Molecular Biology and Genetics: Theory, Methodology and Practice

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Prof. Fuat Bozok, Ph.D.

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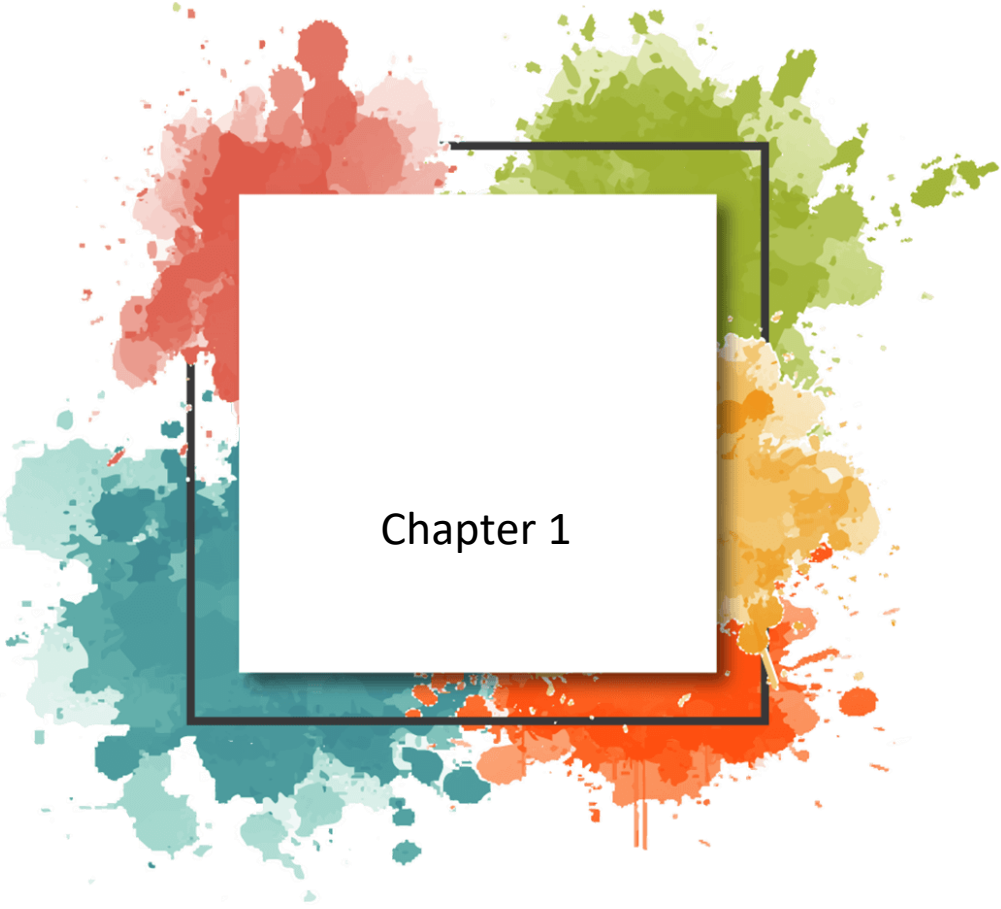
Chapter 1

Extracellular Vesicles as Therapeutic Carriers: Current Advances, Challenges, and Future Perspectives

***Hatice Esenkaya*[1,2]**

1. Introduction

Extracellular vesicles (EVs) have emerged as pivotal players in a rapidly evolving field at the intersection of cell biology and therapeutic innovation. No longer viewed as mere cellular debris, EVs are now understood to be key mediators of intercellular communication, influencing a spectrum of physiological and pathological processes across virtually all biological systems (Hanayama, 2021). Their discovery in diverse biofluids and tissue environments has opened new avenues for diagnostics, disease monitoring, and, increasingly, therapeutic intervention (Aloi et al., 2024).

What makes EVs particularly compelling is their natural biological function and technological potential. Unlike conventional drug delivery systems, EVs are inherently equipped for targeted, efficient, and biocompatible transport of molecular cargo. These qualities, however, are only part of the story. Rapid advancements in molecular profiling, vesicle engineering, and computational modelling have elevated EVs from passive biomarkers to active components in the development of biologically inspired therapies (Du et al., 2023).

Recent research has also begun to explore the synergies between EVs and emerging technologies such as CRISPR-based genome editing, RNA therapeutics, and machine learning–guided design. These integrations suggest a future in which EVs may serve as personalised delivery vectors, tailored to individual patients and disease states (Ghosal et al., 2025).

This chapter examines the transformative potential of EVs in therapeutic development. It provides a critical analysis of their engineering and cargo dynamics, evaluates their functional applications across disease models, and explores the translational hurdles that must be overcome to realise their clinical

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promise. In doing so, we aim to position EVs not simply as delivery tools, but as foundational elements of a new class of precision therapeutics.

2. Biogenesis and Classification of Extracellular Vesicles

EVs are broadly categorised into three principal subtypes: exosomes, microvesicles, and apoptotic bodies, based on their size, biogenesis pathways, and molecular cargo (Akers, Gonda, Kim, Carter, & Chen, 2013). While all EVs share a common role in facilitating intercellular communication, their distinct origins endow them with specific structural and functional attributes, influencing both their physiological relevance and therapeutic utility (Wessler & Meisner-Kober, 2025).

2.1 Exosomes (30–100 nm)

Exosomes are the smallest and most well-characterised class of EVs **(Figure 1B)**. They originate from the endosomal system through a multistep process involving the inward budding of the limiting membrane of early endosomes, leading to the formation of intraluminal vesicles (ILVs) within multivesicular bodies (MVBs). Upon fusion of MVBs with the plasma membrane, ILVs are released into the extracellular milieu as exosomes **(Figure 1A)** (Hushmandi et al., 2024).

This biogenesis is tightly regulated by the endosomal sorting complex required for transport (ESCRT) machinery, comprising four major protein complexes (ESCRT-0, -I, -II, and -III) and associated accessory proteins like ALIX and VPS4 (Schuh & Audhya, 2014). These complexes coordinate the sorting of ubiquitinated proteins into ILVs and membrane scission events (Shields & Piper, 2011). In parallel, ESCRT-independent pathways involving tetraspanins (CD9, CD63, CD81, etc.) and lipid raft microdomains also contribute to exosome formation, underscoring the complexity and redundancy of this process(H. Wei et al., 2020).

The molecular composition of exosomes reflects their endosomal origin, featuring a conserved set of markers including TSG101, ALIX, and heat shock proteins (HSP70, HSP90), in addition to the aforementioned tetraspanins(Yi et al., 2022). Their cargo includes a diverse repertoire of mRNAs, miRNAs, long non-coding RNAs, lipids, and proteins, often selectively packaged depending on cell type and physiological state **(Figure 1A,B)**. Functionally, exosomes are involved in processes ranging from antigen presentation and immune modulation to angiogenesis and metastasis(Lee, Shin, & Chae, 2024).

2.2 Microvesicles (100–1000 nm)

Also known as ectosomes, microvesicles are produced by the direct outward budding and fission of the plasma membrane, a process fundamentally distinct

from exosomal biogenesis **(Figure 1A)** (Doyle & Wang, 2019). Their formation is often triggered by changes in intracellular calcium levels, leading to the activation of calpain, disruption of the actin cytoskeleton, and redistribution of phospholipids (such as phosphatidylserine) across the membrane bilayer(Taylor, Azimi, Monteith, & Bebawy, 2020). This budding process is regulated by small GTPases such as ARF6, and kinases like ROCK, which coordinate cytoskeletal rearrangements necessary for membrane protrusion and scission(D'Angelo, Stahl, & Raposo, 2025).

Because they bud from the plasma membrane, microvesicles often retain surface proteins from the parent cell, including integrins, selectins, and CD40 ligand, which play roles in cell adhesion, migration, and signalling **(Figure 1A,B)** (Yang, Zou, Jose, & Zeng, 2021). Their cargo is highly variable and can overlap with that of exosomes; however, microvesicles tend to be enriched in cytosolic proteins and certain types of RNAs. Importantly, their larger size and dynamic composition make them potent modulators of inflammation, thrombosis, and tumour progression(Ratajczak & Ratajczak, 2020).

2.3 Apoptotic Bodies (1000–2000 nm)

Apoptotic bodies are the largest type of EVs, formed during the late stages of programmed cell death (apoptosis) **(Figure 1A)** (Shi, Phan, & Poon, 2025). As cells undergo fragmentation, membrane blebbing leads to the generation of vesicles containing fragmented nuclear material, organelles, and chromatin. These vesicles are subsequently released into the extracellular space as part of the cell clearance process (Atkin-Smith & Poon, 2017).

Unlike exosomes and microvesicles, apoptotic bodies are characterised by the externalisation of phosphatidylserine, which acts as an "eat me" signal to phagocytes, promoting their rapid clearance (Wen, Creaven, Luan, & Wang, 2023a). Historically regarded as non-functional debris, apoptotic bodies are now appreciated for their potential roles in autoimmunity, tolerance, and intercellular transfer of genetic material (Yu et al., 2023). However, their application in therapeutic delivery is still underexplored, partly due to their heterogeneity and the risk of transferring unwanted or immunogenic components (X. Li et al., 2022). That said, engineered apoptotic bodies are being investigated for cancer vaccines and immunotherapy, given their immunomodulatory capacity (Z. Li et al., 2025).

Figure 1

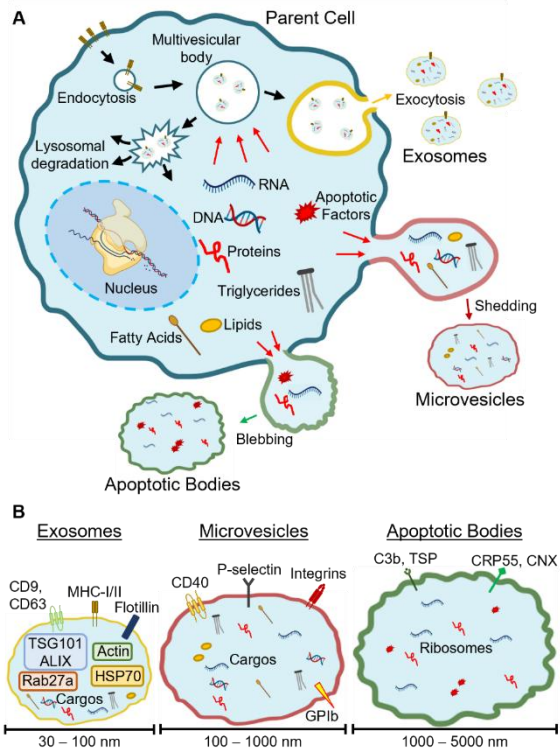

Figure 1 Biogenesis and Cargo Composition of Extracellular Vesicle Subtypes

This schematic illustrates the biogenesis of EV subtypes: exosomes, microvesicles, and apoptotic bodies. Exosomes originate from MVBs and are released via exocytosis, while microvesicles bud directly from the plasma membrane and apoptotic bodies are released during cell death. Each vesicle type carries a range of bioactive cargos, including RNA, DNA, proteins, lipids, and metabolites. The figure also highlights specific markers and structural features associated with each subtype, such as TSG101, Rab27a for exosomes, GPIb for microvesicles, and ribosomal components in apoptotic bodies. These vesicles are key players in intercellular communication and have emerging roles in therapeutic applications. The sizes of each subtype of EVs is shown.

2.4 Functional Implications of Biogenesis Pathways

The distinct cellular origin and biogenetic route of each EV subtype confer specific physical and biochemical properties that directly impact their biological behaviour, therapeutic utility, and pharmacokinetics (Yuchen Li et al., 2020). These features include vesicle size, membrane composition, cargo content, mode of cellular uptake, and downstream signalling capabilities (O'Brien, Ughetto,

Mahjoum, Nair, & Breakefield, 2022). As EV-based strategies advance toward clinical application, understanding these functional consequences has become pivotal for rational vesicle selection, targeting, and engineering.

Exosomes, owing to their endosomal origin, possess a unique surface protein and lipid repertoire enriched in tetraspanins, ceramides, and specific glycoproteins that influence both their circulatory half-life and cellular uptake profile (Kalluri & LeBleu, 2020). Their internalisation by recipient cells primarily occurs through receptor-mediated endocytosis, clathrin-independent pathways, or macropinocytosis. In some contexts, exosomes can directly fuse with the plasma membrane to deliver their cargo into the cytosol (Gonda, Kabagwira, Senthil, & Wall, 2019). The internalisation route affects not only the kinetics of cargo delivery but also the intracellular trafficking and bioavailability of the therapeutic payload. Importantly, the small size of exosomes facilitates their traversal across physiological barriers such as the blood-brain barrier (BBB) and extracellular matrix, making them especially attractive for central nervous system (CNS) applications (Abdelsalam, Ahmed, Osaid, Hamoudi, & Harati, 2023).

In contrast, microvesicles are typically larger and have a more variable composition reflective of the plasma membrane and submembrane cytosolic components of their cell of origin (Ashoub, Salavatipour, Kasgari, Valandani, & Khalilabadi, 2024). These vesicles often express high levels of integrins, selectins, and tissue-specific surface markers, which confer selective adhesion to target tissues (Ratajczak & Ratajczak, 2020). Their uptake mechanisms may involve direct fusion with the plasma membrane, phagocytosis, or lipid raft-mediated endocytosis, depending on both the vesicle composition and the phenotype of the recipient cell. The relatively larger size of microvesicles may restrict their biodistribution to certain tissues, but also allows for higher cargo capacity, which can be leveraged for applications requiring substantial delivery of therapeutic RNA, proteins, or drug molecules (Clancy, Schmidtmann, & D'Souza-Schorey, 2021).

Apoptotic bodies are increasingly recognised for their capacity to engage with phagocytes and antigen-presenting cells via phosphatidylserine exposure and "find me" and "eat me" signals such as CX3CL1 and annexin V (Yu et al., 2023). These interactions are not only critical for maintaining tissue homeostasis and preventing autoimmunity but also offer a potential avenue for designing immunomodulatory therapies, especially in cancer or transplant tolerance contexts. Although less studied than exosomes or microvesicles, emerging research suggests that apoptotic bodies may deliver bioactive nucleic acids and even organelles to recipient cells under certain conditions, providing a largely untapped modality for therapeutic delivery (Battistelli & Falcieri, 2020).

Beyond cellular uptake, the biogenesis of EVs determines their cargo specificity, a factor increasingly appreciated in therapeutic development. The ESCRT machinery, for instance, allows for selective inclusion of proteins, RNAs, and lipids into exosomes, thereby enabling the generation of disease- or stimulus-specific vesicle populations (Gatta & Carlton, 2019). By contrast, microvesicles and apoptotic bodies tend to incorporate cargo via passive mechanisms, such as blebbing or cellular fragmentation, resulting in broader and potentially less predictable molecular profiles. This divergence influences not only therapeutic efficacy but also safety and regulatory considerations, particularly in relation to off-target effects and immunogenicity (Wen, Creaven, Luan, & Wang, 2023b). Another critical implication of EV biogenesis lies in tissue tropism and targeting potential. Surface molecules such as integrins and tetraspanins serve as homing beacons that direct vesicles to specific tissues or cellular microenvironments. For example, exosomes expressing integrin αvβ6 preferentially accumulate in inflamed tissues, while those from brain-derived cells can cross the BBB and deliver therapeutic agents to neurons or glial cells. Engineering the vesicle membrane to express specific ligands, peptides, or antibodies that exploit native targeting pathways is a promising strategy under intense investigation (Dixson, Dawson, Di Vizio, & Weaver, 2023).

Recent technological advances such as single-vesicle RNA sequencing, super-resolution microscopy, flow cytometry-based vesicle sorting, and label-free nanoparticle tracking have revealed that each EV subtype comprises heterogeneous subpopulations with distinct cargo profiles and functional properties (Wang, Huang, Gao, Deng, & Huang, 2024). This complexity challenges the traditional size- and biogenesis-based classification system, suggesting a need for functional taxonomy grounded in molecular identity, biophysical characteristics, and bioactivity. Such refined classification would improve therapeutic design by enabling precise selection of EV subtypes for specific clinical goals.

3. Isolation and Characterisation Techniques

The effective isolation and characterisation of EVs are fundamental prerequisites for their reliable application in both basic research and clinical therapeutics. Given the nanoscale size, heterogeneity, and biological complexity of EVs, there is a critical need for reproducible, scalable, and high-purity techniques that can maintain vesicle integrity while minimising contamination with soluble proteins, lipoproteins, and non-vesicular particles. The selection of isolation and characterisation methods is not only dictated by the biological source and downstream applications but also affects functional readouts, therapeutic efficacy, and regulatory compliance in translational settings.

3.1 Isolation Techniques

The isolation of EVs is typically the first and most crucial step in their analysis and use. Current methods vary widely in their principles, throughput, and yield, with each offering unique advantages and trade-offs:

3.1.1 Differential Ultracentrifugation (DUC):

This method remains the historical and most widely used technique for EV isolation, relying on a series of centrifugation steps at increasing centrifugal forces to remove cells, debris, large vesicles, and finally, small EVs such as exosomes **(Figure 2A)**. Although DUC is capable of processing large sample volumes and does not require specialised reagents, it suffers from several limitations. Among them is the co-isolation of non-vesicular contaminants like protein aggregates, ribonucleoprotein complexes, and lipoproteins. Furthermore, the high-speed spins (>100,000 × g) can induce vesicle aggregation or rupture, thereby compromising functional integrity. Recent efforts have sought to optimise rotor types and pelleting conditions to improve yield and reproducibility (Clos-Sansalvador, Monguió-Tortajada, Roura, Franquesa, & Borràs, 2022).

3.1.2 Density Gradient Centrifugation (DGC):

To address purity concerns in density gradient centrifugation that employs iso-osmotic solutions such as sucrose or iodixanol (OptiPrep) to fractionate vesicles based on their buoyant density rather than size alone **(Figure 2B)**. This approach allows for better separation of EVs from similarly sized non-vesicular components, enhancing purity. However, the method is time-consuming, technically demanding, and generally low-throughput, making it more suitable for analytical purposes than for clinical-scale production (Konoshenko, Lekchnov, Vlassov, & Laktionov, 2018).

3.1.3 Size Exclusion Chromatography (SEC):

SEC has become increasingly favoured for isolating EVs from complex biological fluids, especially plasma and urine **(Figure 2E)**. It works by passing the sample through a porous resin that differentially retains small molecules while allowing larger vesicles to elute earlier. The method maintains vesicle integrity and yields high-purity preparations with minimal loss of biological activity. SEC is especially attractive for clinical-grade EV production due to its scalability, gentle processing conditions, and compatibility with GMP workflows (Walker).

3.1.4 Precipitation-Based Methods:

These commercially available kits, including those using polyethylene glycol (PEG), offer a rapid and user-friendly means to isolate EVs by reducing their solubility and forcing aggregation **(Figure 2D)**. Although attractive for routine

use and small-scale studies, precipitation methods frequently co-precipitate abundant serum proteins (e.g., albumin), immunoglobulins, and polymeric contaminants. This compromises downstream applications such as proteomic analysis or therapeutic use, rendering these approaches less suitable for clinical translation (S. Liu, Yu, Wang, Shen, & Cong, 2020).

3.1.5 Microfluidic Platforms and Immunoaffinity Capture:

Advanced microfluidic devices have revolutionised EV isolation by offering precise, scalable, and automated vesicle capture. Many utilise antibody-functionalised channels or beads targeting EV-specific surface markers (e.g., CD63, CD81) to selectively enrich vesicles of interest **(Figure 2C)**. These platforms offer high specificity and require minimal sample volumes, making them ideal for point-of-care diagnostics and single-vesicle studies. However, their low throughput and dependency on known markers limit their generalisability across EV subtypes (Gao et al., 2023).

Figure 2

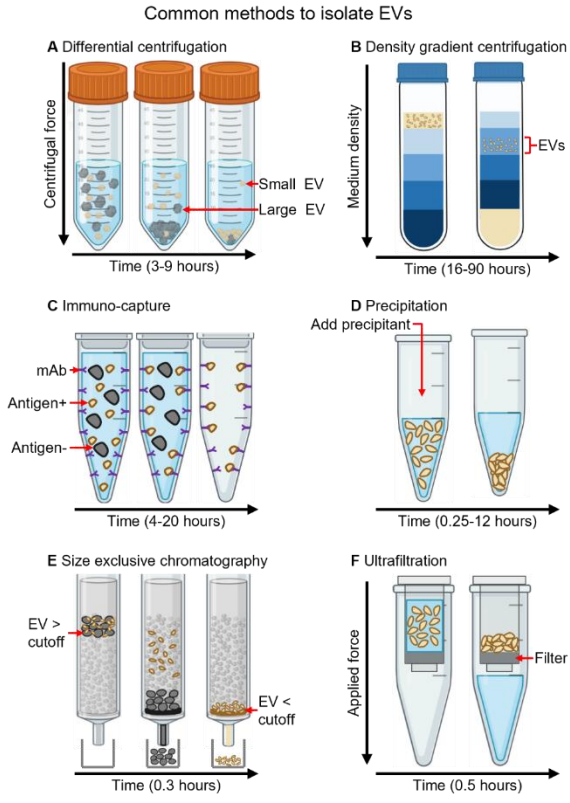

Figure 2 Overview of Cell Separation and Isolation Techniques

This figure illustrates a range of commonly used methods for separating and isolating specific cell populations from complex biological samples. (A) Density gradient centrifugation: Cells are separated based on their density using a medium such as Ficoll or Percoll. After centrifugation, distinct bands corresponding to different cell types can be observed and collected. (B) Differential centrifugation: A sequential centrifugation strategy in which particles are separated according to size and density by increasing centrifugal force. Heavier components sediment first, followed by lighter ones. (C) Magnetic-activated cell sorting (MACS): Cells are labelled with magnetic beads conjugated to specific antibodies. Magnetically labelled cells are retained in a magnetic column, while unlabelled cells are washed away. (D) Filtration-based separation: Cell suspensions are passed through filters or strainers of defined pore sizes to isolate cells based on size exclusion. (E) Immunoaffinity chromatography: Target cells are selectively captured on a column containing antibodies specific to cell surface markers. Unbound cells pass through, while bound cells are later eluted. (F) Column-based filtration (e.g., PluriSelect or other sieve-based methods): Cells are retained or excluded from filter layers or membranes based on size and mechanical properties.

3.1.6 Emerging Technologies:

Recent innovations include tangential flow filtration (TFF) for large-volume processing, acoustic trapping for label-free separation, and magnetic bead-based platforms for scalable immunoisolation. Each of these aims to address the trade-off between purity, scalability, and preservation of bioactivity; key factors in industrial EV manufacturing (Tran et al., 2025).

3.2 Characterisation Techniques

Characterising EVs post-isolation is essential for confirming vesicle identity, assessing sample purity, quantifying particle concentrations, and verifying biological functionality. A combination of orthogonal methods is typically employed to meet the minimal information standards for EV studies (MISEV guidelines).

3.2.1 Nanoparticle Tracking Analysis (NTA):

This technique uses light scattering to track the Brownian motion of individual vesicles in a suspension, providing estimates of particle size distribution and concentration **(Figure 3)**. NTA is widely used due to its accessibility and throughput, although it cannot distinguish between vesicles and similarly sized contaminants, and results can vary depending on sample refractive index and viscosity (Maguire, Rösslein, Wick, & Prina-Mello, 2018).

3.2.2 Transmission Electron Microscopy (TEM):

TEM remains the gold standard for morphological validation, offering nanometer-scale resolution to visualise vesicle shape, size, and bilayer structure **(Figure 3)**. Negative staining or cryo-TEM preparations provide essential visual confirmation of EV identity. However, TEM is low throughput, requires skilled operators, and may introduce artifacts during sample processing.

3.2.3 Western Blot and Flow Cytometry:

These techniques are used to detect canonical EV markers (e.g., CD9, CD63, CD81, TSG101, Alix) and confirm the presence or absence of cellular contaminants (e.g., GM130 for Golgi, calnexin for ER) **(Figure 3)**. Bead-based flow cytometry has improved the sensitivity of detecting vesicle-associated proteins and allows limited multiplexing, though distinguishing EV subtypes remains challenging (Gul, Syed, Khan, Iqbal, & Ahmad, 2022).

3.2.4 Mass Spectrometry (MS):

Proteomic analysis of EV cargo via MS provides deep insights into protein composition, post-translational modifications, and potential functional roles **(Figure 3)**. Label-free or isotopic quantification strategies are often used in comparative studies across disease states, cell types, or bioengineering approaches. However, MS requires extensive sample preparation and purification, especially when working with low-yield fluids like cerebrospinal fluid or urine (Mallia et al., 2020).

3.2.5 RNA Sequencing (RNA-seq):

RNA-seq enables comprehensive profiling of EV-associated small RNAs, mRNAs, and long non-coding RNAs **(Figure 3)**. These signatures not only offer diagnostic potential but also provide insights into vesicle-mediated signalling and regulatory networks. Standardisation of RNA isolation protocols and normalisation strategies remains an active area of development.

Figure 3

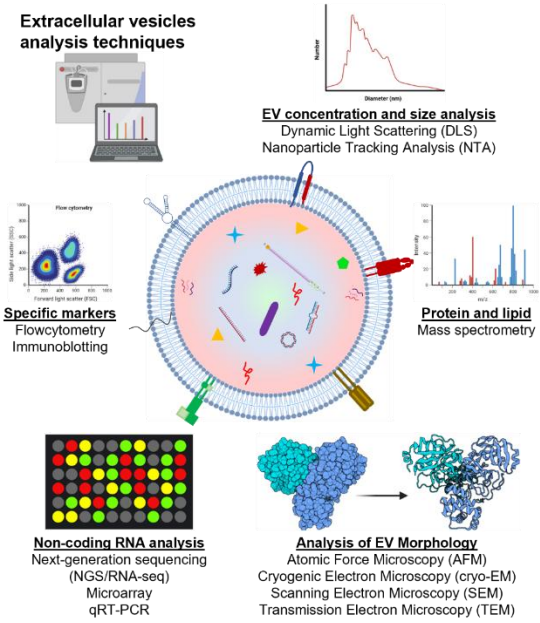

Figure 3 Analytical Techniques for Characterisation of EVs

This figure summarises the major analytical methods used to characterise the physical, molecular, and functional properties of EVs, including exosomes and microvesicles. NTA: Measures the size distribution and concentration of EVs based on Brownian motion and light scattering. Flow cytometry: Enables high-throughput analysis of EV size, surface markers, and population heterogeneity using light scattering and fluorescence signals. Mass spectrometry (MS): Provides detailed proteomic, lipidomic, and metabolomic profiling of EV content, allowing identification of biomarkers and functional molecules. Microarray-based analysis: High-throughput screening of surface proteins, RNAs, or other biomolecules present in EVs, often using antibody or oligonucleotide arrays. Cryo-electron microscopy (Cryo-EM): Allows high-resolution imaging of EV ultrastructure and identification of protein complexes associated with the vesicle membrane or cargo. Western blotting: A commonly used technique to detect specific EV proteins (e.g., CD63, CD81, TSG101) for validation and quality control. The central schematic represents an EV with diverse biomolecular cargo, including proteins, lipids, RNA species (e.g., miRNA, mRNA), DNA, and metabolites. The surrounding panels depict the diverse methodologies that enable comprehensive analysis of EV characteristics in research and clinical applications.

3.2.6 Emerging Characterisation Tools:

New technologies including super-resolution fluorescence microscopy, Raman spectroscopy, atomic force microscopy (AFM), and surface plasmon resonance (SPR) are beginning to fill the gaps in single-vesicle analysis, interaction kinetics, and mechanical property assessment.

4. Molecular Cargo and Functional Properties

EVs serve as intercellular communication vectors through the selective packaging and delivery of a broad spectrum of biologically active molecules. The molecular cargo encapsulated within or embedded in EVs including proteins, nucleic acids, lipids, and metabolites reflects their cell of origin, the environmental conditions during biogenesis, and the mode of vesicle formation. This compositional diversity underlies the extensive functional repertoire of EVs and forms the molecular basis for their application as therapeutic delivery systems.

4.1 Protein Cargo

EV-associated proteins are central to their structural identity and biological functionality. These proteins can be classified broadly into four categories: (1) structural proteins, (2) signalling and enzymatic components, (3) cell adhesion molecules, and (4) cargo-sorting proteins.

Common structural proteins include tetraspanins (CD9, CD63, CD81), ESCRT-associated proteins (TSG101, Alix), and heat shock proteins (Hsp70, Hsp90), which serve as canonical markers for exosomes. These proteins not only stabilise the vesicle membrane but also contribute to cargo sorting and vesicle formation. In addition, EVs often carry cytosolic enzymes such as GAPDH, metabolic regulators, and kinases that can modulate cellular metabolism upon delivery to recipient cells (Yokoi & Ochiya, 2021).

Tumour-derived EVs frequently harbour oncogenic proteins such as EGFRvIII, HER2, and mutant KRAS, which have been shown to facilitate tumour invasion, angiogenesis, and immune evasion by reprogramming stromal or immune cell behaviour. Moreover, vesicular proteins such as MHC-I/II and PD-L1 can regulate immune activation or suppression, suggesting EVs as both immune stimulators and immune escape mediators depending on the context (Kumar et al., 2024).

4.2 Nucleic Acid Cargo

One of the most compelling features of EVs is their ability to transport nucleic acids including messenger RNAs (mRNAs), microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and even fragmented genomic and mitochondrial DNA

between cells. These nucleic acids are protected from enzymatic degradation within the vesicular lumen, allowing them to remain intact and functionally active in extracellular environments (Maas, Breakefield, & Weaver, 2017).

miRNAs are among the most extensively studied EV cargo. They function as post-transcriptional regulators of gene expression by binding to the 3' untranslated region (UTR) of target mRNAs. Vesicle-contained miRNAs such as miR-21, miR-155, and miR-1246 have been implicated in modulating immune responses, promoting epithelial-to-mesenchymal transition (EMT), and enhancing tumour proliferation and metastasis. For instance, EV-mediated transfer of miR-21 can downregulate PTEN in recipient cells, thereby activating the PI3K/Akt pathway, a common oncogenic signalling cascade (Ye Li, Tan, Miao, & Zhang, 2021).

EVs also carry full-length mRNAs capable of being translated into functional proteins in recipient cells, a property that has attracted significant attention in the context of mRNA-based therapeutics. Furthermore, lncRNAs such as HOTAIR and MALAT1, when delivered via EVs, have been linked to chromatin remodelling and gene expression regulation in various pathological conditions (Payandeh et al., 2024).

4.3 Lipid Composition

The lipid composition of EV membranes is distinct and functionally significant. Unlike the parent cell membrane, EV membranes are enriched in cholesterol, sphingomyelin, ceramides, glycosphingolipids, and phosphatidylserine. This unique lipid profile contributes to vesicle rigidity, resistance to shear stress, and long-term stability in biological fluids.

Ceramide plays a crucial role in the ESCRT-independent formation of exosomes via the generation of inward membrane curvature. Phosphatidylserine, typically localised on the inner leaflet of the plasma membrane, is externalised in EVs and recognised by phagocytic receptors, facilitating uptake by recipient cells. These lipid signatures also influence biodistribution, membrane fusion efficiency, and interactions with serum proteins and immune cells, affecting both the pharmacokinetics and targeting specificity of EV-based therapeutics (Elsherbini & Bieberich, 2018).

4.4 Functional Implications of EV Cargo

The bioactivity of EVs is predominantly governed by their cargo and delivery mechanisms. EVs interact with target cells through multiple routes, including ligand-receptor interactions, direct membrane fusion, endocytosis (clathrin-dependent and -independent), and macropinocytosis. These interactions facilitate

the delivery of encapsulated molecules to the cytoplasm, nucleus, or even mitochondria of recipient cells (Y. J. Liu & Wang, 2023).

Functionally, EVs have been shown to (Y. J. Liu & Wang, 2023):

Modulate Immune Responses: EVs from antigen-presenting cells (APCs) such as dendritic cells can present antigens via MHC molecules, stimulating T cell responses. Conversely, tumour-derived EVs may express immunosuppressive ligands such as PD-L1, promoting immune evasion.

Promote Tissue Regeneration: Stem cell-derived EVs are rich in regenerative factors (e.g., Wnt proteins, VEGF, and TGF-β) and have been reported to enhance angiogenesis, suppress apoptosis, and promote proliferation of resident cells in injured tissues.

Facilitate Disease Progression: In cancer, EVs can prepare pre-metastatic niches by remodelling extracellular matrix, recruiting suppressive immune cells, and inducing vascular leakiness in distant organs. In neurodegenerative diseases, EVs may disseminate misfolded proteins such as α-synuclein or tau, thereby propagating pathology.

Deliver Therapeutics: EVs have been engineered to carry small-molecule drugs, siRNAs, mRNAs, and CRISPR/Cas9 components, offering a natural, biocompatible alternative to synthetic vectors. Their membrane structure allows for efficient encapsulation and reduced immunogenicity, making them suitable for repeated administration.

Collectively, the diverse molecular payload of EVs and their dynamic modes of interaction with recipient cells underpin their promise as both biomarkers and therapeutic delivery platforms. As our understanding of vesicular cargo selection and functional targeting continues to evolve, the design of precision-engineered EVs tailored to specific clinical applications is likely to become a central strategy in next-generation nanomedicine.

5. Engineering Strategies for Therapeutic Applications

While EVs offer numerous inherent advantages such as biocompatibility, immune tolerance, and natural tropism, their native forms are often suboptimal for targeted therapeutic use. Enhancing the therapeutic efficacy, cargo specificity, delivery precision, and scalability of EVs has thus necessitated the development of various bioengineering strategies. These approaches aim to augment their cargo-carrying capacity, enable specific targeting to diseased tissues, and extend systemic circulation time while maintaining vesicular integrity and biological function (Y. J. Liu & Wang, 2023).

5.1 Cargo Loading Approaches

5.1.1 Passive Loading

Passive loading refers to the incubation of isolated EVs with therapeutic agents under physiological or optimised conditions that facilitate spontaneous diffusion across the vesicle membrane **(Figure 4)**. This approach is particularly effective for small, lipophilic molecules, such as certain chemotherapeutics (e.g., paclitaxel, curcumin), which integrate into the lipid bilayer or diffuse into the vesicular lumen due to their hydrophobic nature. While this method is simple and minimally disruptive to EV structure, its utility is limited by low encapsulation efficiency and poor suitability for hydrophilic or large macromolecules.

5.1.2 Active Loading

To improve the loading of larger, hydrophilic, or charged molecules such as siRNAs, mRNAs, CRISPR/Cas9 components, or proteins active loading methods are employed **(Figure 4)**. These include:

Electroporation, which uses controlled electric pulses to transiently permeabilise the vesicle membrane, allowing charged macromolecules to enter the vesicle lumen. However, electroporation can lead to nucleic acid aggregation and membrane destabilisation if not carefully optimised.

Sonication, which applies ultrasonic energy to temporarily disrupt the membrane integrity, facilitating cargo incorporation. This method allows for higher loading efficiencies but may compromise EV stability and protein conformation.

Extrusion, which involves forcing EVs and cargo through nanoporous membranes, resulting in vesicle reformation around the therapeutic cargo. While effective for uniform encapsulation, this technique can alter membrane composition and potentially reduce biological activity (Shao et al., 2024).

Figure 4

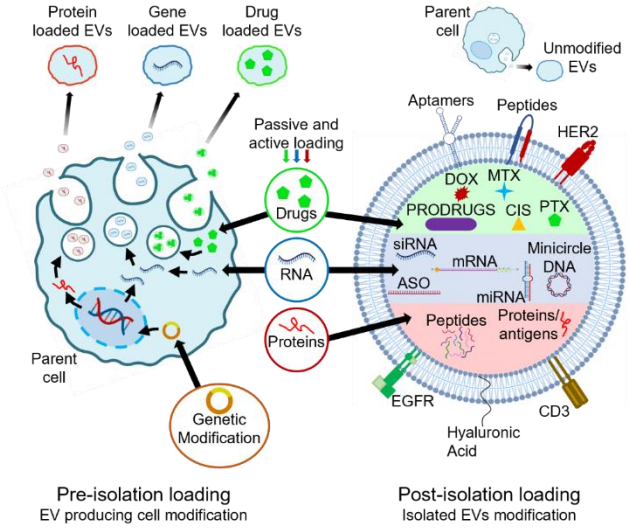

Figure 4 Engineering EVs for Multifunctional Therapeutic Delivery

This schematic illustrates the biogenesis, engineering strategies, and therapeutic cargo loading of EVs for advanced delivery applications. EVs originate from intracellular endosomal pathways and can be naturally secreted or modified through genetic engineering or exogenous loading. Various therapeutic agents, including proteins, RNAs (e.g., siRNA, miRNA, mRNA, ASOs), small-molecule drugs (DOX, MTX, CIS, PTX), and prodrugs, can be incorporated into EVs through endogenous synthesis or post-isolation methods. EVs can be engineered to express surface ligands (e.g., HER2, EGFR-targeting peptides) for enhanced cellular targeting. This multifunctional platform enables the delivery of diverse therapeutic payloads with potential applications in cancer therapy, gene regulation, and immunomodulation. The figure also highlights the integration of minicircle DNA for stable expression systems and the use of antigens and peptides for immunotherapeutic strategies.

5.1.3 Genetic Engineering of Donor Cells

One of the most precise methods for cargo incorporation involves modifying the vesicle-producing (donor) cells at the genetic level **(Figure 4)**. This strategy leverages the endogenous sorting machinery to package therapeutic nucleic acids or proteins into EVs during their natural biogenesis. For instance, cells can be transfected with plasmids encoding specific miRNAs, mRNAs, or fusion proteins, which are then selectively sorted into MVBs and released as exosomal content. Engineered MSCs, for example, have been shown to produce EVs enriched in anti-inflammatory miRNAs (e.g., miR-146a, miR-223), which exert

therapeutic effects in inflammatory and autoimmune disease models. This approach ensures high bioavailability of the intended therapeutic and preserves the natural integrity of the EV membrane (Tran et al., 2025).

5.2 Surface Modification and Targeting Strategies

The natural biodistribution of EVs can be broad and non-specific, limiting their targeting efficiency to diseased tissues. Surface engineering techniques are therefore employed to decorate EV membranes with targeting moieties, enhancing cell-specific uptake and minimising off-target effects (Jayasinghe et al., 2021).

5.2.1 Ligand Display

Surface modification with targeting ligands such as antibodies, peptides, aptamers, or receptor ligands can direct EVs to specific tissues or cell types. For example, the incorporation of Arg-Gly-Asp (RGD) peptides onto EV surfaces enables selective binding to integrins overexpressed in tumour vasculature. This is typically achieved by genetic fusion of targeting peptides to EV membrane proteins such as Lamp2b, CD63, or PDGFR, ensuring their display on the vesicle exterior (Ly et al., 2024).

5.2.2 Chemical Conjugation and Click Chemistry

Covalent attachment of functional groups to EV membranes using biorthogonal chemistry (e.g., click chemistry) offers an alternative to genetic modification. Through reactions such as azide-alkyne cycloaddition, EVs can be functionalised with PEG, fluorescent dyes, or targeting ligands post-isolation. PEGylation has been widely used to increase the half-life of nanoparticles by reducing renal clearance and opsonization, and similar benefits have been observed for PEG-modified EVs. These methods, however, must be optimised to prevent interference with natural receptor-ligand interactions critical for EV uptake (Cabrera-Quiñones, López-Méndez, Cruz-Hernández, & Guadarrama, 2025).

5.3 Synthetic and Hybrid Vesicle Platforms

To overcome limitations related to scalability, heterogeneity, and cargo inconsistency in native EV populations, researchers have developed synthetic EV mimetics and hybrid vesicles. These platforms aim to replicate the desirable biological features of EVs while enabling scalable and controllable production (Park & Jung, 2025).

5.3.1 Synthetic EV-Mimetics

Synthetic EVs are engineered using bottom-up approaches such as self-assembly of lipids, polymers, or proteins to mimic the size, structure, and

functionality of natural EVs. These vesicles can be loaded with defined cargos and functionalised with surface ligands for targeting. Their composition can be tightly controlled, and they are often easier to manufacture under GMP conditions. However, mimetic EVs may lack certain endogenous signalling molecules and membrane markers that contribute to biological activity (López et al., 2025).

5.3.2 Cell-Derived Nanovesicles (CDNs)

Another approach involves mechanically disrupting cells through extrusion or microfluidics to generate nanovesicles that retain cellular membrane proteins and lipids. These CDNs retain many of the membrane-associated properties of natural EVs, including antigen presentation and targeting potential, but can be produced in higher yields and with greater reproducibility (Cheng et al., 2023).

5.3.3 Liposome–EV Hybrids

Hybrid vesicles that integrate synthetic liposomes with components of natural EVs combine the advantages of both systems: the customisable loading and manufacturing scalability of liposomes with the targeting and immune-compatibility features of EVs. These hybrids are typically formed through membrane fusion techniques and can be engineered to deliver a variety of therapeutic cargos, including mRNA, small-molecule drugs, or protein therapeutics (Sato, Zhang, Baba, Chung, & Teramura, 2024).

In summary, the engineering of EVs whether through intracellular manipulation of donor cells or post-isolation vesicle modification represents a critical area of development for transforming EVs into precision nanocarriers. These strategies address key limitations in native EV biology, including suboptimal cargo loading, non-specific targeting, and manufacturing variability, thereby enhancing their potential for clinical translation across a broad range of disease states.

6. EVs as Drug Delivery Platforms: Preclinical and Clinical Evidence

Numerous preclinical studies and an expanding body of early-phase clinical trials have provided compelling evidence for the therapeutic applicability of EVs across a wide spectrum of disease domains. The use of EVs as delivery platforms is supported by several favourable features: (i) their ability to traverse biological barriers such as the BBB, (ii) their low immunogenicity compared to synthetic nanocarriers, (iii) their natural tropism influenced by the parental cell type and surface marker repertoire, and (iv) their stability in circulation, which prolongs the therapeutic window of encapsulated agents. These characteristics have been leveraged in both experimental and clinical settings, as detailed in the subsections below.

6.1 Cancer Therapy

Cancer remains the most extensively studied area for EV-based drug delivery, with promising data across multiple tumour types and therapeutic modalities. EVs derived from both tumour and immune cells have been used to deliver chemotherapeutics, gene-silencing molecules, and immune activators directly to the tumour microenvironment. For example, exosome-encapsulated doxorubicin has demonstrated enhanced anti-tumour efficacy and reduced systemic toxicity in murine breast cancer models. The lipid bilayer of exosomes not only stabilises doxorubicin in circulation but also facilitates its preferential accumulation in tumour tissues via the enhanced permeability and retention (EPR) effect and integrin-mediated uptake. These features significantly mitigate off-target effects such as cardiotoxicity, which is a major limitation of free doxorubicin.

Immune cell-derived EVs are being evaluated as novel cancer immunotherapies. In early-phase clinical trials, dendritic cell (DC)-derived EVs have been administered to patients with advanced non-small cell lung cancer and melanoma. These vesicles, enriched in MHC class I and II molecules and co-stimulatory proteins, can present tumour antigens and stimulate antigen-specific cytotoxic T cell responses. Although these trials have primarily established safety and immunogenicity, therapeutic efficacy has been modest thus far, pointing to the need for improved antigen loading and adjuvant co-delivery strategies. Additionally, ongoing work explores EVs as carriers for siRNAs targeting oncogenes such as KRAS, EGFR, or BCL2, with encouraging tumour suppression observed in xenograft models (Uddin et al., 2024).

6.2 Regenerative Medicine

EVs secreted by MSCs have shown remarkable potential in promoting tissue regeneration and functional recovery across multiple organ systems. These effects are attributed to the delivery of regenerative cargo particularly microRNAs, growth factors, and anti-apoptotic proteins that modulate cellular proliferation, angiogenesis, and inflammation in the damaged tissue.

In cardiac repair, preclinical studies have demonstrated that MSC-derived EVs can reduce infarct size and preserve cardiac function following myocardial infarction. The beneficial outcomes are mediated by the delivery of pro-survival and pro-angiogenic factors, including miR-21, miR-126, and vascular endothelial growth factor (VEGF), which enhance endothelial proliferation and attenuate cardiomyocyte apoptosis (Joladarashi & Kishore, 2022).

In musculoskeletal disorders, EVs derived from adipose- or bone marrow-derived MSCs have accelerated bone and cartilage regeneration in animal models of osteoarthritis and bone fractures. These vesicles promote chondrocyte

differentiation, suppress inflammatory cytokines, and enhance extracellular matrix production.

Clinical studies are currently underway to evaluate the safety and efficacy of MSC-EVs in human patients with ischemic heart disease, chronic kidney injury, and orthopaedic injuries. Notably, phase I trials have reported good tolerability and no adverse immune responses, further supporting the feasibility of EV-based regenerative therapies.

6.3 Neurological Disorders

EVs are uniquely capable of crossing the BBB, a formidable obstacle for most therapeutic agents, making them highly attractive candidates for central nervous system (CNS) drug delivery. Preclinical studies have utilised both endogenous and engineered EVs to deliver neuroprotective and gene-silencing molecules in models of neurodegeneration, traumatic injury, and stroke. One of the landmark studies demonstrated that exosomes engineered to express RVG peptide (which targets neuronal acetylcholine receptors) could deliver siRNA against BACE1, an enzyme implicated in amyloid-beta generation in Alzheimer's disease. The treatment significantly reduced plaque burden and improved cognitive function in transgenic mouse models, highlighting the capacity of EVs to deliver functional nucleic acids into brain tissue.

In Parkinson's disease models, EVs carrying anti-oxidative enzymes or anti-apoptotic miRNAs have been shown to preserve dopaminergic neurons and improve motor outcomes. Similarly, in ischemic stroke, MSC-derived EVs reduced infarct volume and improved functional recovery by modulating neuroinflammation and promoting angiogenesis. Although most of these applications remain preclinical, they form a strong basis for translational efforts aimed at targeting neurodegenerative and neuroinflammatory diseases (Ramos-Zaldívar et al., 2022).

6.4 Immunomodulation

EVs derived from immune and stromal cells possess intrinsic immunomodulatory capacities that can be harnessed for the treatment of autoimmune diseases, inflammatory disorders, and as vaccine delivery systems. In autoimmune models, such as rheumatoid arthritis and systemic lupus erythematosus, EVs enriched with anti-inflammatory miRNAs (e.g., miR-150, miR-223) have attenuated disease progression by suppressing pro-inflammatory cytokines (e.g., TNF-α, IL-6) and modulating macrophage polarisation toward an M2 phenotype. These findings suggest EVs could serve as next-generation immunosuppressive agents with fewer systemic side effects.

EV-based vaccines have also gained attention. Tumour-derived EVs carrying tumour-associated antigens have been used to prime dendritic cells and stimulate antigen-specific CD8+ T cell responses. Similarly, EVs derived from antigen-loaded dendritic cells or genetically modified cells expressing viral or bacterial antigens have shown efficacy in preclinical infectious disease models. These vesicles mimic pathogen-associated molecular patterns (PAMPs) and induce robust humoral and cellular immune responses.

The modularity of EV composition and their biocompatibility make them ideal platforms for customised immunotherapy applications, particularly when combined with adjuvants or immune checkpoint inhibitors (Kalluri, 2024).

7. Comparison with Conventional Drug Carriers

EVs offer a compelling alternative to traditional synthetic nanocarriers such as liposomes, polymeric nanoparticles, dendrimers, and micelles. While synthetic carriers have long been utilised for drug delivery due to their customisable properties and scalable manufacturing, EVs possess several unique biological advantages that make them particularly attractive for next-generation therapeutic applications (Chen et al., 2025).

7.1 Biocompatibility and Immunological Stealth

One of the most significant advantages of EVs lies in their endogenous origin. Being naturally secreted by human cells, EVs are inherently biocompatible and generally well tolerated in vivo. Unlike synthetic nanoparticles that can activate complement pathways or trigger rapid clearance by the mononuclear phagocyte system (MPS), EVs can circulate for longer durations and exhibit reduced immunogenicity. This immunological stealth is largely due to the presence of self-markers such as CD47 (“don’t eat me” signal) on their membranes, which inhibit phagocytic uptake by macrophages. These features minimise off-target toxicity and immune-related adverse effects common limitations observed with conventional systems (La-Beck, Islam, & Markiewski, 2021).

7.2 Intrinsic Targeting Capabilities

Synthetic drug carriers often require extensive surface modification with targeting ligands (e.g., antibodies, peptides) to achieve selective tissue delivery. In contrast, EVs naturally express membrane proteins and adhesion molecules derived from their parent cells, granting them intrinsic tissue tropism. For instance, integrins on tumour-derived EVs mediate preferential localisation to specific organ sites such as lungs or liver. This inherent targeting capability reduces the need for additional engineering and improves biodistribution, particularly in complex in vivo environments. Furthermore, EVs derived from

immune, neural, or mesenchymal stromal cells can preferentially home to inflamed, injured, or hypoxic tissues, further enhancing therapeutic specificity.

7.3 Capacity for Complex and Multifunctional Cargo

Unlike most synthetic nanocarriers, which are optimised for loading small molecules or nucleic acids, EVs can simultaneously carry a diverse repertoire of functional biomolecules. These include mRNAs, microRNAs, long non-coding RNAs, DNA fragments, proteins, lipids, and even metabolites. Their lipid bilayer structure provides protection against enzymatic degradation and preserves the functional integrity of labile molecules such as RNA. This multifaceted cargo-carrying capacity enables EVs to modulate multiple cellular pathways simultaneously, making them well-suited for complex diseases like cancer, neurodegeneration, and autoimmunity. In contrast, the encapsulation efficiency of synthetic systems is typically limited to specific types of cargo (e.g., hydrophobic drugs for liposomes, siRNA for polyplexes), and co-delivery of different modalities often requires separate formulation steps.

7.4 Limitations and Manufacturing Challenges

Despite their many biological advantages, EVs face critical challenges that currently limit their widespread clinical adoption (Moleirinho, Silva, Alves, Carrondo, & Peixoto, 2020):

Heterogeneity: EV populations are highly heterogeneous, with variations in size, surface markers, and cargo content depending on cell source, isolation method, and physiological state. This contrasts with synthetic carriers, which can be produced with uniform size and composition.

Low Yield and Scalability: Large-scale EV production remains a bottleneck. Conventional isolation methods such as ultracentrifugation are labour-intensive and poorly scalable. In comparison, liposomes and polymeric nanoparticles can be manufactured at industrial scale with high reproducibility.

Cargo Characterization: Analysing and quantifying the internal contents of EVs is technically demanding, given the diversity and complexity of their molecular cargo. Techniques like mass spectrometry, RNA-seq, and single-vesicle analysis are advancing but are not yet standardised or widely accessible.

Regulatory Ambiguity: Regulatory frameworks for EV-based therapeutics are still in development, whereas synthetic drug carriers benefit from well-established pathways for clinical approval.

7.5 Synthetic Nanocarriers: Advantages and Trade-Offs

Synthetic systems remain valuable, especially for applications requiring precise control over particle size, surface charge, and drug release kinetics. Their

ability to encapsulate high payload concentrations, combined with established protocols for PEGylation and ligand attachment, makes them suitable for large-scale production and commercialisation. Moreover, synthetic carriers are often more amenable to GMP compliance and batch-to-batch reproducibility, key requirements for regulatory approval.

However, their shortcomings such as potential immunogenicity, low targeting specificity, and poor cargo stability in circulation continue to hinder their therapeutic performance, especially in complex biological systems.

While synthetic nanocarriers provide design flexibility and scalable production, EVs offer a biologically evolved system with superior compatibility, multi-cargo loading, and intrinsic targeting. The integration of EV biology with nanotechnology may ultimately yield hybrid systems that harness the best of both platforms. Bridging the gap between biological sophistication and manufacturing control will be key to realizing the full therapeutic potential of extracellular vesicle-based drug delivery.

8. Future Perspectives and Emerging Trends

The field of extracellular vesicle (EV)-based therapeutics is advancing rapidly, driven by novel insights into EV biology, technological innovations in vesicle engineering, and increasing translational momentum. While early research established the foundational understanding of EVs as intercellular messengers, the current trajectory is defined by their convergence with genome editing, immunoengineering, systems biology, and precision medicine. As this therapeutic paradigm evolves, several emerging trends and future directions are reshaping the landscape.

8.1 Integration with Gene Editing Technologies

One of the most promising frontiers is the use of EVs as delivery vehicles for genome editing tools, particularly CRISPR/Cas9 systems. Unlike viral vectors, EVs offer a safer, non-immunogenic, and transient mode of delivering Cas9 protein, mRNA, or guide RNAs into target cells. Recent studies have demonstrated the successful packaging and delivery of Cas9 ribonucleoprotein complexes within EVs for gene knockout or correction in vitro and in vivo, including liver, retinal, and muscular tissues. This approach could revolutionise treatments for inherited disorders, cancer, and infectious diseases, bypassing concerns associated with viral integration or persistent expression (Su, Wang, Li, & Chen, 2025).

8.2 EVs for mRNA and RNA-Based Therapies

In the post-pandemic era of RNA therapeutics, EVs are being reimagined as natural vectors for mRNA delivery. Unlike LNPs, which carry risks of

inflammation and hepatotoxicity, EVs offer endogenous biocompatibility and enhanced stability in circulation. They can be engineered to encapsulate therapeutic mRNAs coding for proteins such as insulin, VEGF, or therapeutic antibodies. This strategy is particularly appealing for transient protein replacement therapies, including applications in metabolic disorders, cardiovascular disease, and regenerative medicine (Su et al., 2025).

8.3 Personalised and Autologous EV Therapies

With advances in autologous EV production, personalised medicine is poised to benefit significantly from EV technology. EVs derived from a patient's own stem cells, immune cells, or fibroblasts can be loaded with therapeutic cargo tailored to their specific disease profile. This strategy minimises immunogenic risk and optimises compatibility, particularly in treating rare or refractory conditions. Integration with multi-omics platforms allows comprehensive profiling of EV cargo, enabling the selection or engineering of vesicles with optimal therapeutic payloads.

8.4 Artificial Intelligence and Predictive Modelling

Artificial intelligence (AI) and machine learning are beginning to play a critical role in decoding the complexity of EV biology. Algorithms trained on EV datasets including proteomics, transcriptomics, lipidomics, and uptake kinetics can predict cargo sorting mechanisms, biodistribution patterns, and cellular targeting. AI-driven platforms also facilitate the design of optimal surface modifications and engineering strategies to enhance therapeutic index. Such predictive models could accelerate candidate selection for clinical trials, reducing time and cost in drug development.

8.5 Synthetic EVs and Biomimetic Platforms

Another significant trend involves the development of synthetic EV mimetics; engineered vesicles that replicate the structural and functional features of natural EVs while allowing scalable and controlled manufacturing. These include liposome-EV hybrids, cell-membrane coated nanoparticles, and exosome-mimicking nanovesicles. These platforms aim to combine the targeting precision of natural EVs with the reproducibility of synthetic systems. Future generations of hybrid vesicles may incorporate programmable features, such as stimuli-responsive release or intracellular trafficking tags, enhancing delivery efficiency and specificity (X. Wei, Liu, Cao, Wang, & Chen, 2023).

8.6 Regulatory Harmonisation and GMP-Compliant Production

Despite scientific progress, regulatory challenges remain a major bottleneck in EV clinical translation. The next phase of development will require harmonised international guidelines defining EV classification, purity criteria, potency

assays, and long-term safety. Advances in microfluidic bioreactor technology and tangential flow filtration (TFF) are facilitating scalable, GMP-compliant EV production. Furthermore, the emergence of centralised EV banks and contract manufacturing organisations (CMOs) may streamline supply chains and reduce batch variability (Anklam et al., 2022).

8.7 Expansion into New Therapeutic Areas

Beyond oncology and neurology, EVs are now being investigated for applications in infectious diseases (e.g., EV-based vaccines), reproductive medicine (e.g., endometrial repair), and metabolic disorders (e.g., insulin delivery). In ophthalmology, EVs have shown potential in restoring retinal function and modulating intraocular inflammation. In dermatology, EVs are being studied for scar reduction and psoriasis treatment. These expanding indications reflect the versatility of EVs as a universal delivery platform adaptable to diverse clinical needs (Su et al., 2025).

9. Conclusion

EVs have evolved from mere biological curiosities to sophisticated, clinically promising delivery vehicles with wide-ranging therapeutic implications. Their unique properties including innate biocompatibility, natural targeting capabilities, ability to traverse physiological barriers, and complex molecular cargo position them as a transformative modality in precision medicine. Across cancer therapy, neurodegeneration, immunomodulation, and regenerative medicine, EVs have demonstrated significant therapeutic potential in both preclinical models and early-phase clinical trials. Engineering approaches have further expanded the functional landscape of EVs. Techniques such as passive and active cargo loading, genetic manipulation of donor cells, and surface ligand display allow for precise control over payload, targeting, and biodistribution. These advances, in combination with novel synthetic and hybrid vesicle technologies, have improved scalability and modularity, making EVs viable for industrial production and regulatory scrutiny.

However, several challenges must still be addressed before widespread clinical adoption can be achieved. These include standardisation of isolation and characterisation protocols, quality control during large-scale production, cargo heterogeneity, and long-term safety assessment. Moreover, regulatory frameworks must be adapted to accommodate the unique properties of EVs as biologically derived nanotherapeutics. Collaborative efforts among scientists, clinicians, regulatory agencies, and biotech companies will be essential to navigate these complexities and bring EV-based therapies to the clinic. Looking ahead, the convergence of EV technology with genome editing, RNA-based therapeutics, and artificial intelligence opens unprecedented opportunities for

personalised and adaptive medicine. Autologous EVs, equipped with gene-modifying tools or regulatory RNAs, could one day treat genetic disorders, modulate immune responses, or even reverse neurodegeneration with unmatched specificity and safety. As multi-omics technologies refine our understanding of EV cargo and function, the design of bespoke vesicles tailored to individual patient profiles becomes an attainable goal.

EVs represent not only a new frontier in drug delivery but a paradigm shifts in therapeutic design moving from synthetic constructs to biologically inspired systems that harness the body's own communication networks. As the field matures, EVs are poised to become integral components of next-generation therapies, offering novel solutions for diseases that remain untreatable with current modalities.

Author Contributions

Hatice Esenkaya solely conceived and designed the study, data collection, and image analysis, and was responsible for the interpretation of sections. The manuscript was written and finalised entirely by the author.

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Competing Interest Disclosure

The author declares no competing financial or non-financial interests relevant to the content of this study. All research activities were conducted by the author at Kilis 7 Aralik University, Türkiye.

Ethical Disclosure

This study did not involve human participants or animal experiments and therefore did not require ethical approval.

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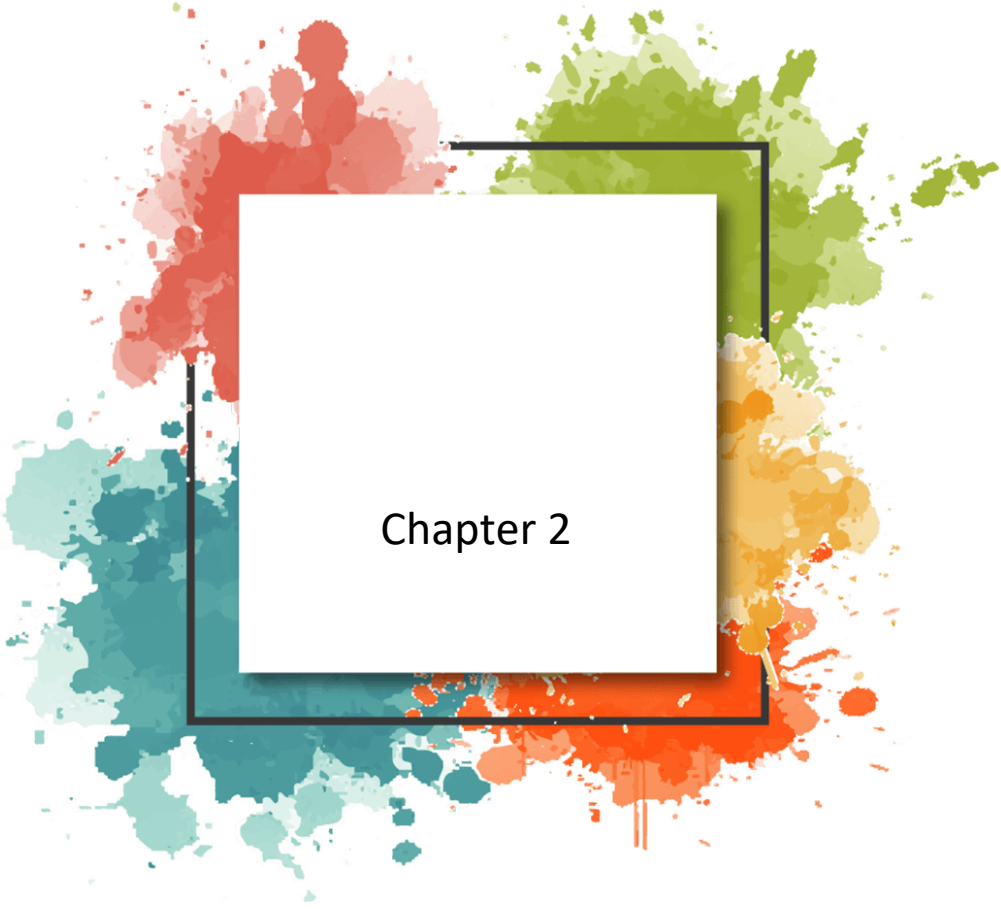
Chapter 2

Telomerase and Cancer İmmunotherapy

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Aliye Ezgi Güleç Taşkıran[3]

1. Introduction:

Cancer is a multifaceted disease arising from the complex interplay of genetic and epigenetic alterations at the cellular level, which transform normal cells into a malignant phenotype characterized by uncontrolled proliferation (Ayhan & Ogawa, 2013). Activation of proto-oncogenes, such as RAS, promotes aberrant cellular proliferation (Downward, 2003), while inactivation of tumor suppressor genes, including TP53, impairs DNA damage responses and apoptosis (Kandoth et al., 2013). Additionally, epigenetic modifications—such as DNA methylation and histone alterations—can silence tumor suppressor genes, further driving tumorigenesis (Baylin & Jones, 2016). These genetic and epigenetic changes collectively disrupt key signaling pathways, establishing a molecular foundation for cancer development and progression.

Among the mechanisms that enable sustained proliferation, telomere maintenance plays a pivotal role. Telomeres are specialized nucleoprotein structures that cap the ends of linear chromosomes, preserving genomic stability and preventing DNA damage responses that could otherwise be triggered by exposed chromosomal termini (Blackburn, Greider, & Szostak, 2006). Due to the inherent limitations of conventional DNA polymerases, telomeres shorten progressively with each round of DNA replication, ultimately leading to replicative senescence or apoptosis (Hayflick & Moorhead, 1961). Telomerase, a ribonucleoprotein enzyme composed of the catalytic subunit telomerase reverse transcriptase (TERT) and the telomerase RNA component (TERC), counteracts this attrition by adding tandem telomeric repeats to chromosome ends (Greider & Blackburn, 1985; Lingner et al., 1997). This activity maintains telomere length and confers replicative potential, particularly in germline and stem cells. In contrast, most somatic cells repress telomerase; however, approximately 85–90% of human cancers reactivate telomerase, enabling unlimited proliferation and

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establishing telomerase as a hallmark of cancer (Blasco, Funk, Villeponteau, & Greider, 1995a; Kim et al., 1994; Jerry W. Shay & Wright, 2010).

The prevalence of telomerase activation across diverse malignancies, coupled with its restricted expression in normal tissues, renders it an attractive therapeutic target. Early approaches—including small-molecule inhibitors, antisense oligonucleotides, and gene therapies—faced challenges related to specificity, delivery, and delayed therapeutic effects (Bacchetti & Counter, 1995; Harley, 2008). More recently, advances in cancer immunotherapy have opened new avenues for telomerase targeting. Telomerase-derived peptides can serve as tumor-associated antigens capable of eliciting cytotoxic T-cell responses, and engineered T-cell strategies as well as telomerase-based vaccines are actively being explored in preclinical and clinical settings (Bernhardt et al., 2006; Mizukoshi & Kaneko, 2019; Patel & Vonderheide, 2004). Thus, integrating insights into cancer genetics, epigenetics, and telomerase biology is essential for the development of innovative, targeted therapeutic strategies.

2. Importance of Telomere and Telomerase Biology

2.1 Telomere Structure

Telomeres are repetitive nucleotide sequences located at the termini of eukaryotic chromosomes that do not encode genetic information (Blackburn, 1991). These regions end with a single-stranded 3' overhang, approximately 50 to 300 nucleotides in length, extending beyond the double-stranded DNA segment (Wellinger & Zakian, 2012). The 3' overhang facilitates the formation of a T-loop structure, in which the telomeric DNA folds back on itself (de Lange, 2005; Griffith et al., 1999). This configuration stabilizes chromosome ends and prevents the activation of DNA damage response pathways by masking them from being misrecognized as double-stranded breaks (Y. Xu & Komiyama, 2023).

Telomeric DNA is safeguarded by a specialized protein complex known as shelterin. This complex is composed of six subunits: TRF1, TRF2, POT1, TPP1, TIN2, and RAP1. Shelterin plays a critical role in protecting telomeres from being misidentified as sites of DNA damage by repair machinery, regulating telomere length, and modulating the activity of the telomerase enzyme to maintain telomere regions (Cai et al., 2024; Klump et al., 2023). The structure of telomeric DNA is explained in Figure 1.

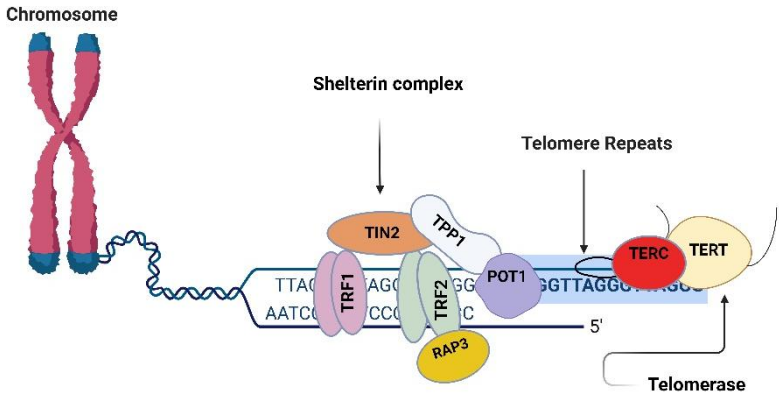

Figure 1. Structure of Telomeric DNA. Repetitive nucleotide sequences located at the termini of eukaryotic chromosomes form a single-stranded 3' overhang which facilitates the formation of a T-loop structure (folding of telomeric DNA back on itself). Shelterin complexed composed of TRF1, TRF2, POT1, TPP1, TIN2, and RAP1 subunits protects the telomeric DNA.

2.2 Telomere Shortening and Cellular Aging

During cell division, the DNA at the 3' end of linear chromosomes cannot be fully replicated due to inherent limitations of DNA polymerase, a phenomenon referred to as the "end replication problem" (Bonnell, Pasquier, & Wellinger, 2021). This leads to progressive telomere shortening with each round of replication (Ohki, Tsurimoto, & Ishikawa, 2001). The telomerase ribonucleoprotein complex counteracts this process by extending the 3' overhang through the catalytic activity of telomerase reverse transcriptase (TERT). Suppression of TERT expression results in continuous telomere attrition during successive cell divisions (Greider & Blackburn, 1985). Once telomeres shorten beyond a critical threshold, they activate the DNA damage response (DDR), leading to cell cycle arrest and ultimately triggering apoptosis (Moix, Sadler, Kutalik, & Auwerx, 2024).

2.3 Telomerase Mechanism of Action

Telomerase was initially discovered in retroviruses and is classified as a specialized DNA polymerase, or reverse transcriptase, capable of synthesizing DNA from an RNA template (Temin & Mizutani, 1970). The mechanism of telomerase action was first characterized in 1985 by Carol Greider and Elizabeth Blackburn in the protozoan Tetrahymena (Greider & Blackburn, 1985). Since then, telomerase has been identified in a wide range of eukaryotic organisms, and the genes encoding its RNA component have been successfully cloned in Tetrahymena, yeast, mice, and humans (Blasco, Funk, Villeponteau, & Greider,

1995b; Feng et al., 1995; Greider & Blackburn, 1989; Singer & Gottschling, 1994). The telomerase RNA component is complementary to the telomeric repeat sequences specific to each organism. In proliferating cells, telomerase plays a critical role in maintaining telomere length (Greider & Blackburn, 1985).

Telomerase is a ribonucleoprotein enzyme that counteracts telomere shortening by adding tandem telomeric repeats to the 3′ ends of chromosomes (Lee & Pellegrini, 2025). Its catalytic subunit, TERT, utilizes the telomerase RNA component (TERC) as a template to extend the G-rich overhang, thereby resolving the "end-replication problem" (Rubtsova et al., 2019). The enzyme binds to the single-stranded telomeric overhang, aligns the RNA template, and synthesizes new repeats (TTAGGG in humans) by reverse transcription. After one repeat is added, telomerase translocate and repeats the process, generating multiple repeats in a processive manner (Alaguponniah et al., 2020). Subsequently, conventional DNA polymerases fill in the complementary C-rich strand. Importantly, the G-rich telomeric DNA has the intrinsic ability to fold into G-quadruplex (G4) structures, which can act as potent physical barriers to telomerase binding and elongation (Jansson et al., 2019). Figure 2 explains the mechanism of action for telomerase activity. While G4s are thought to serve as protective elements that regulate telomerase activity and telomere accessibility, their stabilization has also been explored as an anticancer strategy to inhibit telomerase-dependent telomere maintenance (Figueiredo, Mergny, & Cruz, 2024). Thus, the interplay between telomerase and telomeric G4s represents a critical regulatory mechanism in chromosome end protection and cancer cell immortality (Y. Xu & Komiyama, 2023).

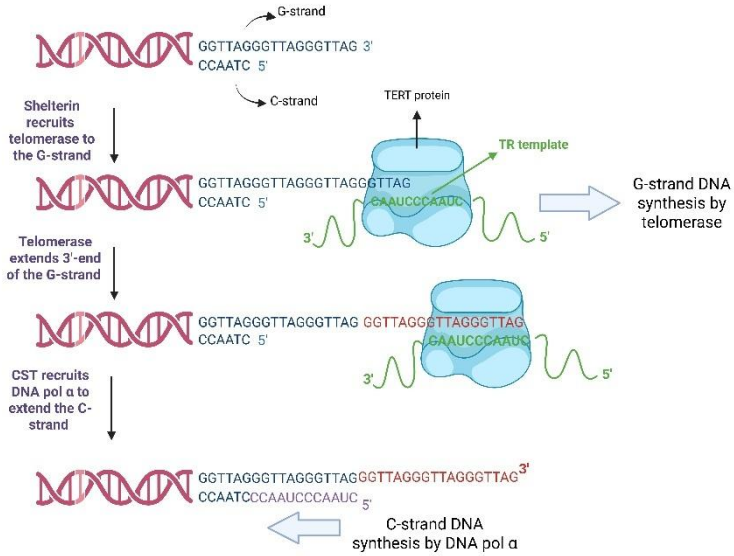

Figure 2. Mechanism of Action for Telomerase Activity. TERT binds to the single-stranded telomeric overhang, aligns the TR template, and synthesizes new repeats by reverse transcription repeatedly. Subsequently, conventional DNA polymerases fill in the complementary C-rich strand. The G-rich telomeric DNA has the intrinsic ability to fold into G4 structures, which can act as potent physical barriers to telomerase binding and elongation.

Additionally, elevated levels of TERC expression have been observed in various cancers, including cervical, ovarian, and head and neck lung carcinomas, underscoring its potential as a therapeutic target (Baena-Del Valle et al., 2018; Cao, Bryan, & Reddel, 2008).

3. The Relationship Between Telomerase Activity and Cancer

3.1 Telomere Length and Cancer

Telomere length in most tumor cells is typically shorter than that observed in corresponding normal tissues (Barthel et al., 2017). Cancer cells exhibit markedly elevated and unregulated proliferative capacity compared to normal somatic cells (Hanahan & Weinberg, 2000). Due to the "end-replication problem" problem telomeres undergo progressive shortening with each cell division (Wynford-Thomas & Kipling, 1997). The accelerated rate of proliferation in cancer cells amplifies this effect, resulting in more pronounced telomere shortening relative to that seen in normal cells (Jerry W. Shay, 2014). Although, majority of cancer types represent shorter telomeres, longer telomeres in gliomas and sarcomas, as opposed to normal tissue are observed as well (Barthel et al., 2017). And longer telomeric regions are mostly attributed to elevated TERT activity due to

mutational state of promoter regions, amplification and genomic rearrangements (Barthel et al., 2017).

Even though highly proliferative cells have shorter telomeres, and this is accepted as a poor health status, longer telomeres due to increased cell growth potential have been shown to be associated with cancer-initiating somatic mutations (Maciejowski & de Lange, 2017). The efficacy and timing of anticancer responses to telomerase inhibitors are believed to be influenced by the initial telomere length, as the onset of telomere dysfunction is dictated by the shortest telomere within the cell (Hemann, Strong, Hao, & Greider, 2001).

3.2 Telomerase Activity Profile According to Cancer Types

The level of telomerase activity varies considerably depending on the tissue of origin and the biological characteristics of the tumor (Kim et al., 1994). Among cancers, solid tumors exhibit the highest and most prevalent telomerase activity. For instance, telomerase activity is detected in approximately 85–95% of lung, breast, prostate, and colorectal cancers (Jerry W. Shay & Wright, 2011). In hematological malignancies, telomerase activity is particularly elevated in acute leukemias (AML and ALL), where it is thought to correlate with disease progression (Allegra et al., 2017). In contrast, telomerase activity is generally lower in chronic leukemias, such as chronic lymphocytic leukemia (CLL), although it can increase significantly in advanced or progressive disease stages (Jebaraj & Stilgenbauer, 2021).

Notably, certain tumor types maintain telomere length through a telomerase-independent mechanism known as alternative lengthening of telomeres (ALT) (Cesare & Reddel, 2010). This mechanism is especially prominent in tumors such as soft tissue sarcomas, gliomas, and pancreatic neuroendocrine tumors, where telomerase activity is often low or undetectable, indicating reliance on the ALT pathway for telomere maintenance (Reddel, Bryan, Colgin, Perrem, & Yeager, 2001).

3.3 Interaction of Telomerase with Signaling Pathways Such as p53, MYC, and RAS

Telomerase exerts pro-oncogenic effects that go far beyond its canonical role in telomere elongation. The catalytic subunit hTERT interacts with multiple intracellular signaling pathways—including c-MYC, WNT/β-catenin, and NF-κB—to regulate cell survival, proliferation, and transformation. These interactions reveal a complex network of cross-talks, which not only supports tumorigenesis but also suggests that hTERT functions as a central modulator of oncogenic signaling. The continuous identification of new hTERT-mediated signaling interactions underscores its multifaceted role in cancer progression and

highlights its potential as a therapeutic target (Pestana, Vinagre, Sobrinho-Simões, & Soares, 2017)

Telomerase activity is tightly regulated by multiple oncogenic and tumor suppressor pathways. Among these, the p53, c-MYC, and RAS signaling axes are particularly important due to their widespread alterations in human cancers (Grzes et al., 2020). These pathways not only regulate hTERT transcription but also influence post-transcriptional and post-translational modifications, thereby modulating both nuclear and extranuclear functions of telomerase (Horikawa, Fujita, & Harris, 2011; Koh et al., 2015; Ram et al., 2009)

3.4 Interaction of p53 and Telomerase

The tumor suppressor p53 functions as a vital detector of genomic stress, including abnormalities such as telomere shortening or dysfunction (Lane, 1992). When activated, p53 can initiate cell cycle arrest, senescence, or apoptosis, thereby preventing the division of cells with compromised genomic stability(Levine, 2020). In addition to its traditional role in maintaining genomic integrity, p53 also acts as a negative regulator of telomerase by directly suppressing the transcription of hTERT, the enzyme's catalytic subunit (D. Xu et al., 2000). Mechanistically, p53 either binds to defined promoter regions or recruits co-repressor proteins to inhibit hTERT expression (Yao, Bellon, Shelton, & Nicot, 2012). In many cancer types, loss-of-function mutations in p53 eliminate this regulatory checkpoint, resulting in uncontrolled telomerase activation and enabling cells to proliferate indefinitely (Vousden & Prives, 2009).

3.5 Interaction of c-MYC and Telomerase

The oncogenic transcription factor c-MYC is a key regulator of telomerase expression. It binds directly to E-box sequences in the hTERT promoter, stimulating its transcription and thereby enhancing telomerase activity (Goueli & Janknecht, 2003). This regulatory effect is especially significant in rapidly proliferating cells, including stem cells and aggressive tumor cells (Khattar & Tergaonkar, 2017).

Recent studies using chromatin immunoprecipitation (ChIP) have shown that MYC-driven activation of hTERT is also modulated by epigenetic mechanisms, such as histone acetylation and methylation, which alter chromatin structure to make the promoter more accessible for transcription (Dang, 2012). Moreover, c-MYC can interact with other transcription factors and signaling pathways, including RAS and PI3K/AKT, to maintain elevated telomerase activity in cancer cells (Wang, Lisanti, & Liao, 2011).

3.6 RAS Pathway and Telomerase Interaction

RAS proteins are small GTP-binding enzymes that control multiple cellular functions, including cell proliferation, differentiation, and survival (Xiao et al., 2023). Oncogenic mutations in RAS are frequently observed in human cancers, resulting in persistent activation of downstream signaling pathways such as PI3K/AKT and MAPK/ERK (Benoit et al., 2022)

Through these pathways, RAS signaling indirectly regulates telomerase by affecting hTERT transcription, its post-translational modifications, and nuclear localization (Goueli & Janknecht, 2004; Uno et al., 2023). Phosphorylation of hTERT by AKT facilitates its transport into the nucleus and boosts its enzymatic activity, while ERK-mediated signaling stabilizes hTERT protein levels and further enhances telomerase function (Jeong et al., 2015).

3.7 Multiple Signaling Pathway Interactions

Recent studies show that p53, MYC, and RAS form an interconnected network regulating telomerase (Wisman et al., 2003). Mutant p53 can upregulate MYC, enhancing hTERT transcription, while RAS influences MYC and hTERT modifications, stabilizing telomerase and supporting extranuclear roles like mitochondrial regulation and metabolic adaptation (Prasad, Mishra, Kumar, & Yadava, 2022). These pathways also coordinate ROS levels, DNA repair, and epigenetic changes, promoting tumor survival and chemoresistance (García-Guede, Vera, & Ibáñez-de-Caceres, 2020; Porter et al., 2017). This highlights telomerase not only as a telomere-maintenance enzyme but also as a key mediator of oncogenic signaling and cellular stress responses.

The signaling pathways involved the telomerase activity regulation is summarized in Figure 3.

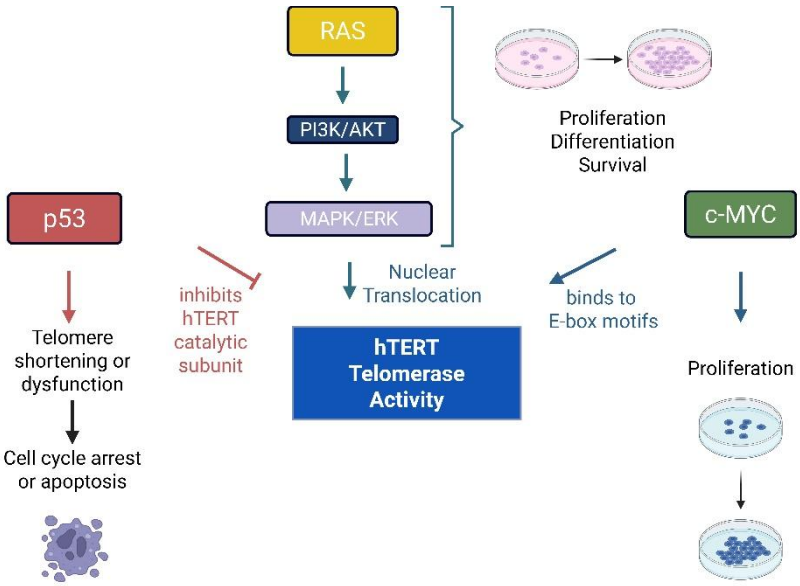

Figure 3. Signaling pathways involved in the regulation of telomerase activity. p53 is involved inhibits hTERT catalytic subunit leading telomere dysfunction followed by cell cycle arrest and cell death. c-MYC enhances hTERT expression through binding to E-box sequences in hTERT promoter region.

4. Telomerase-Directed Treatment Strategies

Over the past two decades, telomerase has been widely investigated as a therapeutic target in cancer. One key strategy involves inhibiting its RNA component hTR using imetelstat (GRN163L), a lipidated oligonucleotide that competitively blocks telomerase activity (Röth, Harley, & Baerlocher, 2010). In vitro studies have shown that GRN163 induces cellular senescence or apoptosis depending on telomere length and effectively suppresses tumor growth in xenograft models (Asai et al., 2003; Shea-Herbert, Pongracz, Shay, & Gryaznov, 2002). While its efficacy in solid tumors has been limited, imetelstat has been successfully repurposed for treating myeloproliferative disorders, demonstrating benefits in certain hematologic patients (Olschok et al., 2023; Tefferi et al., 2015). Another promising approach leverages the high telomerase activity in cancer cells by incorporating nucleoside analogs such as 6-thio-2'-deoxyguanosine (6-thio-dG) and 5-fluoro-2'-deoxyuridine (5-FdU) triphosphate, which trigger telomere dysfunction, DNA damage, and selective death of telomerase-positive cells (Baraniak, Baranowski, Ruszkowski, & Boryski, 2016; Mender, Gryaznov, Dikmen, Wright, & Shay, 2015). Furthermore, the telomerase catalytic subunit

TERT serves as a tumor-associated antigen that stimulates adaptive immune responses, and TERT-targeted peptide vaccines, particularly when combined with immune checkpoint inhibitors, have shown encouraging antitumor effects in preclinical studies (Baraniak et al., 2016; Reyes-Uribe et al., 2018; Sengupta et al., 2018).

Due to the existence of differences in the manner of telomerase activity and telomere length, different strategies can be employed for different cancer types to successfully suppress cancer progression. Telomerase activity inhibition is one of the methods to be employed for cancer types with elevated telomerase activity(Ganesan & Xu, 2017) . The efficacy and timing of anticancer responses to telomerase inhibitors are believed to be influenced by the initial telomere length, as the onset of telomere dysfunction is dictated by the shortest telomere within the cell (Hemann et al., 2001). Telomerase-targeted immunotherapy is another form of therapy which can be employed for cancer types with active telomerase as well. In telomerase-activated immunotherapy DNA vaccines deliver DNA that encodes a modified version of TERT into cells to help the body develop an immune response against telomerase-expressing cells, which then enables the immune system to target cancer cells (Mizukoshi & Kaneko, 2019). Induction of telomere dysfunction is another option to exploit rapid telomere synthesis and to induce DNA damage and immune activation (Mender et al., 2015). In a strategy targeting telomeric DNA secondary structures, DNA damage and cell death induction is carried out via G-quadruplex binders to stabilize G-quadruplex, and to displace shelterin proteins (Salvati et al., 2007). Shelterin targetting rise as another approcah of anti-cancer treatment option in which shelterin proteins are supressed with different methodoliges such as miRNA-mediated down regulation (Vertecchi, Rizzo, & Salvati, 2022). This option is mostly appliciable for cancer cell types characterized by high expression of shelterin proteins. For example, TRF2 is overexpressed in different human cancer types such as breast carcinomas, liver hepatocarcinomas, and lung carcinoma (Blanco, Muñoz, Flores, Klatt, & Blasco, 2007). TERRA targetting is another strategy since it is a long non-coding RNA that regulates telomere maintenance and telomerase activity, processes critical for cancer cell immortality. Dysregulation of TERRA in tumors contributes to telomere instability, making it a potential anticancer target. Strategies such as antisense oligonucleotides, small molecules, or modulation of TERRA transcription aim to disrupt telomere maintenance, selectively impairing cancer cell proliferation while sparing normal cells (Rivosecchi & Cusanelli, 2023).

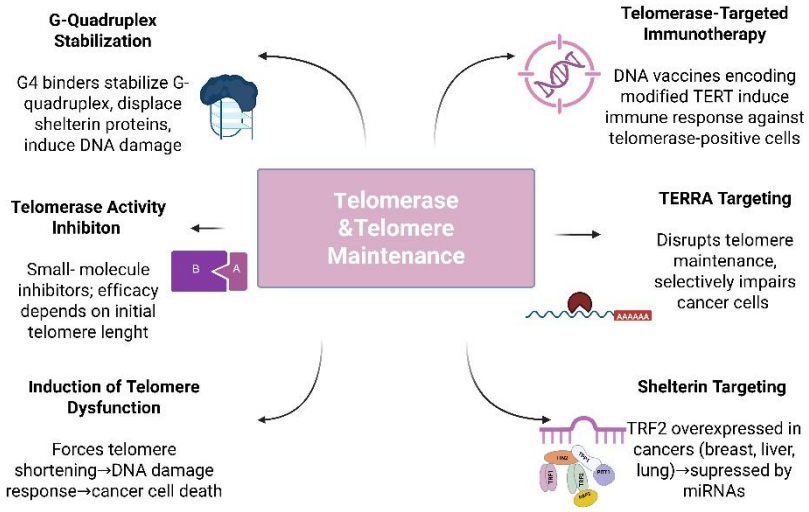

Figure 4. Telomerase-Directed Cancer Treatment Strategies. G-Quadruplex stabilization strategy employs g4 binders to stabilize G4s and displace shelterin proteins. Telomerase inhibition strategy employs small molecules inhibiting the catalytic activity of telomerase. Induction of telomere dysfunction strategy induces forced telomere shortening. Telomerase targeted immunotherapy employs DNA vaccines encoding modified TERT to induce immune response against telomerase-positive cells. TERRA targeting disrupts telomere maintenance by targeting TERRA. Shelterin targeting strategy employs miRNAs to target TRF2 component of shelterin complex.

5. Use of Telomerase as an Immunological Target

Human telomerase reverse transcriptase (hTERT) contains peptides that can be processed and presented by both MHC class I and II molecules, allowing the activation of both $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ helper T cells (Hernández et al., 2002). Accordingly, peptide-based vaccines targeting hTERT (e.g., GV1001, GRNVAC1) and T-cell receptor (TCR)-based approaches that recognize hTERT-derived peptides have been clinically investigated in both hematologic and solid tumors (Lanna, 2025).

Telomerase-based vaccines have recently emerged as promising strategies in cancer immunotherapy. These vaccines are being developed on various platforms, including DNA-based, peptide-based, and recombinant protein-based formats (Vahidi & Zabeti Touchaei, 2024). When used in combination with immune adjuvants or immune checkpoint inhibitors, they have demonstrated synergistic potential in enhancing anti-tumor immune responses (Vahidi & Zabeti Touchaei, 2024) DNA vaccines are advantageous due to their safety, stability, and ease of production (Lu et al., 2024). Several optimization strategies have been

explored to enhance their efficacy, such as codon optimization, incorporation of strong viral promoters, fusion with immunoglobulin sequences, and increasing the number of CpG motifs in the plasmid backbone (Calvet et al., 2014). Recent examples of peptide-based telomerase vaccines include UV1, UCPVax, Vx-001, GX301, GV1001, and PNDV (Vahidi & Zabeti Touchaei, 2024).

Beyond vaccines, recent findings have revealed novel immune-related roles of telomeres, notably the phenomenon of "telomere transfer" between T cells and antigen-presenting cells (APCs) (Lanna et al., 2022). This process begins when naive or central memory T cells with antigen specificity form immunological synapses with APCs (Lanna et al., 2022). During synapse formation, telomeres packaged in vesicles are transferred from short-lived APCs to long-lived T cells. This transfer protects T cells from senescence and promotes a stem-like phenotype, contributing to long-term immune memory (Aureli, Cardenas, Raniolo, & Limongelli, 2023).

Telomere transfer mechanisms may also play a role in tumor biology. Certain antineoplastic agents inhibit ceramidase activity in T cells, preventing telomere acquisition from antigen-presenting cells (APCs) and thereby promoting T cell senescence (Lai et al., 2017). Suppressing this process to induce T cell aging may present a novel strategy for limiting tumor growth. However, this approach has limitations. The potential mutagenic effects of recombinogenic telomeres remain to be fully characterized. Moreover, uncontrolled telomere transfer may trigger inflammatory responses or exacerbate autoimmune diseases if telomeres are misincorporated into recipient T cells (Lanna et al., 2022). To overcome these limitations, bioengineered telomeric vesicles have been developed that can safely deliver telomeres to T cells, prevent senescence, and restore stem-like properties. These vesicles can be customized to fit individual immune profiles, as the direct use of autologous vesicles may not always yield optimal outcomes without engineering support (Lanna, 2025).

Recently, telomeres, long considered passive elements of the genome, have been shown to exhibit dynamic movement within the nucleus (Cho, Dilley, Lampson, & Greenberg, 2014). Advances in super-resolution microscopy have also revealed the presence of extracellular telomeric sequences in body fluids (Lanna et al., 2022). Circulating telomeres hold potential as novel biomarkers for tumor progression, early cancer detection, and monitoring therapeutic responses. Moreover, when paired with tumor-specific antigens from vesicles derived from healthy donor APCs, it may be possible to achieve tumor-specific telomere tracking (Lanna, 2025).

In the context of immunotherapy, telomerase-targeted T cell therapies are also gaining attention. These include chimeric antigen receptor (CAR)-T cells that directly recognize and eliminate cancer cells and immune checkpoint inhibitors

that restore the function of exhausted T cells. Both approaches represent novel directions for telomerase-associated immune modulation (Kirouac et al., 2023; Labanieh & Mackall, 2023; Ledergor et al., 2024).

In conclusion, the newly discovered immunological functions of telomeres not only enhance the potential of telomerase-based strategies in cancer immunotherapy but also illuminate previously unexplored roles of telomere biology in cancer progression.

6. Future perspectives and limitations

Telomerase represents a promising therapeutic target, capable of directly limiting tumor growth and potentially enhancing the efficacy of chemotherapy and immunotherapy. In contexts such as minimal residual disease or chemoprevention, telomerase inhibitors may be particularly effective (J W Shay & Wright, 2001). Developing long-term, low-toxicity, and clinically feasible treatment regimens remains essential.

Telomerase has emerged as a critical target in cancer biology and aging research (Jerry W. Shay & Wright, 2011). Future studies offer numerous opportunities in both clinical and basic science domains. Firstly, the development and optimization of telomerase inhibitors remain of paramount importance (Ganesan & Xu, 2017). Designing potent and specific inhibitors targeting cancer cells, employing peptide nucleic acids (PNA) and modified nucleotides, can enhance therapeutic efficacy while minimizing side effects. Additionally, developing target-specific, encapsulated, and stable formulations of these inhibitors will improve clinical applicability (Baylie et al., 2025; Kageler & Aquilanti, 2024)

The use of telomerase as a diagnostic and prognostic biomarker is another promising research avenue. Techniques such as TRAP, RT-PCR, and immunohistochemistry can be further refined to become faster and more quantitative, enabling routine clinical use in cancer diagnosis and monitoring. Early assessment of telomerase activity is particularly relevant in cancers such as bladder, breast, endometrial, and brain tumors (Loukopoulou, Nikolouzakis, Koliarakis, Vakonaki, & Tsiaoussis, 2024).

The role of telomerase in aging and age-related diseases is also an expanding research area. Extending telomeres in somatic cells may slow cellular aging, prevent age-associated diseases, and improve immune system function. Detailed investigation of the relationships between telomere length, aging, and disease progression is therefore essential (Shao et al., 2020).

Gene therapy and targeted treatment strategies provide another perspective for expanding the clinical applications of telomerase. Utilizing the hTERT promoter

in cancer cell-specific gene therapy systems, targeting cells with telomerase activity, and combining inhibitors with other anti-cancer therapies may offer innovative strategies for future cancer treatment (Liljenfeldt, Dieterich, Dimberg, Mangsbo, & Loskog, 2014; Shou et al., 2025).

Furthermore, a better understanding of telomerase mechanisms, molecular investigation of ALT pathways, and exploration of telomerase's natural nuclear functions will provide guidance for both basic science and clinical applications. Detecting telomerase activity in body fluids such as urine, pleural fluid, bronchoalveolar lavage, and peritoneal fluid could facilitate the development of non-invasive diagnostic methods (Vahidi & Zabeti Touchaei, 2024)

Finally, large-scale clinical studies are essential to evaluate telomerase activity across diverse patient populations and obtain statistically reliable data, which will validate both therapeutic strategies and diagnostic approaches (Loukopoulou et al., 2024; Shou et al., 2025).

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