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Hypoxia-Dependent Inhibition of Tumor Cell Susceptibility to CTL-Mediated Lysis Involves NANOG Induction in Target Cells

Meriem Hasmim,* Muhammad Zaeem Noman,* Jessica Lauriol, † Houssem Benlalam,* Aude Mallavialle,‡ Filippo Rosselli,§ Fathia Mami-Chouaib,* Catherine Alcaide-Loridan, † and Salem Chouaib*

Hypoxia is a major feature of the solid tumor microenvironment and is known to be associated with tumor progression and poor clinical outcome. Recently, we reported that hypoxia protects human non-small cell lung tumor cells from specific lysis by stabilizing hypoxia-inducible factor-1α and inducing STAT3 phosphorylation. In this study, we show that NANOG, a transcription factor associated with stem cell self-renewal, is a new mediator of hypoxia-induced resistance to specific lysis. Our data indicate that under hypoxic conditions, NANOG is induced at both transcriptional and translational levels. Knockdown of the NANOG gene in hypoxic tumor cells is able to significantly attenuate hypoxia-induced tumor resistance to CTL-dependent killing. Such knockdown correlates with an increase of target cell death and an inhibition of hypoxia-induced delay of DNA replication in these cells. Interestingly, NANOG depletion results in inhibition of STAT3 phosphorylation and nuclear translocation. To our knowledge, this study is the first to show that hypoxia-induced NANOG plays a critical role in tumor cell response to hypoxia and promotes tumor cell resistance to Ag-specific lysis. The Journal of Immunology, 2011, 187: 4031–4039.

The tumor microenvironment is an integral part of tumor physiology, structure, and function, playing a critical role in tumor cell survival and growth and contributing to cell transformation and tumor development. Indeed, it is well established that a disrupted relationship between tumoral and stromal cells is essential for tumor cell growth, progression, and development (1), suggesting that an improved understanding of this interaction may provide new and valuable clinical targets for controlling tumor progression.

Tissue oxygenation is an important component of the microenvironment and can acutely alter cell behavior through the direct regulation of genes involved in cell survival, apoptosis, glucose metabolism, and angiogenesis (2). Evidence indicates that hypoxia, defined as oxygen deprivation, is an important feature of the microenvironment of a wide range of solid tumors. Its critical role in radio- and chemoresistance and its significance as an adverse prognosis factor have been well established during the last decades (2). Hypoxia has been shown to induce the loss of differentiation markers of several tumor types while increasing the expression of embryonic markers such as the transcription factors OCT4, SOX2, and NANOG, octamer-binding transcription factor (OCT) 4, SOX2, and the Notch ligand (3, 4). This reprogramming of non-stem cancer cells toward a cancer stem phenotype is associated with increased tumorigenic capacity (4).

At a cellular level, hypoxia evokes a complex molecular response mainly characterized by changes in gene expression that are mediated by hypoxia-inducible factors (HIFs) (2). The HIFs are heterodimers that consist of α and β subunits. Three isoforms of the α subunit are described: HIF-1α, HIF-2α, and HIF-3α (2). Under hypoxic conditions, the α subunit is stabilized and activated and binds the HIFβ isoform, which is not regulated by oxygen levels and is constitutively present in the nucleus (2). A broad range of tumors respond to hypoxia by stabilizing the HIF-1α subunit (5).

In a previous study, we reported that tumor hypoxia decreases tumor susceptibility to CTL-mediated lysis via HIF-1α stabilization and STAT3 phosphorylation (6). Although it has been reported that tumor stem cells were intrinsically susceptible to immune effector cell cytotoxicity (7), the effects of hypoxia-driven tumor dedifferentiation on the response to CTL-mediated lysis have not been reported.

NANOG, a homeodomain-containing protein expressed in embryonic cells, germ cells, and pluripotent stem cells, has the ability when overexpressed to inhibit in vitro embryonic stem cell differentiation and maintain their self-renewal capability independently of the LIF/gp130/STAT3 pathway (8). In human cancers, NANOG expression has been found in germ cell tumors where NANOG functions are required, and it has been reported to increase in several tumor tissues (breast, prostate, kidney) compared with matched benign tissues (9). Although accumulating evidence suggests that the protumoral functions of NANOG rely on promoting tumor cell proliferation (9), stimulation of STAT3 transcriptional activity has also been reported, leading to tumor resistance to chemotherapy and improved cell survival (10).

In line with these reports, NANOG expression in head and neck cancers has recently been associated with unfavorable clinical
outcome (11). However, whether NANOG interferes with tumor cell susceptibility to CTL-mediated cytotoxicity is not yet known.

The present study shows that hypoxia increases NANOG expression in non-small cell lung carcinoma cells and that this induction contributes to hypoxia-induced tumor target resistance to CTL-mediated lysis. Such induction is also associated with the regulation of cell replication and of nuclear translocation of STAT3. Our findings provide new insights into understanding the role of hypoxia-induced NANOG in regulating the cellular response to hypoxia and tumor target susceptibility to CTL-dependent killing.

Materials and Methods

Culture of tumor cells and CTL

The IGR-Heu lung carcinoma cell line was derived and maintained in culture as described previously (12). Briefly, the cells were grown in DMEM/F12 medium supplemented with 10% heat-inactivated FCS, 1% Ultroser G, 1% penicillin-streptomycin, and 1 mM sodium pyruvate (Life Technologies, Cergy Pontoise, France). The Heu161 cytototoxic T cell clone was derived from autologous tumor-infiltrating lymphocytes (13).

Reagents and Abs

Protease inhibitors and sodium orthovanadate were purchased from Roche Molecular Biochemicals. Abs against CD133 and OCT4 were from Abcam. Abs against NANOG were purchased from Santa Cruz Biotechnology (for Western blotting) and R&D Systems (for confocal microscopy). Ab against HIF-1α was from BD Transduction Laboratories. Abs against β-actin and GAPDH were from Sigma-Aldrich.

Hypoxia treatment

For hypoxia treatment, cell cultures were incubated in a hypoxia chamber (Invivo2 400 hypoxia workstation; Ruskinn) with a humidified atmosphere containing 5% CO2, 0.1% O2, and 94.8% N2 at 37˚C (14). Cells were exposed to these hypoxic conditions for 24, 48, and 72 h. Cells for protein analysis were harvested while inside the hypoxia workstation and were not reoxygenated before harvesting.

Flow cytometry analysis

Flow cytometry analysis was performed using a FACSCalibur flow cytometer. Data were processed using CellQuest software (BD Biosciences). Flow cytometry analysis was performed using a FACSCalibur flow cytometer. Data were processed using CellQuest software (BD Biosciences).

RNA extraction and quantitative real-time PCR

Total RNA was extracted using TRizol (Sigma-Aldrich) following the manufacturer’s instructions. For quantification of OCT4, NANOG, and hairy and enhancer of split (HES) 1 expression, cDNA was synthesized from 1 μg mRNA using an Applied Biosystems kit (N808-0234). Real-time PCR analysis was performed using a LightCycler (Roche Applied Science, Meylan, France) and the LightCycler-FastStart DNA Master SYBR Green I mix (Roche Applied Science) according to the manufacturer’s instructions with an initial denaturation step at 95˚C for 10 min, followed by 45 cycles at 95˚C for 10 s and 72˚C for 10 s. Primers used for NANOG, OCT4, and HES1 amplification were: NANOG forward, 5'-CAGCCCTGTGCTACT-CAATGATAGA-3’, reverse, 5'-ACACATGTCTATTTCTCGGCAGC-3’; OCT4 forward, 5’-ACATCAAGGCTTCGAGAAAGACT-3’, reverse, 5’-CTGAATACCTTCCAAATAGAACCC-3’; HES1 forward, 5’-AGGC-CCGACCTTGGGAAAGT-3’, reverse, 5’-CGTACCTCCCACGAC-ACCT-3’ (at a 63˚C annealing temperature). Specificity of PCR amplicons was confirmed by melting curve analysis. Expressions of target genes were normalized to that of RPL13. Relative expression of tested genes was calculated using the target threshold cycle value (Ct) and the 2-ΔΔCt method. For each gene, values were averaged over three independent measurements and the relative transcript level was calculated.

For quantification of apoptosis-related genes, reverse transcription was performed using a high-capacity cDNA reverse transcription kit (Ambion). Real-time PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG w/ROX (Invitrogen) and an Applied Biosystems 7900HT Fast real-time PCR system. Apoptosis-dedicated arrays were elaborated by the PETC platform of INSERM Unité 576.

Western blotting

Adherent tumor cells were washed in 1× PBS and lysed in plates with lysis buffer (62.5 mM Tris-HCl [pH 6.8], 2% w/v SDS, 10% glycerol, 1 mM sodium orthovanadate, 2 mM PMSF, 25 μM leupeptin, 5 mM benzamidine, 1 μM pepstatin, 25 μM aprotinin). Lysates were sonicated on ice, resolved by SDS-PAGE electrophoresis (60 μl/glane), and transferred onto nitrocellulose membranes. After incubation in blocking buffer, the membranes were probed overnight at 4˚C with the indicated Abs. The labeling was visualized using peroxidase-conjugated secondary Abs and with an ECL kit (Amersham International). Blots were scanned and processed by Adobe Photoshop 7.0 software.

Cytotoxicity assay

Four-hour chromium release assays were performed as described previously (6). Briefly, different E:T ratios (6:1 to 1:1) were used on effector cells per well (round-bottom 96-well plates). After 4 h coculture, the supernatants were transferred to LunarPlate-96 wells (PerkinElmer), dried down, and counted on a Packard Instrument TopCount NXT. Percentage-specific cytotoxicity was calculated conventionally as described earlier (6). All cytotoxicity experiments with [3H]Cr were performed under normoxic conditions.

TNF-β production assay

TNF-β release was measured as described previously (16). Briefly, TNF-β was detected by measuring the cytotoxicity of the culture medium on the TNF-sensitive WEHI-164c13 cells with an MTT colorimetric assay.

Confocal microscopy

Cells were plated in 8 well μ-slide (ibidi) and incubated for 24, 48, and 72 h at 37˚C and 0.1% partial pressure of O2 (pO2). Afterwards, cells were washed twice in 1× PBS, fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked for 45 min with 0.1% Triton X-100, 10% FCS, and 1% BSA. After blocking, cells were incubated overnight with diluted anti-NANOG primary Abs, followed by incubation with Alexa 488 and Alexa 546 secondary Abs (Molecular Probes), respectively, in the dark for 1 h at room temperature. Wells were covered with Fluoromount-G (SouthernBiotech, Birmingham, AL) and analyzed the following day by a Zeiss laser scanning confocal microscope (LSM-510; Zeiss, Jena, Germany). Z-projection of slices was performed using LSM Image Examiner software (Zeiss).

Gene silencing

Gene silencing of NANOG was performed using sequence-specific small interfering RNA (siRNA; Santa Cruz Biotechnology). Briefly, 8 × 106 cells were electroporated twice in 48 h in serum-free medium with 20 μM siRNA in an EasyJet Plus electroporation system (Equibio; 260 V, 450 μF) and then allowed to grow for 24, 48, and 72 h in hypoxia. siRNA targeting luciferase was used as a negative control (5'-GCAAGCGUGCACC- CUAGGAUUCU-3'). Gene-specific targeting was evaluated by quantitative real-time PCR (RT-qPCR) or Western blot.

Cell fractionation

For isolation of cytoplasmic and nuclear proteins, the ProteoJet cyttoplasmic and nuclear protein extraction kit (Fermentas) was used following the manufacturer’s instructions. Briefly, hypoxic or normoxic cells were harvested after trypsinization and solubilized in lysis buffer with protease inhibitors and DTT. After centrifugation, the cytoplasmic fraction was harvested after trypsinization and solubilized in lysis buffer with protease inhibitors and DTT. After centrifugation, the cytoplasmic fraction was isolated. Nuclear pellets were washed twice and then lysed in nuclear lysis buffer added with protease inhibitors and DTT. After centrifugation, total DNA was isolated.

Cell cycle

Electroporated IGR-Heu cells were incubated in normoxia or hypoxia (01% pO2) for 72 h. In the last 10 min, BrdU was added to the culture medium. Then, cells were harvested and fixed in 80% ice-cold ethanol. For BrdU detection, DNA was first denatured in a pepsine buffer (0.5 mg/ml pepsine, 2 N 30 mM HCl) for 20 min at 37˚C. Cell pellets were then resuspended in 2 N HCl for 20 min at room temperature. After washing in ice-cold 1× PBS, detection of replicating cells was performed by BrdU staining. Cells were washed in BrdU detection buffer (0.5% FCS, 0.5% Tween 20, 20 mM HEPES in 1× PBS) and BrdU was detected in the dark using a specific primary Ab for 45 min followed by a FITC-conjugated secondary Ab. To detect total DNA, cell pellet 1000 target resuspended in a propidium iodide solution (25 μg/ml propidium iodide, 50 μg/ml RNase A in 1× PBS) and incubated in the dark at room temperature for 30 min. Cells were then analyzed by flow cytometry.
Statistical analyses

Data were analyzed with GraphPad Prism. A Student t test was used for single comparisons. A p value of <0.05 was considered statistically significant.

Results

The resistance of hypoxic tumor cells to CTL is not associated with the acquisition of a stem cell-like phenotype

As previously reported, data depicted in Fig. 1A show that exposure of the lung tumor cell line IGR-Heu to hypoxic treatment resulted in an inhibition of its susceptibility to autologous CTL-mediated killing. We next examined the relationship between hypoxia-induced resistance to specific lysis and the adoption of a stem cell-like phenotype. Indeed, cancer stem cells have been shown to resist cell death induced by apoptotic and chemo- or radiotherapeutic agents (17). For this purpose, we first investigated the influence of hypoxic stress on the expression of the cell surface protein CD133 and the cytosolic enzyme aldehyde dehydrogenase (ALDH) 1. These two markers are respectively associated with lung cancer stem cell proliferation and chemoresistance (18–20).
Flow cytometry analysis indicated that hypoxia did not affect the cell surface expression of CD133 (Fig. 1Ba). Most of the IGR-Heu cells express CD133 constitutively, suggesting that CD133 may not be an exhaustive marker for detection of stem cell-like tumor cells. Total cellular extracts were analyzed by Western blot, and expression levels of CD133 (Fig. 1Bb) and ALDH1 (Fig. 1Bc) were not influenced by exposure to hypoxic stress. Because epithelial to mesenchymal transition (EMT) has been reported to be associated with both acquisition of an immature phenotype (21) and hypoxic stress (22), we investigated the induction of EMT in hypoxic IGR-Heu cells. The expression of the epithelial marker E-cadherin and of the EMT activator Snail, which is also an important regulator of E-cadherin expression (23), was analyzed. Fig. 1Ca shows that the total expression of E-cadherin, as well as surface E-cadherin expression (data not shown), remained unchanged after 72 h exposure to hypoxia. In contrast, Snail expression was significantly decreased after 24 h hypoxia (Fig. 1Ca). Because EMT is associated with morphological changes resulting in a distinct elongated phenotype, cell morphology of hypoxic IGR-Heu cells was therefore visualized by phalloidin staining. Data shown in Fig. 1Cb indicate that hypoxia had no effect on IGR-Heu morphology for up to 72 h. Phalloidin staining also showed the absence of cell rounding, which is a morphological feature associated with the acquisition of immature phenotypes (24). These results indicate that hypoxia-induced tumor resistance was not associated with the acquisition of a stem cell-like phenotype in IGR-Heu cells.

**NANOG is selectively induced in hypoxic tumor cells**

Although hypoxic stress did not result in phenotypic changes in IGR-Heu cells, hypoxia-induced dedifferentiation may be associated with altered expression of specific genes. Thus, to evaluate the potential role of dedifferentiation genes in hypoxic tumor cells, we selected OCT4 and NANOG genes, as well as HES1, a Notch target gene, based on their increased expression under hypoxia (4, 25, 26) as well as their reported role in the control of stem cell self-renewal and multipotency (27–29). Using RT-qPCR, we measured their respective mRNA levels under hypoxic conditions and found that OCT4 and NANOG mRNAs but not HES1 mRNA markedly increased following exposure to hypoxia for 24, 48, and 72 h (Fig. 2A). Whereas Western blot analysis did not reveal OCT4 expression (data not shown), fitting with its marginal mRNA levels under hypoxia, NANOG protein was induced up to 72 h hypoxia (Fig. 2B). This was confirmed by confocal

**FIGURE 2.** Hypoxic stress leads to selective induction of NANOG expression via HIF-1α. A, Hypoxia effects on mRNA expression levels of OCT4, NANOG, and HES-1 genes measured by RT-qPCR. B, IGR-Heu tumor cells were incubated in normoxia or hypoxia for different intervals. After hypoxia exposure, total cell lysates (60 μg) were subjected to SDS-PAGE, blotted, and probed with specific Abs against HIF-1α, HIF-2α, and NANOG, as indicated. β-actin was used as the loading control. C and D, Western blot analysis of NANOG expression levels under hypoxia following specific inhibition of HIF-1α (C) or HIF-2α (D) expression by siRNA. Each experiment was repeated at least three times. NE, non-electroporated; siH1, anti–HIF1-α siRNA1; siH2, anti–HIF1-α siRNA2; siLuc, anti-luciferase siRNA.
microscopy analysis, which revealed increased staining of NANOG along with the time of exposure to hypoxia (data not shown). Fig. 2B also showed that the induction of NANOG under hypoxia correlated with HIF-1α and HIF-2α stabilization. To delineate the potential link between hypoxia-induced NANOG and HIF-1α or HIF-2α, NANOG protein expression was analyzed following HIF-1α or HIF-2α knockdown using specific siRNAs. As shown in Fig. 2C, specific silencing of HIF-1α resulted in the inhibition of hypoxia-induced NANOG after 24, 48, and 72 h of hypoxic stress whereas HIF-2α silencing had no effect (Fig. 2D). These results indicate that hypoxia-induced NANOG was dependent on HIF-1α and raise the possibility that NANOG may play a role in the acquisition of tumor cell resistance to CTL lysis under hypoxic conditions.

Attenuation of hypoxia-induced tumor resistance to CTL-mediated killing following NANOG knockdown

To delineate the role of NANOG induction in hypoxia-mediated tumor cell resistance to specific lysis, we investigated the susceptibility of NANOG-targeted IGR-Heu cells using siRNA under hypoxic conditions. The inhibition of NANOG in hypoxic tumor cells was performed using specific siRNA and confirmed by RT-qPCR, showing an efficient and specific inhibition of NANOG mRNA expression after 72 h hypoxia, whereas luciferase siRNA used as a control had no effect on NANOG mRNA level (Fig. 3A). As shown in Fig. 3B, NANOG knockdown in hypoxic IGR-Heu cells was associated with sensitization of IGR-Heu cells to CTL-mediated lysis following 48 h exposure to hypoxia as compared with control hypoxic cells. We also show that the targeting of NANOG in hypoxic IGR-Heu cells was not associated with an alteration in autologous CTL reactivity, as TNF-β secretion by CTL was not affected (Fig. 3C). Moreover, such targeting did not alter MHC class I expression in IGR-Heu cells (Fig. 3D). This is consistent with the fact that attenuation of hypoxia-induced tumor cell resistance following NANOG targeting was not due to an alteration in CTL reactivity and target cell recognition.

NANOG targeting increases cell death and interferes with cell cycle transition in hypoxic tumor cells

To investigate the molecular mechanisms associated with the attenuation of target cell resistance to CTL following NANOG targeting under hypoxic conditions, we analyzed the expression of a series of antiapoptotic and proapoptotic genes using an apoptosis-related array of 94 genes. NANOG knockdown in hypoxic tumor

**FIGURE 3.** NANOG knockdown restores tumor cell susceptibility to lysis by CTL in hypoxia. A. NANOG expression was silenced using specific siRNA administered by electroporation. Silencing efficiency was assessed by RT-qPCR after 48 h hypoxia. B. Role of NANOG in CTL-mediated lysis toward autologous IGR-Heu tumor cells. IGR-Heu tumor cells were electroporated with NANOG or luciferase siRNAs and kept in normoxia (21% pO2) and hypoxia (01% pO2) for 48 h. Cytotoxicity was determined by a conventional 4-h 51Cr-release assay at different ratios. Heu161 CTL clone cells were used as effectors. Data show the percentage of IGR-Heu-specific lysis of three independent experiments ± SD. C. TNF-β production by the autologous T cell clone in response to stimulation by non-electroporated, control, and NANOG-deficient IGR-Heu cells. CTL clone Heu161 cells were cocultured in the presence of IGR-Heu cells placed in normoxia (21% pO2) versus hypoxia (01% pO2) for 48h. The amount of TNF-β produced by the CTL clone was measured using the TNF-sensitive WEHI-164c13 cells. Each bar is the mean of three independent experiments ± SD. D. Comparative analysis of surface expression of HLA class I (W6/32) conducted on control and NANOG-deficient IGR-Heu tumor cells kept in hypoxia (01% pO2) for 48 h. Isotypic control of mAb was included (IgG). Results are representative of three independent experiments. NE, nonelectroporated; siRNA Luc, luciferase siRNA.
cells revealed an increase in the expression of proapoptotic and antiapoptotic genes (Fig. 4A). However, NANOG depletion in hypoxic cells resulted in a limited but consistent increase in both apoptotic (sub-G₁) and nonreplicating (S-BrdU-negative) cells, suggesting that one of the roles of NANOG in hypoxia is to sustain cell viability (Fig. 4B). Additionally, flow cytometry analysis of BrdU-labeled cells revealed that hypoxia modified the overall level of nucleotides incorporation (Fig. 4Bb). Indeed, we

<table>
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<tr>
<th>Gene symbol</th>
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<tr>
<td>Pro-apoptotic genes</td>
<td></td>
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<tr>
<td>CYCS</td>
<td>NM_08947</td>
<td>1.5-1.7</td>
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<td>DR5</td>
<td>NM_003842</td>
<td>2.9-1.2</td>
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<tr>
<td>BID</td>
<td>NM_197966</td>
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| Anti-apoptotic genes | | |
| TGFβ1 | NM_000660 | 0.4-0.4 |
| BIRC2 | NM_001166 | 1.5-1.9 |
| BIRC3 | NM_001165 | 2.5-2.5 |
| BIRC4 | NM_001167 | 1.5-1.9 |
| FAIM | NM_00103030 | 1.8-1.7 |

**FIGURE 4.** NANOG knockdown increases cell death and restores BrdU incorporation in hypoxic tumor cells. A, Pro- and antiapoptotic gene transcript quantification by qPCR using a 94 apoptosis dedicated array. B, Sub-G₁ cell quantification by flow cytometry based on propidium iodide incorporation in hypoxic control and NANOG knocked down IGR-Heu cells. A statistically significant difference in sub-G₁ cell percentage between hypoxic control and NANOG-targeted cells was observed. *p < 0.05. Bb, Cells were gated according to their DNA content, propidium iodide incorporation (x-axis), and their capability to incorporate BrdU during S phase (y-axis). Red indicates cells in G₀/G₁ (lower left) and G₂/M (lower right). Cells in blue indicate S-phase BrdU-negative cells, that is, cells showing an S-phase DNA content but unable to replicate their DNA and incorporate BrdU. Green and purple identify replicating S-phase cells. The gated BrdU-positive cells were arbitrarily separated in two fractions as a function of their capability to incorporate BrdU. Green indicates cells with low replication capabilities, as a consequence of the accumulation of slowing DNA replication forks, the decrease in the number of active DNA replication forks, or both. Purple represents the fraction of cells able to replicate their DNA at a normal rate. Numeric values correspond to the percentage of gated cells that have incorporated BrdU at a normal and a low level. They are indicated with the corresponding color. Results are representative of two independent experiments.
noticed the appearance of a cell subpopulation characterized by lower BrdU incorporation. This subpopulation represented the majority of S-phase cells in hypoxic conditions. Importantly, NANOG depletion restored a cell cycle profile similar to that observed in normoxic conditions. These results indicate that NANOG knockdown in hypoxic tumor cells correlates with increased replication levels, an event associated to and responsible for DNA damage and apoptosis (30).

Regulation of STAT3 phosphorylation and nuclear translocation by NANOG

We have recently shown that hypoxia-induced resistance of IGR-Heu cells to specific lysis was critically dependent on STAT3 phosphorylation (6). Additionally, a functional cooperation between STAT3 and NANOG for gene transcription has been reported in cancer drug resistance (10). Therefore, we asked whether a functional interaction exists between NANOG and

FIGURE 5. NANOG knock down inhibits hypoxia-induced STAT3 phosphorylation and its nuclear translocation. A, Western blot analysis of the influence of NANOG silencing on STAT3 and Src phosphorylation during hypoxic stress. After 48 h exposure to hypoxia, 60 μg total protein extracts were run with β-actin used as the loading control. B, Luciferase siRNA- and siRNA-NANOG–electroporated IGR-Heu cells were kept in hypoxia for 24, 48, and 72 h and cell lysates were fractionated in cytoplasmic and nuclear fractions following the manufacturer’s instructions. GAPDH was used for technical control and, as expected, it was respectively found mainly in the cytosol for both luciferase siRNA- and siRNA-NANOG–electroporated IGR-Heu cells. Equal loading was confirmed by β-actin blotting. C, Confocal microscopy analysis of pSTAT3-Y705 staining in NANOG-deficient IGR-Heu tumor cells kept in normoxia (21% pO₂) versus hypoxia (01% pO₂), followed by immunofluorescence staining with pSTAT3-Y705–specific Ab. Nuclei were counterstained with To-Pro-3 iodide. Rainbow panel indicates staining intensity (blue to red correspond, respectively, from low to high intensity). The confocal scanning fluorescence micrographs shown are representative of most of the cells analyzed (blue, nucleus; green, pSTAT3-Y705). Experiments were performed at least three times. Scale bars, 20 μm. siRNA Luc, luciferase siRNA.
STAT3 upon hypoxic stress. Fig. 5A indicates that silencing of hypoxia-induced NANOG resulted in an inhibition of STAT3 phosphorylation on Tyr705, a crucial event for STAT3 dimerization and translocation to the nucleus (31). We next examined in hypoxic NANOG-downregulated cells the phosphorylation state of JAK2 and Src, two kinases involved in STAT3 phosphorylation (32) (33). Results indicate that NANOG downregulation under 48 h hypoxia resulted in selective inhibition of Src (Fig. 5A) but had no effect on JAK phosphorylation (data not shown).

Phosphorylation of STAT3-Y705 is crucial for STAT3 translocation to the nucleus (31). However, unpolarized nuclear forms of STAT3 have already been described (34). Therefore we investigated the role of NANOG on STAT3 nuclear translocation in hypoxic IGR-Heu cells using siRNA-targeting NANOG. Fig. 5B indicates that knocking down NANOG in hypoxic NANOG-targeted IGR-Heu cells suppressed STAT3 phosphorylation and its subsequent translocation into the nucleus after 24, 48, and 72 h of hypoxic stress. In contrast, in control hypoxic cells, phosphorylated STAT3 was induced and located in nuclear fractions. It also shows that in NANOG-targeted and control cells, hypoxia-induced NANOG did not translocate to the nucleus but remained in the cytoplasm (Fig. 5B). Confocal microscopy analysis indicated a constant decrease of pSTAT3-Y705 nuclear staining in siRNA-NANOG–treated IGR-Heu cells under 24, 48, and 72 h hypoxia compared with control cells (Fig. 5C). This further points to the role of NANOG in STAT3 phosphorylation on Y705.

Discussion
It is well established that microenvironmental factors, in particular hypoxia, act to shift the normal balance toward malignancy. Accumulating evidence indicates that cancer cells that remain viable in hypoxic conditions often possess an increased survival potential and tend to grow particularly aggressively. Hypoxia induces tumor progression through multiple mechanisms, including several transcriptional programs (2). In this study we show, in human non-small cell lung carcinoma cells, that hypoxic stress selectively induces the stem cell marker NANOG, which is involved in the hypoxia-mediated resistance to specific lysis. Hypoxia-induced NANOG has the ability to control the expression of a series of proapoptotic genes, to regulate cell replication, and to contribute to STAT3 and Src phosphorylation.

The selective increase in NANOG expression under hypoxia was not associated with alteration of CD133 or ALDH1 expression, two reported markers for lung cancer stem cells (20). Additionally, IGR-Heu cells did not lose their epithelial features under hypoxia, as Snail expression was decreased and E-cadherin level remained unchanged in IGR-Heu hypoxic cells. Whether Snail decrease is due to transcriptional repression or increased degradation following hypoxic stress is currently under investigation. Nonetheless, this suggests that in our experimental system, tumor cells did not undergo a phenotypic dedifferentiation but indeed displayed increased expression of NANOG, a transcription factor associated with stemness. NANOG induction in hypoxia has been recently reported (4, 35, 36), suggesting its putative involvement in cell adaptation to hypoxic stress. In this regard, it has been shown that NANOG possesses proangiogenic activities by controlling vascular endothelial growth receptor-2 expression in endothelial cells (37). Although induction of this transcription factor under hypoxia is usually described to be dependent on HIF-2 in cancer stem cells (38, 39) and embryonic stem cells (35), our results clearly indicate that only HIF-1α selectively regulates NANOG induction in our experimental model.

To determine the functional relevance of NANOG in hypoxic tumor resistance to CTL-mediated lysis, we targeted NANOG and found that it resulted in the attenuation of target resistance to CTL-mediated killing. How this transcription factor regulates tumor susceptibility to lysis is not yet established. Nevertheless, our data considering the relevance of NANOG induction following hypoxic stress support the idea that NANOG is a significant mediator of the adaptive cellular response to hypoxia. NANOG has been reported to have antiapoptotic functions by inhibiting apoptosis in transformed human stem cells (40) and choriocarcinoma cells (41). NANOG targeting significantly increased the percentage of sub-G1 dead cells, suggesting that hypoxia-induced NANOG has a protective role on tumor cells. By analyzing cell cycle transition, we show a decrease in BrdU incorporation under hypoxia. This could be related to the activation of DNA repair processes subsequent to DNA damage known to be induced by exposure to hypoxia (42). Additionally, a major event in shifting cells from normoxia to hypoxia is cell cycle arrest (43) (44). Interestingly, our data show that NANOG knockdown restored BrdU incorporation in hypoxic cells, suggesting that NANOG, under conditions of hypoxic stress, could be involved in the activation of checkpoint signaling resulting in slowing down S phase. This is supported by our finding that hypoxia induced p53 protein accumulation and that this event was inhibited following NANOG knockdown (data not shown). To further delineate the role of NANOG in hypoxia, we explored the effect of NANOG on hypoxia-induced STAT3 activation. Bourguignon et al. (10) demonstrated functional cooperation of NANOG with activated STAT3 in inducing expression of the chemoresistance gene MDRI. Previously, we provided evidence indicating that STAT3 phosphorylation was a major event in the acquisition of hypoxic tumor cell resistance to CTL-mediated killing (6). In the present report, our data point to the potential role of NANOG in regulating STAT3 phosphorylation and its subsequent nuclear translocation. To our knowledge, this is the first demonstration involving a functional interaction between NANOG and STAT3 in the regulation of tumor cell susceptibility to CTL-dependent cytotoxicity. More importantly, we showed that NANOG targeting resulted in the inhibition of hypoxia-induced Src phosphorylation. Because Src is known to be required for STAT3 phosphorylation (32, 33), it is tempting to speculate that NANOG controls STAT3 phosphorylation by a mechanism involving Src phosphorylation under hypoxia. This is in agreement with our recent observations showing that chemical inhibition of Src phosphorylation in hypoxic IGR-Heu cells was able to block hypoxia-induced STAT3 phosphorylation (45).

Along with inhibition of hypoxia-induced delay on cell replication levels, NANOG knockdown seems to allow hypoxic tumor cells to gain a “normoxia-like” state that may shift CTL-resistant hypoxic tumor cells to sensitive ones.

Collectively, these results point to a novel function of NANOG and suggest the existence of a new relationship between stem cell markers, hypoxia, and resistance to CTL-mediated lysis.

Disclosures
The authors have no financial conflicts of interest.

References


