Medicon Medical Sciences
Volume 4 Issue 2 February 2023
Research Article

Relationship of Telomerase Inhibitor 1 (PinX1) and Nucleostemin (GNL3) Expression with Telomerase Reverse Transcriptase (TERT) Concentration in Myeloid and Erythroid Origin Cell Groups

Pinar Akpinar Oktar*

LÖSEV- Tissue Typing and Immunology Laboratory, Lösante Hospital, Ankara, Turkey

***Corresponding Author:** Pinar Akpinar Oktar, LÖSEV- Tissue Typing and Immunology Laboratory, Lösante Hospital, Ankara, Turkey.

Received: January 17, 2023; **Published:** January 30, 2023

DOI: 10.55162/MCMS.04.103

Abstract

It is possible to detect possible changes in the differentiation steps of progenitor cell clones in hematopoiesis with a target protein. In our study, PinX1 (PIN2/TRF1-interacting telomerase inhibitor 1), nucleostemin (guanine nucleotide-binding protein-like 3) expressions were determined in erythroid ($CD71^+$), myeloid ($CD71^-$) cell groups obtained from two different healthy donors prepared for transplantation, chronic myelogenous leukemia cell line; K562 and acute promyelocytic leukemia cell line; HL-60, along with TERT (Telomerase Reverse Transcriptase) levels were determined and their relationships with each other were investigated. According to RT-PCR and TERT concentration results; PinX1 and nucleostemin expression in K562 and HL-60 cell lines were shown to be significantly higher along with TERT concentration. In addition, PinX1, nucleostemin expression and TERT concentration were found to be higher in the erythroid ($CD71^+$) group obtained from healthy donors than in the myeloid ($CD71^-$) group. The findings of this study showed that there may be a relationship between PinX1 and nucleostemin and that PinX1 and nucleostemin could be targets in leukemia patients in prospective drug studies.

Keywords: PinX1 (PIN2/TRF1-interacting telomerase inhibitor 1); nucleostemin (guanine nucleotide-binding protein-like 3); TERT (Telomerase Reverse Transcriptase); Chronic myelogenous leukemia; Acute promyelocytic leukemia

Abbreviations

AML: Acute myeloid leukemia.
CFU: Colony forming units.
CML: Chronic myeloid leukemia.
CSF: Colony stimulating factors.
GNL3L: Guanine nucleotide binding protein-like 3.
HPRT: Hypoxanthine phosphoribosyltransferase 1.
NPM: Nucleophosmin.
NS: Nucleostemin.
Nucleostemin: Guanine nucleotide-binding protein-like 3.
PinX1: PIN2/TRF1-interacting telomerase inhibitor 1.
RT-PCR: Reverse transcription polymerase chain reaction.

SOP: Standard operating procedures.

TERT: Telomerase Reverse Transcriptase.

TRF1: Telomeric repeat binding factor 1.

UPL: Universal probeLibrary.

Introduction

Telomerase, a reverse transcriptase enzyme, is essential for telomere length homeostasis. Telomere length must be sufficient to prevent age-related diseases and premature cellular senescence. In addition, telomere shortening is important in restricting cell proliferation to prevent tumorigenesis. Recent studies on telomerase regulation and how telomere length homeostasis is maintained have increased our understanding of this issue.

The enzyme telomerase plays a role in the maintenance of chromosome ends and prolongs telomere length. PinX1 (PIN2/TRF1-interacting telomerase inhibitor 1) protein is involved in the inhibition of this enzyme. Loss of PinX1 disrupts chromosome dynamics as well as increasing telomere length. Zhou., et al. showed that PinX1 is an important tumor suppressor protein in human tumors and mouse model [16]. It plays a vital role in maintaining telomere length and chromosome stability [8]. Although the exact mechanism of PinX1 in human malignancy is not yet known, it has been reported to be involved in the formation and progression of most tumors. Therefore, it may have the potential to be a diagnostic biomarker and therapeutic target in human tumorigenesis [10].

Nucleostemin (NS) was discovered by Tsai and McKay in 2002 and studies have shown that NS is highly expressed in adult mammalian testis and neural stem cells [13]. In the following years, NS was frequently encountered in studies on cell senescence, tumorigenesis and cell cycle control mechanisms [12]. This is where the "stem" suffix in its name originates from. Subsequent studies have shown its high expression in many stem cell types [13, 1]. NS is essential for the proliferation of neural stem cells and cancer cells but has also been found to be important for the early embryo [13, 3]. It has also been reported that the nucleolar GTP-binding proteins; NS and GNL3L (guanine nucleotide binding protein-like 3) can bind and modulate TRF1 (telomeric repeat binding factor 1). It has been shown that during mitosis, TRF1 protein is stabilized by GNL3L and promotes the transition from metaphase to anaphase [14].

Romanova., et al. demonstrated another function of NS in their study, which is to maintain the integrity of small nucleolar ribonucleoproteins, nucleolar architecture and telomerase complex. The degradation of the components of snoRNPs and the telomerase complex that resulting in a decrease in telomerase activity was due to the depletion of NS. These biochemical, morphological and functional studies have demonstrated the essential role of NS in maintaining nucleolar structure and function [11].

There is few information about PinX1 and NS expressions in leukemia patients. Furthermore, there is no study in the literature on the relationship between PinX1, NS and TERT concentration. Therefore, this study is the first study to combine these topics. In clonal hematologic diseases, studying the relationship between PinX1 and NS with the quantification of TERT in the same cells will provide insight into new molecular markers. Thus, in this study, PinX1 and NS expression profiles were examined in erythroid ($CD71^+$) and myeloid ($CD71^-$) cell groups from healthy donors, as well as in leukemia cell lines. We also examined TERT concentration in $CD71^+$, $CD71^-$ groups and leukemia cell lines and investigated the relationship between PinX1, NS and TERT.

Materials and Methods

Samples

In this study, chronic myelogenous leukemia cell line; K562 and acute promyelocytic leukemia cell line; HL-60 that are obtained from Ankara University Faculty of Medicine, Department of Medical Biology, were used to represent leukemia patients. In addition, bone marrow from 2 healthy donors who applied to Gazi University Faculty of Medicine Department of Hematology were included in the study.

Derivation of hematopoietic precursor cells of erythroid and myeloid origin

It is possible to perform clonogenic analysis from progenitor cells using semi-solid culture media. These cells can be clonally grown as "colony forming units" (CFU) in agar or methylcellulose culture media containing specific growth factors, lymphokines and cellular products called "Colony stimulating factors" (CSF). In our study, to obtain $CD34^+$ hematopoietic stem cells from cells collected from the bone marrow of two different healthy donors prepared for transplantation, cells were washed in PBS and centrifuged at 350xg (1200 rpm) for 5 minutes at room temperature. Then, manufacturer's recommended kit protocol steps were followed for ''EasySep Human CD34 Positive Selection Kit (Catalog no:18056, StemCell Technology Inc.)''. After two days of suspension culture, $CD34^+$ cells were cultured and primary culture was performed in 24-well tissue culture plates with a concentration of $2\text{-}6x10^3$ cells/well (0.5ml cell growth medium/well) in cell growth medium containing methylcellulose. These $CD34^+$ cells were incubated with StemSpan™-XF (Catalog no:100-0073) and StemSpan cc100 (Catalog no:02690), a special medium for hematopoietic cells (StemSpan™-XF) for 2 days at 37°C and 5% CO_2. At the end of incubation, $CD34^+$ cells were washed with DMEM F12 (containing 2% FBS) growth medium and cells were diluted to a final cell concentration of $5x10^6$. Cells were plated in MethoCult growth medium (MethoCult, Catalog no:84434, StemCell Technology Inc. Vancouver, Canada). Erythroid and myeloid clonal cells (CFU-E, BFUE, CFUGM, CFU-GEMM) were generated with MethoCult growth medium containing rh EPO, rh SCF, rh G-CSF, rh GM-CSF, rh IL-3 components.

Cells incubated in culture dishes containing MethoCult were left at +4°C for one hour to collect at the end of day 16. Then cells and semi solid medium were dissolved with 1ml PBS, cells were centrifuged at 350xg for 10 minutes and cells were pelleted. The cells were washed once more with PBS and transferred to DMEM F12 growth medium containing 2% FBS and cell separation was performed. Each of the hematopoietic precursor cell groups, which we divided into two groups as erythroid and myeloid origin, were stored as two separate samples. One part of the separated cells was used for RNA isolation and the other part was used for TERT quantification.

K562; a chronic myelogenous leukemia cell line, and HL-60; an acute promyelocytic leukemia cell line, were used to represent PinX1 and NS expression in leukemic cells. Primary culture of HLA-60 and K562 cell lines was performed with RPMI1640, 10% FCS (heat inactive Fetal Calf Serum), 0.2 mM L-Glutamine, 1% antibiotic (1,000,000U/mL penicillin and 1g/mL streptomycin) in a humidified atmosphere at 37°C and 5% CO2. All experiments were performed in triplicate.

RNA was extracted from the replicated cells and cell pellets were stored at -80°C for TERT quantification.

Analysis of Cell Surface Markers by Flow Cytometry

Immunophenotypic studies were performed on cells grown in MethoCult growth medium and on all healthy donor samples containing mixed erythroid and myeloid cell lines before cell sorting. EuroFlow standard operating procedures (SOPs) were used for instrument calibration, sample preparation, immunostaining and data collection in parallel with local protocols and techniques used for routine diagnosis of hematological malignancies in our center [15, 9]. Stained cells were read at low speed on a BD FACSCalibur™ flow cytometer. The data analysis software program BD FACSCalibur™ software was used for data analysis. Immunophenotypic analysis was performed using cell surface markers; CD71 (Cat No: 555536), HLA-DR (Cat No: 641411), CD33 (Cat No: 561157), CD13 (Cat No: 561599), CD64 (Cat No: 644385) and CD45 (Cat No: 655873) to identify myeloid and erythroid groups.

Separation of erythroid and myeloid-derived cells from healthy donors (SORTING)

Cells were prepared for sorting in DMEM F12 growth medium containing 2% FBS. Before sorting, cells were washed twice in PBS-BSA buffer (PBS, supplemented with 0.5% BSA) (10 min. at 200xg at +4°C). Cells were resuspended in 800 µl PBS-BSA buffer to $20x10^6$ cells and cells were stained with FITC CD71 monoclonal antibody using conventional staining method. Then cells were differentiated according to $CD71^+$ and $CD71^-$ expression according to the manufacturer's protocol. It was evaluated on FACSAria II. Data were obtained using FACSDiva (BD Biosciences). The total cell numbers obtained from two cell groups that we wanted to study as a result of cell differentiation by CD71 positivity are as shown in Table S1.

Reverse transcription polymerase chain reaction (RT-PCR)

Erythroid and myeloid progenitor cells were expanded in semisolid MethoCult growth medium and divided into two separate groups. RNA was isolated from these two groups of cells and K562, HL-60 cell lines. Total RNA samples were extracted from each sample with TRI Reagent® solution (Sigma-Aldrich, USA). The purity and concentration of total RNA samples were confirmed with an ultraviolet spectrophotometer (ThermoFisher Scientific, MA USA) (Table S2). cDNA was synthesized with the ''transcriptor first strand cDNA synthesis kit (Roche Diagnostics, Cat No:04897030001)'' according to the manufacturer's instructions. cDNA synthesis was performed using ''random hexamer primers''. Primers were designed according to Gene Bank sequences and purchased from Sentromer DNA Technologies (Sentromer DNA Technologies, Turkey). RT-PCR primer sequence information is shown in Table 1.

Genes	***Primer sequences***
PinX1	F: 5'- CGCTGGTTGTCGTGGTTT-3'
	R: 5'-TGCATTTTTGGGAAAAGACAG-3'
Nucleostemin (NS)	F: 5'-TATCCATGGGGCTTACAAGG-3'
	R: 5'-CTGGACTTCGCAGAGCAAG-3'
HPRT	F:5'-TGACACTGGCAAAACAATGCA-3'
	R:5'-GGTCCTTTTCACCAGCAAGCT-3'

Table 1: RT-RCR Primer sequences.

The polymerase chain reaction protocol for these primers was 10 minutes at 95°C, after denaturation 30 seconds at 95°C, 30 seconds at 60°C, 30 seconds at 72°C repeated 35 times and 5 minutes at 72°C.

In this study, Universal probeLibrary (UPL) probe #16 (NS) (Roche Diagnostics, Cat No: 04686896001, Mannheim, Germany) and UPL probe #82 (PinX1) (Roche Diagnostics, Cat No: 04689054001, Mannheim, Germany) were used for qRT-PCR of NS and PinX1. UPL human HPRT (hypoxanthine phosphoribosyltransferase 1) gene probe (Roche Diagnostics, Cat No: 05046246001, Mannheim, Germany) was used as housekeeping gene. qRT-PCR was performed using 5 µl of the obtained cDNA synthesis products. The qRT-PCR protocol was performed following the UPL probe (Roche Diagnostics) product technical documentation. PinX1 and NS expressions were quantitatively analyzed.

Determination of TERT levels

TERT concentration was calculated as pg/ml in HL-60, K562 cell lines and in hematopoietic precursor cell lines of erythroid and myeloid origin obtained from healthy donors. ELISA Kit for Telomerase Reverse Transcriptase (TERT) (Uscn life science inc.) was used for concentration measurement. The experiments were repeated three times.

Statistical Analysis

SPSS 25.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The differences between NS and PinX1 expressions in the cell lines and the hematopoietic precursor cell groups of erythroid and myeloid origin obtained from healthy donors were analyzed by Mann-Whitney U test. As a result of the analysis, a statistically significant difference was found between the K562 and HL-60 cell lines used in the study compared to the groups obtained from healthy donors ($U=8.00$, $p=0.009$).

The correlations between the expression data of PinX1 and NS genes were analyzed by correlation test. According to the results of Pearson correlation analysis (Nonparametric Correlations), a significant, strong and negative correlation was found between PinX1 and NS expressions in K562 and HL-60 cell lines and healthy donor groups ($r=-1.00$, $p<0.01$).

Correlations between erythroid and myeloid origin cell groups from healthy donors and TERT concentration data in hematopoietic precursor cell lines were analyzed by correlation test. According to the results of Pearson correlation analysis (Non-Parametric Correlations), a significant, strong and positive correlation was found between K562 and HL-60 cell lines and TERT concentrations in healthy donor groups ($r=0.997$, $p<0.01$).

Results and Discussion

Flow cytometry results after sorting

Cells collected from Mahout cell growth media were analyzed for surface markers CD71, HLA-DR, CD33, CD13 and CD64 after sorting. As expected, erythroid cells were negative for CD13, CD33, HLA-DR and CD64 but positive for CD71. In the myeloid cell group, positivity for CD33, low positivity for HLA-DR and negativity for CD13, CD64 and CD71 were observed. Therefore, these surface antigens proved that we were working with the cell groups that we expected (Table 2).

Cell surface markers	*Erythroid cell group*	*Myeloid cell group*
CD71	%49.36	% 8.0
HLA-DR	% 6,28	% 15.55
CD33	% 9,45	%58.0
CD13	% 2,53	% 13.0
CD64	% 3.1	% 10.6

Table 2: Analysis of some surface markers by flow cytometry in cell lines obtained from healthy donors after sorting.

PinX1 and Nucleostemin gene expression

Expression levels of nuclear proteins; PinX1 and NS were analyzed by RT-PCR. According to these results; mRNA expression of PinX1 and NS was significantly lower in myeloid (CD71-) group obtained from healthy donors. PinX1 and NS expressions were significantly higher in HL-60 and K562 cell lines than in erythroid (CD71+) and myeloid (CD71-) cell lines obtained from healthy donors ($p<0.01$). PinX1 and NS expression was significantly higher in erythroid (CD71+) group than in myeloid (CD71-) group ($p<0.01$) (Figure 1, Table S3).

TERT concentrations

In order to evaluate PinX1 and NS expressions together with TERT, TERT concentrations were measured in HL-60 and K562 cell groups and in erythroid ($CD71^+$) and myeloid ($CD71^-$) cell groups obtained from healthy donors ($p<0.01$). The concentrations of TERT in the erythroid ($CD71^+$) group of healthy donors was higher than that in the myeloid (CD71-) group ($p<0.01$) (Figure 1, Table S4).

K562, HL-60 cell lines had higher PinX1, NS expressions and TERT concentrations than healthy donors. In myeloid ($CD71^-$) cell lines obtained from healthy donors, PinX1, NS and TERT concentration were found to be lower than erythroid ($CD71^+$) origin hematopoietic precursor cells. In addition, another important result of this study is the correlation between PinX1 expression and hTERT concentration in chronic myelogenous leukemia and acute promyelocytic leukemia cell lines. Supporting this finding, Capraro V., et al. in 2011, confirmed our study and reported that PinX1 was overexpressed in acute leukemia patients and telomerase activity was also increased in these patients [5]. Campbell., et al. analyzed PinX1, TRF1 and TRF2 expression levels in 22 chronic myeloid leukemia (CML) patients and showed increased expression levels in most patients [4]. Cela., et al. proved that NS is overexpressed in Acute myeloid leukemia (AML) as well as in various solid and hematologic neoplasms [6].

According to these findings, the interaction between PinX1 and NS may be involved in telomerase regulation. It is not known whether NS affects telomerase activity through a protein-protein interaction or whether it exerts its effect by altering the transcription of telomerase regulators. Banik., et al. reported that PinX1 interacts directly with hTERT [2]. While nucleophosmin (NPM), a nuclear

protein like NS, has been implicated in the formation of the PinX1/NPM/hTERT complex, Cheung., et al. showed that both NPM and PinX1 are associated with the RNA binding domain of hTERT [7].

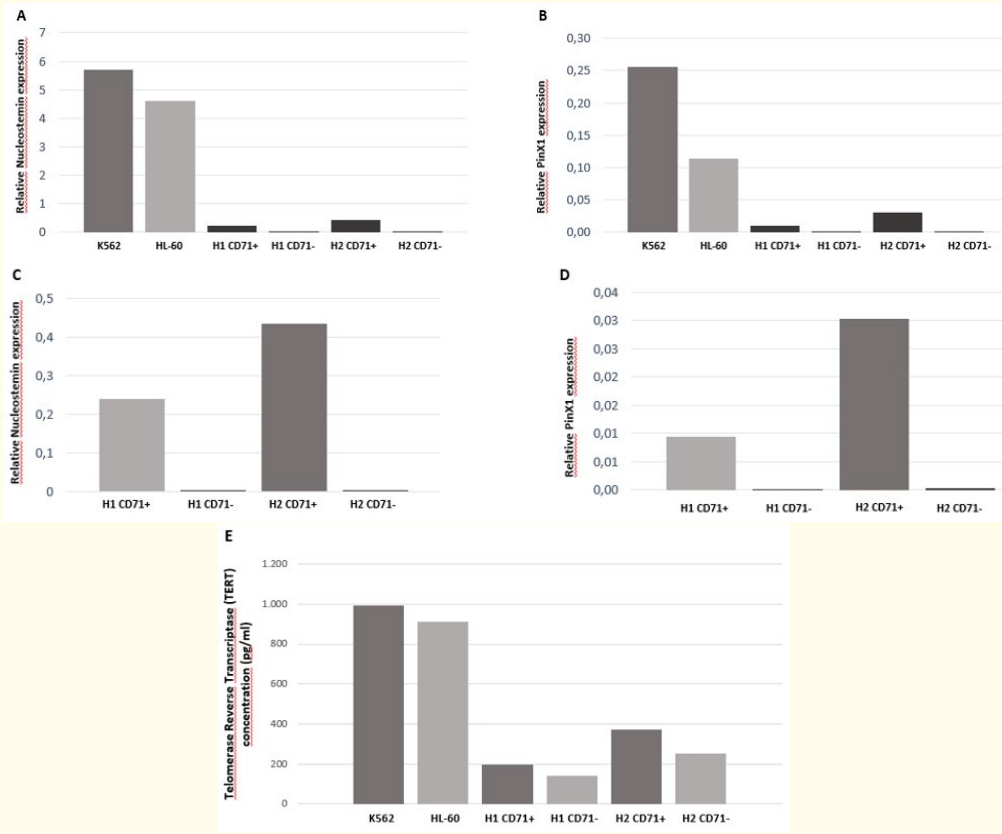

Figure 1: TERT concentration, nucleostemin and PinX1 relative expressions (normalized to housekeeping gene) of K562, HL-60 cell lines and healthy donors. A: Nucleostemin gene expressions, B: PinX1 gene expressions of K562, HL-60 and healthy donors (H1 and H2). C: Nucleostemin gene expressions, D: PinX1 gene expressions of healthy donors (H1 and H2). E: TERT concentration (pg/ml).

Conclusion

In this study, we examined the expression levels of PinX1 and NS nuclear proteins in cell lines representing hematologic diseases (K562 and HL-60) also in erythroid and myeloid cell groups obtained from healthy donors. The results were also correlated with TERT concentration. In the results of our study, it is important to evaluate the changes in NS and PinX1 expression together with changes in TERT concentration and thus telomerase activity. Studies are still needed to prove that subgroup proteins of the telomerase enzyme, such as hTERT, might colocalize with PinX1/NS.

Acknowledgements

I would thank the Tülin Özkan, Dr., Rauf Haznedar, Prof. Dr. for the contributions. Neither this manuscript nor one with similar content has been published or is being considered for publication in any language, except as an abstract or academic thesis. There is no conflict of interest and/or funding disclosure to declare.

Supplemental Material

Cells obtained from MethoCult growth media:	
Healthy Donor 1	Total cell count
$CD71^-$: Myeloid cells	1324823
$CD71^+$: Erythroid cells	1036377

Cells obtained from MethoCult growth media:	
Healthy Donor 2	Total cell count
$CD71^-$: Myeloid cells	1117739
$CD71^+$: Erythroid cells	1821531

Table S1: Total cell counts obtained during sorting.

Cell Groups	*RNA amount*
$CD71^-$ Healthy Donor 1	60 ng/µl
$CD71^+$ Healthy Donor 1	43 ng/µl
$CD71^-$ Healthy Donor 2	56 ng/µl
$CD71^+$ Healthy Donor 2	47 ng/µl
K562	374 ng/µl
HL-60	427 ng/µl

Table S2: Spectrophotometric RNA measurements from cell lines.

Cell Groups	*Relative Nucleostemin expressions*	*Relative PinX1 expressions*
K562	5.76	0.26
HL-60	4.61	0.11
$CD71^+$ Healthy Donor 1	0.24	0.01
$CD71^-$ Healthy Donor 1	0.002	5.67e-05
$CD71^+$ Healthy Donor 2	0.43	0.03
$CD71^-$ Healthy Donor 2	0.0001	0.0004

Table S3: Nucleostemin and PinX1 relative expression values (normalized according to housekeeping gene) of cell groups.

Cell Groups	*OD 450 (mean)*	*pg/ml (mean)*
K562	1.470	994.1190
HL-60	1.352	909.8333
$CD71^+$ Healthy Donor 1	0.275	140.07
$CD71^-$ Healthy Donor 1	0.354	196.73
$CD71^+$ Healthy Donor 2	0.429	250.5
$CD71^-$ Healthy Donor 2	0.601	373.05

Table S4: The average amount of "Telomerase Reverse Transcriptase (TERT)" enzyme obtained for each sample.

References

1. Baddoo Melody., et al. "Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection". Journal of cellular biochemistry 89.6 (2003): 1235-49.

2. Banik Soma SR and Christopher M Counter. "Characterization of interactions between PinX1 and human telomerase subunits hTERT and hTR". The Journal of biological chemistry 279.50 (2004): 51745-8.
3. Beekman Chantal., et al. "Evolutionarily conserved role of nucleostemin: controlling proliferation of stem/progenitor cells during early vertebrate development". Molecular and cellular biology 26.24 (2006): 9291-301.
4. Campbell LJ., et al. "hTERT, the catalytic component of telomerase, is downregulated in the haematopoietic stem cells of patients with chronic myeloid leukaemia". Leukemia 20.4 (2006): 671-9.
5. Capraro Valérie., et al. "Telomere deregulations possess cytogenetic, phenotype, and prognostic specificities in acute leukemias". Experimental hematology 39.2 (2011): 195-202.e2.
6. Cela Ilaria., et al. "Proteomic Investigation of the Role of Nucleostemin in Nucleophosmin-Mutated OCI-AML 3 Cell Line". International journal of molecular sciences 23.14 (2022): 7655.
7. Cheung Derek Hang-Cheong., et al. "Nucleophosmin Interacts with PIN2/TERF1-interacting Telomerase Inhibitor 1 (PinX1) and Attenuates the PinX1 Inhibition on Telomerase Activity". Scientific reports 7 (2017): 43650.
8. Johnson F Brad. "PinX1 the tail on the chromosome". The Journal of clinical investigation 121.4 (2011): 1242-4.
9. Kalina T., et al. "EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols". Leukemia 26.9 (2012): 1986-2010.
10. Li Hai-Long., et al. "PinX1: structure, regulation and its functions in cancer". Oncotarget 7.40 (2016): 66267-66275.
11. Romanova Liudmila., et al. "Novel role of nucleostemin in the maintenance of nucleolar architecture and integrity of small nucleolar ribonucleoproteins and the telomerase complex". The Journal of biological chemistry 284.39 (2009): 26685-94.
12. Tsai Robert YL and Lingjun Meng. "Nucleostemin: a latecomer with new tricks". The international journal of biochemistry & cell biology 41.11 (2009): 2122-4.
13. Tsai Robert YL and Ronald DG McKay. "A nucleolar mechanism controlling cell proliferation in stem cells and cancer cells". Genes & development 16.23 (2002): 2991-3003.
14. Tsai Robert YL. "Nucleolar modulation of TRF1: a dynamic way to regulate telomere and cell cycle by nucleostemin and GNL3L". Cell cycle (Georgetown, Tex.) 8.18 (2009): 2912-6.
15. van Dongen JJM., et al. "EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes". Leukemia 26.9 (2012): 1908-75.
16. Zhou Xiao Zhen., et al. "The telomerase inhibitor PinX1 is a major haploinsufficient tumor suppressor essential for chromosome stability in mice". The Journal of clinical investigation 121.4 (2011): 1266-82.

Volume 4 Issue 2 February 2023