**Exploring the Dynamics of Signal Transmission via Extracellular Vesicles in a Glaucoma Cellular Model.**

**חקר מנגנוני הקינטיקה והדינמיקה שמקורם ב- extracellular vesicle במודל תאי הקשור במחלת הגלאוקומה**

Keywords: Extracellular Vesicles, Exosomes, Signaling, Primary open-angle glaucoma, POAG, Trabecular meshwork, Non-pigmented ciliary epithelium, Oxidative stress

Scientific abstract – ***Exploring the Dynamics of Signal Transmission via Extracellular Vesicles in a Glaucoma Cellular Model.***

This research proposal aims to investigate the role of extracellular vesicles (EVs) in primary open-angle glaucoma (POAG), specifically their function as signaling mediators. While existing knowledge supports the transfer of miRNAs and proteins from non-pigmented ciliary epithelium cell (NPCE)-derived EVs to recipient trabecular meshwork (TM) cells, there is a lack of understanding regarding the dynamic aspects of EVs concentration, the duration of TM cell exposure to NPCE-EVs, and the fate of the initial signal delivered by NPCE-EVs after processing by TM cells.

This study will delve into the signal amplification within TM cells and the subsequent delivery of signals by TM-EVs. It will also explore the potential paracrine and autocrine effects of TM-EVs on unexposed TM cells in response to the original NPCE-EVs. To achieve these objectives, we have developed a Cre-LoxP reporting system that will enable the tracking of NPCE-EV uptake into TM cells and the simultaneous real-time monitoring of TM-EV release. Overall, we aim to uncover dynamic changes in TM cell-derived EVs in the context of POAG.

**We hypothesize that NPCE-derived EVs dynamically influence the cargo of TM cell-derived EVs**, activating specific signaling pathways and promoting the expression of particular miRNAs and proteins. This interaction enhances cellular responses and signaling outcomes, particularly with respect to extracellular matrix (ECM) turnover in HTM cells.

We aim to comprehensively understand the dynamic aspects of EV-mediated signaling between NPCE-derived and TM-derived EVs, with a focus on their role in the management of POAG. To achieve this goal, we propose five Specific Aims:

**Aim 1:** Quantify TM-derived EV release in response to NPCE-derived EVs.

**Aim 2:** Conduct a qualitative analysis of cargo changes in TM-derived EVs after exposure to NPCE-derived EVs, with a focus on miRNAs and proteins.

**Aim 3:** Compare the effects of continuous versus limited exposure to NPCE-derived EVs on TM-derived EV quantity and cargo.

**Aim 4:** Investigate quantitative and cargo modifications in TM-derived EVs after exposure to oxidative stress signals conveyed by NPCE-derived EVs.

**Aim 5:** Assess autocrine and paracrine effects of TM-derived EVs following exposure to NPCE-derived EVs, exploring how modified signals impact initial and neighboring TM cells.

In summary, our research aims to provide a comprehensive understanding of the dynamic changes in TM cell-derived EVs in the context of POAG. Ultimately, this study seeks to shed light on the intricate mechanisms through which EVs mediate signaling between NPCE-derived and TM-derived EVs, offering valuable insights into their role in the pathogenesis of POAG and potentially opening new avenues for treatment and intervention.

**Scientific background**

The primary focus of this research proposal is to delve into the dynamics and kinetics of extracellular vesicles (EVs) as essential signaling mediators. Our investigation will be conducted using a well-established in-vitro model that has been a cornerstone of our lab's research for numerous years, aiming to unravel the pivotal role of ocular drainage system signaling in the context of primary open-angle glaucoma (POAG) disease.

The progressive aging of the population of the Western world is expected to give rise to an increase in the prevalence of POAG, which already affects over 80 million people worldwide [1, 2]. POAG is characterized by optic neuropathy, leading to retinal ganglion cell (RGC) axon loss, RGC death, and blindness associated with pathological processes occurring in the ocular drainage system [3]. Non-pigmented ciliary epithelium cells (NPCEs) continuously produce aqueous humor (AH) which nourishes and cleanses the anterior chamber before being drained out of the eye, with ~80% of this drainage occurring via the conventional outflow pathway comprised of the trabecular meshwork (TM), juxtacanalicular tissue (JCT), and Schlemm's canal [4]. The AH plays an important role in maintaining the spherical structure of the eye and intraocular pressure (IOP). A sustained increase in IOP is a primary risk factor for the development of POAG [5]. Despite intensive research on the regulation of IOP, there exists a gap in the knowledge of the etiological and pathophysiological processes that influence it in the context of disease. Targeted efforts to lower IOP are currently the only strategy available to prevent the loss of vision. Extant pharmacological treatments focus on lowering elevated IOP using drugs that modulate AH production, including β-blockers and carbonic anhydrase inhibitors, or AH drainage, including prostaglandin analogs, α-agonists, and Rho kinase (ROCK) inhibitors [6].

Studies have revealed the significant involvement of TM tissue in the development of AH drainage resistance. An optimal level of extracellular matrix (ECM) remodeling in the TM-JCT region is needed to generate appropriate levels of AH resistance, but excessive ECM deposition can result in increased resistance and elevated IOP [7, 8]. In POAG, characteristic changes occur in the tissue structure of the AH outflow pathway. Major changes in glaucomatous eyes include altered ECM organization in the TM-JCT region and the accumulation of sheath-like plaque materials, leading to altered stiffness [9]. Current evidence suggests that the changes in the TM drainage pathway in POAG involve many regulatory proteins, including matrix metalloproteinases [10], clusterin [11], cathepsin K [12], TGF-β [13], and integrins [14]. This suggests the potential value of using drugs targeting ECM turnover as a standalone therapy or in combination with existing treatments for lowering IOP to protect against POAG progression.

Delivering RNA or proteins that are vulnerable to nucleases, proteases, hydrolysis, phosphorylation, and other natural processes that greatly reduce their levels is a widely acknowledged obstacle. In addition, individual miRNAs and proteins can influence many tissues, and achieving a target-specific response is challenging [15]. Extracellular vesicles (EVs) are nano-sized, double-layer-enclosed vesicles secreted by all cells that can carry parent cell proteins on their surfaces and a mixed cargo of miRNAs, ssDNA, snRNAs, lipids, and proteins within them [16]. This makes EVs an attractive candidate delivery system [17].

EV-focused research has been an active area of investigation over the last decade, with marked progress having been made in the understanding of the mechanisms governing EV biogenesis, release, cargo sorting, and uptake [18]. EV cargo profiles change dynamically in a manner that partially reflects the homeostatic or pathological conditions in the parental cells from which they are derived [19]. Moreover, EVs have been shown to play important roles under both physiologic and pathological conditions [20], potentially participating in both healing processes and the exacerbation of disease.

EV cargo enrichment for specific miRNAs has been reported under specific conditions, and such enrichment can have significant biological effects in cell culture and animal model systems [21]. Our present understanding suggests that changes in EV cargoes in general and miRNAs in particular form a component of signaling pathways that affect adjacent and remote tissues through paracrine effects [22]. EVs have also been suggested to function through autocrine-like effects [23], meaning that EVs released by a specific cell type or tissue affect that same specific cell or tissue, thereby attenuating or stimulating certain intracellular signaling pathways [24, 25].

In recent years, our lab has published several studies demonstrating the ability of NPCE-derived exosomes to modify canonical Wnt/TGF-β signaling, which is a major regulator of ECM modulation, in TM cells [26]. TM cells are exposed to exosomes originating from various ocular tissues, including the cornea, iris, retina, and ciliary muscles. Of these various EV populations, we have focused on NPCE-derived exosomes as the most likely candidate transporters of signals related to IOP regulation to the drainage tissues. NPCE-derived exosomes present with a natural uptake preference by TM cells relative to other ocular cells [27], and exhibit a dose-response effect to exosomes with respect to expression [28]. Recently, we have employed an electroporation approach to load NPCE-derived exosomes with selected siRNAs targeting SMAD7 to modify the Wnt/TGF-β signaling pathway involved in the activation of ECM build-up [29, 30]. The success of this approach highlights the utility of NPCE-derived exosomes as a platform for targeted TM drug delivery. Our results suggest that engineered NPCE exosomes can be loaded with selected cargoes to a degree sufficient to achieve functionally relevant concentrations in target cells.

Oxidative stress is a key facet of POAG pathology [31-34], and our lab has explored the role of exosomes in the delivery of oxidative signals [19, 35, 36]. We have found that EVs derived from NPCEs exposed to oxidative stress significantly protect TM cells against direct oxidative stress.

Many research groups have successfully described the different cells or tissues from which EV cargos originate, and a better understanding of the diversity of EV sizes and contents has been achieved. In living organisms, EV populations are not homogenous, and the vary across a range of typical sizes. Some debate persists among researchers with respect to whether changes in EV size occur in response to particular forms of stress or disease. Microarray analyses of EVs originating from different cells and tissues revealed that thousands of different miRNAs were detectable in a single EV-containing sample, with these miRNA numbers approaching the total number of genes expressed in a tissue [37].

Given the complexity and diversity of EV populations, the extent to which a limited number of EVs containing a specific miRNA can effectively transmit a modifying signal to target recipient cells remains uncertain [38]. For genotypic and phenotypic alterations to manifest, several crucial steps must transpire: an EV carrying a specific miRNA must locate its designated cell target and be internalized, whereupon the miRNA of interest must navigate the target cell's lysosomal compartment, access the target mRNA, and induce changes in gene expression that result in altered protein expression. When this process occurs repeatedly, concurrently, and consistently, the likelihood of EVs exerting modifying effects improves. It is also anticipated that EVs derived from these recipient cells will subsequently exhibit a modified cargo profile, reflecting the new equilibrium state for that target cell. These EVs are then able to influence neighboring cells via paracrine effects and the same cell type via autocrine effects.

Previous research in our lab has shown that NPCE-EVs can influence canonical Wnt, ECM, and oxidative stress-related signaling in TM cells. However, at present, we do not have a complete understanding of how TM cell-derived EVs respond to these NPCE-EVs. In our proposed study, we aim to investigate how NPCE-derived EV signals are transferred to TM cell-derived EVs. To achieve this goal, will use a Cre-LoxP system [39] to analyze the kinetics of TM-EV release after exposure to NPCE-EVs. Our study will clarify the quantitative release of TM-EVs over time at different NPCE-EV:TM cell ratios, thereby helping us understand the changes in stimulated TM-derived EVs.

In this study, we plan to explore the dynamics and kinetics through which EVs function in a second messenger-like manner through an amplification process that intensifies and enhances signaling processes engaged following successful EV uptake by the target cell. This amplification process can manifest as an increase in the release of EVs from these recipient target cells. In addition, these EVs can carry specific cargoes and thereby enable lateral signal transmission to nearby cells and distant tissues. Another potential component of this amplification process may be mediated through autocrine-like mechanisms, thereby increasing the release of EVs from target cells carrying the initial signal consequences. Our working hypothesis is that certain distinct changes in EV cargo profiles, which subsequently trigger amplifying effects, extend beyond the broad spectrum of EV cargo diversity that typically mirrors the cellular state.

In summary, this research proposal seeks to uncover the dynamic alterations in TM cell-derived EVs when exposed to NPCE-EVs. To achieve this goal, we will investigate how signals conveyed by NPCE-derived EVs are transferred to TM cell-derived EVs.

1. **Research Objectives and Expected Significance**

Our primary focus in the proposed study is to gain a detailed understanding of the dynamic and kinetic aspects of EV-mediated signaling between EVs derived from NPCE and those derived from TM cells. This knowledge will serve as a foundation for efforts to expand treatment options for POAG, ultimately advancing the management of this debilitating condition. In particular, our central objective is to delve deeply into the intricate response mechanisms of TM-derived EVs and how they are engaged in parallel with TM cell modifications following exposure to incoming signals carried by NPCE-derived EVs. We are interested in investigating not only the cargos transported by TM-derived EVs but also the multifaceted paracrine and autocrine effects that these signaling interactions may trigger within TM cells. To achieve these objectives, we propose the following Specific Aims:

**Aim 1: Quantify TM-derived EV release in response to NPCE-derived EVs.** There is an ongoing debate surrounding alterations in the release of cell EVs in response to incoming signals. In this Aim, we will investigate the real-time uptake of NPCE-EVs by TM cells and concurrently assess the quantity of TM-EVs released. To achieve this, we have designed a specialized Cre-loxP system that enables us to track Cre+ NPCE-EVs and quantify their uptake by LoxP-TM cells. Additionally, we will monitor TM-derived EVs labeled with fluorescent CD81 as a parallel analytical approach.

**Aim 2: Conduct a qualitative analysis of cargo changes in TM-derived EVs after exposure to NPCE-derived EVs.** In our ongoing research (ISF 1545/20), we have found that the duration of TM cell exposure to NPCE-EVs results in distinct alterations in TM cell mRNA expression patterns. We hypothesize that these modifications in TM genes are, in part, conveyed by the cargoes carried within TM-derived EVs. In this Aim, we therefore plan to investigate the cargo profiles of TM-derived EVs that are released after exposure to NPCE-EVs, with a particular focus on miRNAs and proteins.

**Aim 3: Compare the effects of continuous versus limited exposure to NPCE-derived EVs on TM-derived EV quantity and cargo.** The significant variation in size and content within a single batch of EVs implies that ongoing incoming signals may lead to distinct modifications in the recipient cells. To investigate this further, in this Aim we plan to compare the impact on TM cell mRNA expression when these cells are exposed to a known quantity of NPCE-EVs for a specified duration, as opposed to continuous exposure, using a Boyden system. This comparative analysis will help us assess the changes in TM cell mRNA expression and TM-EVs cargoes more comprehensively.

**Aim 4: Investigate quantitative and cargo modifications in TM-derived EVs after exposure to oxidative stress signals conveyed by NPCE-derived EVs**. Based on our previous findings, we are aware that the responses of TM cells to stress signals conveyed by NPCE-EVs differ from those to "normal" signals. In this Aim, our objective is thus to investigate the dynamic and kinetic aspects of oxidative signals delivered by NPCE-EVs and their impact on TM-derived EVs, as outlined in Aims 1-3. To achieve this, we will expose TM cells to EVs derived from oxidative stress-exposed NPCE cells and subsequently analyze modifications in the cargo profiles of the resultant TM-derived EVs.

**Aim 5: Assess autocrine and paracrine effects of TM-derived EVs following exposure to NPCE-derived EVs.** We posit that EV cargo profiles, to some extent, mirror their parental cells. In this context, when TM cells are exposed to NPCE-derived EVs, TM-derived EVs are likely to carry modified signaling molecules influenced by this exposure. Consequently, these TM-derived EVs have the potential to transmit these modified signals back to the original TM cells (resulting in an autocrine effect) as well as to neighboring TM cells (resulting in a paracrine effect), thereby inducing modifications in their behavior.

1. **Detailed Description of the Proposed Research**

**Working hypothesis**

The effects of EV cargoes on target cells are amplified through two mechanisms: an increase in the release of specific cargo-carrying EVs by the target cells and an autocrine-like effect mediated by the target cells' EVs, which leads to an increase in both the number of self-EVs and the production and release of EV-specific cargoes.

* + 1. Specific hypothesis

We hypothesize that NPCE-derived EVs play a role in dynamically modulating the cargo of TM cell-derived EVs, leading to the activation of targeted signaling pathways. This process is expected to promote the expression of specific miRNAs and proteins associated with these signaling pathways, while also facilitating the turnover of the ECM in HTM cells. This dynamic and kinetic interaction has the potential to enhance cellular responses and signaling outcomes.

**Research design & methods**

**Aim 1**: To comprehensively track the kinetics of NPCE-derived EV uptake by TM cells, we have devised a specialized Cre-LoxP reporting system tailored for precision and accuracy. This system entails several key steps:

1. NPCE Cell Transfection:

We will begin by transfecting NPCE cells with a plasmid that expresses Cre recombinase, genetically fused to BFP (Blue Fluorescent Protein). This tagging allows us to visualize and identify the presence of Cre recombinase. This will result in the expression of BFP-tagged Cre recombinase by these cells, and it will also be present in the NPCE-derived EVs, which we will refer to as [EVsCre+].

1. TM Cell Preparation:

Concurrently, TM cell cells will be transduced with a tailored designed plasmid. This plasmid incorporates LoxP sequences, functioning as genetic markers for NPCE-derived EVCre+ uptake, alongside the EV marker CD81, which will be tagged with a crimson dye to allow for the identification of released TM-derived EVs. Upon the successful uptake of NPCE-derived EVs (EVsCre+), the plasmid within TM cells will undergo a significant fluorescent shift, transitioning from DsRed to GFP. CD81 will be distinctly labeled with a crimson dye and denoted as [TMLoxPCD81].

By following these meticulously designed steps, we can precisely monitor the dynamics of NPCE-derived EV uptake by TM cells, while also monitoring the behavior of TM-derived EVs marked with CD81 and the crimson dye. This integrated approach ensures the comprehensive assessment of both EV uptake and release within our experimental framework, providing invaluable insight into the intricate cellular interactions we aim to explore.

TMLoxPCD81 cells will be exposed to a fixed ratio of NPCECre+-derived EVs (30,000 EVs/cell)[28] for 1, 2, 4, 8, and 24 h, and the TMLoxPCD81 cells will be subjected to continuous live monitoring under a Zeiss Celldiscoverer 7®. This will enable us to precisely quantify the kinetics of EVsCre+ uptake and the subsequent release of TMLoxPCD81-derived EVs.

**Aim 2**: To conduct a qualitative assessment of the changes in TM-derived EV release following exposure to NPCE-derived EVs, we will employ the Cre-LoxP system as described in Aim 1. Additionally, we will collect a sufficient quantity of TMLoxPCD81-derived EVs at designated time points (outlined in Aim 1) for subsequent miRNA microarray analyses.

Our approach will entail correlating the miRNA content of NPCE EVCre+ [19] with the targeted TM mRNA (currently in preparation). We will achieve this correlation by examining TMLoxPCD81-derived EVs, which will serve as indicators of the cellular response.

**Aim 3**: The successful completion of Aims 1 and 2 will empower us to conduct a comprehensive examination of the consequences of the continuous and dynamic exposure of TM cells to NPCE-derived EVs. We will closely investigate both the quantitative aspects and the modifications observed in TM-derived EVs, particularly in terms of their cargo profiles. Rather than administering a fixed amount of EVsCre+ to a known number of TMLoxPCD81 cells for varying durations, we will employ the µ-Slide 2 Well Co-Culture system (ibidi 81806) with the Zeiss Celldiscoverer 7® instrument. This innovative approach enables NPCECre+ and TMLoxPCD81 cells to coexist in separate chambers while sharing EVs and soluble factors. This arrangement ensures that both cell types are exposed to the EVs released by one another, creating a more dynamic and representative experimental environment.

**Aim 4**: Aims 1-3 collectively seek to unveil the intricate dynamics and kinetics of NPCE-derived EVs during their interaction with TM cells. These interactions will manifest as observable modifications in TM cell-derived EVs, providing us with a comprehensive understanding of the intricate cellular communication processes involved.

To delve into the related dynamic and kinetic aspects within a pathological context, we will investigate the effects of oxidative stress signals delivered by NPCE-EVs on TM-derived EVs. We will utilize NPCEcre+ cells exposed to both acute and chronic oxidative stress (OS-NPCECre+)[19] to extract OS-EVCre+. These OS-EVCre+ particles will then be assessed for their uptake kinetics into TMLoxPCD81 cells, building upon the results achieved in Aim 1.

For the qualitative evaluation of the alterations observed in TMLoxPCD81-derived EVs, we will conduct miRNA microarray analyses. The resultant data will be scrutinized in the context of relevant pathways, integrating information from NPCE-derived EVs [19] and TM mRNA data (currently in preparation).

To investigate the transfer and potential amplification of stress signals generated within TMLoxPCD81 cells following exposure to OS-EVsCre+, we will assess the efficiency of TMLoxPCD81-derived EVs in transferring these stress signals to a new culture of naïve TM cells. Our analysis will involve examining changes in gene expression and antioxidant capabilities within these naive TM cells. In summary, we aim to evaluate the efficient transmission of 'second-hand' stress signals originating from OS-NPCE EVs and their modification of TMLoxPCD81 cells. Subsequently, we will investigate the capacity of these TMLoxPCD81-derived EVs to deliver stress signals to a third set of naïve TM cells.

**Aim 5:** In the context of phenotypic changes occurring within the ocular drainage system, one of the most significant alterations involves either ECM accumulation or degradation. To assess the potential of EV-mediated signaling cascades to modify TM cells through autocrine or paracrine effects, we will conduct an evaluation of TM ECM changes.

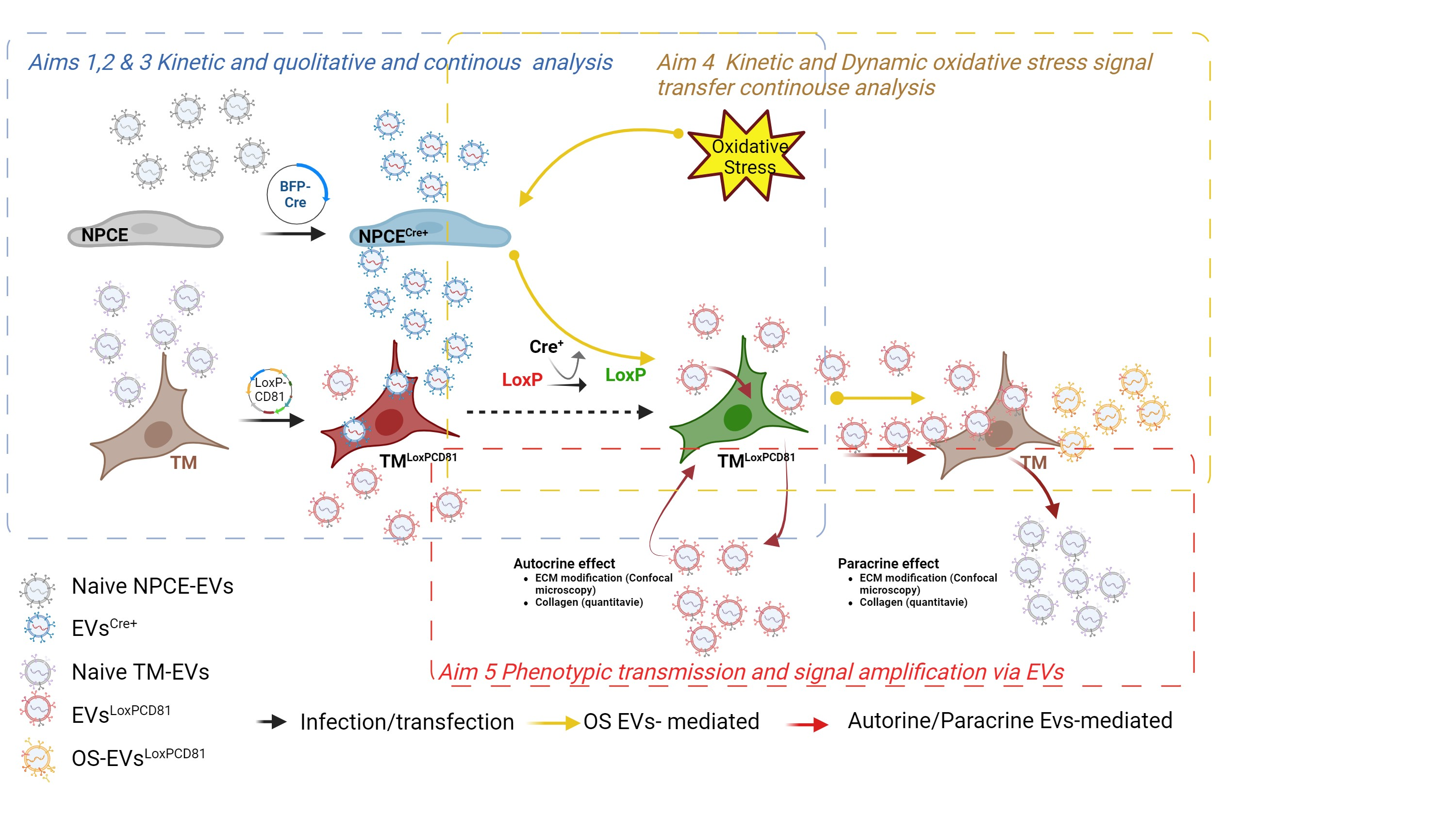
Following the exposure of TMLoxPCD81 to EVsCre+ at optimal time points (Aims 1-3), TM-derived EVs will be collected. These EVs will then be used to treat control TMLoxPCD81 cells, reflecting paracrine-mediated effects. We will employ a soluble collagen colorimetric detection assay to quantitatively measure changes in TM ECM collagen.

To analyze autocrine effects, TMLoxPCD81 cells, after medium replacement, will be observed in the presence of their own EVsLoxPCD81. This will allow us to assess the impact of TM-derived EVs on ECM collagen.

Additionally, similar experiments conducted on slides will enable us to perform specific ECM staining for ECM markers such as F-actin, Integrin, Cadherin, clusterin, and vinculin. These stained samples will be analyzed via confocal microscopy, providing us with a detailed visualization of ECM alterations.

The novelty of the proposed study stems from the remarkable realization that EVs possess dual effects: they directly impact target cells, and also have the power to affect EVs within those target cells. This intricate interplay of EVs within a given cellular environment leads to specific signal amplification, thereby significantly expanding our appreciation for their critical role in this context.

Research plan graphical abstract



**Specific Methods**

Cell culture: An immortalized NPCE cell line and a normal TM cell line were kindly donated by Professor M. Coca-Prados (Yale University, CT, USA and Alcon Research Ltd USA, respectively). Cells will be grown in DMEM with 10% FBS exosome-depleted serum and 50 μg/ml gentamycin at 37°C in a 5% CO2 incubator. Cells at 80% cells will be used in all studies.

EV isolation and depleted serum: EV isolation, separation, and characterization will be performed according to the guidelines of the International Society for Extracellular Vesicles [40]. We will use serum-depleted EVs for cell growth, and a series of ultra-centrifugation steps and qEV Isolation Columns (IZON NZ) for EV isolation. Nanoparticle Tracking Analysis (Malvern UK) and Tunable Recessive Pulse sensing (qNano IZON, NZ) approaches will be used for analyses of EV size and concentrations. EV characteristic markers will be analyzed using an Exo-Check™ Antibody Array (SBI, Mountain View, CA) and western blotting.

Cre-LoxP CD81 reporting system: To address our research objectives, we have collaborated with Dr. Shiran Dror, the head of the Center for Advanced Genomics at Ben-Gurion University, to design a Cre-LoxP system that incorporates fluorescently labeled EVs from NPCE-TM cells. This system is built upon the foundation of commercial plasmids, specifically (i) pLV-CMV-LoxP-DsRed-LoxP-eGFP (Addgene #65726) for LoxP, (ii) pCMV-Sport6-CD81-pHuji (Addgene #130904) for CD81, and (iii) pEB2-E2-Crimson (Addgene #104010) for the crimson fluorescent dye. Dr. Tamar Unger from the Life Sciences Core Facilities at the Weizmann Institute of Science will be responsible for the preparation of an infectious plasmid, ensuring that it meets all the necessary quality control standards

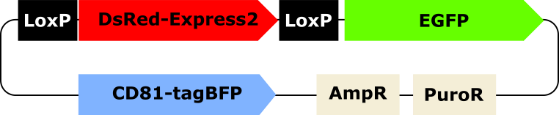
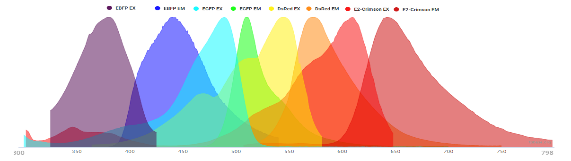
Dynamics and kinetics analyses: TMLoxPCD81 cells will be seeded in 6 mm sterile cell culture dishes (2x106 cells in 2 ml per dish). After 24 h, growth medium will be replaced with fresh EV-depleted medium. Following this medium exchange, NPCEcre+-derived EVs will be introduced to the TM LoxPCD81 cells at a ratio of 30,000 EVs per cell [28]. For Aim 1, different incubation times (1, 2, 4, 8, and 24 h) will be tested for EVsCre+ (BFP, 381:445, nm) uptake by TMLoxPCD81 fluorescent color shift (DsRed, 554:591, nm to eGFP, 488:507) and the release of TM LoxPCD81-derived EVs with labeled CD81 (crimson, 611:646), using the ZEISS Celldiscoverer 7® that enables stable incubations under controlled temperature, CO2, and O2 conditions at the Ilse Katz Institute for Nano-Science and Technology (BGU).

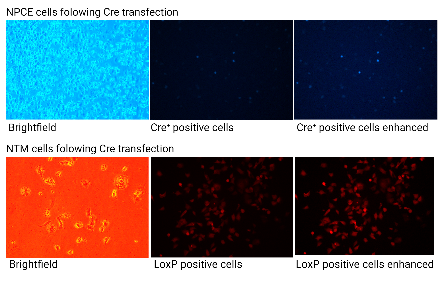
Continuous TMLoxPCD81 Cell Exposure to EVsCre+: To ensure the continuous exposure of TMLoxPCD81cells to EVsCre+ (Aim 3), we will employ the µ-Slide 2 Well Co-Culture system (81806, ibidi, Germany). This system exhibits (i) the ability for shared EV and soluble factor exposure while maintaining separate cellular growth environments; (ii) minimal evaporation tendencies, making it well-suited for extended and long-term assays; (iii) Excellent optical quality such that it can also be seamlessly integrated with the ZEISS Celldiscoverer 7® platform for advanced imaging capabilities. This choice of co-culture system ensures a robust and controlled environment for our experiments, enabling continuous exposure and precise monitoring of cellular interactions.

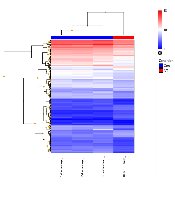
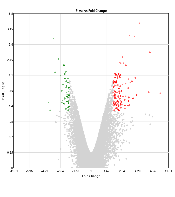
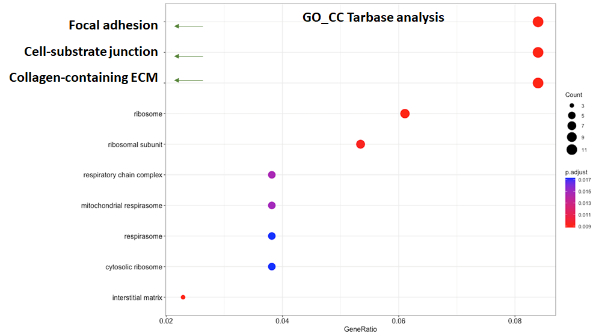
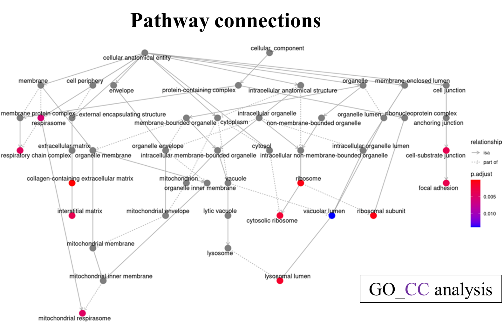
EVsLoxPCD81 Qualitative Analyses: To analyze the miRNA content of EVsLoxPCD81, we will employ a streamlined process using a total exosomal RNA purification kit (cat: 17200, Norgen Biotek Corp., Canada). A brief overview of this process is summarized as follows: *Lysis:* A volume of 200 µL of EVsLoxPCD81 suspended in PBS will be subjected to lysis for 1 minute by vortexing in the presence of lysis buffer; *Separation:* miRNA separation will be facilitated using spin columns, with a centrifugation step at 3500 ×g for 1 minute; *Washing:* The columns will undergo four wash cycles, each at 14,000×g for 1 minute; *Elution:* The purified miRNA will be eluted in a final volume of 50 µL, with a final centrifugation step at 600×g for 2 minutes; *Quality Assessment:* The quality and integrity of the extracted miRNA will be assessed using the Bioanalyzer 2100 (Agilent Technologies, CA, USA), and only samples with miRNA concentrations exceeding 130 ng/µL will proceed to further analysis; *Characterization:* The miRNA content of EVs will be comprehensively characterized using a suitable microarray chip analysis, such as the GeneChip® miRNA 4.0 Array and Flashtag™ Bundle–ThermoFisher (Cat 902445).

Oxidative Stress:(Aim 4) To induce oxidative stress in NPCECre+ cells, we will utilize 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH), which is a free radical-generating azo compound. AAPH is capable of initiating oxidation reactions via the continuous production of peroxyl radical followed by alkoxyl radicals through nucleophilic and free radical mechanisms [19, 41]. NPCE cells will be exposed to acute oxidative stress (15 mM AAPH for 90 min) or chronic oxidative stress (1.5 mM AAPH for 24 h).

ECM Modification (Aim 5): For immunohistochemistry and confocal microscopy analyses, before fixation, SiR-actin (Cytoskeleton, Inc.) and 10 µM verapamil will be added, and staining will follow Keller and Kopczynski's protocol [42]. TM cells will undergo fixation, permeabilization, and blocking. Primary antibodies (see Supplementary Table S2) will be applied and incubated. After thorough washing, appropriate secondary antibodies will be added. Subsequently, coverslips will be mounted onto silicone membranes using DAPI-containing ProLong Gold (Invitrogen). Image acquisition will be carried out with an Olympus FV1000 confocal microscope, ensuring consistent acquisition settings for all treatments. To quantitatively analyze integrin activation, Image J® will be used to measure total fluorescence within a designated field while simultaneously counting the DAPI-stained nuclei. The total fluorescence associated with activated integrins will be normalized to the number of nuclei.

LoxP-CD81 plasmid design and Excitation and Emission Profiles of Chosen Fluorescent Markers. The current research proposal is centered around the successful design of plasmids for transfecting and infecting NPCE and NTM cell lines. This design aims to enable these cells to report on three key aspects: the release of NPCE-EVs, the uptake of these EVs by recipient TM cells, and simultaneously, the release of EVs originating from these TM cells. To accomplish this, it is essential to incorporate distinct, non-overlapping fluorescent dye emission signals. To achieve this goal, the following plasmid has been developed.

Imaging of NPCE and TM cells following Cre transfection or LoxP infection (X100). We have successfully transfected NPCE cells with the commercial pcDNA3.1-CMV-CFP;UBC-Cre25nt plasmid (Addgene #65727) and infected NTM cells with the commercial pLV-CMV-LoxP-DsRed-LoxP-eGFP plasmid (Addgene #65726). Through these experiments, we have established the most optimal conditions for transfection and transduction, including the best drug concentrations for selection.

Exploring TM Gene Expression Changes Induced by NPCE-Derived EVs. We employed a gene-array approach to assess the miRNA content within NPCE-derived EVs and monitored the resulting impact on TM cell mRNA expression profiles following NPCE-derived EV treatment. We also compared these assays with the effects of pre-exposing NPCE cells to oxidative stress. These experiments allowed us to discern TM-expressed genes under normal conditions. We then performed a predictive Gene Ontology (GO) analysis to pinpoint the relevant pathways affected by these changes (*unpublished*). These results will underpin our future investigations into the autocrine and paracrine effects of TM-derived EVs. For instance, our GO\_CC analysis of differentially expressed genes (DEGs) and predicted targets revealed the substantial up-regulation of the highlighted targets (bold & indicated by arrows) in TM cells exposed to chronic oxidative stress.

**Resources Available for the Proposed Research**

Professor Beit-Yannai's research facilities are well-equipped to support this research endeavor. The available resources include qNano nanoparticle analysis, UV-Vis spectrophotometer, ELISA reader, cooled centrifuge, and cell culture amenities such as a laminar biohazard safety hood type II and tissue culture incubator. PCR and Western blotting instruments, supplies, kits, and power supplies are also available. The laboratory also has access to a range of departmental equipment, including ultracentrifuges, a -80°C freezer, and both inverted fluorescent and confocal microscopes. Furthermore, the proposed project will be bolstered by the extensive capabilities of the Ilse Katz Institute for Nano-Science and Technology at Ben-Gurion University, which offers Bio-Imaging and Flow Cytometry resources, TEM and Cryo-EM facilities, spectroscopy capacities, and Bioinformatic services, including statistical support

Professor Beit-Yannai brings extensive expertise in EVs research, encompassing all the necessary aspects for the successful execution of the proposed study. This proficiency is underlined by a consistent track record of publications on EVs communication between NPCE and TM cells. Furthermore, Professor Beit-Yannai has effectively mentored both Ph.D. and M.Sc. students over the past decade, contributing to their academic and research achievements.

**Expected Results and Pitfalls**

Our study is designed to test specific hypotheses related to the dynamic interplay between NPCE-derived EVs and TM cell-derived EVs. We anticipate several key outcomes: (i) Kinetic Interactions: Throughout our study, we will focus on the kinetic aspects of this interaction. We expect to observe temporal changes in the cargo profiles of TM cell-derived EVs, emphasizing the dynamic nature of EV-mediated signaling and its implications for cellular responses over time; (ii) Enhanced EV Cargo Modulation: We expect to observe a substantial increase in the modulation of TM cell-derived EV cargoes due to the influence of NPCE-derived EVs. This enhanced modulation is anticipated to result in the activation of targeted signaling pathways within TM cells, consequently promoting the expression of specific miRNA and proteins associated with these pathways; (iii) Signaling Pathway Activation: As a direct consequence of the altered cargoes within TM cell-derived EVs, we predict the significant activation of specific signaling pathways. These pathways are integral to the regulation of crucial cellular functions in the HTM cells. This activation is expected to contribute to a more efficient cellular response and optimized signaling outcomes; (iv) Extracellular Matrix Turnover: A noteworthy result of the dynamic interaction between NPCE-derived EVs and TM cell-derived EVs is the facilitation of ECM turnover in HTM cells. We anticipate observing a measurable impact on ECM components, reflecting a higher turnover rate that influences tissue remodeling and homeostasis. These expected results will provide a comprehensive understanding of how the interplay between different cell-derived EVs influences cellular signaling and the extracellular matrix within HTM cells. Ultimately, our findings will contribute to a more in-depth comprehension of the intricate mechanisms underlying these cellular interactions.

**Practical Implications:** (i) Enhanced Understanding of Cellular Signaling Dynamics: The kinetic interactions observed in our study shed light on the dynamic nature of EV-mediated signaling. This enhanced understanding can lead to more precise control and manipulation of cellular responses over time. Researchers can utilize this knowledge to investigate similar interactions in various cellular contexts, broadening our comprehension of EV-mediated communication. (ii) Improved Targeted Therapies: The enhanced modulation of TM cell-derived EV cargoes influenced by NPCE-derived EVs signifies an opportunity to develop targeted therapies. By manipulating EV cargoes in a controlled manner, potential treatment strategies could be devised to activate specific signaling pathways or suppress undesired cellular responses. This approach may hold promise for the development of precision medicine interventions. (iii) Optimized Cellular Responses: The activation of specific signaling pathways within HTM cells, resulting from altered cargoes in TM cell-derived EVs, has practical implications for optimizing cellular responses. This insight may be applied to enhance drug delivery systems, especially in the context of ocular diseases such as glaucoma, where precise targeting of signaling pathways is critical. (iv) Tissue Remodeling and Homeostasis: The facilitation of ECM turnover in HTM cells has implications for tissue remodeling and homeostasis. This knowledge may lead to strategies for promoting tissue repair and regeneration. Understanding the role of EV-mediated ECM turnover is relevant not only in ocular health but also in various tissue regeneration approaches.

**Potential Limitations**

The results from the proposed project are poised to significantly advance our understanding of the dynamic effects of EVs. However, the interpretation of our results must be performed while considering the context in which the research was conducted, recognizing the inherent limitations of cell lines. The application of these findings to primary cells should be treated as a potential avenue for future exploration rather than something that will translate directly.

Potential Pitfalls and Alternative Approaches

1) Transfection and Toxicity Issues: When faced with challenges such as obtaining a low yield of NPCECre+ cells or encountering NPCE cell toxicity following transfection, our standard protocol typically entails the use of Lipofectamine 2000 for transfection. However, if persistent NPCE cell toxicity or consistently low transfection yields are encountered, we maintain the flexibility to explore alternative transfection reagents. Options include Lipofectamine 3000, Fugene, RNAiMAX, and Lipofectin. Each of these alternative transfection reagents can vary in a cell type-dependent manner with respect to performance.

2) Quantitative Analyses of EVs: Achieving quantitative analyses of NPCE-derived EVs impacting TM-derived EVs can be accomplished by using different time points instead of relying solely on stimulant-based analyses. To address the challenge of distinguishing between NPCE-derived EVs and TM-derived EVs, we can leverage the µ-Slide 2 Well Co-Culture system (81806, ibidi, Germany). This system offers several advantages: (i) It facilitates shared EVs and soluble factor interactions while maintaining separate growth environments for the two cell types. (ii) It exhibits minimal evaporation tendencies, making it highly suitable for extended and long-term assays. In the event that real-time monitoring of TMLoxPCD81 cells using Zeiss Celldiscoverer 7® does not yield satisfactory results, we have an alternative approach. We can utilize the FACSAria III Fluorescence-Activated Cell Sorting (FACS) method for sorting and analyzing EVs in liquid suspension as a complementary analytical method. This dual-pronged strategy will enhance the robustness of our quantitative analyses of how EVs impact behavior in different cell types.

3) Qualitative Analyses of EVs: For qualitative analysis of EVs, we have the option of employing microarray or RNA sequencing techniques. Both methods require a minimal amount of miRNA, although the specific amount can vary depending on the specific RNA sequencing platform and protocol being used. In general, most RNA sequencing methods require nanogram quantities of RNA. Nevertheless, some technological advances, specialized protocols, and kits now permit RNA sequencing from smaller RNA inputs, including picogram or even femtogram quantities of miRNA. These low-input RNA sequencing methods are especially valuable when dealing with limited starting material, as is often the case when working with a limited amount of EVs. However, as we plan to work with larger Petri dishes in this study, achieving nanogram miRNA yields to better enable these experiments should be achievable.

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**Time Schedule**

|  |  |  |
| --- | --- | --- |
| **Objective** | **Beginning** | **End** |
| Establishing the NPCECre+ and TMLoxPCD81 reporting systems (Aim 1) | 01.10.24 | 30.09.25 |
| Quantify TM-derived EV release upon exposure to NPCE-derived EVs (Aim 1) | 01.10.25 | 30.06.26 |
| Perform a qualitative analysis of TM-derived EV alterations following NPCE-derived EV exposure (Aim 2) | 01.07.26 | 30.06.27 |
| Compare continuous and limited exposure effects on TM-derived EVs in terms of quantity and cargo changes (Aim 3) | 01.10.27 | 30.09.28 |
| Investigate quantitative and cargo modifications in TM-derived EVs post-exposure to oxidative stress signals from NPCE-derived EVs (Aim 4) | 01.07.27 | 30.06.28 |
| Assess autocrine and paracrine effects of TM-derived EVs after NPCE-derived EV exposure (Aim 5) | 30.10.28 | 30.09.29 |

**Explanatory Notes:**

*Establish NPCECre+ and TMLoxPCD81 reporting systems (Aim 1 - 01.10.24 to 30.09.25):* Over this period, the primary focus will be on developing the cellular system needed for the study. This entails creating and validating plasmids and cell lines with specific genetic markers for tracking EVs.

*Quantify TM-derived EV release (Aim 1 - 01.10.25 to 30.06.26):* This objective aims to measure the quantity of EVs released by TM cells upon exposure to NPCE-derived EVs. We will investigate how the presence of NPCE-derived EVs affects this release.

*Perform a qualitative analysis of TM-derived EV alterations (Aim 2 - 01.07.26 to 30.06.27):* During this phase, we will meticulously examine the quality and content of EVs released by TM cells after exposure to NPCE-derived EVs to assess any cargo changes.

*Compare continuous and limited exposure effects (Aim 3 - 01.10.27 to 30.09.28):* This objective involves exploring how different durations of exposure to NPCE-derived EVs influence both the quantity and the composition of EVs released by TM cells.

*Investigate quantitative and cargo modifications (Aim 4 - 01.07.27 to 30.06.28):* Here, the primary focus is on understanding how oxidative stress signals conveyed by NPCE-derived EVs impact both the quantity and the cargo of EVs released by TM cells.

*Assess autocrine and paracrine effects (Aim 5 - 30.10.28 to 30.09.29):* This objective involves studying how EVs released by TM cells following their exposure to NPCE-derived EVs affect TM cells themselves (autocrine effects) and other neighboring cells (paracrine effects). These effects will be evaluated to gain insights into the involved signaling pathways.

**Budget details**

**Personnel**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Name (last, first) | Role in  project | % time  devoted | Salaries (in NIS) | | | | |
| 1st year | 2nd year | 3rd year | 4th year | 5th year |
| Beit-Yannai Elie | PI | 30 | 0 | 0 | 0 | 0 | 0 |
| To be named | Post-Doc | 100 | 154200 | 154200 | 154200 |  |  |
| To be named | PhD student 1 | 100 | 138600 | 138600 | 138600 | 138600 |  |
| To be named | PhD student 2 | 100 |  | 138600 | 138600 | 138600 | 138600 |
| To be named | M.Sc. student | 100 |  |  |  | 105300 | 105300 |
|  |  |  | **292800** | **431400** | **431400** | **382500** | **243900** |

**Justification for requested Personnel:**

Post-Doctoral Researcher:

* + The Post-Doc will lead the integration of the Cre-LoxP reporting system into NPCE and TM cells (Aim 1). This is a pivotal step in our research as it forms the foundation for subsequent experiments.
  + He will be responsible for quantifying the release of TM-derived EVs upon exposure to NPCE-derived EVs with Cre+ markers (Aim 1). This quantification is essential to understand the dynamics of EV-mediated cell-to-cell communication.
  + The Post-Doc will also assess the autocrine and paracrine effects of TM-derived EVs following exposure to NPCE-derived EVs (Aim 5), shedding light on the functional consequences of EV interactions in these systems.

Ph.D. Student (1):

* + This student's primary responsibility is the qualitative analysis of alterations in TM-derived EVs after exposure to NPCE-derived EVs (Aim 2). Qualitative analysis provides valuable insights into structural and morphological changes.
  + Additionally, he will investigate the effects of continuous and limited exposure to NPCE-derived EVs on TM-derived EVs, focusing on changes in quantity and cargo (Aim 3). This quantitative aspect is crucial for understanding the kinetics of EV alterations.

Ph.D. Student (2):

* + The second Ph.D. student will focus on investigating quantitative and cargo modifications in TM-derived EVs after exposure to oxidative stress signals from NPCE-derived EVs. This research direction is vital for understanding how stress-induced signals impact EV cargo composition and quantity.

M.Sc. Student:

* + The M.Sc. student's primary focus will be on the extracellular matrix (ECM) modifications in TM-derived EVs mediated through autocrine or paracrine effects (Aim 5). Investigating ECM changes is key to understanding how EV interactions influence the cell microenvironment.

Technician:

* + The technician, working 50% of his time, will provide essential technical support to the research students. Their collaboration is integral to ensuring the successful execution of various techniques and experiments. Their expertise will be invaluable in maintaining lab equipment and troubleshooting issues as they arise.

In summary, each team member's role is well-defined and critical to the success of our research project. Their collective efforts will contribute to advancing our knowledge of EV-mediated cell communication, the Cre-LoxP system, and the broader implications of these findings in the context of our research aims.

**Supplies and Materials**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Item | Requested sums (in NIS) | | | | |
| 1st year | 2nd year | 3rd year | 4th year | 5th year |
| General lab tools, plasticware, glassware, and consumables including blotting, qNano membranes, EV purification columns | 50000 | 50000 | 50000 | 50000 | 50000 |
| Chemicals, fluorescent dyes, solvents and reagents | 10000 | 10000 | 10000 | 10000 | 15000 |
| Cell lines, media, and sera for tissue culture | 30000 | 30000 | 30000 | 30000 | 30000 |
| Antibodies, specific Inhibitors, assay kits for miRNA or RNA Isolation, qPCR, | 20000 | 40000 | 40000 | 40000 | 40000 |
| Gene array kit |  | 20000 | 20000 | 20000 |  |
|  | **110000** | **150000** | **150000** | **150000** | **135000** |

**Justification for requested Supplies & Materials:**

General Lab Supplies and Reagents:

*Plasticware, Glassware, and Consumables:* These items are essential for everyday lab operations, including sample preparation, storage, and experiments. Sufficient funding ensures a seamless workflow, minimizing disruptions and frequent restocking.

*Blotting, qNano Membranes, and EV Purification Columns:* These tools are critical for protein and vesicle analysis, enabling precise separation, quantification, and EV purification. Adequate funding enhances research reliability.

*Chemicals, Fluorescent Dyes, Solvents, and Reagents:* Fundamental for experiments, these components facilitate sample labeling, staining, and assays. A robust budget ensures access to high-quality materials for precise and reproducible results.

*Cell Lines, Media, and Sera:* These are crucial for tissue culture experiments, supporting cell maintenance and growth, fundamental to our research goals.

*Antibodies, Inhibitors, Assay Kits:* These specific reagents are vital for various experiments, such as miRNA and RNA isolation, qPCR, and gene array analysis. Funds allocated for these reagents enable precise and targeted investigations, contributing to research success.

In summary, the requested budget for lab supplies and reagents is indispensable for maintaining a well-equipped and efficient laboratory. Adequate funding in these areas is pivotal for data quality and reliability, allowing us to conduct precise experiments and achieve valuable scientific advancements.

**Services**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Item | Requested sums (in NIS) | | | | |
| 1st year | 2nd year | 3rd year | 4th year | 5th year |
| Plasmid preparation at the Life Sciences Core Facilities at the Weizmann Institute of Science | 11000 |  |  |  |  |
| miRNA Profiling at Genomic Technologies Facility, The Hebrew University | 10000 | 10000 | 10000 | 10000 | 10000 |
| Bio-imaging, NTA, Cryo-TEM & TEM analysis at Ilse Katz Institute for Nanoscience and Technology, BGU | 20000 | 20000 | 20000 | 20000 | 20000 |
| Gene seq |  | 2500 | 2500 | 2500 | 2500 |
|  | **41000** | **32500** | **32500** | **32500** | **32500** |

**Justification for requested Services:**

To enhance our research capabilities, we are planning to engage specific service providers. We will contract the Plasmid co-cloning service from the Structural Proteomics Unit at the Weizmann Institute. Additionally, we will procure expression analysis and miRNA profiling services from The Alexander Silberman Institute of Life Science at HUJI.

Furthermore, some of our essential research equipment, including NTA, Zeta sizer, Bio-Imaging, confocal microscopy, and cryo-TEM, incur usage fees at the Ilse Katz Institute for Nanoscale Science and Technology (IKI) at Ben-Gurion University. This budget request is intended to cover the associated expenses related to the utilization of these critical instruments.

**Other Expenses**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Item | Requested sums (in NIS) | | | | |
| 1st year | 2nd year | 3rd year | 4th year | 5th year |
| Post-Doc travel expenses for scientific meetings abroad or in Israel | 5000 | 5000 | 5000 |  |  |
| PhD student (1) travel expenses for scientific meetings abroad or in Israel | 1500 | 5000 | 1500 | 5000 |  |
| PhD student (2) travel expenses for scientific meetings abroad or in Israel |  | 1500 | 5000 | 1500 | 5000 |
| M.Sc. student travel expenses for scientific meetings in Israel |  |  |  | 1500 | 1500 |
| qNano maintenance & repair | 40000 |  |  |  |  |
|  | **46500** | **11500** | **11500** | **11500** | **6500** |

**Justification for requested Other Expenses:**

*Justification for 40,000 NIS for qNano Maintenance*:

Our qNano instrument has been a cornerstone of our laboratory's research for over a decade, and its continued reliability is vital for ongoing EV analysis. The requested funds are essential to support this crucial instrument's maintenance, ensuring its longevity and consistent performance. This investment not only safeguards our research quality but also maintains the invaluable asset that has been integral to our work for over 10 years.

A segment of the requested budget is earmarked for covering the travel expenses of our graduate students. This funding will facilitate their engagement in data presentations and lectures at scientific gatherings, with a preference for their attendance at prestigious international conferences such as the ARVO Meeting, the International Symposium on Ocular Pharmacology and Therapeutics abroad, as well as local conferences hosted by the ISREV (Israel Society for Extracellular Vesicle) and the ISVER (Israeli Society for Vision and Eye Research). This opportunity for our students to share and gain knowledge on a global stage is a significant investment in their academic and professional growth.

**Computers**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Item | Requested sums (in NIS) | | | | |
| 1st year | 2nd year | 3rd year | 4th year | 5th year |
| Personal computer for the researcher |  |  | 8000 |  |  |
| Personal computer for the lab technician/students | 6000 |  |  | 6000 |  |
| Software | 750 | 750 | 750 | 750 | 750 |
|  | **6750** | **750** | **8750** | **6750** | **750** |

**Justification for requested Computers:**

Upgrading two older lab computers will streamline the tasks of both the technician and the students engaged in the research project.

We kindly request the replacement of the Principal Investigator's laptop computer to enhance the efficiency of their research work.

To efficiently handle and manage PDF files for reporting, an annual Adobe Acrobat Reader license is essential.

**Miscellaneous**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Item | Requested sums (in NIS) | | | | |
| 1st year | 2nd year | 3rd year | 4th year | 5th year |
| Photocopies and office supplies | 1000 | 1000 | 1000 | 1000 | 1000 |
| Publication charges in scientific journals (including editing and translation) | 6000 | 12000 | 12000 | 12000 | 12000 |
| Memberships in scientific associations | 1500 | 1500 | 1500 | 1500 | 1500 |
|  | **8500** | **14500** | **14500** | **14500** | **14500** |

**Justification for requested Miscellaneous items:**

The main expense will be dedicated to covering publication fees associated with sharing our research findings. The sum requested also includes scientific associations' annual membership fees, photocopies, and office supplies.

**Equipment**

|  |  |
| --- | --- |
| **Item** | **Price (in NIS)** |
|  |  |
| **Total Price (NIS)** |  |
| Other expenses (including shipping, installation, customs and taxes): |  |
| **Total** |  |
| **Funds requested from ISF:** |  |
|  |  |
|  |  |

**Justification for requested equipment:**

**Additional Funding - Cooperation and Exchanges**

|  |  |
| --- | --- |
| **Description** | **Total (in NIS)** |
| Post-Doc students travel to |  |
| PhD students travel to |  |
|  |  |
|  |  |

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9. Clark, A.F., *The cell and molecular biology of glaucoma: biomechanical factors in glaucoma.* Investigative ophthalmology & visual science, 2012. **53**(5): p. 2473-2475.

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