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RESEARCH ARTICLE

TESCALCIN OVER-EXPRESSION INCREASES KERATIN 18 AND 19 IN K562 CELLS

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ABSTRACT

Purpose: Tescalcin (TESC) has been shown to be essential in mammalian cells as a regulator of intracellular Ca^{2+}. Ca^{2+} is a second messenger functioning in many metabolic pathways as well as in cell differentiation, cell size and the cell cycle. K562 cells over-expressing TESC change their morphology and adopt adherent properties. Considering differences in morphology may have been reflected in changes of the cytoskeleton, we focused on the expression levels of keratins, which are cytoskeletal intermediate filaments in epithelial cells and also expressed in K562. We over-expressed the TESC gene via lentiviral transduction and analyzed keratin 8 (K8), keratin 18 (K18), and keratin 19 (K19) expression.
Methods: K562 cell line were stably transduced using a lentiviral vector (LentiTESC) carrying the TESC gene and green fluorescent protein. mRNA and protein expression of K8, K18 and K19 were qRT-PCR and western blot.
Result: Over-expression of TESC lead to a significant increase of K18 and K19 mRNA and protein levels; whereas K8 expression showed no significant change when compared to control K562 cells.
Conclusion: TESC may have a role in regulating cell morphology through keratins via interacting with intermediate molecules such as DST. This is the first study reporting a correlation between TESC and keratin expression.

Keywords: Keratins, TESC, K562

INTRODUCTION

Tescalcin (also called Calcineurin B homologous protein 3) is a 24-kDa EF-hand, Ca2+-binding protein that has recently been discovered to regulate growth and cell differentiation (1). In vitro studies have shown that tescalcin functions as a regulator of the Na+/H+ exchanger via direct protein-protein interactions (2). Tescalcin has interactions with several proteins like dystonin (DST) by which neural intermediate filaments are anchor to the actin cytoskeleton in brain and muscular tissue; and keratin-containing intermediate filaments anchor to hemidesmosomes in epithelial tissue (3,4). Thus, it is plausible that tescalcin may directly play a role in cellular morphology and motility through its direct interaction with DST. The expression level of tescalcin changes dramatically during development and high expression of tescalcin has been reported in several cancers (1). The genetic and epigenetic alterations leading to malign transformation of a cell not only includes differentiation, cell death and proliferartion pathways, but also involves changes in the expression of various metabolic and structural proteins such as cytoskeletal proteins and intermediate filaments (5).

In mammalian cells, intermediate filament proteins (IFs) are the largest of the three major cytoskeletal protein groups. IFs are a wide collection of cytoplasmic and nuclear proteins that are expressed cell-specifically and are encoded by ~70 human genes, including keratins, lamins, vimentin ect. Keratins are located in specifically in epithelial cells, whereas it is replaced by vimentin in mesenchymal

cells; glial fibrillary acidic protein in glial and stellate cells; desmin myocytes; and lamins in the cell nucleus. Keratin has a ~310 amino acid common helical rod domain flanked by non-helical head and tail domains of various lengths and contains sequences with several phosphorylation sites. When keratins are phosphorylated, they become water-soluble, which allows keratin patterns to be re-formed during cell division, cytokinesis, cell differentiation, and migration (6). Keratins have a number of important functions, including its high expression protecting from cell death, cellular and subcellular mechanical integrity, protein targeting to subcellular compartments, scaffolding and regulation of cell signaling events (7). Most cells usually express only one kind of IF protein. Normal erythroid cells and many leukemic cells have solely vimentin IFs, which is consistent with their mesenchymal origin. However, cytokeratin positivity in malignant cells of hematopoietic origin has been reported. Järvinen et al. showed that undifferentiated K562 cells (a chronic myeloid leukemia cell line) contain typical glandular epithelial cell characteristics such as expression of cytokeratins along with vimentin. (6).

We previously reported that morphological changes accompanied imatinib resistance in K562 cells where cells gained an adherent phenotype. Transcriptome analysis revealed a significant increase in the expression of tescalcin in these imatinib resistant cells (9). We hypothesized that tescalcin expression may have induced the morphological changes observed in imatinib resistant K562 cells. Viral transduction of the tescalcin gene was performed into K562 cells resulting in an epithelial-like morphology when compared to the suspended ancestral K562 cells. We addressed whether keratins –a major cytoskeleton element of epithelial cells- were responsible for the observed morphological changes. Keratins 8, 18 and 19 were previously reported to be expressed in K562 leukemic cells (6). We aimed to examine how their expression is effected after tescalcin transduction.

MATERIAL AND METHODS

Cell Culture

Both control and LentiTESC transduced K562 cells were cultured in 25 and 75 cm2 surface area flasks, in RPMI-1640 medium containing 10% fetal bovine serum, 2 mM L-Glutamine and 1 unit/mL penicillin G, and 1 mg/mL streptomycin (Thermo Fischer, 15140122), in a humid incubator at 37ºC and 5% partial CO_2 pressure. Additionally, LentiTESC transduced K562 cells were under selection of 8 ug/ml Puromycin (Sigma, P7130) antibiotic. All manipulations were performed in a BSL 2 laminar flow cabinet.

Transduction K562 with TESC

Lentiviral particles with the inserts of tescalcin and GFP (green fluorescent protein) coding genes were purchased from Origene (RC229652L4V). Viruses contained a puromycin resistance gene. We initially performed an antibiotic selection kill curve to determine the concentration of puromycin. $5x10^4$ cells of K562 was seeded in 24-well plates. Puromycin (Sigma) solutions were prepared as 0, 0.25, 1, 2, 5, and 10 µg/mL and given to cells after 24 hours. Cells were observed for 120 hours. Puramycin was added to the culture media every 24^{th} hour. 100 µl were cell collected, stained with trypan blue (Sigma-Aldrich, St. Louis, MO, USA) and counted using a Neubauer chamber, as 8 replicates.

For the viral transduction of the TESC gene. 150000 K562 cells/well were cultivated in 24 well plates, in RPMI 1640 supplemented with 10% FBS, 1 unit/mL penicillin G, and 1 mg/mL streptomycin in a 37˚C, 5% CO_2 humidified incubator for 24 hours. Cells were centrifuged 200g for 5 minutes. TESC lentiviral particle (Origene RC229652L4V) and polybrene (Sigma 1003) was added to one well, while the other wells were polybrene, puromycın and non-treatment controls. All cells were incubated in at 37˚C in a 5% CO2 incubator for 24 hours. Medium was refreshed and 4 µg/ml puromycin was added. Efficiency rates of transduced cells were analyzed by measuring GFP expression using fluorescent inverted microscopy and flow cytometry.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

$3x10^6$ non-treated and TECS transduced cells were collected and total RNA was isolated by RNeasy extraction kit (Qiagen 74106). Quality of RNA was measured and calculated by OD260/OD280 nm absorption ratio (MultiskanGo, ThermoScientific). The isolated RNAs was converted to complementary DNA by reverse transcription (OneScript Plus G236) according to the datasheet prescription. The cDNAs were amplified by RT-PCR (Roche LightCycler 480) with usage of forward and reverse primers of K8, K18 and K19 genes. Cycling conditions were 95ºC for 5

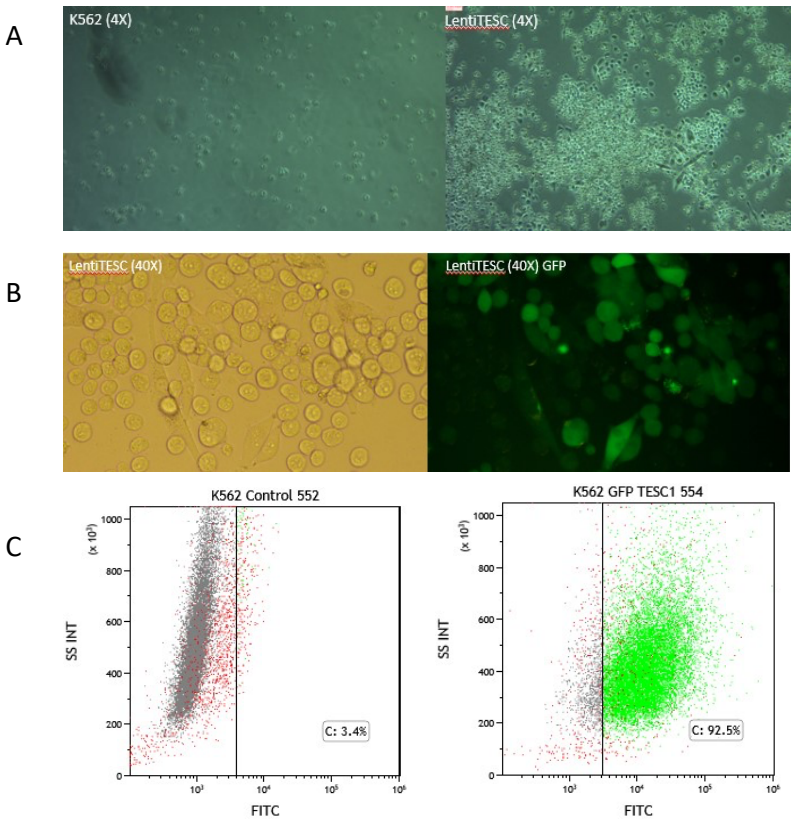

Figure 1. A) Morphology of K562 (control) and LentiTESC transduced K562 cells under brightfield inverted microscopy. LentiTESC cells are growing adherently and have different morphology when compared to control K562 cells. B) Brightfield and florescent light microscopy images of LentiTESC transduced K562 cells. Nearly all cells are observed to express GFP. C)Flow cytometry analysis of LentiTESC transduced k562 cells, transduction efficiency is observed to be 92,5%

min in order to polymerase activation/denaturation and 35 cycles (95ºC for 15 sec and 60ºC for 60 sec) for amplification followed by a dissociation process (65ºC for 5 sec then 5 sec each at 0.5ºC for increases between 65-95ºC). GAPDH was used as reference gene. ΔΔCt method was used for calculating mRNA expression relatively.

Western Blot Analysis

To evaluate the effect of tescalcin overexpression on K8, K18 and K19 filaments, K562 cell lysates were prepared, measured total protein concentrations and calculated as 40 ug/ml per well. Then loaded on sodium dodecyl sulfate polyacrylamide gel and run by electrophoresis. Separated proteins were transferred onto polyvinylidene fluoride (PVDF, Merck Millipore, IPVH00005) membranes from gel and blotted with primary antibodies specific to K8, K18, K19 and GAPDH followed by incubation with goat anti-mouse IgG antibody-HRP conjugate for K8, K18, K19 and GAPDH as secondary antibody. Protein bands were visualized by using enhanced chemiluminescence (ECL) reagent (BioRad, 1705060).

Statistical Analysis

Student's t-test (GraphPad Prism 5) were used to compare means. Data were expressed as mean ± S.E.M (n=3-5, each performed in triplicate), and a p value < 0.05 was accepted as statistically significant.

RESULTS

TESC Transduction Induced an Adherent Morphology in K562 Cells

K562 cells are hematopoietic cells that grow in suspension culture. K562 cells that have been transduced and express high levels of TECS were observe to change their morphology and gain adherent properties (Figure 1A). Transduction efficiency was analyzed by both flow cytometry and fluorescent microscopy (Figure 1B). Flow cytometry, revealed a %92,5 transduction efficiency based on GFP positivity (Figure 1C).

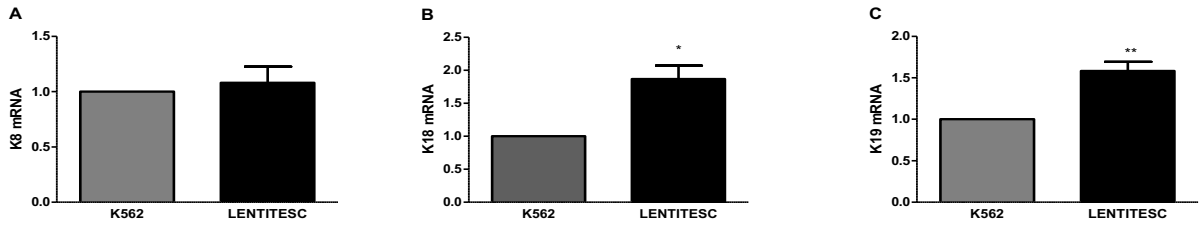

Figure 2. qPCR analysis of K8, K18 and K19: A) K8 Statical Analysis (n=5). B) K18 Statical Analysis (n=3). C) K19 Statical Analysis (n=5).

Keratin 18 and 19 Expressions Significantly Increases in LentiTESC Transduced K562 Cells

qPCR analysis of K8, K18 and 19 showed that while there is no significant change in K8 mRNA expression, K18 and 19 mRNA expressions have significant increased LentiTESC transduced K562 cells (Figure 2A, B and C).

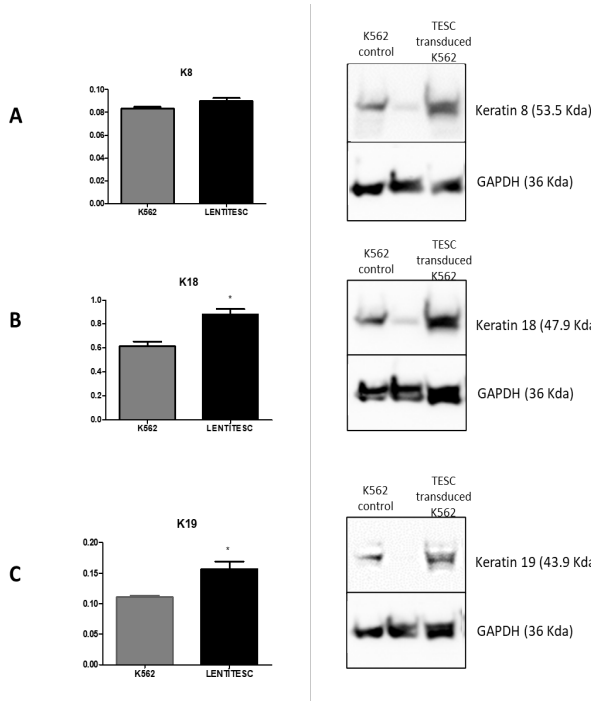

Figure 3. Western Blot images and image analysis of proteins: A) Statistical analysis of bands via Fiji (n=4) (left), western blot image of keratin 8 protein (right). B) Statistical analysis of bands via Fiji (n=3) (left), western blot image of keratin 18 protein (right). C) Statistical analysis of bands via Fiji (n=3) (left), western blot image of keratin 19 protein (right)

qPCR analysis of K8, K18 and 19 expression was verified by western blot. No significant change in K8 protein expression was observed (Figure 3A). On the other hand 18 and 19 protein expressions were significant increased in LentiTESC transduced K562 cells (Figure 3B and C).

DISCUSSION

In this study we aimed to detect changes in the expression of keratin 8,18 and 19 levels in TESC transduced K562 cells. The study was designed based on the hypothesis that morphological changes observed in TESC over-expressing K562 cells might have also lead to changes in the cytoskeleton. In epithial cells the main intermediate filaments that give the cells their shape and integrity are keratins. Keratins, are replaced by vimentins in cells of mesenchymal origin. However, Järvinen et al reported that keratin and vimentin were expressed simultaneously in K562 cells (6). Keratins have dense networks close to the membrane of epithelial cells and bind to desmosomes, forming the characteristic regular arrangement of epithelial cells and reinforcing cell-cell connections. Due to these properties, it is obvious that keratins can provide a disadvantage in mesenchymal cells that do not necessarily spend their life cycle connected to a basal membrane or another cell

In cancer, features such as the invasive properties and subsequent migration are directly related to the ability of cells to change their morphology and move; and are essential for carcinogenesis. Changes in the cytoskeleton are prominent features of cancerous cells. As mentioned previously, an important factor that enable cells to change their cytoskeleton is their dynamic intermediate filament composition. Changes in the intermediate filament composition during subsequent stages of carcinogenesis such as, epithelial mesenchymal transition and mesenchymal epithelial transition have been reported. (8)

Different studies have shown that tescalcin, has a yet to be identified role in the invasion and metastasis of cancer cells. We previously reported that a form of tyrosine kinase resistance observed in chronic myeloid leukemia K562 cells is a result of an adaptive phenotypic shift. A change in phenotype which includes adopting epithelial characteristics, confers cancer cells therapy resistant (9). In the experimental

context of K562 cells, this adaptive phenotypic shift was accompanied with high TESC expression. Changes in cellular morphology, invasive capacity and mobility are associated with changes in cytoskeletal elements.

CONCLUSION

Keratin 8, 18 and 19 proteins were previously reported to be expressed in K562 cells. We analyzed changes in the expression of these keratins at mRNA and protein levels, when TESC is over-expressed. We showed that TESC over-expression leads to predominant morphological changes in K562 cells. Keratin 18 and 19 mRNA and protein levels were significantly increased in LentiTesc transduced cells when compared to control K562 cells. Keratin 8 expression did not show any significant difference. It is plausible that TESC may have a role in regulating cell morphology through keratins via interacting with intermediate molecules such as DST. This is the first study reporting a correlation between TESC and keratin expression.

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