6.3.4.Scientific:

6.3.4.1. Title and keywords:

• Fields of research – as entered in the registration form; this can be edited.

• Research title (in Hebrew and in English) – as entered in the registration form; this can be edited.

**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 – To help search in scientific databases during the evaluation process, keywords, both general and specific, should be listed, including synonyms. If the keyword is already in the ISF’s database, the system will automatically complete the search phrase.

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

6.3.4.2. Abstract and program:

• Scientific abstract – a file of up to one page (limited to 200 KB in PDF format only). The title “Scientific abstract” should appear at the top of the abstract, followed by the name of the first principal investigator (PI), proposal number and research title. The template file of the abstract should be downloaded and then re-uploaded in this section. Make sure to adhere to the technical guidelines that appear in section 7 below, and here.

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 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-EVs uptake into TM cells and simultaneous real-time monitoring of TM-EVs release. Overall, the research aims to uncover the 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 miRNA and proteins. This interaction enhances cellular responses and signaling outcomes, particularly in ECM turnover within HTM cells.

This study aims 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. The specific objectives are as follows:

1. Quantify TM-derived EV release in response to NPCE-derived EVs using a specialized tracking system.
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.
3. Compare the effects of continuous versus limited exposure to NPCE-derived EVs on TM-derived EV quantity and cargo.
4. Investigate quantitative and cargo modifications in TM-derived EVs after exposure to oxidative stress signals conveyed by NPCE-derived EVs.
5. Assess autocrine and paracrine effects of TM-derived EVs following exposure to NPCE-derived EVs, exploring how modified signals impact both the original TM cells and neighboring ones.

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 of EV-mediated signaling between NPCE-derived and TM-derived EVs, offering valuable insights into their role in managing POAG and potentially opening new avenues for treatment and intervention.

• Research Program – a single file (limited to 16 MB) that includes the research plan, figures, and bibliography, in PDF format only. The name of the first principal investigator and proposal number should appear at the beginning of the research program. The research proposal should be integrative so that the various parts of it converge into one study and not several independent parts.

• The program should include the following sections:

1. **Scientific background** – including a review of the research carried out on the proposed topic.

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 Western world's aging population suggests an increase in the prevalence of POAG, a disease affecting over 80 million people worldwide[1, 2]. POAG involves optic neuropathy, leading to retinal ganglion cell (RGC) axon loss, RGC death, and blindness, with a pathological process occurring in the ocular drainage system [3]. Non-pigmented ciliary epithelium cells (NPCE) produce aqueous humor (AH) continuously nourishing and cleansing the anterior chamber and finally drained out of the eye predominantly (80%) via the conventional outflow pathway comprising of the trabecular meshwork (TM), juxtacanalicular tissue (JCT), and the Schlemm's canal [4]. The AH plays an important role in maintaining the spherical structure of the eye and the intraocular pressure (IOP). A sustained elevation in IOP is a primary risk factor for the development of POAG[5]. Despite intensive research on IOP regulation, there exists a gap in the knowledge of the etiology and pathophysiology of the disease. Targeting to lower the IOP is the only strategy available to prevent the loss of vision. Present pharmacological treatment focuses on reducing elevated IOP by drugs modulating the AH production including -blockers and carbonic anhydrase inhibitors or the AH drainage including prostaglandin analogs, -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 balance in extracellular matrix (ECM) remodeling in the TM-JCT region is needed for the generation of AH resistance, but excess ECM deposition results 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 are excessive alterations in ECM organization in the TM-JCT region and the accumulation of sheath-like plaque materials, leading to altered stiffness[[9]. Our understanding of the changes in the trabecular meshwork (TM) drainage pathway in POAG involves numerous regulatory proteins, such as MMPs[10], Clusterin[11], Cathepsin K[12], TGFβ[13], Integrin[14], and others. This suggests the potential effectiveness of drugs targeting extracellular matrix (ECM) turnover as a standalone therapy or in combination with existing treatments for lowering intraocular pressure (IOP)

Delivering RNA or proteins vulnerable to nucleases, proteases, hydrolysis, phosphorylation, and other natural processes that greatly reduce their levels is a widely acknowledged obstacle. In addition, selected miRNA and proteins influence many tissues, and achieving target-only response is challenging[15]. EVs might be the answer to these challenges. EVs are naturally released, nano-sized, and double layer vesicles that can carry parent cell proteins on their surface and a mixed cargo of miRNA, ssDNA, snRNA, lipids, and proteins within them[16]. This makes them as an attractive and an optimal delivery system[17].

EVs biology research attracted an increasing number of researchers during the last decay. The EVs biologist successfully described EVs biogenesis and release mechanisms, cargo sorting, and uptake pathways[18]. The EVs cargo is subjected to changes reflecting partially the originating cells' homeostasis or pathology conditions[19]. Moreover, EVs were shown to be involved in many pathologic conditions playing a role in homeostasis and maladies[20]. EVs have been shown to participate in healing processes on one hand and, in pathology exacerbate on the other hand.

Studies have shown EVs enrichment in specific miRNA content under specific conditions, with significant biological effects in cell culture, and animal studies[21]. Our present understanding suggests that the changes in EVs cargo and specifically miRNA content are part of signaling pathways affecting adjacent and remote tissues known as paracrine effect[22]. Another suggested effect of EVs was autocrine-like[23], meaning that EVs released by a specific cell type or tissue affect the same specific cell or tissue. Mechanisms were autocrine-like effects attenuated or stimulated intracellular signaling pathways were shown[24, 25].

Our lab have published couple of papers along the last years describing the ability of NPCE derived exosomes to modify TM cells canonical Wnt/TGF signaling pathway, a major regulator of ECM modulation[26]. We acknowledge that trabecular meshwork (TM) cells are subjected to exosomes originating from various ocular tissues, such as the cornea, iris, ciliary muscles, and even retinal exosomes. Among these, we have singled out NPCE-derived exosomes as the most likely candidates for transporting signals related to intraocular pressure (IOP) regulation to the drainage tissues. NPCE derived exosomes has a natural uptake preference by TM cells over other ocular cells[27], and exhibit a dose-response effect to exosomes on the expression[28]. Lately, we have used the electroporation approach to load NPCE derived exosomes with selected siRNA targeting SMAD7 to modify the Wnt/TGF signaling pathway involved in the activation of ECM build-up[29, 30]. Thus, showing the ability of NPCE derived exosomes to serve as targeted TM drug delivery systems. Our results suggest that engineered NPCE exosomes can be loaded with selected cargo to a degree capable of achieving effective concentration in the target cells.

Oxidative stress is one of the key factors in POAG pathology[31-34] and exosomes role in delivery of oxidative signals explored in our lab[19, 35, 36]. Our finding has shown that EVs derived from NPCE exposed to oxidative stress significantly protect TM cells from direct oxidative stress.

These days while many researchers successfully have described the different cells or tissues originating EVs cargo, the diversity of the EVs' size and content is much clearer. A homogenate population of EVs doesn’t exist and a typical range of EVs sizes are released. Researcher debate regarding, generally average EVs size changes taking place under stress or diseases. Microarray analysis performed over many EVs originating from different cells and tissues reported thousands of different miRNAs detected for a single batch of EVs. These numbers of miRNAs approached the total number of genes expressed in a tissue[37].

Hence, the extent to which a limited number of extracellular vesicles (EVs) containing a specific miRNA can effectively transmit a modifying signal to their target cell remains uncertain [38]. For a genotypic and subsequently phenotypic alteration to manifest, several crucial steps must transpire: the EV carrying the specific miRNA must locate its designated cell target, be internalized, navigate through the target cell's lysosomal process successfully, 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 amplifies. Furthermore, it is anticipated that EVs from target cells will possess modified cargo, reflecting the new target cell equilibrium. These EVs can now 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 signaling, extracellular matrix (ECM), and oxidative stress signals in TM cells. However, we still don't fully understand how TM cell-derived EVs respond to NPCE-EVs. In our current study, we aim to investigate how NPCE-derived EV signals are transferred to TM cell-derived EVs. We will use a Cre-LoxP system[39] to analyze the kinetics of TM EV release after exposure to NPCE EVs. Our study will assess the quantitative release of TM-EVs over time at different NPCE-EV:TM cell ratios, helping us understand the changes in stimulated TM-derived EVs.

Along the present research, we would like to explore the dynamics and kinetics EVs' effects resembling second messenger biology. Namely, an amplification process intensifying and enhancing the signal process taking place following successful EV uptake by the target cell. The amplification process can be expressed by increasing the release of EVs from the target cells. In addition, these EVs can carry a specific cargo and thus enable lateral signal transmission to nearby cells and distant tissues. Another possible component of the amplification process can be via autocrine-like manner, and thus increase the target cell release of EVs carrying the initial signal consequences. Our working hypothesis suggests that certain distinct alterations within the cargo of EVs, which subsequently trigger significant amplification effects, extend far beyond the broad spectrum of EVs 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. We will investigate how signals conveyed by NPCE-derived EVs are transferred to TM cell-derived EVs.

1. **Research objectives and expected significance**

Overall objective

Our primary focus is to gain a profound 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 expanding treatment options and ultimately achieving substantial advancements in the management of POAG. In particular, our central objective is to delve deeply into the intricate response mechanisms of TM-derived EVs in parallel to TM cells modifications, when exposed to incoming signals carried by NPCE-derived EVs. We are keen to investigate not only the cargo transported by TM-derived EVs but also the multifaceted paracrine and autocrine effects that these signaling interactions might trigger within the TM cells.

* **Aim 1- To quantify the release of TM-derived EVs when exposed to NPCE-derived EVs.** There is an ongoing debate surrounding the alteration in the release of cell EVs in response to incoming signals. In this study, we aim to 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 CD-81 fluorescence, release as part of this parallel assessment.
* **Aim 2 -** **To conduct a qualitative analysis of alterations in the release of TM-derived EVs when exposed to NPCE-derived EVs.** In our ongoing research (ISF 1545/20), we have found that the exposure duration of TM cells 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 cargo carried within TM-derived EVs. Therefore, our goal is to investigate the cargo of TM-derived EVs, with a particular focus on miRNAs and proteins, which are released after exposure to NPCE-EVs.
* Aim 3- **To thoroughly compare the effects of continuous versus limited exposure of TM cells to NPCE-derived EVs on both the quantity and the modifications observed in TM-derived EVs, particularly regarding their 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, we plan to compare the impact on TM cell mRNA expression when exposed to a known quantity of NPCE-EVs for a specified duration, as opposed to continuous exposure using the Boyden system. This comparative analysis will help us assess the changes in TM cell mRNA expression and TM-EVs cargo more comprehensively.
* **Aim 4- to investigate both the quantitative changes and cargo modifications in TM-derived EVs after exposing TM cells 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. Hence, our objective is 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, 2, and 3. To achieve this, we will expose TM cells to EVs derived from oxidative stress NPCE cells and subsequently analyze the modifications in the cargo of TM-derived EVs.
* **Aim 5- to assess the autocrine and paracrine effects of TM-derived EVs after TM cells have been exposed to NPCE-derived EVs.** Our hypothesis is that EVs, to some extent, mirror their parent 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, including**:
   * **Working hypothesis**

The effects of EV cargo 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 content.

* + 1. Specific hypothesis

It is hypothesized 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 miRNA and proteins associated with the signaling pathway, while also facilitating the turnover of the extracellular matrix (ECM) in HTM cells. This dynamic and kinetic interaction aims to enhance cellular responses and signaling outcomes.

* + **Research design & methods** this section should also include reference to the approvals from authorities if required for conducting the research.

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

NPCE Cell Transfections:

* + 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.

As a result, the Cre recombinase, now tagged with BFP, will also be present in the NPCE-derived EVs, which we will refer to as [EVsCre+].

TM Cell Preparation:

* Concurrently, TM cell will conduct infection process using a tailored designed plasmid. This plasmid incorporates LoxP sequences, functioning as genetic markers for NPCE-derived EVCre+ uptake, alongside the EVs marker CD81, which will be tagged with a crimson dye allowing TM-derived EVs released identification.
* Upon successful uptake of NPCE-derived EVs (EVsCre+), the plasmid within TM cells will undergo a significant fluorescent shift, transitioning from DsRed to GFP. alongside an exosomal marker, CD81. This CD81 marker 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 keeping a close eye on the behavior of TM-derived EVs marked with CD81 and the crimson dye. This integrated approach ensures a comprehensive assessment of both EV uptake and release within our experimental framework, providing invaluable insights into the intricate cellular interactions we aim to explore.

TMLoxPCD81 cells will be exposed to a fix ratio of NPCECre+ derived EVs (30000 EVs/cell)[28] for 1,2,4,8 and 24h, and the TMLoxPCD81 cells will be continuously live monitor under Zeiss Celldiscoverer 7®. This will enable us to precisely quantify the kinetics of EVsCre+ uptake and the subsequent release of TMLoxPCD81-derived EVs. This will allow us quantitative kinetics of EVsCre+ uptake and TM LoxPCD81-derived EVs release.

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 (also outlined in aim 1) for subsequent miRNA microarray analysis.

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

Aim 3: The successful fulfillment of aims 1 and 2 will empower us to conduct a comprehensive examination of the consequences of 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. Rather than administering a singular quota of EVsCre+ to a known count of TMLoxPCD81 cells for varying durations, we will employ the µ-Slide 2 Well Co-Culture system (ibidi 81806) under Zeiss Celldiscoverer 7®. 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 the other, creating a more dynamic and representative experimental environment.

Aim 4: (4a) Aims 1, 2, and 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 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 a qualitative evaluation of the alterations observed in TMLoxPCD81-derived EVs, we will conduct miRNA microarray analysis. The data obtained will be scrutinized in the context of relevant pathways, integrating information from NPCE-derived EVs[19] and TM mRNA data (currently in preparation).

(4b) 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 extracellular vesicles (EVs) in transferring these stress signals to a new culture of naive 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 naive TM cells.

Aim 5**:** In the context of phenotypic changes occurring within the ocular drainage system, one of the most significant alterations involves either the accumulation or degradation of the extracellular matrix (ECM). 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.

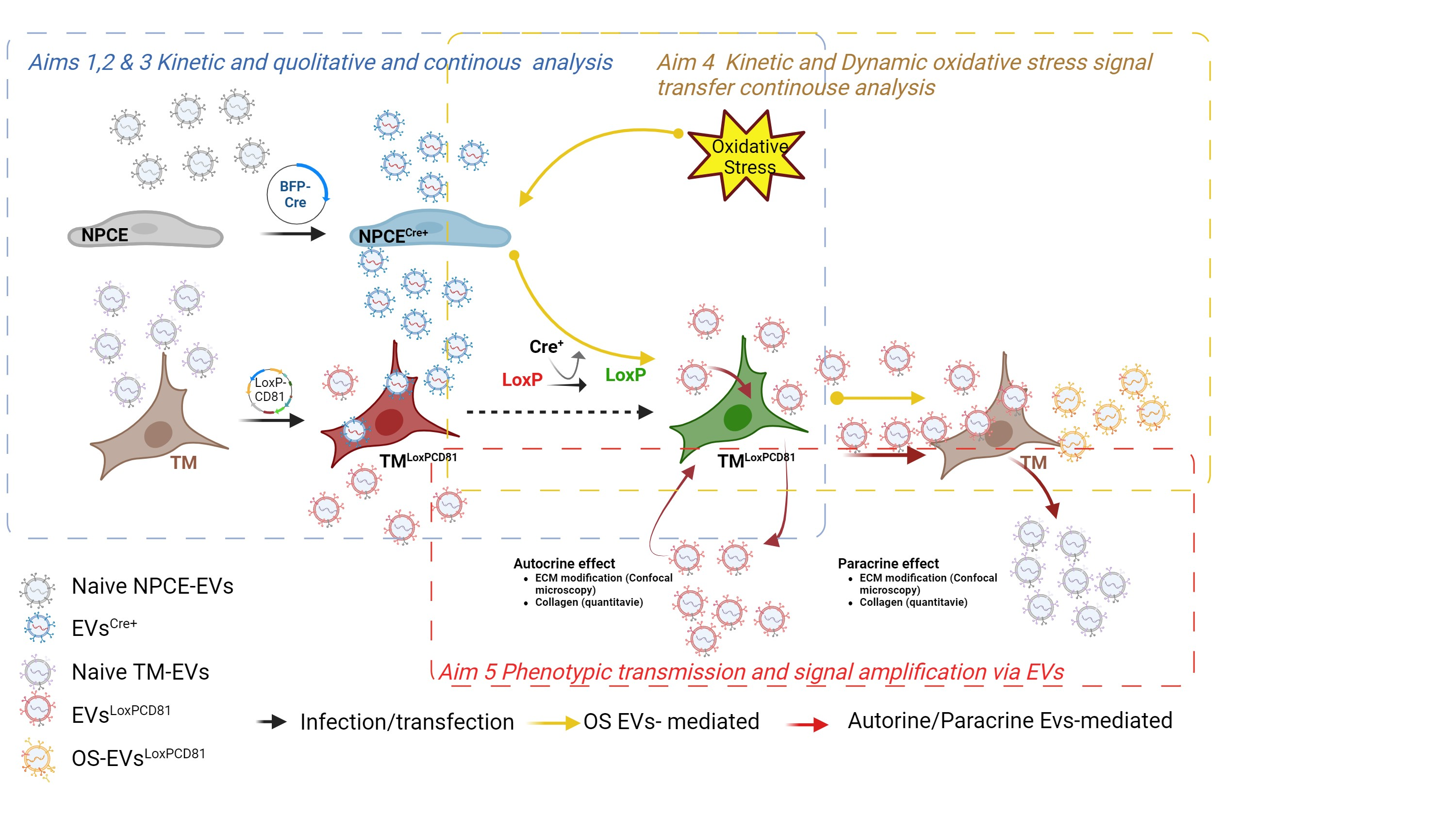
Following the exposure of TMLoxPCD81 to EVsCre+ at optimal time points (Aims 1, 2, & 3), TM-derived EVs will be collected. These EVs will then be used to treat control TMLoxPCD81 cells, reflecting paracrine-mediated effects. We will employ a soluble collagen colorimetric detection assay to quantitatively measure changes in TM ECM collagen.

For 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, including ECM markers such as F-actin, Integrin, Cadherin, clusterin, vinculin, and others. These stained samples will undergo confocal microscopy analysis, providing us with a detailed visualization of ECM alterations.

The concept's novelty stems from the remarkable realization that EVs possess dual influence—they directly impact target cells and also have the power to affect EVs within the very target cells. This intricate interplay of EVs within the cellular environment leads to specific signal amplification, thereby significantly expanding our comprehension of their pivotal role.

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 exosomes depleted serum and 50μg/ml gentamycin at 37°C in 5% CO2. 80% confluence cells will be used through the studies.

EVs isolation and depleted serum. EV isolation, separation and characterization will be performed according to The International Society for Extracellular Vesicles[40]. We will use serum depleted EVs for cell growth, series of ultra-centrifugations and qEV Isolation Columns (IZON NZ) for EVs isolation, Nanoparticle Tracking Analysis (Malvern UK) and Tunable Recessive Pulse sensing (qNano IZON, NZ) for EVs size and concentration analysis. EVs characteristic markers will be analyzed using Exo-Check™ Antibody Array (SBI, Mountain View, CA) and western blot analysis.

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 marked 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

Dynamic and kinetics analysis: TMLoxPCD81 cells will be seeded in 6 mm sterile cell culture dishes (2x106 cells in 2 ml per dish). 24 h later, growth medium will be replaced with fresh EVs 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 time 1,2,4,8 and 24h will be tested for EVsCre+ (BFP, 381:445, nm) uptake by TMLoxPCD81 fluorescent color shift (DsRed, 554:591, nm to eGFP, 488:507) and TM LoxPCD81-derived EVs CD81 labeled (crimson, 611:646) release, using the ZEISS Celldiscoverer 7® that allows stable Incubation with temperature, CO2 and O2 control at the Ilse Katz Institute for Nano-Science and Technology (BGU).

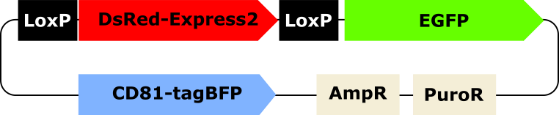
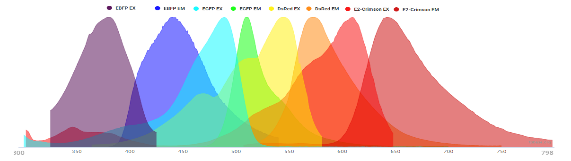
Continuous TMLoxPCD81 Cell Exposure to EVsCre+: To ensure the continuous exposure of TMLoxPCD81cells to EVsCre+ (aim 3), we will employ the µ-Slide 2 Well Co-Culture system (81806, ibidi, Germany). This system offers (i) a shared EVs and soluble Factors while maintaining separate growth environments. (ii) minimal evaporation tendencies, making it well-suited for extended and long-term assays. (iii) Excellent Optical Quality: 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.

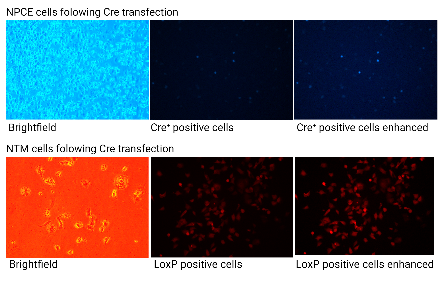
EVsLoxPCD81 Qualitative Analysis**:** To analyze the miRNA content of EVsLoxPCD81, we will employ a streamlined process using a total exosomal RNA purification kit (cat: 17200, Norgen Biotek Corp., Canada). Here's a concise overview of the procedure: *Lysis:* A volume of 200 µL of EVsLoxPCD81 suspended 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). 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 the suitable microarray chip analysis, such as the GeneChip® miRNA 4.0 Array and Flashtag™ Bundle–ThermoFisher (cat 902445).

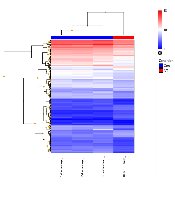
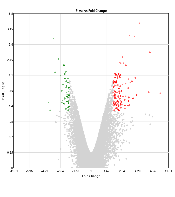
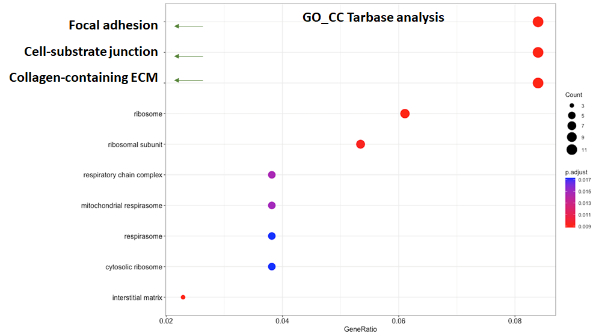
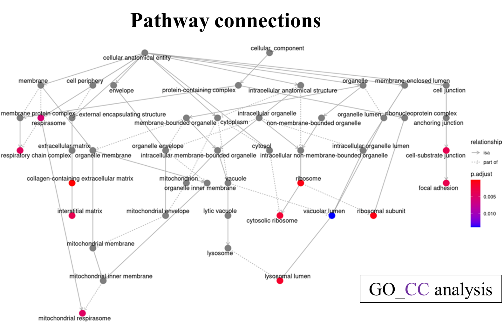
Oxidative Stress:(Aim 4) To induce oxidative stress in NPCECre+ cells, we will be utilized 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH), which is a free radical-generating azo compound. AAPH is capable of initiating oxidation reactions by continuous production of peroxyl radical followed by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

ECM Modification (Aim 5) - Immunohistochemistry and Confocal Microscopy: 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 (refer to 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.

* + Preliminary **results** (if relevant)

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, their uptake by NTM cells, and simultaneously, the release of EVs originating from NTM cells. To accomplish this, it is essential to incorporate distinct, non-overlapping fluorescent dye emission signals. As part of this effort, the following plasmid has been developed.

NPCE & TM cells following Cre transfection or LoxP infection (X100). We initiated the transfection of NPCE cells with the commercial plasmid pcDNA3.1-CMV-CFP;UBC-Cre25nt (Addgene #65727) and NTM infection cells with the commercial plasmid pLV-CMV-LoxP-DsRed-LoxP-eGFP (Addgene #65726). The primary conditions for transfection and infection were established, including the determination of optimal 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 mRNA expression post-NPCE-derived EVs treatment. Furthermore, we compared these assays with the effects of pre-exposing NPCE cells to oxidative stress. The results from these investigations allowed us to discern TM-expressed genes under normal conditions. Subsequently, we conducted a predictive Gene Ontology (GO) analysis to pinpoint the relevant pathways affected by these changes(unpublished), 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 (DEG) and predicted targets revealed a substantial up-regulation of the highlighted targets (bold & indicated by arrows) in TM cells exposed to chronic oxidative stress.

* + **The researcher’s resources for conducting the research**: description of personnel and infrastructure –accessibility and availability, including for materials and archives if necessary. Proposals submitted in the humanities should also note the level of mastery of languages essential for the research.

Professor Beit-Yannai's research facilities are well-equipped to support this research endeavor. These 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 cell culture incubator. Additionally, there are PCR and Western blot tools, running and blotting kits, and a power supply. The laboratory also has access to various departmental equipment, including ultracentrifuges, a -80°C freezer, and both inverted fluorescent and confocal microscopes. Furthermore, the research is 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.

* + In the research program, it is recommended to address the **expected results and pitfalls** and proposing alternative approaches and methods in case the proposed method/experiment does not work as expected.

expected results

Our study is designed to test the 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 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 cargo due to the influence of NPCE-derived EVs. This enhanced modulation is anticipated to result in the activation of targeted signaling pathways within the 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 cargo within TM cell-derived EVs, we predict a 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 extracellular matrix (ECM) turnover in HTM cells. We anticipate 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 extracellular vesicle (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 cargo influenced by NPCE-derived EVs signifies an opportunity to develop targeted therapies. By manipulating EV cargo 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 cargo 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 extracellular matrix (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** Acknowledge any potential limitations or uncertainties in your expected results.**:** The research results are poised to significantly advance our understanding of EVs' dynamic effects. The interpretation of the findings must consider 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 exploration rather than a direct translation.

Challenges and Potential Solutions:

1. Transfection and Toxicity Issues: When faced with challenges such as obtaining a low yield of NPCECre+ or encountering NPCE cell toxicity following transfection, our standard protocol typically suggests using Lipofectamine 2000. However, if difficulties persist, including persistent NPCE cell toxicity or consistently low transfection yields, we maintain the flexibility to explore alternative transfection reagents. Options include Lipofectamine 3000, Fugene, RNAiMAX, and Lipofectin. It's worth noting that the performance of these alternative transfection reagents can vary depending on the specific cell types under investigation.

2. Quantitative Analysis of EVs: Achieving a quantitative analysis of NPCE-derived EVs impacting TM-derived EVs can be accomplished by using different time points instead of relying solely on stimulant-based analysis. 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 enhances the robustness of our quantitative analysis of EVs' impact and behavior in different cell types.

3. Qualitative analysis of EVs: For qualitative analysis of EVs, we have the option of employing microarray or RNA sequencing techniques. In both methods, the minimal amount of miRNA needed can vary depending on the specific RNA sequencing platform and protocol being used. In general, most RNA sequencing methods typically require nanograms quantities of RNA. Nevertheless, with the advancement of technology, specialized protocols, and kits now permit RNA sequencing from smaller RNA inputs, including quantities as low as picogram or even femtogram 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. As we plan to work with larger Petri dishes, the approximate nanograms of miRNA required for these methods are well within reach with our current

* + Bibliography with \* – a file should be uploaded with the same bibliography that appears in the research program. The publications that are closest in subject area to the proposed topic (and are not written by those submitting the proposal!) should be marked with an asterisk (\*). The list should cite the **full names of the articles and all their authors**. The author’s first name may be cited by its initial letter. This file will only be used by the members of the committee. Make sure to include the proposal number and PI name at the top of the page. If the bibliography includes publications in the Hebrew language, they must be separated under an English title: Hebrew Publications.

Bibliography with \*

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* + Articles that were accepted but not yet published – in exceptional cases, when an article is cited in the bibliography that was **accepted for publication** but not yet published, and its content is deemed essential for evaluating the research proposal, an electronic copy of the article should be attached. It should be noted in the body of the research proposal that the article can be provided by the ISF upon special request by the reviewer. Articles that have not yet been accepted for publication should not be attached.

6.3.4.3. Time schedule – the different stages of the research should be specified in a table. A short explanation of the time schedule must be included in the Explanatory Notes section. In cases of theoretical research that cannot be divided into stages, you can indicate that a time schedule is not relevant to this research. In any case the explanation is mandatory.

|  |  |  |
| --- | --- | --- |
| **Objective** | **Beginning** | **End** |
| Establishing the NPCECre+ and TMLoxPCD81 reporting system. (aim1) | 01.10.24 | 30.09.25 |
| Quantify TM-derived EV release upon exposure to NPCE-derived EVs. (aim1) | 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. (aim3) | 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 system (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

Personal

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| 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 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. His 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 EVs purification column | 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 EVs 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 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 EVs 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 researcher's laptop computer to enhance the efficiency of the Principal Investigator's 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:**

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 |  |
|  |  |
|  |  |

1. Quigley, H.A. and A.T. Broman, *The number of people with glaucoma worldwide in 2010 and 2020.* British journal of ophthalmology, 2006. **90**(3): p. 262-267.

2. Tham, Y.-C., et al., *Global prevalence of glaucoma and projections of glaucoma burden through 2040: a systematic review and meta-analysis.* Ophthalmology, 2014. **121**(11): p. 2081-2090.

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