Cell-permeant recombinant Nanog protein promotes pluripotency by inhibiting endodermal specification

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Abstract   A comprehensive understanding of the functional network of transcription factors establishing and maintaining pluripotency is key for the development of biomedical applications of stem cells. Nanog plays an important role in early development and is essential to induce natural pluripotency in embryonic stem cells (ESCs). Inducible gain-of-function systems allowing a precise control over time and dosage of Nanog activity would be highly desirable to study its vital role in the establishment and maintenance of pluripotency at molecular level. Here we engineered a recombinant cell permeable version of Nanog by fusing it with the cell penetrating peptide TAT. Nanog-TAT can be readily expressed in and purified from *E. coli* and binds to a consensus Nanog DNA sequence. At cellular level it enhances proliferation and self-renewal of ESCs in the absence of leukemia inhibitory factor (LIF). Nanog-TAT together with LIF acts synergistically as judged by enhanced clonogenicity and activation of an Oct4-promoter-driven GFP reporter gene. Furthermore Nanog-TAT, in the absence of LIF, promotes pluripotency by inhibiting endodermal specification in a Stat3-independent manner. Our results demonstrate that Nanog protein transduction is an attractive tool allowing control over dose and time of addition to the cells for studying the molecular control of pluripotency without genetic manipulation.

Introduction

Embryonic stem cells (ESCs) differ from somatic cells by indefinite symmetrical cell divisions and retaining the ability to differentiate into all cell types of the organism (termed pluripotency). A comprehensive understanding of the underlying molecular network of cellular factors (Loh et al., 2006), extrinsic stimuli (Smith et al., 1988; Williams et al., 1988), intracellular signaling pathways (Ermakov et al., 2012), cell cycle regulation (Coronado et al., 2013) and microenvironment (Fernandes et al., 2010), establishing and maintaining pluripotency and controlling differentiation is key for the development of biomedical applications of stem cells. Stimulation by extrinsic factors has been reported to be essential for self-renewal and pluripotency. The cytokine leukemia inhibitory factor (LIF) is required for the culture of mouse ESCs in serum-supplemented media (Smith et al., 1988; Williams et al., 1988) activating the signal transducer and activator of transcription-3 (STAT3). Withdrawal of LIF from the culture medium can be partially compensated by inducible activation of STAT3 as has been shown by employing a tamoxifen-inducible STAT3-estrogen-receptor fusion protein (Matsuda et al., 1999). LIF in concert with BMP4 has been demonstrated to sustain pluripotency and self-renewal in the absence of serum (Ying et al., 2003). Both extrinsic signals are integrated into the transcriptional machinery maintaining stem cell properties via intrinsic messenger molecules. Overexpression of the homeodomain transcription factor Nanog is sufficient to maintain mouse ESC self-renewal independent of LIF and feeder cells, suggesting a role for Nanog as an important downstream regulator of extracellular signals (Chambers et al., 2003; Mitsui et al., 2003). Although Nanog plays a vital role in the establishment of pluripotency its particular role at molecular level is still not fully understood.

Inducible gain-of-function systems allowing a precise control over time and dosage of Nanog activity would be highly desirable to study stemness and nuclear reprogramming pathways. Recently, biologically active recombinant cell-permeant versions of the pluripotency factors Oct4, Sox2, Klf4 and c-Myc proteins have been reported by us and other groups (Bosnali & Edenhofer, 2008; Kim et al., 2009; Zhang et al., 2012; Zhou et al., 2009). Protein transduction involves the generation of recombinant fusion proteins consisting of the protein-of-interest and a so-called cell penetrating peptide (CPP), e.g. derived from the trans-activator of transcription (TAT) protein encoded by Tat gene in HIV or arginine-rich peptides (Bosnali & Edenhofer, 2008; Kim et al., 2009; Zhang et al., 2012; Zhou et al., 2009). Several reports demonstrate the expression and purification of recombinant Nanog protein from mouse, human, luxi cattle and chicken, either as an authentic full-length protein (Ha et al., 2009; Hu et al., 2012; Loh et al., 2006; Yang et al., 2009; Yang et al., 2011; Yu et al., 2013; Zhang et al., 2012) or as a truncated version (Jauch et al., 2008). Although these studies report that recombinant Nanog is able to bind to DNA (Ha et al., 2009; Jauch et al., 2008; Loh et al., 2006; Yang et al., 2009; Yang et al., 2011), the overall biological activity remained unclear.

In this study we demonstrate the expression and purification of recombinant cell-permeant Nanog in fusion with the TAT. Nanog-TAT translocates to both, cytoplasm and nucleus, and is stable under cell culture conditions. Nanog-TAT promotes mouse ESC proliferation, self-renewal, inhibits endodermal specification in a Stat3-independent manner and promotes pluripotency in the absence of LIF. Our results demonstrate that Nanog protein transduction is a powerful method to study molecular mechanisms underlying pluripotency without genetic manipulation.

Materials and methods

Cell culture

Mouse ESCs were cultured on gelatin-coated dishes in high glucose DMEM (Gibco) with 15% FCS, 1% non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine and 100 μM β-mercaptoethanol and 1000 U/ml LIF (Chemicon) or Advanced DMEM (Gibco) with 5% Fetal calf serum (FCS), 2 mM L-glutamine, 100 μM β-mercaptoethanol and 1000 U/ml LIF. For standardization, differentiated cells were counter-selected from Oct4-GiP ESCs (Oct4-GiP; (Ying et al., 2002)) by adding 1 μg/ml puromycin for at least 1 week before an experiment. Cells were passaged every 2–4 days using TrpLE (Trypsin-like Enzyme, Invitrogen) for dissociation. For aggregation experiments $2 \times 10_5$ Oct4-GiP ESCs were transferred into Petri dishes (Nunc). In all experiments medium was changed daily. The LIF inhibitor hLIF-05 (Vernallis et al., 1997) was produced in COS-7 cells electroporated with the hLIF-05 expression plasmid and the supernatant was tested for its ability to block LIF signaling (Fig. S1). In all LIF-free experiments the inhibitor was used at a concentration that blocked at least 20 U/ml LIF. CV1 fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) containing 10% fetal calf serum, 1% non-essential amino acids, 1% sodium pyruvate and 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Plasmid construction and preparation of recombinant fusion proteins

For Nanog-TAT, PCR fragments encompassing the open reading frames for NLS-Nanog-TAT-H6 flanked by NcoI-XhoI sites were inserted into the NcoI-XhoI sites of pTriEx1.1 (Novagen). The TAT-Nanog plasmid (pTriEx-HTNNanog) was generated by insertion of PCR amplified Nanog cDNA flanked by a HindIII and a nuclear localization sequence (NLS) (N-terminal) and a XhoI site (C-terminal) into HindIII and XhoI sites of pTriEx-HTNCre. For bacterial over-expression, overnight cultures (LB containing 0.5% glucose and 50 μg/ml Carbenicillin) were inoculated with freshly transformed BL21 (DE3) GOLD cells (Stratagene) and cultured at 30°C. Expression cultures (TB containing 0.5% glucose and 100 μg/ml Ampicillin) were grown at 37°C and induced at an OD_{600} of 1.5 with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h. Pellets were resuspended in lysis buffer (2 mM imidazole, 500 mM NaCl, 50 mM Na_2HPO_4, 5 mM Tris, pH 7.8) and lysozyme (Sigma) and benzonase (Novagen) was sequentially added, each for 20 min at 4°C. After a centrifugation step the supernatant was incubated for 1 h with 1 ml Ni-NTA slurry (Qiagen) per liter initial culture. The resin was packed in a gravity column, washed (5 mM imidazole, 500 mM NaCl, 50 mM

Na_2HPO_4, 5 mM Tris, pH 7.8) with 6 bed volumes and eluted (250 mM imidazole, 500 mM NaCl, 50 mM Na_2HPO_4, 5 mM Tris, pH 7.8) with 8 bed volumes. Eluted fractions were successively dialyzed against PBS followed by non-supplemented KnockOut-DMEM (Gibco).

Nanog protein transduction

Culture media for transduction experiments were prepared by mixing Nanog dialysates 1:1 with double supplemented medium (AdvDMEM additionally supplemented with 2% FCS, 1% insulin-transferrin-selenium (ITS) (Invitrogen), 1% non-essential amino acids, 4 mM glutamine and 200 μM β-mercaptoethanol). The mixture was incubated in a water bath for 2 h at 37°C and cleared from precipitations by centrifugation and sterile filtration. FCS was then added to a final concentration of 5%. After this treatment the concentration for Nanog-TAT is approximately 100 nM. Final Nanog-TAT concentration in FCS containing medium was determined via dot blot analysis with Nanog-TAT dialysates serving as standard.

EB and teratoma formation

Oct4-GiP ESCs were cultured for 12 passages without LIF in the presence of Nanog-TAT and the LIF-Inhibitor hLIF-05. Control cells without Nanog-TAT differentiated within the first 4 passages and stopped to proliferate. To remove differentiated cells from Nanog-TAT cultures 1 μg/ml puromycin was added 2 days before cell aggregation. The suspension cultures were kept for 2 weeks in Petri-dishes with LIF-free ES medium. For teratoma formation Nanog-TAT cultures were kept for 11 passages without LIF. LIF was then added to expand the cells and cells were splitted (1:4 or 1:8) every 2–4 days when they reached confluency of around 70–80% for 5 passages. 1×10^6 of these expanded cells was then injected into a severe combined immunodeficiency (SCID)-beige mouse. After 8 weeks, the developed teratoma was isolated and subjected to histological examination with hematoxylin and eosin staining.

Electrophoretic mobility shift assay (EMSA)

Binding of 100 ng recombinant Nanog-TAT protein and 0.5 ng biotinylated oligonucleotides was performed in 20 μl binding buffer (10 mM Tris, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, 1 μg poly(dI-dC), 0.05% NP-40). Binding reactions were separated by native PAGE, subsequently blotted on a positively charged nylon membrane (Roche) and analyzed with the LightShift Chemiluminescent EMSA kit (Pierce) according to manufacturer's specifications. Signals were detected with the CCD camera of a ChemiDoc XRS documentation system (Biorad).
    Oligo sequences:

    5'-TCGACACCCTTCGCCGATTAAGTACTTAAG (sense)
    5'-TCGACTTAAGTACTTAATCGGCGAAGGGTG (antisense)

RT-PCR

RNA from aggregates or somatic cells was isolated with the SV Total RNA Isolation System (Promega) or Trizol (Invitrogen),

respectively, and reverse-transcribed with M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega). PCR reactions were performed using GoTaq (Promega).

    Oct4-F: 5'-AGTGGAAAGCAACTCAGAGGG
    Oct4-R: 5'-GGTTCTCATTGTTGTCGGCTT
    Nanog-F: 5'-AGGGTCTGCTACTGAGATGCTCTG
    Nanog-R: 5'-CAACCACTGGTTTTTCTGCCACCG
    Rex-1-F: 5'-CGTGTAACATACACCATCCG
    Rex-1-R: 5'-GAAATCCTCTTCCAGAATGG
    GATA6-F: 5'-GCAATGCATGCGGTCTCTAC
    GATA6-R: 5'-CTCTTGGTAGCACCAGCTCA
    TTR-F: 5'-CTCACCACAGATGAGAAG
    TTR-R: 5'-GGCTGAGTCTCTCAATTC
    GAPDH-F: 5'-ACGACCCCTTCATTGACCTCAACT
    GAPDH-R: 5'-ATATTTCTCGTGGTTCACACCCAT

Flow cytometry analysis

Flow cytometry analysis was performed with a FACSCalibur (BD Biosciences) and data was analyzed using CellQuest Acqusition software (BD Biosciences) as described in the FACSCalibur System User Guide.

Immunohistochemistry and staining

For immunostaining of embryoid bodies (EBs) paraffin-embedded slices were stained in a DakoCytomation Autostainer (DakoCytomation) according to the manufacturer's instructions using the following antibodies: mouse anti-pan cytokeratin (CK, Chemicon), mouse anti-α-desmin and rabbit anti-human α-1-fetoprotein (AFP, both from DakoCytomation). For visualization the EnVision System (DakoCytomation) with horseradish-coupled secondary antibodies against mouse and rabbit was used and diaminobenzidine (DAB) as chromogenic substrate. For immunostaining cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells were successively treated with the following antibodies: anti-SSEA1 (mouse IgM, 1:80; Hybridoma Bank), biotinylated anti-mouse IgM (1:200, DAKO) in combination with FITC-Avidin, anti-Oct4 (rabbit IgG, 1:400, Santa Cruz Biotechnology, Inc.), Cy3 goat anti-rabbit IgG (1:400, Jackson Immuno Research). Cells were also stained with Sox2 (mouse mAb, 1:100, MAB2018; R&D Systems), Rex-1 (Rabbit pAb, 1:500; ab28141; Abcam) and FoxD3 (mouse mAb, 1:500; ab107248; Abcam). Secondary Alexa488- or Alexa555-labeled antibodies (1:1000; Life technologies, Carlsbad, CA) were used to detect and visualize the primary antibodies.

    For intracellular staining, 0.1% Triton was added to the antibody solutions. DAPI was used for nuclear counterstaining. Alkaline phosphatase (AP) activity was detected with the Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Inc.) or the Alkaline Phosphatase Detection Kit (Chemicon).

Western blotting

SDS-PAGE separated protein samples were blotted on a nitrocellulose membrane. Immunoblots against total Stat3 and Tyrosine 705 phosphorylated (pY-705) Stat3 were performed with rabbit anti-Stat3 (9132; Cell Signaling Technology) and rabbit anti-Phospho-Stat3 (Tyr705) (9131; Cell Signaling Technology), respectively. As secondary antibody

we utilized HRP-linked anti-rabbit IgG (Cell Signaling Technology). Detection was carried out with SuperSignal West Pico Chemiluminescent Substrate (PIERCE).

Results

Purified recombinant Nanog-TAT specifically binds to consensus sequence

To achieve induction of Nanog activity by direct protein delivery rather than genetic manipulation, we intended to generate cell-permeable biologically active Nanog protein. Based on our previous experience with the cell-permeable Cre recombinase H6-TAT-NLS-Cre (Peitz et al., 2002), we decided to construct a fusion gene of Nanog together with the highly basic transduction peptide TAT derived from the HIV transactivator of transcription. Using the same approach, we have generated transducible versions of Oct4 and Sox2 proteins (Bosnali & Edenhofer, 2008), and demonstrated that protein transduction could replace invasive viral transduction approach to generate induced puripotent stem (iPS) (Thier et al., 2010; Thier et al., 2012a) and induced neural stem (iNS) (Thier et al., 2012b) cells. To identify a recombinant Nanog version with superior biochemical properties, we screened several variants of Nanog fusion proteins with different tag (NLS, TAT, His) combinations at both N- and C-terminal ends of Nanog (data not shown). Out of those variants, we determined NLS-Nanog-TAT-His (designated as Nanog-TAT hereafter) exhibiting the best combination of yield, purity, stability at cell culture conditions and its ability to translocate to the nucleus, and was therefore chosen for further analysis. Nanog-TAT is soluble under cell culture conditions and is able to translocate to both, cytoplasm and nucleus, of more than 95% of cultured mammalian cells when added to the cell culture media (Fig. S2 A). Immunostaining of Nanog-TAT treated mammalian cells revealed the presence of Nanog protein in the nucleus (Fig. S2 A). However, it also showed that cellular uptake of Nanog-TAT results in persistent endosomal entrapment in the periphery of the nucleus of all cells (Fig. S2 A). Nanog-TAT concentration reduced to approximately 50% within 48 h in cell-free conditions and 24 h when co-incubated with cultured cells (Fig. S2 B). Based on these observations we decided to replace Nanog-TAT-supplemented media every 24 h in cell culture assays.

To assess the biological activity of Nanog-TAT *in vitro* we performed an EMSA employing an oligonucleotide containing a Nanog binding DNA sequence (Pan & Pei, 2005). Specificity of the binding was analyzed by addition of excess competitor, i.e. unlabeled DNA. Recombinant Nanog-TAT protein induced a shift of the labeled DNA that could be blocked by unlabeled oligonucleotides (Fig. 1A) confirming the specificity of the binding of Nanog-TAT to the DNA.

Nanog-TAT promotes ESC proliferation and self-renewal

To analyze the functionality of Nanog-TAT in ESC proliferation, we used 3 different murine ESC lines namely, Bruce-4 (C57BL/6), Oct4-GiP (129/MF1) and RF8 (129/TerSv) and cultured them in the absence of LIF but in the absence or presence of Nanog-TAT for at least 14 to 17 days. To rule out stimulation from endogenous LIF, we employed the LIF

inhibitor hLIF-05 which has been reported to block the activity of all known LIF receptor ligands (Chambers et al., 2003; Vernallis et al., 1997) (Fig. S1). At each passage, the cells were counted and the cumulative numbers of cells were determined. Cells were passaged at the ratio 1:4 or 1:8 when they reached the confluency of around 70–80%. After removal of LIF, untreated cells rapidly differentiated and ceased to proliferate within 2–4 passages. In contrast, Nanog-TAT-treated cells kept proliferating (Figs. 1 B, C, D) for more than 20 passages (data not shown). Withdrawal of Nanog-TAT from these Nanog-TAT-treated LIF-free cultured ESCs resulted in complete differentiation within few passages. Moreover, we observed an enhanced proliferation upon combined application of LIF and Nanog-TAT in Oct4-GiP ESCs (Fig. 1E).

We next examined the biological activity of Nanog-TAT on ESCs to modulate ESC self-renewal. For this, we cultured Bruce-4 (C57BL/6) ESCs in the absence of LIF on gelatinized plates. Under these conditions control cells rapidly differentiated within few days. We found no undifferentiated colony after 3 days of LIF-withdrawal as judged by morphology (Fig. 2A). Even in the presence of LIF we observed a large fraction of spontaneously differentiated cells predominantly adopting a primitive endoderm-like morphology (Fig. 2A). In contrast, Nanog-TAT-treated cells exhibited significantly reduced differentiation in the absence of LIF. Moreover, both LIF and Nanog-TAT nearly completely inhibited spontaneous differentiation and induced growth of colonies exhibiting morphologies resembling ESCs cultured on feeder layers (Fig. 2A). AP staining after 3 days further confirmed that Nanog-TAT-treated cultures maintained an undifferentiated state of these cells (Fig. 2B). In order to quantify the effect of Nanog-TAT on murine ESCs AP stained cells were classified into three categories. The first category, termed 'undifferentiated', encompassed compact colonies with sharp edges strongly staining for AP. The second category comprised partially compact colonies, which lost the sharp edges and stained partially for AP. This category was named 'mixed'. The third category was named 'differentiated' and contained colonies, which already lost the compact, three-dimensional structure and did not stain for AP. In the presence of LIF the amount of undifferentiated ESC colonies increases from 60% to over 95%. In the presence of LIF, differentiation of ESCs is inhibited. In the presence of Nanog-TAT, 25% of mixed colonies are still observed, whereas the complete absence of Nanog-TAT leads to full differentiation (Fig. 2C). We observed similar observations with Oct4-GiP ESCs when these cells were cultured either in the presence or absence of the cytokine LIF together with 100 nM of Nanog-TAT (Fig. 2D). When exposed to both, Nanog-TAT and LIF, both ESC lines formed tightly compacted and loosely attached colonies and the cultures were nearly devoid of any differentiated colonies (Figs. 2 A, D). In order to determine putative differences in self-renewal upon Nanog-TAT transduction we performed a clonogenicity assay in which ESCs cultured for 4 passages with Nanog-TAT with or without LIF, were clonally plated in normal ESC medium and enriched for Oct4-positve cells by puromycin selection. Under LIF-withdrawal conditions no single colony was found in the untreated control cells, whereas a small percentage of Nanog-TAT-treated cells gave rise to colonies (1.5 ± 0.2%). LIF-stimulation was more potent resulting in 12.3 ± 0.8% colony formation. However, additional Nanog-TAT
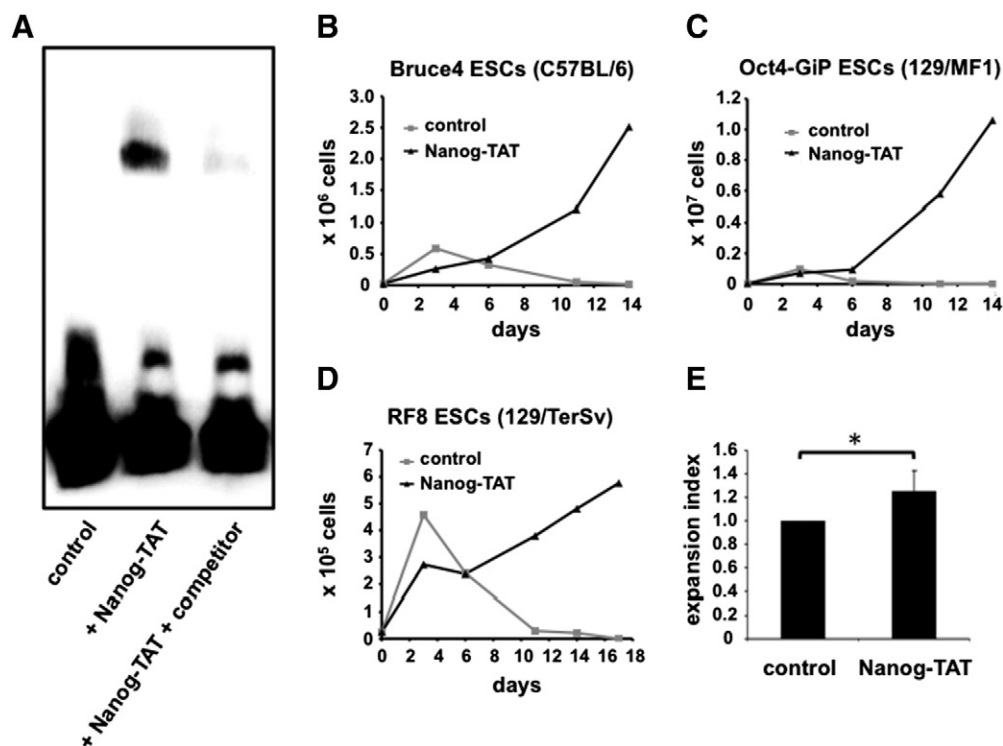
Figure 1 Purified recombinant Nanog-TAT specifically binds to consensus DNA sequence and enhances proliferation of mouse ESCs. A) Nanog-TAT was subjected to an EMSA employing a specific Nanog consensus DNA sequence (Pan & Pei, 2005). A shift of the protein–DNA complex is observed. The shift can be prevented by the addition of excess unlabeled DNA (competitor). The binding reaction containing 100 ng of recombinant protein was subjected to a native PAGE exhibiting 5% (bis)acrylamide. After the gel run was completed the protein–DNA complexes were transferred onto a membrane and the shift was detected using streptavidin–HRP conjugates. B–D) ESCs were cultured in the presence of the LIF-inhibitor hLIF-05 (Vernallis et al., 1997) to block LIF signaling and with or without Nanog-TAT. Cumulative cell numbers at each passage were determined for at least 14 days for Bruce-4 (C57BL/6) (B), Oct4-GiP (C) and at least 17 days for RF8 (D) ESCs. Cells were splitted at a ratio of 1:4 or 1:8 on day 3, 6, 11 and 14 for (B,C) and 3, 6, 11, 14 and 17 days for (D) when they reached the confluency of around 70–80%. E) Oct4-GiP ESCs cultured in the presence of LIF with or without Nanog-TAT. Cells were cultured for 2 days and then counted. The relative cell expansion within this time period is presented. Statistical analysis to calculate p-value was carried out using a two-tailed test; * p value <0.05. Data are means +/− s.e.m. n = 5.

treatment induced a further increase in cloning efficiency (15.5 ± 0.8 %) (Fig. 2E).

Nanog-TAT-treated ESCs maintain pluripotency

We next monitored the Oct4 transcriptional activity of Nanog-TAT-treated cells by flow cytometry analysis employing the Oct4-GiP ESC line. Oct4-GiP ESCs serve as a straightforward model system since GFP fluorescence and puromycin resistance is driven by regulatory sequences of the mouse *Pou5f1* (Oct4) gene and thus the transgene is expressed only in pluripotent and germline cells (Yeom et al., 1996; Ying et al., 2002). For standardization, we counter selected differentiated cells by culturing Oct4-GiP ESCs at least 1 week in the presence of puromycin prior to experiments. Puromycin-selected Oct4-GiP ESCs exhibit a bright GFP expression resulting in a sharp peak of GFP-high-expressing cells (99%) (Fig. 3A, middle). Withdrawal of LIF results in broadening of the peak and concomitant decrease of GFP-high-expressing cells to about 60% (Fig. 3A, bottom). After three passages in the absence of LIF the control cells ceased to proliferate and eluded further analysis. Nanog-TAT-treated

cells in contrast could be further proliferated and exhibited a distinct pattern of GFP fluorescence (Fig. 3A, top). Low passage numbers of Nanog-TAT-treated cells displayed heterogeneous GFP-expression containing a modest proportion of GFP-high-expressing cells (47%). However, after continuous passaging in the presence of Nanog-TAT the population of GFP-high-expressing cells constantly increased forming a second distinct population of cells. After seven passages GFP expressing cells reached to almost 90% of all Nanog-TAT-treated cells (Fig. 3A). Application of both LIF and Nanog-TAT resulted in a high fluorescence intensity similar to that of puromycin-selected ESCs (Fig. 3B).

To further characterize the Nanog-TAT-treated ESCs we analyzed pluripotency-associated markers after seven to eight passages in the absence of LIF. Immunocytochemical analysis revealed that Nanog-TAT-treated cells stained positive for AP activity, SSEA-1 and Oct4 (Fig. 3C) and also stained positive for other pluripotency-associated markers Sox2, Rex-1 and FoxD3 (Fig. S3). Cells cultured in the presence of Nanog-TAT and LIF also stained positive for all the pluripotency-associated markers (Fig. S3). Next we examined whether Nanog-TAT-treated cells were still pluripotent by assaying their ability to differentiate into the three germ
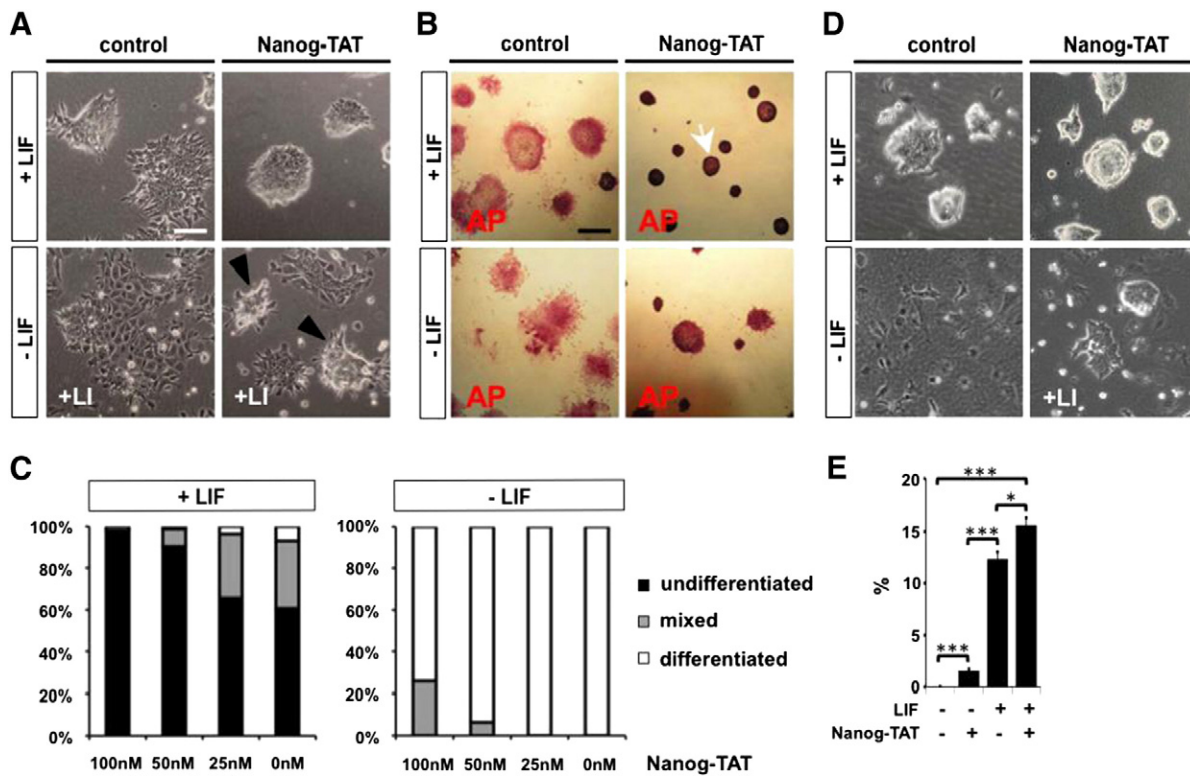
Figure 2 Nanog-TAT sustains pluripotency in ESCs in the absence of LIF. A) Nanog-TAT suppresses spontaneous ESC differentiation of Bruce-4 (C57BL/6) ESCs. ESCs were cultured on gelatin-coated dishes with 100 nM Nanog-TAT for 3 days in the presence or absence of LIF as indicated. LIF-withdrawn cultures additionally contained the LIF inhibitor hLIF-05, since LIF agonistic activity occurs upon spontaneous differentiation in a paracrine manner. Over a 3-day period cells cultured without LIF but in the presence of Nanog-TAT started to differentiate but still showed colonies with a partially ES-like morphology (black arrowhead). ESCs cultured with control medium lacking both, LIF and Nanog-TAT, completely differentiated into flat cells forming a monolayer. LIF: leukemia inhibitory factor, LI: LIF inhibitor; Scale bar: 50 μm. B) After 3 days treatment with 100 nM Nanog-TAT, Bruce-4 (C57BL/6) ESCs were fixed and stained for AP. In the presence of both, LIF and Nanog-TAT, cultures contained almost exclusively undifferentiated ES-like colonies, whereas ESCs cultured in the absence of LIF but in the presence of Nanog-TAT contained mixed colonies as well. AP: alkaline phosphatase; Scale bar: 100 μm. C) A quantification of the morphological scoring of Bruce-4 (C57BL/6) ESC colonies cultured for 5 days in different concentrations of Nanog-TAT in the presence or absence of LIF. For classification see text. D) Nanog-TAT suppressed spontaneous ESC differentiation of Oct4-GiP ESCs (Ying et al., 2002). ESCs were cultured on gelatin-coated dishes with Nanog-TAT for 3 passages in the presence or absence of LIF as indicated. LIF-withdrawn cultures contained the LIF inhibitor hLIF-05. Cells were splitted at a ratio of 1:4 or 1:8 every 2–4 days when they reached the confluency of 70–80%. After 3 passages, Oct4-GiP ESCs cultured in the absence of LIF and in the presence of 100 nM of Nanog-TAT still exhibit mixed colonies. E) Nanog-TAT treatment increased clonogenicity of Oct4-GiP ESCs. ESCs were cultured for 4 passages in the presence or absence of LIF and 100 nM Nanog-TAT. Cells were splitted at a ratio of 1:4 or 1:8 every 2–4 days when they reached the confluency of 70–80%. Cells were then clonally plated in ESC medium with LIF and puromycin for 6 days and originated colonies were counted. Statistical analysis to calculate p-value was carried out using a two-tailed test; * p value <0.05; *** p value <0.001. Data are means +/− s.e.m. $n = 3$.

layers *in vitro*. For that, Nanog-TAT-treated ESCs cultured for 12 passages in the absence of LIF were aggregated to form EBs. After 2 weeks of culture, EBs were analyzed exhibiting widespread differentiation as determined by staining against the germ layer markers *α-1-fetoprotein* (endoderm), *desmin* (mesoderm) and *cytokeratin* (ectoderm) (Fig. 3D). Moreover we assessed the capacity of Nanog-TAT-treated cells to induce teratoma formation. For that, Nanog-TAT cultures kept for 11 passages without LIF and 5 more passages in normal ESC medium were injected into SCID-beige mice resulting in teratoma formation displaying various differentiated tissues (Fig. 3E). Finally we analyzed the developmental potential of Nanog-TAT-treated cells by blastocyst injection. For that, cells that had been cultured with Nanog-TAT for 8 passages in the absence of LIF were expanded in normal ESC medium for 5

more passages and subsequently injected into blastocysts. PCR analysis of offspring revealed presence of the GFP-transgene in various tissues of the living offspring (data not shown). For teratoma as well as blastocyst injection we expanded the cells first in Nanog-TAT for 11 and 8 passages, respectively, in absence of LIF, and then in normal ESC medium. We did this for technical reasons since we observed that Nanog-TAT protein tended to clog the injection needle yielding overall low cell survival.

Nanog-TAT suppresses endoderm specification in ESCs

In order to examine whether Nanog-TAT maintains pluripotency autonomously from the JAK/Stat pathway we employed chemical inhibition to the JAK/Stat signaling pathway
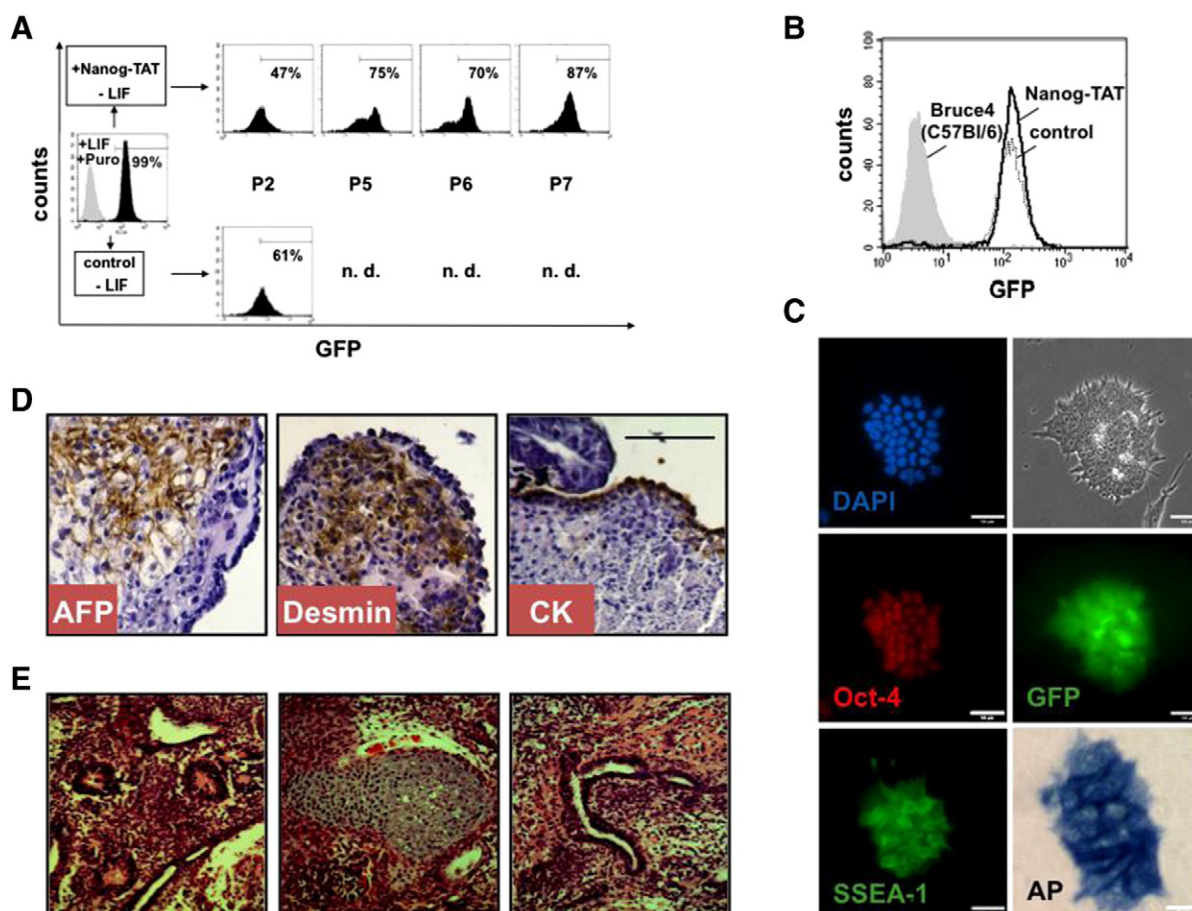
Figure 3 Nanog-TAT sustains pluripotency in ESCs on further expansion in the absence of LIF. A) Flow cytometry analysis of Oct4-GiP ESCs in response to Nanog-TAT. Even after 7 passages in the absence of LIF, Nanog-TAT induces a proliferating population of GFP-positive cells (top panel). Untreated cells completely differentiate to non-proliferating cultures within 3 passages, which could not be further analyzed by flow cytometry (bottom panel). Grey: wildtype Bruce-4 (C57BL/6) ESCs; n. d., not determined. B) Flow cytometry analysis of Oct4-GiP ESCs cultured for 4 passages in the presence of LIF with (black line) or without Nanog-TAT (dotted line). Grey, Bruce-4 (C57BL/6) control cells. C) Immunocytochemical analysis of Oct4-GiP ESCs cultured in the absence of LIF with Nanog-TAT at passage 8. Colonies appeared monolayered and pluripotent state is maintained by Nanog-TAT as judged by staining against pluripotency-associated markers like Oct4, SSEA-1, alkaline phosphatase (AP). Moreover, native GFP fluorescence yielding from the Oct4 promoter-driven GFP reporter construct, is shown. Scale bar: 50 μM (except AP, 25 μM). D) Immunohistochemical analysis of embryoid bodies (EBs) derived from Oct4-GiP ESCs that had been cultured for 12 passages in the absence of LIF but presence of Nanog-TAT protein. Sections of EBs shown stained for markers of all the three germ layers: AFP, α-1-fetoprotein; Desmin; CK, cytokeratin. Scale bar: 200 μm. E) Histological analysis of a teratoma obtained after injection of Nanog-TAT-treated Oct4-GiP ESCs into SCID-beige mice. Before injection Nanog-TAT-treated ESCs were cultured in the absence of LIF for 11 passages and expanded in normal ESC medium for further 5 passages. Cells were splitted at a ratio of 1:4 or 1:8 every 2—4 days when they reached the confluency of 70—80%. Various tissues including primitive neuroepithelium, cartilage tissue, and adenoid tissue were observed. Scale bar: 200 μm.

(Thompson et al., 2002). The JAK inhibitor induced massive differentiation in untreated control cells whereas in Nanog-TAT-treated cultures more than 50% of the colonies remained undifferentiated. Even in the presence of a threefold higher inhibitor concentration, Nanog-TAT was still able to maintain undifferentiated colonies (Fig. 4A). To rule out unspecific stimulation of the JAK/Stat pathway by recombinant Nanog-TAT preparations we assayed the phosphorylation status of Stat3. Immunoblots of ESC lysates against phosphorylated Tyrosine 705 of Stat3 showed no enhanced phosphorylation in the presence of Nanog-TAT (Fig. 4B).

It has been shown that overexpression of Nanog in aggregated ESCs blocks differentiation into primitive endoderm (Hamazaki et al., 2004). Therefore we asked whether Nanog-TAT

influences the differentiation potential of ESC aggregates in a similar manner. To this end, Oct4-GiP ESCs were aggregated and cultured for 4 days in the presence or absence of Nanog-TAT and LIF, respectively, to form EBs. Nanog-TAT-treated cultures exhibit less EBs with defined, sharp borders as compared to the control EBs (Fig. 4C). We assessed the gene expression patterns of pluripotency markers and early endodermal lineage specification genes by RT-PCR. Control ESCs upon EB formation showed reduced expression of pluripotency markers and upregulation of endodermal markers (Fig. 4D; (Coronado et al., 2013)). Upon Nanog-TAT treatment and in the absence of LIF we observed maintenance of pluripotency markers Rex-1, Oct4 as well as Nanog (Fig. 4D). In contrast, endodermal markers such as GATA6

and transthyretin were found to be downregulated in Nanog-TAT-treated cells (Fig. 4D). In the presence of LIF neither GATA6 nor transthyretin-specific RNA was detected irrespective of the presence of Nanog-TAT (Fig. 4D). Transcription of the pluripotency-associated genes Rex-1, Oct4 and Nanog was efficiently induced by LIF; however, Nanog-TAT induced a further increase in transcriptional activity of these pluripotency genes (Fig. 4D).

Discussion

The robust expression and purification of stable and biologically active eukaryotic proteins represent a bottleneck of protein transduction technology (Pan et al., 2010; Patsch & Edenhofer, 2007; Yang et al., 2011). In this study, we demonstrate recombinant expression and purification of a cell-permeant version of biologically active Nanog protein. Although several studies have reported purification of recombinant Nanog from different species including mouse (Jauch et al., 2008; Loh et al., 2006), human (Ha et al., 2009; Yang

et al., 2009; Yang et al., 2011), *Luxi* cattle (Hu et al., 2012) and chicken (Yu et al., 2013) the biological functionality, however, remained unclear. Recombinant Nanog proteins have been reported to bind to a consensus DNA sequence using EMSA or EMSA-like technique (Ha et al., 2009; Jauch et al., 2008; Loh et al., 2006; Yang et al., 2009; Yang et al., 2011). One study employing CPP-fused Nanog protein further reports that recombinant Nanog could translocate to the nucleus (Yang et al., 2009); however, no further data was shown to demonstrate the functionality of the protein.

Our study shows that our purified recombinant mouse Nanog protein from *E. coli* is stable at cell culture conditions, translocates to both cytoplasm and nucleus and is biologically active. Proof of functionality is based on i) the DNA-binding ability to a well-described consensus sequence, ii) enhancement of proliferation and iii) supporting self-renewal of mouse ESCs in the absence of LIF but in the presence of LIF inhibitor to abrogate residual LIF signaling. DNA binding ability insufficiently determines the functionality of pluripotency-associated transcription factors. Yang et al. reported that although recombinant Oct4 and Sox2 proteins bind to the
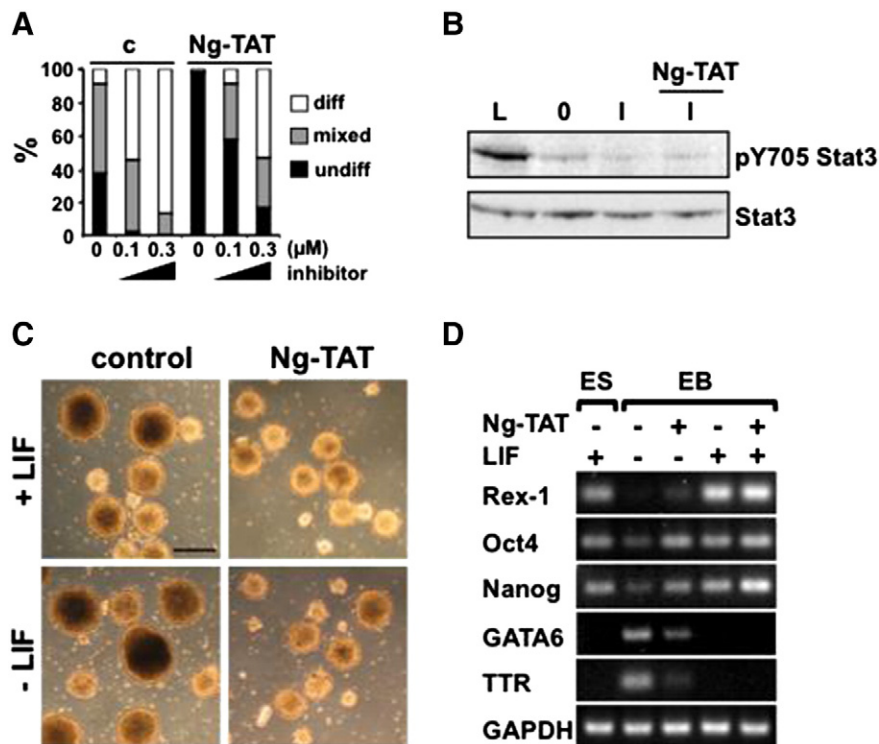

Figure 4 Nanog-TAT inhibits endodermal differentiation in a Stat3-independent manner. A) Self-renewal is sustained by Nanog-TAT even in the presence of a JAK inhibitor. Oct4-GiP ESCs were cultured with or without Nanog-TAT and with 0.1 and 0.3 μM of JAK-inhibitor at clonal density. After 5 days cells were stained for AP and scored by classifying into three categories: undifferentiated, mixed and fully differentiated as described in the text. B) Recombinant Nanog-TAT does not induce Stat3 phosphorylation. Tyrosine 705 of Stat3 was assessed for its phosphorylation status by immunoblotting in response to Nanog-TAT. L, LIF; 0, without LIF; I, inhibitor hLIF-05; Ng-T, Nanog-TAT; pY705, Tyrosine 705 phosphorylated Stat3. C) Morphology of aggregated Oct4-GiP ESCs, cultured in suspension for 4 days in the presence or absence of Nanog-TAT and LIF. Scale bar, 200 μm. D) Nanog-TAT inhibited endodermal specification. RT-PCR analysis of ESC cultured in both monolayer and in suspension for 4 days in the presence or absence of Nanog-TAT and LIF (see (C)). RT-PCR analysis of ESCs cultured in monolayer is to demonstrate that the starting population of ESCs used for aggregate formation under different conditions did express pluripotency markers and showed no expression of endodermal differentiation markers investigated. EB, embryoid bodies; Ng-T, Nanog-TAT; TTR, transthyretin.

consensus DNA sequence, the proteins are not fully biologically active as they fail to regulate most of the target genes investigated (Yang et al., 2011). Further, we did not observe any mixed or undifferentiated colonies on the basis of morphological analysis in the absence of LIF and Nanog-TAT, but we did observe mixed colonies upon treatment with Nanog-TAT and in the absence of LIF which were AP positive. Surprisingly, we did not observe any undifferentiated colonies in the latter condition although the translocation of Nanog into the cells was high. The reason for this could be that the amount of Nanog protein was not ample enough to generate undifferentiated colonies but was sufficient to enhance proliferation and self-renewal of subpopulations in ESCs. These mixed colonies expressed pluripotency-associated markers and remained pluripotent in the absence of LIF when compared to cells cultured in the absence of LIF and Nanog-TAT. The fact that for technical reasons we had to culture Nanog-TAT-treated cells prior to teratoma as well as blastocyst injection in normal ESC medium raises the possibility of a selective outgrowth of a rare subpopulation of cells that may be responsible for formation of teratoma and chimera. Although we cannot rule out, we consider this a rather unlikely scenario since before that Nanog-TAT-treated cells were cultured for up to 11 passages in the absence of LIF and in the presence of LIF inhibitor. As demonstrated, control cells expanded in the absence of LIF and Nanog and in the presence of LIF inhibitor could not maintain their self-renewal and pluripotency characteristics, ceased to proliferate and differentiated within 2-4 passages only. These results suggest that continuous application of Nanog-TAT kept the pluripotency network active and maintained self-renewal as well as pluripotency of these cells in the absence of LIF. In addition, our data indicates that Nanog suppresses endoderm differentiation indicating that Nanog exerts its intracellular function in a Stat3-independent manner. We found endodermal genes such as GATA6 and transthyretin downregulated upon Nanog gain-of-function by protein transduction. Thus, Nanog protein transduction data presented here is in line with other studies reporting that Nanog suppresses differentiation of ESCs by inhibiting endodermal specification (Chambers et al., 2003; Darr et al., 2006; Hamazaki et al., 2004; Mitsui et al., 2003). Our finding that Nanog-TAT not only increases proliferation but also clonogenicity of ESCs might build a basis for enhanced suspension culture as a prerequisite for biomedical applications (Chen et al., 2012; Singh et al., 2010). In conclusion, our results readily recapitulate previously published reports employing conventional genetic Nanog overexpression (Chambers et al., 2003; Mitsui et al., 2003) to enhance proliferation and self-renewal and maintenance of pluripotency of ESCs. This confirms that our recombinant Nanog protein from *E. coli* is biologically active and provides an effective model to study the molecular control of pluripotency by modulating stem cell properties in target cells by non-genetic means.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2014.02.006.

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