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Polycomb YY1 is a critical interface between epigenetic code and miRNA machinery after exposure to hypoxia in malignancy

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ABSTRACT

Yin Yang 1 (YY1) is a member of polycomb protein family involved in epigenetic modifications and transcriptional controls. We have shown that YY1 acts as positive regulator of tumor growth and angiogenesis by interfering with the VEGFA network. Yet, the link between polycomb chromatin complex and hypoxia regulation of VEGFA is still poorly understood. Here, we establish that hypoxia impairs YY1 binding to VEGFA mRNA 3′UTR ($p < 0.001$) in bone malignancy. Moreover, RNA immunoprecipitation reveals the formation of triplex nuclear complexes among YY1, VEGFA DNA, mRNA, and unreached about 200 fold primiRNA 200b and 200c via Dicer protein. In this complex, YY1 is necessary to maintain the steady-state level of VEGFA expression while its silencing increases VEGFA mRNA half-life at 4 h and impairs the maturation of miRNA 200b/c. Hypoxia promotes histone modification through ubiquitination both of YY1 and Dicer proteins. Hypoxia-mediated down-regulation of YY1 and Dicer changes post-transcriptional VEGFA regulation by resulting in the accumulation of primiRNA200b/c in comparison to mature miRNAs ($p < 0.001$). Given the regulatory functions of VEGFA on cellular activities to promote neoangiogenesis, we conclude that YY1 acts as novel critical interface between epigenetic code and miRNAs machinery under chronic hypoxia in malignancy.

1. Introduction

Tumor adaptive response to hypoxia involves a variety of epigenetic, transcriptional and posttranscriptional events [1]. Several lines of evidence establish that hypoxia is an important regulator of histone modifications, miRNA biogenesis and function [1,2].

The evolutionary conserved Polycomb proteins (PcGs) modify chromatin structure promoting histone methylation, or monoubiquitination [3,4] and preserve epigenetic memory [5,6]. In addition, PcGs control transcriptional programs, [7] acting as repressors in normal tissues [7,8]. PcGs can form two complexes the repressive complex 1 (PRC1) which includes proteins such as Yin Yang 1 (YY1), BMI1, HPH1–3, HPC proteins, RING1A–1B [9] and PRC2/3/4 with core protein EZH2 [10,11].

YY1 is associated with both complexes [12] and it is the only PcG protein with a sequence-specific DNA binding element (CCATnTT) and RNA binding properties [13]. Although, YY1 is mainly described as a transcription factor [13], the association between YY1 and PRC1/2 provides a direct link between the chromatin-PcG complex and the DNA of target genes [14].

YY1 has been shown to be involved in the development and/or progression of several solid and hematological tumors by interacting with cell cycle regulation. Indeed, YY1 overexpression is associated with unchecked cellular proliferation, resistance to apoptotic stimuli, tumorigenesis and metastatization [15–17]. Our previous findings have identified that YY1 is overexpressed in bone malignancy favoring tumor growth [18–20]. Silencing of YY1 can reduce vessel formation and neoangiogenesis [20–22]. Since the vascular endothelial growth factor A (VEGFA) is a powerful effector of neoangiogenesis under hypoxic condition [23] and it is the most frequently duplicated gene in malignancy of the bone, [24–27], it would be interesting to define the regulative cross talk between YY1/VEGFA.

MicroRNAs (miRNAs) are small RNA molecules with a regulatory function and tissue specificity that modulate several target genes involved in cellular processes such as proliferation, differentiation and apoptosis. They predominantly target the 3′UTR mRNA and are frequently deregulated in cancer. In this context, YY1 has shown to be the target of several miRNAs in different tumor types thus acting as tumor activator or suppressor [28,29].

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In our experimental conditions, we show that polycomb YY1 in the partecipates posttranscription regulation of VEGFA by providing a critical interface between chromatin remodelling and miRNA complexes.

2. Results

2.1. Role of polycomb YY1 on VEGFA expression during hypoxia

To understand whether YY1 could regulate specific VEGFA isoforms, we analyzed VEGFA mRNA isoforms 121, 165, and 189 in osteoblasts and osteosarcoma cell lines. In normal osteoblastic cells, VEGFA 165 mRNA was the predominant isoform, accounting for 78.8% of total VEGFA transcripts whereas in osteosarcoma cells it varied between 3 and 20% of total mRNAs, as well as VEGFA 121 (Fig. 1A). Despite changes in the expression of different isoforms, the amount of total VEGFA mRNA was higher in normal cells vs. osteosarcomas (Fig. 1B). As expected, the ratio between pro- and anti-angiogenic VEGFA isoforms was shifted towards the pro-angiogenic in osteosarcoma cells while in normal cells the two isoforms were balanced (Suppl. Fig. 1). The effect of YY1 and its undistinguished homologous YY2 on VEGFA mRNA isoforms were evaluated by using stable cell lines silenced for YY1/2, (Suppl. Fig. 2A, B). As indicated in Fig. 1B, silencing of YY1/2, both in Saos and U2os cells, determined a significant accumulation of VEGFA mRNAs 121/165 which was more evident as protein accumulation monitored by ELISA (Fig. 1C) and western blots vs. controls Saos and U2os pBlock-it cells (Fig. 1D). No VEGFA protein accumulation was observed in osteoblasts (Fig. 1C and D). Hypoxia, which stimulates tumor cells to release VEGFA, increased very low VEGFA transcripts in Saos pBlock-it cells (Fig. 1E). In contrast, hypoxia determined a four- fold higher protein accumulation vs. normoxia in Saos pBlock-it cells (Fig. 1F and G). These phenomena were less evident in shYY1/2 cells (Fig. 1F and G and Suppl. Fig. 2C). Interestingly, HIF-1α was down-regulated in shYY1/2 cells in both hypoxic and normoxic conditions (Fig. 1F).

2.2. YY1 influences the stability of VEGFA 121/165 mRNA

During normoxia, the silencing of YY1/2 increases VEGFA protein more than its mRNA. We hypothesized that YY1 could affect its stability. Indeed, osteoblasts (Ost) and cancer cells were treated with actinomycin D to block transcription. VEGFA 121/165 mRNAs were then measured at the indicated time points. The real time PCR data confirmed the general knowledge that VEGFA 121 and 165 mRNA half-life were 20 min in normal cells (Fig. 2A) and 15 min in tumor cells (Fig. 2B and C). In contrast, the half-life of these two VEGFA isoforms was prolonged up to about 4 h in both U2os (Fig. 2D) and Saos cells silenced for YY1/2 (Fig. 2E). Therefore, it is likely that the stabilization of VEGFA transcripts accounts for the higher steady state level of VEGFA mRNA and in accordance with the accumulation of VEGFA protein in response to YY1/2 silencing (Fig. 1C, D, F, and G). Yet, VEGFA mRNA stability measurements which are known to be dependent on phosphorylation of posttrascriptional regulator proteins [30–33] were also performed in the presence of PD98059 and SB239063 inhibitors MAPKinase pathway. Both inhibitors increase VEGFA 121/165 mRNAs half life in normal and tumor cells (Fig. 2A–C). Interestingly, PD98059 and SB239063 did not affect VEGFA mRNA stability in shYY1/2 cell lines (Fig. 2D and E). These findings suggest that YY1 could interfere with the same pathway. Finally, hypoxia increases stability of VEGFA 121 and 165 mRNAs in Saos pBlock-it cells (Fig. 2F) while did not in shYY1/2 cells (data not shown). Thus, YY1 could play different roles in the posttranscriptional regulation of VEGFA in normoxia vs. hypoxia conditions.

2.3. VEGFA chromatin status in normoxia and hypoxia

VEGFA is posttranscriptionally controlled by the miRNA machinery and through the binding of stabilizing and destabilizing proteins to the AU-rich element (ARE) domains located in the 3′UTR and 5′UTR of its mRNA [30–33]. In order to investigate the mechanism underlying the influence of polycomb YY1 on VEGFA mRNA stability, VEGFA gene was scanned for YY1 consensus binding sites with MatInspector Professional 8.0 software (http://www.genomatix.de). The analysis revealed several YY1 binding sites in the 3′UTR region (Fig. 3A). To investigate for possible physical interactions with genomic DNA, we carried out ChIP assays using anti-YY1 antibody and real time PCR on pulled-down material in Saos cells. Among all predicted binding sites, the amplicons of two of them, located approximately at positions CHR6:43752323 + 43752800 and CHR6:43753242 + 43753823 of the VEGFA 3′UTR (called region 1 and 2 respectively), were enriched after YY1 immunoprecipitation (Fig. 3A). We performed such experiments in normoxic and hypoxic conditions by investigating epigenetic marks due to the involvement of YY1 in epigenetic modifications. In normoxia, data reported as fold change over non-immunoprecipitated DNA, revealed that histone methylation (H3K27me3) was 7 fold higher in region 1 than to region 2 (Fig. 3B and Suppl. Fig. 3A, B) and YY1 protein enriched (about 3 fold) region 1 compared to region 2 (Fig. 3B). No enrichment was observed with IgG antibodies showed as semiquantitative gel analysis (Suppl. Fig. 3A, B). During hypoxia, YY1 protein enriched region 2 and histone H2A was ubiquitinated compared to region 1, (Fig. 3C and Suppl. Fig. 3A, B). Silenced of YY1/2 did not influence histone methylation of region 1 (Suppl. Fig. 3A, B) while affected H2A ubiquitination at region 2 (Fig. 3A and Suppl. Fig. 3B) suggesting that YY1 may be involved in epigenetic modification during hypoxia.

2.4. Polycomb YY1 is recruited on VEGFA mRNA during normoxia and hypoxia

Since polycomb YY1 protein can bind both DNA and RNA, [34,35] and because we observed a binding to the 3′UTR region of the VEGFA gene, we hypothesized that YY1 may bind also VEGFA mRNA (Fig. 4A). To investigate interactions between YY1 and VEGFA mRNA, we performed RNA immunoprecipitation (RNA ChIP) with anti-YY1, anti-HIF-1α and anti-TTP antibodies following crosslinking of RNA to protein. We analyzed these interactions both in normoxia and hypoxia. Real time PCRs of YY1 pull-down material (performed with primer sets on 5/7 exons junction see Table 1) showed significant co-immunoprecipitation of VEGFA mRNA in normoxia (Fig. 4B) also showed as semiquantitative gel analysis (Suppl. Fig. 4A). Data, reported as fold enrichment over non-immunoprecipitated samples, indicated that interaction between VEGFA mRNA and YY1 was specific. This was evident from the absence of VEGFA mRNA in shYY1/2 cells, IP with anti-YY1 antibodies which was restored in sh.scramble cells (Suppl. Fig. 4A and B). Additionally, the interaction was not detected without crosslinking, in RT-negative samples and when non-specific IgG antibodies were used (Fig. 4B and Suppl. Fig. 4A). During hypoxia, YY1 became ubiquitinated, TTP occupancy was very low (Fig. 4C and Suppl. Fig. 4A, B) and HIF-1α antibody enriched VEGFA mRNA by 160 fold in hypoxia compared to control (Fig. 4C and Suppl. Fig. 4B). Interestingly, silencing of YY1/2 reduced the occupancy of VEGFA mRNA by both TTP and HIF1α proteins in both normoxic and hypoxic conditions (Fig. 4B, C and Suppl. Fig. 4A, B). This finding supports the hypothesis that YY1 is necessary to stabilize HIF-1α and TTP on VEGFA mRNA. Therefore, we tested whether there was a direct interaction among these factors, and western blots, following immunoprecipitation, confirmed that YY1 bound both HIF-1α and TTP proteins (Fig. 4D). The interaction was not detected when IgG antibodies were used (Fig. 4D). Silencing of TTP determined an increase of VEGFA 165/121 mRNAs stability and a decrease of protein suggesting that TTP regulated the translational machinery of VEGFA (Suppl. Fig. 5).

2.5. YY1 recruits RNAi machinery component on VEGFA mRNA

In attempt to determine the mechanism of the YY1-dependent VEGFA mRNA decay, we dosed mature and primiRNA 16 as well as