A sister of \textit{NANOG} regulates genes expressed in pre-implantation human development

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The \textit{NANOG} homeobox gene plays a pivotal role in self-renewal and maintenance of pluripotency in human, mouse and other vertebrate embryonic stem cells, and in pluripotent cells of the blastocyst inner cell mass. There is a poorly studied and atypical homeobox locus close to the \textit{Nanog} gene in some mammals which could conceivably be a cryptic paralogue of \textit{NANOG}, even though the loci share only 20\% homeodomain identity. Here we argue that this gene, \textit{NANOGNB} (\textit{NANOG Neighbour}), is an extremely divergent duplicate of \textit{NANOG} that underwent radical sequence change in the mammalian lineage. Like \textit{NANOG}, the \textit{NANOGNB} gene is expressed in pre-implantation embryos of human and cow; unlike \textit{NANOG}, \textit{NANOGNB} expression is restricted to 8-cell and morula stages, preceding blastocyst formation. When expressed ectopically in adult cells, human \textit{NANOGNB} elicits gene expression changes, including downregulation of a set of genes that have an expression pulse at the 8-cell stage of pre-implantation development. We conclude that gene duplication and massive sequence divergence in mammals generated a novel homeobox gene that acquired new developmental roles complementary to those of \textit{Nanog}.

1. Background

The \textit{Nanog} gene was originally described independently by Wang \textit{et al.} [1], Mitsui \textit{et al.} [2] and Chambers \textit{et al.} [3] and has been placed within the ANTP class of homeobox genes. \textit{NANOG} is highly expressed in mouse and human embryonic stem cells (ESCs) and during pre-implantation stages of embryo development from the 8-cell stage to the blastocyst, notably in the blastocyst inner cell mass which contributes to all somatic and germ-line tissues of the embryo. In mouse and human, the \textit{Nanog} gene facilitates self-renewal of ESCs in culture and plays a central role in maintaining ESCs in a pluripotent state [2–4]. The gene is thought to have an analogous role in the embryo, being essential for maintenance of pluripotency by cells of the inner cell mass [2].

Although initially thought to be a singleton gene and not part of a gene family, some vertebrate species possess a second \textit{Nanog} gene. Booth \& Holland [5] reported that human \textit{NANOG} has 11 pseudogenes, comprising 10 processed pseudogenes dispersed around the genome plus one duplication pseudogene (\textit{NANOGP1}) generated by a segmental duplication involving \textit{NANOG} and an adjacent solute carrier gene. \textit{NANOGP1} was independently named \textit{NANOG2} by Hart \textit{et al.} [6] and this name was adopted as evidence accumulated that the locus was under selection and produced a protein [6–8]. The \textit{NANOG2} locus is shared by humans and chimpanzees [7,9]. An independent duplication of \textit{NANOG} was also reported in chicken [10], and more recently this duplication was found to be prevalent across birds. \textit{Nanog} gene duplications have been noted in other species including guinea pig, coelacanth and gar [9]. In each
NANOG Neighbour in 2010 by the Human Gene Nomenclature Committee. The name NANOGNB was chosen to reflect chromosomal position with no inference made about evolutionary origin. Although the two loci are closely linked physically (in head to tail orientation), the deduced homeodomain of NANOGNB shares only 20% sequence identity with that of NANOG (12/60 amino acids); far below the normal extent of protein sequence similarity for duplicates within a homeobox gene family. Zhong & Holland [11] placed NANOGNB in a separate gene family to NANOG, along with three retroposed pseudogenes, and considered the homeodomain sequence to be so divergent that it could not even be placed in one of the 11 classes of bilaterian homeobox genes (by comparison, NANOG is clearly in the ANTP class [11,12]). In HomeoDB2, it was stated that if NANOGNB evolved by tandem gene duplication from NANOG, then it must have undergone ‘massive sequence divergence’ [13]. There is no orthologue in mouse.

Scerbo et al. [9] included the Nanognb locus in an analysis of synteny around the Nanog gene in vertebrates. These authors noted Nanog as present and adjacent to Nanog in human, chimpanzee, dog and elephant genomes. This is a far more restricted phylogenetic distribution than the Nanog gene itself, which is present in teleost fish, gor, coelacanth, anole lizard, turtle, birds and mammals [9]. Although secondarily lost from Xenopus, the Nanog gene is also present in urodele amphibians [14]. The Nanog gene, therefore, dates back at least to the base of ostechthyans, over 420 million years ago (Ma); in contrast, a recognizable Nanog has only been reported from eutherian mammals, for which the crown-group common ancestor may be as recent as 61 Ma [15]. If Nanog was indeed derived by duplication from Nanog, then the massive sequence divergence must have occurred in a relatively short period of time: just 20% homeodomain sequence identity now remains. By contrast, Hox genes such as HOXAI and HOXBI, separated by duplication over 450 Ma, share 88% homeodomain identity. Even the highly divergent ARGFX homeobox gene, dating to the base of eutherian mammals [16], shares 53% homeodomain identity with its progenitor CRX. The extremely low sequence similarity between NANOG and NANOGNB raises questions over whether the two neighbouring genes really share a recent common ancestry. However, it should also be noted that NANOGNB shares low sequence similarity with every other homeobox gene yet must have evolved from some progenitor; NANOG is perhaps the best candidate due to its physical proximity.

Here we investigate the origin and function of the NANOGNB homeobox gene. We show that NANOGNB is an extremely divergent duplicate (cryptic paralogue) of NANOG and that the human NANOGNB gene is expressed predominantly at the 8-cell to morula stages of development, initially concomitantly with NANOG but silenced earlier. Ectopic expression of NANOGNB in adult cells causes gene expression changes including downregulation of a set of genes which have a sharp expression peak at the 8-cell stage of pre-implantation development, presaging pluripotency. We suggest that gene duplication and massive divergence generated a novel homeobox gene that acquired new developmental roles in mammals complementary to those of NANOG.

2. Results

2.1. NANOGNB is a cryptic paralogue of NANOG

To examine if NANOG and NANOGNB are cryptic paralogues, with recent evolutionary history obscured by extensive amino acid sequence divergence, we deployed two strategies: identification of shared motifs and molecular phylogenetic analysis.

To search for shared motifs, we compared not only eutherian mammal Nanog and Nanognb, but also the single and duplicated Nanog genes of non-mammalian vertebrates (birds, reptiles and ray-finned fish). In total, 147 deduced protein sequences were included; full alignment data have been deposited [17]. Manual inspection revealed a mosaic of alignable and non-alignable sequences (figure 1a). Eutherian mammal Nanog proteins could be aligned well with each other, as could reptile/bird/fish Nanog proteins, but alignment between the two groups was limited outside the homeodomain despite their undoubted common ancestry. Short shared motifs were found both N-terminal and C-terminal to the homeodomain. Eutherian Nanognb proteins showed less similarity to either group, and the homeodomain sequence was markedly different. However, two small motifs N-terminal to the Nanognb homeodomain were identified as similar to motifs in Nanog proteins: the first motif (blue in figure 1a) is putatively shared with all Nanog proteins; the second (red in figure 1a) is identifiable in Nanog proteins of reptiles and birds, but not eutherian mammals. These motifs are located further from the homeodomain in Nanognb proteins due to a putative insertion. The presence of shared motifs is suggestive of common ancestry rather than definitive proof. We suggest that these motifs were present in a common ancestral vertebrate Nanog protein, and the mosaic pattern of conservation reflects differential retention in different genes and lineages. Comparison to the known functional domains of eutherian mammal Nanog suggests that NANOGNB does not contain the C-terminal transactivation and dimerization domains, while an N-terminal repressor domain is partially conserved (figure 1b).

For phylogenetic analysis, we first examined placement of NANOGNB within a phylogenetic tree of ANTP class homeodomains. If NANOGNB is a cryptic duplicate of NANOG, it would be expected to group with NANOG within the NKL subclass of ANTP genes. Phylogenetic analysis using all ANTP class homeodomains of chicken, human and zebrafish supported this prediction, with a distinct clustering of NANOG and NANOGNB sequences within the NKL subclass (electronic supplementary material, file S1: figure S1). However, this result is sensitive to sampling because when non-ANTP class homeodomains are included, the placement of NANOGNB is disrupted, perhaps due to long-branch effects (electronic supplementary material, file S1: figure S2). A phylogenetic analysis involving only NANOG and NANOGNB proteins permitted a longer sequence alignment to be used (exons 2 and 3) and is particularly revealing (figure 1c). When rooted using the single Nanog genes of teleost fish, the most logical root position, NANOGNB is placed nested within the amniote NANOG genes consistent with an
origin from NANOG. A series of putative gene duplication events can be deduced. The two Nanog genes of reptiles and birds (denoted Nanog1 and Nanog2) form deeply separated clades, although with Pelodiscus sequences are aberrantly placed; this suggests that an ancient gene duplication generated these genes prior to the radiation of extant reptiles and birds. Interestingly, the two genes are not grouped together to the exclusion of mammalian Nanog, suggesting that the duplication may be older than the reptile/mammal divergence. In this scenario, mammalian Nanog can only be orthologous to one of the reptile/bird genes, raising the question of what is the mammalian orthologue of the other reptile/bird Nanog? In our analysis, Nanognb falls as a sister group to the reptile/bird Nanog1 gene, albeit on a long branch, and is suggested to be the missing orthologue of reptile/bird Nanog1 (or conceivably of Nanog2), which underwent extensive sequence change specifically in eutherian mammals.

Hence, we propose that a single ancestral Nanog gene underwent tandem gene duplication before the divergence of extant reptiles, birds and mammals. After the origin of the mammalian lineage, one of these genes underwent radical sequence divergence to become Nanognb. Placement of sequences from the platypus (Ornithorhynchus, a monotreme) suggests some of this sequence divergence occurred before divergence of monotremes and therian mammals, with divergence further pronounced in the eutherian mammals. Mouse has lost the Nanognb gene secondarily. Interestingly, Nanognb was not identified in marsupial mammals (Sarcophilus, Monodelphis), but two Nanog genes in Sarcophilus (Tasmanian devil) are both closer to Nanog in our phylogenetic analysis, possibly reflecting gene conversion or a separate gene duplication. Within fish, gar apparently has an independent duplication.

2.2. NANOGNB expression is more temporally restricted than NANOG expression

Genes closely linked to NANOG in human and mouse, notably GDF3 and DPPA3, are similarly expressed in pluripotent embryonic stem cells and blastocysts [18], and evidence is accumulating for a chromatin domain with shared regulatory input around the NANOG gene extending at least as far as GDF3 [19–21]. Since the human (and cow) NANOGNB gene lies within this region, between NANOG and DPPA3, we asked whether the gene shares a temporal expression profile with NANOG. Mapping of RNA-Seq reads for human and cow pre-implantation stages to a revised annotation of coding genes revealed that NANOGNB is expressed...
Figure 2. Expression analysis of genes in the chromosomal vicinity of NANOGNB in human and syntenic regions in mouse and cow. (a) Region surrounding NANOGNB on the short arm of human chromosome 12. Expanded view shows expression of 35 genes spanning a 2 Mb region (O, oocyte; Z, zygote; 2C, 2-cell embryo; 4C, 4-cell embryo; 8C, 8-cell embryo; M, morula; B, late blastocyst; ESC, embryonic stem cell). Adult tissues, left to right: adipose, adrenal, brain: parietal lobe, brain: substantis nigra, brain: whole, breast, CD34þ cells, CD4þ cells, CD8þ cells, colon, duodenum, endometrium, oesophagus, fallopian tube, gallbladder, heart, kidney, liver, lung, lymph node, macular retina, macular RPE, monocytes, natural killer cells, neutrophils, ovary, pancreas, placenta, prostate, salivary gland, skeletal muscle, skin, small intestine, smooth muscle, spleen, stomach, testes, thymus, thyroid, tonsil, whole blood. (b) Expression of genes in the syntenic regions in cow and mouse. MI and GV are oocyte stages. Expression values are normalized to the sample in each species where each gene is most highly expressed. Expression values below a FPKM of 2 are treated as unexpressed.

2.3. Targets down-regulated by NANOGNB are enriched for pre-implantation genes

Determining putative downstream targets of NANOGNB is complicated by the inaccessibility to experimentation of 8-cell and morula stages of human development, absence of expression in ESCs and lack of an orthologue in mice. In this situation, ablation of function by gene targeting is not a viable option for investigating function. Hence we used ectopic expression of V5-tagged human NANOGNB in primary adult human fibroblast cells to test if NANOGNB could elicit transcriptomic changes. We identified a total of 1070 upregulated and 1155 down-regulated genes with at least 1.3-fold expression change compared to cells transfected with empty vector control (electronic supplementary material, file S3).

To determine the biological significance of the transcriptome changes, we deployed a method to examine overlap with temporal profiles of gene expression in human development. This enables attention to be focused on those NANOGNB-responsive genes most likely to be direct or indirect targets of NANOGNB during pre-implantation development. Using RNA-Seq data from seven stages from oocyte to late blastocyst, we generated 69 distinct expression profiles with at least 1.3-fold expression change compared to cells transfected with empty vector control (electronic supplementary material, file S3)

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downregulated genes (profiles C19, C31, C61; combined Fisher’s exact test $p = 1.5 \times 10^{-11}$) (electronic supplementary material, file S1; figure S3). The enrichment for downregulated genes in profile C61 is far larger than for any other profile, up- or downregulated (Fisher’s exact test $p = 6.7 \times 10^{-9}$). The profile is also depleted for upregulated genes (Fisher’s exact test $p = 5.2 \times 10^{-7}$). It is striking that this profile describes genes with a sharp peak in expression at the 8-cell stage in human development, decreasing rapidly in expression level precisely at the time when $\text{NANOGNB}$ is increasing in expression (figure 3). This temporal correlation, together with the experimentally demonstrated transcriptomic effect, is consistent with a model in which $\text{NANOGNB}$ represses the activity of genes showing a marked pulse of pre-morula and pre-blastocyst expression.

Temporal profile C61 contains 594 human genes, of which 76 are downregulated by ectopic $\text{NANOGNB}$ in adult cells. To investigate the function of these 76 putative targets, we plotted their normal expression patterns in human early development and in adult tissues. An expected strong peak of expression at the 8-cell stage is clearly observed, but it is also evident that most of the putative targets are deployed again in adult tissues, with particular sets of genes expressed in testis and in the immune system (B cells, T cells, NK cells, monocytes, neutrophils; figure 4). The genes code for a wide range of proteins including a dual-specificity kinase CLK4 and several

![Figure 3. Enrichment of $\text{NANOGNB}$-responsive genes within gene expression profiles generated from embryo expression data. (a) Expression plots for individual genes and profile averages for the eight initial profiles which were combined to create combined profile C61. (b) Plots for all individual gene expression and profile average for combined profile C61. (c) Overlap of genes from the up- and downregulated genes after $\text{NANOGNB}$ overexpression in fibroblast Fisher’s exact test $p$-values. Grey lines represent standardized expression patterns for individual genes. Red lines represent the average standardized expression pattern for each profile. The blue line shows the relative standardized expression pattern for $\text{NANOGNB}$ superimposed on profile C61. O, oocyte; Z, zygote; 2C, 2-cell embryo; 4C, 4-cell embryo; 8C, 8-cell embryo; M, morula; B, late blastocyst.](http://rsob.royalsocietypublishing.org/)

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| down-regulated genes | up-regulated genes |
|----------------------|--------------------|
| observed             | expected           | $p$-value         |
| 76                   | 16                 | 38                |
|                      |                     | $8.6 \times 10^{-3}$| depleted        |
|                      |                     | 40                | $1.2 \times 10^{-5}$| enriched   |
transcription factors (IRX1, HEY1 and RELB). The roles of the majority of the 76 genes in pre-implantation embryos are unknown.

3. Discussion

The NANOGNB gene has long been an enigma. First identified in human but absent in mouse, the gene was subsequently found in several other mammals. Its evolutionary origins were obscure because, although neighbouring the well-known NANOG gene, the homeodomain of NANOGNB is extremely divergent from any other homeodomain sequence known. Here we argue that NANOGNB was generated by tandem duplication of the NANOG gene followed by extensive, indeed dramatic, protein sequence divergence. Furthermore, we propose that the sequence divergence did not occur in all evolutionary lineages inheriting the duplicate genes, with birds/reptiles retaining two quite similar Nanog genes, while in mammals one paralogue diverged to become Nanognb.

Extensive sequence divergence after tandem duplication has been reported for other homeobox genes, although not as extreme as with Nanog. For example, the human PRD class genes ARFX, DPRX, TPRX1, TPRX2 and LEUTX are divergent duplicates of the CRX gene, a member of the Otx gene family [16]. As with NANOG and NANOGNB, the ‘parental’ gene retained a sequence similar to that of the unduplicated condition, while the paralogues accumulated extensive sequence change. Such ‘asymmetric’ evolution of duplicated homeobox genes has also been reported in other taxa, such as duplicated Hox genes of Lepidoptera and TALE class genes of molluscs (reviewed by [22]).

It is interesting that ARFX, DPRX, TPRX and LEUTX genes are, like NANOGNB, expressed in pre-implantation human embryos, and can regulate the expression of embryonic genes when ectopically expressed in adult cells and ESCs [16,23,24]. This may reveal a propensity of novel genes to be recruited to early developmental stages; for example, co-option to pathways regulating the formation of distinct cell lineages for embryo and placenta. This finding is also compatible with the phylotypic egg-timer model in which early and late stages of development are more amenable to evolutionary modification [25]. Alternatively, recruitment to early developmental stages may simply be a product of a paralogue inheriting or sharing cis-regulatory information with its progenitor gene. In this context, the cis-regulatory landscape around the Nanog gene is relevant.

It has long been recognized that three genes expressed in pluripotent human and mouse embryonic stem cells map as chromosomal neighbours: Gdf3, Dppa3 and Nanog [18]. There is accumulating evidence for a degree of co-regulation of these...
genes. Mapping of local three-dimensional chromatin configuration around the human NANOG promoter using DNase Hi-C revealed a single chromatin domain in human ESCs encompassing GDF3, DPPA3 and NANOG, plus two other genes located between them: NANOGNB and CLEC4C [19]. Examination of chromosomal contacts using 3C in mouse ESCs has shown that the syntenic 160 kb domain folds into a physical loop from Nanog to Gdf3 [20]. These authors also demonstrated that the looped region is enriched for binding sites for transcription factors involved in ESC self-renewal and that the genes are co-regulated. The latter study [20] and a recent functional dissection of the region by Blinka et al. [21] used mouse ESCs. In this species the Nanog gene has been lost, leaving intergenic DNA between Nanog and Dppa3. If equivalent looping and co-regulation occurs in human ESCs, as suggested by DNase Hi-C [19], then it is expected that human NANOGNB would share some cis-regulatory input with NANOG.

Although NANOGNB and NANOG most probably share cis-regulatory input, their expression patterns are not identical. Specifically, expression of NANOGNB drops dramatically before the blastocyst is formed (FPKM value decreasing by over 90% from the morula stage) and is undetectable in human ESCs. The peak of expression for NANOGNB is at the morula stage, and hence its functions must be different from those of NANOG which has a pivotal role in self-renewal of pluripotent cells in the inner cell mass of the blastocyst. Ectopic expression of NANOGNB reveals that the protein is capable of modulating gene expression, with a particularly strong effect being downregulation of genes that peak at the 8-cell stage of pre-implantation development. Hence, we conclude that the in vivo function of NANOGNB is to regulate a sharp pulse of gene expression that occurs at the 8-cell to morula stage, before cell fate commitment, comprising a suite of genes involved in transcription, intracellular signalling and cell division.

4. Methods

4.1. Embryo and adult tissue RNA-Seq profiling

Mapped and processed RNA-Seq expression data for normal human adult tissues and developmental stages and mouse developmental stages are from Dunwell & Holland [26], including a corrected gene model for human NANOGNB. RNA-Seq reads from cow embryonic stages were aligned to the bovTaue8 reference genome using the STAR RNA-Seq aligner using the default settings with the addition of --outSAMstrandField intronMotif (electronic supplementary material, file S4; [27]). Expression levels for each sample were generated in the form of FPKM (fragments per kilobase per million reads) using CUFFLINKS [28].

4.2. Motif and phylogenetic analysis

Predicted peptide sequences were obtained from NCBI, Ensembl and HomeoDB2 [13], and are listed in the electronic supplementary material, file S5. Amino acid sequence alignments were generated using MAFFT v. 7.123b [29] and putatively homologous peptide motifs identified by eye; phylogenetic analysis was performed using RAxML v. 8.2.4 [30], after manual removal of poorly aligned regions, using settings: -T 30 -f a -k -x 12345 -p 12345 -d # 500 -m PROTGAMMALG.

4.3. Ectopic NANOGNB gene expression

The human NANOGNB coding sequence was synthesized by GenScript USA and cloned in-frame with a C-terminal V5 tag under the control of a CMV promoter in a GFP co-expressing vector (pSF-CMV-Ub-daGFP Ascl, Oxford Genetics #OG244). Electroporation into primary adult fibroblasts was performed as described [16]; cells were cultured for 48 h, harvested by trypsinization and resuspended in sorting buffer (2 mM EDTA, 25 mM HEPES, 0.5% BSA in Mg2+/Ca2+-free PBS). GFP-positive cells were enriched by sorting using a BD FACSARIA III, collecting 75 000–283 000 cells per replicate, and RNA was extracted using RNeasy Mini Kit (Qiagen). Three biological replicates were performed for NANOGNB and empty vector transfections. TrueSeq (Illumina) libraries were prepared using 400 ng RNA per replicate at the Oxford Genomics Centre and sequenced using the Illumina HiSeq4000 platform generating between 42.8 and 61.3 million paired-end reads per replicate. Reads were aligned to the human reference genome GRCh38, raw read counts were generated with featureCounts and differential gene expression analysis in DESeq2 [31,32]. FPKM expression values for protein-coding genes were also generated using CUFFLINKS. Differentially expressed genes identified by DESeq2 were filtered by applying the following criteria: adjusted p-value ≤ 0.05 (Benjamini–Hochberg correction); upregulated to FPKM ≥ 2 or downregulated from FPKM ≥ 2; fold change ≥ 30% increase or decrease. Raw RNA-Seq reads and mapped featureCounts data have been deposited to NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) under accession GSE94053.

4.4. Embryo temporal profile enrichment analysis

To reveal which NANOGNB-responsive genes were probable in vivo targets, we examined overlap with temporal profiles of human gene expression, modified from the method described by Maeso et al. [16]. Gene expression values (FPKM) for seven human developmental time points (oocyte, zygote, 2-cell, 4-cell, 8-cell, morula and late blastocyst) were filtered to retain all expressed genes with a variance ≥ 5, resulting in a total of 8837 genes. The genes were then initially grouped into a total of 160 different expression profiles by Mrunz based on expression changes across embryo stages, profile IDs 1–160 [33]. Clusters with similar temporal profiles, those with a pairwise Pearson correlation ≥ 0.95, were combined to generate a final collection of 69 distinct profiles, as designated collapsed profile IDs C1–C69.

A stepwise test was used to identify temporal profiles enriched or depleted for genes affected by NANOGNB ectopic expression. First, for each of the up- or downregulated genes, we identified in which of the 69 developmental profiles it was present; genes not present in any profile were removed. Second, a Pearson’s χ2 test tested if NANOGNB-responsive genes were differentially assigned among profiles. As a statistically significant difference was seen (p-value ≤ 0.05), the Pearson’s statistic for each profile was calculated to reveal which profiles contributed most to the difference. Third, to verify that identified profiles were responsible, a Pearson’s χ2 test on the remaining profiles tested for significant difference among them. Fourth, temporal profiles found to be enriched or depleted in NANOGNB-responsive genes were combined and the significance of enrichment or depletion was determined using Fisher’s exact test.

Data accessibility. Raw transcriptome data (sequencing reads) are deposited on SRA – GEO accession GSE94053 https://www.ncbi.nlm.nih.gov/geo
Authors’ contributions. T.L.D. conceived the study and performed experiments and data analysis. P.W.H.H. and T.L.D. participated in project design. T.L.D. and P.W.H.H. wrote, reviewed and approved the manuscript.

Competing interests. We declare we have no competing interests.

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