Smad-Interacting Protein-1 and MicroRNA 200 Family Define a Nitric Oxide–Dependent Molecular Circuitry Involved in Embryonic Stem Cell Mesendoderm Differentiation

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Objective—Smad-interacting protein-1 (Sip1/ZEB2) is a transcriptional repressor of the telomerase reverse transcriptase catalytic subunit (Tert) and has recently been identified as a key regulator of embryonic cell fate with a phenotypic effect similar, in our opinion, to that reported for nitric oxide (NO). Remarkably, SIP1/ZEB2 is a known target of the microRNA 200 (miR-200) family. In this light, we postulated that Sip1/ZEB2 and the miR-200 family could play a role during the NO-dependent differentiation of mES.

Methods and Results—The results of the present study show that Sip1/ZEB2 expression is downregulated during the NO-dependent expression of mesendoderm and early cardiovascular precursor markers, including Flk1 and CXCR4 in mES. Coincidently, members of the miR-200 family, namely miR-429, -200a, -200b, and -200c, were transcriptionally induced in parallel to mouse Tert. This regulation occurred at the level of chromatin. Remarkably, miR-429/miR-200a overexpression or Sip1/ZEB2 knockdown by short hairpin RNA interference elicited a gene expression pattern similar to that of NO regardless of the presence of leukemia inhibitory factor.

Conclusion—These results are the first demonstrating that the miR-200 family and Sip1/ZEB2 transcription factor are regulated by NO, indicating an unprecedented molecular circuitry important for telomerase regulation and early differentiation of mES. **(*Arterioscler Thromb Vasc Biol*. 2011;31:898-907.)**

Key Words: biology, developmental ■ gene expression ■ molecular biology ■ nitric oxide ■ vascular biology ■ differentiation ■ epigenetics ■ microRNA

The balance between self-renewal and lineage-directed differentiation of embryonic stem cells has recently been linked to the presence of epigenetic marks believed to play a key role in this process.[1] The progression of stem cells toward the differentiation process is, in fact, characterized by striking alterations in the organization of chromatin architecture leading to gene expression regulation.[2,3] It has recently been demonstrated that nitric oxide (NO), the gaseous product of the NO family of synthases, is a potent pleiotropic epigenetic modifier.[4,5] In mammals, NO plays a pivotal role in the physiological regulation of adult cell proliferation, metabolism, survival, and gene expression. We recently reported that in mouse embryonic stem cells (mES), this molecule determines mesendoderm and vascular differentiation, an event associated with a global wave of chromatin remodeling paralleled by a decreased expression of stemness-associated genes, including Oct4, Nanog, and KLF4, and the appearance of lineage specific mesodermal markers.[6] During the progress of these studies, we noticed that, similar to adult cells,[7] mES exposed to the NO donor diethylenetriamine (DETA)/NO expressed high levels of the catalytic subunit of the mouse telomerase reverse transcriptase (mTert). Although a large body of information is available on the transcriptional regulation of Tert, little is known about its regulation in embryonic stem cells exposed to high levels of NO.[8]

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Telomerase activity is important for mES growth. In fact, deletions leading to loss of either mouse telomerase RNA subunit (mTr) or its telomerase reverse transcriptase catalytic subunit (mTert) subunits determined the progressive loss of telomeres, genomic instability, aneuploidy, and telomeric fusion, with negative effects on the growth rate.[9] In addition, mTert overexpression enhanced self-renewal and improved resistance to apoptosis and oxidative stress, thus increasing proliferation.[10] Although the function of Tert during differentiation is still uncertain, several lines of evidence have recently reported data in favor of a role for this molecule promoting cellular differentiation.[8,10–14] Tert, in fact, is abundantly expressed in embryonic or undifferentiated cells. Although its expression and activity weakens with aging, leading to a gradual shortening of telomeres in a wide variety of highly differentiated somatic cells,[10,15] Tert expression and activity has effects that are important for differentiation in cells of mesodermal origin, such as endothelial cells,[16,17] and during the differentiation of cardiac precursors derived from mES.[11]

Tert promoter is regulated by multiple positive and negative transcription regulators that are responsible for the fine tuning of Tert expression in different cell types and organs during growth, development, or transformation.[8,9,13] Among these regulators, it has recently been reported that human Tert promoter is repressed by the transcription factor Smad-interacting protein-1 Sip1/ZEB2.[18,19] The best characterized role of Sip1/ZEB2 is during the induction of epithelial-to-mesenchymal transition, a phenomenon occurring normally during embryonic development, wound healing, and carcinogenesis, which leads to cell transformation and metastasization.[20] This process is efficiently counteracted by the microRNA 200 family (miR-200), which prevents Sip1/ZEB2 expression.[21–23] Intriguingly, Chng et al,[24] characterizing the effect of SIP1 overexpression/knockdown in human embryonic stem cells, found that SIP1, important for neuroectodermal commitment, negatively regulates mesendoderm, thus affecting mesoderm and endoderm formation. Whether NO regulates mesendoderm and vascular gene expression through the induction of miR-200 family members and SIP1/ZEB2 downregulation is currently unknown. In an attempt to answer this question, the present work established the following experimental points: (1) SIP1/ZEB2 has a role in the NO-dependent developmental program of mES; (2) NO represses Sip1/ZEB2 expression via the induction of specific miR-200 family members, namely miR-429, -200a, -200b, and -200c; and (3) the overexpression of representative members of the miR-200 family, such as miR-429 or miR-200a, as well as the direct Sip1/ZEB2 knockdown by short hairpin RNA (shRNA) interference, is sufficient to induce the expression of mTert and that of mesendoderm and vascular markers, thus reproducing the effect of NO alone seen in embryonic and adult cells.[6,17] These results were achieved regardless of the presence or absence of the leukemia inhibitory factor (LIF), suggesting that in the presence of NO, both Sip1/ZEB2 and the miR-200 family are parts of a molecular circuitry that dominantly regulates stemness and early differentiation in mES.

Materials and Methods

Cell Culture and Treatments

Cells were cultured according to previously reported protocols.[6] See Supplemental Methods, available online at http://atvb.ahajournals.org, for further details.

MicroRNA Detection

Details are provided in the Supplemental Methods.

Real-Time PCR Detection of mRNA

For mRNA analyses, cDNA was primed from 2 μg of total RNA using the Superscript III first strand (Invitrogen) according to the manufacturer's protocol. mRNA levels were analyzed using the SYBR-GREEN qPCR method (Invitrogen) and quantified with an ABI Prism 7000 SDS (Applied Biosystems). Further details are provided in the Supplemental Methods.

Primary miR Detection

Details are provided in the Supplemental Methods.

Western Blot Analysis of Protein Expression

Details are provided in the Supplemental Methods.

Transfection of MicroRNA Precursors and Antagomirs

Details are provided in the Supplemental Methods.

Telomere Repeat Amplification Protocol Assay

Telomerase activity was measured by the telomere repeat amplification protocol method. The assays were repeated at least 3 times with 3 different preparations of cell lysates, as previously described.[25]

Staining of Flk1, SM22α, and Sip1

Details are provided in the Supplemental Methods.

Confocal Microscopy

Details are provided in the Supplemental Methods.

SIP1/ZEB2 Interference

Stable knockdown of SIP1/ZEB2 was carried out through infection with pLKO.1-shSip1/Zeb2 lentivirus (Sigma). Further details are provided in the Supplemental Methods.

The Mouse Embryonic Stem Cell RT^2 Profiler PCR Array Profile

Total mRNA was treated with on column DNase treatment (Qiagen RNeasy Mini Kit) and then with RT2 First Strand Kit (C-03) both to remove any and all residual contamination from RNA samples and to convert it in cDNA. cDNA template was mixed with the ready-to-use PCR master mix, and equal volumes were aliquoted to each well of the same plate to perform the real-time PCR cycling program. Further details are provided in the Supplemental Methods.

Chromatin Immunoprecipitation

Details are provided in the Supplemental Methods.

Fluorescence-Activated Cell Sorter Analysis

Details are provided in the Supplemental Methods.

Statistical Analysis

Statistical analyses were carried out by analysis of variance. Statistical significance was evaluated by a 2-tailed unpaired Student *t* test. A probability value <0.05 was considered statistically significant.

Results

NO Induces Tert Expression and Function During the Early Differentiation of mES

NO is a potent regulator of Tert expression and function.[7] It is unclear, however, whether this process occurs in embryonic stem cells induced to differentiate by NO-donors.[6] This question is particularly relevant in light of the evidence that Tert not only plays a role in telomere length but is also linked to proliferation, survival, and differentiation processes that may be crucial in the early embryonic stem cell lineage differentiation and in their applications to tissue regeneration.[6] To address this important question, real-time polymerase chain reaction (qRT-PCR) and telomerase repeat elongation protocol experiments were performed on mES treated with the NO donor DETA/NO, during a time course from 6 to 24 hours. The exposure to DETA/NO determined a rapid expression of Tert (Figure 1A), whose activity was significantly increased at the 6-hour time point after treatment; it remained elevated at 24 hours (Figure 1B). LIF deprivation alone determined a transient decrease of both Tert expression (Figure 1A) and activity (Figure 1B), which was dramatically increased at the 6-hour time point after DETA/NO treatment. Under these conditions, in spite of Tert induction, cell cycle distribution and annexin V analyses of LIF-deprived NO-treated mES revealed an enrichment in G_0/G_1 paralleled by a slight increase in apoptosis (see Supplemental Figure IA). Additional experiments performed in the presence of the Tert inhibitor 3′-azido-3′-deoxythymidine showed a significant reduction in the expression of vascular markers, suggesting, in our experimental context, that Tert function is more involved in differentiation[8,13,15,26] than proliferation or survival control[27,28] (see Supplemental Figure IB). This evidence prompted us to further explore those mechanisms potentially involved in the NO-dependent regulation of Tert.

SIP1/ZEB2, a transcriptional target of the transforming growth factor-β pathway,[29] mediates the transforming growth factor-β-regulated repression of human Tert.[19] A series of experiments was performed to explore whether Sip1/ZEB2 could be regulated in mES exposed to DETA/NO. Western blotting and immunofluorescence analyses revealed that in the presence of DETA/NO, Sip1/ZEB2 protein decreased within 24 hours (Figure 1C to 1E). Under control conditions (−LIF), however, no significant changes in SIP1/ZEB2 protein content were observed, suggesting that the presence of NO was important for this process.

NO Regulates miR-200 Family Gene Expression at the Transcription Level

The miR-200 family comprises 5 members: miR-200a, -200b, -200c, -141, and -429. They are located within 2 clusters on separate chromosomes. Interestingly, the 5 members can be subdivided into 2 subgroups according to their seed sequences. MiR-200a and miR-141 make up group I, and miR-200b, -200c, and miR-429 make up group II.[30,31] The most prominent targets of the miR-200 family are 2 E box binding transcription factors, ZEB1 and SIP1/ZEB2. The regulation of members of this microRNA (miRNA) family was independently evaluated in mES cultured for 24 hours in the presence or absence of DETA/NO after LIF deprivation (Figure 2A). Cells cultured in the presence of LIF were used as control. Under control conditions, none of the miR-200 family members were significantly expressed. However, in the presence of DETA/NO, on LIF deprivation, a significant and sustained increase in miR-200a, -200b, -200c, and -429 expression was observed, whereas miR-141 was only transiently modulated. Similar results were obtained in mES cultured in the presence of LIF either after NO induction by DETA/NO (Figure 2A) or after expression of a constitutively active form of the endothelial nitric oxide synthase[32] ($eNOS^{Ser1177Ala}$, data not shown). These data suggest that the NO-dependent regulation of miR-200 family members is independent of the differentiation status of the cells and may represent an important step in the mES differentiation determined by NO.[6] Additional experiments revealed that the effect of NO was at the transcription level, as indicated by the induction of pri-miR transcripts, which were already upregulated at 3 hours and remained significantly higher at the 24-hour time point (Figure 2B). Chromatin immunoprecipitation experiments performed to evaluate the remodeling of chromatin at the miR-200b, -200a, and -429 promoter (Figure 2C) showed that the presence of DETA/NO determined the panacetylation of histones H3 (H3Ac) in the nucleosomes of this region, a pattern that correlated well with the expression of this cluster being regulated at transcription level.

MiR-200a and miR-429 Repress Sip1/ZEB2 Expression, Inducing mES Differentiation

NO is a pleiotropic agent able to modify gene expression and function in a large variety of cell types.[4–6] In our experimental system, gene array analysis demonstrates that, besides genes related to the vascular lineage, NO had a broad effect on the expression of mesendoderm genes. This effect has been observed in the presence of LIF, after LIF deprivation, in cells treated with NO alone, and after miR-200a/429 overexpression or the shRNA interference of SIP1/SEB2 (see Supplemental Tables I to XII).

The NO-dependent regulation of miR-200 family suggested a role for these miRs during mES commitment to mesendoderm. To verify this hypothesis, 2 representative miR-200 family members with different seed sequences, miR-200a and miR-429, both of which are able to target Sip1/ZEB2 mRNA, were overexpressed, at high and low copy numbers, as pre-miRs in undifferentiated mES. Overexpression of miR-200a and miR-429 was verified by qRT-PCR (see Figure 3A and Supplemental Figure IIA). The on-target effect of miR-429/200a overexpression was determined by Western blotting (Figure 3B) and immunofluorescence analyses (Figure 3C). Both miR-200a and miR-429 significantly reduced Sip1/ZEB2 expression, eliciting an increase of markers associated with the vascular phenotype, including Flk1 and SM22α (Figure 3D). A large panel of mesodermal and nonmesodermal markers, in addition to Flk1, Sm22α, mTert, smooth muscle actin, Brachyury, Nestin, and NeuroD, was evaluated by qRT-PCR (shown in Figure 3E, Supplemental Figures IV and V, and Supplemental Tables V and VI). The results revealed a clear positive

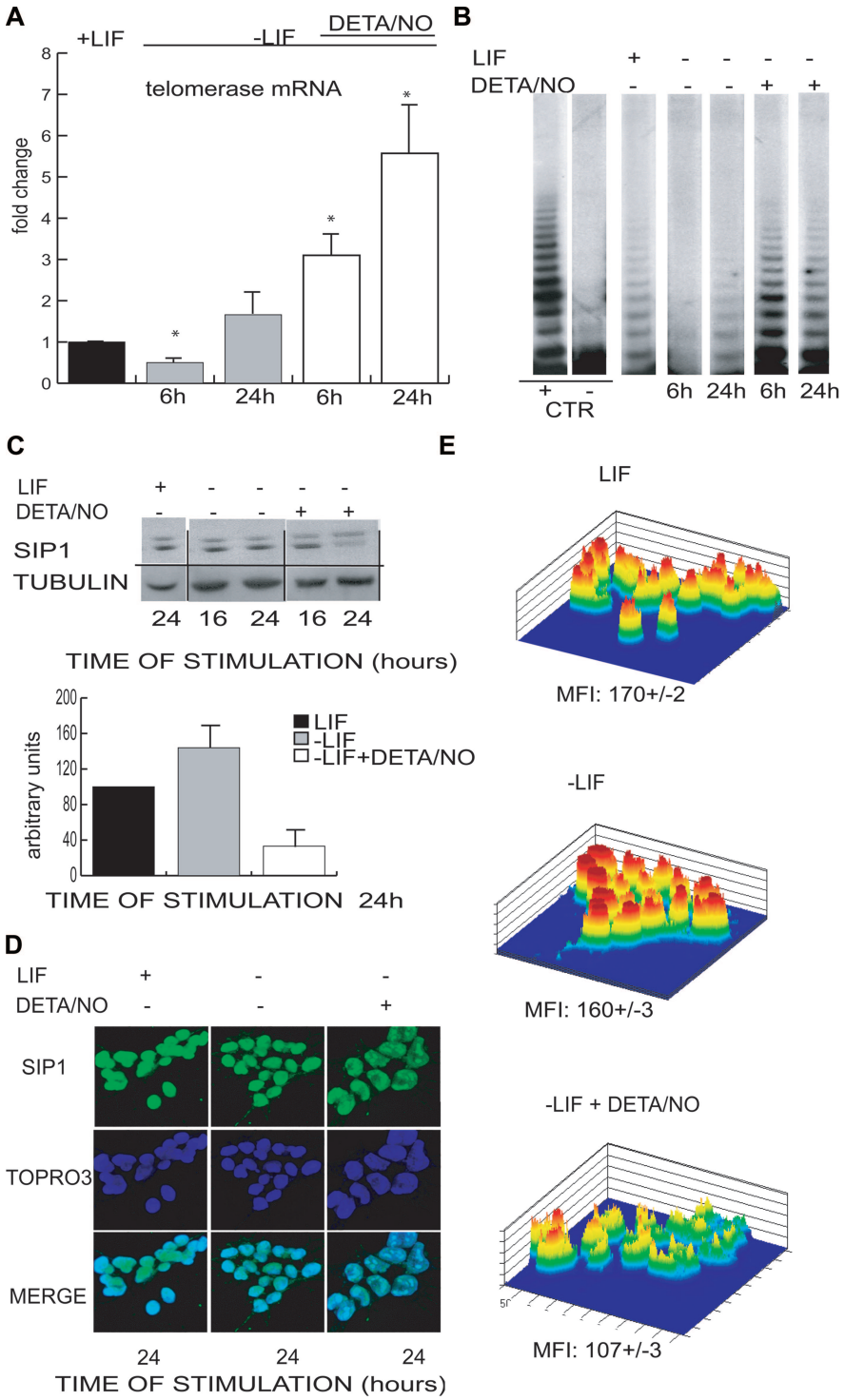

Figure 1. Mouse telomerase is upregulated during NO-dependent differentiation of mES. A, mTert expression. Total RNA was extracted from mES induced to differentiate in the absence of LIF with or without DETA/NO and analyzed by qRT-PCR. Data represent the mean±SD of 3 independent experiments normalized to controls cultured in the presence of LIF. B, mTert activity. A telomere repeat amplification protocol assay was performed in extracts from mES exposed to DETA/NO or kept under control conditions from 6 to 24 hours. Positive and negative control (heat inactivated): cell extracts from HeLa cells. C, Western blot analysis of Sip1/ZEB2 expression level in total cellular extracts from mES cultured for 24 hours in the absence of LIF and in the presence or absence of DETA/NO. Tubulin was used as a loading control. This result is representative of 3 independent experiments. D, Immunofluorescence analysis. Left panels: cells were stained with Sip1 antibodies and examined by confocal microscopy. Nuclei were counterstained with Topro3. E, The graphs shows the mean fluorescence intensity (MFI) of Sip1/ZEB2 expression in mES cultured with or without LIF or DETA/NO.

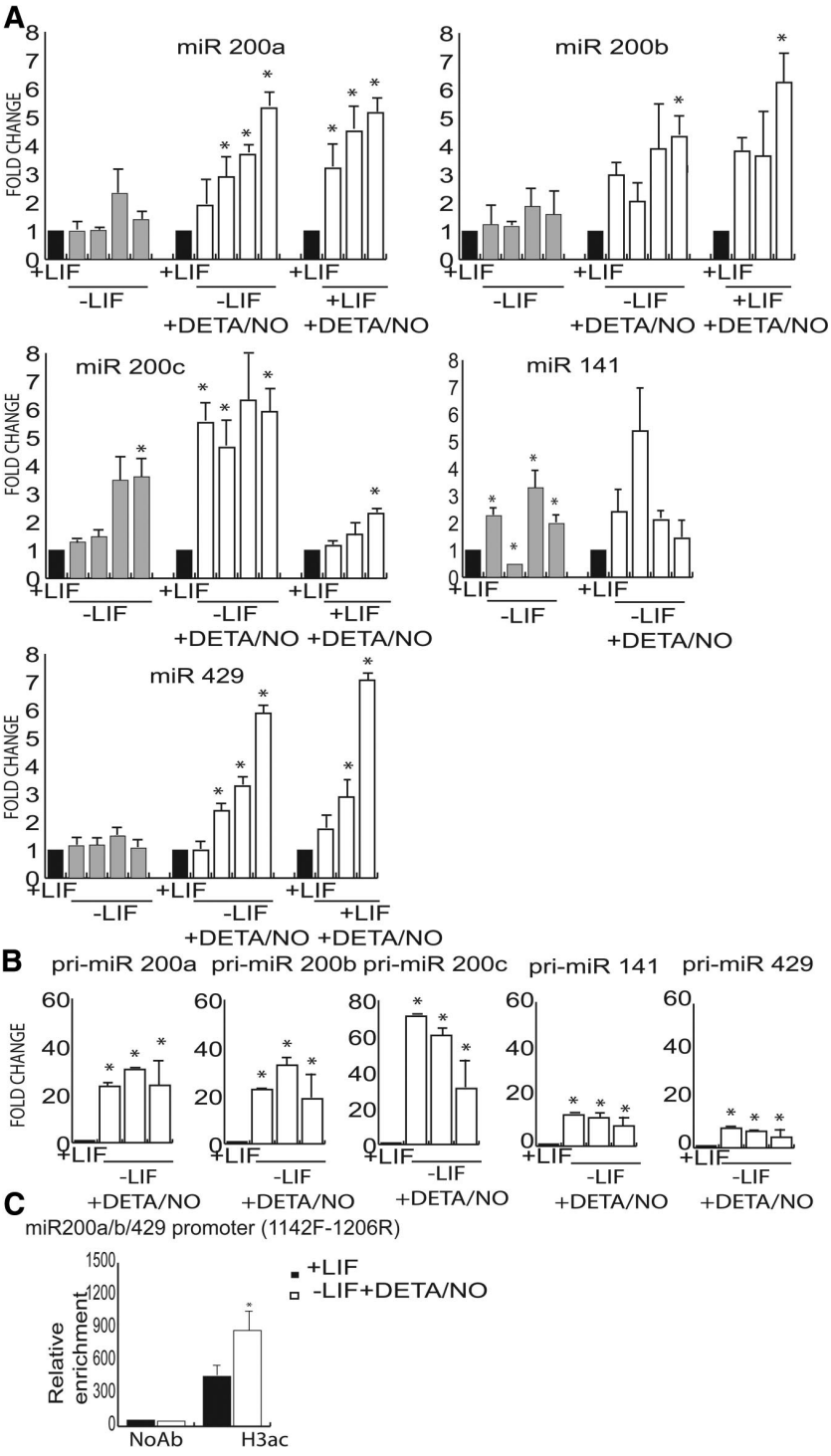

Figure 2. NO upregulates selective members of the miR-200 family. A, qRT-PCR evaluation of miR-200 family levels in DETA/NO-treated mES with or without LIF. Values were normalized to miR-16 expression. Six independent experiments in duplicate were performed. Time course: 3, 6, 16, and 24 hours for DETA/NO-treated mES without LIF; 6, 16, and 24 hours in presence of LIF. B, Pri-miR detection in DETA/NO treated cells. Cells were collected at 2, 6 and 24 hours after DETA/NO addition. Data represent the mean±SD from 3 independent experiments. Values were normalized by GADPH. The value for the control was normalized to 1, and all other values were calculated accordingly. C, Chromatin immunoprecipitation analysis of the miR-200a/b/429 cluster promoter. Cells were treated overnight with DETA/NO in the absence of LIF. Immunoprecipitations were performed using antibodies to H3Ac (Abcam) or in the no-antibody (NoAb) condition as negative control. Data are mean±SD of 3 independent experiments and relative enrichment over control. Bars represent SD. *$P<0.005$.

effect on mesendoderm, mesoderm, and endoderm, greater than that of neuroectodermal markers. Similar results were obtained in experiments in which the expression level of miR-429 was kept in the range of 4- to 5-fold above controls (see Supplemental Figure IIA and IIB). Furthermore, the expression of miR-200a or -429 antagomirs (Supplemental Figure IIIA) significantly reduced the effect of NO on OCT4, Flk1, and CXCR4 expression levels (Supplemental Figure IIIB and IIIC).

Sip1/ZEB2 Silencing Modifies Chromatin Structure and Promotes Differentiation

SIP1/ZEB2 has recently been found involved in the regulation of human embryonic stem cell self-renewal or neuroec-

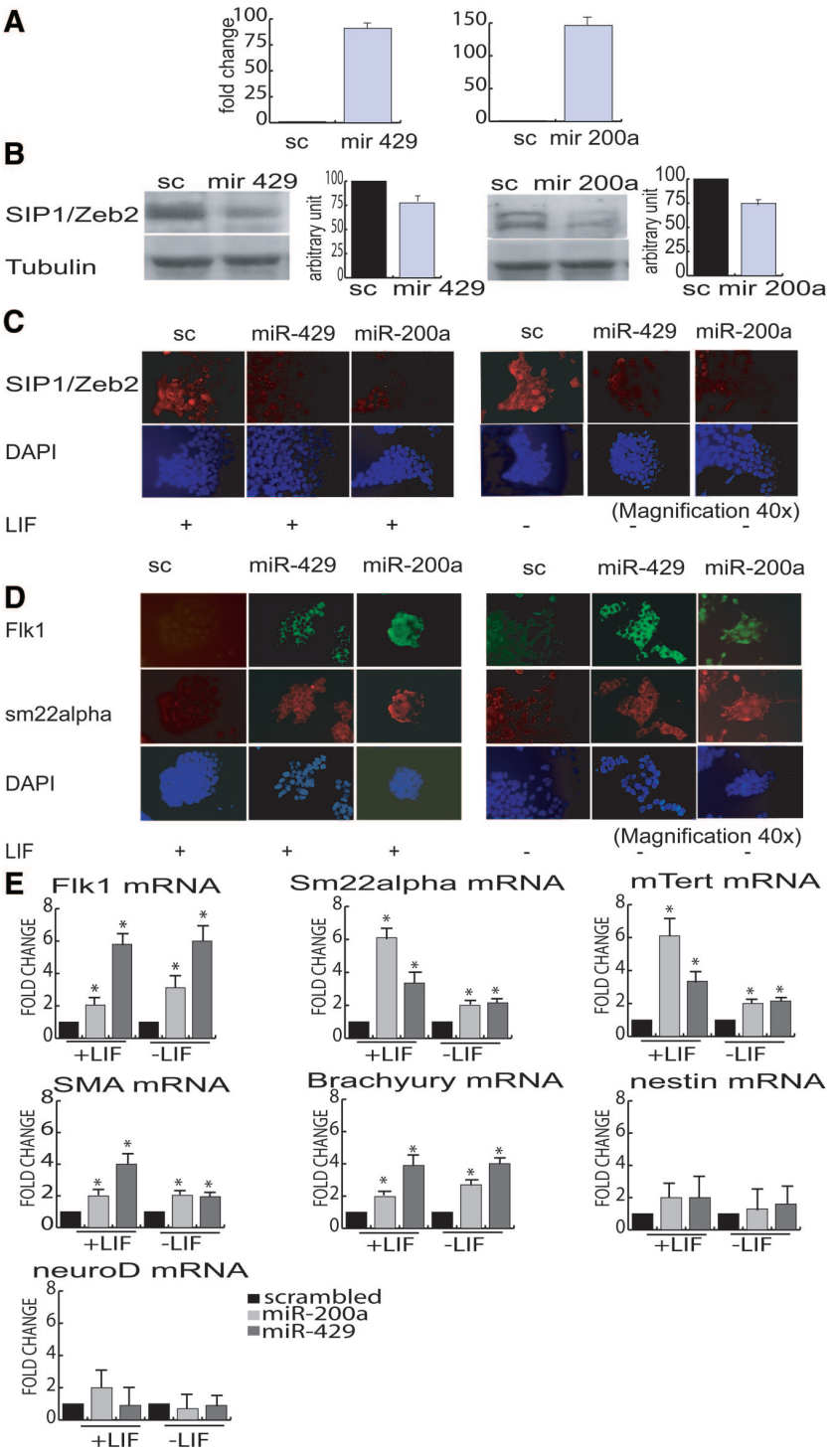

Figure 3. miR-200s overexpression stimulates mesodermal gene expression. A, Result of qRT-PCR showing pre-miR (mir)-429 or -200a expression. B, Representative immunoblotting of Sip1/ZEB2 protein levels in transfected cells. Three independent experiments were performed. C, Result of immunofluorescence analyses performed on mES transfected with a scrambled control (sc) or miR-429 and -200a. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. This result is representative of at least 3 independent experiments. D, Immunofluorescence analysis of Flk1 (upper panel) and SM22α (middle panel) expression in mES transfected with pre-miR-429 or pre-miR-200a. Results are representative of 3 independent experiments. E, qRT-PCR analysis of differentiation markers in cells transfected with miRNA precursors. Data represent mean±SD of 3 independent transfections. *$P<0.005$.

todermal differentiation, allowing mesendoderm-associated gene expression to occur only when its expression decreases.[24] To evaluate the direct role of Sip1/ZEB2 in the context of the NO-dependent differentiation of mES, a series of stable transfectants was generated with different shRNA vectors targeting mouse Sip1/ZEB2. The results of these experiments identified 2 shRNA clones, clones 1 and 2, that effectively downregulated Sip1/ZEB2 at protein level (Figure 4A). Phase contrast microscopy showed that the shRNA-dependent downregulation of Sip1/ZEB2 caused dramatic morphological changes in mES, although cultured in the presence of LIF (Figure 4B). Specifically, cells with reduced Sip1/ZEB2 content became spindle-shaped and acquired a stromal-like phenotype compared with controls. Under these conditions, similarly to the NO treatment, the expression of pluripotency genes, including OCT4 and Nanog, were significantly de-

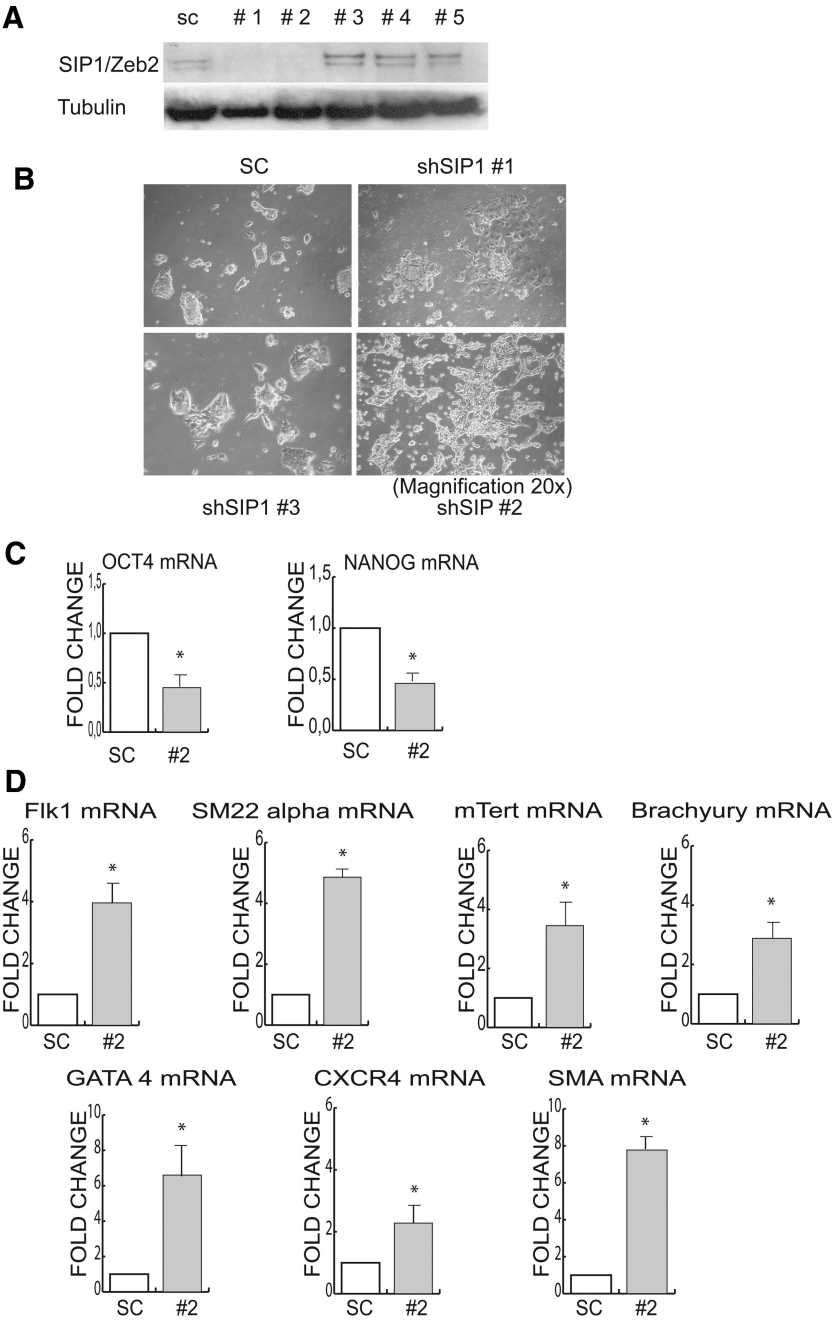

Figure 4. Sip1/ZEB2 shRNA induces the appearance of mesodermal markers in mES. A, Western blot analysis after Sip1/ZEB2 interference (shSIP1 #1) compared to scrambled control (sc). B, Phase contrast microscopy showing mES morphology after Sip1/ZEB2 interference. C, qRT-PCR analysis of Oct4 and Nanog mRNA in shSIP1 clone 2. D, qRT-PCR analysis of mTert and early mesoendoderm/vascular genes in shSIP1 clone 2. Data represent mean±SD. *$P<0.005$.

creased (Figure 4C). Conversely, early mesendoderm genes and markers associated with vascular precursors, including Flk1, SM22α, mTERT, Brachyury, GATA4, CXCR4, and smooth muscle actin, were increased (Figure 4D). These results suggest that Sip1/ZEB2 downregulation is important for the NO-dependent early commitment of mES to a mesendoderm/vascular-like phenotype[6] (see also Supplemental Figures IV and V and Supplemental Tables VII, IX, and X). Notably, the mES differentiation determined by Sip1/ZEB2 knockdown was obtainable in the presence of LIF (Figure 5A), further suggesting that appropriate levels of this factor are required for stemness maintenance. These findings were further confirmed by qRT-PCR and gene array analyses performed in the presence or absence of NO (Figure 5B and Supplemental Tables I to X). Interestingly, this process was paralleled by global and local changes in the chromatin landscape. Figure 5C shows that, in cells exposed to NO or in clone 2 bearing a reduced Sip1/ZEB2 content, the global H3Ac was increased, a process that was paralleled by a significant upregulation in histone H4 lysine 20 trimethylation (H4K20me3). Intriguingly, it has recently been reported that the low density of H4K20me3, typically present in chromatin domains of telomeric and subtelomeric regions, may be an epigenetic mark associated with stemness.[33] Therefore, a global increase in H4K20me3 levels, which is suggestive of an ongoing differentiation process,[34] could be

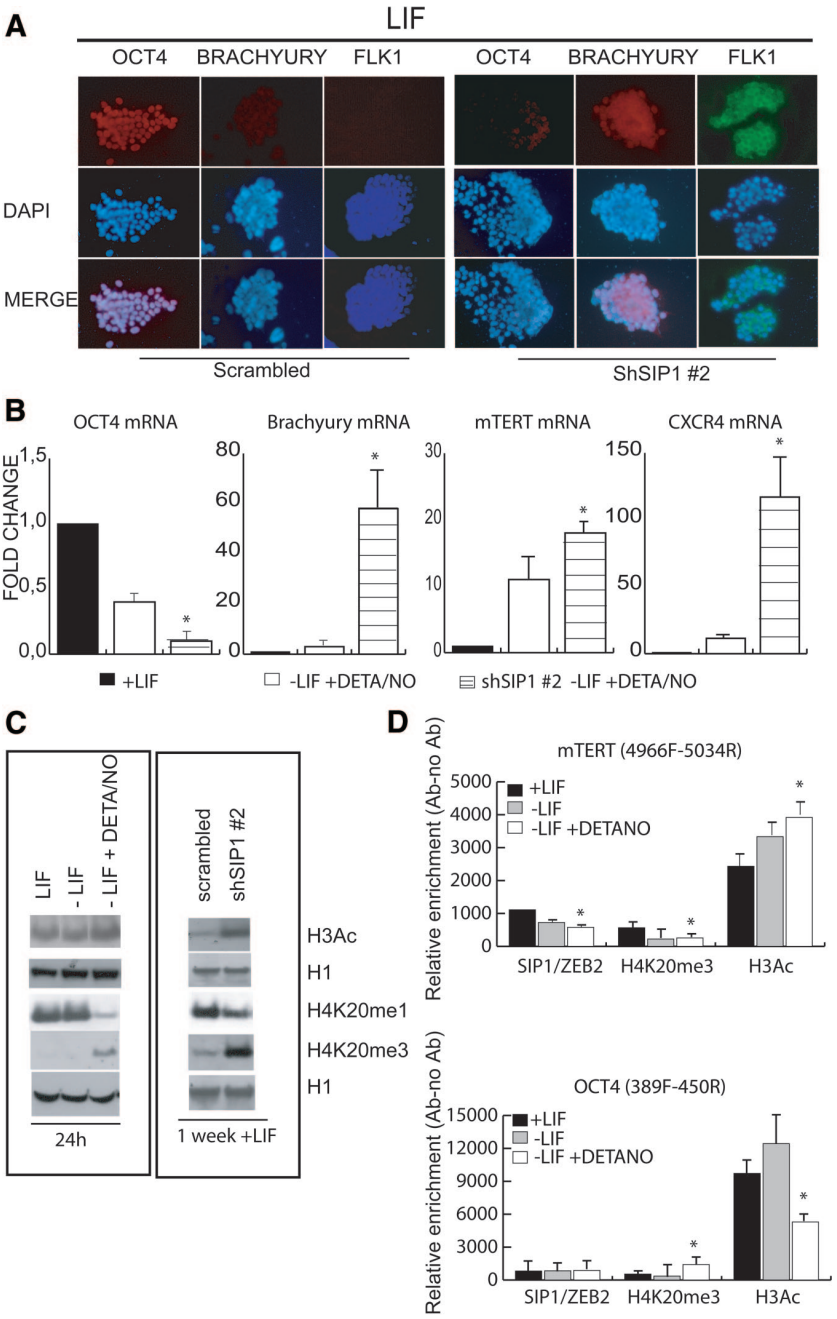

Figure 5. SIP1/Zeb2 shRNA interference reproduces NO effects in undifferentiated mES. A, Immunofluorescence analysis of differentiation marker expression in shSIP1 clone 2. B, qRT-PCR analysis of marker expression in shSIP1 clone 2. Cells were cultured in the presence of DETA/NO. C, Evaluation of H4 Lysine 20 mono- and trimethylation and total H3 acetylation in cells evaluated with or without DETA/NO or after Sip1/ZEB2 interference. Panels are representative of 3 independent experiments. D, chromatin immunoprecipitation analysis performed on mTert and Oct4 promoters. Cells were treated for 16 hours in the absence of LIF with or without DETA/NO. Immunoprecipitations were performed using antibodies to Sip1/ZEB2, H4K20me3, H3Ac. The no-antibody (NoAb) condition was used as negative control. Data are mean±SD of 3 independent experiments and represent relative enrichment over control. *$P<0.005$.

proposed as a further hallmark of the NO dependent commitment of mES to differentiate. The coincident presence of H3Ac, which is typically a transcription activation-prone signal, and H4K20me3 may either be consequence of the embryonic dual labeling of histones present in regions poised for transcription,[35] as well as possibly reflecting the structure of different portions of the genome, which could be activated or repressed during differentiation. To foster this possibility, chromatin immunoprecipitation experiments were performed on OCT4 and mTert promoter regions. Figure 5D shows that acetylation of histone H3 increased on mTert nucleosomes, whereas H4K20me3 significantly decreased, paralleled by a reduction in Sip1/ZEB2 binding. Conversely, H4K20me3 was significantly upregulated and H3Ac reduced in the promoter region of OCT4 that was repressed by NO. Taken together, these results suggest that chromatin remodeling is at the basis of the expression of miRs and other genes during mES differentiation in the presence of NO.

Discussion

In the present work, the following experimental points were established: (1) the NO-dependent expression of the miR-200 family; (2) the NO-dependent downregulation of Sip1/ZEB2 protein level, which coincided with (3) mTert upregulation and mesendoderm, mesoderm, and endoderm gene expression; and (4) that the direct expression of miR-200 family

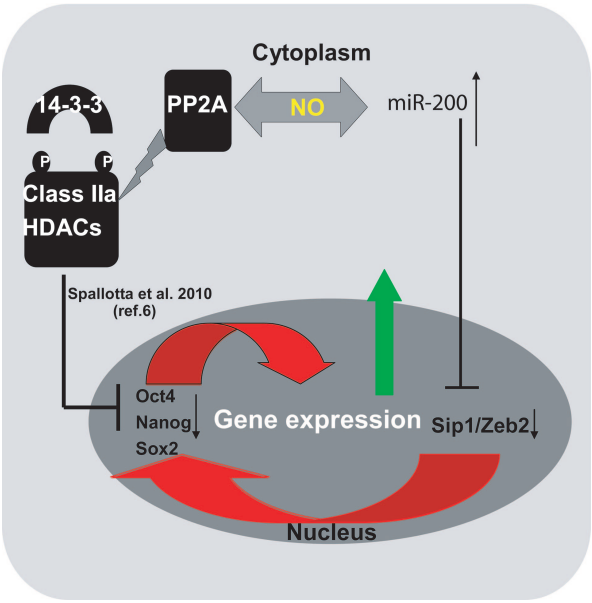

Figure 6. Schematic representation of NO mechanism of action in mES. The exposure of mES to a source of NO activates 2 pathways implicated in mesendoderm differentiation. (1) On LIF withdrawal, NO activates protein phosphatase 2A (PP2A) and class IIa histone deacetylases (HDACs) to localize into the nucleus, a phenomenon associated with the transcriptional repression of stem genes and the activation of a mesoderm developmental program.[6] (2) NO upregulates the miR-200 family, which targets Sip1/Zeb2 mRNA. Sip1/Zeb2 decrease determines repression of stemness genes in either the presence or the absence of LIF, mTert activation, and mesendoderm, mesoderm, and endoderm differentiation. Under these conditions, the expression of vascular precursors-associated markers is also promoted.

members or the knockdown of SIP1/ZEB2 recapitulated the biological effects of NO, providing evidence for an unprecedented NO-dependent multiplayer molecular circuitry controlling embryonic cell fate (Figure 6). NO, in fact, determines miR-200a, -200b, -200c, and -429 expression, which induced mesendoderm and precursor vascular marker expression. This process was characterized by Sip1/ZEB2 downregulation, which is a well-established target of the miR-200 family.[22] Remarkably, the direct targeting of Sip1/ZEB2 reproduced or enhanced the NO-dependent phenotype even in cells cultured in the constant presence of LIF. These findings suggest that NO activated a molecular mechanism dominant in LIF inhibition and depending on the activation of the miR-200 family or Sip1/ZEB2 reduction. The role of these molecular effectors in embryonic stem cells is still controversial and requires further investigation. Specifically, an antidifferentiation effect has been associated with the miR-200 family,[36] but, consistent with our results, the expression of Sip1/ZEB2 has been found to counteract the mesendoderm differentiation of human embryonic stem cells.[24] In this context, we define an unprecedented molecular network in which the presence of NO triggers the expression of members of the miR-200 family, mastering downstream effects that are important for the early mES commitment to mesendoderm differentiation.

We previously reported that shear stress–activated or NO-treated mES formed capillary-like structures on Matrigel and were angiogenic in vivo.[6,37] In the present work, however, NO-treated mES or cells with a reduced Sip1/Zeb2 content failed to form capillary-like structures on Matrigel (not shown), suggesting that their differentiation process is incomplete and that the capacity to form in vitro tubular structures is not yet present at this stage. Further work is required to elucidate this point.

Although Tert contribution to mesendoderm and early vascular differentiation is still uncertain, our evidence indicates that it may act as a molecular switch important for the transition from undifferentiated mES to early committed cells, reflecting its property to influence cell differentiation [8,10,11,13–15,17]. Because NO induces mES to differentiate preferentially into cells expressing mesendoderm, mesoderm, and endoderm genes with weak or no effect on neuroectoderm markers, it is conceivable that Tert activity could be required for the progression of that specific developmental program.

miRNAs are important regulators involved in the establishment and maintenance of pluripotency or in the regulation of common sets of target genes in the early stages of embryonic development.[38] Sip1/ZEB2 is among the best characterized targets of the miR-200 family, and it has been demonstrated to play a pivotal role in epithelial/mesenchymal transition.[22] A link between NO and the regulation of the miR-200/Sip1/ZEB2 circuitry has not yet been demonstrated. To investigate the role of Sip1/ZEB2 during mES differentiation, shRNA experiments were performed. The results raised questions about a potential novel function of this molecule in mES biology. In fact, Sip1/ZEB2 knockdown was sufficient to negatively regulate the expression of stemness genes, enhancing the density of epigenetic markers associated with the control of gene expression or differentiation, such as H3Ac and H4K20me3, even in the presence of the potent inhibitor LIF. This observation is further supported by the evidence that NO transcriptionally upregulated miR-200a, -200b, -200c, and -429 and by the fact, reported by others, that Sip1/ZEB2 expression preferentially differentiated human embryonic stem cells along a neuroectodermal lineage.[24] Altogether, our findings shed light on a novel role of the Sip1/ZEB2 protein, which either represents a negative regulator of mES mesendoderm commitment or, more intriguingly, is part of the transcriptional circuitry regulating mES stemness, as its removal had consequences similar to those determined by Nanog deletion.[39] In summary, our work describes a novel NO-dependent molecular mechanism ruling mES mesendoderm and early vascular differentiation, which may occur in parallel with the histone deacetylase-dependent pathway previously described[6] (Figure 6). It further represents the first demonstration of a direct NO-dependent regulation of miRNAs and ascribes a role to the NO/miR-200 family/Sip1/ZEB2 circuitry at the crossroads of early cell fate decisions in mES.

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Disclosures

None.

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