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Eradicating the Root of Cancer:

- Targeted Therapy of Cancer Stem Cells via Exosomal DNA Delivery-

Background to this Drug Discovery Project and/or Biotechnology (1)

Challenges to Be Addressed

Glioblastoma Multiforme (GBM) is the most aggressive and lethal form of primary brain tumor, affecting approximately 3 out of every 100,000 individuals annually. It is characterized by extremely rapid proliferation, diffuse infiltration into surrounding brain tissue, and profound resistance to existing therapies. Even with maximal surgical resection, radiotherapy, and chemotherapy using the alkylating agent temozolomide (TMZ), the median survival remains only 12 to 15 months, and the 5-year survival rate is less than 10%. Virtually all patients experience recurrence.

One of the major contributors to this dismal prognosis is the intrinsic cellular heterogeneity of GBM, especially the presence of a highly treatment-resistant subpopulation known as **cancer stem cells (CSCs)**, which exhibit the following properties:
Possess stem cell-like features, including self-renewal and multilineage differentiation potential;
Show intrinsic resistance to apoptosis, oxidative stress, and DNA-damaging agents;
Survive standard therapies and drive tumor regrowth, invasion, and metastasis.

Moreover, CSCs contribute to shaping the tumor microenvironment, evading immune surveillance, and promoting angiogenesis—further exacerbating treatment resistance.

In addition, the delivery of therapeutics to the brain is severely hindered by the **blood–brain barrier (BBB)**, a highly selective physiological barrier that blocks the passage of most macromolecules and therapeutic agents, particularly nucleic acid-based and biologic drugs.

Given these challenges, **there remains a critical unmet medical need** for:
Therapeutic approaches that can selectively and effectively eliminate CSCs;
Practical drug delivery systems (DDS) capable of crossing the BBB and safely delivering CSC-targeting agents to tumor sites in the brain.

Unless we overcome both of these challenges—targeting CSCs and penetrating the BBB—significant improvement in GBM prognosis will remain elusive, and the vicious cycle of **treatment** → **recurrence** → **progression** will persist.

Background to this Drug Discovery Project and/or Biotechnology (2)

NANOG – A Master Regulator of Cancer Stemness in Glioblastoma

Recent studies, including a substantial body of evidence from our group, have established that **NANOG**, a master transcription factor essential for maintaining pluripotency in embryonic stem cells, also plays a pivotal role in regulating cancer stemness in **glioblastoma (GBM)** and promotes the drug resistance of **cancer stem cells (CSCs**) by upregulating their protective mechanisms.

Key Milestones and Supporting Evidence:
2003: We were the first to demonstrate that overexpression of **NANOG** in mesenchymal stem cells induces the expression of **OCT4** and **SOX2**, successfully generating pluripotent-like cells.
2010: Our group reported, for the first time, that **NANOG is highly expressed in GBM-derived cancer stem cells (CSCs)**. This expression was directly linked to stem-like characteristics and tumor-initiating potential.

NANOG regulates key genes and pathways involved in:

- Self-renewal and multilineage differentiation
- Resistance to chemotherapy
- Apoptosis evasion
- Invasion and metastatic behavior

2019: We demonstrated that **exosomal NANOG DNA** can serve as a **non-invasive diagnostic and prognostic biomarker** for multiple cancers, including GBM.
2023: We further showed that **NANOG knockdown** in GBM CSCs significantly **reduced chemoresistance**, **enhanced apoptotic cell death**, and improved therapeutic response to **temozolomide (TMZ)**.

Clinical Significance:
NANOG is critically involved in modulating several resistance pathways, including:

- **MGMT expression**, a key determinant of TMZ sensitivity
- **PI3K/AKT** and **STAT3** signaling, which support CSC survival and proliferation
- **ABCB1-mediated drug efflux**, reducing intracellular concentrations of therapeutic agents

These findings strongly position **NANOG** as a highly selective and actionable therapeutic target for overcoming treatment resistance and preventing recurrence in glioblastoma. Targeting NANOG may offer a novel and effective strategy in combating this highly aggressive brain tumor.

Breaking Through Biological Barriers: The Rationale Behind Exosome-Based Drug Delivery Systems

Even with promising molecular targets like **NANOG**, effective delivery to the brain remains the greatest bottleneck in glioblastoma (GBM) therapy. Key challenges that must be addressed include:
The Blood–Brain Barrier (BBB) severely limits the brain penetration of most therapeutic agents, especially nucleic acid-based drugs such as DNA and RNA, which cannot cross the BBB unaided.
Viral vectors, while widely used, cannot naturally traverse the BBB and pose serious concerns such as low delivery efficiency, toxicity, and immunogenicity. Their systemic use for CNS disorders like GBM remains controversial.
Lipid nanoparticles (LNPs) also struggle to pass the BBB and tend to accumulate non-specifically in the liver and spleen. Furthermore, they exhibit immunostimulatory effects and limited endosomal escape capacity.

Our solution: Exosome-based DDS (Drug Delivery System)
Exosomes are natural cell-derived nanovesicles (30–150 nm) with high biocompatibility and low immunogenicity. They can intrinsically cross the BBB and transport biologically active molecules such as RNA, DNA, and proteins. We have established the following proprietary technologies to enhance their therapeutic potential:

- **Endogenous loading of nucleic acid therapeutics** such as shRNA and CRISPR/Cas constructs during exosome biogenesis in producer cells.
- **Exosomal Localization Signals (ELS):** In 2023, we identified and patented a short peptide motif that directs therapeutic nucleic acids to be efficiently loaded into the exosomal lumen.
- **Membrane engineering with targeting peptides:** We developed methods to genetically modify donor cells to express targeting peptides on the exosome surface for selective delivery to target tissues.
- **Brain Homing Peptides (BHPs):** In 2025, we demonstrated that BHPs expressed on exosome membranes enable highly efficient delivery to the brain after peripheral administration.

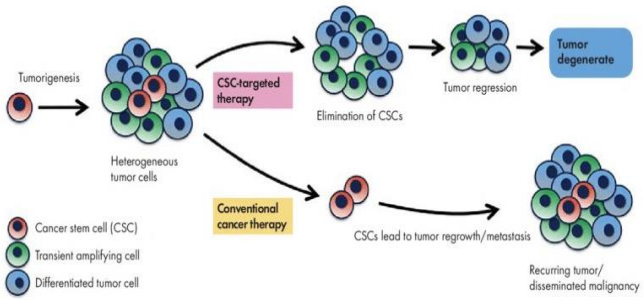
Therapeutic Advantages:

- Precision targeting of GBM tumor tissues
- Non-viral, low-toxicity gene delivery modality
- Compatible with intravenous, subcutaneous, and intranasal routes of administration

Our exosome DDS technology overcomes the dual challenge of targeting **cancer stemness (via NANOG inhibition)** and **crossing the BBB**, offering a transformative new therapeutic paradigm for glioblastoma.

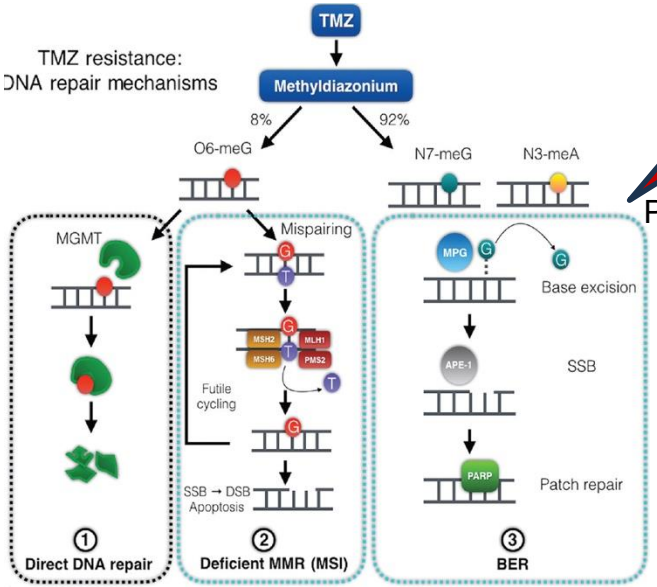
Summary of this Drug Discovery Project and/or Biotechnology (1)

Conventional therapy cannot eliminate cancer stem cells (CSCs), which become seeds to produce another cancer.

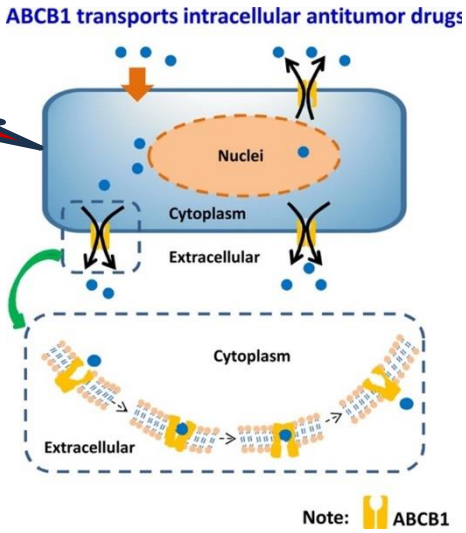


Tumorigenesis initiates with CSCs, which generate a heterogeneous tumor cells. CSC-targeted therapy eliminates CSCs, leading to tumor regression and degeneration. In contrast, conventional cancer therapy primarily eliminates non-CSCs while sparing CSCs, which survive to drive tumor regrowth, metastasis, and recurrence. This highlights the critical importance of targeting CSCs for durable therapeutic outcomes.

NANOG, which promotes drug resistance CSCs, represents a critical therapeutic target for effective cancer therapy.



NANOG is not expressed in normal adult cells but is upregulated in CSCs, where it enhances drug resistance by activating protective mechanisms. Its selective suppression can boost chemotherapy efficacy without off-target side effects.



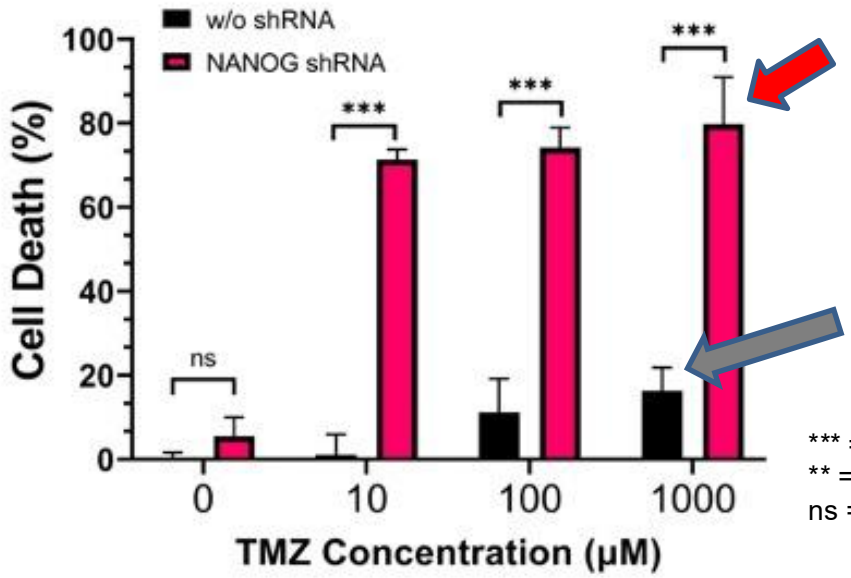
•**NANOG**, originally known as a gene specific to embryonic stem cells, was discovered by our group to be specifically expressed in **cancer stem cells (CSCs)** of glioblastoma. *(Clin Neurosurg. 2010; 57:151–159)*

•We identified that **distinct DNA sequences within exosomes** can serve as novel biomarkers for CSCs. *(PLOS ONE. 2020; 13(5): e0197782, patent pending)*

Summary of this Drug Discovery Project and/or Biotechnology (2)

NANOG Inhibition Overcomes Chemoresistance in Glioblastoma CSCs

Treatment of CD133+ cancer stem cells (CSCs) derived from GBM tumor tissue with temozolomide (TMZ) alone induced cell death in a dose-dependent manner; however, even at 1 mM, less than 20% of cells were eliminated. In contrast, co-treatment with shRNA targeting NANOG markedly enhanced TMZ-induced cell death, indicating that NANOG suppression increases the chemosensitivity of GBM CSCs.



A combination therapy of exosomal delivery of nucleic medicine reducing the drug resistance mechanisms of GBM and TMZ killed almost 100% of cancer stem cells with the TMZ treatment.

Conventional therapy, Temozolomide (TMZ) can only kill 25% of cancer stem cells, even at a very high (1mM) dose.

*** = $p < 0.001$
** = $p < 0.01$
ns = not significant

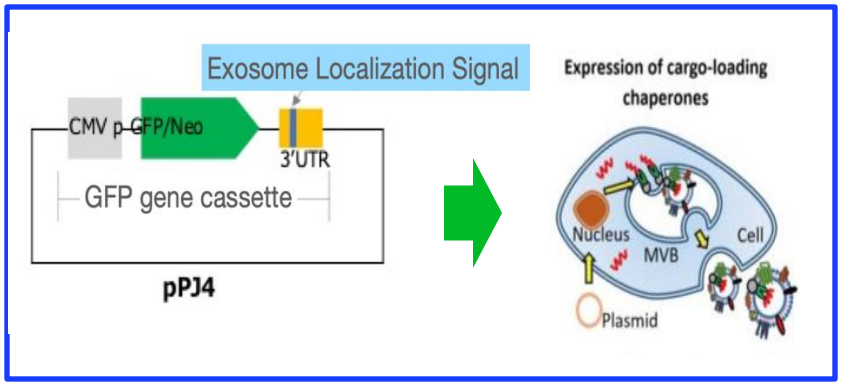
This exosome-based NANOG knockdown strategy significantly improves response to TMZ and may help overcome recurrence in GBM.

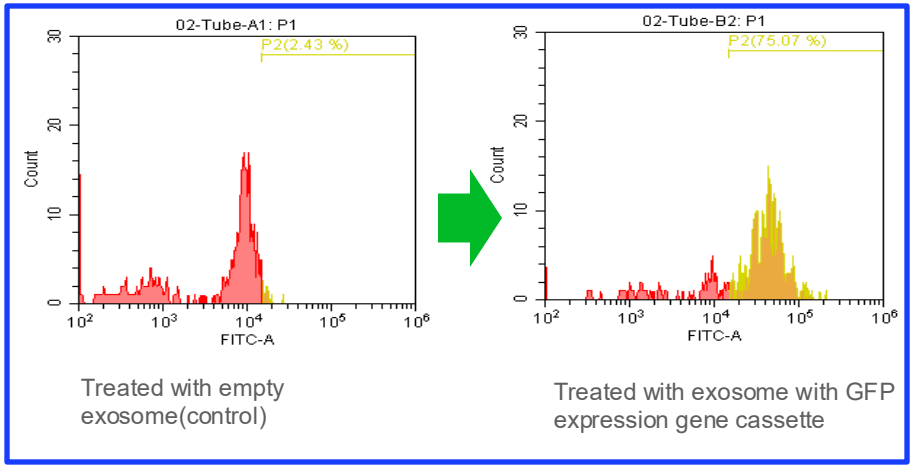
Suppression of NANOG Expression Reduces Drug Resistance of Cancer Stem Cells in Glioblastoma. *Genes*.; 14(6):1276

Patent: Delivery of gene expression modulating agents for therapy against cancer and viral infection PCT/US2021/021674; filed March 10, 2021, Claims priority to USAN 62/987,483; filed 3/10/2020

Breakthrough in Exosome Engineering: ELS Enables DNA Loading During Production

Key Advantages:
Non-invasive loading: Avoids physical or chemical transfection methods such as electroporation or lipofection
Scalable integration: Seamlessly compatible with high-throughput exosome manufacturing systems
Therapeutic precision: Supports targeted, efficient delivery of nucleic acid-based drugs





This slide highlights our proprietary **Exosomal Localization Signal (ELS)**, a unique 3'-UTR sequence that enables efficient packaging of plasmid DNA into exosomes during endogenous production by host cells. The schematic on the left demonstrates the ELS inserted downstream of a GFP expression cassette, facilitating exosome loading without the need for physical or chemical transfection methods.

The flow cytometry data on the right confirms successful delivery: exosomes carrying the ELS-GFP cassette induce GFP expression in recipient cells, whereas control exosomes do not. This represents a **novel, non-invasive platform for nucleic acid drug delivery** using engineered exosomes.

Patent: JP5220103834 A novel method to load the desired nucleic acid into exosomes as a nucleic acid drug DDS (3/15/2022)

This study was supported by Kobe Life Science Gap Fund ($50K).

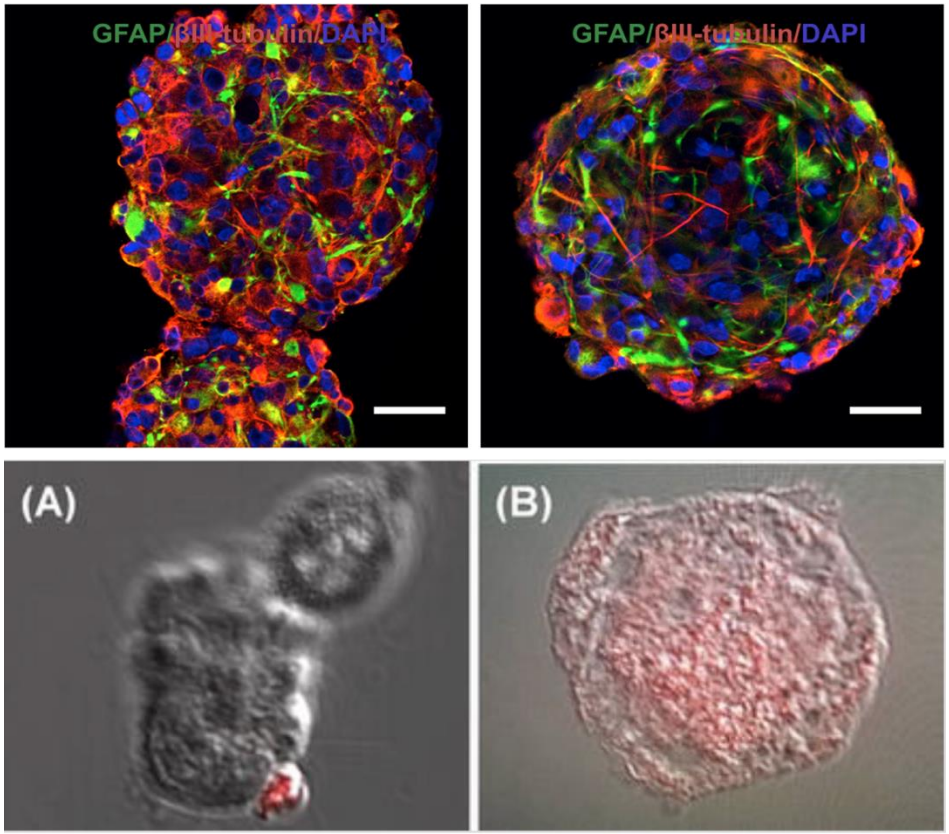
3′-UTR Sequence of Exosomal NANOGP8 DNA as an Extracellular Vesicle-Localization Signal. *Int. J. Mol. Sci.* **2024**, *25*, 7294.

Summary of this Drug Discovery Project and/or Biotechnology (4)

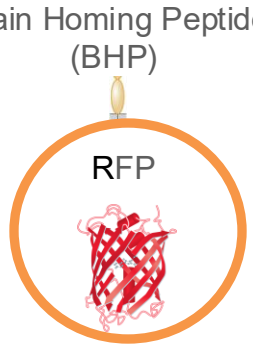
Modified exosomes facilitate efficient RFP protein transfer to human mini-brain tissue

This slide demonstrates our successful delivery of a fluorescent model protein, **RFP (Red Fluorescent Protein)**, to **human brain organoids (mini-brains)** using **exosomes engineered** to encapsulate RFP and display **Brain Homing Peptide (BHP)** on their surface.

Neural cells are among the most difficult cell types to transfect using conventional non-viral methods. Our exosome-based system bypasses this limitation by enabling **non-viral, targeted protein delivery** to brain tissues, offering a safer and more scalable alternative to viral vectors.



Human brain organoids derived from induced pluripotent stem cells immunostained with:
GFAP (Green, astrocytes)
βIII-tubulin (Red, neurons)
DAPI (Blue, nuclei).

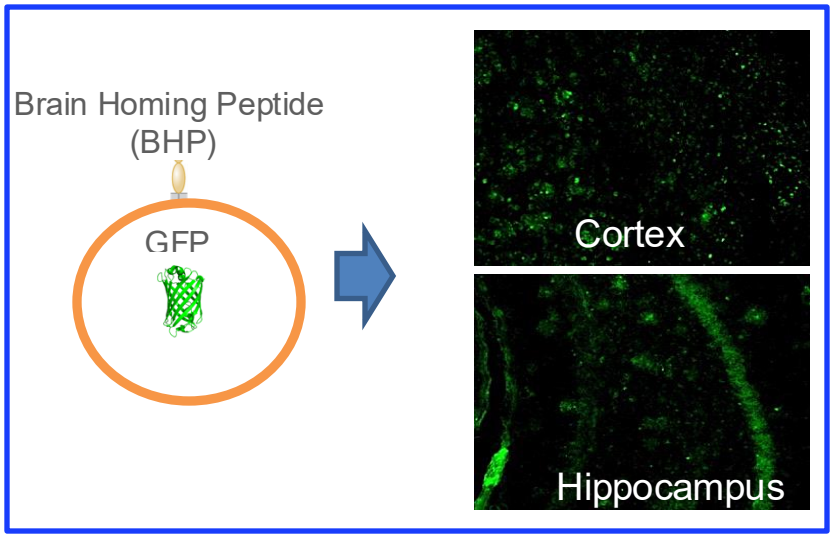


Key Highlights:

- **Proof-of-concept** for therapeutic protein delivery to brain tissue without invasive methods.
- **Modification** of exosome surface enables **target specificity and enhanced uptake.**
- Paves the way for **non-viral delivery of** therapeutic proteins, enzymes, or gene editors (e.g., CRISPR/Cas9) to the brain.

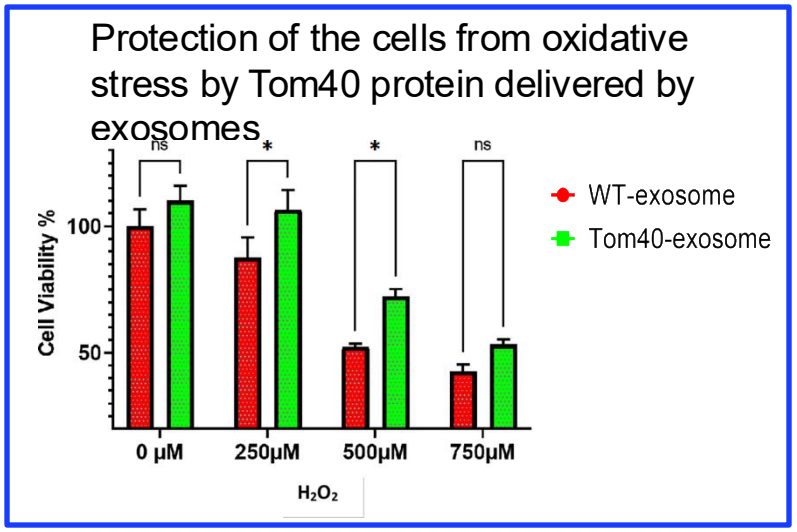
Summary of this Drug Discovery Project and/or Biotechnology (5)

Engineered Exosomes Enable Targeted Brain Delivery of Functional Cargo



Systemically Administered Exosomes Efficiently Cross the BBB and Deliver Cargo to the Brain

Exosomes modified with a brain-homing peptide (BHP) and loaded with GFP were administered to mice via subcutaneous injection. In the **top panel**, GFP expression was detected in neural cells surrounding the **ventral posteromedial thalamus**. In the **bottom panel**, strong GFP signals were observed in **hippocampal pyramidal cells** and adjacent regions. These results demonstrate that peripherally administered, BHP-modified exosomes can efficiently cross the **blood-brain barrier (BBB)** and deliver molecular cargo to central nervous system (CNS) tissues—highlighting their promise as a non-invasive platform for targeted **therapeutic delivery to the brain**.



Tom40-Exosomes Protect HEK293 Cells from H_2O_2-Induced Oxidative Stress

HEK293 cells were pretreated with Tom40-loaded exosomes for 16 hours, followed by exposure to hydrogen peroxide (H_2O_2) for 4 hours. Cell viability was assessed using the MTT assay, which measures mitochondrial metabolic activity. Data represent mean ± SEM from four independent replicates. Statistical significance was determined by two-way ANOVA, with correction for multiple comparisons using the false discovery rate (FDR) method (*P < 0.05).

Sayeed N, Sugaya K. (2022) Exosome-mediated Tom40 delivery protects against hydrogen peroxide-induced oxidative stress by regulating mitochondrial function. PLoS One. ;17(8):e0272511. doi: 10.1371/journal.pone.0272511.

Smith, J., Field, M., & Sugaya, K. (2025). Brain-Homing Peptide Expression on the Membrane Enhances the Delivery of Exosomes to Neural Cells and Tissue. *Neuroglia*, *6*(1), 3.

Advantage of this Project and/or Biotechnology (1)

Non-Viral, Targeted Delivery of Therapeutic Biomolecules Across the Blood-Brain Barrier

This biotechnology platform utilizes engineered exosomes modified with a brain-homing peptide (BHP) to achieve efficient, non-invasive delivery of functional proteins, nucleic acids, or small molecules directly to the central nervous system. Unlike viral vectors or conventional transfection methods, which pose safety risks or show poor transfection efficiency in neural cells, this approach offers:

- **High biocompatibility and low immunogenicity**
- **Ability to cross the blood-brain barrier (BBB) after peripheral administration**
- **Efficient delivery to hard-to-transfect neural tissues**
- **Scalability for clinical translation using a cell-free delivery system**

This exosome-based strategy opens new avenues for treating neurological disorders such as Alzheimer's disease, glioblastoma, and Parkinson's disease, while minimizing off-target effects and invasive procedures.

Proprietary Platform with Strong IP Protection and Scalable Manufacturing

This project is supported by a robust intellectual property portfolio, including a granted Japanese patent covering the Exosomal Localization Signal (ELS) for efficient nucleic acid loading, as well as additional filings for brain-homing exosome surface modifications. Notably, two key U.S. patents/applications further strengthen the platform's translational value:

US11,193,174 B2 covers the use of exosomal NANOG DNA as a **non-invasive biomarker** for cancer diagnosis and prognosis. It can be used to **monitor disease progression and treatment response** in clinical trials, offering a valuable companion diagnostic for exosome-based therapies.

US20230212566A1describes a method to load gene-modulating agents (e.g., shRNA, mRNA, CRISPR/Cas components) into exosomes for **targeted therapeutic delivery**. In the context of this project, it enables the development of **GBM** and potentially other cancers or infectious diseases.

These proprietary technologies provide a **dual advantage**: enabling precise diagnostics and enabling functional, tissue-targeted therapy. Furthermore, the system is designed for seamless integration into scalable, cell-based exosome production workflows, ensuring batch-controlled, GMP-compliant manufacturing.

Because the platform avoids harsh methods such as electroporation or lipofection, it preserves exosome integrity, improves consistency, and enhances safety—making it suitable for clinical application.

This combination of **freedom to operate**, **diagnostic-therapeutic synergy**, and **manufacturing scalability** positions the technology for rapid clinical translation and strategic partnerships.

Advantage of this Project and/or Biotechnology (2)

Addresses a Critical Unmet Need in Glioblastoma Treatment

This project offers a transformative, non-viral delivery approach using **engineered exosomes** capable of crossing the **blood-brain barrier (BBB)** and delivering therapeutic cargo—such as **functional proteins, shRNA, or chemotherapeutic agents**—directly to **glioblastoma (GBM)** cells, including drug-resistant cancer stem cells. **Brain-homing peptide** modifications enhance tumor specificity, while the **non-immunogenic, cell-derived nature** of exosomes minimizes systemic toxicity. This platform addresses one of the most pressing challenges in neuro-oncology: the **precise, non-invasive delivery of therapeutics to intracranial tumors**.

Importantly, although originally developed for GBM, the **modular architecture** and **biological compatibility** of this platform make it highly adaptable to a broad spectrum of cancers and other intractable diseases—especially those where conventional delivery methods are hindered by biological barriers, poor targeting, or safety limitations.

In **oncology**, exosomes can be surface-modified with **tumor-targeting peptides or antibodies**, and loaded with therapeutic RNA, proteins, or drugs, enabling targeted treatment of cancers such as **breast, lung, pancreatic, ovarian, and liver cancer**, as well as **metastatic and drug-resistant tumors**.

In **neurodegenerative diseases**, exosomes that cross the BBB can deliver therapeutic payloads to the CNS. This includes **gene-silencing strategies** such as shRNA or CRISPR to suppress mutant genes (e.g., **HTT in Huntington's disease**) and delivery of neuroprotective proteins for **Alzheimer's and Parkinson's disease**.

In the context of **infectious diseases**, exosome delivery enables targeted application of **CRISPR/Cas or shRNA constructs** to eliminate persistent or hard-to-reach viral reservoirs, offering a novel therapeutic strategy for infections like **HIV, COVID-19, and hepatitis**.

Strategic Collaborations for Scalable, Clinical-Grade Manufacturing

A key advantage of this project is the proactive establishment of industry collaborations to support **clinical translation and commercial scalability**. We are already working with a **materials science company** to co-develop an **automated exosome production system** optimized for **high-throughput, closed-loop biomanufacturing**. This system is designed to ensure batch consistency, sterility, and compliance with GMP standards—critical for therapeutic applications.

In parallel, we are partnering with a **bioprocess media company** to create a **serum-free, xeno-free culture medium** specifically tailored for exosome production intended for **clinical use**. This will eliminate the variability and safety concerns associated with animal-derived components, supporting regulatory approval and reproducibility.

These collaborations position the project for **rapid scale-up**, **regulatory alignment**, and **efficient clinical deployment**, setting it apart from early-stage academic platforms that lack translational infrastructure.

Reference and/or Patent(s)

1) Potential target disease and/or therapeutics area on this proposal

The primary target of this proposal is GBM, highly malignant brain tumor characterized by treatment resistance and recurrence driven by CSCs. Existing therapies are largely ineffective at eliminating CSCs, making meaningful improvement in patient outcomes extremely challenging.Beyond GBM, this technology is also expected to be applicable to other CSC-driven cancers with high NANOG expression, including hepatocellular carcinoma, ovarian cancer, breast cancer, and colorectal cancer.

2) Key paper and/or

- Field M, Alvarez AA, Bushnev S, and Sugaya K*,(2010) Embryonic Stem Cell Markers Distinguishing Cancer Stem Cells From Normal Human Neuronal Stem Cell Populations in Malignant Glioma Patients Clinical Neurosurgery, Volume 57, 151-159
- Alvarez AA, Field M, Bushnev S, Longo MS, Sugaya K*. (2015) The effects of histone deacetylase inhibitors on glioblastoma-derived stem cells. J Mol Neurosci. 55(1):7-20
- Vida M, Bacchus M and Sugaya K*. (2019) Differential sequences of exosomal NANOG DNA as a potential diagnostic cancer marker. PLOS ONE13(5): e0197782. https://doi.org/10.1371/journal.pone.0197782
- Vaidya, M.; Sugaya, K.*. (2020) Differential sequences and single nucleotide polymorphism of exosomal SOX2 DNA in cancer PLoS ONE 15(2): e0229309. https://doi.org/10.1371/journal.pone.0229309
- Sayeed N, Sugaya K. (2022) Exosome-mediated Tom40 delivery protects against hydrogen peroxide-induced oxidative stress by regulating mitochondrial function. PLoS One. ;17(8):e0272511. doi: 10.1371/journal.pone.0272511.
- Vaidya M, Smith J, Field M, Sugaya K. (2023)Analysis of regulatory sequences in exosomal DNA of NANOGP8. PLoS One. 25;18(1):e0280959. doi: 10.1371/journal.pone.0280959. PMID: 36696426; PMCID: PMC9876286.
- Smith J, Field M, Sugaya K. (2023) Suppression of NANOG Expression Reduces Drug Resistance of Cancer Stem Cells in Glioblastoma. *Genes*.; 14(6):1276. https://doi.org/10.3390/genes14061276
- Vaidya M, Kimura A, Bajaj A, Sugaya K.(2024) 3'-UTR Sequence of Exosomal NANOGP8 DNA as an Extracellular Vesicle-Localization Signal. Int J Mol Sci. Jul 2;25(13):7294. doi: 10.3390/ijms25137294. PMID: 39000405; PMCID: PMC11242200.
- Smith, J., Field, M., & Sugaya, K. (2025). Brain-Homing Peptide Expression on the Membrane Enhances the Delivery of Exosomes to Neural Cells and Tissue. *Neuroglia*, *6*(1), 3. https://doi.org/10.3390/neuroglia6010003

3) Patent and its status

- Sugaya K, et al. **Exosomal NANOG DNA as a diagnostic cancer marker.** United States patent US11,193,174 B2. Issued December 7, 2021.
- JP5220103834 目的とする核酸を搭載した、核酸医薬品としてのエクソソームの新規製造方法 filed 3/15/2022
- Sugaya K, et al. **Delivery of gene expression modulating agents for therapy against cancer and viral infection.** United States patent application US20230212566A1. published July 6, 2023.