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Evaluation of the gene/protein expression levels of the potential stem cells marker TMEM121 in Thy1 positive cells

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Abstract

Introduction: Transmembrane protein 121 (TMEM121) is a conserved integral membrane protein highly expressed in the rat adrenal sub-capsular niche, where it co-localises with the proliferation marker Ki67 and is implicated in stem/progenitor cell self-renewal and tissue homeostasis. Thy-1$^+$positive cells (Thy1cells), isolated from rat adrenal cortex, exhibit mesenchymal stem cell characteristics early in culture but undergo spontaneous immortalisation with prolonged passaging. The dynamics of TMEM121 expression across this transition remain uncharacterised.

Materials and Methods: Thy1cells were isolated by Magnetic-Activated Cell Sorting (MACS) and monitored over nine months. TMEM121 mRNA expression was assessed by RT-qPCR using the ΔΔCt method with β-Actin as reference. Protein expression was evaluated by dual immunocytofluorescence using anti-TMEM121 and anti-Thy1 antibodies, with fluorescence area (μm²) and signal intensity quantified in >100 cells/condition using Fiji/ImageJ. Data are presented as mean ± SEM; group differences were analysed by unpaired Student's t-test.

Results: Thy1cells displayed slow, fibroblast-like colony growth in early culture, then underwent a marked phenotypic shift between months 4 and 6, with reduced substrate adherence and accelerated proliferation, indicating immortalisation. RT-qPCR showed a significant reduction in TMEM121 mRNA in late-passage (9-month) cells (1.1±0.3-fold) versus early-passage (2-month) cells (1.9±0.2-fold). Immunocytofluorescence confirmed a significant ($p < 0.001$) decline in protein expression, with 46.3% reduction in expression area and 63.6% reduction in signal intensity in late-passage cells.

Discussion: The progressive loss of TMEM121 parallels the transition from a homeostatic, niche-dependent state to unrestrained proliferation, mirroring its downregulation in human malignancies where it correlates with poor prognosis and dysregulation of the AKT1/p27/cyclin axis. Its context-dependent role—tumour-suppressive in some cancers yet oncogenic in others—suggests TMEM121 operates as a microenvironment-sensitive regulator whose silencing may confer a competitive proliferative advantage in vitro.

Conclusion: TMEM121 is significantly downregulated at both mRNA and protein levels during spontaneous immortalisation of Thy1cells, identifying it as a candidate marker of the stem-like homeostatic state and a potential target for further investigation in adrenocortical pathology.

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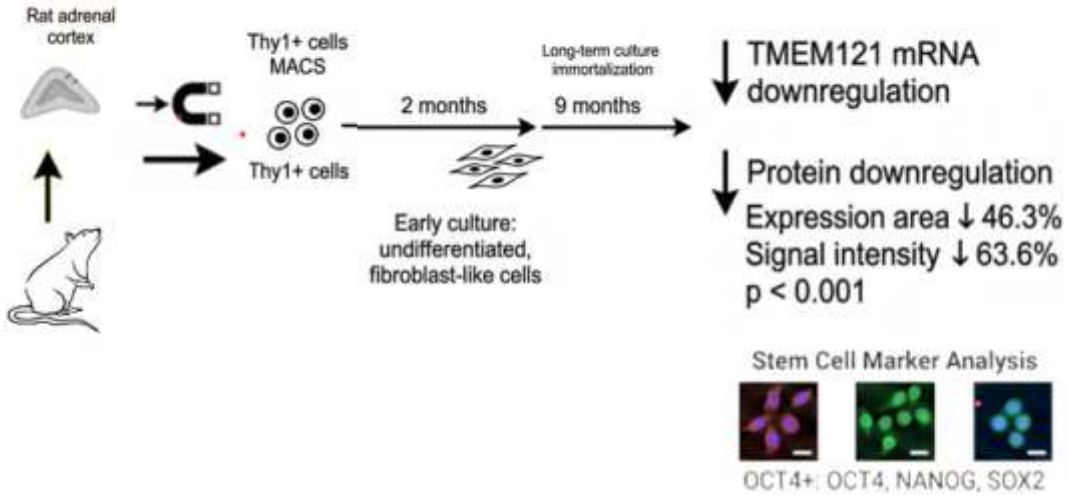
Graphical Abstract



Keywords:
TMEM121; Thy-1⁺ positive cells; RT-qPCR; immunocytofluorescence; spontaneous immortalisation; adrenal cortex; stem cell marker.

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Introduction

TMEM121 is a member of highly conserved proteins (TMEM), and also identified as (integral membrane protein), which have ambiguous cellular functions. The role of TMEM121 protein have been proposed to be associated with embryonic stem cell development [1]. This developmental role of TMEM121 suggests an important activity in adult tissue repair. For instance, TMEM121 demonstrated a role in wound healing activity of human endothelial cells, which potentially aids angiogenesis [2]. In the adrenal cortex of the rat, TMEM121 is highly expressed in the capsule and sub-capsular regions [3]. This specific localization in the sub-capsular area represents a niche for adrenocortical stem/progenitor cells [4–6]. A high co-localization rate with the proliferation marker Ki67 suggests a strong, though associated, link between TMEM121 and active cell division [3]. Others suggested TMEM121 as a potential suppressor of the Mitogen-Activated Protein Kinase (MAPK) signaling pathways [1]. Several research recorded TMEM121 localization within the endoplasmic reticulum (ER), where it might regulate ER morphology and/or organelle contact sites [7, 8]. Regarding ER's role in protein synthesis and calcium homeostasis, these findings consider TMEM121 as an influencer to tumorigenesis by managing cellular stress and/or metabolic reprogramming. Recent findings has challenged the model of TMEM121 as a universal driver of cell division. Because when the MDA-MB-231 breast cancer cells were transfected with the full coding sequence of TMEM121, cells demonstrated no significant increase in the proliferation rate [9]. It is proposed that the highly dysregulated or hypermethylated state of certain cancer genomes might render them unresponsive to TMEM121's regulatory functions [10, 11]. Cancer bioinformatics findings specify that TMEM121 was often downregulated in tumors, which correlates with poor prognosis in kidney and liver cancers, suggesting a tumor-suppressive role [12]. On the contrary, TMEM121 appears to act as an oncogenic facilitator in gastric cancer, as its suppression by miR-148a-3p inhibits cell invasion and growth [13]. This study aims to quantify and localize TMEM121 mRNA and protein expressions within isolated Thy1 cells at two different stages in culture to determine the cellular behavior regarding this marker during the process of immortalization.

Materials and Methods

Thy1cells culture and passaging

The isolated Thy1cells were isolated as previously described [14]. Initially, Cultures were maintained

at 37°C in a 5% CO_2 humidified atmosphere and subcultured using TrypLE™ (Gibco) upon reaching 70–90% confluence [15]. .the cells were requiring a period of 8-10 days to transition from a floating or semi-attached state to full, strong attachment to the culture plastic. This effective attachment required subsequent detachment for passaging that necessitated a long incubation of 20-25 minutes with TrypLE™ reagent. Once established, the cell culture was maintained by replenishing the growth medium every week. At early stages of culture, the cells were passaged at a low frequency, approximately every 4 weeks, only when they reached 90-100% confluence. The attached population exhibited a characteristic fibroblast-like morphology, forming cellular projections and, consistently from the first month in culture, a tendency to grow in colonies. However, at late stages of culture (6-12 months of culture), the cells were propagating and reach confluent faster, hence the changing of media and passaging were conducted more often.

RNA purification

RNeasy Mini Kit (Cat no. 74104) from QIAGEN was used for RNA purification. The procedure was conducted according to the manufacturer's instructions, which started by harvesting cells with TrypLE reagent, washing them with PBS, and pelleting them via centrifugation. The cell pellet is then lysed using Buffer RLT and homogenized. After adding ethanol, the lysate is applied to an RNeasy spin column and centrifuged. An on-column DNase digestion step is performed using a mix of DNase I and buffer RDD. The next step included washing the column with buffers RW1 followed by RPE. Then RNA was eluted with 50µl nucleases-free water. NanoDrop™ 2000/2000c was used to assess RNA's concentration and purity.

TMEM121 gene expression

Reverse transcription quantitative PCR (RT-qPCR) was employed to estimate TMEM121 gene expression. The reaction started with reverse transcription of mRNA, using 300 ng of total RNA to synthesize first-strand cDNA. EasyScript® kit SuperMix was used with oligo-T primers According to the manufacturer's instructions with modification [16]. RNA from biological replicates was pooled prior to this synthesis. The cDNA diluted 1:30 to be used as the template for the subsequent quantitative PCR (qPCR) reactions. These reactions were designed for relative quantification of TMEM121 expression. A specific set of primers were used for this process (Forward primer 5-3: CAGGACCTCGTCCCGCTTT; Reverse primer 5-3: TAGTCCAGCGTCTGTGCGGC). The housekeeping gene β-Actin was selected as an internal reference to confirm the normalized results using β-Actin forward primer 5-3: CACCCGCGAGTACAACCTTC; Reverse primer 5-3: CCCATACCCACCATCACACC). The mix of real time PCR was in volume of 20µl. The ingredients of this reaction included 0.5µL of each primer (10µM), 10µl of 2x QuantiTect SYBR Green PCR master mix (Qiagen), and 5 µl of the diluted cDNA. Then 4 µl of nuclease-free water to complete the volume to 20µl. The qPCR program started with initial denaturation at 95°C for 15 minutes. Then 40 cycles of denaturation at 95°C for 15 seconds, Annealing at 59°C for 1 minute. The reaction was ended with melting the PCR product, a step was conducted to show melting curve of the PCR product. The qPCR was performed using a computerized Rotor-Gene device. Gene expression of TMEM121 was relatively (with β-Actin) calculated as fold changes using the comparative ΔΔCt quantification method [17]. Gene expressions in the experimental samples were compared in both early and late stages of Thy1 cell culture.

Immunocytofluorescence

Immunocytofluorescence was performed using polyclonal mouse anti TMEM121(Abcam) and rabbit monoclonal anti Thy1(produced in house) primary antibodies. A dual- Immunocytofluorescent assay was used to quantify and localize their expression in Thy1 cells. Following Al-Bedhawi., (2018) procedure the assay included: culturing the cells on coverslips inside the wells of four or six well plate. After reaching the desired confluence, the cells were washed with PBS, fixed with 4% paraformaldehyde, then blocked with 10% goat serum. They were then incubated overnight at 4°C with the primary antibodies, diluted in a 1% goat serum solution[14]. Then cells were washed and treated with fluorescent Alexa-Fluor conjugated secondary antibodies (Abcam) (anti-mouse and anti-rabbit) for 30 minutes. Finally, after a final wash, the samples were mounted with (4',6-diamidino-2-phenylindole (DAPI)) to stain nuclei. The signals of Alexa Fluor (AF) 488 green and AF 546 yellow were visualized using a Zeiss-Axio Imager microscope. Fiji/ImageJ was used as described by Schindelin et al. [18]. This method was used to assess the expression area and intensity considering

consistent imaging settings for all samples and preventing pixel saturation to maintain quantifiable data and employ high resolution for clear visualization of cells.

Statistical Analysis
Quantifiable data were presented as mean ± standard error of the mean (SEM). Differences in TMEM121 mRNA and protein expression (area and intensity) between early and late-passage cells, were analyzed using an unpaired Student's t-test. Statistical significance was defined as P-value. All analyses and graphing were conducted using GraphPad Prism software.

Results

Characterization and Maintenance of Thy1Positive Cell Culture

Thy1positive cells displayed an extended primary adaptation phase during the first two weeks of culture, transitioning from a floating or partially adherent state to strong attachments to the culture plastic surface. The adherent cells population showed a characteristic of fibroblastic morphology, forming cellular projections and growing in colony formations from the first few weeks in culture. Consequently, cells were maintained with weekly medium changes and were passage at low frequency, approximately every 4 weeks upon reaching confluence. Thy1cells were transformed gradually during their 4^{th} to 6^{th} month of culture exhibiting less attachment to plastic and more propagating level as their confluence would reach from 30% to 100% within 5-6 days. These changes mainly indicate immortalization of Thy1cells.

Analysis of TMEM121 Gene Expression via RT-qPCR
Investigating TMEM121 expression, total RNA was successfully purified from Thy1cells using the RNeasy Mini Kit, with reliable total RNA concentration (150-400ng/µl) and purity of (260/280= 1.8-2.0). For RT-qPCR analysis, RNA from biological replicates was pooled, and 300ng was successfully reverse-transcribed into cDNA. Quantitative PCR was performed on diluted cDNA (1:30) using gene-specific primers for TMEM121, with β-Actin that served as the endogenous reference gene. Expression levels were calculated by relative quantification using ΔΔCt method. The RT-qPCR analysis demonstrated a significant reduction in the relative TMEM121 mRNA expression in Thy1 cells during the late stages of culture 1.1±0.3 in comparison to the early stages 1.9±0.2 (figure1).

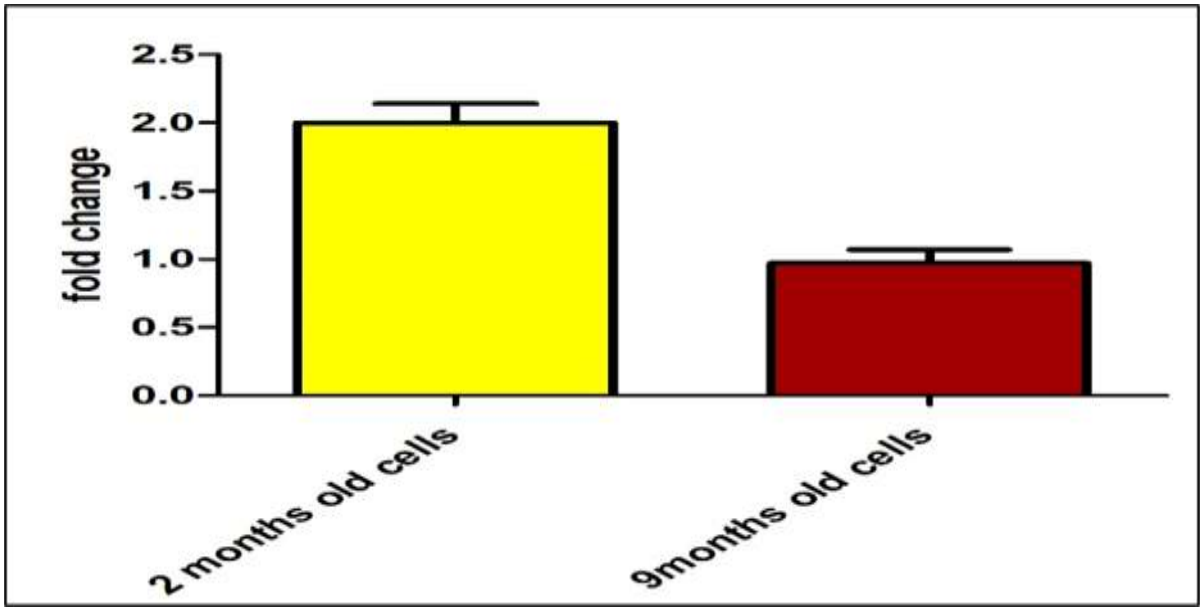

Figure 1. RT-qPCR relative quantification represented fold changes of TMEM121 mRNA expression after 2 and 9 months of cell cultures. Three biological replicates were used in this experiment.

Analysis of TMEM121 protein expression via immunocytofluorescence
Protein expression of TMEM121 was successfully assessed in parallel with detection of Thy1protein using specific antibodies by immunocytofluorescence assay (figure 2). TMEM121 Protein expression was estimated using two criteria (expression area and signal intensity) Expression area, which presented

by total fluorescent area per cell and the mean signal intensities of the expression after subtracting the background were quantified for >100 cells/condition using Fiji/ImageJ software. Data were presented as mean ± SEM in (table 1). The results were in harmony with the transcriptional data, immunocytofluorescence analysis revealed a significant reduction in TMEM121 protein expression in late-passage cells (9 months old) compared to their early-passage counterparts (2months old).

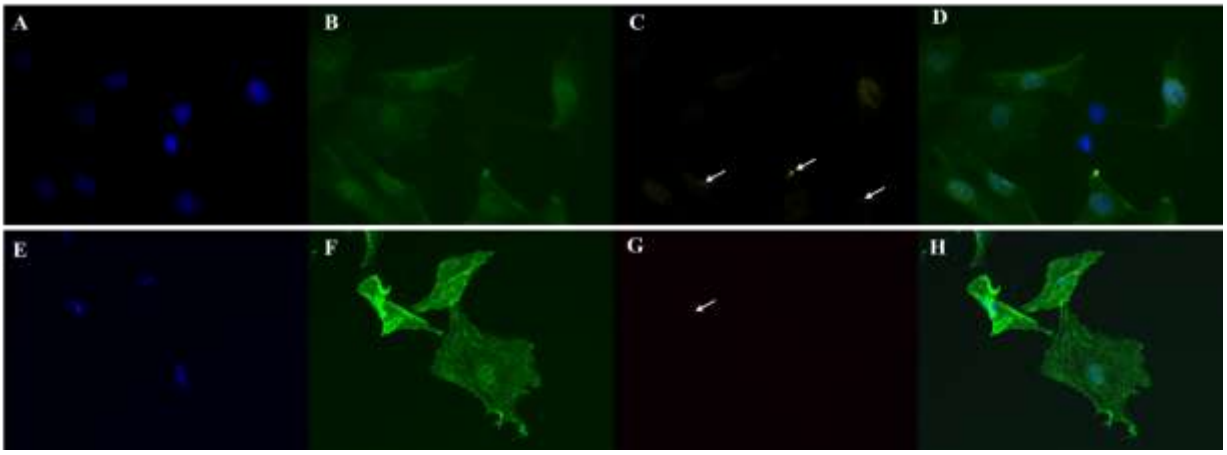

Figure 2. Dual-Immunocytofluorescence on 2months and 9month old cultures of Thy1cells. Cells were fixed with 4%PFA in PBS. Cells were incubated with mouse anti-Thy1antibody (B and F) or incubated with a rabbit anti-TMEM121 antibody (Abcam) (C and G) before detection with anti-mouse Alexa 488 and anti-rabbit Alexa 546 respectively. All samples were mounted, and cell nuclei were stained with DAPI in (A and E). Merged images of (A), (B) and (C) are shown in (D) and of (E), (F) and (G) in (H). The upper row of photos are 2months old cells (A, B, C and D). The lower row of photos are 9months old cells (E, F, G and H).

Table 1. Quantitative Analysis of TMEM121 Expression by Immunocytofluorescence

Parameter	**Early Passage (2 mo)**	**Late Passage (9 mo)**	**% Reduction**	**Statistical Significance**
Expression Area (µm²) (Mean ± EM; *n* cells)	147.4 ± 8.2 (n = 125)	78.7 ± 5.1 (n = 145)	46.3%	**p < 0.001**
Mean Signal Intensity (A.U.) (Mean ± SEM)	1024 ± 120	373 ± 85	63.6 %	**p < 0.001**

Discussion

The downregulation of TMEM121 in late-passage Thy1 cells reflects a significant shift in cellular identity, which might be driven by the transition from a homeostatic state to an unrestrained proliferation state. In its native environment, the adrenal sub-capsular niche, TMEM121 is suggested to function within a specific molecular pattern mainly required for regulation of self-renewal and tissue homeostasis [4, 5]. The co-localization of TMEM121 with the proliferation marker Ki67 in tissue sections further supports its role in maintaining a tightly controlled proliferative compartment [3]. However, the gradual adaptation of the cultured cells to *in vitro* conditions forces a change from their niche-dependent regulation to a loss of mRNA and protein expression that reveals a spontaneous immortalization. In this perspective, TMEM121 might affect growth through certain pathways associated with tumor suppression and cell cycle regulation. For example, TMEM121 was downregulated in malignant cancers like cervical and prostate cancer. The expression level of TMEM121 was positively correlated with the expression level of AKT1 and p27 (cell cycle regulator) while negatively correlated with the expression levels of BCL-2 (anti- apoptotic protein), cyclin D1, and cyclin E2 PIK3CB, CASP3(caspase-3) and E-cadherin (CDH1) [19]. These findings connect this downregulation with the elevation of tumor progression and reduced clinical outcomes. While TMEM121 experimental activation inhibits viability and migration [19, 20]. Thus, TMEM121 might be suggested to have a restraining effect on these cancer cell cycles, or work as a modulator of growth factor signaling. When considering the environment of a culture medium, cells live in rich medium and spatially unrestrained. In such environment, cells, which silence these inhibitory signals would gain a competitive advantage. The functional role of TMEM121 exhibits significant context dependency,

particularly within oncogenically transformed cell states. This is exemplified by its dichotomous effect on tumor progression. In pancreatic adenocarcinoma, elevated TMEM121 expression is correlated with a more favorable prognosis, indicating a potential tumor-suppressive function[21]. Conversely, in fully malignant, aggressive models such as the MDA-MB-231 breast cancer line, the forced overexpression of TMEM121 demonstrates no measurable impact on cellular proliferation [9]. This suggests that the regulatory pathways which TMEM121 modulates under homeostatic conditions may become fundamentally disrupted or entirely bypassed in advanced disease stages. Collectively, these observations support a model in which TMEM121 operates as a contextual homeostatic regulator, with its biological output being critically determined by cues from the tumor microenvironment [13]. The recorded decline in TMEM121 expression across the period of spontaneous immortalization of Thy1 cells, reveals a fundamental molecular adaptation within the culture. This adaptation likely represents a strategic shift, where the cellular program is reconfigured to prioritize proliferative fitness and survival, potentially at the cost of its initial stem-like properties.

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Availability of Data and Materials
All data generated or analyzed during this study are included in this published article. The raw datasets supporting the conclusions of this article, including RT-qPCR data and immunofluorescence images, are available from the corresponding author upon reasonable request. The Thy1-positive cell line used in this study was isolated and characterized as previously described (Al-Bedhawi, 2018), and samples may be made available subject to institutional and ethical approval.

Authors' Contributions
Mohammed Abdalmalek Ali Al-Bedhawi (MAAA): Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing - original draft, Writing - review and editing, Visualization, Project administration. MAAA was responsible for the isolation and culture of Thy1-positive cells, performed all RT-qPCR experiments, conducted immunocytofluorescence analyses, performed statistical analyses, and drafted the manuscript.
Zainab H. Abood[2] **&Riyam Basim Ali** Conceptualization, Methodology, Resources, Supervision, Writing - review and editing, Validation. RSA provided expert guidance on experimental design, contributed to data interpretation, provided critical intellectual input, supervised the project, and reviewed and edited the manuscript. Both authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate
All animal procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Baghdad. The Thy1-positive cells were isolated from rat adrenal cortex following approved protocols as previously described (Al-Bedhawi, 2018). All experimental protocols were reviewed and approved by the Ethics Committee of the Institute of Genetic Engineering and Biotechnology, University of Baghdad (Approval Number: [Insert approval number if available]). Animals were handled and sacrificed humanely in accordance with institutional guidelines and international standards for animal welfare.

Patient Consent for Publication
Not applicable. This study did not involve human subjects, human data, or human tissue samples. All experiments were conducted using animal-derived cell cultures.

Competing Interests

The authors declare that they have no competing interests. There are no financial or non-financial interests that could be perceived as influencing the objectivity, integrity, or interpretation of this research. Neither author has any affiliations, memberships, or financial holdings that might be perceived as affecting the objectivity of this study.

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