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Article

Oxygen-sensitive TERT promoter methylation regulates telomerase activity

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Abstract: Telomere repeats at the ends of human chromosomes protect chromosomes from degradation, and telomerase has a prominent role in telomere maintenance. Telomerase also affects cell proliferation, DNA replication, differentiation, and tumourigenesis. TERT (telomerase reverse transcriptase enzyme) is the catalytic subunit of telomerase and is critical for enzyme activity. TERT promoter mutations and promoter methylation are strongly associated with increased telomerase activation in cancer cells. Notably, TERT and telomerase are downregulated in stem cells during their differentiation. Therefore, the link between differentiation and telomerase provides a valuable tool for studying the epigenetic regulation of TERT enzyme. Oxygen tension affects several cellular behaviours including proliferation, metabolic activity, stemness, and differentiation. The role of oxygen tension in driving promoter modifications of the TERT gene in embryonic stem cells (ESCs) is poorly understood either in vitro or in vivo. We adopted a monolayer ESCs differentiation model to explore the role of low, physiological, oxygen (physoxia) in the epigenetic regulation of telomerase and associated genes, including TERT, DNMTs, and HDACs. Cells were cultured in either air, a 2% O2 incubator, or a 2% O2 oxygen workstation to provide a fully defined 2% O2 environment. Pre-gassed media (pre-conditioned to 2% O2 in a HypoxyCool unit) was used in all 2% O2 experimentation. As anticipated, physoxia culture increased the proliferation rate and stemness of ESCs and a slower onset of differentiation in physoxia was evident. Further, downregulated TERT expression was correlated to reduced telomerase activity during differentiation. TERT expression and telomerase activity remained significantly elevated in physoxia during differentiation. A substantial increase in TERT promoter methylation levels was noted during differentiation. Chemical inhibition of DNMT3B reduced TERT promoter methylation and was associated with increased TERT gene and telomerase activity during differentiation. DNMT3B CHiP demonstrated that downregulated TERT expression and increased proximal promoter methylation were associated with DNMT3B binding to the promoter. In conclusion, we have demonstrated that DNMT3B can directly bind TERT promoter, change its methylation levels, and contribute to regulation of telomerase activity.

Keywords: telomerase, TERT promoter, DNMT3B, pluripotent stem cells, characterization, epigenetic, methylation, physiological oxygen, DNA methyltransferase

1. Introduction

Embryonic stem cells (ESCs) have inherent capacities of self-renewal, high proliferation capacity, pluripotency, telomerase activity, and long telomeres [1], [2]. ESCs can differentiate into multiple tissue cell types of representatives of endoderm, ectoderm, and mesoderm germ layers [3]. Telomerase is highly expressed in ESCs, and through maintenance of telomere length plays a role in maintaining ESCs pluripotency, proliferation and self-renewal [4], [5]. Somatic cells express very low or no detectable levels of telomerase [6].

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Telomerase enzyme consists of protein and RNA (TERC) essential components and is the primary mechanism for telomere maintenance and elongation [7], [8]. TERT, the catalytic protein subunit, is a rate-limiting factor for telomerase activity [9]. Knockdown of TERT results in loss of pluripotency, loss of clonogenicity, and spontaneous differentiation of ESCs [10]. TERT transcriptional silencing during the differentiation of stem cells and activation during the transformation into cancer cells is still poorly understood. It is clear that genetic and epigenetic changes play a role in the regulation of TERT gene, including promoter mutations, DNA methylation, histone modification, and non-coding RNAs.

Epigenetic modifications have a crucial role in cell fate including upregulation of lineage-specific genes and decreased expression of self-renewal related genes [11], decreased TERT expression and telomerase enzyme activity [12], [13] during differentiation. Undifferentiated ESCs have a unique epigenetic signature compared to differentiated and somatic cells [14]–[17]. DNMT3A and DNMT3B are highly expressed in undifferentiated ESCs compared to somatic cells and down-regulated during differentiation [18]–[20]. DNMT1, DNMT3A, and DNMT3B are three major DNA methyltransferases responsible for methylation in mammals [21], [22]. DNMT1 can distinguish hemimethylated DNA during DNA replication and maintains global methylation patterns following replication [23]. DNMT3A and DNMT3B de nova methyltransferases establish DNA methylation patterns during gametogenesis, embryogenesis and somatic tissue development working in coordination with DNMT1 [24]. DNMT3L (DNA methyltransferase 3-like) cooperates with DNMT3A and DNMT3B to stimulate their catalytic activity and is highly expressed in ESCs [25]. Somatic cells and ESCs display distinct DNA methylation signatures associated with lineage specification [17], [26], [27]. GC islands at distal TERT promoter are critical for telomerase expression. Silencing of DNMT3B with chemotherapeutic drugs, adriamycin and azacytidine reduced hTERT expression, and led to an increase in senescence-associated beta-galactosidase activity in glioma cell lines in the zebrafish [28]. They showed that DNMT3B together with GC islands in distal TERT promoter plays an important role in the regulation of telomerase expression [28], [29]. In our study, we observed a decreased methylation at distal and proximal promoter regions and increased TERT expression after nanaomycin A treatment (DNMT3B selective inhibitor) in human ESCs.

Physiological oxygen tensions vary across organs and tissue components and typically range from 1–14 % in vivo for most tissues depending on the distance away from the vascular system [30]–[32]. The physiological normoxia (physoxia) environment for ESCs typically ranges from 2 to 5% [33]. Oxygen affects epigenetic modifications, and epigenetics is essential for initiation of hypoxic response pathways [34]. Cells cultured in air oxygen conditions have higher oxidative stress, DNA damage, genomic instability, and senescence due to the formation of reactive oxygen species [31]. Reduced oxygen tension changes stem cell characteristics and physiology, such as proliferation, differentiation, pluripotency, genomic stability and DNA methylation [35]–[39]. Low oxygen culture conditions decrease global DNA methylation levels across a range of cancer and stem cells [42], [43]. Therefore, investigating the role of physiological oxygen on discrete epigenetic profiles is increasingly relevant for mammalian stem cell culture.

Here, we have investigated the effect of physoxia on stem cell differentiation and telomerase activity. We have explored the association between TERT expression and gene promoter methylation. In summary, we noted a higher proliferation rate of ESCs in reduced oxygen culture. Monolayer differentiated cells expressed mesodermal markers predominantly with slower onset of differentiation in physoxia. Telomerase activity, TERT expression, and telomere length were decreased during differentiation following increased methylation on the TERT promoter. We report higher telomerase activity, TERT expression, and telomere length in physoxia. CHIP (Chromatin immunoprecipitation) data revealed that decreased TERT expression and increased proximal promoter methylation were associated with DNMT3B binding to the promoter region. Our data suggested that methylation, oxygen environment, DNMTs and histone methyltransferases could play a crucial role in TERT regulation during stem cell differentiation.

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2. Results

Characterization of Monolayer Differentiated ESCs

Cell proliferation and viability were higher in reduced oxygen conditions (Fig S1). The expression of pluripotency markers (Oct-3/4, Nanog, SSEA-1, SSEA-4, and alkaline phosphatase) were analyzed in undifferentiated and differentiated ESCs using immunofluorescence staining. There was a gradual decrease in expression of Oct-3/4, Nanog, SSEA-4, and alkaline phosphatase and increased SSEA-1 during differentiation of ESCs (Fig S2).

Gene expression associated with three germ layer differentiation was analyzed by qRT-PCR, including Nestin, TBXT (T-Box Transcription Factor T), KDR (Kinase Insert Domain Receptor) and AFP (Alpha-fetoprotein). Differentiated SHEF1 cells displayed higher ($p<0.001$) Nestin expression at day 5 in 21% AO versus 2% PG and 2% WKS. A downregulation in Nestin gene expression was observed on day 10 ($p<0.001$) and day 20 in all conditions ($p<0.001$) versus undifferentiated cells (Fig 1.A). Nestin expression was higher in 21% AO versus 2% WKS ($p<0.01$) microenvironment in undifferentiated SHEF2. Reduced Nestin expression was noted in differentiated SHEF2 at day 5 in 2% PG ($p<0.05$) and days 10, 20 in 2% WKS ($p<0.05$) versus 21% AO. There was a significant decrease in Nestin expression on days 20 and 40 ($p<0.001$) in all conditions compared to undifferentiated cells (Fig 1.B). Pooled data showed a significant decrease in Nestin expression at day 10 ($p<0.01$), 20 ($p<0.001$) and 40 ($p<0.001$) in all conditions when compared to undifferentiated cells. There was a significant decrease in 2% PG, 2% WKS on day 5 ($p<0.01$) and 2% WKS on day 10 ($p<0.05$) when compared to air oxygen (Fig 1.C).

A significant increase in KDR gene expression was observed in monolayer differentiated cells at days 10 ($p<0.01$), 20 ($p<0.001$) and 40 ($p<0.001$) in all conditions compared to undifferentiated SHEF1 cells. KDR gene expression was decreased at day 5 in 2% PG ($p<0.05$) and 2% WKS ($p<0.01$) versus 21% AO (Fig 1.A). A significant increase in KDR expression was noted on days 5, 10, 20, and 40 ($p<0.001$) in all conditions compared to undifferentiated SHEF2 cells. Undifferentiated and day 5 differentiated SHEF2 cells in 2% PG ($p<0.05$) displayed less KDR expression than air oxygen. Reduced expression of KDR was reported in 2% WKS at days 10 ($p<0.05$) and 20 ($p<0.01$) compared to 21% AO. (Fig 1.B). Combined data showed a significant decrease in 2% WKS ($p<0.05$) compared to 21% AO. KDR expression was decreased in differentiated SHEF at day 5 in 2% PG and 2% WKS ($p<0.001$) and at day 20 in 2% WKS ($p<0.01$) versus 21% AO. Overall, there was a significant increase in gene expression in all conditions during differentiation at days 10 ($p<0.01$), 20 ($p<0.001$) and 40 $p<0.001$) compared to undifferentiated cells (Fig 1.C).

Elevated mesodermal marker, TBXT, expression was noted in undifferentiated 21% AO SHEF1 vs. 2% PG ($p<0.05$) and 2% WKS. TBXT expression was significantly elevated in differentiated SHEF1 at days 5, 10, 20, and 40 ($p<0.001$) vs. undifferentiated cells (Fig 1.A). Similarily, undifferentiated SHEF2 cells showed a significant reduction in TBXT expression in 2% PG ($p<0.05$) and 2% WKS ($p<0.01$) compared to 21% AO. TBXT expression was significantly higher on day 5, 10, 20, and 40 ($p<0.001$) of differentiated SHEF2 compared to undifferentiated cells. Differentiated SHEF2 cells showed a significant decrease at 20 day in 2% PG ($p<0.01$) and 2% WKS ($p<0.01$) and at day 40 in 2% PG ($p<0.01$) compared to AO (Fig 1.B). Pooled data from undifferentiated ($p<0.01$) and 20 days ($p<0.05$) differentiated cells showed significantly less TBXT expression in physoxia versus AO. Further, TBXT expression was higher in all conditions at 5, 10, 20 and 40 days ($p<0.001$) versus 21% AO (Fig 1.C).

Endodermal marker, AFP, gene expression in monolayer differentiated SHEF1 and SHEF2 cells displayed no increase in the mean value during 5, 10, 20 or 40 days of differentiation (Fig 1). Pooled data showed decreased AFP expression at day 5, ($p<0.01$) day 10 ($p<0.001$), 20 ($p<0.01$) and 40 ($p<0.001$) in all conditions compared to undifferentiated SHEF cells (Fig 1.C).

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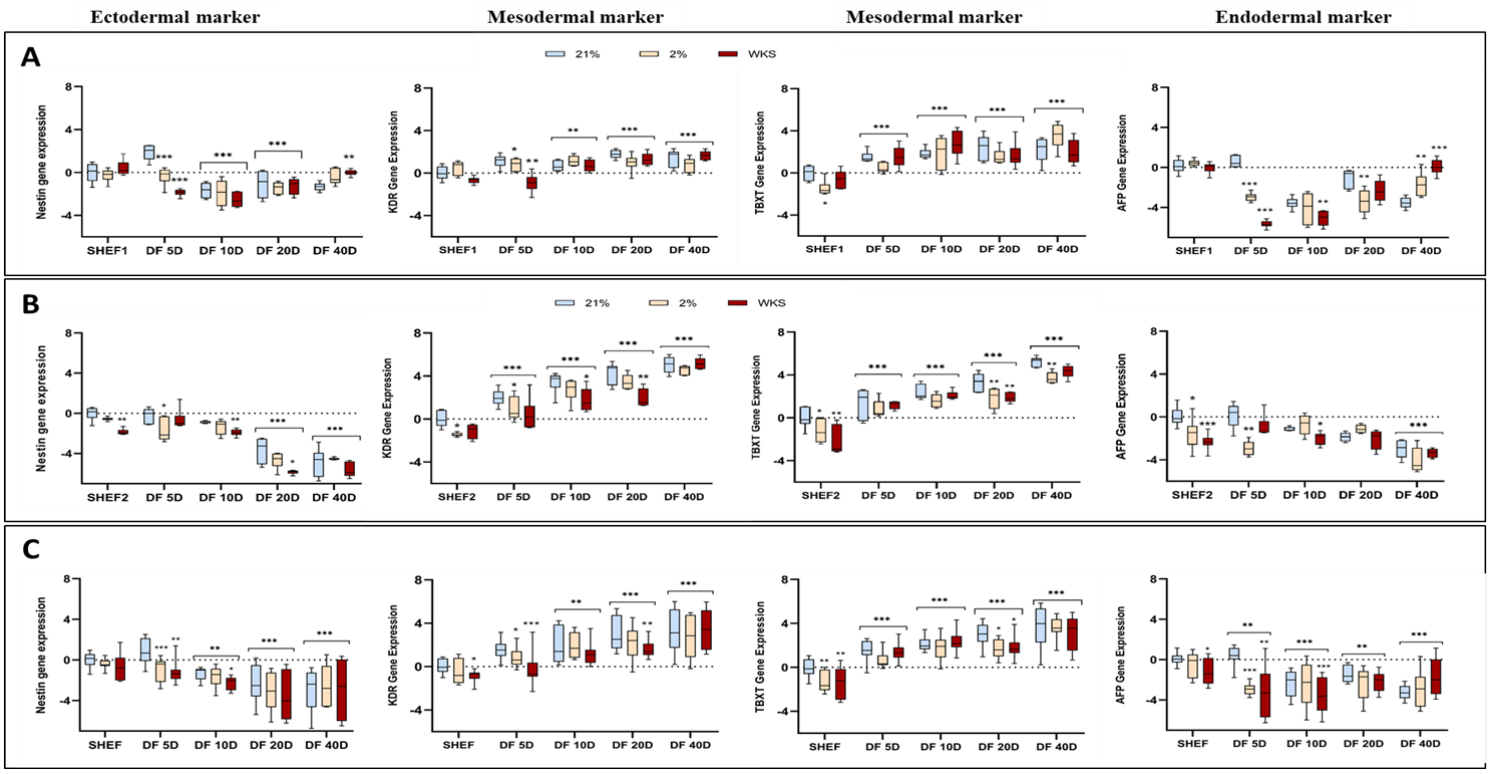

Figure 1. **Increased mesodermal markers expression in monolayer differentiated cells.** Expression of three germ layer differentiation markers was analyzed in SHEF1 (**A**), SHEF2 (**B**), and pooled data (**C**) in 21% AO, 2% PG and 2% WKS. The RT-qPCR expression ($2^{-\Delta\Delta CT}$) of the genes normalized to the expression of GAPDH. Data are represented as n=3x3, *p<0.05, **p<0.01, ***p<0.001 vs air oxygen.

Decreased TERT Expression, Telomerase Activity and telomere length during differentiation

TERT expression, telomerase activity and telomere length were determined in undifferentiated and differentiated cells. TERT expression decreased at differentiation days 5 (-1.01, p<0.001), 10 (-2.3, p<0.001), 20 (-3.6, p<0.001), and 40 (-7.3, p<0.001) in comparison to undifferentiated SHEF1 cells in 21% AO. Elevated TERT expression was noted at days 5 (-0.4, p<0.05), 10 (-1.4, p<0.001), and 20 (-3.0, p<0.05) in 2% PG vs. 21% AO. Consistently, undifferentiated SHEF1 (0.7, p<0.01), and differentiation days 5 (-0.2, p<0.01), (-1.2, p<0.001), 20 (-1.5, p<0.001), and 40 (-5.4, p<0.001) in 2% WKS displayed elevated TERT expression vs. 21% AO (Fig 2.A). TERT expression levels in undifferentiated SHEF2 were higher in 2% PG (0.9, p<0.001) and 2% WKS (1.4, p<0.001) vs. 21% AO. Downregulation was subsequently noted at at days 5 (-0.79), 10 (-2.2, p<0.001), 20 (-2.9, p<0.001), and 40 (-4.4, p<0.001) in 21% AO. TERT expression was higher after 5 days differentiation in 2% PG (0.2, p<0.01) and 2% WKS (1.3, p<0.001) in comparison to 21% AO cultured cells (-0.8). Further, SHEF2 in 2% WKS showed significantly higher expression at days 0 (p<0.001), 5 (p<0.001), 10 (p<0.001), 20 (p<0.001), and 40 (p<0.05) (Fig 2.B). Pooled data demonstrated significantly increased expression in undifferentiated stem cells cultured in 2% PG (0.7, p<0.05) and 2% WKS (0.8, p<0.05) in comparison to AO. Further, 2% PG and 2% WKS displayed elevated expression as undifferentiated (p<0.05) and after 5 (p<0.01) and 20 (p<0.001) days of differentiation vs. AO (Fig 2.C). Confirmatory TERT protein expression levels were demonstrated in SHEF1 (Fig S3.A) and SHEF2 (Fig S3.B).

Undifferentiated SHEF1 had higher telomerase activity in 2% PG (5.6 ± 0.3, p<0.05) and 2% WKS (5.7 ± 0.4, p<0.01) vs. 21% AO (5.4 ± 0.2). A significant reduction in telomerase was observed across 40 days of differentiation in all conditions. Significantly higher telomerase expression was noted on days 5 and 10 in 2% PG (5.2 ± 0.1, p<0.01 and 4.5 ± 0.1, p<0.001) versus AO. Similarly, higher telomerase expression was observed in SHEF1 in 2% WKS (5.2 ± 0.3, p<0.01, 4.1 ± 0.1, p<0.05 and 4.0 ± 0.1, p<0.001) compared to 21% AO

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on days 5, 10 and 20, respectively (Fig 2.A). There was no significant difference in undifferentiated SHEF2 cells cultured in either 21% AO, 2% PG, or 2% WKS (5.8 ± 0.2, 5.8 ± 0.1, 6.0 ± 0.1, respectively). There was a higher activity in 2% PG and 2% WKS at days 5 (5.4 ± 0.4, $p<0.001$, 5.7 ± 0.2, $p<0.001$), 10 (4.9 ± 0.6, $p<0.001$, 5.2 ± 0.3, $p<0.001$), and 20 (4.2 ± 0.3, $p<0.01$, 4.4 ± 0.4, $p<0.001$) compared to AO (4.3 ± 0.1, 3.7 ± 0.2, 3.3 ± 0.3, respectively). 2% PG condition (3.5 ± 0.5, $p<0.001$) had elevated activity in comparison to AO (2.8 ± 0.3) after 40 days of differentiation (Fig 2.B). Pooled data from ESCs indicated elevated telomerase activity in 2% PG and 2% WKS at days 5 (5.3 ± 0.3, $p<0.001$ and 5.5 ± 0.3, $p<0.001$), 10 (4.8 ± 0.5, $p<0.001$ and 4.9 ± 0.6, $p<0.001$) and 20 (3.8 ± 0.6, $p<0.01$ and 4.3 ± 0.4, $p<0.001$) compared to AO (4.4 ± 0.2, 3.7 ± 0.2 and 3.3 ± 0.2, respectively). Overall, there was a significant difference in telomerase activity between AO and reduced oxygen conditions, 2% PG and 2% WKS, in differentiated SHEF cells (Fig 2.C).

Undifferentiated SHEF1 had telomeres of 12.5 ± 1.1 kb (21% AO), 11.9 ± 1.1 kb (2% PG), and 12.9 ± 1.6 kb (2% WKS). Telomere length was longer at days 5 and 40 in 2% WKS (11.1 ± 0.7, 8.3 ± 0.7, $p<0.05$) versus AO (10.8 ± 0.5). Telomere shortening was consistent after days 5 ($p<0.001$), 10 ($p<0.001$), 20 ($p<0.001$), and 40 ($p<0.001$). Average telomere shortening was ~260 bp per replication in three conditions after 40 days. Undifferentiated SHEF2 had significantly longer telomeres in 2% WKS (12.5 ± 0.8, $p<0.05$) versus 21% AO (11.2 ± 0.8) and 2% PG (11.6 ± 1.0) conditions. Telomere shortening was consistent after days 5 ($p<0.05$), 10 ($p<0.001$), 20 ($p<0.001$), and 40 ($p<0.001$). Average telomere shortening was approximate ~207 bp per replication in three conditions. Pooled data showed a significant difference of telomere lengths in undifferentiated stem cells cultured in 2% WKS (12.7 ± 1.3, $p<0.05$) versus 21% AO (11.9 ± 1.2) and 2% PG (11.8 ± 1.0). Longer telomere lengths were noted in undifferentiated cells vs. day 5 (11.0 ± 0.8, $p<0.05$) and day 40 (8.4 ± 0.8, $p<0.01$) cultured cells in 2% WKS compare to 21% AO. All conditions demonstrated significant telomere shortening after days 5 ($p<0.001$), 10 ($p<0.001$), and 20 ($p<0.001$).

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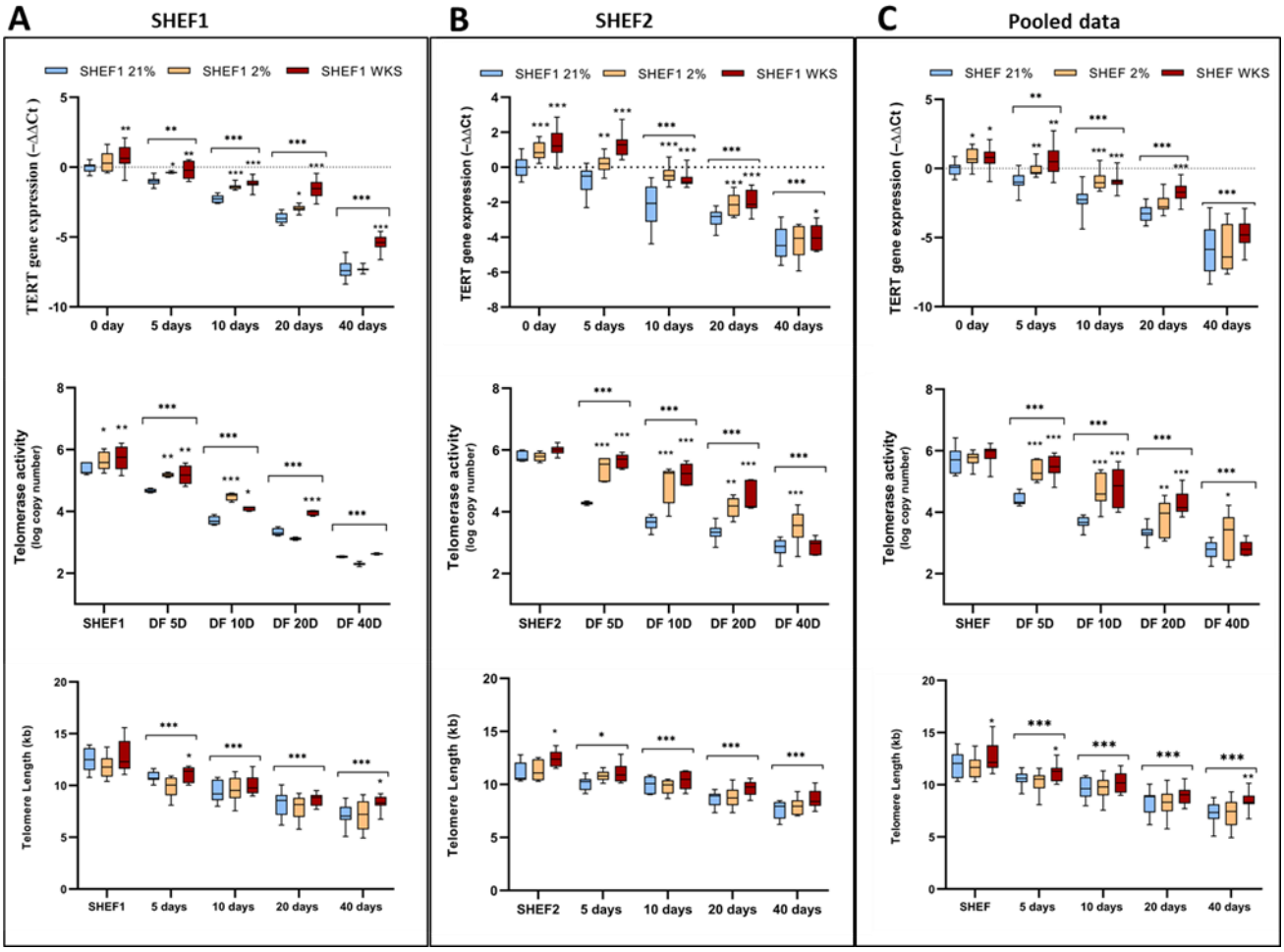

Figure 2. **Decreased telomerase activity in differentiated SHEF1 cells with higher enzyme activity in physoxia**. SHEF1 cells were cultured in air oxygen (21% AO) and physiological oxygen conditions (2% PG and 2% WKS) at different time points. Data are represented as mean ± standard deviation (SD), n=3, *p<0.05, **p<0.01, ***p<0.001.

TERT promoter methylation in hESC

TERT promoter regions were subsequently analysed to determine their extent of CpG methylation including, regions I (-1456, -1495 bp from TSS), II (-674, -717 bp from TSS), III (-315 -348 bp from TSS), IV (-122, -171 bp from TSS), and V (-67, -106 bp from TSS).

Region I was highly methylated in all conditions and during differentiation. SHEF1 showed a gradual increase in methylation across regions II, III, IV, and V with higher methylation levels noted in 21% AO than 2% WKS. Pooled data from regions II, III, IV, and V indicated increased methylation at days 10 (18.6% and 17.5%, p>0.001), 20 (22.8% and 20.4%, p>0.001), and 40 (23.2% and 21.2%, p>0.001) in AO and WKS vs. undifferentiated cells (15.7% and 10.9%), respectively. There was a significant difference between undifferentiated (p>0.001), day 20 (p>0.001), and day 40 differentiated cells (p>0.05) (Fig 3.A). Less methylation in 2%WKS was noted vs. AO in regions II (47.8%, 53.7%, p>0.05), IV (9.8%, 12.1%, p>0.001), and V (5.5%, 7.3%, p>0.001 and combined data from II, III, IV and V (17.7%, 21.1%, p>0.05) (Fig 3.B).

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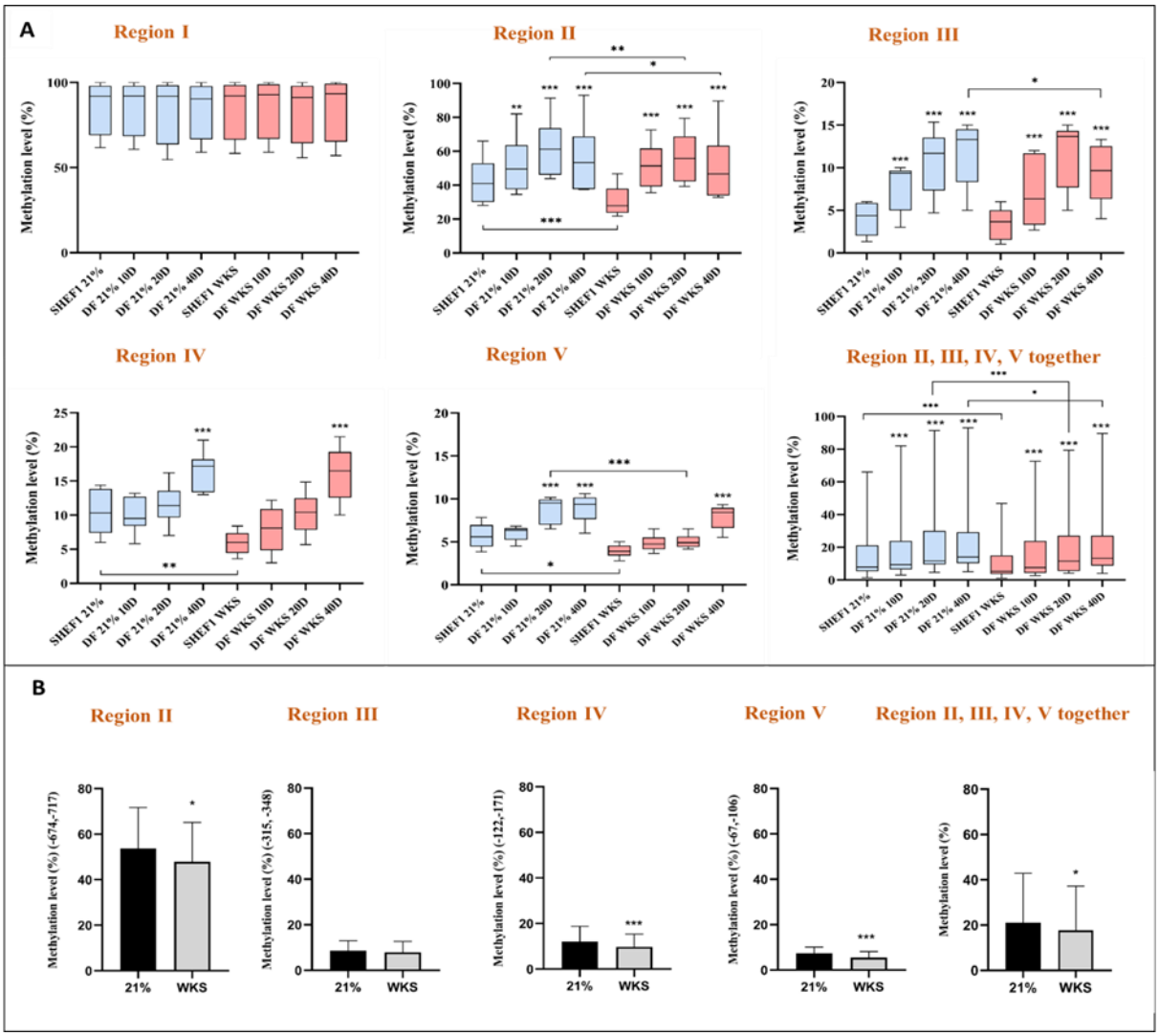

Figure 3. **Increased methylation levels of TERT promoter in air oxygen cultured SHEF1 cells.** Promoter regions relative to TSS were evaluated using pyrosequencing. (A) Y-axis indicates DNA methylation level (%) at CpG sites, and X-axis represents the name of samples. Data presented as median (min-max). (B) The data from differentiated and undifferentiated cells together was indicated in this graph to compare two oxygen conditions. Y-axis indicates DNA methylation level (%) at CpG sites, and X-axis represents the conditions as mean ± SD. n=3, *p<0.05, **p<0.01, ***p<0.001.

SHEF2 cells also demonstrated highly methylated region I. Differentiation process increased methylation in regions II, III, IV, and V and similar to SHEF1 data, higher methylation levels were noted in 21% AO than 2% WKS. Pooled data from regions II, III, IV, and V displayed increased methylation at days 10 (21.1% and 19.6%, p>0.001), 20 (22.9% and 21.1%, p>0.001), and 40 (23.0% and 22.1%, p>0.001) in AO and WKS vs. undifferentiated cells (17.8% and 14.0%), respectively (Fig 4.A). We also compared differentiated and undifferentiated methylation data for each region to compare the differences between 21% AO and 2% WKS. Reduced methylation levels in 2% WKS was noted in regions II (55.6%, 50.0%, p>0.01), III (16.6%, 13.3%, p>0.01), IV (10.1%, 8.9%, p>0.05), V (6.3% 5.8%, p>0.05) and data pooled from the four regions (20.9%, 18.0%, p>0.05) vs. AO (Fig 4.B).

Pooled data from differentiated cells showed increased methylation in regions II, III, IV and V and methylation levels in 21% AO vs. 2% WKS. Regions II, III, IV, and V combined data showed increased methylation at days 10 (19.4% and 18.5%, p>0.001), 20 (23.1% and 20.8%, p>0.001), and 40 (23.2% and 21.4%, p>0.001) in AO and WKS compared to undifferentiated cells (16.8% and 12.4%), respectively. Methylation of undifferentiated (p>0.001), 20 (p>0.001) and 40 days differentiated cells (p>0.01) had decreased methylation

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in 2% WKS vs 21% AO (Fig 5.A). Combined data from differentiated and undifferentiated cells indicated that regions II (48.8% and 54.5%, p>0.001), III (10.5% and 12.9%, p>0.01), IV (9.4% and 11.2%, p>0.001), V (5.6% and 6.9%, p>0.001) and data pooled from the four regions (21.0%, 17.8%, p>0.01) demonstrated a significant decrease in 2% WKS vs. 21% AO (Fig 5.B).

Figure 4. **Increased methylation levels of TERT promoter in air oxygen cultured SHEF2 cells.** Promoter regions relative to TSS were evaluated using pyrosequencing. (A) Y-axis indicates DNA methylation level (%) at CpG sites, and X-axis represents the name of samples. Data presented as median (min-max). (B) The data from differentiated and undifferentiated cells together was indicated in this graph to compare two oxygen conditions. Y-axis indicates DNA methylation level (%) at CpG sites, and X-axis represents the conditions as mean ± SD. n=3, *p<0.05, **p<0.01, ***p<0.001.

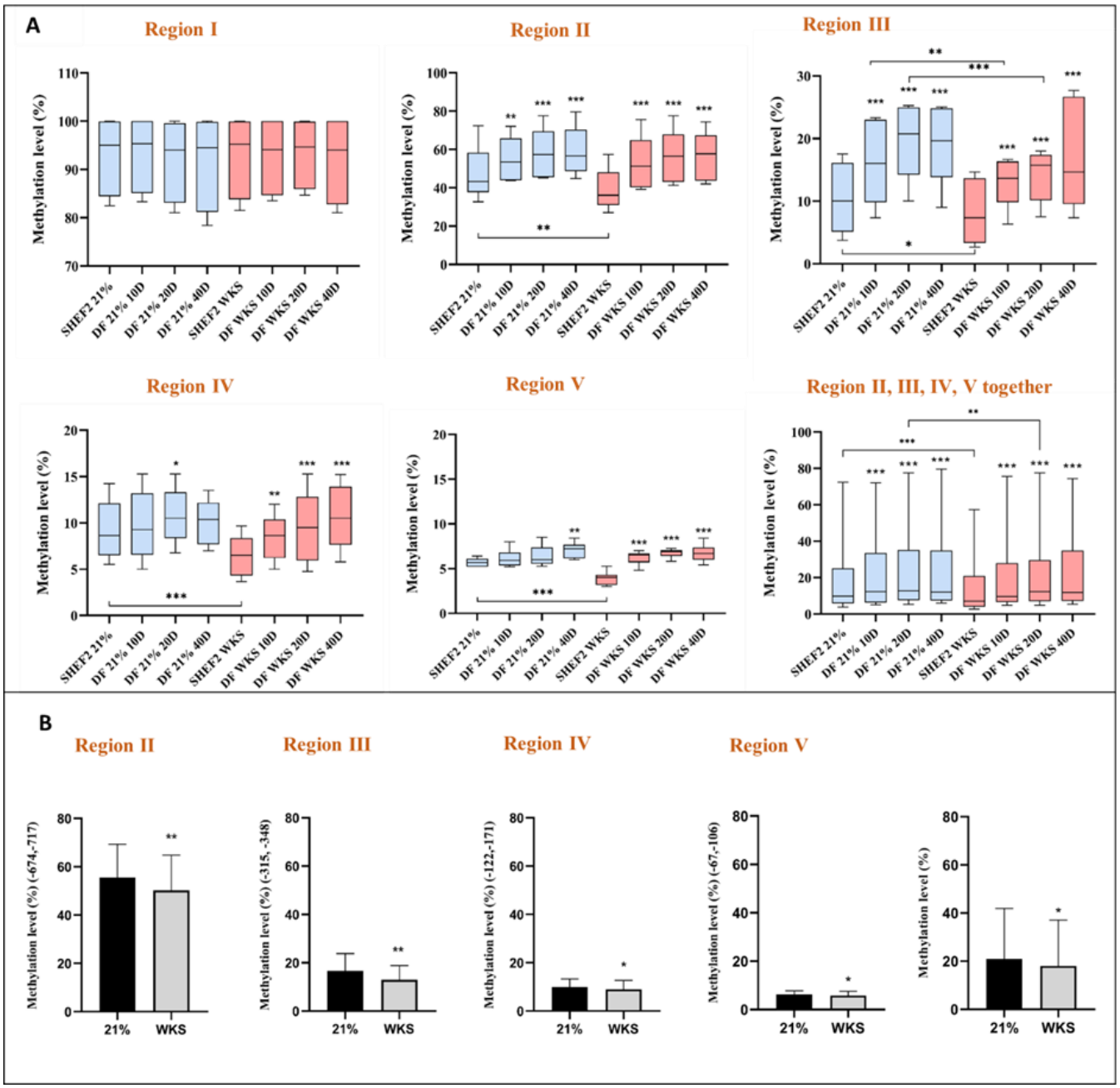

Figure 5. **Pooled data from SHEF1 and SHEF2 showed increased methylation levels of TERT promoter in air oxygen.** Promoter regions relative to TSS were evaluated using pyrosequencing. (A) Y-axis indicates DNA methylation level (%) at CpG sites, and X-axis represents the name of samples. Data presented as median (min-max). (B) The data from differentiated and undifferentiated cells together was indicated in this graph to compare two oxygen conditions.

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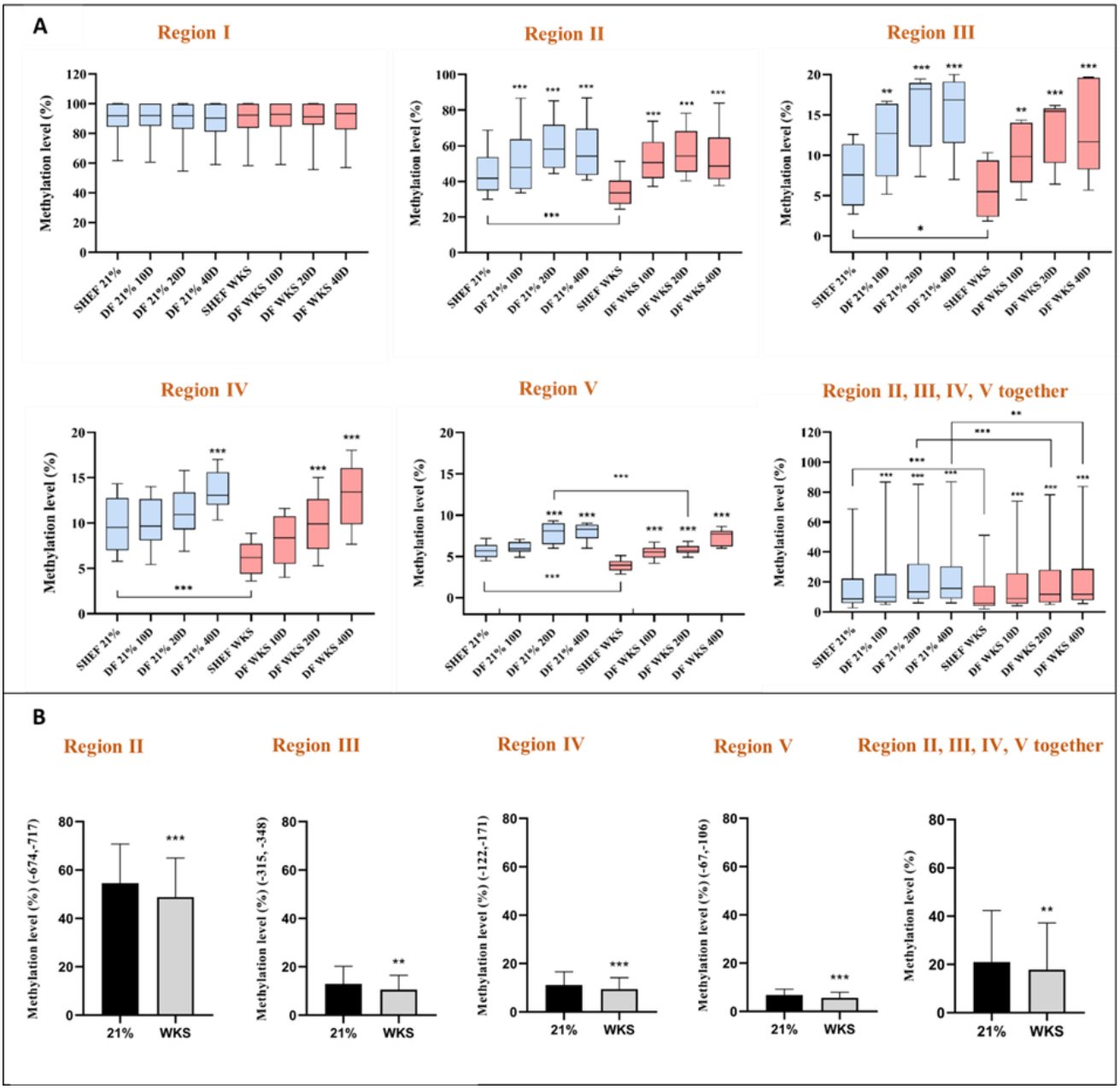

Y-axis indicates DNA methylation level (%) at CpG sites, and X-axis represents the conditions as mean ± SD. n=3, *p<0.05, **p<0.01, ***p<0.001.

DNMTs expression in PSCs and their differentiation progeny in response to physiological oxygen

Differentiated SHEF1 had a significant decrease in DNMT1 expression on days 10 and 40 versus undifferentiated cells. Pooled data from differentiated and undifferentiated cells indicated decreased expression in 2% WKS (-1.13, $p<0.05$) vs. 21% AO (-0.37) (Fig 6.A). SHEF2 cells displayed a significant reduction in 2% WKS (-1.58, $p<0.01$) vs. AO (-0.46) at day 10. DNMT1 expression was decreased significantly in differentiated cells ($p<0.001$) versus undifferentiated cells (Fig 6.B).

DNMT3A expression decreased significantly after 10 ($p<0.05$), 20 ($p<0.01$), and 40 days differentiation ($p<0.001$) vs. undifferentiated SHEF1. Differentiated and undifferentiated SHEF1 indicated a significant decrease in 2% WKS (-1.46, $p<0.05$) vs. AO (-0.52) (Fig 6.E). SHEF2 samples displayed significantly reduced DNMT3A expression after 10, 20, and 40 days of differentiation ($p<0.001$) vs. undifferentiated cells (Fig 6.F).

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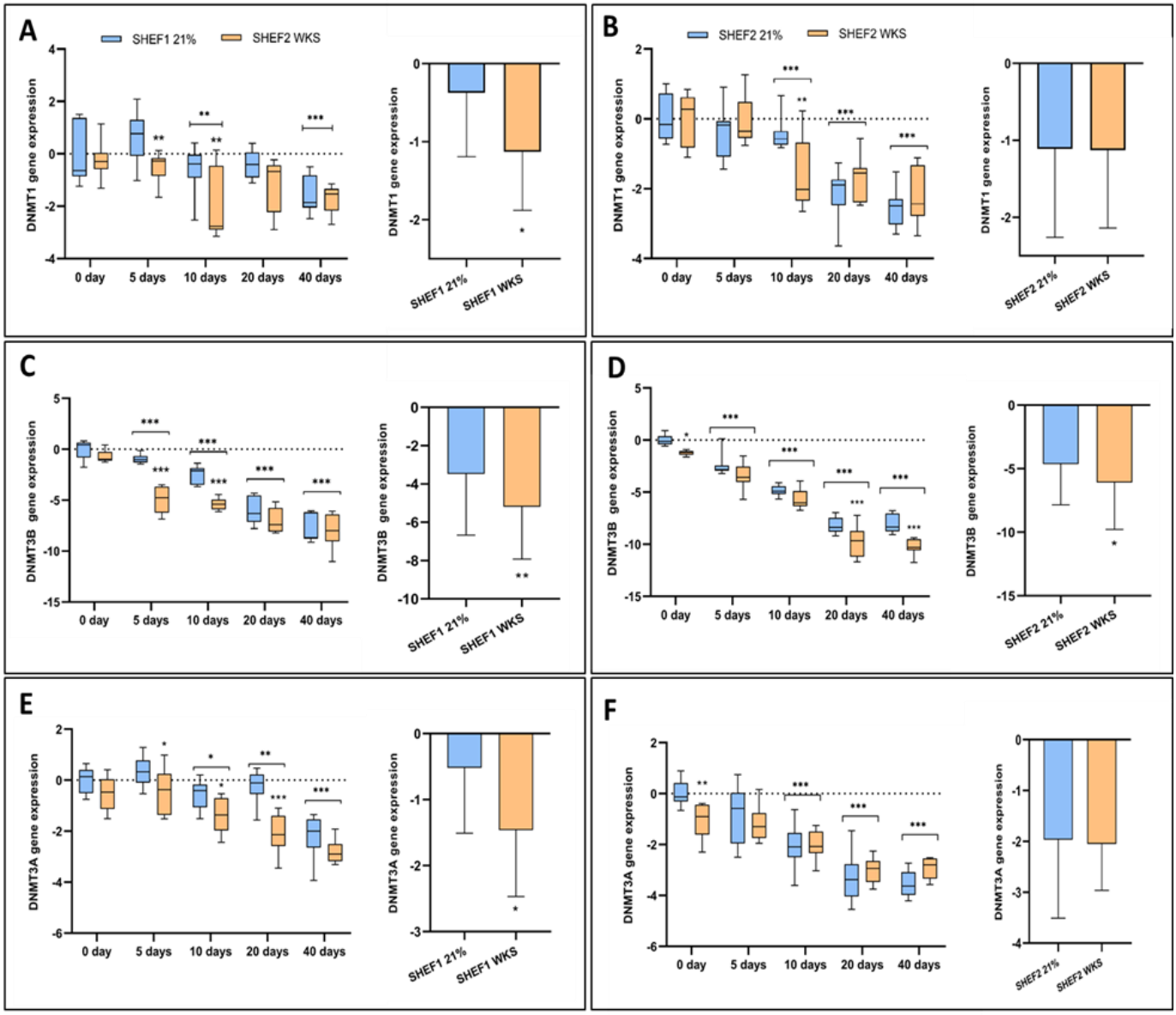
DNMT3B expression significantly decreased during 5, 10, 20, and 40 days of differentiation ($p<0.001$) vs. undifferentiated ESCs. Combined data showed a significant decrease in 2% WKS (-5.19, $p<0.01$ and -6.08, $p<0.05$) vs. AO (-3.47 and -4.63, fold change $2^{-\Delta\Delta Ct}$) (Fig 6.C-D). Overall, there was a decrease in DNMT expression during differentiation.

Figure 6. **DNMTs gene expression in ESCs cells.** DNMT1 (A, B), DNMT3B (C, D) and DNMT3A (E, F) data were obtained from SHEF1 and SHEF2 undifferentiated and differentiated cells in 21% AO and 2% WKS. The RT-qPCR expression of the DNMTs normalized to the expression of GAPDH. Data are represented as mean ± standard deviation (SD), n=3, *p<0.05, **p<0.01, ***p<0.001 vs 21% AO.



NA treatment decreased DNMT3B gene expression

DNMT3B selective inhibitor, NA was used to determine the impact of DNMT3B on methylation and epigenetic regulation of TERT. Non-toxic dose evaluation determined 40nM as the maximum tolerated by undifferentiated SHEF2, and cells cultured in the WKS hypoxia condition were more sensitive to NA (Fig 7.A). Differentiated cells displayed a higher tolerance to NA with a maximum non-toxic drug dose determined at 310 nM (Fig 7.B).

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DNMT3B expression in undifferentiated SHEF2 was significantly decreased after NA treatment in 21% AO (-3.3, p<0.001) and 2% WKS (-3.5, p<0.001). A significant decrease was noted at day 5 in 21% AO (p<0.001) and 2%WKS (p<0.01). In contrast to above, NA treated cells displayed an increase in gene expression at days 20 and 40 in 21% AO (p<0.01) and 2%WKS (p<0.01) in comparison to untreated cells. (Fig 7.C). Differentiated and undifferentiated SHEF2 data together displayed a significant decrease in 2% WKS (-6.1, p<0.05) conditions versus AO (-4.6) (Fig 7.D).

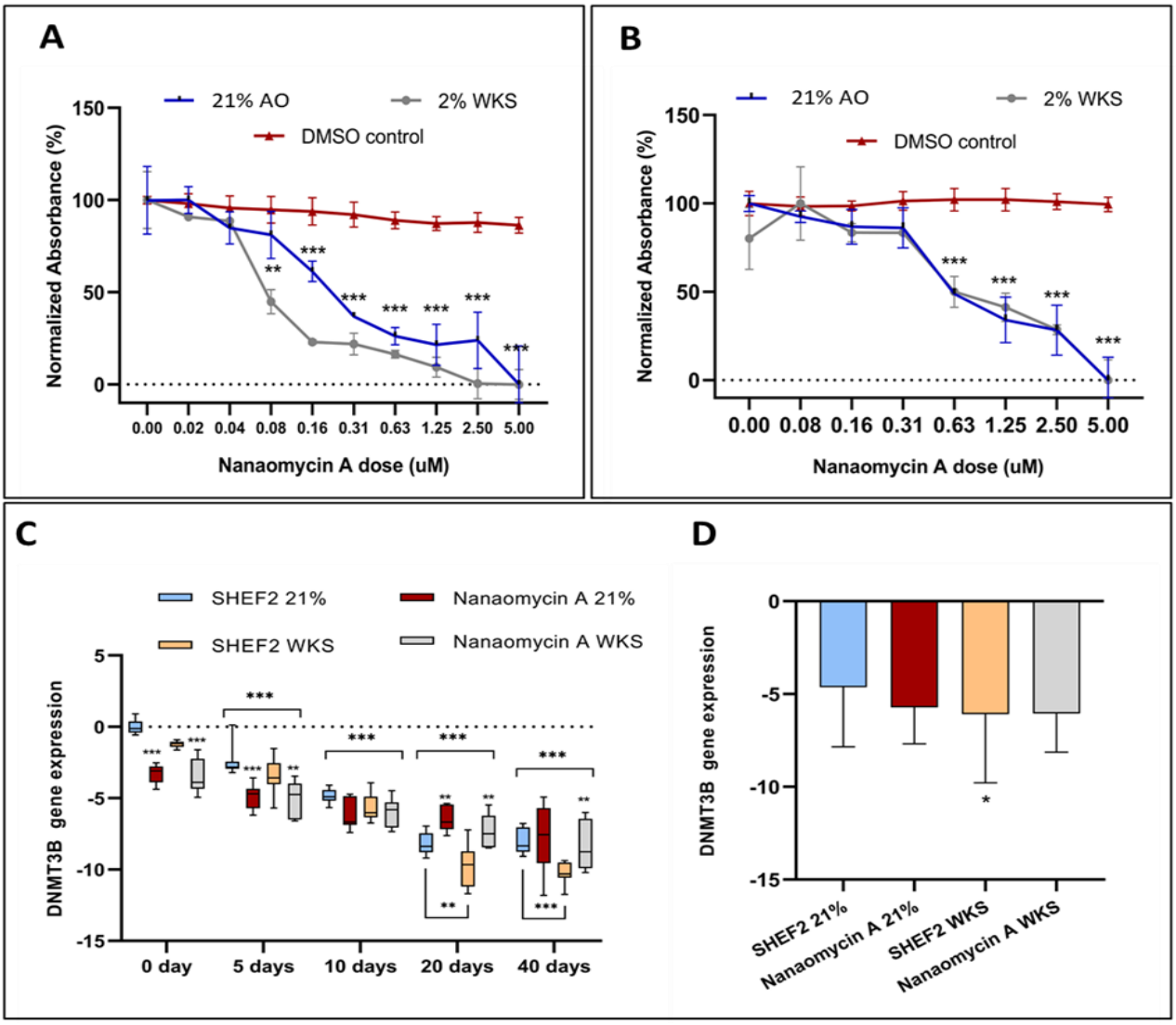

Figure 7. **Non-toxic NA dose and DNMT3B expression in SHEF2 cells.** Undifferentiated (A) and differentiated SHEF2 cells (B) were treated with increasing doses of NA ranging from 20 nM to 5 μM for seven days. Cell viability was plotted against NA concentrations in μM. (C) The RT-qPCR expression of the DNMT3B normalized to the expression of GAPDH. (D) Differentiated and undifferentiated SHEF2 data were pooled together. Data are represented as mean ± standard deviation (SD), n=3, *p<0.05, **p<0.01, ***p<0.001 vs 21% AO.

Inhibition of DNMT3B increased TERT expression in SHEF2

A significant reduction in DNMT3B activity was noted days 10 (p<0.001), 20 (p<0.001), and 40 (p<0.001) differentiation when compared to undifferentiated SHEF2. NA treated undifferentiated SHEF2 cells displayed decreased DNMT3B activity (p<0.05). Further, NA treated differentiated cells demonstrated decreased enzyme activity at days 5 (0.55, p<0.05), 20 (0.35, p<0.01), and 40 (0.19, p<0.01) in comparison to untreated samples (0.87, 0.54 and 0.48) (Fig 8.A).

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NA treatment resulted in increased TERT expression and telomerase activity during long term differentiation. NA treated cells had higher TERT expression at days 20 (-0.59, $p<0.01$) and 40 (-2.08, $p<0.001$) in comparison to untreated cells (1.96 and -4.05) in 2% WKS (Fig 8.B). Differentiated SHEF2 cells showed a significantly higher telomerase activity (4.22 ± 0.19, $p<0.001$) after 40 days NA treatment in comparison to untreated cells (2.84 ± 0.29) in 2% WKS. There was no significant difference in telomerase activity in undifferentiated and days 5, 10, and 20 differentiated SHEF2 cultured in 2% WKS versus 21% condition (Fig 8.C).

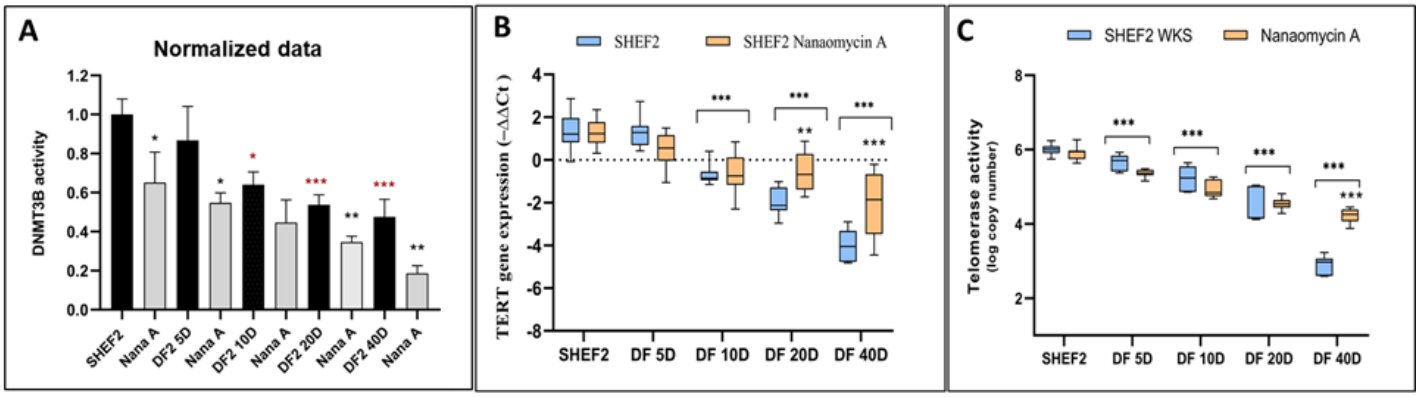

Figure 8. **Decreased DNMT3B enzyme activity after NA treatment in 2% WKS.** (A) DNMT3B enzyme activity, red indicated a comparison between undifferentiated and differentiated cells (B) TERT expression and (C) telomerase enzyme activity. Data are represented as mean ± standard deviation (SD), n=3, *p<0.05, **p<0.01, ***p<0.001 vs 21% AO.

Decreased methylation on TERT promoter after NA treatment and DNMT3B Binding on TERT promoter

Overall, NA treatment decreased TERT promoter methylation. Data pooled from undifferentiated and differentiated SHEF2 demonstrated that region I showed a decreased methylation level after NA treatment (69%, $p>0.001$) versus untreated samples (93%). Data pooled from region II displayed significantly reduced methylation levels in NA-treated cells (15.9%, $p>0.001$) compared to untreated cells (50%). Combined data from region III displayed significantly reduced methylation levels in NA treated (6.9%, $p>0.001$) compared to untreated cells (13.3%). However, treated and untreated samples displayed no significant changes in methylation levels in region IV (9.5% and 8.8%) and also region V (5.6% and 5.9%), respectively. Overall, pooled data from all regions (I, II, III, IV and V) showed a significant decrease in methylation levels in region V following NA treatment (18.5%, $p<0.01$) when compared to untreated cells (29.4%) (Fig 9.A).

CHIP identified that DNMT3B binding to TERT promoter region II was increased in differentiated SHEF2 on days 10 (3.1, $p<0.01$), 20 (3.5, $p<0.001$), and 40 (3.9, $p<0.001$) when compared to undifferentiated SHEF2 (2.5). Elevated DNMT3B binding on region III was noted after 20 (4.6, $p<0.001$) and 40 days (4.9, $p<0.001$) differentiation vs. undifferentiated (3.7). Similarly, region IV had elevated DNMT3B binding after 20 (3.0, $p<0.001$) and 40 days (3.1, $p<0.001$) vs. undifferentiated (2.5). Overall, we noted increased DNMT3B binding on TERT promoter at days 10 (2.5, $p<0.01$), 20 (2.7, $p<0.001$), and 40 (2.8, $p<0.001$) in 2% WKS when compared to undifferentiated (2.1). Pooled data from all regions showed increased DNMT3B binding to the TERT proximal promoter region during 10 (3.1, $p<0.05$), 20 (3.4, $p<0.001$) and 40 (3.7, $p<0.001$) days differentiation compared to undifferentiated (2.7 ± 0.7) (Fig 9.B).

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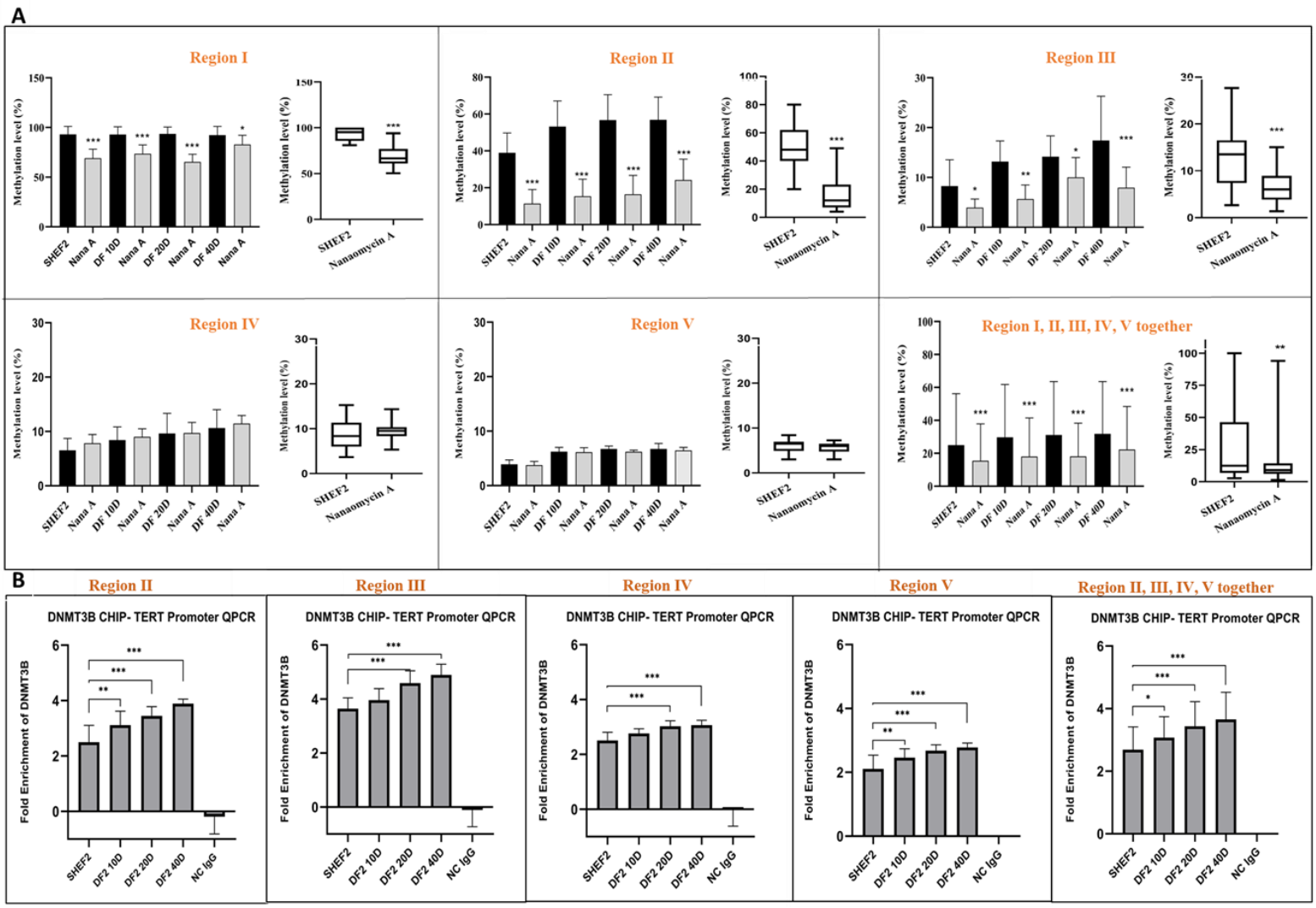

Figure 9. **Reduced methylation levels of TERT promoter following NA treatment in 2% WKS.** Regions I, II, III, IV, V and pooled data from all regions (A) were analyzed using pyrosequencing. Y-axis indicates DNA methylation level (%) at CpG sites, and X-axis represents the name of cells. (B) Binding of DNMT3B enzyme on TERT promoter regions using CHIP qPCR. Data are represented as mean ± standard deviation (SD), n=3, *p<0.05, **p<0.01, ***p<0.001.

3. Discussion

With limitless proliferative potential and ability to differentiate into a wide range of cell types, pluripotent stem cells have the potential to produce human tissues, treat genetic illnesses, and be utilised across a broad range of developmental and fundamental biology questions [42], [43]. Pluripotent ESCs hallmark characteristics include high proliferation, colony formation, Oct-4 and Nanog expression, alkaline phosphatase activity, telomerase activity, and long telomeres [1]. However, telomerase activity and TERT gene expression are reduced during differentiation indicating that telomerase regulation plays an important role in embryonic development [44]–[47]. In this study, we have shown that telomerase regulation is oxygen sensitive during differentiation and may be epigenetically regulated by DNMT3B enzyme.

ESCs and iPSCs exhibit high proliferation, colony formation, high expression of Oct-4 and Nanog, alkaline phosphatase activity and they lose pluripotency marker expression as they differentiate [48]–[50]. We also observed that ESCs monolayer differentiation is accompanied by downregulation of pluripotency genes (Fig S2). ESCs, isolated from pre-implantation blastocysts, can differentiate into all embryonic lineages [52] in vivo and in vitro where 3D aggregates, embryoid bodies, express upregulated Nestin (ectodermal)

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[53], Brachyury (mesodermal) and AFP (endodermal) markers [54]. Similar to previous studies, we observed increased expression of differentiation markers after monolayer differentiation of stem cells in vitro and increased expression of mesodermal markers. Three germ layer differentiation markers were used to assess the ESCs' monolayer differentiation status. Monolayer differentiated ESCs showed no ectodermal or endodermal marker expression. There was an increased expression of mesodermal markers, TBXT and KDR expression in hESC during differentiation with significantly less expression in physiological oxygen culture than AO.

Decreased expression of TERT in differentiated ESCs resulted in decreased proliferation, increased number of cells in the G1 phase, promoted differentiation, and a failure to produce stable ESC sublines [10], [44]–[47], [55]. Consistent with previous literature, telomerase assay and immunofluorescent staining of TERT protein data confirmed a gradual downregulated telomerase during 40 days of differentiation in both ESCs. Telomerase activity was considerably higher in both ESCs cultivated in physiological oxygen niches. Correlated to these results, we found a significant drop in TERT expression and telomere length during differentiation and greater gene expression in low oxygen cultured cells.

The physiological oxygen environment is a critical component of stem cell biology and cell fate [35], [40], [56], [57]. Embryos adapt to reduced oxygen concentrations due to the absence of vascularization in early development, and ESCs can be maintained in the range of 2–5% physiological normoxia [35], [38], [57]–[59]. We observed that reduced oxygen culture (2% PG and 2% WKS) increased the proliferation rate of ESCs in comparison to AO (Fig S1). Many researchers have looked at how epigenetic mechanisms like DNA methylation and histone changes affect cellular processes, including differentiation and embryonic developmental programming [17], [24]. DNA methylation and hydroxymethylation have essential roles in PSCs functions, differentiation, cell fate, and maintenance of characteristics in low oxygen environments [17], [60]–[62]. DNMT1 is a maintenance methylase during replication with an affinity for hemimethylated DNA. DNMT3A and DNMT3B collaborate with DNMT1 to perform de novo methylation, including during early development [63]. DNMT3A and DNMT3B are highly expressed in undifferentiated ESCs [19], [64], [65] but downregulated during differentiation [18]. We observed a significant reduction in the expression of maintenance and de novo methyltransferases DNMT1, DNMT3B and DNMT3A during the differentiation of ESCs. Our results showed a sharp decrease in the expression of DNMT3B during pluripotent stem cell differentiation in line with previous studies [66].

Methylation alteration of the TERT promoter is strongly linked to its regulation [67]. ES and somatic cell methylation is distinct and distinguishes pluripotent stem from somatic cells [17]. Previous data showed lower methylation signature in ESCs similar to our observations [68]–[70]. We observed that the distal promoter (region I) has a high methylation level in all conditions with no significant difference during differentiation. The methylation status on proximal promoter regions II , III, IV and V was more responsive to change, with emphasis on regions II and III. Our data showed a strong link between telomerase activity and oxygen-sensitive TERT proximal promoter methylation. There was a substantial increase in methylation level during differentiation.

Molecular docking studies of DNMT3B suggest that NA forms hydrogen bonds with Glu697, Arg731, Arg733 and Arg832 for stabilization of the protein-ligand complex [71], [72]. NA (5 μmol/L) induced no changes in transcript levels of DNMT3B or DNMT1, but selectively inhibited DNMT3B methylase activity. [71]. We investigated the effect of DNMT3B inhibition on TERT gene expression and telomerase activity. Interestingly, we observed an increase in TERT gene expression after 20 days of differentiation and telomerase activity after 40 days of differentiation compared to untreated cells. Pyrosequencing indicated that DNMT3B inhibition reduced TERT promoter methylation, associated with increased gene and enzyme activity. CHIP-qPCR data revealed that DNMT3B binding to TERT promoter correlated with increased methylation during differentiation and was associated with decreased TERT gene and telomerase activity.

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In conclusion, understanding the mechanism behind reversible silencing of the TERT gene during differentiation, embryonic development, and ageing, or conversely activation in cancer has potential for informing future clinical applications, cancer treatment, diagnosis, prognosis, and cellular ageing research. We have highlighted the link between TERT promoter methylation, TERT expression, and correlated this to telomerase and DNMT3B enzyme. Careful application of stem cell models can contribute to the development of useful epigenetic engineering tools for brand new clinical applications.

4. Materials and Methods

Cell Culture

SHEF1 and SHEF2 were cultured in E8 medium (Life Technologies) with Essential 8 Supplement (1X) in culture vessels coated with 5mg/ml vitronectin (Recombinant Human Protein; Life Technologies, London, UK). Spontaneous differentiation medium comprised Knockout DMEM, 10% FBS, 1% NEAA, 1% L-glutamine and β-mercaptoethanol. Cells were maintained in either air oxygen (21% AO), a fully defined 2% O2 environment (workstation) (2% WKS), and a standard 2% O2 incubator (2% PG) where samples were handled in a standard class II biological safety laminar flow cabinet. Media utilized in either 2% O2 setting was deoxygenated to a 2% O2 level using defined Hypoxycool (Baker Ruskinn, Bridgend, UK) cycle settings. Cell proliferation was assessed using WST1 assay (Sigma-Aldrich, UK) in three oxygen conditions (Fig S1). Non-toxic nanaomycin A (NA) dose was determined with WST1 in three oxygen conditions to determine maximum drug doses. The DNMT3B selective inhibitor (nanaomycin A) was purchased from Adooq Bioscience (USA) and dissolved in DMSO. Cell viability was determined with serial dilution of drugs ranging from 20 μM to 80 nM over seven days.

Characterization of ESCs

Undifferentiated and differentiated hESC were characterized using the Human Pluripotent Stem Cell Marker Antibody Panel (R&D systems, UK) at ; days 5, 10, 20 and 40 (Fig S2).

Gene expression

RNA was isolated from ESCs with the RNeasy® Mini Kit (Qiagen, Manchester, UK), and concentration quantified using a NanoDropTM 2000/2000c Spectrophotometer (Thermo Scientific). qRT-PCR was performed using the QuantiFast SYBR Green OneStep qRT-PCR kit (Qiagen, Manchester, UK). PCR primers were from Sigma-Aldrich, UK. Primer sequences and product sizes are listed in Supplemental Information 1. PCR amplification was performed on 25ng of isolated RNA, and relative quantification of gene expression was measured using the 2-ΔΔCT method.

Telomerase Activity

The TRAPeze® RT Telomerase Detection Kit (Millipore, USA) was used with Amplifluor® primers to detect telomerase activity. Protein extracts from cell pellets were prepared using CHAPS Lysis Buffer and stored at -85°C to -75°C. qRT-PCR followed manufacturer protocol. The standard curve of the control template was used to quantitate telomerase activity via fluorometric detection.

Telomere Length Quantification

Absolute Human Telomere Length Quantification qPCR assay (Caltag Medsystems Limited, UK) was used to measure telomere length. Single-copy reference primer set recognizes a 100 bp-long region on human chromosome 17 and is used as a reference for each

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sample. Reference genomic DNA sample provided a standard for data normalization with known telomere length for calculation of telomere length of target samples. DNA extraction (Qiagen, UK) was performed for each cell pellet. qRT-PCR followed manufacturer protocol. Data from qRT-PCR were analyzed by the following manufacturer calculations.

Pyrosequencing

EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA) was used for bisulphite conversion of 500 ng genomic DNA. Sequences for TERT gene promoter regions were designed via the PyroMark Q24 Software 2.0. Primer sequences, locations and expected PCR product size are listed in Supplemental Information 2 and supplied by Biomers (Germany).

Converted DNA (2-4 µl) was utilized as a template for PCR amplification. PCR reactions were performed with GoTaq®G2 Flexi DNA Polymerase kit (Promega, Southampton, UK). Initial denaturation was achieved by cycling at 95°C for 5 minutes, followed by touch-down cycling for the first 14 cycles, with the temperature decreasing by 0.5°C each cycle. After that, 35 cycles of 95°C for 45 seconds, annealing at 55–63°C for 45 seconds, elongation at 72°C for 30 seconds, and a final elongation step of 72°C for 5 minutes were performed. The quality of the PCR amplification product was validated using a 2% agarose gel electrophoresis. Combination of PCR products with streptavidin-sepharose beads enabled biotin-labelled PCR amplicon collection (GE Healthcare, US). Following purification, beads with biotin labelled PCR amplicons were released into an annealing mix. PyroMark Gold Q24 Reagents, including the four nucleotides, the substrate, and the enzyme mix, were placed into a pyrosequencing dispensation cartridge which was inserted, along with the Q24 pyrosequencing plate, into the pyrosequencing device. PyroMark Q24 Software 2.0 was used to analyze the data.

DNMT3B Activity Colorimetric Assay

EpiQuik™ DNA Methyltransferase 3B Activity Assay (Insight Biotechnology, UK) was used to analyze DNMT3B activity. After labelling the blank control wells, 3 µl (10 µg) samples were introduced to the relevant wells. After the samples were prepared, each well was filled with 27 µl of DNMT Assay Buffer and 3 µl of diluted Adomet, 8 mM, and incubated at 37°C for 90 minutes. After incubation, wells were washed three times with 150 µl of 1X Wash Buffer. Each well was filled with diluted Capture Antibody solution (50 µl), and the plate was incubated at room temperature for 60 minutes on an orbital shaker (50-100 rpm). Then, wells were aspirated and washed three times with 150 µl of 1X Wash Buffer. A detection antibody (50 µl) was added to each strip well and incubated at room temperature for 30 minutes. Then wells were washed with 150 µl of 1X Wash Buffer four times. Enhancer solution (50 µl) was added to each strip well and incubated at room temperature for 30 minutes. Again, the wells were washed with 150 µl of 1X Wash Buffer four times. A developing solution (100 µl) was added to each well and incubated at room temperature for 2-10 minutes in the dark. Stop solution (50 µl) was added to stop the reaction, and then absorbance was read on a microplate reader at 450 nm within 5-15 minutes.

Chromatin Immunoprecipitation (ChIP)

ChIP-IT® Express Chromatin Immunoprecipitation Kit (Active Motif, USA) was used to investigate the binding of DNMT3B antibody to TERT promoter regions. Cells (1.5 x 107) were fixed with formaldehyde, and protein/DNA complexes stabilized via cross-linking. The cell pellet was resuspended in 1ml cold lysis buffer supplemented with 5 µl PMSF (phenylmethylsulfonyl fluoride, a serine protease inhibitor) and 1 µl Protease Inhibitor Cocktail (PIC). The mixture was vortexed to mix and then incubated on ice for 30 minutes. To pellet the nuclei, samples were centrifuged for 10 minutes at 5,000 rpm at 4°C, and the

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supernatant carefully discarded. The nuclei pellet was resuspended in 350 µl digestion buffer with 1.75 µl PIC and 1.75 µl PMSF and incubated for 5 minutes at 37°C. Enzymatic shearing cocktail was added to the nuclei and incubated for 5 minutes at 37°C. The reaction was stopped via addition of 7 µl cold 0.5 M EDTA and incubated for 10 minutes on ice. ChIP DNA Purification Kit (Active Motif, USA) was used to clean up 50 µl DNA samples and establish concentration.

DNA fragments with specific DNMT3B protein interaction were captured with DNMT3B antibody and protein-G coated magnetic beads. The ChIP-IT® Control Kit (Active Motif, USA) was used to assess the non-specific binding of the DNMT3B antibody. 25 µg of DNA was used for ChIP reactions. ChIP reactions were prepared using 25 µl Protein-G coated magnetic beads, 10 µl ChIP buffer 1, 20-60 µl sheared chromatin (25 µg), 1 µl PIC and dH2O to complete the final volume of 100 µl and 2 µg DNMT3B (mouse IgG) antibody. ChIP reaction positive (2 µg RNA pol II plus 2 µg bridging antibody) and negative control (IgG antibody) were included: 25 µl Protein-G coated magnetic beads, 10 µl ChIP buffer 1, 20-60 µl sheared chromatin (25 µg), 1 µl PIC and dH2O to complete the final volume 100 µl. Following overnight incubation, tubes were spun briefly to collect sample and placed on the magnetic stand to pellet the magnetic beads from the liquid. After washing and reverse cross-linking, DNA was eluted from the beads. Afterwards, proteins are removed, and DNA purified with ChIP DNA Purification Kit for downstream analysis. We used designed primers for TERT promoter regions for ChIP samples Supplemental Information 3 for qRT-PCR. RT-PCR reaction was performed on samples using the SYBR Green Master Mix (Thermofisher, UK). We ran serial dilutions of input DNA for each primer set to produce a standard curve with known DNA quantities, e.g. 0.005 ng, 0.05 ng, 0.5 ng, 5 ng, 50 ng.

Statistical analysis

Experimental data analysis and graphical display of data were performed using statistical software GraphPad Prism 8. A comparison among the groups was assessed using the ANOVA test. The threshold for statistical significance was accepted as $p<0.05$. Data are presented as mean ± SD, and each result represents a replicate of 3 independent experiments (n=3).

Supplementary Materials: Figure S1; S2; S3.

Author Contributions: Conceptualization, F.D. and N.R.F.; Data curation, F.D.; Formal analysis, F.D; Funding acquisition, F.D. and N.R.F.; Investigation, F.D. and N.R.F.; Methodology, F.D. and N.R.F.; Project administration, N.R.F.; Supervision, N.R.F.; Validation, N.R.F.; Visualization, F.D.; Writing—original draft, F.D.; Writing—review & editing, F.D. All authors have read and agreed to the published version of the manuscript.

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