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Nanog transforms NIH3T3 cells and targets cell-type restricted genes

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Abstract

The transcription factor Nanog is uniquely expressed in embryonic stem (ES) cells and in germ cell tumors and is important for self-renewal. To understand the relation between this and cell transformation, we expressed Nanog in NIH3T3 cells, and these cells showed an increased growth rate and a transformed phenotype as demonstrated by foci formation and colony growth in soft agar. This suggests that Nanog possesses an oncogenic potential that may be related to the role it plays in germ cell tumors and to its function in self renewal of ES cells. We studied the transcription targets of Nanog using microarrays to identify Nanog regulated genes. The list of genes regulated by Nanog was unique for each cell type and more than 10% of the Nanog regulated genes, including transcription factors, are primary Nanog targets since their promoters bind Nanog in ES cells. Some of these target genes can explain the transformation of NIH3T3.

Keywords: Self-renewal; Oncogenicity; Microarray; Promoters; Nanog targets

Embryonic stem (ES) cells, derived from the inner cell mass of the blastocyst, can be propagated in culture without differentiation and they are endowed with two defining properties: self renewal through symmetric cell division and pluripotency, allowing for the development of all the cell types present in the adult [1]. ES cells maintain pluripotency by a transcriptional program that suppresses differentiation and this block is removed during embryonic development [2,3]. Recent data suggest that these properties may be regulated by a small number of ES cell-specific transcription factors such as Nanog, Oct4 and Sox2 whose expression is down regulated early during embryogenesis. Deficiency of Oct4 or Nanog in ES cells, by knock-out [3] or knock-down procedures [4], abolish both self renewal and pluripotency and results in differentiation to either trophectoderm lineage in the case of Oct4 [5] or to extra-embryonic endoderm, in the case of Nanog, suggesting different roles in gene silencing for each of the Oct4 and Nanog factors [3,6]. Apparently Oct4 and Nanog function in coordination to maintain ES cell pluripotency [7]. These transcription factors are not usually expressed in adult tissues. In contrast, their expression in germ cell (GC) tumors as part of their transformation process suggests that GC tumors represent a differentiation stage closely related to ES cells [8,9]. Recently Nanog expression was also found in some breast tumors and cell lines such as MCF7 [8]. Interestingly, ectopic expression of Oct-4 in Swiss3T3 cells resulted in transformation and tumor growth in nude mice [10]. Additionally, induction of Oct-4 expression in transgenic mice resulted in epithelial dysplasias [11], perhaps a feature associated with self renewal.

Further understanding of the role of the aforementioned transcription factors in regulating ES cell differentiation was gained by identifying the promoters occupied by these

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factors in ES cells [12]. Collectively they bind to ~10% of the promoters in the human genome (1687, 1271, and 623 for Nanog, Sox2, and Oct4, respectively) and more than one factor binds to promoter regions of hundreds of these targets. However, more than half of these genes, including tissue specific genes, are silenced in ES cells indicating that these factors suppress differentiation genes. Thus, suppressing genes for differentiation and activation of genes needed for maintenance of self renewal is part of the mechanism used by Nanog to support pluripotency [13]. The identification of target genes for Nanog by gene expression analysis is necessary for understanding its role during development and its oncogenic potential.

In this paper we showed that Nanog can be oncogenic and transforms NIH3T3 cells, suggesting that part of the mechanism that supports self-renewal by Nanog may be related to cancer cell proliferation. To determine the target genes of Nanog in differentiated cells, we ectopically expressed Nanog in various cell types (lung, colon, and fibroblasts) and analyzed the expression profiles of genes activated by Nanog using microarrays. Several of the genes activated in NIH3T3 cells are consistent with a potential for oncogenic transformation. We found that at least 10% of the genes found to be regulated by Nanog are likely to be primary targets of Nanog since their promoters were shown to bind Nanog [12] and that the genes activated by Nanog in differentiated cells are unique for each cell-type.

Materials and methods

Plasmids, viruses, and cell culture. The coding sequence of human Nanog was PCR amplified from pPyCAG:hNanog [2] using primers with *Bgl*II/*Eco*RI overhangs and cloned into pTZ58 (Fermentas, Vilnus, Lithuania). The *Bgl*II/*Eco*RI fragment was then subcloned into pBabe-HA (*Bam*HI/*Eco*RI) and pEYFP-C1 (Clontech, Mountain View, CA) (*Bgl*II/*Eco*RI) to generate HA-Nanog and EYFP-Nanog encoding plasmids, respectively. The pBabe-RasV12 construct was a gift from D. Ginsberg (Bar-Ilan university).

Cell culture and viral transductions. COS7 and HEK293T cells were grown in complete DMEM, NIH3T3 cells were grown in DMEM supplemented with 10% bovine serum, H1299 and HCT116 cells stably expressing the murine ecotropic receptor [14] were grown in complete RPMI 1640 plus and McCoy's medium, respectively. The pBabe puro constructs were co-transfected with a helper plasmid into 293T cells using FUGene (Roche, Basel, Switzerland) to produce replication incompetent virions. Culture supernatants were collected, filtered, and frozen. Efficiency of transduction was estimated to be ~50%. Actively dividing cells were transduced with viruses in the presence of polybrene (Sigma–Aldrich, Park Rabin Rehovot, Israel) (4 μg/ml) and the cells were cultured in the presence of puromycin (Sigma–Aldrich, Park Rabin Rehovot, Israel) (HCT116: 0.62 μg/ml; H1299: 1.25 μg/ml; and NIH3T3: 1.25 μg/ml) to generate cell lines stably expressing HA-Nanog. Cell proliferation was measured in 96-well plate at a density of 2000 cells/well. Cell viability was measured every day using the MTT assay as described previously [15].

Foci formation and soft agar assays. Foci assays [16] were done using NIH3T3 fibroblasts infected with viruses encoding Nanog, H-RasV12 or a control plasmid in six-well plates. The cells were grown in DMEM supplemented with 2% bovine fetal serum after infection and cultured for 3 weeks when foci were evident. The soft agar assays were carried out in 60 mm culture dishes as previously described [17]. NIH3T3 cells stably expressing Nanog, H-RasV12 or a control plasmid were suspended at densities of 1000, 5000, and 10,000 cells/dish in DMEM supplemented with 5% defined bovine calf serum (iron supplemented) and 0.3% agar (Difco). This was layered on growth medium containing 0.5% agar. Cells were fed twice a week with 0.5 ml of medium and colonies were photographed microscopically 3 weeks after plating.

Protein analysis and immunofluorescence. Cell lysates were prepared from confluent 10 cm plates using RIPA buffer. HA-Nanog was immunoprecipitated using a mouse monoclonal anti-HA antibody (12CA5) and Western analysis was performed using a rat monoclonal anti-HA antibody (3F10, Roche), followed by HRP-anti Rat IGG (Jackson Lab, West Grove, PA). COS7 cells were transfected with plasmids encoding YFP, YFP-Nanog or HA-Nanog using JET-PEI (Qbiogene, Irvine, CA). On the next day, the cells were plated on cover slips and 24 h later the cells were fixed using 4% paraformaldehyde. Cells expressing HA-Nanog were permeabilized using 2% Triton X-100 and stained with antibodies to HA (3F10, Roche) and Nanog (eBioscience, San Diego, CA). Secondary anti-rat Cy2 and anti-mouse Cy5 were used for visualization, respectively. The coverslips were mounted on slides using Mowiol and images were generated using a UV-fluorescent microscope (Olympus).

RNA extraction, microarray analysis, and annotation. Total RNA was extracted from cells stably expressing Nanog or selected with puromycin after infection with a control plasmid using Trizol (Invitrogen, Carlsbad, CA) from duplicate samples. Samples for hybridization were prepared according to manufacturer's protocols with minor changes. Briefly, 5 μg of mRNA was used to generate first-strand cDNA using a T7-linked oligo(dT) primer. After second-strand synthesis, in vitro transcription was performed with biotinylated UTP and CTP (Enzo Diagnostics), resulting in approximately 300-fold amplification of RNA. The target cRNA generated from each sample was hybridized to the chip using an Affymetrix Gene Chip Instrument System (Santa Clara, CA). When scaled to a target intensity of 150 (using Affymetrix MAS 5.0 array analysis software), scaling factors for all arrays were within acceptable limits (0.63–0.84), as were background, Q values and mean intensities.

The probe sets contained in the Affymetrix Human Hu133A2 oligonucleotide array or in the Affymetrix mouse MOE430A oligonucleotide arrays were processed using MAS 5.0 software. Probe sets which didn't have "present" calls on duplicate chips were discarded. All the expression values below 20 were raised to 20. Fold change for each probe set was calculated from the average expression value in the treated samples divided by the average value in the control samples. We varied the threshold above which fold change was considered significant to determine the highest threshold that gives a statistically significant number of genes that are affected by Nanog and are identified [12] as Nanog-binders.

To determine the Affymetrix probe set ID's of the genes whose promoters were identified as "Nanog-binding," we made use of the coordinates given by Boyer et al. [12] and traced them via the Ensembl BioMart database (http://www.biomart.org/DB_install1.html).

Verification of microarrays data was done on selected samples using DNase treated (Promega, Madison, WI) total RNA, for reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. First strand synthesis was performed with 10 pmol random hexanucleotides and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) in a 30 μL reaction volume, at 42 °C for 80 min.

Standard PCR were performed in 15 μL reaction volume using 10 pmol of specific primers corresponding to the human ID2, MMP2 (H1299 or HCT116 cells) or mouse FGFR1, BTG2, JUN-b, PTN (NIH3T3 cells) genes. PCR primers were selected using the Primer3 package from the Whitehead Institute for Biomedical Research (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and with the criteria that each oligo pair produced a PCR product that spanned in between two different exons. The forward and reverse primer sequence, and annealing temperature for each primer set were as follows: JUN-B, forward primer (FP) 5′-ACGGAGGGAGAGAAAAGCTC-3′, and reverse primer (RP) 5′-TGTTCCATTTTCGTGCACAT-3′; 60 °C, FGFR1, FP 5′-ATGGTT GACCGTTCTGGAAG-3′, RP 5′-GGAAGTCGCTCTTCTTGGTG-3′ and 60 °C; BTG2, FP 5′-GATGGCTCCATCTGTGTCCT-3′ and RP 5′-TATACGGTGGCCTGTTGTCA-3′, 60 °C, ID2, FP 5′-CGT GAG GTC CGTTAGGAAAA-3′ and RP 5′-ATAGTGGGATGCGAGTCCAG-3′;

58 °C, MMP2, FP 5′-ATGACAGCTGCACCACTGAG-3′ and RP 5′-ATTTGTTGCCCAGGAAAGTG-3′, 60 °C.

PCR was also conducted with primers specific for the housekeeping gene GAPDH FP 5′-CCATGGAGAAGGCTGGGG-3′ and RP 5′-CAAAGTTGTCATGGATGACC-3′, with annealing temperature of 56 °C, as an internal control for equivalent amounts of cDNA per reaction.

PCR cycling conditions included 94 °C for 5 min, 26, 28, 30 or 35 cycles of 94 °C for 30 s, selected annealing temperature for 45 s, 74 °C for 30 s. At the end of the cycling, a 74 °C step for 7 min was applied.

Results

Ectopically expressed Nanog localizes to the nucleus

HCT116 a colorectal carcinoma derived cell line, H1299 a non-small cell lung carcinoma cell line and NIH3T3 murine fibroblasts were transduced with viruses encoding Nanog or a control virus and stable clones were selected using puromycin. Pooled clones were analyzed for Nanog expression by Western analysis. Nanog was detected with an anti-HA antibody as a band of ~34 kDa in the three cell lines (Fig. 1A).

In ES cells, Nanog was found to be localized to the nucleus [18]. To verify the sub-cellular localization of ectopically expressed Nanog, we generated a construct encoding Nanog fused to Enhanced Yellow Fluorescent protein (EYFP-Nanog). COS7 cells were transfected with plasmids encoding EYFP-Nanog, or EYFP and plated on cover slips. The cells were later fixed, mounted on slides and analyzed under a fluorescent microscope (Fig. 1B). Alternatively, cells were transfected with pBabe plasmid encoding HA-Nanog, fixed, permeablilized, and stained using anti-HA or anti-Nanog antibodies (Fig. 1C). EYFP was localized all over the cell, while both EYFP-Nanog and HA-Nanog was localized only in the nucleus. Our result suggests that Nanog is a nucleus resident protein and encodes a nuclear localization signal.

Nanog accelerates NIH3T3 cell proliferation

Ectopic expression of Nanog in NIH3T3 cells resulted in increased number of cell protrusions and a tendency to make cell contacts (Fig. 2A) while no obvious changes were seen in H1299 or HCT116. We next measured the effect of Nanog expression on the proliferation of the three cell lines using the MTT assay [15]. Equal numbers of cells were plated in a 96-well plate and cell viability was measured up to 4.5 days post-plating. Expression of Nanog enhanced the rate of NIH3T3 cell proliferation in comparison to control

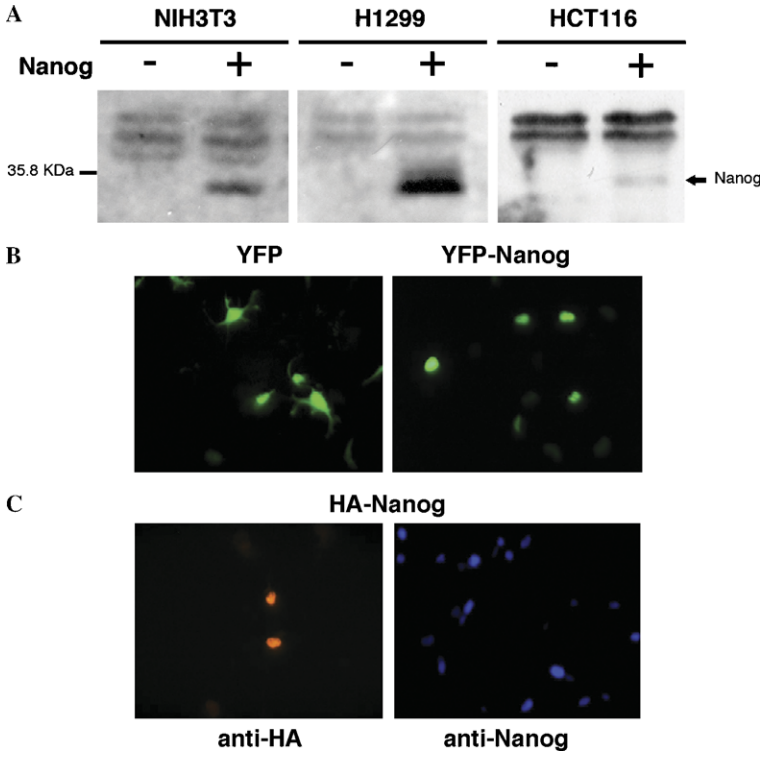

Fig. 1. Ectopically expressed Nanog is located in the nucleus. (A) Confluent 10 cm plates of NIH3T3, HCT116, and H1299 containing pooled clones selected after transduction with a control virus or a virus encoding Nanog, as indicated, were lysed using RIPA buffer. HA-tagged proteins were immunoprecipitated from the lysates using a mouse anti-HA antibody immobilized on anti-mouse Ig-Sepharose and a Western blot was performed using a rat anti-HA antibody followed by anti-Rat IgG-HRP. (B) COS7 cells were transfected using a plasmid encoding EYFP-Nanog or EYFP as indicated. The cells were grown on coverslips, fixed using 4% paraformaldehyde and mounted on slides. Fluorescence microscopic photographs were taken using the fluorescence microscope. (C) COS7 cells transfected with a plasmid encoding HA-Nanog were fixed, permeablilized and stained using anti-HA or anti-Nanog antibodies as indicated. Secondary anti-rat IgG Cy2 or anti-mouse IgG Cy5 were used for visualization.

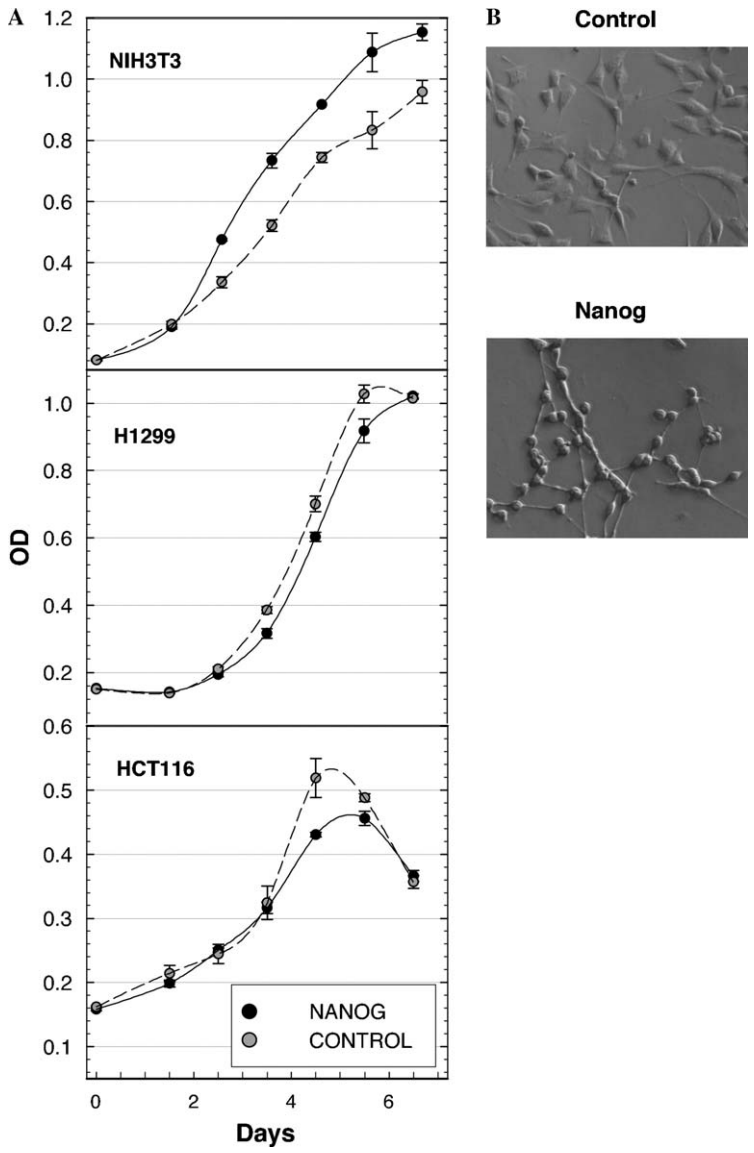

Fig. 2. Nanog alters proliferation in a cell-type specific manner. (A) NIH3T3, HCT116, and H1299 cells stably expressing Nanog or infected with the control virus were plated in a 96-well plate at a density of 2000 cells/well in triplicates. After each 24 h period, the cells were incubated with the MTT reagent and cell viability was measured using an ELISA reader. Average values at each time point and the standard errors are plotted with respect to time. (B) Microscopic pictures of NIH3T3 cells expressing Nanog.

cells, as was also shown recently by others [19]. On the other hand no significant effect was observed in HCT116 and H1299 cells ectopically expressing Nanog (Fig. 2B).

Nanog transforms NIH3T3 cells

We next analyzed if the expression of Nanog transforms NIH3T3 cells. For this, we performed foci formation and soft agar colony formation assays. NIH3T3 cells were plated at low density (20% confluence) and infected with retroviruses encoding Nanog, RasV12 or a control virus. The cells were then allowed to grow for 2 weeks under low serum (2%) condition. Cells expressing Nanog as well as RasV12 formed foci after 2 weeks while there were no foci seen in the control cells. In comparison to RasV12, foci formed by Nanog expressing cells were smaller in size but more abundant (Fig. 3A).

To further characterize the altered phenotype resulting from expression of Nanog, we performed a colony formation assay in soft agar. NIH3T3 cells stably expressing Nanog, RasV12 or a control virus were resuspended in medium containing 0.3% agar, at a density of 5000 cells/dish, and layered on medium containing 0.5% agar. The plates were incubated for 3 weeks and the number and size of colonies were analyzed. Cells expressing Nanog as well as RasV12 formed colonies while no colonies were observed in the control (Fig. 3B). Expression of Nanog resulted in 950 colonies/dish in comparison to RasV12 where 2700 colonies/dish were formed. Further the colonies formed with Nanog were smaller in size compared to

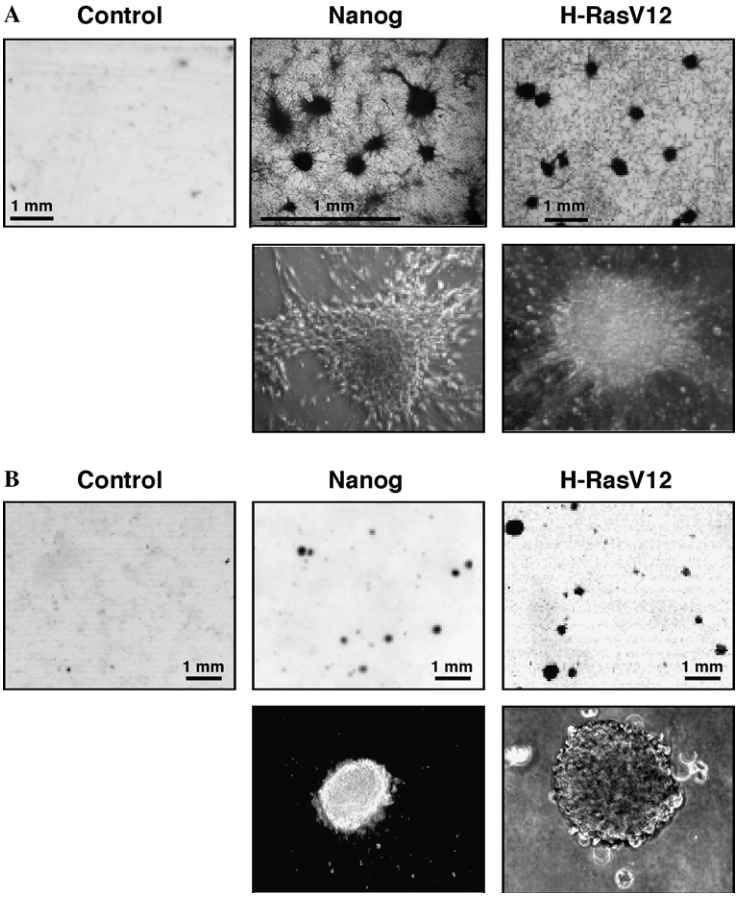

Fig. 3. Nanog transforms NIH3T3 cells. (A) NIH3T3 cells were plated at low density and infected with viruses encoding Nanog, RasV12 or a control virus and grown for 2 weeks in DMEM supplemented with 2% FBS. The plates were then analyzed for foci formation. Representative pictures of a plate at low resolution as well as a magnified focus are shown. (B) NIH3T3 cells stably expressing Nanog, RasV12 or a control virus were trypsinized and 5000 cells/dish were mixed with agar to a final concentration of 0.3% and layered on 0.5% Agar in 60 cm plates. The plates were irrigated with DMEM twice per week for 3 weeks. Representative pictures of the colonies formed, as well as magnified colonies are shown.

that of RasV12. These results confirm that Nanog is a transforming gene for NIH3T3, as cells expressing Nanog are not contact inhibited and can grow in an anchorage independent manner.

Targets of Nanog are cell-type restricted

We used microarray analysis to determine gene expression profiles of cells expressing Nanog in comparison to cells expressing a control vector for each of the three cell lines. We focused on targets in NIH3T3 that may help explaining the transformation of these cells by Nanog. Varying the fold-change threshold between 1.5 and 2.1 we registered the number of up and down regulated genes that appear also in the lists of Boyer et al. [12]. This resulted in a list of 294, 136, and 518 genes in NIH3T3, H1299, and HCT116, respectively. None of the identified genes were common in all three cell types although few (~5%) common genes between pairs of cells were identified, suggesting that Nanog regulates a unique set of genes in each cell line. We then compared these lists with the list of 1687 genes whose promoters were found to be occupied by Nanog in ES cells [12]. This resulted in 24, 18, and 71 genes for NIH3T3, H1299, and HCT116, respectively. These genes (Table 1), that comprise about 10% of the genes regulated by Nanog in our cell lines can be considered as primary targets of Nanog, since it was shown that Nanog interacts with their promoters [12]. It is interesting that a significant part of the direct targets of Nanog are transcription factors and regulators of gene expression (Table 1). For example the transformation of NIH3T3 by Nanog, can be partly explained by activation of STAT3 (Table 1) which is known to be involved in cell transformation, as an oncogene [20]. STAT3 is also known to be required for self-renewal in murine ES cells, where it is activated by LIF but exogenous LIF can be replaced by overexpression of Nanog [2]. Additionally, in NIH3T3 cells JUN-B is upregulated (Table 1). JUN-B is an oncogene and contribute to cell transformation and focus formation in rat embryo fibroblasts [21].

The expression patterns of selected genes from the samples analyzed by microarrays were subsequently compared by RT-PCR (Fig. 4) and validated the direction and amount of the fold change registered with the microarrays.

Table 1
Functional classification of Nanog target genes in different cell lines[a,b,c,d]

	NIH3T3	HCT116	H1299
Transcription regulation	JUN B, ZFP36L1, GLI3, JADE1*, KLF5*, STAT3	PBX1, FLJ13782, ZNF217, ISGF3G, HOXB9, ODAG, MED12, ID2, HBP1, JUN, TNRC11, TAF4B, ID1, KLF5, CALR*	COPEB, JUN, CITED2, ING4, TLE2, ID2
Receptor/ligand	PTN, FGFR1, CTGF*	ROR1, KITLG	FGF2*
Signaling	RRAS	SEMA3A, SNX1, FZD7	CHN2, XCL2
Protein synthesis/degradation	ADAMTS5	DDX17, BFSP1, FLJ14281, ENPEP, LSM5*, MEP50*, C13orf7*	
Cell adhesion	LAMA4, COL4A6, CSPG2	COL4A6, SEPP1, COL4A5, COL7A1, SERPINH1*, CSPG2*	MMP2
Cell growth/cancer	HAS2	SKIL, TIA1, MLH1, PKMYT1*, ORAOV2*, SGK*	
Other	RBP1, TNF, AIP6, OTUB1, DHCR7, CNN3, CYR61,THBS1, HIST1H3D, ANXA8*, TARBP2*	SLC7A11, LHPP, CYP24A1, MAP1LC3B, FLJ22471, KIAA0903, CPS1, HBG1, C5orf4, NEBL, GALNT3, C6orf111, CLIC4, BCKDHA, KIAA0974, C20orf23, CDH1, ENSA, LOC169611, KIAA0738, FLJ20010, P4HA1, SCNN1A, LOC90379*, HIST1H4F*, SS18L2*, DHFR*, PMSCL1*, EST1B*, FTL*	STC1, GDAP1L1, HIST2H2AA, RAB15, LOH11CR2A, HRB2, SLC7A11*, FLJ22688*

[a] The Nanog target genes are those that overlap with those reported by Boyer et al. [12], except JUN-B that is upregulated in NIH3T3. The symbols are according to the NCBI listings.
[b] * labels denote genes that were downregulated in our experiments.
[c] Genes are ordered in each group according to its fold change from higher to lower, from left to right.
[d] Underlined genes indicate that they are present in two different cell lines.

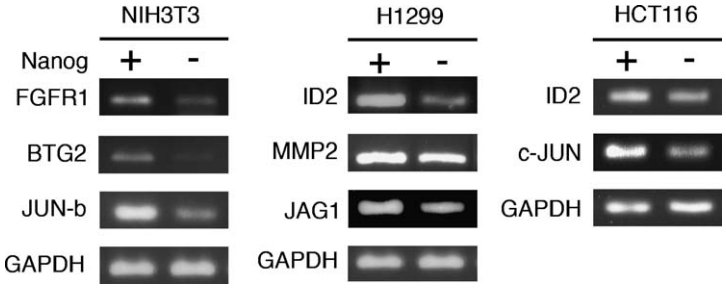

Fig. 4. RT-PCR validation of microarrays. RT-PCR amplification of selected Nanog regulated genes is shown. Differentially expressed mRNAs analyzed in NIH3T3 cells were FGFR1 (164 bp, 32 cycles), BTG2 (197 bp, 28 cycles), and JUN-b (223 bp, 28cycles). In H1299 cells were ID2 (237 bp, 30 cycles), JAG1 (194 bp, 30 cycles), and MMP2 (174 bp, 28 cycles) were amplified. In HCT116 cells were ID2 (237 bp, 30 cycles), and c-JUN (168 bp, 30 cycles). GAPDH (198 bp, 28 cycles) was tested in all the cell lines as an internal control.

Discussion

In this study we ectopically expressed Nanog in cells from differentiated tissues in order to analyze properties that may be related to its contribution for self renewal of ES cells. Molecular mechanisms that regulate self renewal may be shared to some extent between stem cells and cancer cells. Nanog was shown recently to be expressed in primordial germ cells tumors and may contribute to malignant transformation of these cells in certain cancers of the gonads (GC tumors, seminoma) and also in few other tumor types like MCF7 and osteosarcoma [8,9].This suggests that Nanog may be a transforming gene but because its expression is shut-off in adult tissues it was not found to be expressed widely in human tumors. We show here that by expressing Nanog in NIH3T3 it enhances their growth rate and confers on them focus formation and anchorage independent growth, hallmarks of transforming oncogenes. Similar properties were described for Oct4, another ES cells transcription factor, when expressed in Swiss 3T3 cells [10] or in vivo, when expressed in transgenic mice in epithelial cells [11]. These results suggest that oncogenic transformation is consistent with Nanog's role in self renewal of ES cells and provides a mechanistic explanation for the recent finding of Nanog overexpression in seminoma and other tumors of the breast and bone [8,9].

We were also interested in the genes regulated by Nanog, in particular in NIH3T3, that may be consistent with cell transformation. By using Affymetrix microarrays, we determined the genes that are regulated by Nanog in cell lines representing three tissues (lung, colon, and fibroblasts). Approximately 10% of the genes in these lists (Table 1) were reported to be occupied on their promoters by Nanog in ES cells [12] and therefore are very likely primary targets of Nanog. The annotation of these genes suggests that a relatively significant portion of them are

transcription regulators that may activate other genes. Surprisingly very little overlap was found between the three lists of genes in Table 1, suggesting that Nanog target genes are accessible in a tissue specific manner. We speculate that in ES cells where the genome is demethylated [22], the chromatin is accessible for Nanog to occupy its target sites in all promoters [12]. However, during implantation and remethylation the differentiation process involves down-regulation of many genes that were expressed in ES cells as well as up-regulation of genes needed for the prospective tissue [9,13]. This reprogramming of methylation and other chromatin modifications may result in blocking the accessibility of promoters in genes that are not usually expressed in that tissue and therefore will not be accessible for Nanog regulation. Examination of the genes regulated by Nanog in NIH3T3 suggest that some of them may be involved in cell transformation. For example JUN-B and STAT3 are such candidates. It is of interest that STAT3, which is involved in self renewal of ES cells may also be involved in the transformation of NIH3T3 studied here.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.02.152.

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