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NANOG in cancer stem cells and tumor development: An update and outstanding questions

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Abstract

The homeobox domain transcription factor NANOG, a key regulator of embryonic development and cellular reprogramming, has been reported to be broadly expressed in human cancers. Functional studies have provided strong evidence that NANOG possesses protumorigenic attributes. In addition to promoting self-renewal and long-term proliferative potential of stem-like cancer cells, NANOG-mediated oncogenic reprogramming may underlie clinical manifestations of malignant disease. In this review, we examine the molecular origin, expression, biological activities and mechanisms of action of NANOG in various malignancies. We also consider clinical implications such as correlations between NANOG expression and cancer prognosis and/or response to therapy. We surmise that NANOG potentiates the molecular circuitry of tumorigenesis, and thus may represent a novel therapeutic target or biomarker for the diagnosis, prognosis and treatment outcome of cancer. Finally, we present critical pending questions relating NANOG to cancer stem cells and tumor development.

Keywords

NANOG; cancer stem cells; tumor development; self-renewal

Introduction

The master transcription factor NANOG confers self-renewal and ground state pluripotency to embryonic and reprogrammed cells. NANOG regulates embryonic stem cell (ESC) pluripotency and cell-fate specification through complex interactions with a myriad of factors, including OCT4, SOX2, and KLF4 [1]. Cellular reprogramming of somatic cells to induced pluripotent stem (iPS) cells via the forced expression of ESC self-renewal factors, including NANOG, has unveiled the potency of aberrant expression of developmental programs [2-4]. Unlike engineered reprogramming for the purposes of regenerative

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medicine, uncontrolled and spontaneous acquisition of stem cell programs has profound pathophysiological implications, particularly in regards to cancer.

Oncogenic transformation mirrors cellular reprogramming. The acquisition of developmental programs has been shown to correlate with tumorigenic cells that possess unlimited self-renewal (i.e., cancer stem cells; CSCs). For example, SOX2 has been detected in squamous cell carcinoma and non-small cell lung cancer and SOX2 levels correlate with CSC attributes in glioblastoma [5], breast cancer [6], and Ewing's sarcoma [7]. NANOG, the focus of this review, has been broadly detected in primary human tumors of diverse origin, including those arising in the brain, breast, esophagus, colon, ovary and prostate, among others.

Functional studies have provided compelling evidence that NANOG plays a vital role in malignant disease, correlating with cell proliferation and various malevolent properties such as clonogenic growth, tumorigenicity, invasiveness, and therapeutic resistance. Among the earliest work, ectopic expression of mouse and human NANOG in NIH3T3 cells, respectively, promoted entry into S-phase and foci formation in soft agar [8, 9]. Furthermore, *Rb1*$^{-/-}$ mouse fibroblasts cultured under sphere-forming conditions upregulated *Nanog* mRNA concurrently with reprogramming to a CSC phenotype, including the acquisition of a cytotoxic-drug effluxing Side Population (SP), increased expression of stem cell (and CSC) markers (e.g., CD44 and CD133) and tumor-initiating cell capabilities upon transplantation [10]. Overexpression of NANOG in immortalized but benign HEK-293 cells promoted malignant transformation, accompanied by enhanced proliferation, anchorage-independent growth in soft agar and, importantly, tumor formation in athymic nude mice [11]. Taken together, these findings provide evidence that NANOG possesses oncogenic potential.

Despite this evidence, however, NANOG's role in cancer is somewhat enigmatic, as NANOG does not appear to function as a classical oncogene. For example, unlike transgenic mouse models in which Oct4 overexpression caused dysplastic and aggressive tumor-like growths in a remarkably short time frame in the skin and intestinal epithelia [12], Nanog overexpression in two similar doxycycline-inducible transgenic mouse models induced only modest hyperplastic outgrowths in the intestinal and colonic epithelium [13] and stratified epithelium of the forestomach and esophagus [14]. In a parallel study, we reported human NANOG overexpression in the K14-compartment in transgenic mice to be insufficient to elicit tumor development, despite signs of skin and lingual hyperplasia in early life [15]. In another transgenic mouse model overexpressing murine Nanog in adult mammary tissues, Nanog alone was also found to be insufficient to elicit tumor formation, even after prolonged expression [16]. However, when co-expressed with Wnt-1, Nanog enhanced mammary tumorigenesis and metastasis [16]. Consequently, NANOG seems to function as a cooperating or potentiating protumorigenic molecule in the appropriate context.

NANOG origins in cancer: biochemical and regulatory implications

Elucidating the origins of *NANOG* transcripts in human cells has been confounded by the presence of multiple and, in some cases, highly similar paralogs, as a consequence of

retrotransposition [17]. Recently, the location and genomic organization of all human *NANOG* loci have been clarified, including the evolutionary source of *NANOG* (referred to as *NANOG1*) with classical intron/exon structure and located on chromosome 12, a tandem duplication referred to as *NANOG2* (aka *NANOGP1*) and 9 other intronless retrogene derivatives [18, 19]. *NANOGP8*, located on chromosome 15, is the only retrogene with an intact open reading frame (**Fig. 1A**), with the remainder considered pseudogenes as they harbor indels (resulting in frame shifts) and/or deleterious truncations [15, 20].

It is currently unknown to what degree the three full-length NANOG protein variants potentially encoded by *NANOG1*, *NANOG2* and *NANOGP8* loci possess unique biochemical activities or biological properties. Although *NANOG2* mRNA is quite distinct from that derived from either of the other 2 loci due to alternative 5' exon usage (encoding a shorter NANOG2 protein with an alternative N-terminus), NANOG1 and NANOGP8 only differ by a single conserved amino acid (aa), with both encoding proteins of 305 aa and NANOGP8 harboring a Q253H substitution in the C-terminal transactivation domain (**Fig. 1A**). At this moment, there are only a few reliable strategies to distinguish between the two mRNA species (**Fig. 1A-B**). One strategy relies on direct sequencing of the open reading frame to detect the 759G>C that results in the Q253H aa change in NANOGP8 (**Fig. 1A**). Of note, a 22-bp deletion in the 3'UTR is polymorphic in *NANOG1* and monomorphic in *NANOGP8*, and thus should not be used as a definitive feature to distinguish between transcripts [21]. Another distinguishing strategy takes advantage of the synonymous 144G>A in *NANOGP8*, a nucleotide change detectable by virtue of RFLP (restriction fragment length polymorphism) due to the introduction of an AlwNI cut site (**Fig. 1A**). Subsequent gel electrophoresis banding patterns of digested PCR products implicate NANOG origins, such that *NANOG1* gives rise to undigestible fragments and *NANOGP8* is subject to AlwNI fragmentation (as illustrated in **Fig. 1B**; refer to **[22]**). Finally, there is a stretch of ~18 bp sequence at the 5'-untranslated region (5'-UTR) following the transcription start site (TSS) that is unique to *NANOG1* or *NANOGP8* mRNA, which can theoretically be exploited to distinguish between the two transcripts (**Fig. 1**).

Using these strategies, the primary source of NANOG in cancer has been reported by numerous groups to be the retrotransposed locus *NANOGP8* [21-27]. This preferential expression may be due to the fact that the *NANOG1* locus is transcriptionally silenced during cell-fate specification early in embryogenesis. Nevertheless, *NANOG1* has been reported to be the origin of NANOG in certain cancer types, such as hepatocellular carcinoma [28] and some colorectal cancers [23]. It should be born in mind that as a retrogene derivative, *NANOGP8* possesses distinct promoter elements relative to *NANOG1*. For example, TRANSFAC analyses of the *NANOGP8* promoter *in silico* fail to identify OCT4/SOX2 elements present in the promoter of *NANOG1* (**Fig. 1C,** Jeter et al, unpublished observations). Thus, as a consequence of *cis*-element differences, trans factors regulating *NANOG* mRNA transcriptional activation or repression in cancer cells will vary depending on the locus-of-origin for *NANOG* expression and the cellular context.

Structurally, NANOG1 protein has an N-terminal "interference" domain, a homeodomain essential for DNA binding, and a C-terminal transactivation domain with a tryptophan-rich region involved in NANOG dimerization (**Fig. 1D**). Of note, enforced expression of the

murine Nanog1 dimer, but not the monomer, has been found to functionally replace wild-type Nanog to sustain cytokine-independent self-renewal of mouse ESCs [29]. Although both NANOG1 and NANOGP8 have been demonstrated to have similar reprogramming capabilities [30] (and thus may have overlapping roles in promoting malignant disease), some biochemical distinctions between the two proteins have been reported [31]. It will be very interesting to determine both the shared and potentially distinct biological functions between NANOG1 and NANOGP8.

NANOG as a regulator of proliferation and chromatin remodeling in ESCs

The balance between self-renewal and differentiation in dividing stem (and progenitor) cells is fundamental to development, tissue homeostasis and tumorigenesis. Both mouse and human ESCs proliferate rapidly, largely by virtue of an abbreviated G1 phase in the pluripotent state [32, 33] Considering that NANOG1 overexpression increases cell proliferation and shortens the G1-S transition in human ESCs, NANOG appears to function as a vital transcription factor regulating cell-cycle progression in ESCs [34]. Chromatin immunoprecipitation combined with reporter-based transfection assays have demonstrated that NANOG1 can bind to the regulatory regions of *CDK6* and *CDC25A* genes, thereby positively regulating their transcription. The effects of NANOG1 overexpression on S-phase entry could be mitigated by the siRNA-mediated down-regulation of *CDK6* or *CDC25A* transcripts (and resultant proteins) alone, suggesting that CDK6 and CDC25A are downstream cell cycle effectors of NANOG1 during the G1 to S transition in human ESCs [34].

Using fluorescent, ubiquitin-sensitive cell cycle reporters, human ESCs were recently shown to be particularly susceptible to differentiation in G1, such that altering the cell cycle of ESCs facilitates changes in cell specification [35]. Although these data convincingly demonstrate that differentiation and the cell cycle are intimately linked in ESCs, whether a dividing ESC remains pluripotent or gives rise to differentiated progeny is dictated at the molecular level. In addition to the regulatory activities of master transcription factors, cell state transitions during embryogenesis are governed by the epigenetic landscape in a given cell. Thus, the interplay of pluripotency-maintaining transcription factors together with chromatin modifiers collaboratively represses differentiation and maintains the primitive and renewing stem cell state. Endogenous murine Nanog1 and Oct-4 protein complexes have been found to interact with each other and associate with proteins from multiple transcriptional repression complexes, including the NuRD, Sin3A and Pml complexes [36, 37]. Although immunoprecipitation failed to detect Mbd3 among the components of the nuclear remodeling and histone deacetylase complex (e.g., Mta1, Mta2, Hdac1, etc.) pulled down with Nanog1 in murine ESCs [37], overexpression of Mbd3–the essential scaffold of the NuRD complex– has been found to augment Nanog-mediated reprogramming of murine MEFs [36]. Nevertheless, even in $Mbd3^{-/-}$ mouse ES cells, Nanog1 and Oct-4 can communicate with distinct repression complexes (termed Nanog and Oct4 associated deacetylase [NODE]) to control gene transcription and ESC differentiation [37]. In human ESCs, NANOG co-occupies and represses developmental genes in concert with lysine specific demethylase 1 (LSD1), a component of NuRD and the transcriptional repression complex coREST [38]. The NANOG/OCT4/SOX2 interactome is also thought to encompass

members of the Polycomb group (PcG) family of transcriptional repressors and SetDB1 (reviewed in [39]). In addition to this myriad of chromatin remodeling complexes associated with transcriptional repression, NANOG has also been found to associate with transcriptional activators such as components of the SWI/SNF nucleosome remodeling complex and Wdr5 of the trithorax group [37, 40]. In mesenchymal stem cells (MSCs), NANOG and OCT4 transactivate expression of the DNA methyltransferase DNMT1, which subsequently downregulates the cyclin-dependent kinase inhibitors p16 and p21 and represses differentiation genes to maintain the self-renewal of MSCs [41]. Furthermore, more recent research has shown that members of the genomic methylation regulatory ten-eleven translocation (TET) family, specifically the methylcytosine hydroxylases TET1 and TET2, are recruited by Nanog1 to activate the expression of pluripotency genes and fulfill somatic cell reprogramming [42, 43].

Conceptual overview of the pro-tumorigenic effects of NANOG

Oncomine analysis reveals that *NANOG* mRNA is elevated in many types of cancer relative to matched benign tissues (**Fig. 2A**). Also, as we shall describe below, immunohistochemistry (IHC) shows that NANOG protein is heterogeneously expressed in both the nucleus and cytoplasm in a wide variety of primary human patient tumors. The presence of NANOG in neoplastic cells suggests a functional role for this molecule in tumor development or disease progression. However, given NANOG's apparent lack of direct oncogenic activity in transgenic animal models (13-16), how does this pluripotency factor execute its protumorigenic properties? Compelling evidence suggests that NANOG may foster CSC traits by imbuing subsets of cancer cells with self-renewal potential, thereby bolstering the immortality of the entire tumor population. *FIRST*, NANOG mRNA and protein are enriched in many CSC populations such as the $CD44^+$ breast [44], prostate [25] and oral squamous [45] cancer cells, $CD133^+$ prostate [25], brain [46, 47] and ovarian [48] cancer cells, and $CD24^+$ hepatocellular carcinoma cells [28], among others. Of clinical relevance, elevated NANOG expression has been frequently associated with worse clinical outcome in numerous epithelial malignancies (see below). *SECOND*, enforced NANOG expression increases the frequency of CSCs such as $CD133^+$ and $AldeFLUOR^+$ breast cancer cells [49]. *THIRD*, in contrast, RNAi-mediated *NANOG* knockdown leads to attenuated CSC properties such as sphere formation and clonogenic efficiency in breast and prostate cancer cells [25].

A positive correlation between NANOG levels and proliferation has been frequently reported in cancer cells. Although it is currently unknown whether this phenomenon is directly associated with cell fate (as in ESCs), increased proliferation is a hallmark of neoplastic disease. NANOG knockdown in human gastric cancer cells reduced the proliferative, invasive and migratory capacity of cancer cells, associated with increased apoptosis and cell cycle arrest at the S phase [50]. Similar scenarios have been reported in response to NANOG inhibition in a variety of other cell types, such as glioblastoma [51] and breast [52] and prostate [25] carcinoma cells. Interestingly, NANOG knockdown in breast cancer cells appeared to modulate cell cycle progression by inducing G_0/G_1 arrest correlating with decreased levels of the cell cycle regulatory protein cyclin D1 [52].

Molecular oncogenesis can be thought of as a process of spontaneous cellular reprogramming. Unlike engineered reprogramming to generate iPS cells, deregulated and abnormal expression of NANOG (and/or other stem cell related factors) could foster 'oncogenic reprogramming' facilitating dynamic acquisition of states enhancing the adaptability of tumor cells to the gauntlet of challenges neoplastic cells face during tumor development and disease progression. Biologically plastic, renewing tumor cells may be intrinsically resistant to anti-cancer therapeutics and enriched upon experimental and clinical treatments. Thus, NANOG-expressing cancer cells have been observed to mediate therapy resistance, tumor recurrence, and distant metastasis. For example, NANOG has been observed to promote chemoresistance, increased cell migration, and epithelial mesenchymal transition (EMT) [49, 53, 54], a reversibly acquired cell state associated with metastasis. Microarray and quantitative real-time PCR analysis showed a parallel, elevated expression of *NANOG* and *OCT4* in lung adenocarcinoma. Double knockdown of NANOG and OCT4 suppressed the expression of *Slug*, a key EMT regulatory transcription factor, reversed the EMT process and blocked the tumorigenic and metastatic ability, thereby greatly improving the mean survival time of lung carcinoma cell-transplanted immune-compromised mice [53]. IHC analysis demonstrated the presence of NANOG, OCT4 and Slug in high-grade lung adenocarcinoma, with triple positivity potentially indicating a worse prognostic outcome, and providing rationale to therapeutically manipulate NANOG/OCT4 signaling to control EMT, repress tumor-initiating ability and inhibit metastatic spread [53].

Another crucial hurdle in the gauntlet cancer cells face is immunity. NANOG expressing cancer cells purportedly possess enhanced capabilities to evade the immune system. Hypoxia-induced NANOG in non-small cell lung cancer protects against cytotoxic T lymphocyte mediated tumor cell killing, possibly via a mechanism involving the signal transducer and activator of transcription 3 (STAT3) [55]. Vaccine-induced evolution and immune evasion of TC-1 human papillomavirus cervical cancer cells has also been shown to depend upon NANOG expression, as NANOG knockdown rendered xenograft tumors susceptible to immune surveillance *in vivo* [56]. Mechanistically, NANOG induced CSC phenotypes and immune evasion through T cell leukemia/lymphoma 1A/Akt (Tcl1a/Akt) in human cervical cancer, a signaling axis potentially conserved in a variety of other cancer types [57]. Furthermore, NANOG expression levels correlate with stage and prognosis of cervical cancer in patients, suggesting that NANOG may foster the development and progression of cervical cancer by facilitating immune evasion capabilities among CSCs [57].

NANOG expression and function in somatic human cancers

Here, we shall briefly describe clinical and xenograft studies implicating NANOG in the development of a variety of human malignancies and organized according to the tissue of origin. Although amplification of the short arm of chromosome 12 encoding NANOG is a 'hotspot' for oncogenic transformation and considered pathognomonic in male germ cell tumors (for a review, see [58]), our focus here is on somatic human cancers.

Prostate cancer

The pro-tumorigenic functions of NANOG in prostate cancer (PCa) have been clarified by functional studies [25, 49, 59]. Working on PCa cell lines, xenografts and primary tumor

specimens, we first demonstrated that NANOG short-hairpin RNA (shRNA) inhibited PCa sphere formation, clonal growth, and tumor development [25]. A gain-of-function strategy was then employed in which tetracycline-inducible PCa cell lines with NANOGP8 overexpression were established to further investigate the functions and mechanisms of NANOG in prostate tumorigenesis, and we found that NANOG induction phenotypically and functionally reprogrammed PCa cells and led to the emergence of castration-resistant PCa [42]. Substantiating these findings, NANOGP8 induction has been observed in some PCa cell and xenograft models [49, 60, 61]. In contrast, knocking down NANOG in undifferentiated, $PSA^{-/lo}$ CSCs inhibited xenograft tumor regeneration [62]. These findings suggest that NANOG might be a key regulatory factor mediating castration resistance and may therefore represent a critical, clinically relevant target for treatment of lethal, late-stage PCa. In support of this suggestion and of potential interest, *NANOG* mRNA is elevated in some PCa metastases (**Fig. 2B**, left) and in PCa harboring ETS2 deletion (**Fig. 2**; right).

NANOG protein is heterogeneously expressed as a gradient in PCa cells and enriched in $CD44^+$ and $CD44^+CD133^+$ cells (compared to marker-negative cells) and in primary tumor samples (compared to long-term cultured cells [25]). Interestingly, NANOG appeared to inversely correlate with expression of androgen receptor [25], suggesting a possible mechanism by which NANOG may promote castration resistance. Castration-tolerant PCa repopulating cells from early passage xenografts have also been reported to express NANOG, which appeared to largely localize to the cytoplasm [63]. NANOG protein was induced by hypoxia and positively correlated with hypoxia-inducible factor 1 α (HIF1α) in primary prostate tumors [64]. These findings were corroborated by independent observations of hypoxia-mediated upregulation of *NANOGP8* mRNA in Du145 and PC3 PCa cells [65].

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) exhibits cellular heterogeneity and stemness-related genes are preferentially expressed in NANOG-positive CSCs [66, 67]. However, it remains unclear whether or how these CSCs contribute to HCC initiation and progression. Using a chemoresistant HCC xenograft model, CD24 was shown to mark relatively quiescent NANOG-expressing tumor cells with serial sphere- and tumor-forming capabilities, metastatic potential and the capacity to differentiate *in vitro* [28]. That NANOG was epistatic to CD24 and critical for the tumorigenicity of these cells was demonstrated by the ability of NANOG overexpression to rescue tumor development in CD24 knockdown cells and to enhance serial sphere formation [28].

In primary tumor specimens, increased expression of NANOG was found to correlate with a worse clinical outcome in HCC [67]. Using a NANOG promoter reporter system, a small fraction of liver cancer cells exhibiting enhanced self-renewal, clonogenicity and tumor initiation were isolated [66]. These $NANOG^+$ CSCs were invasive, metastatic and resistant to therapeutic agents (e.g., sorafenib and cisplatin). Furthermore, NANOG knockdown reduced self-renewal, accompanied with decreased expression of stemness-related genes and increased expression of mature hepatocyte-specific genes [66]. In a separate study, a significant correlation was noted between NANOG expression and the expression of NODAL, P-SMAD3 and SNAIL [54]. The co-expression of NANOG and P-SMAD3 may

be a potential predictor of poor prognosis for HCC patients. Additionally, HCC cells in the tumor edge areas displayed higher NANOG expression than cells in the tumor center, which might suggest an important role for NANOG in HCC invasion and metastasis [54]. Finally, in tissue microarray analyses of 2 cohorts of HCC patients (n= 323) the co-expression of stemness markers NANOG and OCT4 in HCC concurred with aggressive tumor behaviors and predicted worse clinical outcome [67].

Leukemia

Transcripts of the retrogene derivative *NANOG2* were reported in mixed lymphocytic leukemia (MLL), suggesting that *NANOG2* could be involved in regulating leukemic stem cell functions [68]. More recently, Cao *et al.* used a sequencing-based method encompassing the crucial distinguishing 759G>C transition to demonstrate that *NANOGP8* is the predominant source of NANOG in acute T cell lymphoblastic leukemia (T-ALL), including primary patient samples [58]. RNAi-mediated NANOG attenuation in T-ALL cells was associated with loss of proliferation, reduced self-renewal, and increased apoptosis via blocking cell cycle progression through p53 signaling [69].

Glioblastoma multiforme

Glioblastoma multiforme (GBM) is a highly invasive and incurable brain tumor [70, 71]. In GBM, an important signaling pathway implicated in tumor growth, CSC expansion and specific expression of ESC-like stemness signature is the Hedgehog-GLI (HH-GLI), which appears to exert its function through direct regulation of NANOGP8 [46]. Using a loss-of-function approach, NANOGP8 was shown to be a HH-GLI mediator essential for GBM formation and sustenance as well as the survival and expansion of CD133-positive GBM CSCs [46, 70]. It is noteworthy that 3 GLI-*cis* elements are present upstream of *NANOG1* and 2 in *NANOGP8* regulatory regions, and functional analysis has revealed that NANOG is regulated by GLI, and vice versa, forming a positive feedback loop that is negatively regulated by p53 [46]. Analysis of tissue microarrays of 80 low-grade (WHO Grade II) and 98 high-grade human gliomas (WHO Grades III and IV) revealed higher protein levels of NANOG, KLF4, OCT4 and SOX2 in high-grade gliomas, as compared to low-grade ones [72]. NANOG was subsequently identified as an independent prognostic factor in the subgroups of low-grade astrocytoma, high-grade astrocytoma and glioblastomas [72].

Colorectal cancers

Both NANOGP8 and NANOG1 have been implicated in colorectal cancer (CRC) [22, 23]. In clinical CRC samples, the NANOG protein is expressed only in a small fraction of cancer cells; however, single *NANOG1*-positive CRC cells isolated via promoter-tracking constructs could form spheres similar to embryoid bodies derived from ESCs [23]. *NANOG1* expression appeared to be regulated by c-Jun and β-catenin/TCF4 as c-Jun could bind to the *NANOG1* promoter via the octamer M1 DNA element [23]. Interestingly, AlwN1 DNA fingerprinting (**Fig. 1A-B**) revealed that *NANOGP8* transcripts were detected in all CRC specimens tested, unlike *NANOG1* mRNA species, which were detected in only some CRC samples [23]. Using the same RFLP strategy with AlwN1 digestion, *NANOGP8* mRNA was

detected in CRC liver metastases and NANOGP8 functionally promoted the clonogenic potential and tumorigenic capacity of CRC cells [22].

IHC analysis in 175 CRC samples demonstrated that high levels of NANOG protein strongly correlated with poor prognosis, lymph node metastasis and Dukes classification [73]. NANOG protein was higher in CD133-positive CRC cells and overall 72 of the 360 cases (20%) positively expressed NANOG protein [74]. Univariate and Spearman correlation analyses associated *NANOGP8* expression with histological grade, lymph node metastasis, TNM stage, and liver metastasis [74]. Consequently, NANOGP8 might be considered a significant biomarker for postoperative liver metastasis of CRC patients.

Lung cancer

In a study of 163 lung cancer patients, the expression levels of NANOG protein in lung cancer tissues were upregulated compared to the normal lung tissues and positively correlated with clinical stages [64]. Furthermore, NANOG overexpression predicted a worse prognosis for lung cancer patients [75]. In another study [65], *NANOGP8* mRNA was detected in 84.8% (39 out of 46) of lung cancer samples and was found to be expressed at high levels even in the early clinical stages, suggesting that *NANOGP8* mRNA detection could represent a new tool to help diagnose lung cancer irrespective of the clinical stage.

Breast cancer

In a study comprising 100 breast cancer patients, patients with strong NANOG expression had significantly lower disease-free and overall survival rates than those with weak NANOG expression [76]. As discussed above, NANOG expression has been frequently correlated with CSC marker expression, functional properties, and therapy resistance in breast cancer cells. For example, NANOG knockdown in MCF-7 cells inhibited tumor growth, sphere formation and drug resistance [25] and blocked cell cycle progression, colony formation and migration [52]. NANOG-mediated chemoresistance in MCF-7 cells apparently occurred via complex formation with STAT-3 downstream of hyaluronan-induced CD44 activation, resulting in the expression of the multi-drug transporter MDR1 (ABCB1) [44]. Protein kinase Cε has also been proposed to be an intermediate in NANOG-mediated drug resistance in breast cancer cells by direct phosphorylation of NANOG leading to increased miR-21 levels and upregulation of anti-apoptotic proteins including IAPs and drug resistance mediators such as MDR1 [77]. Regardless of the underlying mechanisms, there exists solid evidence linking NANOG to breast cancer chemotherapy resistance.

Pancreatic cancer

In a tissue microarray analysis of 43 human pancreatic cancer, IHC for NANOG and OCT4 followed by Kaplan-Meier analysis revealed that high NANOG (and OCT4) expression predicted a worse prognosis and inversely correlated with patient survival [78]. Double knockdown of *NANOG* and *OCT4* significantly reduced proliferation, migration, invasion, chemoresistance, and tumor regeneration capacity of Panc-1 cells [68]. In a separate study of a small cohort of pancreatic ductal adenocarcinoma (PDAC) patient samples, NANOG was found to be co-expressed with the adult stem cell marker LGR5 (leucine-rich repeat-

containing G-protein coupled receptor 5), which might mark the cell-of-origin for PDAC [79].

Ovarian cancer

Ovarian cancer (OC) is the most lethal in all gynecological malignancies. *NANOG* mRNA and NANOG protein were enriched in OC cells with sphere-forming, tumor regeneration, and chemodrug resistance properties [80]. IHC examination of a large cohort of OC patients revealed increased nuclear NANOG protein in OC specimens (compared with benign tissues) correlating with pathological grade and tumor stage [81]. More recently, NANOG expression was shown to be significantly associated with risk of high-grade cancer development, severe histological subtypes, chemotherapeutic resistance, and poor overall and disease-free survival [82]. shRNA-mediated NANOG depletion impeded OC cell proliferation, migration and invasion associated with an increase in mRNA expression of *E-cadherin*, *caveolin-1*, *FOXO1*, *FOXO3a*, *FOXJ1* and *FOXB1*, whereas NANOG overexpression enhanced OC cell migration and invasion [82]. Of note, microRNAs may also be involved in *NANOG* posttranscriptional regulation, as miR-214 has been shown to regulate ovarian cancer stem cell (OCSC) properties by targeting the p53/NANOG axis [48]. Specifically, miR-214 levels showed a positive relationship with the frequency of OCSCs and NANOG protein such that the sphere-forming potential and the percentage of $ALDH1^+$ OCSC population were enhanced by enforced expression of miR-214 and attenuated by inhibition of miR-214 in a p53 wild-type background. Further, p53 was directly repressed by miR-214 whereas miR-214 regulation of NANOG appeared to occur indirectly through p53 as forced p53 expression abrogated miR-214 induced NANOG [48]. p53 is known to be a negative regulator of *NANOG1* transcription in ESCs, suggesting that *NANOG1* might be a primary locus-of-origin for NANOG in OC cells. Regardless, these data demonstrate a critical role for miR-214 in modulating OCSC properties by regulating the p53-Nanog axis and suggest that both miR-214 and NANOG could represent therapeutic targets for OC [48].

Conclusions, perspectives, and outstanding questions

Numerous investigations so far have causally linked NANOG to and also shed light on the role of NANOG in tumorigenesis, with implications in cancer prognosis and anticancer therapeutics. Since multiple oncogenic signal transduction pathways appear to modulate chemoresistance, EMT, metastasis, and other CSC properties through NANOG, this powerful reprogramming and stem cell-associated factor may represent a crucial molecular nexus underlying malignant disease. These findings demonstrate that NANOG is a protumorigenic factor that may serve in the clinic as a biomarker for cancer diagnosis, prognosis and predictor of anticancer therapeutic efficacy. Furthermore, NANOG itself may represent a therapeutic target as its elimination is predicted to ablate CSC self-renewal and root out the cause of tumor recurrence and metastasis.

Nevertheless, there are also many critical outstanding questions about the involvement and mechanisms of NANOG in tumorigenic processes. Answers to these questions will facilitate the design of novel cancer therapeutics targeting NANOG.

1) Why is NANOG, unlike OCT4, non-tumorigenic or only weakly tumorigenic by itself in transgenic animal models [12-16]? In what contexts does it function as a potentiating or cooperating oncogene? What cooperating oncogenic pathways does NANOG converge with in order to elicit transformation? The observations that NANOG, when co-expressed with Wnt-1, enhanced mammary tumorigenesis and metastasis [16] support the notion that the oncogenic functions of NANOG require synergistic cooperation with other genes/pathways. This caveat also appears to apply to SOX2, which has been found to induce the transformation of squamous basal stem cells of the esophagus and forestomach, only when co-expressed with activated STAT3 [83]. It is also conceivable that other classical brakes to transformation, such as the tumor suppressor p53 may play a role in restricting the oncogenic properties of NANOG. In support, p53 negatively regulates a reciprocal loop between GLI1 and NANOG (NANOGP8) in glioblastoma [46] and represses NANOG (NANOG1) expression in ovarian cancer cells, a p53-NANOG regulatory axis antagonized by the oncogenic microRNA miR-214 [48]. Thus, p53 may impinge upon NANOG-mediated oncogenic reprogramming in pre-neoplastic or cancerous cells and further analysis of correlations between NANOG expression and p53 mutation remain outstanding.

2) Are there distinct biochemical differences between NANOG1 and NANOGP8 in the context of regulating tumor development? In more advanced disease stages, could NANOGP8 expression preferentially potentiate metastatic propensity or resistance to conventional therapy? As discussed earlier, somatic cancer cells seem to predominantly express the retrogene *NANOG8* rather than ESC-specific *NANOG1*. In fact, there is evidence that cancer cells shut down NANOG1 expression [25]. Then what is the advantage of expressing NANOGP8? Although NANOGP8 and NANOG1 seem to be equipotent in reprograming normal [30] and cancer [49] cells, preferential expression of NANOGP8 in cancer cells argues for at least some distinct mechanisms or biological properties of this protein. At a global level, context-dependent NANOG-induced malignant phenotypes may also be dictated by the presence of NANOG-interacting proteins, such as other transcription factors that could function to decode chromatin occupancy, or given the epigenome reprogramming proclivities of *NANOG1*, chromatin-remodeling factors. To date, the majority of mechanistic studies have been based on studies *in vitro*. A systems biology approach will ultimately be needed to permit deeper understanding of the temporal and intensity dynamics of NANOG-associated regulatory networks in somatic cancer cells. Practically, as most commercial antibodies are raised against NANOG1 protein and thus do not distinguish between NANOG1 versus NANOGP8 [31], some potentially NANOGP8-unique functions will only be uncovered when high-quality NANOGP8-specific antibodies become available.

3) Along this line of discussion, how is *NANOGP8* transcriptionally (and post-transcriptionally) regulated in somatic cancer cells? This is obviously an interesting question as *NANOGP8*, being a retrogene, is regulated differently than *NANOG1* (**Fig. 1C**). Elucidating the upstream regulators will also help develop therapeutics to target NANOG, which appears to function as an essential self-renewing molecule that fuels tumor maintenance, metastatic spread, and drug resistance.

Supplementary Material

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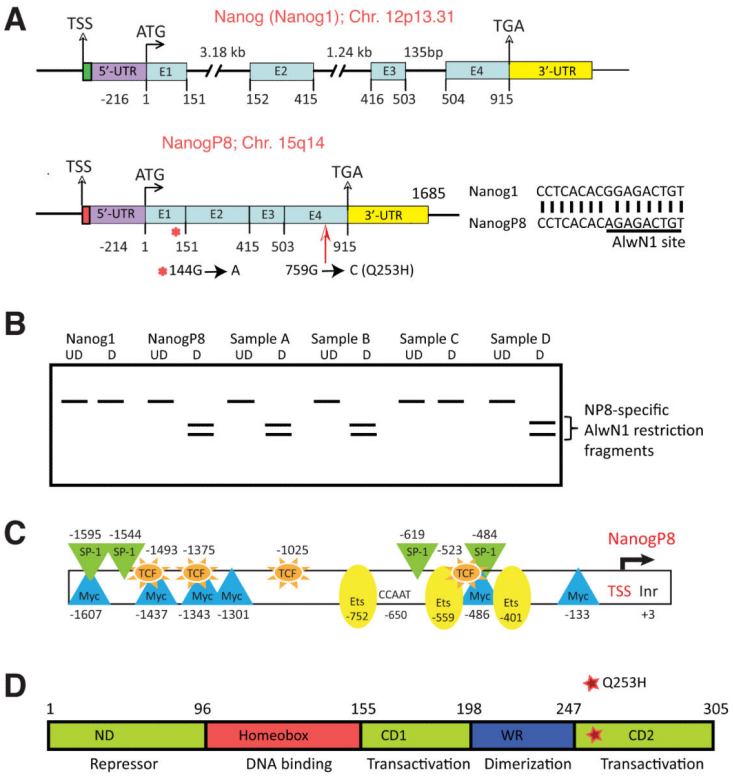

Figure 1. Genomic architecture and functional domains of NANOG

(**A**) *NANOG1* has a classical intron/exon structure with 4 exons (E), whereas *NANOGP8* is a retrotransposed gene and thus lacks introns. Both genes possess a 915-bp open reading frame, nearly identical between the 2 loci except for the 144G>A transition often used to discriminate between *NANOG1* and *NANOGP8* mRNA species (see B, below), and the 759 G>C giving rise to the single conserved aa change (Q253H). The 5'-UTRs (untranslated regions) and 3'UTRs are also highly conserved, except for the first ~18-bp, which are unique to each gene (marked by a green and red rectangle) and could theoretically be exploited to differentiate between the *NANOG1* vs. *NANOGP8* mRNA species. TSS, transcriptional start site.

(**B**) The 144G>A transition can be used for DNA fingerprinting, giving unique AlwN1 digestion fragments for *NANOGP8* (NP8). The sequences in this region can be employed to design RT-PCR primers flanking the AlwN1 cut site, and then digested (D) versus undigested (UD) PCR products separated by gel electrophoresis (shown is a representation of anticipated fragments) should reveal unique digestion fragments for each NANOG variant, corresponding to the locus of origin.

(**C**) The proximal promoter (2 kb upstream of TSS) of *NANOGP8* was analyzed using the Transcription Element Search System online tool to identify candidate transcription factor binding sites based on TRANSFAC motifs. The nucleotide positions for the indicated motifs are shown relative to the TSS. Four putative *NANOGP8* promoter-binding factors include SP1, MYC (c-MYC), TCF and ETS.

(**D**) NANOG protein has an N-terminal 'interference' domain to which co-repressors may bind (ND), homeodomain important for DNA binding, and a C-terminal transactivation domain containing two subdomains (CD1 & CD2) and a tryptophan-rich (WR) domain

involved in dimerization and activation. The asterisk indicates the conserved aa change (Q253H) in NANOGP8

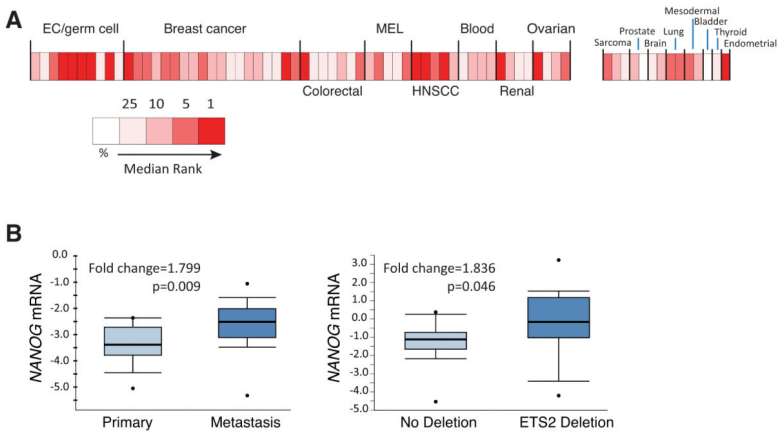

Figure 2. *Oncomine* analysis of *NANOG* transcripts in malignant diseases

(**A**) *Oncomine* analysis of *NANOG* mRNA expression in malignant diseases, filtered according to a threshold of >1.5X upregulated and P<0.05. The heat map indicates the median gene rank for expression in the indicated dataset/tissue type (scale shown below), where white indicates that NANOG was not among the top 25%. Datasets (72 in total; Supplementary Table S1) were clustered by tissue type into the indicated categories, with expression in embryonal carcinoma (EC)/germ cell tumors (positive control) placed at the front and the remaining tissues presented according to the relative frequency of datasets with a positive correlation. MEL, melanoma; HNSCC, head and neck squamous cell carcinoma. (**B**) Box plot presentation of 2 prostate cancer datasets (from A, above). Gene expression was normalized using total intensity, median centered and log(2) transformed to give equal weight to expression values relative to the median for analysis. The box plot on the left and right are based on the *Chandran (Prostate, BMC Cancer, 2007*) and *Grasso (Prostate, Nature, 2012*) datasets (Supplemental Table S1), respectively.