Aim 1: Development and Characterization of Exosome-based Therapeutic for PARPi and CSF-1R Inhibitors.

Sub-aims:

1.1. Generate exosomes from iPSC-MSCs and load them with PARPi cargo.

1.2. Conjugate the surface of the exosomes with CSF-1R inhibitors and markers specific to TAMs (CD68, CD163) and cancer cells (MM2, EpCam, HER2, CA125).

Based on the research findings highlighted by La Greca et al. [1], the proteomic composition of extracellular vesicles (EVs) derived from induced pluripotent stem cells (iPSCs), iPSC-derived mesenchymal stem cells (iPSC-MSCs), and conventional mesenchymal stem cells (MSCs) varies significantly. This suggests a nuanced evolution of protein content as iPSCs transition into iPSC-MSCs, with the resulting EVs exhibiting a distinct proteomic signature that is more specific and likely reflects their specific functions within the stem cell microenvironment. This includes roles in supporting stem cell maintenance, facilitating differentiation, and mediating intercellular communication within tissues.

Given these insights, our approach will involve harnessing iPSC-MSCs to generate EVs for therapeutic purposes. To meet the demands for high yield and potency necessary for clinical applications, we will employ innovative culture strategies, particularly bioreactors, which offer continuous culture capabilities and enable real-time monitoring of crucial parameters such as oxygen levels and pH. Recent work by Cao et al. [2] has demonstrated that EVs derived from 2D cultures and hollow fiber bioreactor (HFB)-cultured MSCs exhibit comparable surface marker profiles, size, and morphology, with the latter yielding up to a 19.4-fold increase in production.

For the culture of EVs, our methodology will involve utilizing a bioreactor system with a 48-hour harvest interval supplemented with human platelet lysate (HPL) as a culture medium. HPL not only supports xeno-free MSC culture, aligning with clinical trial requirements, but also enhances translational potential. It's worth noting that while HPL contains exogenous serum derived EVs along with other nanoparticles such as growth factors and protein aggregates, it still represents a superior serum alternative within this context.

After the EVs have been released into the culture medium, for EV isolation, we will use a microfluidic system which can isolate exosomes with high purity, minimizing contamination form other extracellular vesicles or protein aggregates. The process is more efficient and requires less time than ultracentrifugation techniques (gold standard), it can be scaled up and the same system can be used for exosome modifications.

Using Western Blot, Elisa, or Sem analysis, we will then proceed to the characterization of the MSCs assessing the assessment of protein markers, including CD9, CD63, CD81, CD59, as well as cytosolic proteins such as ALIX, TSG101, and Hsp70/90 [3] (Fig. 1 and 2).

A comparison of a normal and a normal event

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**Figure 1.** Flow cytometry analysis of the positive marker at the surface of the

exosomes – Ref: [4]

A close-up of a grey surface

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**Figure 2.** SEM image of exosome with 500 and 200 nm scale bars – Ref: [4]

To enhance the specificity of iPSC-MSC-derived exosomes for targeting tumor-associated macrophages (TAMs) and cancer cells, we must delicately balance the need for efficient TAM targeting with the imperative to evade uptake by macrophages and leukocytes in the Mononuclear Phagocyte System (MPS) organs. This optimization is critical to ensure that the engineered exosomes maintain sufficient circulation time to effectively reach and target tumors.

Given the limitations associated with PEGylation, we are exploring alternative strategies such as "Self" peptide conjugation. This approach involves modifying the exosome surface with self-peptides that mimic endogenous proteins, potentially reducing recognition by MPS cells. A study by Pial et al. [5], demonstrated an inverse correlation between nanobead uptake by the immune system and in vivo persistence. This indicated that “self” CD47 nanobeads had longer bloodstream circulation and likewise, our engineered exosomes, designed to minimize immune cell uptake, will be more likely to evade the MPS and reach their target tissue (Fig. 3 and 4).

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| A screenshot of a computer screen  Description automatically generated  **Figure 3** - Binding of hCD47 and Self peptide increases phosphor-SIRP.  Ref: [5] | A graph of a graph showing the results of a nanobeans  Description automatically generated with medium confidence **Figure 4 -** Inverse correlation between in vivo persistence ratio and in vitro inhibition of phagocytosis by hCD47 and Self peptide at 45 min – Ref: [5] |

The next steps in our research will involve:

* **Combining CD47 expression:** and incorporating TAM-specific ligands, such as antibodies against TAM markers (e.g., CD68, CD206, CD163), on the exosome surface along with CD47 expression. This dual targeting approach enables selective TAM targeting despite CD-47-mediated immune evasion.
* **Conjugating the exosome surface with CSF-1R inhibitors** to enrich the M1 population of TAMs and modulate TAM recruitment and distribution However, the largest macrophage polarization effects have been observed for agonists of the toll-like receptors 7 and 8 (TLR7/8) [6], and we may want instead to investigate these therapeutics. These small molecules can be modified for conjugation with exosomes, enhancing their antitumor efficacy.
* **Fine-tuning CD47 Expression Levels**: on exosomes to balance immune evasion with TAM targeting. This optimization can enhance exosome biodistribution and maximize TAM targeting.
* **Integrating a pH-Sensitive components:**

Like in the design of a micelle for drug delivery by Pia et al. [7], we will modify the PARPi cargo to include a functional group compatible with click chemistry; and attach it via a peptide linker to matrix metalloproteinase-2 (MMP-2), a protein only found in tumors. This will allow selective release of the PARPi cargo near the cancer cells.

Aim 2: In Vitro Evaluation of Therapeutic Efficacy and Specificity

Sub-aims:

2.1. Assess the cytotoxic effects of the engineered exosomes on a panel of cancer cell lines in vitro.

2.2. Evaluate the recruitment and activation of immune cells by treated cancer cells, focusing on CD4+ and CD8+ T cells and the impact on TAMs.

We will prime with a variety of human cancer cell lines, including A549 lung cancer, SK-OV-3 ovarian cancer, and MDA-hyb1 breast cancer cells, with our engineered exosomes treated with sub-lethal doses PARPi for 24 hours. Using LC-MS/MS, we will quantify the amount of PARPi delivered to the cancer cells by the MSC-derived exosomes. This measurement will be compared to the expected standard dosage of PARPi, as demonstrated in previous research by Merlzer et al [8]. This analysis will provide insights into the efficacy of our engineered exosomes as PARPi delivery vehicles across different cancer cell lines (Fig. 5).

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Figure 5 – Ref: [8]

1. Characterization of the four different MSC investigated populations.
2. Kinetic of exosomes production by MSCGFP,

progressive exosome release increase to reach a plateau after 24h.

1. Quantification of exosome produced per cell within a specific timeframe.
2. Apoptotic/necroptotic subG1 phase cells remained at equally low levels in control and Taxol-treated MSC populations, confirming no detectable cytotoxic effects.

Based on study from [9], hematolymphoid humanized mouse models are the most promising animal models to test the antitumor effects of checkpoints blockers and immunotherapy strategies. If needed to align our therapeutic design with the unique characteristics of a cancer tissue, we may opt for different murine models, such as a GEMM, including the KPC (KrasLSL-G12D/+; Trp53R172H/+; Pdx-1-Cre) model for pancreatic cancer or the APC (adenomatous polyposis coli) model for colorectal cancer.

After injecting

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