FINAL SCIENTIFIC REPORT

AGE RELATED CEREBRAL MICROVASCULAR PATHOLOGIES AND THERAPEUTICAL APPROACHES USING ENDOTHELIAL PRECURSOR CELLS

Execution Stage: Months 1-36

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I. GENERAL OBJECTIVES OF THE PROJECT

Aging and age-related comorbidities represent rapidly growing medical and socioeconomic issues in the EU. Impairment of brain functions is responsible for a substantial portion of age-related health problems. The functional state of the central nervous system (CNS) is largely dependent on the quality of the microvasculature, which is determined by endothelial cells in capillaries forming a lining and closely interacting with pericytes and astrocytes. These cells have limited regenerative capacity and are prone to senescence. For these reasons, the central hypothesis of the project is that endothelial renewal can substantially contribute to maintaining the functional integrity of the CNS, and in this process, endothelial precursor cells (EPC) can play a crucial role.
By using advanced in vitro and in vivo models, the main objectives of the project are:
- Determining key aspects of the incorporation of endothelial precursor cells into the brain's capillary network.
- Identifying determinant elements in the communication between endothelial precursor cells and pre-existing endothelial cells, as well as other components of the neurovascular unit (pericytes, astrocytes).
- Evaluating the benefits of endothelial renewal on the functions of the neurovascular unit.
Our research can provide new insights for treatments aimed at improving neurovascular functions in aging and age-related diseases.

II. SPECIFIC OBJECTIVES

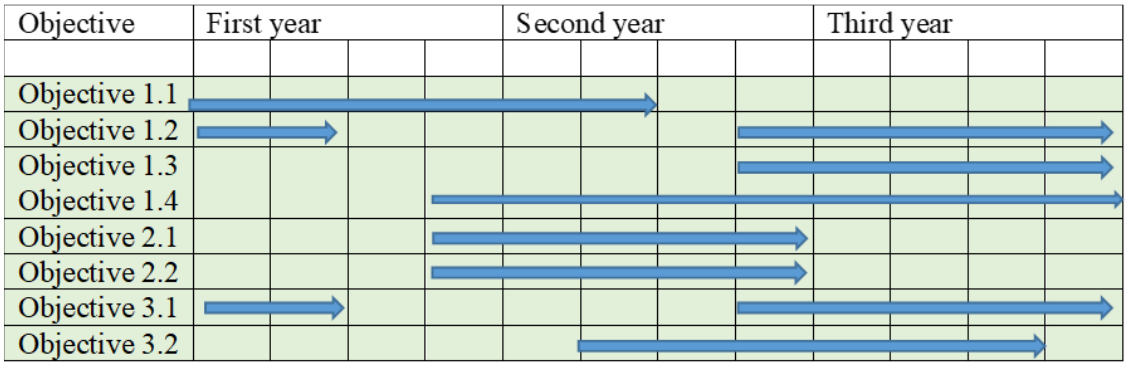

Fig.1. GANTT Chart of the Project

Objective 1.1. Dynamics of endothelial precursor cell integration
Objective 1.2. Dynamics of EPC integration in elderly animals
Objective 1.3. Senolysis effect on EPC incorporation
Objective 1.4. Dynamics of EPC integration under pathological conditions
Objective 2.1. Visualization of extracellular vesicles
Objective 2.2. Identification of cells that uptake extracellular vesicles
Objective 3.1. Effect of EPC on the barrier formed by the neurovascular unit
Objective 3.2. Structure of tight junctions in EPCbiectiv 1.1. Dinamica integrarii celulelor endoteliale precursoare

III. RESULTS
EXECUTION STAGE I

Milestone 1. Establishment of mouse colonies for aging
Milestone 2. Establishment of an EPC cell line genetically marked

Dynamics of Endothelial Precursor Cell Integration (Objective 1.1, part 1)

In the first set of experiments, immortalized mouse endothelial precursor cells (EPCs), designated MagEC 10.5, were labeled with Cell-tracker red (Thermo Fisher). Subsequently, 4*10^5 of these fluorescent cells were injected into the internal carotid artery of 10-12 weeks old BALB/c mice under isoflurane anesthesia. This experimental model allowed us to start monitoring the immediately incorporated precursor cells without waiting for the development of genetically marked precursor cells. However, this method only allowed short-term monitoring.

The number of MagEC cells residing in the tissue on a section from each mouse was determined, selecting sections from the same brain region. The counted cells were verified by calculating fluorescence intensity against the background and expressed as the number per cubic millimeter of tissue. Controls had 54.6+/- 14.7 cells/mm3 of brain tissue at 4 hours, a number that decreased to 30.2+/- 9.2 cells/mm3 of brain tissue at 24 hours and 29.8+/-10.2 cells/mm3 of brain tissue at 48 hours.

To determine if there are preferred sites for EPC adhesion in the capillary bed, spatial localization was analyzed by evaluating the fraction of EPCs adhering to vascular junctions. 4.4% of all observed EPC cells were located at microvascular junctions. This number was 9.4% at 4 hours, and after 24 and 48 hours, it decreased to 2% and 3.1%, respectively. This suggests that cell adhesion is preferred on straight vascular sections compared to capillary junctions. The adhesion of all MagEC cells was observed at the microvascular level, not in larger arterioles or venules. A verification step was included to differentiate red fluorescent cells in the capillary lumen from possible blood clots. Thus, near each detected cell, green autofluorescence was observed. Interestingly, this also proved to us that at 4 hours after injection, more than 80% of MagEC cells had adjacent green/red fluorescence, indicating residual blood after perfusion. This number significantly decreased to 20% after 24 hours, indicating better perfusion, suggesting that precursor cells no longer completely filled the capillary vascular lumen but began to flatten, possibly indicating the beginning of the integration process.

To study the areas where EPCs adhere to the capillary bed and begin to integrate into vessels, we used immunofluorescence microscopy with antibodies marking specific components of the neurovascular unit (NVU) (PECAM, Claudin-5, Collagen IV, AQP4, CD13). These studies showed that EPC cells can integrate into the existing vascular bed and form tight junctions with the microvascular endothelium.

To monitor EPC cells in the long term, cell lines expressing fluorescent proteins were created. The first cell line expresses the soluble fluorescent protein tdTomato, producing intense fluorescent cells; the second cell line expresses tdTomato fused with CD9, a transmembrane protein enriched with extracellular vesicles, to study their role in intercellular communication.

The soluble tdTomato protein was transfected using the standard plasmid transfection methodology and puromycin selection, followed by cloning by dilution. The CD9-tdTomato fusion transgene was transfected using a lentiviral vector (LP771-025, GeneCopoeia) at an MOI of 10 using puromycin selection and cloning by dilution. Both fluorescent cell lines were characterized to compare them with the parental cell line (Fig. 2).

These cells could be detected at 7 days post-injection (Fig. 3), at which point the number of adhesion sites where precursor cells could be identified was approximately 10 times lower compared to the number observed for cells marked with Cell tracker red at 4 hours.

For in vivo monitoring, cranial windows were installed in FVB/Ant:TgCAG-yfp_sb #27 mice expressing the yellow fluorescent protein Venus in endothelial cells. After postsurgical recovery of the mice, red-labeled EPC cells were injected into circulation. Using two-photon microscopy, we could visualize red-fluorescent EPC cells within the yellow-fluorescent cerebral vasculature at different time points (Fig. 4). The observed volume of brain tissue was adapted to conditions, generally starting with a scan of 700 x 700 x 350-400 micrometers. Red fluorescent cells were observed shortly after injection into cerebral vasculature at depths of 150-300 micrometers below the pia mater. Some of these cells migrated into the cerebral circulation during observation. At 24 hours post-injection, the cells either remained in the same location as initially detected, moved slightly, or disappeared completely. In some cases, new cells appeared in the monitored space. A significant number of EPC cells attached to capillary branching points or in their vicinity, suggesting that this might be the preferred adhesion point in the superficial layers of the cortex.

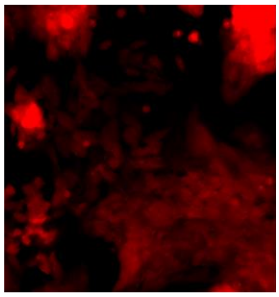

Fig. 2. MagEC 10,5 tdTomato cells, epifluorescence image

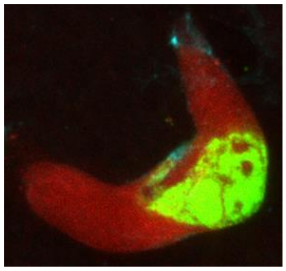

Fig. 3. MagEC 10.5 tdTomato Cells (red) Adhering to a Cerebral Capillary in Fixed Brain Tissue (Claudin-5 marked in cyan, nuclear labeling in green)

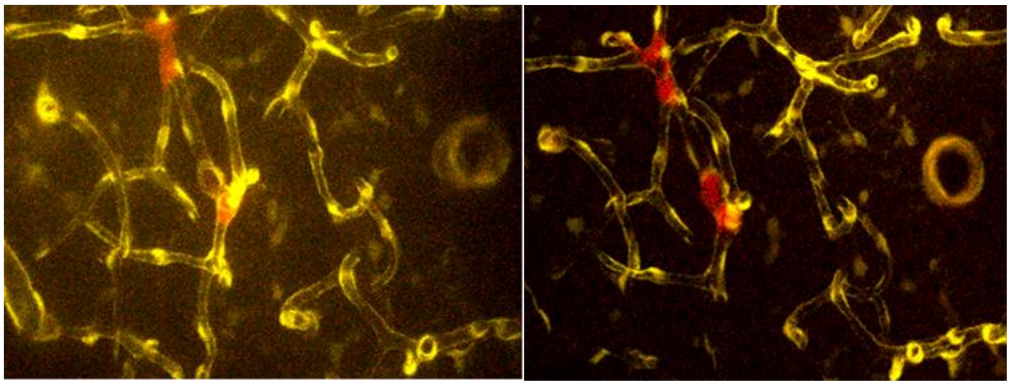

Fig. 4. In vivo two-photon microscopy image, MagEC 10.5 tdTomato cells at 1 hour and 24 hours after injection

The Effect of EPC on the Blood-Brain Barrier (Objective 3.1, Part 1).

The mouse colony for aging has been established. We optimized the method for assessing blood-brain barrier permeability using two-photon microscopy and fluorescein as a tracer (Fig. 5.) (Nyul-Toth et al., Am J Physiol Heart Circ Physiol. 2021 Apr 1;320(4):H1370-H1392).

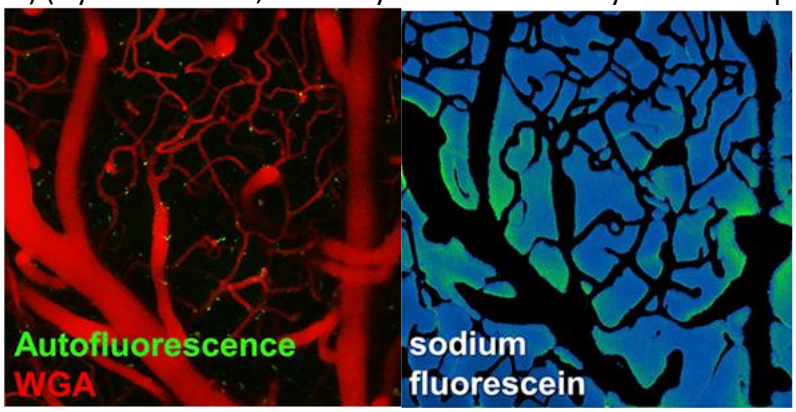

Fig. 5 Permeability of the BBB for fluoresceine

EXECUTION STAGE II

Milestone 3. Determination of the temporal and spatial distribution of endothelial precursor cells (EPCs).
Milestone 4. Identification of neurovascular unit cells communicating with endothelial precursor cells through extracellular vesicles.

The dynamics of endothelial precursor cell integration (Objective 1.1, Part 2). The structure of tight junctions in EPCs (Objective 3.2, Part 1).

Using 3D volumetric scanning with two-photon microscopy, we determined the adhesion points' location for a large number of EPCs in vivo. This confirmed that adhesion at microvascular branching points is preferred in the in vivo cortex compared to what we observed ex vivo when examining adhesion in the sectioned brain tissue, which includes both cortical and subcortical regions. Furthermore, what could not be observed in ex vivo

examinations was that in in vivo examinations, a significant number of EPCs were found transiently in meningeal blood vessels.

In a paper published in 2022, we presented the MagEC 10.5 endothelial precursor cell line as a therapeutic platform for neurodegenerative diseases (Thinard, Farkas et al. https://doi.org/10.3390/pharmaceutics14071418). The orientation, adhesion, and integration of cells into cerebral vasculature. Detection of MagEC 10.5 EPCs labeled with red cell tracker in brain tissue at 4.5 hours post-intracarotid injection. Detection on 80µm sections. EPCs appear as white dots in the sections (Fig. 6a).

Identification of astrocytes, pericytes, endothelial cells of the vascular wall, and collagen IV in the extracellular matrix allowed the localization of MagEC 10.5 endothelial progenitors inside the vessels along with their insertion into the blood-brain barrier (BBB) (Fig. 6b). Immunolabeling for PECAM-1 and fluorescence laser scanning microscopy were used to delineate cerebral capillary microvessels around fluorescent EPCs. Shortly after injections, the EPCs imaged in brain sections seemed to fill the capillary lumen. Already at 28 hours post-injection, it was observed that the EPCs were flattened and adhering to the vessel walls (Fig. 6c). In longer-term experiments, MagEC 10.5 cells were transformed to express the red fluorescent protein, tdTomato (called MagEC 10.5 RT), and similarly, were injected into BALB/c mice. Cells were observed at 7 days post-injection to be labeled with the tight junction protein, claudin 5. These results showed the formation of tight junctions between injected MagEC 10.5 RT and resident unlabeled endothelial cells (Fig. 6d, e). We concluded that injected MagEC 10.5 RT integrates into the capillary bed.

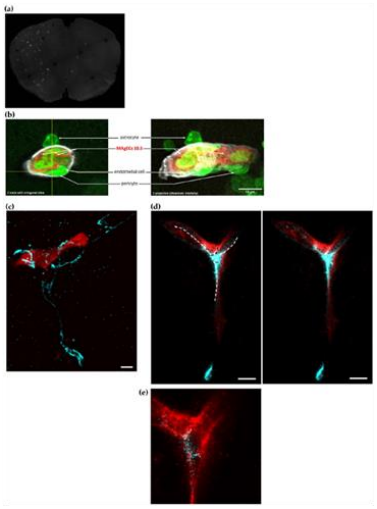

Fig. 6. Adhesion and integration of injected cells into the blood vessels of the brain. (a) Detection of MagEC 10.5 EPCs labeled with the red cell tracker marker in the brain at 4.5 hours after intracarotid injection. (b) Microscopic detection of MagEC 10.5 cells labeled in red (Cell Tracker Red). Syto 13 (green), which stains all nuclei, allows approximate identification of cells by the position and shape of the nuclei. (c) At 28 hours after ICA injection of MagEC 10.5 (labeled with Cell Tracker Red, red), there are EPCs that do not completely block the lumen of cerebral microvessels, indicating possible integration of EPCs into the vascular wall. The cerebral capillary is delineated by PECAM-1 immunostaining (cyan). The image is a maximum intensity projection of a confocal z-stack. (d) MagEC 10.5 RT transduced (tdTomato, immunostained with anti-RFP, red) were observed in the brain blood vessels at 7 days after ICA injection, showing integration into existing blood vessels. The upper right branch of the capillary shows a lumen that appears to be lined with an endothelial cell differentiated from the transduced EPC. The lower branch seems to contain only partially fluorescent red cells. Claudin 5 immunostaining (cyan) shows tight junctions between two edges of the same endothelial cell derived from the EPC in both upper branches and between an endothelial cell derived from the EPC (red) and another unlabeled endothelial cell (white dashed lines). Between adjacent endothelial cells, the tight junction runs around the circumference of the vessel, sealing the junction of the two cells (black dashed lines); the right panel shows the non-overlaid image. The image is a maximum intensity projection of a confocal z-stack. (e) STED image from a single optical section of the stack presented in (d) reveals the structure of the tight junction filament at a higher resolution (pixel size 40 nm). Scale bars represent 5 µm. Scale bars are 10 µm.

Furthermore, we demonstrated that precursor cells can be used to express antibodies against amyloid-beta and TDP43 proteins, which play a central role in Alzheimer's disease and ALS, respectively. Daughter cell lines expressing the antibody fragment (Fab) were

developed by our collaborators and tested on brain organoids. We showed that when MagEC cells expressing Fab are injected into the vasculature of mice, they can orient themselves towards cerebral vasculature and remain there for several days (Fig. 7.).

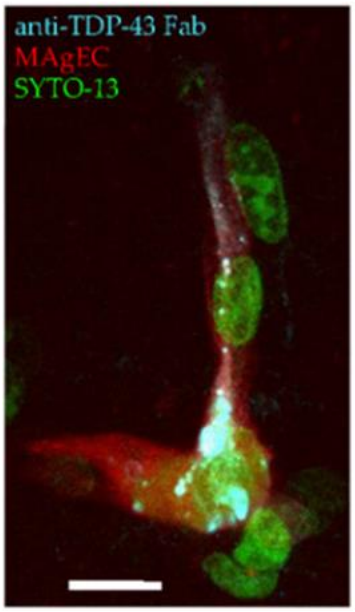

Fig. 7. Fab fragment expressed by EPC in vivo. The scale is 10 µm.

Visualization of extracellular vesicles (Objective 2.1.) and identification of cells that absorb extracellular vesicles (Objective 2.2.).

We used two techniques to visualize intercellular communication based on extracellular vesicles originating from injected EPCs. First, we created an EPC cell line expressing the red fluorescent fusion protein CD9-tdTomato to monitor the localization of extracellular vesicles derived from EPCs. We co-cultured yellow fluorescent endothelial cells (isolated from FVB/Ant:TgCAG-yfp_sb #27 mice) together with EPCs expressing CD9-tdTomato, and we identified a clear localization of extracellular vesicles derived from EPCs inside endothelial cells.

Secondly, we generated another EPC cell line expressing the GFP-Cre fusion protein. These cells were injected into the circulation of Ai14 Cre-dependent tdTomato reporter mice. The hypothesis was that these cells would store the Cre-expressing recombinase in extracellular vesicles, so intercellular communication mediated by extracellular vesicles would activate the reporter gene in target cells, turning them fluorescent red (Fig. 8).

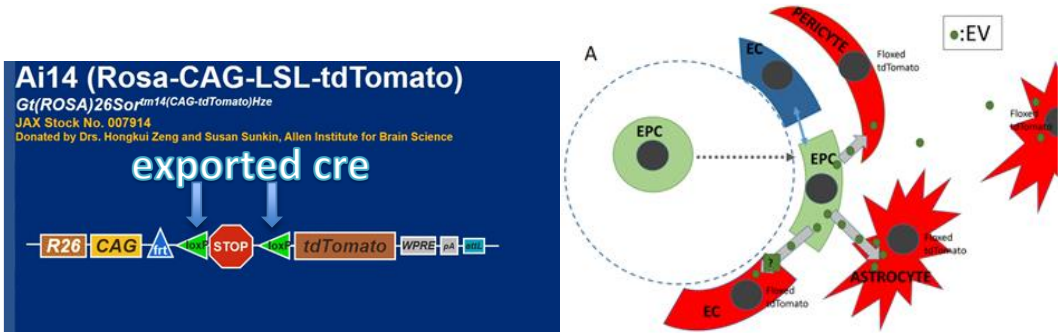

Fig. 8. The strategy for identifying cells that take up extracellular vesicles

At 5 days after the injection of EPCs, brain sections containing red fluorescent cells were categorized based on the morphology of reporter cells and were subsequently immunostained. For example, co-staining CD13+ Claudin5 for vessels. Out of 274 tested cells, 36.9% were positive for NeuN, indicating neurons, 48.9% were positive for GFAP, indicating astrocytes, 4.7% were positive for CD13, indicating pericytes, 4.4% were positive for Claudin 5, indicating endothelial cells, and 5.1% remained unidentified.

Integration dynamics of EPCs under pathological conditions (Objective 1.4., part 1).

Analyzing the molecular mechanisms that modulate the orientation and adhesion of precursor endothelial cells under pathological conditions, precursor cells were pre-treated with recombinant TNF-alpha or VEGF. Optimal concentrations were determined in adhesion tests where EPCs were seeded on top of a confluent monolayer of endothelial cells.

EXECUTION STAGE III

Milestone 5. Determining the temporal and spatial distribution of endothelial precursor cells in aged animals (EPCs).
Milestone 6. Identifying the effect of senolytic substances.
Milestone 7. Determining the effect of EPCs on the functionality of the blood-brain barrier (BBB) and tight junctions (TJs).
Milestone 8. Studying the role of pathological conditions.

Determining the temporal and spatial distribution of endothelial precursor cells in aged animals (Objective 1.2.) The effect of senolysis on the incorporation of EPCs (Objective 1.3.).

We determined the dynamics of EPC adhesion in the cerebral vasculature of aged animals and studied the effect of senolytic substances. Using the senolytic navitoclax, we showed that the number of adherent EPCs increased following senolysis after 48 hours in young BALB/c mice. Once the mice reached the age of two years, we conducted a more detailed analysis, expanding the experimental setup with an additional senolytic and using both young and old mice. The two groups were aged 8-12 weeks and 20-26 months, respectively. The senolytic treatments were as follows: 100 mg/kg navitoclax once daily for 5 days via oral gavage or 5 mg/kg dasatinib combined with 50 mg/kg quercetin in a single administration with a 2-day or 7-day rest period after treatment for navitoclax or dasatinib + quercetin, respectively. The pre-treatment was followed by intracarotid injections of EPCs. The graphs show boxplots of the number of adherent EPCs in each animal from each treatment group, normalized to a volume of 1 mm^3 of tissue each.

In young mice, we observed an increase in the number of adherent EPCs to the brain vasculature in response to both senolytic treatments (navitoclax or dasatinib/quercetin). This effect was already noticeable at 24 hours. Moreover, the observed decline in the number of adherent EPCs over time was rescued by senolysis. Our results indicate that EPCs adhere less to the vasculature of old mice compared to young mice. However, if we normalize the number

of adherent cells to their respective controls, the relative increase in adhesion in response to senolysis was comparable in old and young mice after 48 hours, as shown in the following figure.

Efectul EPC asupra barierei hemato-encefalice formate de unitatea neurovasculara (Obiectiv 3.1. partea 2).

Evaluarea funcțională a BBB și a TJ a fost efectuată prin monitorizarea permeabilității in vivo folosind microscopie cu doi fotoni. Șoarecii tineri și bătrâni FVB/Ant:TgCAG-yfp_sb #27, care exprimă proteina fluorescentă galbenă Venus în celulele endoteliale au fost supuși implantării ferestrei craniale pentru microscopie cu doi fotoni. Șoarecii au fost injectați cu EPC-uri care exprimau tdTomato. Șoarecii care prezentau EPC-uri fluorescente roșii în fereastra craniană în ziua următoare injectării EPC-urilor au fost apoi canulati în artera femurală, reintroduși în microscopul cu doi fotoni și injectați cu Na-fluorescein și Evans blue (markeri cu greutate moleculară mică și mare) înregistrând imagini cu microscopul cu doi fotoni. Intensitatea fluorescenței pentru markeri a fost măsurată în regiunile de interes (ROI) aproape de vasele de sânge care conțineau EPC-uri și vasele de control care nu conțineau EPC-uri. Având în vedere că distanța medie între capilarele din creier este de aproximativ 40 de microni, un ROI se afla imediat lângă vas, în timp ce al doilea se afla la 20 de microni mai departe într-o direcție în care știam din imagini tridimensionale anterioare că nu exista alt vas în apropiere. Rezultatele noastre au arătat că permeabilitatea BBB pentru Na-fluorescein și Evans blue-albumin nu s-a schimbat atât la șoarecii tineri, cât și la cei bătrâni la 3 zile după injectarea EPC-urilor (Fig. 9.).

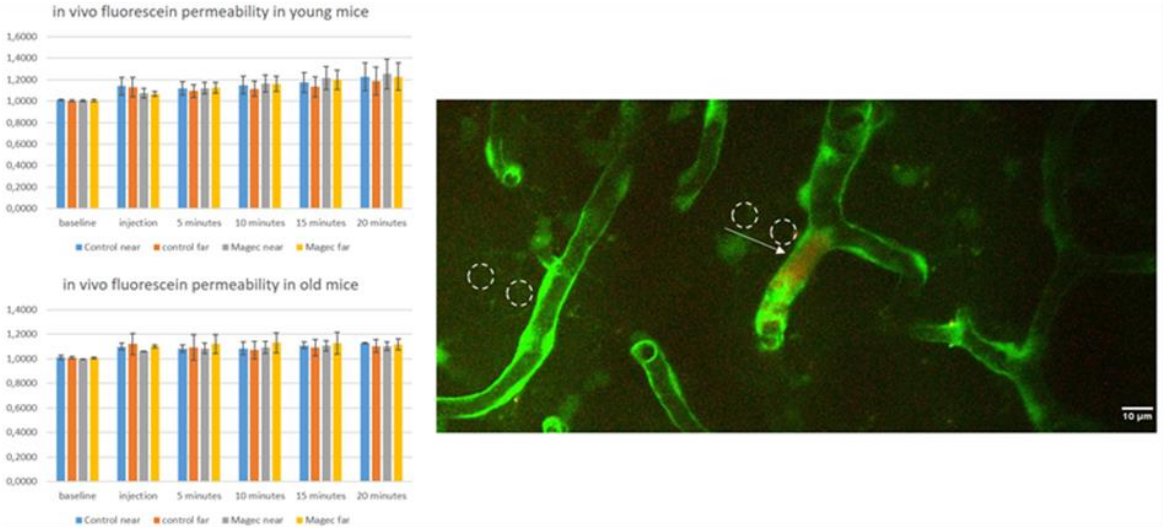

Fig. 9. In vivo measurement of BBB function. The image shows a representative image with ROIs marked as dotted circles and the position of the labeled EPC marked with an arrow. The bar graph shows the average relative intensity (+- S.E.M.) at points close and far from vessels after EPC injection compared to baseline fluorescence..

Tracking the integration of EPCs under pathological conditions (Objective 1.4, part 2) and the structure of tight junctions in EPCs (Objective 3.2, part 2)

The effect of hypoxic injury was studied using multiple models. Microinfarcts were modeled either by ablating branches along a capillary or by injecting fluorescent microspheres into

circulation. It was expected that hypoxic injury following the ablation of several capillary branches through laser irradiation would result in the adhesion of EPCs at our selected sites, allowing for a detailed study of individual adhesion and integration events. However, we did not observe the adhesion of EPCs at the site of injury in several experiments.
When we used fluorescent microspheres to induce multiple small hypoxic injuries, the sites of the injuries were easily identifiable, and EPCs were often observed near the microspheres blocking the capillaries. At the same time, both the microspheres and associated EPCs were not observed to remain in the same location for an extended period.

We further studied the integration of EPCs and the effects of integration in the overall hypoxia model using the occlusion of two vessels (2VO - bilateral occlusion of the common carotid artery). The area presenting neural lesions in response to hypoxia was detected with TTC. In these experiments, healthy tissue was colored in red, and damaged tissue in white. The injection of EPCs led to a significantly faster recovery after hypoxic injury, as evidenced by the reduction in tissue lesion area after 7 days compared to sham controls. Already at 5 days after hypoxic injury and EPC injection, an increase in the number of integrated EPCs into the cerebral vasculature was recorded, which may explain the faster tissue healing.

In addition to quantifying the integration of EPCs based on their assuming endothelial morphology, we further verified integration using immunofluorescence. Immunofluorescent microphotographs also revealed that capillaries formed by the integrated EPCs and pre-existing endothelial cells could jointly form the vessel lumen, as seen in the following image. The presence of tight junctions between the two cells and the presence of a vascular lumen strongly suggest that this is a functional blood vessel. This may be the basis for the positive effect that EPCs have on the regeneration of brain tissue following ischemic injury.

Our hypothesis is that the positive effect of EPCs on tissue regeneration can be increased by increasing the number of adherent EPCs in the brain vasculature. Therefore, we sought mechanisms to increase the number of adherent EPCs in brain blood vessels. Pretreating the EPC culture for one hour with 1 or 5 ng/mL TNF-alpha led to an increase in adherence at 24 hours after cell injection. Additionally, cells adhered more at the same site in the vasculature after 120 hours.

IV SUMMARY OF RESULTS, THEIR IMPACT, THE MOST SIGNIFICANT RESULT

The research activities proceeded according to the research plan, and all proposed objectives were achieved.

Objectives 1.1. and 1.2. Characterized, using confocal microscopy and two-photon in vivo microscopy, the temporal and spatial dynamics of adhesion and integration of endothelial precursor cells (EPCs) in the cerebral vasculature in both young and old animals. Experiments revealed a preference for adhesion at microvascular branching points in the cortex.

Objective 1.3. Observed an increase in the number of adherent EPCs to the brain vasculature in response to both senolytic treatments (navitoclax or dasatinib/quercetin) in both young and old animals. The effect of dasatinib/quercetin treatment was more pronounced.

Objective 1.4. Initially studied the effect of hypoxia on EPC incorporation using various models (microinfarcts, laser ablation, 2VO). In the case of 2VO (bilateral occlusion of the common carotid artery), a significant increase in the number of integrated EPCs in the cerebral vasculature was recorded, resulting in significantly faster recovery of nervous tissue after hypoxic injury. Additionally, demonstrated that VEGF and TNF-alpha can increase the number of EPCs incorporated into the cerebral microvasculature.

Objectives 2.1. and 2.2. Used two techniques to visualize intercellular communication based on extracellular vesicles originating from injected EPCs. Firstly, created an EPC cell line expressing the red fluorescent fusion protein CD9-tdTomato. This method was applicable in vitro, but in vivo it was not sensitive enough. Therefore, produced another EPC cell line expressing the GFP-Cre fusion protein, which was injected into the circulation of Ai14 Cre-dependent tdTomato reporter mice. With this method, it was established that the majority of extracellular vesicles released by EPCs are taken up by neurons and astrocytes (of 274 positive cells tested, 36.9% were NeuN-positive neurons, 48.9% were GFAP-positive astrocytes, 4.7% were CD13-positive pericytes, 4.4% were Claudin 5-positive endothelial cells, and 5.1% remained unidentified).

Objectives 3.1. and 3.2. Established a method for determining blood-brain barrier permeability using two-photon microscopy and fluorescein as a tracer. Results showed that BBB permeability for Na-fluorescein and Evans blue-albumin did not change in both young and old mice 3 days after EPC injection. Using immunofluorescence and super-resolution microscopy (STED), demonstrated that capillaries formed by integrated EPCs and pre-existing endothelial cells can jointly form the vascular lumen. The presence of tight junctions between the two cells and the existence of a vascular lumen indicate the functionality of these blood vessels.

Additionally, demonstrated that EPCs can be used to express antibodies against amyloid-beta plaque and TDP43 proteins, central in Alzheimer's disease and ALS, respectively.

The most significant result of the research was the demonstration of the integration of endothelial precursor cells into the cerebral microvasculature, which can alleviate the consequences of pathological situations, such as cerebral hypoxia.

IMPACT

By completing the proposed research plan, we have gained a deep understanding of the mechanisms and dynamics of endothelial precursor cell integration into the cerebral microvasculature and the concomitant changes in the structure and function of the barrier. We revealed age-related alterations in these processes. It was shown that senolysis with some substances already approved for clinical use can increase the incorporation of endothelial precursor cells, providing high translational value to our results (the use of these drugs only requires repositioning). We demonstrated the therapeutic effect of EPCs in a model of cerebral hypoxia. All these results indicate that EPCs can be suitable therapeutic agents for future cellular therapies aimed at improving vascular function in the aging process and treating various diseases of the central nervous system.