

Transactivation test

The p(ATGCAAAT)×3-luc reporter gene was constructed by inserting double-stranded oligonucleotides Oct4BS (5′-CTAGCATGCAAATAA-CAGCGCGCATGCAAATAACAGCGCATGCAAATAACAGCGCCCC-3′) and Oct4BAS (5′-GGGGCGCTGTTATTTGCATGCGCTGTTATT-TGCATGCGCGCTGTTATTTGCATG-3′) into the pGL3 vector (Promega). To construct the pΔPE-luc reporter gene, a 1.4 kb fragment from the *mOct4* distal enhancer was amplified from pGOF18ΔPE-GFP using ODES (5′-GTACGCGTGAATTCAGACAGGACTGCTGGGC-3′) and SVAS (5′-AGCATCACAAATTTCACAAATAAAGAATTCACG-GCTTT-3′) primers (Hong et al., 2004) and subcloned into pGL3. For luciferase assays, ZHBTc4 cells were plated at 1×10^5 cells per well with 2 μg/ml tetracyclin. Twenty-four hours later, 75 ng of reporter plasmid, 150 ng of the test plasmid and 10 ng of the *Renilla* reporter plasmid were co-transfected using 600 ng FuGENE 6 (Roche) and incubated overnight before fresh medium was added with 2 μg/ml tetracyclin. Cell lysates were analysed 48 hours after transfection as described by the manufacturer (Promega).

RNA interference (RNAi) vector construction

pFLΔNeo was obtained by inserting into pBSK the 2 kb PCR-amplified product mU6ΔNeoΔ, derived from the mU6ΔNeo-ΔApaIDXhoI template (Coumoul et al., 2004) using mU6SmaIS (5′-ATCCCGGGGTATATCC-GACGCCGCCAT-3′) and mU6HindIIIAS (5′-ATAAGCTTAACAAG-GCTTTTCTCC-3′) primers. Double-stranded short hairpin (sh) RNA was cloned into pFLΔNeo, generating pFLΔNeo-XshRNA vectors for each gene to be targeted. The oligonucleotides containing the *Hin*dIII and *Xho*I sites used for generating the 21 bp shRNA sequence were: cPouV-shRNA-2S (5′-AGCTTAAGATGTTCAGCCAGACCACCTTCAAGAGAGGTG-GTCTGGCTGAACATCTTTTTTTTC-3′) and cPouV-shRNA-2AS (5′-TCGAGAAAAAAAAGATGTTCAGCCAGACCACCTCTCTTGAAGG-TGGTCTGGCTGAACATCTTA-3′) against *cPouV*; cNanog-shRNA-1S (5′-AGCTTAACAGAAACCTTCAGGCTGTGTTCAAGAGACACAGC-CTGAAGGTTTCTGTTTTTTTTC-3′) and cNanog-shRNA-1AS (5′-TCGAGAAAAAAAACAGAAACCTTCAGGCTGTGTCTCTTGAACA-CAGCCTGAAGGTTTCTGTTA-3′) against *cNanog*; as well as cNanog-shRNA-3S (5′-AGCTTAAGGCCAAGAGCCGCACAGCTTTCAAGA-GAAGCTGTGCGCTCTTGGCCTTTTTTTTC-3′) and cNanog-shRNA-3AS (5′-TCGAGAAAAAAAGGCCAAGAGCCGCACAGCTTCTCTT-GAAAGCTGTGCGGCTCTTGGCCTTA-3′) and cOct6-shRNA-3S (5′-AGCTTAAGCAGCGGCGGATCAAGCTGTTCAAGAGACAGCTTGA-TCCGCCGCTGCTTTTTTTTC-3′) and cOct6-shRNA-3AS (5′-TCGA-GAAAAAAAAGCAGCGGCGGATCAAGCTGTCTCTTGAACAGCT-TGATCCGCCGCTGCTTA-3′) against *cOct6*. The Cre-ERT2 coding sequence (Feil et al., 1997) was cloned into the pCIFL-Hygro vector, derived from pCINeo (Promega), by replacing the neomycin cassette with a hygromycin cassette to produce pCre-ERT2-Hygro.

Cell maintenance and transfection

cESC were maintained and transfected as previously described (Pain et al., 1996; Pain et al., 1999). For kinetic experiments, formation of cEB was achieved by allowing dissociated proliferating cESC to float in bacterial dishes. When used, retinoic acid was added at 10^{-7} M 24 hours after plating and considered as T=0. Cycloheximide and actinomycin D were added to the culture medium at 10 μg/ml for various times as indicated.

ZHBTc4 cells were maintained as described (Niwa et al., 2000). Expression of the endogenous murine *Oct4* can be downregulated by addition of 1 μM doxycyclin (Sigma). For transfection, 5×10^6 cells were electroporated (BioPulser, BioRad) at 575 μF with 25 μg of the various linearised vectors. From twenty-four hours after electroporation, doxycyclin was added at daily intervals, and puromycin was added 72 hours after electroporation at 1 μg/ml and administered daily for 6 days.

RNAi induction and proliferation assay

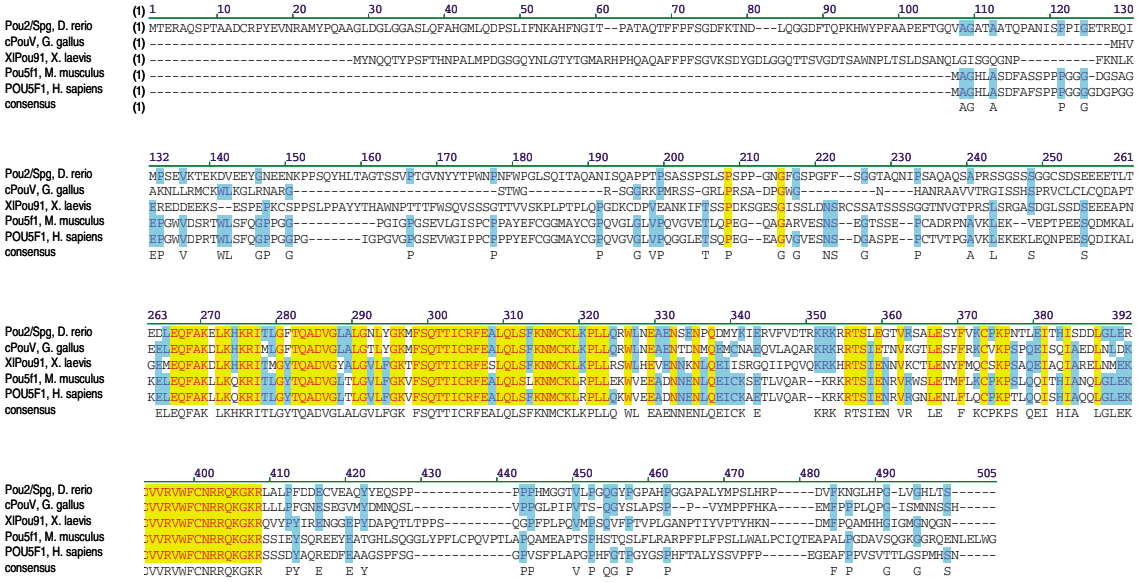
Once transfected and selected with 200 μg/ml neomycin for 7 days, resistant clones of cESC were pooled, transfected with the pCre-ERT2-Hygro vector and selected for 7 days with 0.75 μg/ml hygromycin. Clones were numbered, picked and individually observed during the induction of shRNA expression by adding 1 μM 4-hydroxytamoxifen to the medium. Morphology was assessed by direct microscopic observation and Wright Giemsa staining. For proliferation kinetic assays, clones were picked individually, the cells dispersed and plated in six wells in 250 μl medium. After 24 hours, 4-hydroxytamoxifen was added and proliferation was assessed at different times using two wells per time point using Cell Proliferation Kit II (XTT) (Roche).

RESULTS

Cloning of the chicken homologue of the mammalian *Oct4* gene

Subtractive hybridisation of cDNAs from cESC and chicken embryoid bodies (cEB) resulted in identification of a 228 bp cDNA fragment encoding a partial POU domain. A combination of the results of screening a cDNA library and 5′-RACE using mRNA from cESC, allowed us to define an open reading frame (ORF) of 888 bp coding for a 295 amino acid (aa) protein. Comparative analysis and phylogenetic tree construction using maximal parsimony and neighbour-joining methods revealed that this sequence is statistically more closely related to the other PouV proteins than to the other Pou factors (Fig. 1A) (Felsenstein, 1978; Saitou and Nei, 1987). This novel predicted protein is part of the PouV protein subfamily, which contains XlPou91, the *D. rerio* Pou2 and mammalian Pou5f1 proteins and exhibits high similarity with the other members of the family (Fig. 1B,C).

A



B

Species	Genbank nb	Bt	Ss	Hs	Pt	Mm	Tv	Dr	Gg	Xl		
										Pou25	Pou91	Pou60
B. taurus	NP 777005	100	97	93	93	74	58	32	51	33	34	33
S. scrofa	Q9TSV5		100	95	95	75	58	32	51	32	32	32
H. sapiens	NP 002692			100	100	76	57	31	51	33	35	34
P. troglodytes	Q7YR49				100	76	57	30	51	32	33	32
M. musculus	NP 038661					100	47	26	46	29	31	29
T. vulpecula	AY 345973						100	22	58	27	29	31
D. rerio	NP 571187							100	35	33	32	30
G. gallus	DQ 867024								100	37	37	38
X. laevis (Pou25)	AAA 49996									100	52	40
X. laevis (Pou91)	AAA 49999										100	38
X. laevis (Pou60)	AAA 49997											100

C

Species	Genbank nb	Homeodomain			Bt	Ss	Hs	Pt	Mm	Tv	Od	Dr	Gg	Xl		
		start	end	size										Pou25	Pou91	Pou60
B. taurus	NP 777005	142	288	146	100	100	97	98	92	69	48	67	67	63	65	55
S. scrofa	Q9TSV5	142	288	146		100	97	98	92	69	48	67	67	63	65	55
H. sapiens	NP 002692	142	289	147			100	99	93	69	48	65	67	63	66	56
P. troglodytes	Q7YR49	142	288	146				100	92	69	49	65	67	63	66	56
M. musculus	NP 038661	135	282	147					100	65	49	65	65	63	65	56
T. vulpecula	AY 345973	1	120	119						100	37	50	56	51	51	46
O. dioica	AAT 47873	224	372	148							100	44	48	42	48	46
D. rerio	NP 571187	253	402	149								100	76	66	63	60
G. gallus	DQ 867024	80	229	149									100	76	71	65
X. laevis (Pou25)	AAA 49996	231	380	149										100	76	68
X. laevis (Pou91)	AAA 49999	222	371	149											100	64
X. laevis (Pou60)	AAA 49997	206	355	149												100

D

Gene		Nkx2.1	Nkx2.4	Nkx2.3		Nkx2.5				Nkx2.6	Nanog					Nkx2.2	
Species		Gg	Mm	Gg	Mm	G. g	Xl	Hs	Mm	Mm	Mm	Rr	Hs	Hs-2	Gg	Hs	Mm
	Genbank Nb																
G. gallus, Nkx2.1	NP_989947	100	60	36	34	40	39	38	38	39	27	24	27	28	28	45	45
M. musculus, Nkx2.4	NP_075993		100	35	29	35	34	36	36	35	22	20	19	19	21	43	43
G. gallus, Nkx2.3	CAA66257			100	61	56	54	55	55	46	28	26	26	28	28	45	46
M. musculus, Nkx2.3	NP_032725				100	51	50	51	52	39	24	22	24	24	24	39	39
G. gallus, Nkx2.5	NP_990495					100	82	74	73	52	33	31	33	36	31	47	48
X. laevis, Nkx2.5	AAA19861						100	71	70	50	34	30	33	36	32	47	47
H. sapiens, NKX2.5	NP_004378							100	90	47	31	29	29	30	31	46	46
M. musculus, Nkx2.5	NP_032726								100	48	30	28	30	30	31	46	46
M. musculus, Nkx2.6	NP_035050									100	39	37	37	49	40	49	49
M. musculus, Nanog	BAC76998										100	80	66	59	44	33	33
R. rattus, Nanog	XP_575662											100	62	53	41	30	30
H. sapiens, NANOG	NP_079141												100	81	44	32	33
H. sapiens, NANOG2	AAS57555													100	41	33	33
G. gallus, Nanog	DQ 867025														100	33	33
H. sapiens, NKX2.2	NP_002500															100	99
M.musculus Nkx2.2	NP_035049																100

Fig. 1. See next page for legend.

Fig. 1. The *cPouV* gene encodes a chicken PouV protein. (**A**) Alignment of *D. rerio* Pou2 (NP_571187), *X. laevis* Pou91 (AAA49999.1), *M. musculus* Oct4 (Pou5f1, NP_038661) and *H. sapiens* OCT4 (POU5F1, NP_002692) PouV proteins with the chicken PouV coding sequence (DQ867024) using NTi Clustal software (Invitrogen). (**B**,**C**) Similarity table analysis of the full-length proteins (B) or of their homeodomains (C) of Oct4 homologues was performed with sequences of 360 aa for *B. taurus* (Bt) Oct4 (NP_777005), of 360 aa for *Sus scrofa* (Ss) Oct4 (Q9TSV5), of 360 aa for *H. sapiens* (Hs) OCT4 (NP_002692), of 360 aa for *P. troglodytes* (Pt) Oct4 (Q7YR49), of 352 aa for *M. musculus* (Mm) Oct4 (NP_038661), of 189 aa for *T. vulpecula* (Tv) Oct4 (AAQ24229), of 472 aa for *D. rerio* (Dr) Pou2 (NP_571187), of 448 aa for *X. laevis* (Xl) Pou25 (AAA49996), of 445 aa for *X. laevis* Pou91 (AAA49999) and of 426 aa for *X. laevis* Pou60 (AAA49997). The 295 aa were used for the *G. gallus* (Gg) PouV protein (DQ867024). (**D**) Similarity table analysis of Nkx and Nanog families performed with sequences of 344 aa for *G. gallus* (Gg) Nkx2.1 (NP_989947), of 354 aa for *M. musculus* (Mm) Nkx2.4 (NP_075993), of 323 aa for *G. gallus* Nkx2.3 (CAA66257), of 362 aa for *M. musculus* Nkx2.3 (NP_032725), of 294 aa for *G. gallus* Nkx2.5 (NP_990495), of 299 aa for *X. laevis* (Xl) Nkx2.5 (AAA19861), of 324 aa for *H. sapiens* (Hs) NKX2.5 (NP_004378), of 318 aa for *M. musculus* Nkx2.5 (NP_032726), of 216 aa for *M. musculus* Nkx2.6 (NP_035050), of 305 aa for *M. musculus* Nanog (BAC76998), of 312 aa for *R. rattus* (Rr) Nanog (XP_575662), of 305 aa for *H. sapiens* NANOG (NP_079141), of 232 aa for *H. sapiens* (Hs-2) NANOG2 (AAS57555), of 273 aa for *H. sapiens* NKX2.2 (NP_002500) and of 273 aa for *M. musculus* Nkx2.2 (NP_035049). The 310 aa were used for the chicken Nanog protein (DQ 867025). Red text and yellow highlights indicate a complete aa conservation between tested species; blue text and blue highlights indicate partial conservation between tested species.

At the genome level, the novel gene was mapped to chicken chromosome GGA17, specifically between primers SEQ0256 and SEQ0257 described in the ChickRH6 whole-genome radiation hybrid (WGRH) panel (http://chickrh.toulouse.inra.fr/). Syntenic comparison identified a relationship between this chicken gene, *XlPou91* and zebrafish *pou2*. This relationship appears to be absent, either deleted or displaced, in mammalian species, despite the presence of adjacent syntenic loci on mouse chromosome 2 (data not shown).

In conclusion, our data reveal the existence in the chicken genome of a gene belonging to the *PouV* gene subfamily. We will therefore henceforth refer to this new chicken gene as chicken *PouV* (*cPouV*) (GenBank accession DQ867024).

Cloning of chicken *Nanog* cDNA

A chicken *Nanog* gene was predicted in the chicken genome annotation at reference ID ENSGALG00000014319 on chicken chromosome 1 (GGA1). Primers designed using this sequence were used to isolate a clone from the chicken embryonic stem cell library with a 930 bp ORF (GenBank accession DQ867025). Comparative analysis and phylogenetic tree construction revealed that this sequence is closely related to mammalian *Nanog* genes and that the predicted protein exhibits high similarity with the other Nanog proteins (Fig. 1D). This sequence contains a homeodomain of 57 aa, located between aa 98 and 155, but does not have the WWW repeat in the C-terminus that is characteristic of the mammalian Nanog subfamily (Pan and Pei, 2005). In contrast to the recently reported chicken Nanog sequence identified in silico (Canon et al., 2006), our cloned protein does not indicate the existence of a 112 aa segment after aa 50 that could correspond to a putative alternatively spliced form.

cPouV and *cNanog* are highly expressed in proliferating cESC and downregulated during differentiation of cESC

To determine the expression profiles of *cNanog* and *cPouV*, we performed real-time RT-PCR experiments showing that proliferating cESC express high levels of *cPouV* and *cNanog* (Fig. 2A,D, time 0). cESC can be induced to differentiate either as cEB, by preventing cell attachment, or following treatment with chemical inducers such as DMSO or retinoic acid (RA) (Pain et al., 1996). During a 5-day RA treatment, *cPouV* expression was almost completely abolished in parallel to similar reductions in expression of the markers alkaline phosphatase (*AP*) and telomerase reverse transcriptase (*Tert*) (Fig. 2A). Chicken *Gcnf* expression was also strongly downregulated, as was expression of *Sox2* and *Nanog*, although with a more complex profile. By contrast, *Rarγ* expression was upregulated following RA treatment (Fig. 2A). Treatment with cycloheximide, known to block de novo protein synthesis, did not affect the downregulation of *cPouV* and *Gcnf* transcription, whereas *Nanog* transcription was no longer responsive to RA, suggesting that downregulation of *cPouV* and *Gcnf* transcription are direct transcriptional events following RA treatment (Fig. 2B). Moreover, following actinomycin D treatment, which blocks transcription, a 50% decrease in the expression of *cPouV* and *Gcnf* was observed 8 to 12 hours after addition of the drug. A decrease of greater than 50% in *Nanog* mRNA levels was seen as early as 30 minutes after treatment, suggesting that it has a very short half-life (Fig. 2C).

Expression of *cPouV* and *Nanog* was also strongly downregulated during formation of cEB (Fig. 2D), as was expression of *Sox2* and *AP*. By contrast, *Sox3*, *Cdx2* and *Gata6* were induced, suggesting a complex differentiation process during formation of these three-dimensional bodies (Fig. 2E).

cPouV and *cNanog* are expressed dynamically in early chick embryos

To determine the likely sites at which *cPouV* and *cNanog* function during normal development, transcripts were identified in embryos during progressive stages of development using whole-mount in situ hybridisation. *cPouV* mRNA was found to be ubiquitously expressed in the epiblast of pre-primitive streak stage embryos and in a salt-and-pepper fashion in the forming hypoblast (Fig. 3A,B,B′). As the primitive streak started to form, transcripts were strongly localised in the epiblast of the streak itself (Fig. 3C,C′) and in the mesoderm emerging from it, whereas expression in the lower layer tended to decrease (Fig. 3D-F,D′,F′). Expression in the area opaca was lost by stage 3^+ (Fig. 3F). At later stages, *cPouV* continued to be expressed in the mesoderm, but was undetectable in the endoderm (Fig. 3G-I). At stage 8 and subsequently, *cPouV* was strongly expressed in the neural plate and neural tube with particularly strong expression in the anterior hindbrain/posterior midbrain (Fig. 3I). Later, at stage 9 and subsequently, *cPouV* was still expressed in neural tissue and expression appeared in primordial germ cells (Fig. 3J).

cNanog showed a different pattern. In pre-streak embryos, transcripts were detected in the whole epiblast but not in the forming hypoblast (Fig. 4A,B,B′). As the primitive streak started to form, transcripts disappeared from the primitive streak epiblast but were still expressed throughout the area pellucida epiblast (Fig. 4C-E,C′,D′). At the end of gastrulation (stage 4^-4^+), *cNanog* mRNA was quickly downregulated in the epiblast and persisted in a crescent anterior to the emerging head process (Fig. 4F-H). As the neural plate formed (stages 6-8), expression in the epiblast was restricted to the anterior neural plate (Fig. 4H-J,H′).

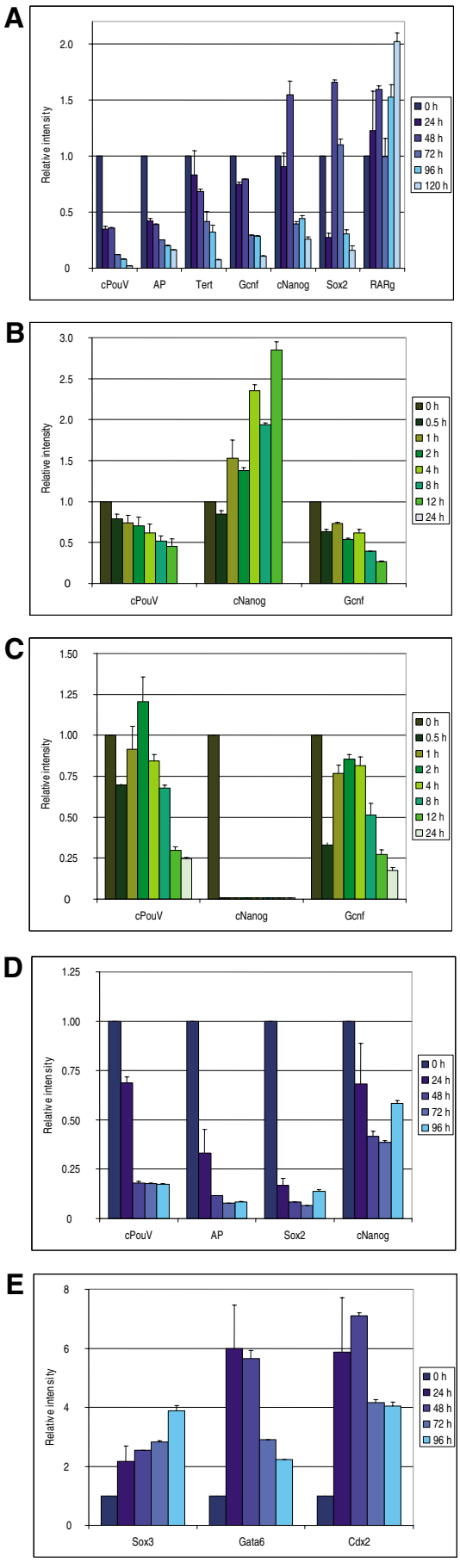


Fig. 2. Kinetics of expression of pluripotency genes during differentiation of cESC. Proliferating cESC were induced to differentiate (**A**) by retinoic acid treatment at 10^{-7} M for 5 days after plating or (**D**,**E**) by embryoid body formation for 4 days. Five independent experiments provided similar results. (**B**,**C**) As in A except that cycloheximide (B) or actinomycin D (C) was added to the culture medium at 10 μg/ml at T=0; two independent experiments provided similar results. Expression of some of the genes analysed, as measured by real-time RT-PCR, was downregulated (D) or upregulated (E). A value of 1 was assigned to expression levels at T=0, i.e. at the start of the induction of differentiation.

In conclusion, following initial, high levels of expression in early pluripotent epiblast cells, *cPouV* and *cNanog* present a very restricted in vivo pattern of expression during early embryonic development.

cPouV and *cNanog* are expressed in the germ cells during late embryonic development

In order to determine the expression profile of *cPouV* and *cNanog* during late embryonic development, quantitative (Q) RT-PCR analysis was performed on chicken embryo tissues at day 16 to 17, including intestine, muscle, kidney, spleen, lung, brain, liver, heart and gonads. Expression was detected in gonads (male and female, data not shown) but at a level 270-fold lower than in proliferative cESC, and also in spleen and brain but 530-fold and 1100-fold lower, respectively, than in cESC (data not shown). In situ hybridisations confirmed that *cPouV* is expressed in gonads, with expression restricted to germ cells. At stage 33 (7 days of incubation), *cPouV* expression was detected in a salt-and-pepper fashion in the forming gonads (Fig. 5A,B). The *cPouV*-positive cells were found to also express the germ-cell-specific markers *Cvh* (by mRNA detection, Fig. 5C-F) and SSEA-1 (by immunostaining, Fig. 5G,H). *Sox2* and *Cvh*, the expression of which is high in embryonic brain and gonads, respectively, were used as control gene markers for tissue specificity (data not shown).

cNanog expression was also detected by QRT-PCR in heart, brain, kidney and gonads, but at levels 20-, 25-, 90- and 100-fold lower, respectively, than in cESC (data not shown). In early embryos, *Nanog* was also expressed in scattered cells in the germinal crescent: these cells are likely to correspond to the future germ cells (Fig. 4I and arrowhead in H′). Later in development, *cNanog* was still expressed in germ cells at stage 33 (Fig. 5I-K) identified as SSEA-1–positive, in a similar manner to the *cPouV*-expressing cells (Fig. 5L,M). However, *cNanog* expression became weaker at this stage compared to the previous stages. This expression profile was observed in both male and female embryos (data not shown).

In conclusion, expression of *cPouV* and *Nanog* becomes restricted to germ cells at later stages of embryonic development.

Overexpression of *Oct4*-related genes in cESC and mESC

In order to compare *cPouV* function with its orthologues, coding sequences of *cPouV*, murine *Oct4* (*mOct4*), *XlPou91* and zebrafish *pou2* were transfected into cESC and mESC. In cESC, overexpression of *cPouV* using the pCAGIP vector impaired the isolation of proliferating clones. Using pCMV-based vectors, gene expression analysis revealed a 4-fold induction of *cPouV,* but a strong decrease in expression of *Nanog* and *Tert*. By contrast,

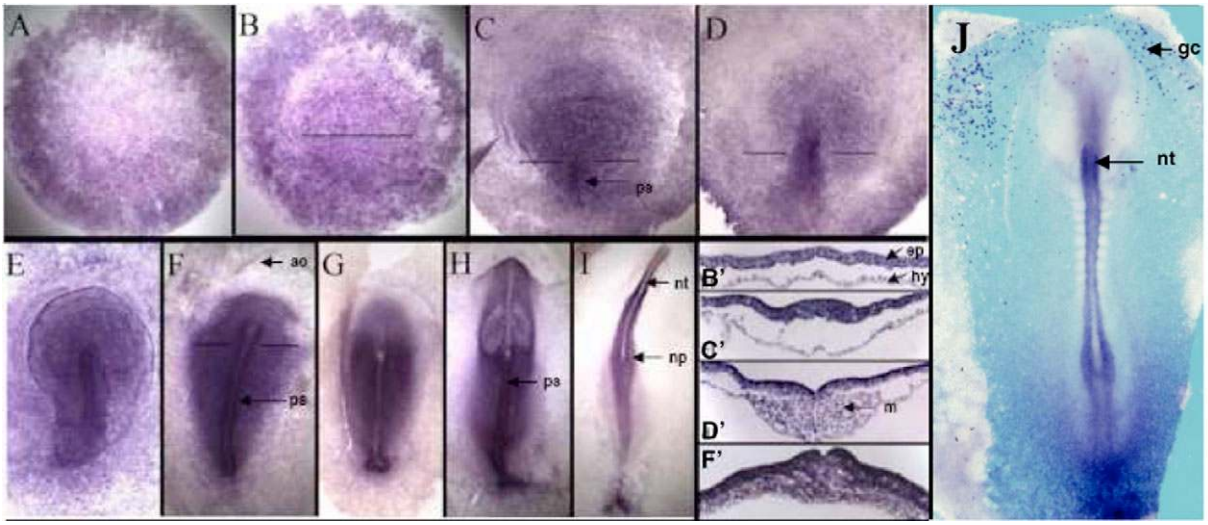


Fig. 3. *cPouV* expression during chick embryo development. (**A-J**) Whole-mount in situ hybridisation to *cPouV* transcripts. Transcripts are detected in the area pellucida and area opaca of the epiblast in pre-streak embryos (A, stage XI; B, stage XIII), and in the hypoblast in a salt-and-pepper manner (B′). At stage XIV (C), the expression is very strong in the area pellucida of the epiblast, especially where the streak is forming (C′). Transcripts are expressed in the ingressing mesoderm at stages 2-3 (D,D′). As the primitive streak elongates and the embryo grows, expression is still detected in the ectoderm and mesoderm (E, stage 3+; F,F′, stage 4+; G, stage 5). At stage 7 (H) and 8 (I), *cPouV* mRNA is detected in the forming neural tube and in the underlying mesoderm, but is absent from the endoderm (data not shown). At stage 9 (J), *cPouV* mRNA is expressed in neural tissue (nt) and presumptive migrating germ cells (gc). B′,C′,D′,F′ are transverse sections of the embryos in B,C,D,F, respectively. ao, area opaca; ep, epiblast; gc, germ cells; hy, hypoblast; m, mesoderm; np, neural plate; nt, neural tube; ps, primitive streak.

strong upregulation of *Gata4*, *Gata6* and *Cdx2*, associated with differentiation, was observed (Fig. 6A). Ectopic expression of *XlPou91* induced a similar expression profile, with an increase in endogenous *cPouV* and of differentiation markers *Gata4*, *Gata6* and *Cdx2*, and a strong decrease in *Nanog* and *Tert* expression (Fig. 6B). By contrast, overexpression of *mOct4* did not modify *cPouV* or *Nanog* endogenous expression levels and induced only a slight increase in endogenous chicken *Cdx2* gene expression (Fig. 6B).

As previously described (Niwa et al., 2002), it was not possible to isolate clones of cells overexpressing *mOct4* after transfection of mESC with the neomycin resistance overexpression plasmid. The same pCAGIP vector was used to overexpress *cPouV* and *XlPou91* in mESC, but clones could only be isolated of mESC expressing *XlPou91*. Endogenous expression of *Oct4* was maintained and expression of *Gata4*, *Gata6* and *Cdx2*, as well as of mesendodermal markers including *Hnf1*, brachyury, *Sox17* and laminin B1, was observed (Fig. 6C). Using a pCMV-based expression vector, *cPouV* expression enabled identification of clones presenting a similar expression profile, i.e. with a maintenance of endogenous *Oct4* expression but only a slight increase in *Gata4*, *Gata6*, *Cdx2*, *Hnf1*, brachyury, *Sox17* and laminin B1 expression (Fig. 6D).

In conclusion, high ectopic expression of *cPouV* impairs the proliferation of both cESC and mESC, but a moderate level of expression of exogenous *cPouV* is tolerated by cESC and mESC with an associated modification of the observed gene expression profile.

cPouV is able to rescue partially *Oct4*-deficient ZHBTc4 mESC

A good test of whether *cPouV* is functionally equivalent to its murine counterpart *Oct4*, is to assay the ability of the chick gene to rescue the ZHBTc4-inducible cells in which endogenous *Oct4* expression is downregulated by addition of doxycyclin. Transfection of ZHBTc4 cells with expression vectors for *mOct4* or *XlPou91* allowed isolation of proliferating clones in the presence or absence of doxycyclin, (Fig. 7A,B) as predicted (Niwa et al., 2000; Morrison and Brickman, 2006). In the presence of doxycyclin, expression of *cPouV* was able to support the growth of slowly proliferating AP-positive colonies (Fig. 7C) with a rescue index (the ratio between the number of clones in the presence versus the absence of doxycyclin) of 0.5 (Fig. 7D), as compared with 1.0 for expression of *mOct4* and 3.5 for *XlPou91*. However, the colonies recovered after *cPouV* expression were limited in their capacity to be passaged or amplified and exhibited a differentiated morphology. No clones were obtained after expression of zebrafish *pou2* in the presence of doxycyclin.

Real-time RT-PCR analysis performed on RNA from the clones generated by *cPouV* complementation revealed a complete loss of endogenous *mOct4* mRNA, but high expression of the exogenous *cPouV* mRNA (data not shown). Expression of pluripotency-associated markers such as *Nanog*, *Sox2*, *Utf1* and *Zfp42* (*Rex1*) was maintained at the same level in cells complemented by *XlPou91* as in cells complemented by *mOct4* (and expression was even higher for *Tert* and *Fgf4*). Expression of these markers was reduced, but detectable, in cells complemented by *cPouV*, with the exception of *Sox2* and *Fgf4* for which no expression could be detected in the presence of *cPouV* (Fig. 7E).

To test the ability of this gene to transactivate specific *Oct4*-responsive elements, promoters containing either the *mOct4* consensus binding site (ATGCAAAT), or the 1.4 kb ΔPE fragment from the *mOct4* promoter (Yeom et al., 1996; Hong et al., 2004), linked to a luciferase reporter, were transfected into ZHBTc4 cells. These promoters were activated in ZHBTc4 cells treated with doxycyclin in the presence of the expression vectors coding for *mOct4*, *XlPou91* or *cPouV*, as measured by luciferase activity (Fig. 7F). This interesting result suggests that the cPouV protein is able to recognise *mOct4*-response elements and activate transcription.

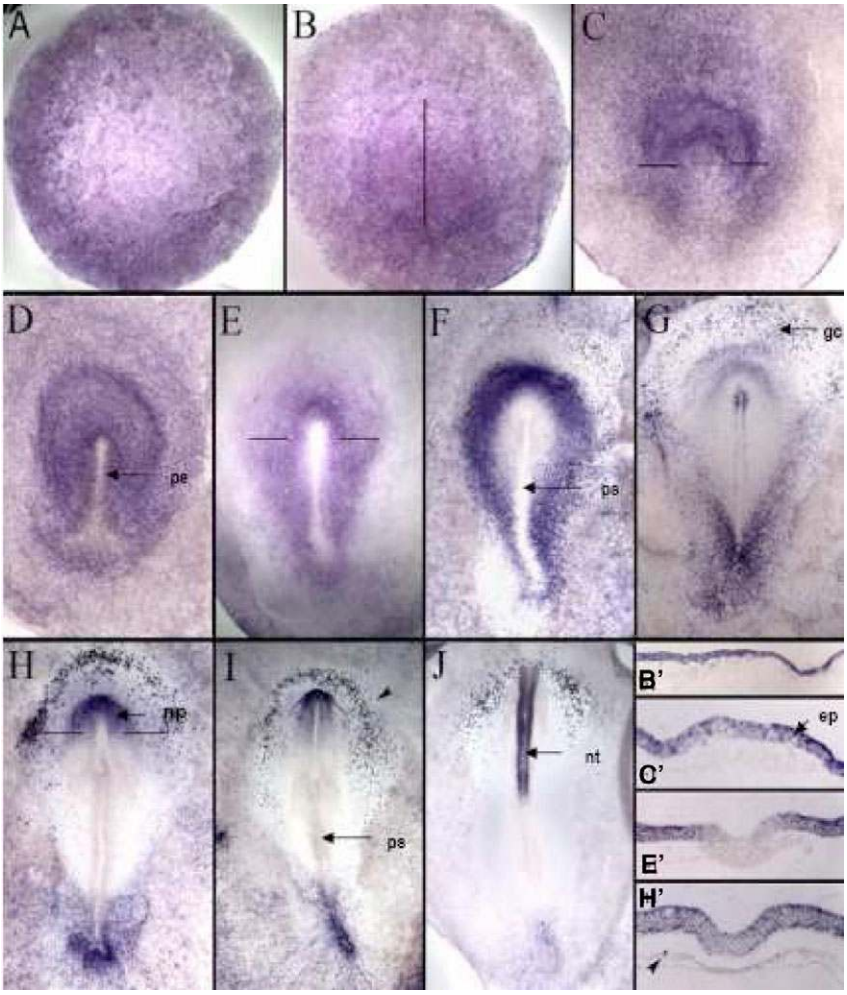


Fig. 4. *cNanog* expression during chick embryo development. (**A-J**) Whole-mount in situ hybridisation to *cNanog* transcripts. *Nanog* transcripts are localised in the epiblast of the area pellucida and area opaca of the pre-streak embryo (A, stage XI; B, stage XII), but not in the hypoblast (B′). From stage XIV, *cNanog* mRNA disappears from the posterior area pellucida (C,C′) and from the growing primitive streak (D, stage 3; E,E′, stage 3+; F, stage 4+). *cNanog* transcripts are downregulated in the epiblast from stage 4+ (F), and are confined anteriorly in a crescent region in the epiblast (G, stage 5+). At stage 6 (H), expression is restricted to the neural plate and the neural tube (I, stage 7; J, stage 8). *cNanog* is also expressed in scattered cells in the germinal crescent from stage 4 (arrowhead in I and H′). B′ is a longitudinal section of the embryo in B, anterior at the right; C′,E′,H′ are transverse sections of embryos in C,E,H, respectively. ep, epiblast; gd, gonad; ms, mesonephros; np, neural plate; nt, neural tube; ps, primitive streak.

In conclusion, these experiments suggest that the c*PouV* gene is able to partially rescue the loss of *mOct4* function in mESC, and does interact with and activate *mOct4*-dependent regulatory elements.

cNanog function in ES cells

Overexpression of mouse Nanog protein in mESC results in growth factor-independent maintenance of the pluripotent cell phenotype. To test whether overexpression of *cNanog* can confer the same growth-factor independence on mESC, proliferation of mESC was assessed in the absence of LIF, after transfection of a *cNanog* expression plasmid. Colonies did form in the absence of LIF, indicating that *cNanog* is able to confer growth factor independence (Fig. 8A). In the absence of LIF, the transfected cells were undistinguishable from the parental cells, on the basis of morphology, AP staining and growth rate (Fig. 8B-G). Real-time RT-PCR analysis of these proliferating clones indicated that expression of pluripotent factors, including *mOct4* and *Sox2*, was maintained, but with the notable exception of *Fgf4*, the expression of which was almost completely abolished (Fig. 8H).

In contrast to mESC, cESC are not dependent on a single cytokine for their proliferation and survival (Pain et al., 1996) (our unpublished results). Overexpression of *cNanog* conferred the ability of the cESC to grow in a low-serum medium in the absence of growth factors and cytokines that are usually required for proliferation (Fig. 8I). The clones obtained proliferated actively and were easily passaged and amplified (data not shown). It was particularly surprising to obtain proliferative avian primary stem cells in the presence of only 1% foetal bovine serum. Interestingly, under these drastic conditions, *mNanog* expression did not have any pronounced effect on the chicken cells. Real-time RT-PCR analysis confirmed overexpression of *cNanog*, the maintenance of *Tert* and reduced, but detectable, expression of *cPouV* and *AP* (Fig. 8J-K).

In conclusion, we have shown that *cNanog* functions in a very similar way to *mNanog* in mESC and has a more dramatic effect in cESC, where overexpression permits maintenance of the stem cell phenotype in the absence of growth factors and in low serum.

Inactivation of *cPouV* or *cNanog* inhibits ES cell proliferation and induces differentiation

To assess *cPouV* and *cNanog* function, constructs expressing shRNAs were designed to knockdown transcripts of these genes, using a tamoxifen-inducible Cre system to activate the expression of the shRNAs. Following induction of Cre recombinase activity by tamoxifen addition, a rapid and dramatic morphological change was observed, involving changes associated with differentiation (Fig. 9A-D). These changes were seen in ~60% of the clones when specific shRNAs were used against *cPouV* and *cNanog* (Fig. 9G) This morphological change was observed even in the presence of growth factors and was accompanied by a loss of AP activity and of SSEA-1 antibody staining (Fig. 9H), plus a growth rate alteration 48 and 96 hours after Cre induction (Fig. 9I). Comparison of the gene

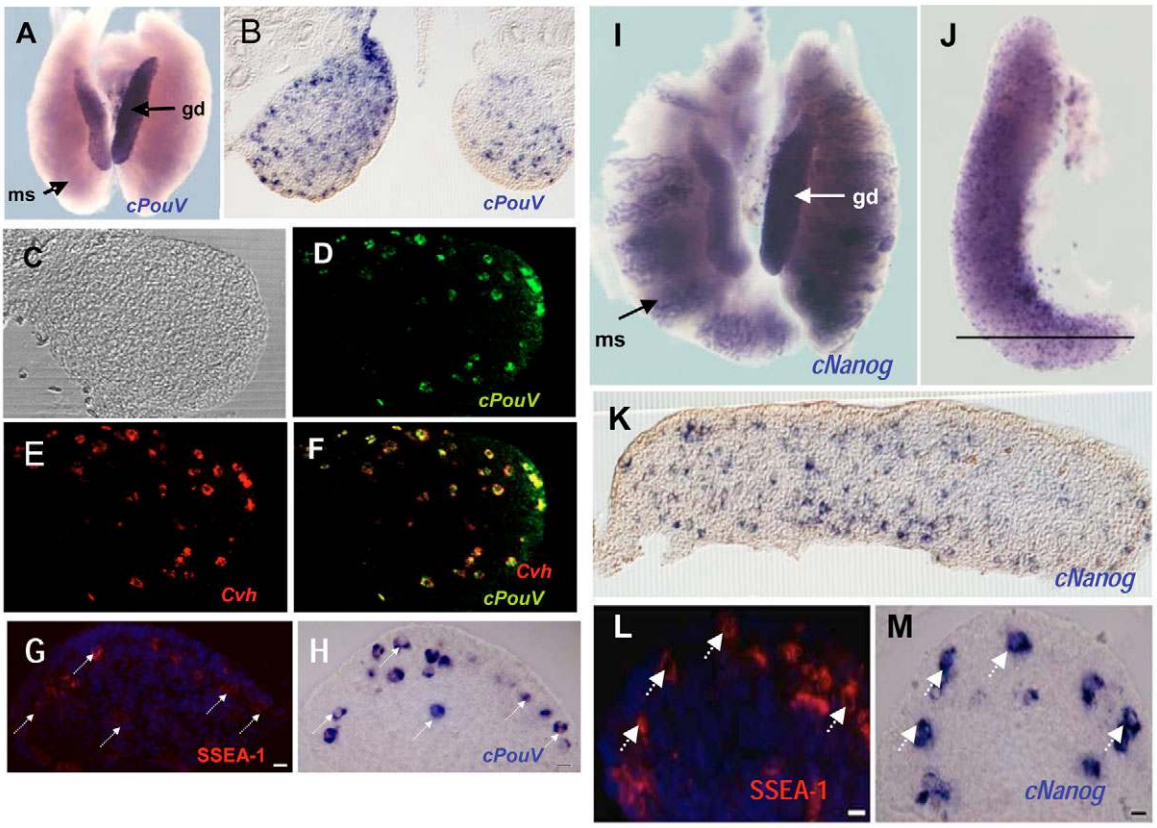


Fig. 5. *cPouV* and *cNanog* are expressed in germ cells during later embryonic development. (**A-H**) At stage 33, *cPouV* mRNA is detected in the developing gonad, which is attached to the mesonephros (A,B), in the germ cells (D), as detected by co-localisation (F) of *cPouV* (D) both with *Cvh* (chicken *Vasa*, E) expression and with SSEA-1-positive cells (G) revealed on adjacent sections counterstained by Hoechst and *cPouV* probe (H, arrows). (B) Section of gonad shown in A. (C) Bright field of the stage 33 gonad used for in situ hybridisation (D,E,F). (**I-M**) At stage 33, *cNanog* is highly expressed in gonads and in mesonophros tubules (I) and gonad (I,J), especially in germ cells (K), as revealed by SSEA-1 staining (L) on the same cells that express cNanog in adjacent sections (M, arrow) counterstained by Hoechst (L). (K) Section of dissected gonad from the urogenital tract (J). ms, mesonephros; gd, gonads. Scale bars: 15 μm.

expression profiles between differentiated clones and clones that continued to proliferate revealed strong inhibition of endogenous *cPouV* expression as well as of *cNanog* and *Gata4* and strong induction of *Gata6* (Fig. 9J). No upregulation of *Cdx2* was detected, nor of other mesendodermal markers such brachyury or *Hnf3β*. Similar experiments involving inhibition of another POU-domain gene, *Oct6* (Levavasseur et al., 1998), did not change the endogenous level of *cPouV* and proliferating clones were obtained (Fig. 9G,J).

When a similar analysis was performed using shRNA directed against *cNanog* mRNA, a similar process of differentiation occurred, with thin cytoplasmic protrusions (Fig. 9E,F), a loss of AP activity and SSEA-1 staining (Fig. 9G) and reduced proliferation (Fig. 9H). This phenomenon was observed with two distinct sequences, shRNA-1 and shRNA-3. Real-time RT-PCR expression analysis showed a drastic decrease in the expression of *cPouV*, *Gcnf* and *Gata4* (Fig. 9I) and an induction of *Gata6*.

In conclusion, inhibition of either *cPouV* or *cNanog* leads to a loss of proliferation of cESC and to the induction of differentiation.

DISCUSSION

Oct4 is established as one of the key factors controlling pluripotency and the unique self-renewing property of mammalian ESC (Chambers and Smith, 2004). Both overexpression and disruption of *Oct4* in mESC leads to a loss of pluripotency and induces the cells to differentiate into primitive endoderm, characterised by high *Gata6* expression (Li et al., 2004), and into trophectoderm expressing *Cdx2* (Niwa et al., 2000; Strumpf et al., 2005; Niwa et al., 2005; Tolkunova et al., 2006). In vivo, it is now thought that complex regulatory mechanisms lead to restricted expression in early pregastrulation embryos (Gu et al., 2005a; Boiani et al., 2002) and in the germ line (Kehler et al., 2004; Yeom et al., 1996).

Nanog, a homeodomain transcription factor, was identified as another key factor maintaining the pluripotency of mammalian ESC (Chambers et al., 2003; Mitsui et al., 2003; Hart et al., 2004). In mESC, *Nanog* overexpression has been shown to substitute for the requirement for growth factors in the maintenance of self-renewal. Disruption of *Nanog* leads to a loss of pluripotency and to induction of differentiation towards an endoderm-like state (Mitsui et al., 2003).

The existence and equivalent functions of homologues of these genes in non-mammalian vertebrates are still debated. Functional assays were used to identify the zebrafish *pou2* gene as the *Oct4* homologue (Burgess et al., 2002), but this gene appears to be mainly involved in the endoderm-specification cascade (Reim et al., 2004; Lunde et al., 2004). In *Xenopus*, *XlPou91*, a *PouV* gene, plays a significant role in the maintenance of pluripotent cells during early development and was shown to rescue *Oct4* depletion in mESC.

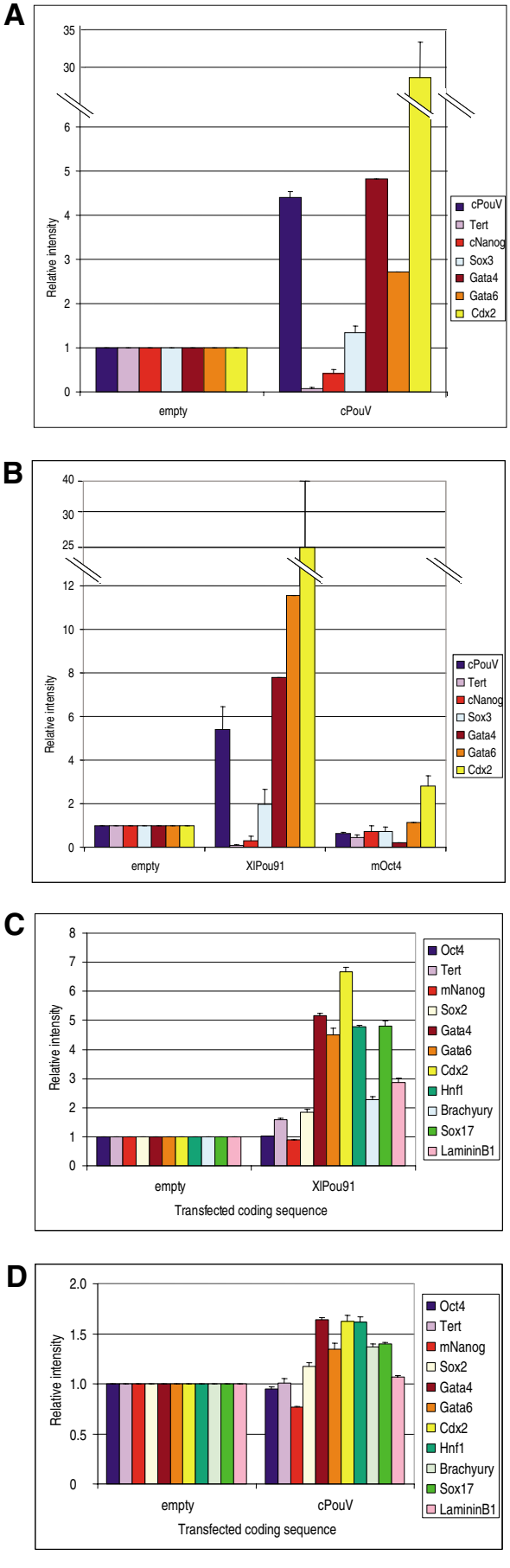


Fig. 6. Overexpression of *cPouV* in cESC and mESC. (**A**) Following transfection of the different *PouV* coding sequences, overexpression of *cPouV* in cESC revealed loss of endogenous *cNanog* expression and an increase in *Gata4*, *Gata6* and *Cdx2* expression. (**B**) A similar profile was observed for *XlPou91*, but only a slight increase in *Cdx2* when *mOct4* is transfected in cESC. (**C**,**D**) Upregulation of *Gata4*, *Gata6* and *Cdx2* is strong (C) when *XlPou91* is overexpressed in mESC with an induction of other markers (*Hnf1*, brachyury, *Sox17* and laminin B1), in contrast to a moderate induction when *cPouV* is overexpressed in mESC (D). A value of 1 was given to the gene expression level obtained in clones transfected with the empty vector (empty). Two independent experiments provided similar results.

XlPou91 knockdown in vivo using morpholinos induces expression of *Xcad3*, which is considered to be the *Xenopus* homologue of *Cdx2* (Morrison and Brickman, 2006). These data are consistent with the idea that *PouV* family members, including murine *Oct4*, could act to prevent premature commitment of pluripotent cells present in vertebrate embryos prior to and during gastrulation.

cESC have been isolated and maintained in culture for long periods (Pain et al., 1996; Petitte et al., 2004; Van de Lavoir et al., 2006). These cells were derived from the culture of pre-primitive streak blastodermal cells and are characterised by the presence of typical ESC markers such as AP, Tert activity and reactivity with particular antibodies including ECMA-7, SSEA-1, SSEA-3 and EMA-1 (Pain et al., 1996; Petitte et al., 2004).

In a differential screen from proliferative cESC and cEB, we identified a new coding sequence containing a POU domain. Several strands of evidence support the view that this gene is the chicken homologue of mammalian *Oct4*. First, comparative analysis and phylogenetic tree construction reveal that this sequence belongs, with high probability, to the *PouV* subfamily. Genomic analysis also demonstrates a clear syntenic conservation of the different loci between the non-mammalian species. We therefore refer to this gene as chicken *PouV* (*cPouV*). Second, this gene is expressed in vitro only in proliferating ESC. Its expression is rapidly downregulated once differentiation is induced by RA or during formation of cEB. This downregulation is maintained in the presence of cycloheximide, suggesting a direct effect of RA on transcription.

Third, *cPouV* is expressed in a complex pattern in the embryo, being expressed widely in the early epiblast and later becoming restricted to specific regions, including the mesoderm and nervous system. This initial expression in multipotent epiblast cells, which then becomes restricted once the cells start to be committed, is also shared by the zebrafish, *Xenopus* and mouse homologues, which have been implicated in regulation of early neural development and patterning (Ramos-Mejia et al., 2005; Burgess et al., 2002; Reim and Brand, 2002; Morrison and Brickman, 2006). During late development, *cPouV* expression becomes more restricted to migrating and proliferating germ cells, as demonstrated by co-localisation with *Cvh*-positive cells in the developing gonads. This germ-line-restricted expression is a feature shared with its murine counterpart, in contrast to the zebrafish and *Xenopus* homologues. We conclude that *cPouV* plays a similar role to its mammalian and non-mammalian homologues in pregastrulating embryos, but functions more like the mammalian homologue in germ cells.

Another feature of this chicken gene is its ability to induce differentiation when overexpressed in cESC. Expression of *cPouV* in cESC and mESC alters the morphology and reduces the growth

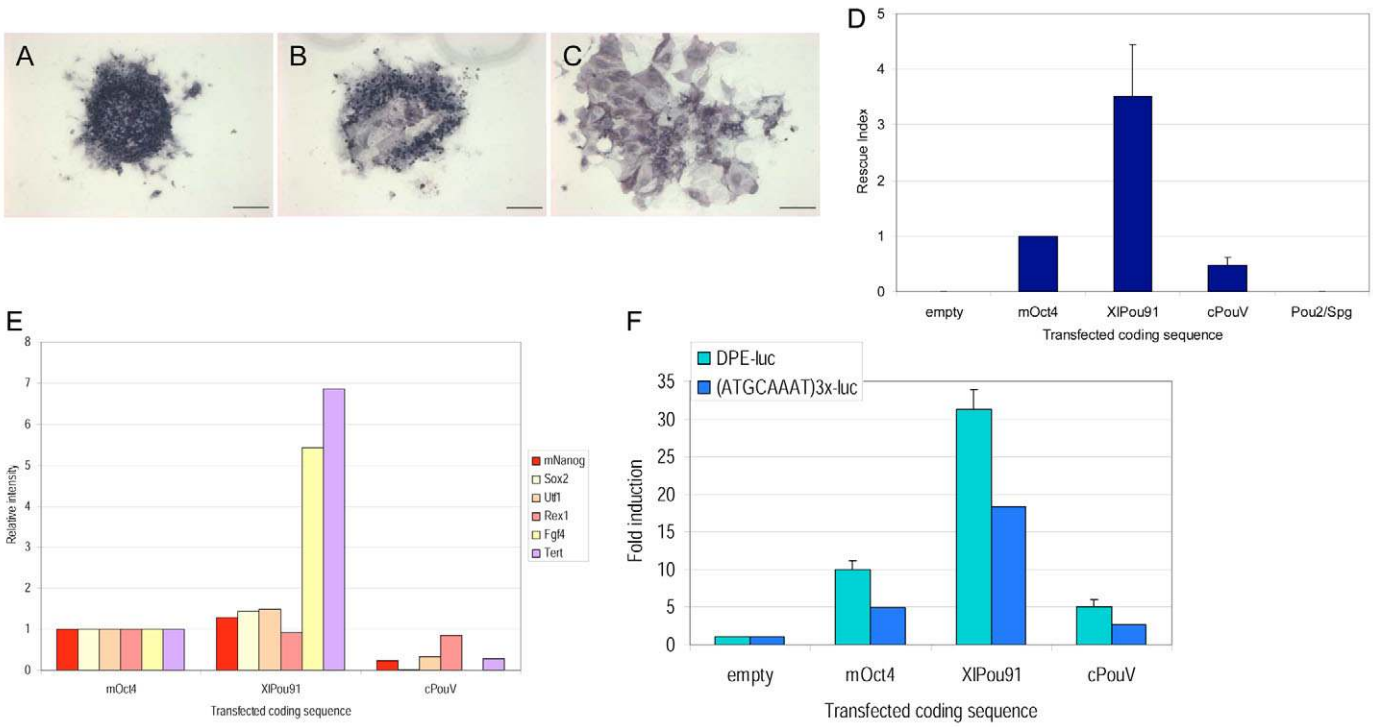


Fig. 7. *Oct4*-deficient ZHBTc4 mESC are only partially rescued by *cPouV* expression. ZHBTc4 cells transfected with *mOct4*, *XlPou91*, *cPouV* or *pou2* expression vectors were treated with doxycyclin after selection of stable clones. AP-positive clones were obtained with *mOct4* (**A**), *XlPou91* (**B**) and *cPouV* (**C**), but no clones were isolated with empty control vector (empty) or *pou2*. A rescue index (RI, the ratio between the number of clones in the presence versus the absence of doxycyclin) of 1 is given in the presence of *mOct4* (**D**). This RI is the result of two independent experiments with a total number of clones of 114/0, 82/19, 123/95, 74/8 and 82/0, respectively, in the absence/presence of doxycylin for the empty vector, the *mOct4*, *XlPou91*, *cPouV* and *pou2* expression vectors. (**E**) Expression of pluripotency-associated genes in ZHBTc4 complemented clones was analysed by real-time RT-PCR. A value of 1 was given to the level detected in the *mOct4*-complemented clones. *Nanog*, *Utf1*, *Zfp42* (*Rex1*) and *Tert* expression was lower in clones complemented by *cPouV* than in those complemented with *XlPou91*. (**F**) ZHBTc4 cells were co-transfected in the presence of luciferase reporter gene driven either by the ΔPE promoter or the Oct4 consensus sequence (ATGCAAAT). A value of 1 was given to the empty vector. Each result is the average of four wells per condition, and two independent experiments provided similar results. *cPouV* expression activated both promoters.

rate of the ESC, and inhibits isolation of clones from cells in which *cPouV* is expressed from a very strong promoter. Expression using a moderate CMV promoter induces expression of differentiation markers such as *Gata4*, *Gata6* and *Cdx2*. In mouse, these markers are associated with endodermal and trophectodermal lineages, but their function during early chicken development is still unknown. A similar profile of gene expression is obtained in parallel experiments with *XlPou91*, suggesting common target genes. Overexpression of *cPouV* in mESC led to a more moderate phenotype, with a slight induction of differentiation markers such as *Gata6* and *Cdx2*, and also of *Hnf1*, brachyury and *Sox17*, which are known in mouse to be strongly expressed in mesendoderm structures (Tada et al., 2005; Yasunaga et al., 2006). *cPouV* is able to trigger a differentiation programme when overexpressed in both mESC and cESC.

cPouV is only able to partially rescue the phenotype of *mOct4*-deficient cells. In ZHBTc4 ES cells, *cPouV* expression can restore limited proliferation, in contrast to the zebrafish *pou2* gene but in agreement with recent findings regarding *XlPou91* function (Morrison and Brickman, 2006). In the presence of *cPouV*, endogenous mouse *Nanog* expression in mESC is maintained at a low but detectable level. Expression of *Utf1*, *Zfp42* (*Rex1*) and *Tert* is maintained, but expression of *Sox2* and *Fgf4* is completely abolished. These observations could explain the limited ability of these clones to be passaged and amplified. However, under these same conditions, the various factors are able to transactivate specific promoters containing either the *Oct4* consensus binding site or the mouse endogenous *Oct4* promoter, suggesting that *cPouV* is able to substitute functionally for mouse *Oct4*. The N-terminal domains of the mouse and chicken genes are highly diverged. It is probable that stringent interprotein interactions are required for full activity of the chicken protein. A first attempt to test this hypothesis, by constructing molecular chimaeras between the mouse *Oct4* N-terminus and the *cPouV* homeodomain and C-terminus, proved unable to restore complete ZHBTc4 cell proliferation (data not shown), suggesting that other mechanisms and/or partners are likely to be required.

We also report the isolation of the chicken functional orthologue of *Nanog* (*cNanog*) from proliferating cESC and demonstrate that *cNanog* also plays a role in the maintenance of self-renewal and pluripotency of cESC. The sequence we cloned is shorter than the one recently reported by Canon et al. (Canon et al., 2006). The *cNanog* expression profile differs both in vitro and in vivo from that of *cPouV*. Specifically, *cNanog* expression is downregulated by differentiation but with a different time-course than *cPouV* in both RA-induced differentiation and cEB formation. In contrast to *cPouV*, *cNanog* transcription is maintained and possibly increased in the presence of cycloheximide and the mRNA half-life appears to be reduced in the presence of actinomycin, suggesting a short half-life

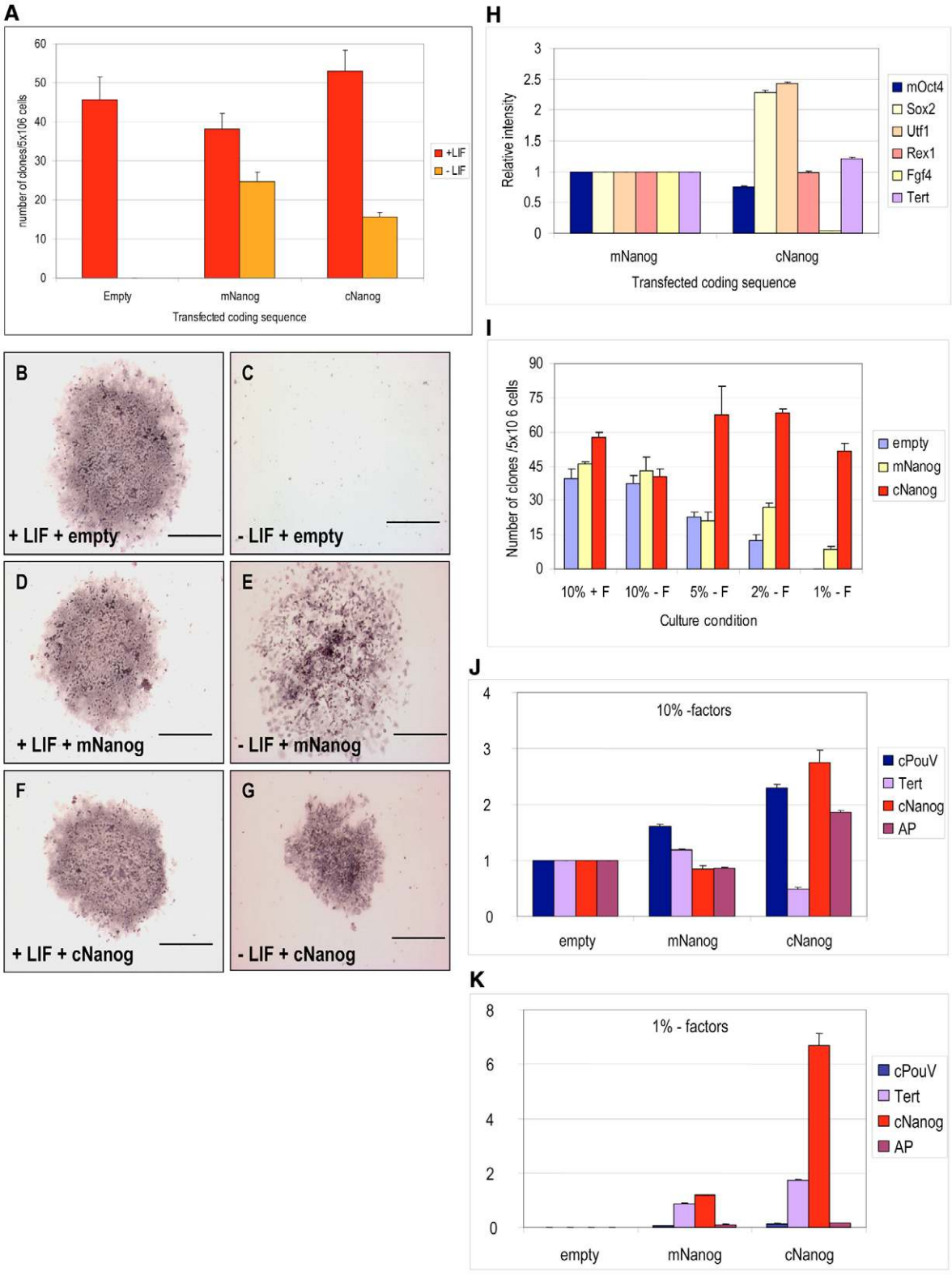


Fig. 8. Overexpression of *cNanog* induces growth factor-independent proliferation in both mESC and cESC. mESC or cESC were transfected with either *mNanog*, *cNanog* or empty expression vector. For mESC, LIF was removed, clones scored (**A**), stained for alkaline phosphatase activity and observed (**B-G**). Similar results were obtained in two independent experiments. Scale bars: 400 μm. (**H**) Gene expression analysis revealed expression of *mOct4* as well as of *Sox2*, *Utf1*, *Rex1* and *Tert*, but with the notable exception of *Fgf4*. A value of 1 was given to the level of expression in the clones obtained without LIF in the presence of *mNanog*. (**I**) For cESC, growth factors and cytokines (bFgf, Scf, Igf1, Il6, Il6R) (–F) were removed, serum concentration reduced as indicated (10% to 1%) and clones scored. (**J**,**K**) Gene expression analysis revealed maintenance of *cPouV* expression in the presence of 10% serum without factors (J), but a complete loss of expression when serum was reduced to 1% without factors (K). Under these conditions, the expression of *cNanog* is upregulated and the cells continue to proliferate.

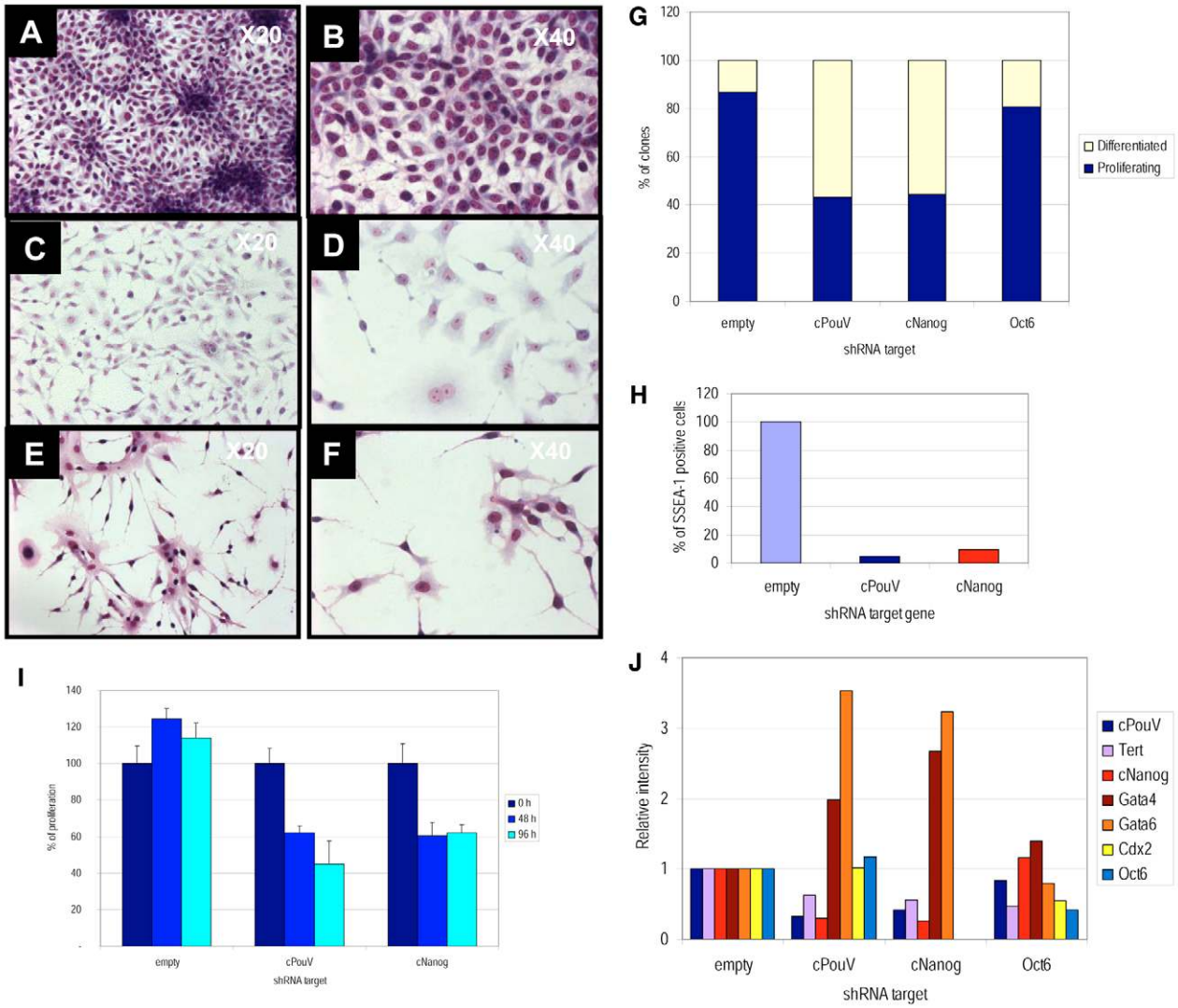


Fig. 9. Inhibition of *cPouV* and *cNanog* expression stops proliferation and induces cESC to differentiate. cESC were transfected with vectors allowing conditional expression of shRNA-2 and shRNA-1 against *cPouV* and *Nanog*, respectively, and of Cre-ERT2 recombinase, the activity of which was induced by 4-hydroxytamoxifen. Similar results were obtained with shRNA-3 against *cNanog*. The morphology of the cells targeted for *cPouV* (**C**,**D**) and *cNanog* (**E**,**F**) was strongly altered, compared with control cells (**A**,**B**) and with targeted cells for cOct6 (data not shown), in 60% of the clones (**G**). In these differentiated cells, the percentage of SSEA-1-positive cells was drastically reduced (**H**). Proliferation was also rapidly reduced as assessed by XTT proliferation on 12 independent clones followed for 4 days after tamoxifen addition (T=0 hours) (**I**). Gene expression analysis (**J**) revealed a strong decrease in expression of target genes *cPouV* and *cNanog* in contrast to a strong upregulation of *Gata4*, *Gata6* and *Cdx2* in cells expressing shRNA against *cPouV*, and the upregulation of *Gata4* and *Gata6* for those cells expressing shRNA against *cNanog*. In the latter case, no *Cdx2* expression could be detected. A shRNA-3 against another Pou family member, *Oct6*, does not alter *cPouV* and *cNanog* expression by comparison with the control vector (empty). *Oct6* expression cannot be detected in the cells targeted by shRNA against *cNanog* (J). Three independent experiments provided similar results.

of the mRNA. In vivo, the expression pattern of *cNanog* is also different from that of *cPouV*, with a more rapid disappearance from the epiblast and a subsequent restriction to the anterior neural plate. *cNanog* expression is detected in migrating germ cells, as was also observed for *Nanog* in the mouse (Yamaguchi et al., 2005), but also in germ cells of developing gonads.

Following overexpression of either mouse or chicken *Nanog* in mESC, the resulting proliferating clones are able to grow in the absence of the cytokine LIF, suggesting a functional complementation between the mouse and chicken genes. A notable exception is an almost complete loss of *Fgf4* expression in clones overexpressing *cNanog*. In cESC, a drastic reduction in serum in the culture medium revealed an important action of *cNanog* in maintaining proliferation of undifferentiated cells, suggesting that *cNanog* is able to stimulate proliferation and cell-cycle machinery in the absence of exogenous growth factors by acting directly on downstream targets.

Finally, inhibition of expression of *cPouV* and *cNanog*, using an inducible knockdown approach, promotes rapid growth arrest within 48 hours of shRNA induction. This inhibition of proliferation is accompanied by an induction of differentiation as detected by altered morphology, loss of SSEA-1 labelling and expression of *Gata6* and *Cdx2*. This suggests that these two genes play a key role in the maintenance of the pluripotent character of cESC.

In conclusion, the identification of chicken *PouV* elucidates some aspects of epiblast proliferation and maintenance of pluripotency in vitro and in vivo, and points the way for a better understanding of germ cell development and proliferation in the chicken embryo. The chicken *Nanog* gene also plays a role in this process and the functional relationship between these two key genes requires further investigation.

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References

Acloque, H., Risson, V., Birot, A. M., Kunita, R., Pain, B. and Samarut, J. (2001). Identification of a new gene family specifically expressed in chicken embryonic stem cells and early embryo. *Mech. Dev.* **103**, 79-91.

Ambrosetti, D. C., Basilico, C. and Dailey, L. (1997). Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct-3 depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding sites. *Mol. Cell. Biol.* **17**, 6321-6329.

Avilion, A. A., Nicolis, S. K., Pevny, L. H., Perez, L., Vivian, N. and Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev.* **17**, 126-140.

Barnea, E. and Bergman, Y. (2000). Synergy of SF1 and RAR in activation of Oct-3/4 promoter. *J. Biol. Chem.* **275**, 6608-6619.

Ben-Shushan, E., Sharir, H., Pikarsky, E. and Bergman, Y. (1995). A dynamic balance between ARP-1/COUP-TFII, EAR-3/COUP-TFI, and retinoic acid receptor:retinoid X receptor heterodimers regulates Oct-3/4 expression in embryonal carcinoma cells. *Mol. Cell. Biol.* **15**, 1034-1048.

Ben-Shushan, E., Thompson, J. R., Gudas, L. J. and Bergman, Y. (1998). Rex-1, a gene encoding a transcription factor expressed in the early embryo, is regulated via Oct-3/4 and Oct-6 binding to an octamer site and a novel protein, Rox-1, binding to an adjacent site. *Mol. Cell. Biol.* **18**, 1866-1878.

Boiani, M., Eckardt, S., Scholer, H. R. and McLaughlin, K. J. (2002). Oct4 distribution and level in mouse clones: consequences for pluripotency. *Genes Dev.* **16**, 1209-1219.

Burgess, S., Reim, G., Chen, W., Hopkins, N. and Brand, M. (2002). The zebrafish spiel-ohne-grenzen (spg) gene encodes the POU domain protein Pou2 related to mammalian Oct4 and is essential for formation of the midbrain and hindbrain, and for pre-gastrula morphogenesis. *Development* **129**, 905-916.

Canon, S., Herranz, C. and Manzanares, M. (2006). Germ cell restricted expression of chick nanog. *Dev. Dyn.* **235**, 2889-2894.

Chambers, I. and Smith, A. (2004). Self-renewal of teratocarcinoma and embryonic stem cells. *Oncogene* **23**, 7150-7160.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S. and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113**, 643-655.

Chew, J. L., Loh, Y. H., Zhang, W., Chen, X., Tam, W. L., Yeap, L. S., Li, P., Ang, Y. S., Lim, B., Robson, P. and Ng, H. H. (2005). Reciprocal transcriptional regulation of PouVf1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. *Mol. Cell. Biol.* **25**, 6031-6046.

Coumoul, X., Li, W., Wang, R. H. and Deng, C. (2004). Inducible suppression of Fgfr2 and Survivin in ES cells using a combination of the RNA interference (RNAi) and the Cre-LoxP system. *Nucleic Acids Res.* **32**, e85.

Dailey, L., Yuan, H. and Basilico, C. (1994). Interaction between a novel F9-specific factor and octamer-binding proteins is required for cell-type-restricted activity of the fibroblast growth factor 4 enhancer. *Mol. Cell. Biol.* **14**, 7758-7769.

Eyal-Giladi, H. and Kochav, S. (1976). From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. *Dev. Biol.* **49**, 321-337.

Feil, R., Wagner, J., Metzger, D. and Chambon, P. (1997). Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem. Biophys. Res. Commun.* **237**, 752-757.

Felsenstein, J. (1978). Cases in which parsimony or compatibility methods will be positively misleading. *Syst. Zool.* **27**, 401-410.

Fuhrmann, G., Chung, A. C., Jackson, K. J., Hummelke, G., Baniahmad, A., Sutter, J., Sylvester, I., Scholer, H. R. and Cooney, A. J. (2001). Mouse germline restriction of Oct4 expression by germ cell nuclear factor. *Dev. Cell* **1**, 377-387.

Gu, P., Goodwin, B., Chung, A. C., Xu, X., Wheeler, D. A., Price, R. R., Galardi, C., Peng, L., Latour, A. M., Koller, B. H. et al. (2005a). Orphan nuclear receptor LRH-1 is required to maintain Oct4 expression at the epiblast stage of embryonic development. *Mol. Cell. Biol.* **25**, 3492-3505.

Gu, P., LeMenuet, D., Chung, A. C., Mancini, M., Wheeler, D. A. and Cooney, A. J. (2005b). Orphan nuclear receptor GCNF is required for the repression of pluripotency genes during retinoic acid-induced embryonic stem cell differentiation. *Mol. Cell. Biol.* **25**, 8507-8519.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Embryol. Exp. Morphol.* **88**, 49-92.

Hanna, L. A., Foreman, R. K., Tarasenko, I. A., Kessler, D. S. and Labosky, P. A. (2002). Requirement for Foxd3 in maintaining pluripotent cells of the early mouse embryo. *Genes Dev.* **16**, 2650-2661.

Hart, A. H., Hartley, L., Ibrahim, M. and Robb, L. (2004). Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human. *Dev. Dyn.* **230**, 187-198.

Hay, D. C., Sutherland, L., Clark, J. and Burdon, T. (2004). Oct4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells. *Stem Cells* **22**, 225-235.

Hong, Y., Winkler, C. and Schartl, M. (1998). Production of medakafish chimeras from a stable embryonic stem cell line. *Proc. Natl. Acad. Sci. USA* **95**, 3679-3684.

Hong, Y., Winkler, C., Liu, T., Chai, G. and Schartl, M. (2004). Activation of the mouse Oct4 promoter in medaka embryonic stem cells and its use for ablation of spontaneous differentiation. *Mech. Dev.* **121**, 933-943.

Kehler, J., Tolkunova, E., Koschorz, B., Pesce, M., Gentile, L., Boiani, M., Lomeli, H., Nagy, A., McLaughlin, K. J., Scholer, H. R. et al. (2004). Oct4 is required for primordial germ cell survival. *EMBO Rep.* **5**, 1078-1083.

Kirchhof, N., Carnwath, J. W., Lemme, E., Anastassiadis, K., Scholer, H. and Niemann, H. (2000). Expression pattern of Oct4 in preimplantation embryos of different species. *Biol. Reprod.* **63**, 1698-1705.

Kuroda, T., Tada, M., Kubota, H., Kimura, H., Hatano, S. Y., Suemori, H., Nakatsuji, N. and Tada, T. (2005). Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression. *Mol. Cell. Biol.* **25**, 2475-2485.

Levavasseur, F., Mandemakers, W., Visser, P., Broos, L., Grosveld, F., Zivkovic, D. and Meijer, D. (1998). Comparison of sequence and function of the Oct-6 genes in zebrafish, chicken and mouse. *Mech. Dev.* **74**. 89-98.

Li, L., Arman, E., Ekblom, P., Edgar, D., Murray, P. and Lonai, P. (2004). Distinct GATA6- and laminin-dependent mechanisms regulate endodermal and ectodermal embryonic stem cell fates. *Development* **131**, 5277-5286.

Looijenga, L. H., Stoop, H., de Leeuw, H. P., de Gouveia Brazao, C. A., Gillis, A. J., van Roozendaal, K. E., van Zoelen, E. J., Weber, R. F., Wolffenbuttel, K. P., van Dekken, H. et al. (2003). POUVF1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors. *Cancer Res.* **63**, 2244-2250.

Lunde, K., Belting, H. G. and Driever, W. (2004). Zebrafish PouVf1/pou2, homolog of mammalian Oct4, functions in the endoderm specification cascade. *Curr. Biol.* **14**, 48-55.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M. and Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113**, 631-642.

Morrison, G. M. and Brickman, J. M. (2006). Conserved roles for Oct4 homologues in maintaining multipotency during early vertebrate development. *Development* **133**, 2011-2022.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H. and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95**, 379-391.

Nishimoto, M., Fukushima, A., Okuda, A. and Muramatsu, M. (1999). The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. *Mol. Cell. Biol.* **19**, 5453-5465.

Niwa, H., Miyazaki, J. and Smith, A. G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* **24**, 372-376.

Niwa, H., Masui, S., Chambers, I., Smith, A. G. and Miyazaki, J. (2002). Phenotypic complementation establishes requirements for specific POU domain and generic transactivation function of Oct-3/4 in embryonic stem cells. *Mol. Cell. Biol.* **22**, 1526-1536.

Niwa, H., Toyooka, Y., Shimosato, D., Strumpf, D., Takahashi, K., Yagi, R. and Rossant, J. (2005). Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. *Cell* **123**, 917-929.

Nordhoff, V., Hubner, K., Bauer, A., Orlova, I., Malapetsa, A. and Scholer, H. R. (2001). Comparative analysis of human, bovine, and murine Oct4 upstream promoter sequences. *Mamm. Genome* **12**, 309-317.

Okumura-Nakanishi, S., Saito, M., Niwa, H. and Ishikawa, F. (2005). Oct-3/4 and Sox2 regulate Oct-3/4 gene in embryonic stem cells. *J. Biol. Chem.* **280**, 5307-5317.

Pain, B., Clark, M. E., Shen, M., Nakazawa, H., Sakurai, M., Samarut, J. and Etches, R. J. (1996). Long-term in vitro culture and characterisation of avian embryonic stem cells with multiple morphogenetic potentialities. *Development* **122**, 2339-2348.
Pain, B., Chenevier, P. and Samarut, J. (1999). Chicken embryonic stem cells and transgenic strategies. *Cells Tissues Organs* **165**, 212-219.
Pan, G. and Pei, D. (2005). The stem cell pluripotency factor NANOG activates transcription with two unusually potent subdomains at its C terminus. *J. Biol. Chem*. **280**, 1401-1407.
Petitte, J. N., Liu, G. and Yang, Z. (2004). Avian pluripotent stem cells. *Mech. Dev*. **121**, 1159-1168.
Pikarsky, E., Sharir, H., Ben-Shushan, E. and Bergman, Y. (1994). Retinoic acid represses Oct-3/4 gene expression through several retinoic acid-responsive elements located in the promoter-enhancer region. *Mol. Cell. Biol*. **14**, 1026-1038.
Ramos-Mejia, V., Escalante-Alcalde, D., Kunath, T., Ramirez, L., Gertsenstein, M., Nagy, A. and Lomeli, H. (2005). Phenotypic analyses of mouse embryos with ubiquitous expression of Oct4: effects on mid-hindbrain patterning and gene expression. *Dev. Dyn*. **232**, 180-190.
Reim, G. and Brand, M. (2002). Spiel-ohne-grenzen/pou2 mediates regional competence to respond to Fgf8 during zebrafish early neural development. *Development* **129**, 917-933.
Reim, G., Mizoguchi, T., Stainier, D. Y., Kikuchi, Y. and Brand, M. (2004). The POU domain protein spg (pou2/Oct4) is essential for endoderm formation in cooperation with the HMG domain protein casanova. *Dev. Cell* **6**, 91-101.
Rodda, D. J., Chew, J. L., Lim, L. H., Loh, Y. H., Wang, B., Ng, H. H. and Robson, P. (2005). Transcriptional regulation of nanog by OCT4 and SOX2. *J. Biol. Chem*. **280**, 24731-24737.
Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Evol. Biol*. **4**, 406-425.
Schoorlemmer, J., van Puijenbroek, A., van Den Eijnden, M., Jonk, L., Pals, C. and Kruijer, W. (1994). Characterization of a negative retinoic acid response element in the murine Oct4 promoter. *Mol. Cell. Biol*. **14**, 1122-1136.
Soodeen-Karamath, S. and Gibbins, A. M. (2001). Apparent absence of Oct 3/4 from the chicken genome. *Mol. Reprod. Dev*. **58**, 137-148.
Strähle, U., Blader, P., Adam, J. and Ingham, P. W. (1994). A simple and efficient procedure for non-isotopic in situ hybridization to sectioned material. *Trends Genet*. **10**, 75-76.
Streit, A. and Stern, C. D. (2001). Combined whole-mount in situ hybridization and immunohistochemistry in avian embryos. *Methods* **23**, 339-344.
Strumpf, D., Mao, C. A., Yamanaka, Y., Ralston, A., Chawengsaksophak, K., Beck, F. and Rossant, J. (2005). Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development* **132**, 2093-2102.
Tada, S., Era, T., Furusawa, C., Sakurai, H., Nishikawa, S., Kinoshita, M., Nakao, K., Chiba, T. and Nishikawa, S. (2005). Characterization of mesendoderm: a diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. *Development* **132**, 4363-4374.
Tokuzawa, Y., Kaiho, E., Maruyama, M., Takahashi, K., Mitsui, K., Maeda, M., Niwa, H. and Yamanaka, S. (2003). Fbx15 is a novel target of Oct3/4 but is dispensable for embryonic stem cell self-renewal and mouse development. *Mol. Cell. Biol*. **23**, 2699-2708.
Tolkunova, E., Cavaleri, F., Reinbold, R., Christenson, L. K., Schöler, H. and Tomolin, A. (2006). The Caudal-related protein Cdx2 promotes trophoblast differentiation of mouse embryonic stem cells. *Stem Cells* **24**, 139-144.
Tomioka, M., Nishimoto, M., Miyagi, S., Katayanagi, T., Fukui, N., Niwa, H., Muramatsu, M. and Okuda, A. (2002). Identification of Sox-2 regulatory region which is under the control of Oct-3/4-Sox-2 complex. *Nucleic Acids Res*. **30**, 3202-3213.
Van de Lavoir, M. C., Mather-Love, C., Leighton, P., Diamond, J. H., Heyer, B. S., Roberts, R., Zhu, L., Winters-Digiacinto, P., Kerchner, A., Gessaro, T. et al. (2006). High-grade transgenic somatic chimeras from chicken embryonic stem cells. *Mech. Dev*. **123**, 31-41.
Velkey, J. M. and O'Shea, K. S. (2003). Oct4 RNA interference induces trophectoderm differentiation in mouse embryonic stem cells. *Genesis* **37**, 18-24.
Yamaguchi, S., Kimura, H., Tada, M., Nakatsuji, N. and Tada, T. (2005). Nanog expression in mouse germ cell development. *Gene Expr. Patterns* **5**, 639-646.
Yasunaga, M., Tada, S., Torikai-Nishikawa, S., Nakano, Y., Okada, M., Jakt, L. M., Nishikawa, S., Chiba, T., Era, T. and Nishikawa, S. (2006). Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. *Nat. Biotechnol*. **12**, 1491-1492.
Yeom, Y. I., Fuhrmann, G., Ovitt, C. E., Brehm, A., Ohbo, K., Gross, M., Hubner, K. and Scholer, H. R. (1996). Germline regulatory element of Oct4 specific for the totipotent cycle of embryonal cells. *Development* **122**, 881-894.