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# EVALUATION OF THE EFFECT OF THE EXPRESSION INDUCTION OF TMEM 121 GENE ON Y1 CELLS VIABILITY, PROPAGATION AND MIGRATION

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**ABSTRACT**
Transmembrane Protein 121 (TMEM121) is spatially expressed in the adrenal capsule which considers a niche for stem/progenitor cells. Yet its biological function remains enigmatic. Overall studies present a dilemma, due to conflicting evidence suggesting TMEM121 may act as either a tumor suppressor or an oncogenic driver. To present a relative resolve to this ambiguity, this study was designed to speculate TMEM121 role in cell division and migration by inducing its expression in a mouse adrenocortical Y1 cell model. The current study employed computerized time-lapse microscopy following the induced expression of TMEM121 and its effect on cellular kinetics. A pIRES2-EGFP vector system was generated by cloning the TMEM121 coding sequence into this vector prior to transfection into Y1 cells. The transfected cells were then monitored over a 40-hour period to quantify the cellular key metrics. The results revealed that TMEM121 acts as a significant negative regulator of Y1 cell behavior ($p < 0.01$). Following transfection, a significant reduction in viable cell counts relative to controls was recorded ($p < 0.05$). However, this suppressive effect proved transient, because the continuous monitoring revealed the inhibition was later subsided, and cell viability converged with control levels within seven days, showing a non-significant difference from the control group. In terms of propagation, the expression of TMEM121 exerted a potent anti-proliferative effect; quantitative analysis showed a marked suppression in population doubling ($p < 0.01$), with the propagation index (60%) lower than the control group. The current study also observed that TMEM121 significantly impaired cell motility ($p < 0.01$) with approximately over 60% reduction. The behavioral motility of the TMEM12 transfected cells record lower directional persistence ($p < 0.01$), often pivoting in place rather than progressing linearly. This might occurs due to the potential downregulation of the PI3K/AKT signaling pathway, which is crucial for cytoskeletal remodeling which might be correlated with TMEM121 expression induction.

**KEYWORDS:** TMEM121, Y1 cells, pIRES2-EGFP, Propagation and migration

## INTRODUCTION

The TMEM family constitutes a vast and functionally diverse group of proteins that span biological membranes. These proteins play important roles as essential mediators of signal transduction, ion transport, and cellular homeostasis [1]. TMEM121 stands out as a highly conserved protein whose biological functions are becoming increasingly significant despite remaining largely unclear. Early characterizations of TMEM121 emphasized its presence during embryonic stem cell development [2], suggesting a role that likely translates into adult tissue repair mechanisms. Intriguingly, in the adrenal gland, TMEM121 exhibits highly specific localization within the capsule and sub-capsular areas [3], which are recognized as critical niches for adrenocortical stem/progenitor cells [4–6]. This stem-like association is supported by observations of a 72% co-localization rate between TMEM121 and the proliferation marker Ki67 in these zones, implying a strong link to active cell division [3, 7]. However, the role of TMEM121 as a driver of cell division is complicated by conflicting of the experimental evidence. Although the molecular characterization suggests it may influence tumorigenesis through stress management or metabolic reprogramming, it mainly failed to change cell growth. For instance, expressing the full coding sequence of TMEM121 in MDA-MB-231 breast cancer cells failed to yield a significant increase/decrease in proliferation rate [8]. This lack of response potentially attributable to the hypermethylated or dysregulated state of specific cancer genomes [9, 10]. Furthermore, bioinformatics data indicate that TMEM121 may actually function as a tumor suppressor in kidney and liver cancers [1]. These apparent paradoxes suggested that TMEM121 operates as a context-dependent regulator, potentially modulating pathways such as the Mitogen-Activated Protein Kinase (MAPK) or PI3K/AKT signaling cascades [1, 2, 11]. To resolve these dynamics, the mouse adrenocortical Y1 cell line offers most similar niche where TMEM121 potentially suggested as a potential stem cell marker [3]. By inducing TMEM121 expression in Y1 cells via the pIRES2-EGFP vector system and quantifying the resulting changes in cell viability, propagation, and migration. This investigation may clarify the role of TMEM121 in the immortalization process and its potential as a pivotal regulator of adrenocortical cell behavior.

## MATERIALS AND METHODS

### Ethics

This study was approved by institutional ethics committee of institute of genetic engineering and biotechnology/university of Baghdad for animal and cell research.

### Animals

Mouse adrenal glands were isolated from adult albino BALB/c mice (8–12 weeks old). Mice were housed under controlled temperature and light conditions and euthanized by $CO_2$ asphyxiation in compliance with relevant animal welfare regulations.

### RNA Isolation

Total RNA was isolated from mouse adrenal tissue starting with homogenizing in GENEzol™ Reagent (Geneaid) and following the manufacturer's protocol. In brief, samples in GENEzol were subjected to chloroform-mediated phase separation the centrifugation. Following centrifugation, the RNA was precipitated with isopropanol, washed with 75% ethanol, and resuspended in RNase-free water. To eliminate potential genomic DNA contamination, the samples underwent DNase treatment. The concentration and quality of the purified RNA was subsequently assessed using a NanoDrop 2000 spectrophotometer (ThermoFisher). RNA integrity was visually confirmed via agarose gel electrophoresis.

### cDNA Synthesis and RT-PCR

Aliquots of total RNA was utilize to generate First-strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). This cDNA template was subsequently used to amplify the full-length coding sequence of mouse *TMEM121* gene (transcript variant X1, accession number: NM_153776.2) using gene-specific primers designed via NCBI Primer-BLAST. The thermal cycling protocol consisted of an initial denaturation at 94°C for 1 min, followed by 39 amplification cycles (denaturation at 94°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 2 min), and concluded with a final extension step at 72°C for 5 min. To isolate the specific amplicon, PCR products were resolved on a 1.5% agarose gel, and the target band was excised and purified using QIAquick Gel Extraction Kit (ID. 28704).

### *TMEM121* Gene Cloning Strategy

*TMEM121* amplicon (after purification) was used as the template for a secondary PCR amplification in order to facilitate directional cloning. The cloning strategy included designing EcoRI and HindIII restriction sites upstreamat the 5′ ends of the forward and reverse primers, respectively. A GC-rich platform sequences (GCG and CGC) were added upstream the restriction sites, which ensure efficient enzymatic cleavage. Hence, the resulting full-length primers used for cloning were:

- EcoRI-Forward: 5′-GCGGAATTCCGGCGCGCTGCATCTG-3′
- HindIII-Reverse: 5′-CGCAAGCTTCGTGGTCCGAGAGATTCCTG-3′

Following amplification by PCR, the PCR product was run by agarose gel and the designated band was purified and subjected to double digestion with EcoRI and HindIII enzymes(Promega). The pIRES2-EGFP expression vector underwent same digestion protocol to create compatible ends. Finally, the purified insert was ligated into the linearized vector using T4 DNA ligase (Promega) during an overnight incubation at 4°C.

### Transformation and Plasmid Verification

The ligation mixture was transformed into Mach1™ *E. coli* competent cells employing the Inoue method. Successful transformants were initially identified via blue-white screening. Plasmid DNA was isolated using the **Qiagen Midiprep kit** and subsequently validated (confirming the structural integrity of the construct) using bidirectional sequencing (Source BioScience). And also through double digestion with EcoRI and HindIII enzymes.

### Cell Culture Conditions

The biological model for this study was mouse adrenocortical Y1 cell line (ATCC® CCL-79™). As several types of cancer cell lines, cultures were maintained and propagated in Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10% fetal bovine serum (FBS) and supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (2 mM). All cells were maintained at 37°C within a humidified incubator containing 5% $CO_2$ [12].

### Transfection of Y1 Cells

To initiate transfection, Y1 cells were seeded into 6-well plates and cultured until they reached 70–90% confluence. Transfection complexes were generated by diluting 3 µg of plasmid DNA which either the target pIRES2-EGFP-TMEM121 construct or an empty pIRES2-EGFP control. The construct was diluted in 300 µL of serum-free DMEM. This solution was complexed with 3 µL of TurboFect transfection reagent and incubated for 20 minutes at room temperature to facilitate DNA binding. The resulting mixture was added dropwise to the wells with cell monolayers.

The transfection medium was exchanged with fresh complete DMEM after 5–6 hours. Transfection efficiency was evaluated 24 hours of transfection by visualizing GFP expression using an AXIO Inverted Epifluorescent Microscope.

**Assessment of Cell Viability and Recovery**

Transfected Y1 populations (TMEM121-expressing and empty vector controls) were expanded under general standard conditions of media supplement. Cultures were maintained at 37°C in a 5% $CO_2$ humidified atmosphere and subcultured using TrypLE™ (Gibco) upon reaching 70–90% confluence. Prior to initiating kinetic assays, baseline cell health was established using trypan blue exclusion staining, with cell densities quantified via a hemocytometer [13]. To evaluate the long-term impact of TMEM121 expression on post-transfection recovery, a distinct experimental cohort was maintained for 7 days. At the conclusion of this period, cell viability and population counts were reassessed to determine survival rates.

**Propagation and migration assays via Time-Lapse Microscopy**

To analyze the impact of TMEM121 expression induction on cells propagation and migration, Cells were seeded into 6wells plate as previously described, when they reached 30-50% confluence, the assay was initiate. A computerized time lapse system (Axio Imager) was used to track cell division. The system was continuously acquiring images every 10 minutes for over 40 hours to a random clear field of each well. The propagation and migration of cells was subsequently analyzed using quantitative digital analysis of the time lapse imagery. By tracking x,y coordinates across frames, the quantitative digital calculated several key metrics to quantify cell behavior. Accumulated distance represents the total path length and indicates overall metabolic activity, while Average Velocity measures speed in micrometer/hour. The system also assess Persistence, the ratio of displacement to total distance, to determine if motion is directional or random. Finally, Mitotic Frequency counts division events per hour to provide a direct measurement of proliferation inhibition.

**Statistical Analysis**

Data are presented as mean ± standard deviation. Differences between groups were analyzed using an unpaired two-sample t-test with one-way ANOVA where appropriate. A p-value $< 0.05$ was considered statistically significant.

**RESULTS**

**Cloning and Expression of TMEM121**

Total RNA was successfully purified from Mouse adrenals, with adequate total RNA concentration (250-780ng/µl) and purity of (260/280= 1.9-2.2). The purified RNA was profiled by agarose gels electrophoresis, which showed clear 28s and 18s bands (figure 1(lane 2,3 and4)) , which represents total RNA with acceptable integrity The full-length coding sequence of mouse TMEM121 was successfully amplified from mouse adrenal gland cDNA using RT-PCR. The PCR product was then modified to incorporate flanking EcoRI and HindIII restriction sites and subsequently cloned into the mammalian expression vector pIRES2-EGFP. Agarose gels electrophoresis of the vector after digestion with the two restriction enzymes ( figure 1 lane 6) and the sequence analysis confirmed the in-frame insertion of the TMEM121 gene with no mutations. Transient transfection of mouse adrenocortical Y1 cells with the pIRES2-EGFP-TMEM121 construct resulted in efficient expression (30-45%), as visualized by widespread EGFP fluorescence 24 hours post-transfection, confirming successful delivery and co-expression of the gene of interest.

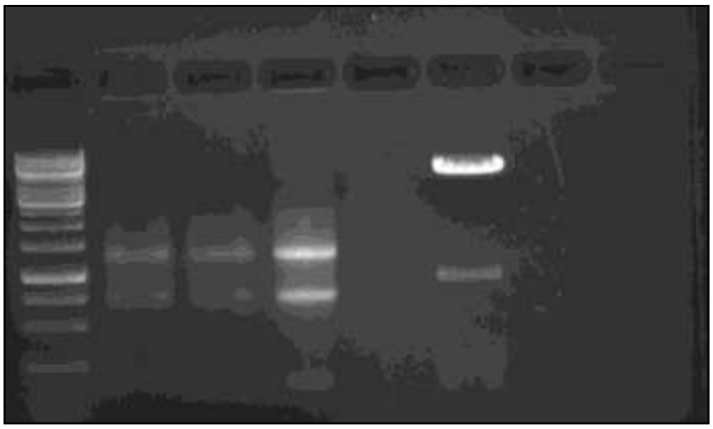

**Figure 1.** Agarose gel electrophoresis: Lane 1 is 1Kb ladder (ThermoFisher). Lanes (2,3 and 4) each is 10µl of isolated total RNA from mouse adrenals. Lane 6 reveals Two bands. The large one represents the mammalian expression vector pIRES2-EGFP after digestion with the two restriction enzymes EcoRI and HindIII and the small one (approximately 1200bp) is the TMEM121 insert.

**Effect of TMEM121 Expression induction on Y1 Cell Viability**

The expression of TMEM121 significantly impacted the viability of Y1 cells in the initial period following transfection. Trypan blue exclusion staining revealed a significant decrease ($p < 0.05$) in viable cell count in the TMEM121 transfected wells compared to wells transfected with the empty vector (control). However, with the extended monitoring, this inhibitory effect on viability was gradually faded over a period of seven days. The viability of TMEM121 transfected cells recovered to levels similar to the control group (table 1).

**Table 1. Detection of Y1 cell viability via Trypan blue exclusion**

| Parameter | Empty Vector Control Group | TMEM121-Transfected Group | Notes / Statistical Significance |
|---|---|---|---|
| Effect on Viability | Baseline Viability | Inhibitory effect observed | Impact was transient |
| Viable Cell Count (Initial Post-Transfection) | Normal | Decreased compared to control | Non-significant decrease *($p < 0.05$)* |
| Viability Trend Over 7 Days | Stable, comparable levels | Recovered to levels comparable to the control group by Day 7 | The initial inhibitor |

**Effect of TMEM121 Expression Induction on Y1 Cell Propagation**

The influence of TMEM121 over expression induction on cell propagation kinetics was analyzed by time lapse microscopy during a 40 hours incubation period. The transfection experiment included either a PIRES2-EGFP control vector (empty) or a PIRES2-EGFP vector containing the TMEM121 coding sequence. The captured images were analyzed with 10 hours' time interval to quantify population expansion and morphological shifts using Quantitative digital analysis of the time lapse imagery. The results revealed a significant ($p < 0.01$) divergence in propagation rates between the two experimental groups. Following the initial time laps (T=0 to T=10 hours), the control group exhibited robust exponential growth, characterized by frequent mitotic events and the formation of expanding colonies (figure 2). By the 40-hour terminal point, the control cells reached high confluency (approximately 85–90%,). In contrast, TMEM121 wells cells demonstrated a marked suppression in population doubling. While initial seeding densities were comparable regardless to the impact of both group transfection effect (which also showed a similar impact on both groups), the TMEM121 expressing population exhibited a sustained reduction in feature density, maintaining a propagation index approximately 60% lower than that of the control group across all subsequent time points (T=10 through T=40). Statistical metrics derived from texture and feature density analysis (Laplacian variance) are summarized in (table 2).

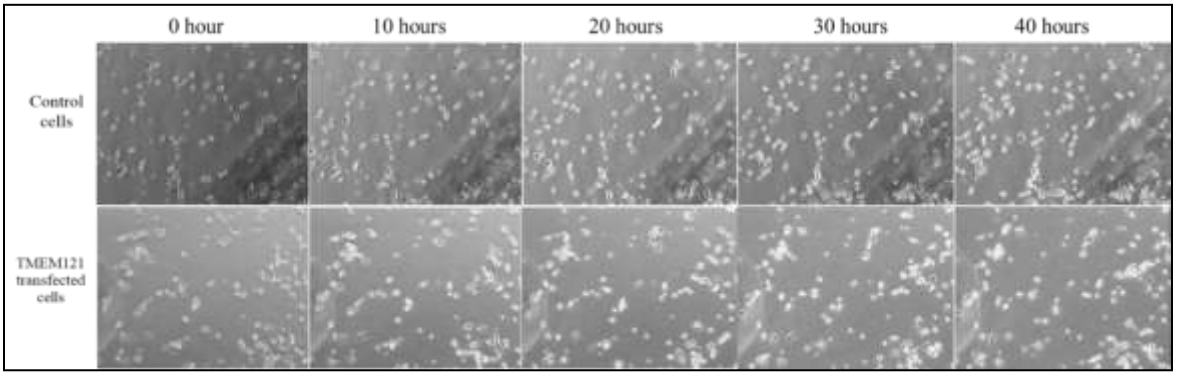


**Figure 2.** Comparison of Y1 cell kinetics by time laps microscopy of cells (time 0-40 hours using 10 hours intervals on 10x power magnification). Cells were cultured to reach 30-50% confluency before treatment. Upper row, transfected with PIRES2-EGFP empty vector (Control). Lower row, transfected with TMEM121coding sequence insert in PIRES2-EGFP vector (treatment).

**Table 2:** Impact of TMEM121 Expression on Relative Cell Density and Population Propagation over a Time Course of 40 hours.

| Time Interval (h) | Control (Relative Density) | TMEM121 (Relative Density) | Inhibition Ratio (%) |
|---|---|---|---|
| 0 | 1.00 | 1.00 | — |
| 10 | 1.42 | 0.58 | 59.1% |
| 20 | 1.88 | 0.64 | 65.9% |
| 30 | 2.15 | 0.72 | 66.5% |
| 40 | 2.44 | 0.81 | 66.8% |

*Note: Relative density is normalized to the baseline state of the control group.*

The data indicates that TMEM121 expression applies an effective proliferative inhibition on Y1 cells. This suggests that TMEM121 induction leads to a significant prolongation of the cell cycle or a reduction in the proportion of actively cycling cells (growth fraction). The plastic surface large gaps in the treatment groups field of vision at 40 hours, compared to the near confluence of the control, confirms that TMEM121 acts as a negative regulator of Y1 cell expansion under these experimental conditions.

**Cells migration**

The kinetic analysis of the time-lapse microscopy demonstrated significant ($p < 0.01$) impairing of TMEM121 to the migration capacity of Y1 cells. While control cells exhibit dynamic typical *in vitro* searching behaviors, which are characterized by rapid lamellipodial extensions and high directional persistence. In the fields of TMEM121 transfected wells, a significant number of cells appear to be more tethered to the plastic. These cells show a marked reduction ($p < 0.01$) in average velocity and accumulated distance. These cells often pivot in place rather than progressing linearly across the field of view. These cells more likely were the ones receiving the TMEM121 vector. Furthermore, TMEM121 transfected cells demonstrate reduced intercellular interaction; they remain largely isolated and fail to form the organized clusters seen in the control group, suggesting a disruption in chemotactic sensing or cell to cell adhesion mechanisms these findings are summarized in (table 3).

**Table 3: The analysis of comparative kinetic of control and TMEM121 transfected Y1 cells**

| Statistic | Control Cells | TMEM121 Transfected | Interpretation |
|---|---|---|---|
| Mean Velocity | ≈25–30 μm/h | ≈8–12 μm/h | Migration speed reduced by >60%. |
| Persistence | High (Linear) | Low (Random/Pivoting) | Loss of directional motility. |
| Motility Pattern | Active Crawling | Vibrational/Stationary | Impaired cytoskeletal remodeling. |
| Cell Morphology | Elongated/Polarized | Rounded/Apolar | Failure to establish a leading edge. |
| Interactions | Dynamic Clustering | Isolated | Reduced "social" sensing/adhesion. |

**DISCUSSION**

The present study revealed the alteration in the activity and viability of Y1 adrenocortical cells after TMEM121 expression induction. Cloning and transfection to induce TMEM121 expression in Y1 cells significantly impair their cellular behavior, mostly on proliferation and dynamic activity. Previous studies on adrenal cortex, might argue these findings without the appropriate physiological backdrop of the adrenal cortex, specifically the capsule or sub capsular niche [3]. This zone is recognized as the adrenocortical stem and/or progenitor cells [4, 5]. The characterizations of TMEM121 as a potential stem cells marker in these zones that reported a high co-localization rate (72%) with the proliferation marker Ki67, suggested TMEM121 as an actively associated factor with cell division in the healthy adrenal cortex [3]. However, our quantitative data in the Y1 cell line revealed the opposite. In contrast to the adrenal capsule where it appears to support renewal, TMEM121 exerts an anti proliferative effect in the Y1 model. The significant suppression of population doubling, migration and the formation of interstitial gaps presented TMEM121 as a negative regulator. This discrepancy aligns with recent findings in other cancer models; for instance, expressing the full coding sequence in MDA-MB-231 breast cancer cells similarly failed to produce a significant increase in proliferation [8]. This behavior of TMEM121 could be described as context dependent because this protein acted as a promoter in stem niches but a restrainer in cancer cell lines. This suggestion is further supported by bioinformatics data that indicate TMEM121 as a tumor suppressor in kidney and liver cancers [1]. The reduction of a proliferative response in Y1 cells after TMEM121 transfection might be attributed to a cascade of events led to hypermethylation or dysregulation state of the cancer genome. These changes can fundamentally alter the functional outcome of gene re-expression [9, 10]. The cellular motility and directional persistence that was impaired by TMEM121 expression induction, were aligned with similar data of cervical cancer where TMEM121 overexpression significantly reduces HeLa cell viability and inhibits migration, which interfere with PI3K/AKT signaling pathway [1]. As the PI3K/AKT pathway plays a crucial role for cytoskeletal remodeling and forward propulsion, its suppression offers a rational explanation for the 60% reduction in velocity and the distinct "pivoting" behavior that was observed in our transfected Y1 cells. It is worth noting that while the initial inhibitory effect on viability was significant, it was also transient, with levels recovering after seven days. This temporal dynamic suggested that while TMEM121 initially acts as a restraint on the cell cycle that potentially by inhibiting pathways like MAPK or PI3K/AKT [1, 2], the cells possess the plasticity to eventually undergo metabolic reprogramming to adapt. In conclusion, this study confirms that TMEM121 has a significant role in modulating the activity of Y1 adrenocortical cells, which was distinct from its role in the adrenal stem/progenitor niche [3]. The dual roles of TMEM121 as growth suppressor *in vitro* using the Y1 cells as a model, and growth stimulator *in vivo* in adrenal cortex present valid data that are fundamental for understanding adrenal physiology and its potential implications in adrenocortical pathologies [7, 14].

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