DOI: 10.5281/zenodo.23594646

CURRENT DEVELOPMENTS AND THEMATIC REVIEWS IN MOLECULAR BIOLOGY AND GENETICS

Editor
Prof. Fuat Bozok, PhD.

Current Developments and Thematic Reviews in Molecular Biology and Genetics

Editor

Prof. Fuat Bozok, PhD.

Publisher
Platanus Publishing®

Editor in Chief

Prof. Fuat Bozok, PhD.

Cover & Interior Design
Platanus Publishing®

The First Edition
October, 2026

ISBN

978-625-6517-44-8

©copyright
All rights reserved. No part of this publication may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopy, or any information storage or retrieval system, without permission from the publisher.

Platanus Publishing®
Address: Natoyolu Cad. Fahri Korutürk Mah. 157/B, 06480, Mamak, Ankara, Turkey.

Phone: +90 312 390 1 118
web: www.platanuspublishing.com
e-mail: platanuskitap@gmail.com



Platanus Publishing®

CONTENTS

CHAPTER 1 ..... *5*

Extracellular Vesicle-Mediated Communication in Breast Cancer Bone Metastasis

Betül Uluca
Betül Yüksel
Ogün Morkoç

CHAPTER 2 ..... *27*

MCL1 Alternative Splicing and Therapeutic Targeting in Cancer from Isoform Regulation to Clinical Translation

Hatice Esenkaya

CHAPTER 3 ..... *45*

Alpha-Interferons: Biology, Recombinant Production, and Delivery Systems

Ogün Morkoç
Betül Uluca

CHAPTER 1

Extracellular Vesicle-Mediated Communication in Breast Cancer Bone Metastasis

Betül Uluca[1]
Betül Yüksel[2]
Ogün Morkoç[3]

[1] Assistant Professor Dr., Department of Molecular Biotechnology, Faculty of Science, Turkish-German University, Istanbul, Türkiye, ORCID: :0000-0001-7139-9010
[2] Department of Molecular Biotechnology, Faculty of Science, Turkish-German University, Istanbul, Türkiye
[3] Research Assistant, Department of Molecular Biotechnology, Faculty of Science, Turkish-German University, Istanbul, Türkiye, ORCID: 0000-0003-0881-8803

Introduction

Breast cancer is the most common cancer among women and accounts for approximately one-third (32%) of all newly diagnosed cancers in women. For localized disease, the 5-year relative survival rate exceeds 99%, whereas this rate falls to approximately 33% in patients with distant metastases (Siegel, Kratzer, Wagle, Sung, & Jemal, 2026).

Breast cancer is also among the malignancies most commonly associated with skeletal involvement. The bone represents the most frequent site of distant spread, and skeletal metastases occur in up to 70% of patients with late-stage breast cancer (Coleman et al., 2020). Such metastases can lead to considerable skeletal morbidity, which is characterized by severe symptoms including hypercalcemia, spinal cord compression, bone pain, and pathological fractures, with substantial consequences for quality of life (Coleman et al., 2020). Metastasis is also a major limitation of effective cancer treatment. In metastatic disease, resistance to therapy remains a major barrier to curative treatment and contributes to increased mortality (X. Chen et al., 2022; Yang et al., 2017).

The selective migration and colonization of cancer cells to specific organs is referred to as organotropism. This distinct trajectory of metastasis is governed by a reciprocal interplay between autonomous tumor-cell traits and the microenvironmental features of the recipient tissues (W. Chen, Hoffmann, Liu, & Liu, 2018; Peinado et al., 2017). Physically, bone serves as a dynamic tissue where formation and resorption are continuously coordinated. Osteoblasts are differentiated from mesenchymal stem cells and mediate de novo bone formation, whereas osteoclasts are multinucleated cells of myeloid origin responsible for the degradation of mineralized bone. Osteocytes are former osteoblasts in the bone matrix and play an important role in coordinating bone remodeling (Raggatt & Partridge, 2010).

The bone microenvironment additionally contains stromal, endothelial, and immune cell populations that interact with bone-resident cells and contribute to tissue homeostasis (Raggatt & Partridge, 2010; Taverna, Giusti, D'Ascenzo, Pizzorno, & Dolo, 2020).

This microenvironment can be modified during breast cancer progression by signals originating from the primary tumor, even before disseminated tumor cells reach the bone. Extracellular vesicles and soluble factors secreted by tumor cells can circulate to distant tissues and modify local cellular and molecular conditions, thereby contributing to the establishment of a microenvironment that may support subsequent metastatic colonization (Peinado et al., 2017; Taverna et al., 2020). The resulting microenvironment can provide signals that support tumor-cell survival and adaptation after dissemination.

Extracellular vesicles (EVs) represent cell-secreted, membrane-enclosed nanoparticles and involved in intercellular communication. They can carry different classes of molecules, including proteins, nucleic acids and lipids, and can transfer these cargo molecules to target recipient cells (Welsh et al., 2024).

In breast cancer, tumor-derived EVs have been implicated in several processes associated with bone metastasis, including pre-metastatic niche formation, bone remodeling, immune modulation, and therapy resistance (Tamura, Yoshioka, Sakamoto, Ichikawa, & Ochiya, 2020; Yang et al., 2017; Yuan et al., 2021).

EV-mediated communication should be considered as one component of the broader network of interactions, linking primary breast cancer cells with the bone microenvironment, alongside soluble factors, extracellular matrix components, and direct cell–cell interactions. The following sections discuss the role of EVs in breast cancer bone metastasis, focusing on the conditioning of the bone microenvironment, tumor-cell homing and colonization, reciprocal tumor–bone communication, dormancy and therapy resistance, and the potential diagnostic and therapeutic applications of EVs.

1. Why Bone? Bone as a Metastatic Soil and Formation of the Pre-Metastatic Bone Niche

Within the framework of Stephen Paget's fundamental 'seed and soil' theory, the targeted migration of mammary carcinoma cells to the bone marrow environment represents a non-random interaction between metastatic progenitors and a permissive niche (Paget, 1889). According to this concept, disseminated tumor cells preferentially grow in organs whose microenvironment provides suitable conditions for their survival and proliferation. This biological compatibility, known as "organotropism," is primarily determined by the complex interaction between tumor cells and the specific microenvironment of the target tissue (W. Chen et al., 2018). As a dynamic tissue that undergoes continuous cellular and extracellular remodeling, bone provides a microenvironment in which tumor cells can interact with resident cells, extracellular matrix components, chemokines, and growth factors (Coleman et al., 2020).

Bone mass in the adult skeleton is maintained through the coordinated activity of numerous cellular factors involved in bone formation and resorption (Raggatt & Partridge, 2010). This process depends mainly on the activities of three major bone cell types. Osteoblasts, which originate from mesenchymal stem cells and develop under the control of the RUNX2 transcription factor, form new bone tissue through the synthesis of type I collagen and mineralization of the extracellular matrix (Bailey, Sims, & Knott, 2002; Ducy, Schinke, & Karsenty, 2000). Osteoclasts are multinucleated cells of myeloid origin specialized in the resorption of mineralized bone matrix (Boyle, Simonet, & Lacey, 2003; Clarke, 2008). Osteocytes are former osteoblasts within the bone matrix. They constitute approximately 90–95% of bone cells and form an extensive mechanosensory network that contributes to the regulation and repair of bone tissue (Bonewald, 2007).

Under physiological conditions, skeletal homeostasis relies on a finely tuned equilibrium that coordinates osteoclastic tissue degradation and osteoblast-mediated matrix deposition (J.-M. Kim, Lin, Stavre, Greenblatt, & Shim, 2020). An important molecular regulator of this balance is the RANKL/OPG system.

Receptor activator of nuclear factor κB ligand (RANKL), produced by osteoblasts, osteocytes, and activated T cells, together with macrophage colony-stimulating factor (M-CSF), is essential for osteoclast differentiation. RANKL binds to receptor activator of nuclear factor κB (RANK) on osteoclast precursor cells and promotes their differentiation and activation (Boyce & Xing, 2008; Hanley, Adachi, Bell, & Brown, 2012; J.-M. Kim et al., 2020). In contrast, osteoprotegerin (OPG) acts as a competitive decoy receptor that sequesters RANKL, preventing its interaction with RANK and thereby suppressing uncontrolled bone resorption (Lacey et al., 1998; Raggatt & Partridge, 2010; Simonet et al., 1997).

The physiological relevance of this RANKL/RANK/OPG axis translates directly into clinical practice; specifically, targeting RANKL via the monoclonal antibody denosumab serves as a therapeutic cornerstone for mitigating both osteoporosis and the osteolytic skeletal events secondary to bone metastases (Pageau, 2009).

This basic organization of bone remodeling is important for understanding why bone can provide a suitable microenvironment for metastatic breast cancer cells. The persistent remodeling of the bone matrix and the activity of osteoblasts and osteoclasts generate a dynamic environment containing numerous signaling molecules that can influence disseminated tumor cells. The bone matrix also contains hydroxyapatite and a variety of matrix-associated signaling molecules, including transforming growth factor beta (TGF-β), insulin-like growth factors (IGFs), and bone morphogenetic proteins (BMPs), which can influence the behavior of both resident and metastatic cells (Juárez & Guise, 2011; Raggatt & Partridge, 2010).

On the other hand, breast cancer is a heterogeneous disease, and the frequency and pattern of distant metastasis differ among molecular subtypes. Luminal tumors show a particular propensity for bone metastasis, whereas basal-like tumors more frequently develop visceral metastases (Kennecke et al., 2010). These differences indicate that bone tropism depends on both tumor-cell properties and the characteristics of the target tissue.

One important molecular mechanism involved in bone homing is the CXCL12/CXCR4 axis. CXCL12 is produced by cells within the bone marrow microenvironment, whereas CXCR4 is expressed by many breast cancer cells. Their interaction can promote tumor-cell migration and retention within the bone marrow niche. The CXCL12/CXCR4 axis is therefore one component of the molecular interactions that contribute to bone homing (Kang et al., 2003; Müller et al., 2001).

The bone microenvironment is therefore not simply a passive target for circulating tumor cells. Its cellular composition, extracellular matrix, chemokine profile, vascular properties, and bone-remodeling activity can influence whether disseminated tumor cells successfully establish a metastatic lesion.

The soil, however, is not necessarily established only after tumor-cell arrival. The primary tumor can modify distant tissues before disseminated tumor cells reach them, generating a pre-metastatic niche (PMN) that may facilitate subsequent metastatic colonization (Peinado et al., 2017). The concept of the pre-metastatic niche (PMN), first described by Kaplan et al. in 2005, refers to the establishment of a tumor-supportive microenvironment at a future metastatic site before the arrival of disseminated tumor cells (Kaplan et al., 2005). Subsequent studies have shown that this process can be influenced by soluble tumor-derived factors and extracellular vesicles released by the primary tumor (X. Chen et al., 2022; Peinado et al., 2017). During PMN formation, tumor-derived soluble factors and EVs can modify vascular and stromal compartments, including endothelial barrier function, and thereby alter the accessibility and local conditions of future metastatic sites. This can be accompanied by the recruitment of bone marrow-derived immunosuppressive cells and the activation of local stromal cells, which contribute to extracellular matrix (ECM) remodeling (X. Chen et al., 2022; Peinado et al., 2017). These coordinated changes prepare the target tissue for subsequent tumor-cell arrival and are considered an important component of the organotropic dissemination of breast cancer to bone.

Following the arrival and subsequent colonization of tumor cells, interactions between these cells and the bone microenvironment can further alter bone remodeling, thereby impacting tumor cell survival and metastatic growth.

2. EV-Mediated Conditioning of the Bone Microenvironment

The formation of a pre-metastatic niche requires coordinated changes in the target tissue that involve multiple cellular compartments. Extracellular vesicles released by breast cancer cells can contribute to this process by transferring proteins and nucleic acids to recipient cells within distant tissues (Giacchi et al., 2026). In the bone microenvironment, experimental studies have provided evidence that tumor-derived EVs can alter the phenotype and activity of bone-forming and bone-resorbing cells (Wu et al., 2021; Yuan et al., 2021). Other studies have identified EV-mediated effects on stromal, endothelial, immune, and metabolic compartments, although the extent to which these mechanisms are specific to bone remains less well established (X. Chen et al., 2022; Fong et al., 2015; Zhou et al., 2014) (Figure 1).

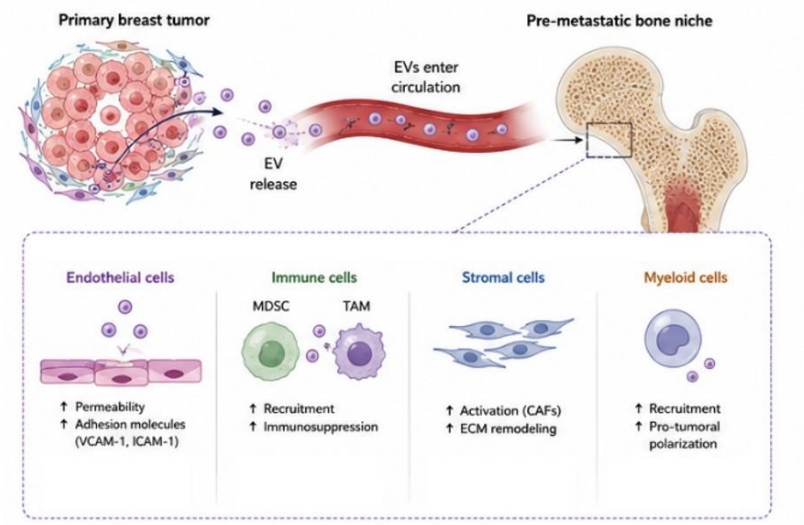


Figure 1. Primary tumor-derived EVs condition the bone microenvironment. Breast cancer-derived EVs can alter endothelial, immune, stromal, and myeloid cell functions within the bone microenvironment, including vascular permeability, immune-cell recruitment, stromal activation, and ECM remodeling. These changes may contribute to tissue conditioning before tumor-cell colonization. *(EV, extracellular vesicle; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; TAM, tumor-associated macrophage; MDSC, myeloid-derived suppressor cell; CAFs, cancer associated fibroblasts)*

2.1 EV-Mediated Reprogramming of Bone-Forming and Bone-Resorbing Cells

Tumor-derived EVs can influence both sides of bone remodeling. Some EV-associated signals promote osteoblastic changes, whereas others enhance osteoclast differentiation and activity. The available evidence indicates that EV-mediated conditioning of the bone microenvironment involves multiple cargoes and recipient-cell populations rather than a single pathway (Figure 2).

One of the most direct examples was reported by Li et al. (2022). EVs released by RUNX2-overexpressing (OE) breast cancer cells were shown to interact with osteoblasts and promote an osteogenic phenotype. EV-associated cadherin 11 (CDH11) contributed to EV recognition and uptake by osteoblasts, whereas integrin α5 (ITGA5) contributed to the osteogenic response. In the experimental model, EVs isolated from RUNX2-OE MDA231 breast cancer cells were administered to mice before MDA231 RUNX2-OE cancer cell inoculation, and EV treatment promoted an osteogenic pre-metastatic niche and subsequent bone colonization by cancer cells (Li, Zhang, Lu, Yue, & Huang, 2022). The study therefore provides evidence linking EV-mediated changes in the bone microenvironment to subsequent tumor-cell colonization. However, the EV exposure was experimentally administered and therefore may not reproduce the continuous endogenous release of EVs from a primary tumor.

Another study showed that EV-associated miR-940 derived from bone-metastatic prostate cancer cells promotes an osteogenic phenotype in mesenchymal stromal cells (Hashimoto et al., 2018). The authors also used MDA-MB-231 breast cancer cells experimentally overexpressing miR-940 and found that EVs from these cells induced an osteoblastic rather than the osteolytic phenotype typically associated with this model. ARHGAP1 and FAM134A were identified as functional targets of miR-940 involved in this response. However, miR-940 was experimentally overexpressed in the MDA-MB-231 cells, and the study does not establish miR-940 as an endogenous EV cargo associated with breast cancer bone metastasis.

EV-mediated communication can also reduce bone-forming activity. Liu et al. (2018) identified miR-218 as an EV-associated miRNA enriched in bone-tropic breast cancer cells. Furthermore, elevated circulating levels of miR-218 were specifically detected in the sera of breast cancer patients with bone metastases, underscoring its clinical relevance as a bone-tropic biomarker**.** EV-associated miR-218 was transferred to preosteoblasts and reduced COL1A1 expression and type I collagen deposition. In vivo, systemic administration of EVs from miR-218 overexpressing MDA-231-miR-218 cells significantly reduced the bone formation marker Procollagen type 1 amino terminal propeptide (P1NP) in mouse serum without altering bone resorption markers (Xuxiang Liu et al., 2018).

The selective loading of miRNAs into EVs may also influence the effects of breast cancer-derived vesicles on bone cells. In a recent study, knockdown of the lysine-specific demethylase 1 (LSD1) in breast cancer cells altered the miRNA composition of secreted exosomes, with a marked reduction in exosomal miR-6881-3p despite little change in the corresponding cellular miRNA levels (Z. Liu et al., 2023).The RNA-binding protein hnRNPA2B1 was identified as a regulator of miR-6881-3p sorting into exosomes. Exosomes with reduced miR-6881-3p inhibited osteoblast differentiation and promoted osteoclast differentiation, whereas restoration of miR-6881-3p reversed these effects. In vivo, exosomes derived from the LSD1 KD breast cancer cells induced osteolysis and promoted bone metastasis, supporting a role for EV miRNA sorting in tumor–bone communication (Z. Liu et al., 2024).

Other studies have expanded the range of EV-associated miRNAs implicated in the regulation of osteoclast lineage cells. Kim et al. (2023) identified miR-494-3p, miR-4508, and miR-6869-5p as exosomal miRNAs that may contribute to the osteoclastogenic effects of MDA-MB-231 cells and RAS-activated breast cancer models. Notably, serum concentrations of these miRNAs were significantly elevated in patients with HER2-positive luminal breast cancer compared with those diagnosed with triple-negative breast cancer. Functional experiments revealed that administration of a miR-494-3p mimic promoted osteoclast differentiation, which was associated with suppression of LGR4, a negative regulator of RANKL-mediated osteoclastogenesis. Conversely, inhibition of miR-494-3p diminished the ability of tumor-derived exosomes to stimulate osteoclast differentiation. The study also demonstrated that miR-494-3p negatively affected BMP2-mediated osteoblast differentiation through targeting

semaphorin 3A (SEMA3A). Consistent with these findings, inhibition of miR-494-3p in vivo attenuated the osteolytic activity induced by breast cancer-derived exosomes (O. Kim et al., 2023). These findings further support the concept that breast cancer EV cargo can contribute to an imbalance in bone remodeling, although the extent to which this occurs during the pre-metastatic phase requires further investigation.

The effects of breast cancer-derived EVs on the regulation of osteoclasts provide another direct mechanism. SCP28 breast cancer cells release EVs enriched in miR-21. These EVs suppresses programmed cell death protein 4 (PDCD4) and promotes NFATc1-associated osteoclast differentiation. Decreasing miR-21 levels in the donor breast cancer cells altered the osteoclast-promoting activity of the resulting EVs, supporting a cargo-dependent mechanism. In vivo, tumor-derived EV administration was associated with increased osteoclast activity and bone loss. Serum exosomal miR-21 was also higher in patients with breast cancer bone metastases than in patients without bone metastases (Yuan et al., 2021).

Another mechanism involves exosomal miR-20a-5p. Guo et al. (2019) showed that MDA-MB-231-derived exosomes transfer miR-20a-5p to bone-marrow-derived macrophages (BMMs), promoting their proliferation and differentiation toward osteoclasts by targeting the SRC kinase signaling inhibitor 1 (SRCIN1). Consistent with these experimental findings, miR-20a-5p was more highly expressed in breast tumor tissues than in adjacent normal tissues and was further increased in tumor samples from patients with bone metastasis. However, the study did not include an in vivo bone-metastasis model and therefore provides mechanistic evidence for cancer cell EV-mediated regulation of osteoclast precursors (Guo et al., 2019).

Exosomal miR-19a provides another mechanistic example in estrogen receptor-positive breast cancer. Wu et al. (2021) showed that EVs released by bone-metastatic MCF7BoM2 cells are taken up by osteoclast precursors, where miR-19a suppresses PTEN and activates NF-κB and AKT signaling, thereby promoting osteoclastogenesis. The study also showed that the tumor-derived soluble factor integrin-binding sialoprotein (IBSP) is required for the observed phenotype, demonstrating that EV-associated and soluble tumor-derived factors cooperate in remodeling the bone microenvironment (Wu et al., 2021).

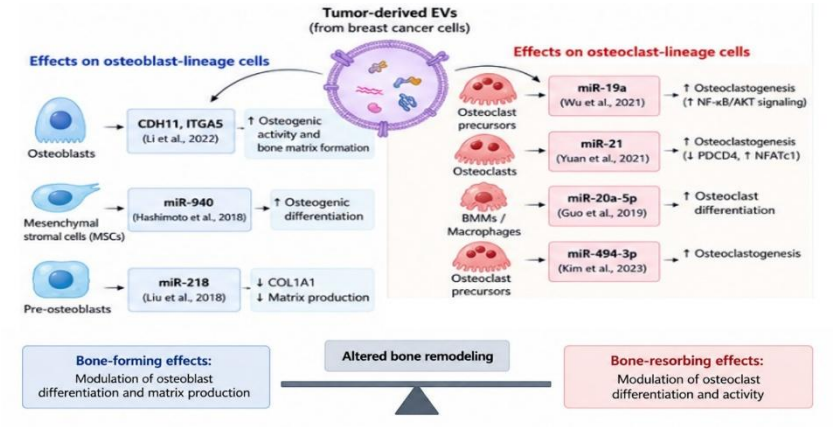


Figure 2. EV-mediated reprogramming of bone forming and bone resorbing cells. Breast cancer-derived EVs can modulate osteoblast-lineage and osteoclast-lineage cells through distinct protein- and miRNA-mediated mechanisms, altering osteogenic differentiation, bone matrix production, osteoclastogenesis, and bone resorption. *(CDH11, cadherin 11; ITGA5, integrin subunit alpha 5; BMMs, bone marrow-derived macrophages; COL1A1, collagen type I alpha 1 chain; PDCD4, programmed cell death protein 4; NFATc1, nuclear factor of activated T cells 1; NF-κB, nuclear factor kappa B; AKT, protein kinase B; miR, microRNA)*

Additional evidence has implicated EV-associated long non-coding RNAs in osteoclast regulation. Xiaoya Liu et al., (2025) reported that breast cancer-derived exosomes contain lncRNA-MIR193BHG, which, upon uptake by osteoclast precursor cells, decreases miR-489-3p levels and relieves miR-489-3p-mediated repression of DNMT3A, thereby promoting osteoclast differentiation. Decreasing MIR193BHG expression in donor breast cancer cells attenuated osteoclast differentiation and bone-resorptive activity in recipient cells and in vivo (Xiaoya Liu et al., 2025).

On the other hand, EV cargo can also have opposing effects on bone metastasis. Although a direct EV-mediated transfer remains to be fully elucidated, Puppo et al. (2024) identified miR-24-2-5p as a highly enriched miRNA within breast cancer cell derived small EVs. increasing miR-24-2-5p levels reduced breast cancer cell aggressiveness and suppressed osteoclast differentiation and bone-metastatic progression in experimental models. These findings indicate that EV-associated miRNAs may have context-dependent effects, and that not all EV-associated miRNAs identified in breast cancer promote bone metastatic progression.

Together, these studies indicate that breast cancer-derived EVs can influence both bone formation and bone resorption, whereas evidence that these EV-mediated changes are sufficient to establish a bone pre-metastatic niche before tumor-cell arrival is more limited and varies among experimental models (Table 1).

Table 1. Representative EV-mediated mechanisms relevant to the breast cancer bone metastatic microenvironment.

Reference	Breast cancer model	EV cargo	Recipient cell	Main effect	Experimental evidence
Li et al., 2022	RUNX2-OE Breast Cancer Cells	CDH11 / ITGA5	Osteoblasts	Induction of an osteogenic pre-metastatic niche	In vitro + in vivo; PMN/bone colonization
Yuan et al., 2021	SCP28 & MDA-MB-231	miR-21	Osteoclasts	↑ osteoclastogenesis	In vitro + in vivo; PMN/bone remodeling + patient serum association
X. Liu et al., 2018	Bone-tropic MDA-MB-231	miR-218	Preosteoblasts / osteoblasts	↓ type I collagen expression and deposition by osteoblasts	In vitro + in vivo; bone niche conditioning + patient serum association
Hashimoto et al., 2018	MDA-MB-231	experimentally OE miR-940	Mesenchymal stromal cells	↑ osteogenic differentiation	In vitro + in vivo tumor-cell model
Wu et al., 2021	MCF7BoM2	miR-19a	Osteoclast precursors	↑ osteoclastogenesis	In vitro + in vivo + patient serum association
Kim et al., 2023	MDA-MB-231 & RAS activated Breast Cancer Cells	miR-494-3p	Osteoclast precursors + Osteoblast precursors	↑ osteoclastogenesis ↓ osteoblastogenesis	In vitro + in vivo + patient serum association
Guo et al., 2019	MDA-MB-231	miR-20a-5p	Bone marrow macrophages / osteoclast precursors	↑ osteoclastogenesis	In vitro + patient **tissue** association
X. Liu et al., 2025	BoM-1833	lncRNA-MIR193BHG	Bone marrow macrophages / osteoclast precursors	↑ osteoclastogenesis	In vitro + in vivo

2.2 EVs as One Component of Pre-Metastatic Niche Formation

One example is EV-associated miR-105, which is released by breast cancer cells and can be transferred to endothelial cells (Zhou et al., 2014). miR-105 targets the tight-junction protein ZO-1 and weakens endothelial barrier integrity, thereby facilitating vascular permeability and tumor-cell dissemination. Although this mechanism provides evidence that breast cancer EVs can modify the vascular barrier during metastatic progression, it has not been established as a bone-specific mechanism of PMN formation.

Metabolic conditioning represents another EV-mediated component of the pre-metastatic program. Breast cancer-derived EV-associated miR-122 reduces glucose uptake by non-tumor cells by suppressing the glycolytic enzyme pyruvate kinase, thereby increasing glucose availability for metastatic tumor cells (Fong et

al., 2015). In a related mechanism, breast cancer-derived EV-associated miR-105 targets MXI1 in cancer-associated fibroblasts, activating MYC signaling and reprogramming glucose and glutamine metabolism to support tumor growth (Yan et al., 2018). These findings support the capacity of breast cancer EVs to reprogram the metabolism of distant or stromal cells, but neither mechanism has been demonstrated specifically in the bone microenvironment.

Exosomes derived from highly metastatic murine breast cancer cells have also been shown to modulate systemic immunity to orchestrate pre-metastatic niche formation in distant organs. For instance, these EVs can suppress CD4+ and CD8+ T-cell proliferation and reduce NK-cell cytotoxicity, thereby creating a permissive, immunosuppressive environment in common metastatic sites such as the lungs (Wen et al., 2016). More direct evidence for immune modulation in the context of breast cancer bone metastasis has been provided by a study of tumor-derived exosomal Intercellular adhesion molecule 1 (ICAM1) (M. Chen, Fu, & Wu, 2024) reported that ICAM1-enriched exosomes released by triple-negative breast cancer cells interacted with LFA-1 on CD8+ T cells, suppressed T-cell activation and proliferation, and promoted an exhausted phenotype. Blocking ICAM1 reduced exosome binding to CD8+ T cells and attenuated the immunosuppressive effect. These findings suggest that tumor-derived EVs may contribute to immune escape in the bone metastatic setting.

Thus, EVs may be considered as one component of the broader PMN-forming network where soluble tumor-derived factors, EVs, extracellular matrix remodeling, vascular changes, immune-cell recruitment, and metabolic alterations can act together to modify the distant microenvironment. However, the mechanisms described above should be distinguished from the bone-specific effects, because many have been demonstrated in models of general metastatic dissemination or in organs other than bone (X. Chen et al., 2022; Peinado et al., 2017). For breast cancer bone metastasis, the most direct EV evidence currently concerns reprogramming of bone-forming and bone-resorbing cell populations, whereas the contribution of EVs to endothelial, immune, and metabolic remodeling in the bone PMN remains less well defined**.**

3. Bone Homing, Colonization, and Reciprocal Tumor–Bone Communication

The formation of a pre-metastatic niche does not itself constitute metastasis. Disseminated tumor cells must enter the circulation, reach the bone marrow, extravasate, and survive within the new microenvironment. Following invasion and intravasation, breast cancer cells can disseminate as circulating tumor cells (CTCs). Their localization to bone is influenced by interactions between tumor-cell surface molecules and chemokines, adhesion molecules, and other signals produced by the bone microenvironment.

The CXCL12/CXCR4 axis is one of the best-characterized mechanisms involved in bone homing. CXCL12, also known as stromal cell-derived factor-1 (SDF-1), is expressed by cells within the bone marrow microenvironment, whereas C-X-C chemokine receptor type 4 (CXCR4) is expressed by many breast

cancer cells. Their interaction can promote migration and retention of tumor cells within the bone marrow niche (Kang et al., 2003; Müller et al., 2001). Tumor cells must subsequently adhere to the bone-marrow vasculature, cross the endothelial barrier, and adapt to the local microenvironment.

Successful bone metastasis therefore requires more than physical entry into the bone. Disseminated tumor cells must establish reciprocal interactions with resident bone and stromal cells that support their survival and subsequent growth.

3.1 Tumor–Bone Communication After Colonization

Once breast cancer cells have established themselves in bone, the interaction becomes reciprocal. Tumor cells release soluble factors that alter bone remodeling, while bone-resident cells and the bone matrix provide signals that can support tumor-cell survival and proliferation.

A central mechanism involves parathyroid hormone-related protein (PTHrP). PTHrP released by breast cancer cells acts on osteoblast-lineage cells and increases receptor activator of nuclear factor κB ligand (RANKL) while reducing osteoprotegerin (OPG). Increased RANKL signaling through receptor activator of nuclear factor κB (RANK) promotes osteoclast differentiation (Käkänen & Mundy, 2003; Thomas et al., 1999). Osteoclast-mediated bone resorption subsequently releases matrix-associated factors, including transforming growth factor beta (TGF-β) and insulin-like growth factor 1 (IGF-1), as well as calcium (Käkänen & Mundy, 2003). These signals can promote tumor-cell proliferation and survival and can stimulate further production of osteolytic factors.

EVs can also contribute to this established tumor–bone interaction. The EV-associated miR-21, miR-19a, and miR-20a-5p mechanisms described above provide examples of tumor-derived EVs that can enhance osteoclast differentiation and activity.

Thus, the osteolytic vicious cycle is maintained by a self-reinforcing interaction in which breast cancer cells stimulate osteoclast-mediated bone resorption, while the resulting release of matrix-associated factors further supports tumor-cell growth and the production of osteolytic signals.

4. Tumor–Stromal EV Crosstalk, Dormancy, and Therapy Resistance

Bone marrow provides a specialized microenvironment in which disseminated breast cancer cells can remain in a non-proliferative or slowly proliferating state for prolonged periods, whereas some cancer cells can proliferate and establish clinically detectable metastatic lesions. Dormant cells are clinically important because their low proliferative activity can reduce their sensitivity to treatments that preferentially target actively dividing cells.

Dormancy is not simply a passive state of tumor-cell inactivity. It is maintained through interactions between disseminated tumor cells and the surrounding bone-marrow microenvironment. EV-mediated communication is one component of this bidirectional interaction. (Ono et al., 2014). demonstrated that bone-marrow MSC-derived exosomes can induce dormant phenotypes in

metastatic breast cancer cells. In the BM2 model, MSC-derived exosomes were taken up by breast cancer cells and reduced proliferation and invasion. miR-23b was identified as an important cargo, and was associated with suppression of myristoylated alanine-rich C-kinase substrate (MARCKS), a protein involved in motility and cell cycling. Increased miR-23b and reduced MARCKS were also detected in metastatic breast cancer cells isolated from patient bone marrow samples (Ono et al., 2014).

A complementary mechanism was reported by (Bliss et al., 2016). Breast cancer cells were shown to modify bone-marrow MSCs and alter the miRNA content of MSC-derived exosomes. Exosomes containing miR-222 and miR-223 promoted quiescence in a subset of breast cancer cells and contributed to chemotherapy resistance. In vitro experiments linked MSC-derived exosomes to breast cancer-cell quiescence, whereas in vivo experiments used MSCs modified with antago-miRs against miR-222/223 to alter the stromal EV-mediated effect. Systemic administration of these modified MSCs increased the sensitivity of dormant breast cancer cells to carboplatin in an immunodeficient mouse model (Bliss et al., 2016).

Together, these studies demonstrate that EV-mediated communication in the metastatic bone marrow is bidirectional. Tumor cells can modify stromal cells and their EV cargo, while stromal cells can subsequently influence disseminated tumor-cell behavior through their EVs. This reciprocal communication provides a potential mechanism by which the bone-marrow microenvironment can regulate both tumor-cell dormancy and treatment response.

This model is particularly relevant to treatment resistance. Dormant cells may be less sensitive to conventional chemotherapy because they are not actively cycling. However, therapy resistance should not be attributed exclusively to EV-mediated dormancy. Tumor-cell intrinsic properties, immune surveillance, stromal signaling, metabolic adaptation, and other features of the bone-marrow niche also contribute to treatment persistence.

The transition from dormancy to metastatic outgrowth is clinically critical. Bone-marrow niche factors, including TGF-β2, BMP7, immune signals, and changes in the extracellular matrix, have been implicated in the maintenance and release of tumor-cell dormancy (Nobre et al., 2021; Risson, Nobre, Maguer-Satta, & Aguirre-Ghiso, 2020). However, the specific contribution of EV cargo to the transition from dormancy to metastatic outgrowth remains insufficiently defined.

5. Clinical Translation, Biomarker Potential, and Current Limitations

The mechanisms described above raise two major translational questions: whether EVs can be used as biomarkers of breast cancer progression and bone metastasis, and whether EV-mediated communication can be therapeutically manipulated.

5.1 EVs as Potential Biomarkers

EVs are attractive candidates for liquid biopsy because they can be detected in blood and contain proteins, lipids, and nucleic acids that may reflect the molecular state of their cells of origin. EV-associated molecules have also been investigated as potential biomarkers of breast cancer progression and bone metastatic disease. For example, increased serum exosomal miR-21 has been reported in patients with breast cancer bone metastasis (Yuan et al., 2021). However, this finding represents a patient-level association rather than clinical validation of an EV-based biomarker. Clinical studies and reviews have highlighted substantial interest in blood-derived EV biomarkers in breast cancer, but also major methodological heterogeneity and the need for prospective validation, standardized EV isolation, and consistent reporting. Currently available EV isolation and characterization methods do not always allow reliable discrimination of breast-cancer-derived EVs from the much larger population of circulating EVs in blood (Schwich & Rebmann, 2018).

5.2 EVs as Therapeutic Targets and Delivery Systems

EV-mediated mechanisms may also be therapeutically targeted in two general ways. First, tumor-derived EV release, uptake, or specific cargo functions could be inhibited. Second, EVs could be engineered or loaded with therapeutic molecules and used as delivery vehicles (X. Chen et al., 2022).

The preclinical studies described above mainly provide examples of the first approach. Inhibition of miR-21, miR-19a, miR-20a-5p, or stromal miR-222/223 signaling can modify osteoclast activity or tumor-cell dormancy in experimental systems (Bliss et al., 2016; Guo et al., 2019; Wu et al., 2021; Yuan et al., 2021). Similarly, targeting stromal EV cargo can alter the response of dormant breast cancer cells to chemotherapy (Bliss et al., 2016). However, most of these approaches remain preclinical. Demonstrating that inhibition of an EV-associated molecule changes a phenotype in cultured cells or a mouse model does not establish clinical efficacy in patients (Table 2).

Table 2. Methodological features and limitations of representative EV studies in breast cancer bone metastasis.

Reference	EV Isolation Method	Functional manipulation	Major limitation
Li et al., 2022	Ultracentrifugation	CDH11 OE&KD / ITGA5 OE&KD	Exogenous EV administration may not reproduce endogenous exposure
Yuan et al., 2021	2x Ultracentrifugation	Donor-cell miR-21 siRNA and KD	Patient data show association, not yet clinically validated
X. Liu et al., 2018	Ultracentrifugation + density gradient ultracentifugation	Stable miR-218 overexpression	Exegenous EVs administered at defined doses; endegenous exposure remains uncertain
Hashimoto et al., 2018	Ultracentrifugation	miR-940 experimental overexpression model	Relies on **experimentally** increased miR-940 overexpression
Wu et al., 2021	Ultracentrifugation +RNAse	miR-19a KO loss-of-function	Soluble IBSP also contributes to the phenotype
Kim et al., 2023	2x Ultracentrifugation	miRNA inhibitor/mimic	Experimental EV treatment may not reflect endogenous EV exposure
Guo et al., 2019	Ultracentrifugation	miRNA OE & inhibitor	No in vivo bone-metastasis validation
X. Liu et al., 2025	Ultracentrifugation	lncRNA-MIR193BHG KD	only TCGA association, no clinical validation

Note: EV, extracellular vesicle; OE, overexpression; KD, knockdown; KO, knockout

5.3 Evidence Quality and Methodological Limitations

Despite the increasing number of studies linking EVs to breast cancer bone metastasis, the strength of the evidence varies substantially between studies. A major limitation is the heterogeneity of EV isolation and characterization procedures. Differential ultracentrifugation, precipitation, size-exclusion chromatography, density-based methods, and combinations of these approaches can produce preparations with different degrees of EV enrichment and different levels of co-isolated non-vesicular material (van Niel, D'Angelo, & Raposo, 2018; Welsh et al., 2024). This methodological heterogeneity can complicate direct comparison between studies and may affect the interpretation of functional experiments. Consequently, a biological effect observed after administration of a

preparation described as “exosomes” cannot automatically be attributed exclusively to EVs or to a specific EV cargo. Throughout this chapter, the term “exosome” is retained when referring to the terminology used in the original study, whereas “EV” is used as the broader term when the specific biogenesis or EV subtype has not been established.

The Minimal Information for Studies of Extracellular Vesicles 2023 (MISEV2023) guidelines emphasize the need to report EV separation and characterization procedures in sufficient detail and to consider the possibility of co-isolated proteins, lipoproteins, nucleic-acid aggregates, and other non-vesicular components. The guidelines also recommend appropriate controls when assigning biological effects to EVs or EV-associated molecules (Welsh et al., 2024). This issue is particularly relevant when comparing studies using exogenously administered EV preparations with studies examining endogenous EV release in spontaneous tumor models. Many experimental studies rely on purified or enriched EV preparations that are added directly to recipient cells or administered to animals at experimentally defined doses. An observed phenotype therefore demonstrates the activity of the administered preparation, but does not by itself establish that the same quantity, composition, or exposure occurs during endogenous tumor progression (van Niel et al., 2022).

Cargo-specific loss-of-function experiments can substantially strengthen causal interpretation. For example, miR-21 knockdown in donor SCP28 breast cancer cells altered the osteoclast-promoting activity of their EVs (Yuan et al., 2021), while depletion of miR-19a in donor breast cancer cells reduced the ability of tumor-derived exosomes to activate osteoclast-related signaling (Wu et al., 2021). Similarly, the CDH11/ITGA5 study connected EV-associated proteins with osteoblast reprogramming and subsequent bone colonization (Li et al., 2022).

Overall, the most convincing studies are those that combine: (i) a well-characterized EV preparation, (ii) evidence for EV uptake by recipient cells, (iii) identification of a candidate cargo, (iv) cargo-specific gain- or loss-of-function experiments, (v) appropriate non-EV controls, and (vi) an in-vivo model that links the cellular phenotype to bone metastasis.

6. Conclusions and Future Directions

EVs provide a mechanistic link between the primary breast tumor, the bone microenvironment, and disseminated tumor cells. Tumor-derived EVs can modify bone-forming and bone-resorbing cells and contribute to the conditioning of the bone microenvironment before metastatic colonization. After tumor-cell arrival, EVs can participate in reciprocal communication that regulates bone remodeling and tumor growth. In the bone marrow, stromal-cell-derived EVs can further influence disseminated tumor cells and contribute to dormancy and therapy resistance. The current evidence therefore supports a model in which EVs function as components of a dynamic, bidirectional communication network involving tumor cells, stromal cells, osteoblasts, osteoclasts, and other components of the bone microenvironment.

The translational potential of EV-based approaches is promising, particularly for liquid-biopsy biomarker development and, at the preclinical stage, for strategies targeting EV cargo or EV-mediated communication. However, methodological heterogeneity, incomplete assessment of EV purity, the predominance of exogenous EV administration in experimental models, limited evidence from endogenous EV systems in breast cancer bone metastasis, and limited clinical validation remain important barriers to translation.

Statement on the Use of Artificial Intelligence Artificial intelligence tools were used to assist in the preparation of illustrative figure included in the chapter, AI-based tools were also used for grammatical editing, and improvement of sentence clarity and flow. The authors reviewed, revised, and verified the generated material and remain fully responsible for the scientific content of the chapter.

References

Bailey, A. J., Sims, T. J., & Knott, L. (2002). Phenotypic expression of osteoblast collagen in osteoarthritic bone: production of type I homotrimer. *The International Journal of Biochemistry & Cell Biology*, *34*(2), 176–182. https://doi.org/10.1016/S1357-2725(01)00107-8

Bliss, S. A., Sinha, G., Sandiford, O. A., Williams, L. M., Engelberth, D. J., Guiro, K., … Rameshwar, P. (2016). Mesenchymal Stem Cell–Derived Exosomes Stimulate Cycling Quiescence an d Early Breast Cancer Dormancy in Bone Marrow. *Cancer Research*, *76*(19), 5832–5844. https://doi.org/10.1158/0008-5472.CAN-16-1092

Bonewald, L. F. (2007). Osteocytes as Dynamic Multifunctional Cells. *Annals of the New York Academy of Sciences*, *1116*(1), 281–290. https://doi.org/10.1196/annals.1402.018

Boyce, B. F., & Xing, L. (2008). Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Archives of Biochemistry and Biophysics*, *473*(2), 139–146. https://doi.org/10.1016/j.abb.2008.03.018

Boyle, W. J., Simonet, W. S., & Lacey, D. L. (2003). Osteoclast differentiation and activation. *Nature*, *423*(6937), 337–342. https://doi.org/10.1038/nature01658

Chen, M., Fu, Z., & Wu, C. (2024). Tumor-derived exosomal ICAM1 promotes bone metastasis of triple-negative breast cancer by inducing CD8+ T cell exhaustion. *The International Journal of Biochemistry & Cell Biology*, *175*, 106637. https://doi.org/10.1016/j.biocel.2024.106637

Chen, W., Hoffmann, A. D., Liu, H., & Liu, X. (2018). Organotropism: new insights into molecular mechanisms of breast cancer metastasis. *Npj Precision Oncology*, *2*(1), 4. https://doi.org/10.1038/s41698-018-0047-0

Chen, X., Feng, J., Chen, W., Shao, S., Chen, L., & Wan, H. (2022). Small extracellular vesicles: from promoting pre-metastatic niche form ation to therapeutic strategies in breast cancer. *Cell Communication and Signaling*, *20*(1), 141. https://doi.org/10.1186/s12964-022-00945-w

Clarke, B. (2008). Normal Bone Anatomy and Physiology. *Clinical Journal of the American Society of Nephrology*, *3*(Supplement_3), S131–S139. https://doi.org/10.2215/CJN.04151206

Coleman, R. E., Croucher, P. I., Padhani, A. R., Clézardin, P., Chow, E., Fallon, M., … Costa, L. (2020). Bone metastases. *Nature Reviews Disease Primers*, *6*(1), 83. https://doi.org/10.1038/s41572-020-00216-3

Ducy, P., Schinke, T., & Karsenty, G. (2000). The Osteoblast: A Sophisticated Fibroblast under Central Surveillance. *Science*, *289*(5484), 1501–1504. https://doi.org/10.1126/science.289.5484.1501

Fong, M. Y., Zhou, W., Liu, L., Alontaga, A. Y., Chandra, M., Ashby, J., … Wang, S. E. (2015). Breast-cancer-secreted miR-122 reprograms glucose metabolism in premet astatic niche to promote metastasis. *Nature Cell Biology*, *17*(2), 183–194. https://doi.org/10.1038/ncb3094

Giacchi, L., Ucci, A., Pucci, E., Lancia, L., Pulcini, F., Delle Monache, S., … Ponzetti, M. (2026). Extracellular Vesicles from Osteotropic Triple-Negative Breast Cancer Cells Transfer miRNAs to Bone Cells Reducing Collagen Expression and Bone Matrix Mineralisation. *Pharmaceutics*, *18*(3), 317. https://doi.org/10.3390/pharmaceutics18030317

Guo, L., Zhu, Y., Li, L., Zhou, S., Yin, G., Yu, G., & Cui, H. (2019). Breast cancer cell-derived exosomal miR-20a-5p promotes the proliferat ion and differentiation of osteoclasts by targeting SRCIN1. *Cancer Medicine*, *8*(12), 5687–5701. https://doi.org/10.1002/cam4.2454

Hanley, D. A., Adachi, J. D., Bell, A., & Brown, V. (2012). Denosumab: mechanism of action and clinical outcomes. *International Journal of Clinical Practice*, *66*(12), 1139–1146. https://doi.org/10.1111/ijcp.12022

Hashimoto, K., Ochi, H., Sunamura, S., Kosaka, N., Mabuchi, Y., Fukuda, T., … Sato, S. (2018). Cancer-secreted hsa-miR-940 induces an osteoblastic phenotype in the bone metastatic microenvironment via targeting ARHGAP1 and FAM134A. *Proceedings of the National Academy of Sciences*, *115*(9), 2204–2209. https://doi.org/10.1073/pnas.1717363115

Juárez, P., & Guise, T. A. (2011). TGF-β in cancer and bone: Implications for treatment of bone metastase s. *Bone*, *48*(1), 23–29. https://doi.org/10.1016/j.bone.2010.08.004

Käkänen, S.-M., & Mundy, G. R. (2003). Mechanisms of osteolytic bone metastases in breast carcinoma. *Cancer*, *97*(S3), 834–839. https://doi.org/10.1002/cncr.11132

Kang, Y., Siegel, P. M., Shu, W., Drobnjak, M., Kakonen, S. M., Cordón-Cardo, C., … Massagué, J. (2003). A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell*, *3*(6), 537–549. https://doi.org/10.1016/S1535-6108(03)00132-6

Kaplan, R. N., Riba, R. D., Zacharoulis, S., Bramley, A. H., Vincent, L., Costa, C., … Lyden, D. (2005). VEGFR1-positive haematopoietic bone marrow progenitors initiate the pr e-metastatic niche. *Nature*, *438*(7069), 820–827. https://doi.org/10.1038/nature04186

Kennecke, H., Yerushalmi, R., Woods, R., Cheang, M. C. U., Voduc, D., Speers, C. H., … Gelmon, K. (2010). Metastatic Behavior of Breast Cancer Subtypes. *Journal of Clinical Oncology*, *28*(20), 3271–3277. https://doi.org/10.1200/JCO.2009.25.9820

Kim, J.-M., Lin, C., Stavre, Z., Greenblatt, M. B., & Shim, J.-H. (2020). Osteoblast-Osteoclast Communication and Bone Homeostasis. *Cells*, *9*(9), 2073. https://doi.org/10.3390/cells9092073

Kim, O., Tran, P., Gal, M., Lee, S., Na, S., Hwangbo, C., & Lee, J.-H. (2023). RAS-stimulated release of exosomal miR-494-3p promotes the osteolytic bone metastasis of breast cancer cells. *International Journal of Molecular Medicine*, *52*(3), 84. https://doi.org/10.3892/ijmm.2023.5287

Lacey, D. L., Timms, E., Tan, H.-L., Kelley, M. J., Dunstan, C. R., Burgess, T., … Boyle, W. J. (1998). Osteoprotegerin Ligand Is a Cytokine that Regulates Osteoclast Differe ntiation and Activation. *Cell*, *93*(2), 165–176. https://doi.org/10.1016/S0092-8674(00)81569-X

Liu, Xiaoya, Ma, R., Wei, F., Wang, M., Jiang, Y., Zheng, P., & Cao, Z. (2025). Tumor-derived exosomal lncRNA-MIR193BHG promotes bone metastasis of br east cancer by targeting the miR-489-3p/DNMT3A signaling axis in osteo clasts. *Journal of Translational Medicine*, *23*(1), 142. https://doi.org/10.1186/s12967-025-06156-4

Liu, Xuxiang, Cao, M., Palomares, M., Wu, X., Li, A., Yan, W., … Wang, S. E. (2018). Metastatic breast cancer cells overexpress and secrete miR-218 to regu late type I collagen deposition by osteoblasts. *Breast Cancer Research*, *20*(1), 127. https://doi.org/10.1186/s13058-018-1059-y

Liu, Z., Xin, B., Zhang, N., An, P., Shi, Y., Yang, J., … Hu, X. (2024). LSD1 modulates the bone metastasis of breast cancer cells through hnRN PA2B1-mediated sorting of exosomal miRNAs. *Cell Death Discovery*, *10*(1), 115. https://doi.org/10.1038/s41420-024-01897-5

Liu, Z., Zhang, N., Xin, B., Shi, Y., Liang, Z., Wan, Y., & Hu, X. (2023). Exosomes from LSD1 knockdown breast cancer cells activate osteoclastog enesis and inhibit osteoblastogenesis. *International Journal of Biological Macromolecules*, *235*, 123792. https://doi.org/10.1016/j.ijbiomac.2023.123792

Li, X.-Q., Zhang, R., Lu, H., Yue, X.-M., & Huang, Y.-F. (2022). Extracellular Vesicle–Packaged CDH11 and ITGA5 Induce the Premetastati c Niche for Bone Colonization of Breast Cancer Cells. *Cancer Research*, *82*(8), 1560–1574. https://doi.org/10.1158/0008-5472.CAN-21-1331

Müller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., … Zlotnik, A. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature*, *410*(6824), 50–56. https://doi.org/10.1038/35065016

Nobre, A. R., Risson, E., Singh, D. K., Di Martino, J. S., Cheung, J. F., Wang, J., … Aguirre-Ghiso, J. A. (2021). Bone marrow NG2+/Nestin+ mesenchymal stem cells drive DTC dormancy via TGF-β2. *Nature Cancer*, *2*(3), 327–339. https://doi.org/10.1038/s43018-021-00179-8

Ono, M., Kosaka, N., Tominaga, N., Yoshioka, Y., Takeshita, F., Takahashi, R., … Ochiya, T. (2014). Exosomes from bone marrow mesenchymal stem cells contain a microRNA th at promotes dormancy in metastatic breast cancer cells. *Science Signaling*, *7*(332). https://doi.org/10.1126/scisignal.2005231

Pageau, S. C. (2009). Denosumab. *MAbs*, *1*(3), 210–215. https://doi.org/10.4161/mabs.1.3.8592

Paget, S. (1889). THE DISTRIBUTION OF SECONDARY GROWTHS IN CANCER OF THE BREAST. *The Lancet*, *133*(3421), 571–573. https://doi.org/10.1016/S0140-6736(00)49915-0

Peinado, H., Zhang, H., Matei, I. R., Costa-Silva, B., Hoshino, A., Rodrigues, G., … Lyden, D. (2017). Pre-metastatic niches: organ-specific homes for metastases. *Nature Reviews Cancer*, *17*(5), 302–317. https://doi.org/10.1038/nrc.2017.6

Puppo, M., Croset, M., Ceresa, D., Valluru, M. K., Canuas Landero, V. G., Hernandez Guadarrama, M., … Clézardin, P. (2024). Protective effects of miR-24-2-5p in early stages of breast cancer bone metastasis. *Breast Cancer Research*, *26*(1), 186. https://doi.org/10.1186/s13058-024-01934-2

Raggatt, L. J., & Partridge, N. C. (2010). Cellular and Molecular Mechanisms of Bone Remodeling. *Journal of Biological Chemistry*, *285*(33), 25103–25108. https://doi.org/10.1074/jbc.R109.041087

Risson, E., Nobre, A. R., Maguer-Satta, V., & Aguirre-Ghiso, J. A. (2020). The current paradigm and challenges ahead for the dormancy of disseminated tumor cells. *Nature Cancer*, *1*(7), 672–680. https://doi.org/10.1038/s43018-020-0088-5

Schwich, E., & Rebmann, V. (2018). The Inner and Outer Qualities of Extracellular Vesicles for Translational Purposes in Breast Cancer. *Frontiers in Immunology*, *9*. https://doi.org/10.3389/fimmu.2018.00584

Siegel, R. L., Kratzer, T. B., Wagle, N. S., Sung, H., & Jemal, A. (2026). Cancer statistics, 2026. *CA: A Cancer Journal for Clinicians*, *76*(1), e70043. https://doi.org/10.3322/caac.70043

Simonet, W. S., Lacey, D. L., Dunstan, C. R., Kelley, M., Chang, M.-S., Lüthy, R., … Boyle, W. J. (1997). Osteoprotegerin: A Novel Secreted Protein Involved in the Regulation o f Bone Density. *Cell*, *89*(2), 309–319. https://doi.org/10.1016/S0092-8674(00)80209-3

Tamura, T., Yoshioka, Y., Sakamoto, S., Ichikawa, T., & Ochiya, T. (2020). Extracellular Vesicles in Bone Metastasis: Key Players in the Tumor Mi croenvironment and Promising Therapeutic Targets. *International Journal of Molecular Sciences*, *21*(18), 6680. https://doi.org/10.3390/ijms21186680

Taverna, S., Giusti, I., D'Ascenzo, S., Pizzorno, L., & Dolo, V. (2020). Breast Cancer Derived Extracellular Vesicles in Bone Metastasis Induct ion and Their Clinical Implications as Biomarkers. *International Journal of Molecular Sciences*, *21*(10), 3573. https://doi.org/10.3390/ijms21103573

Thomas, R. J., Guise, T. A., Yin, J. J., Elliott, J., Horwood, N. J., Martin, T. J., & Gillespie, M. T. (1999). Breast Cancer Cells Interact with Osteoblasts to Support Osteoclast Formation1. *Endocrinology*, *140*(10), 4451–4458. https://doi.org/10.1210/endo.140.10.7037

van Niel, G., D'Angelo, G., & Raposo, G. (2018). Shedding light on the cell biology of extracellular vesicles. *Nature Reviews Molecular Cell Biology*, *19*(4), 213–228. https://doi.org/10.1038/nrm.2017.125

Welsh, J. A., Goberdhan, D. C. I., O'Driscoll, L., Buzas, E. I., Blenkiron, C., Bussolati, B., … Witwer, K. W. (2024). Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. *Journal of Extracellular Vesicles*, *13*(2). https://doi.org/10.1002/jev2.12404

Wen, S. W., Sceneay, J., Lima, L. G., Wong, C. S. F., Becker, M., Krumeich, S., … Möller, A. (2016). The Biodistribution and Immune Suppressive Effects of Breast Cancer–Derived Exosomes. *Cancer Research*, *76*(23), 6816–6827. https://doi.org/10.1158/0008-5472.CAN-16-0868

Wu, K., Feng, J., Lyu, F., Xing, F., Sharma, S., Liu, Y., … Watabe, K. (2021). Exosomal miR-19a and IBSP cooperate to induce osteolytic bone metastas is of estrogen receptor-positive breast cancer. *Nature Communications*, *12*(1), 5196. https://doi.org/10.1038/s41467-021-25473-y

Yang, S., Wang, D., Li, J., Xu, H., Shen, H., Chen, X., … Tang, J. (2017). Predictive role of GSTP1-containing exosomes in chemotherapy-resistant breast cancer. *Gene*, *623*, 5–14. https://doi.org/10.1016/j.gene.2017.04.031

Yan, W., Wu, X., Zhou, W., Fong, M. Y., Cao, M., Liu, J., … Wang, S. E. (2018). Cancer-cell-secreted exosomal miR-105 promotes tumour growth through the MYC-dependent metabolic reprogramming of stromal cells. *Nature Cell Biology*, *20*(5), 597–609. https://doi.org/10.1038/s41556-018-0083-6

Yuan, X., Qian, N., Ling, S., Li, Y., Sun, W., Li, J., … Li, Y. (2021). Breast cancer exosomes contribute to pre-metastatic niche formation an d promote bone metastasis of tumor cells. *Theranostics*, *11*(3), 1429–1445. https://doi.org/10.7150/thno.45351

Zhou, W., Fong, M. Y., Min, Y., Somlo, G., Liu, L., Palomares, M. R., … Wang, S. E. (2014). Cancer-Secreted miR-105 Destroys Vascular Endothelial Barriers to Prom ote Metastasis. *Cancer Cell*, *25*(4), 501–515. https://doi.org/10.1016/j.ccr.2014.03.007

CHAPTER 2

MCL1 Alternative Splicing and Therapeutic Targeting in Cancer from Isoform Regulation to Clinical Translation

***Hatice Esenkaya*[1]**

[1] Department of Molecular Biology and Genetics, Kilis 7 Aralık University, Kilis, Türkiye, ORCID 0000-0001-9357-4975

1. Introduction

Failure to undergo apoptosis allows malignant cells to persist despite intracellular stress and exposure to anticancer treatment. A major point of control in this process lies at the mitochondria, where interactions between anti-apoptotic and pro-apoptotic members of the BCL-2 family determine whether mitochondrial outer-membrane permeabilization proceeds (1). MCL-1 occupies an especially dynamic position within this network. Unlike relatively stable survival proteins, MCL-1 can change rapidly in abundance because both its transcript and protein are subject to tight turnover. Consequently, alterations in cellular signaling or stress can modify MCL-1-dependent survival over relatively short periods (2).

An additional level of regulation arises from alternative processing of MCL1 pre-mRNA. Exon 2 usage is particularly important because it separates two functionally contrasting outcomes. Inclusion of this exon favors production of the longer, anti-apoptotic MCL-1L isoform, whereas exon 2 exclusion generates MCL-1S, a shorter isoform associated with pro-apoptotic activity. Thus, alternative splicing does more than alter transcript structure in this system; it can redirect the functional output of the same gene. The frequent predominance of MCL-1L in malignant cells has therefore created interest in therapeutically modifying MCL1 at both the RNA and protein levels (3).

Therapeutic approaches to MCL-1 can intervene at several stages of its expression and function. One strategy directly disrupts the interaction between MCL-1 and pro-apoptotic BH3-containing proteins by targeting its binding groove (4) . Other approaches act earlier in the pathway by limiting transcription or translation, accelerating protein loss, or modifying MCL1 pre-mRNA processing. These mechanisms are not biologically equivalent. Direct protein inhibition can rapidly liberate apoptotic effectors already sequestered by MCL-1, whereas splice-directed intervention requires alteration of RNA processing followed by changes in the abundance of the existing and newly synthesized isoforms (5) . Such kinetic differences are important when selecting treatment schedules, pharmacodynamic measurements, and combination strategies.

This narrative review places alternative splicing within the broader therapeutic biology of MCL-1. It contains no original dataset and does not reproduce unpublished thesis findings. The focus is the published evidence linking MCL1 isoforms to cancer survival and the opportunities and limitations of targeting this pathway therapeutically.

2. MCL1 Gene Protein and Physiological Functions

MCL1 was originally characterized in the context of myeloid-cell differentiation and is located within the chromosome 1q21 region, which is recurrently altered in human malignancies (6). The major long protein isoform, MCL-1L, functions as an anti-apoptotic component of the BCL-2 family. Its structural organization includes BCL-2 homology regions and a C-terminal membrane-associated segment. These features enable MCL-1L to interact with

selected pro-apoptotic BCL-2-family proteins. By retaining these apoptotic partners, MCL-1L limits activation of the mitochondrial death machinery and thereby increases the threshold required for BAX/BAK-mediated membrane permeabilization and subsequent caspase activation (7).

Another defining property of MCL-1 is the speed with which its cellular abundance can change. Multiple post-translational regulatory mechanisms, including phosphorylation and ubiquitin-dependent pathways, influence its stability and proteasomal removal (8). MCL-1 levels can consequently respond rapidly to alterations in growth signaling and cellular stress. This high turnover rate also creates a therapeutic vulnerability: when transcription or protein synthesis is interrupted, MCL-1 can decline more rapidly than longer-lived anti-apoptotic proteins, potentially exposing cells that depend strongly on its continued production (9).

Physiological dependence on MCL-1 is widespread but not uniform. Genetic studies have demonstrated essential functions in hematopoietic stem and progenitor cells, lymphocytes, neutrophils, cardiomyocytes, and other differentiated tissues (10). MCL-1 also participates in mitochondrial dynamics and metabolism beyond its canonical anti-apoptotic function. These roles create a therapeutic paradox. Cancer cells can be acutely dependent on MCL-1, yet complete or sustained inhibition may also injure normal tissues (11).

The clinically relevant target is therefore not simply high MCL-1 expression. It is a state of functional dependence in which pro-apoptotic proteins are held predominantly by MCL-1 and can be released by disrupting that interaction (12). Expression, gene copy number, BH3 profiling, and the abundance of binding partners may each contribute to identifying this state, but no single marker is sufficient across all tumor types (13).

3. Alternative Splicing and the MCL1 Isoform System

Alternative splicing provides MCL1 with an unusual means of generating opposing biological signals from a single gene. The coding sequence is distributed across three exons, with exon 2 serving as a critical determinant of isoform identity. When exon 2 is retained, the transcript encodes the long MCL-1L isoform. Exclusion of exon 2 instead joins exons 1 and 3 and produces MCL-1S (14). Early characterization of MCL-1S demonstrated that this shorter protein lacks structural regions present in MCL-1L, including the BH1, BH2, and C-terminal transmembrane regions, while retaining a BH3-like region (15,16). The splice event therefore produces considerably more than a quantitative change in MCL1 expression; it alters the structural and functional properties of the resulting protein.

This distinction is central to the therapeutic relevance of MCL1 splicing. MCL-1L primarily supports cell survival, whereas MCL-1S has been associated with promotion of apoptosis and can interact with the long isoform. However, the biological effect of increasing MCL-1S cannot be interpreted independently of cellular context. The relative abundance and stability of both isoforms, together

with the expression of other BCL-2-family members and the existing state of mitochondrial priming, influence the final apoptotic response. From a therapeutic perspective, shifting splicing away from MCL-1L is nevertheless particularly interesting because it could combine loss of a survival signal with simultaneous generation of a product favoring apoptosis (17).

MCL1 processing is not restricted to the MCL-1L/MCL-1S pair. MCL-1ES represents an additional transcript generated through a distinct non-canonical splicing event and has also been associated with pro-apoptotic activity (18). It is important not to treat MCL-1ES and MCL-1S as equivalent isoforms, because they originate from different RNA-processing events and encode structurally distinct products. For therapeutic splice manipulation, most experimental attention has therefore centered on the better-characterized exon 2 decision that determines the balance between MCL-1L and MCL-1S (15).

Isoform measurement requires care. Junction-specific RT-PCR or quantitative PCR establishes RNA output but does not demonstrate corresponding protein production (19). MCL-1L turns over rapidly, and the stability and translation of the shorter products may differ. Antibodies must be validated for isoform specificity. A rigorous study therefore measures RNA and protein over time and connects them to mitochondrial priming, BAX or BAK activation, cytochrome c release, and caspase activity (20).

4. Regulation of MCL1 Exon 2 Selection

Selection of MCL1 exon 2 results from the combined influence of splice-site recognition, cis-regulatory RNA sequences, RNA-binding proteins, transcriptional behavior, and the chromatin environment. These components do not act independently, and their relative contribution can vary between cell types. Consequently, manipulation of the same splicing regulator does not necessarily produce an identical MCL1 isoform response in every experimental system (21). This context dependence is important when considering therapeutic intervention, because MCL1 splicing is better viewed as the output of an interacting regulatory network than as a switch controlled by one factor.

Among the regulatory proteins investigated in this network, SRSF1 has received considerable attention. Experimental studies indicate that SRSF1 can favor production of the long MCL-1 isoform, while reduction of SRSF1 activity can shift processing toward MCL-1S in particular cellular settings. SRSF5 has also been implicated in regulation of MCL1 RNA processing. These findings require careful interpretation because SRSF1 participates in additional aspects of RNA metabolism and can influence protein synthesis and stability as well as splice-site selection. The variable MCL-1 responses observed after SRSF1 perturbation in different cell models illustrate both the potential and the limitations of targeting broadly acting splicing regulators therapeutically (22).

Members of the hnRNP family also contribute. hnRNP F, hnRNP H1, and hnRNP K bind endogenous MCL1 pre-mRNA and alter the MCL-1L/MCL-1S balance in breast-cancer cells. Their effects are interdependent and influenced by

the abundance of other splicing regulators (23). Mapping the exact cis-elements through which these proteins act may permit more selective oligonucleotide strategies than systemic inhibition of the proteins themselves.

Spliceosomal components provide another control point. SF3B1 participates in branch-point recognition, and pharmacological or genetic perturbation of SF3B-associated splicing can alter MCL1 processing. MCL1 is particularly sensitive because its survival product is short-lived and must be continuously replenished. However, core-spliceosome modulation changes many transcripts, so cell death cannot be assigned to MCL1 without rescue experiments and transcriptome-wide analysis (24,25).

Splicing is coupled to transcription and chromatin. HDAC1, HDAC2, SRSF1, and KAT2B have been linked to dynamic histone acetylation around MCL1 exon 2. Their RNA-dependent association with the gene body connects nascent RNA processing to the chromatin environment (26). Signaling pathways, long non-coding RNAs, and phosphorylation of splicing factors add further layers. For example, DGCR5 has been reported to promote SRSF1-mediated production of MCL-1L in esophageal squamous-cell carcinoma (27).

5. MCL1 Dysregulation and Therapeutic Dependence in Cancer

Abnormal MCL-1 activity in cancer can result from several molecular alterations rather than from a single mechanism. Depending on tumor context, increased MCL-1L may reflect amplification of the MCL1 locus, enhanced transcription, greater translational efficiency, increased protein stability, or RNA processing that preferentially generates the long isoform (28). More than one of these processes may operate within the same tumor. Total MCL1 transcript abundance alone therefore provides an incomplete description of the mechanism responsible for maintaining anti-apoptotic MCL-1 activity (2).

Dependence on MCL-1 has been documented particularly in several hematological malignancies, including acute myeloid leukemia and multiple myeloma, but it is also present in subsets of solid cancers. Importantly, this dependence can evolve under therapeutic pressure. Blocking another anti-apoptotic protein such as BCL-2 can alter the distribution of pro-apoptotic partners and increase their sequestration by MCL-1, thereby creating an alternative survival route (29). Similar adaptive responses can occur following kinase inhibition or cytotoxic treatment. MCL-1 may therefore function not only as a constitutive survival protein but also as a compensatory mechanism that enables malignant cells to tolerate therapeutic stress (30).

The MCL-1L/MCL-1S ratio may complement total MCL-1 measurement. High MCL-1L has been associated with treatment resistance and adverse features in several cancer types, including oral cancer (31). Nevertheless, an observational isoform ratio is not a validated universal biomarker. Tumor cells with abundant MCL-1L may remain protected by BCL-XL, whereas cells with moderate MCL-1L can be highly dependent if most BIM and BAK are bound to it (32).

Functional assays are therefore valuable. BH3 profiling measures how close mitochondria are to the apoptotic threshold and which pro-survival protein maintains that state. Co-immunoprecipitation or proximity methods can identify the binding partners sequestered by MCL-1. Integrating these results with isoform-specific RNA and protein measurements may provide a more accurate therapeutic biomarker than any single expression value (12).

6. Direct Therapeutic Inhibition of MCL 1

Direct pharmacological targeting of MCL-1 has largely focused on disrupting its interaction with pro-apoptotic BCL-2-family proteins. Small-molecule BH3 mimetics are designed to bind the hydrophobic interaction site of MCL-1 and compete with proteins that would otherwise remain sequestered there. Successful displacement of these partners can restore the ability of BAX and BAK to drive mitochondrial apoptosis in cells that rely on MCL-1 for survival. Although this concept builds on the therapeutic precedent established by BCL-2 inhibition, MCL-1 presents different structural, pharmacological, and normal-tissue constraints (33).

Several selective inhibitors established strong preclinical activity. S63845 showed that potent MCL-1 inhibition could kill MCL-1-dependent tumor cells and produce antitumor effects in vivo (34). Clinical candidates subsequently included tapotoclax (AMG 176), AZD5991 (35), S64315/MIK665, ABBV-467, and PRT1419 (30). These agents differ in scaffold, binding kinetics, dosing schedule, and pharmacokinetic properties, but all seek to disrupt the same central protein-protein interaction (36).

Clinical translation has been difficult. In a first-in-human study of AZD5991 in relapsed or refractory hematologic malignancies, the compound demonstrated pharmacodynamic activity but limited antitumor efficacy, and cardiac safety signals affected development (37). ABBV-467 was active in tumor models but was also associated with cardiac troponin increases in patients (38). Early studies of other MCL-1 inhibitors likewise emphasized the challenge of separating tumor-cell dependence from normal-tissue dependence.

These results do not invalidate MCL-1 as a target. They show that continuous maximal inhibition may be unsuitable and that schedule, exposure, and patient selection are crucial. Short pulses may release sufficient pro-apoptotic proteins in highly primed cancer cells while allowing recovery in normal tissue. Combination therapy may reduce the dose required for each agent. A clinically useful MCL-1 inhibitor will need a pharmacologic window defined by functional biomarkers rather than by potency alone (11).

7. Therapeutic Redirection of MCL1 Splicing

Manipulating MCL1 pre-mRNA processing provides an alternative to directly inhibiting the mature MCL-1L protein. In a splice-directed strategy, an oligonucleotide is designed to interfere with recognition of a selected splice site or regulatory RNA sequence involved in exon 2 usage. Successful redirection toward exon 2 exclusion would be expected to alter the isoform distribution in

two complementary directions: synthesis of the anti-apoptotic MCL-1L product would decline while production of the shorter MCL-1S isoform would increase (39). This dual molecular consequence distinguishes splice redirection from approaches that simply decrease total MCL-1 abundance.

Experimental studies have established that MCL1 pre-mRNA is accessible to sequence-specific manipulation. Antisense morpholino approaches have shifted MCL1 processing toward MCL-1S in cancer-cell models, with the RNA response accompanied by changes in protein expression and increased apoptotic activity (40). Related work has further supported the broader principle that deliberate modification of MCL1 splice choice can interfere with malignant-cell growth (41). These studies provide proof of concept that MCL1 is accessible to sequence-directed splice control. Together, these observations provide a mechanistic foundation for considering exon 2 selection itself as a therapeutic target rather than viewing alternative splicing solely as a biomarker of MCL1 activity.

The greatest advantage of splice-switching oligonucleotides is programmability. A sequence can be selected to block an exon boundary, enhancer, silencer, or splice-factor binding site without changing the genome. Steric-blocking chemistries can alter splice choice without degrading the transcript through RNase H. Multiple candidate sequences can be screened across exon 2 and its flanking introns to identify positions that produce a strong and reproducible isoform shift (42).

The principal barriers are delivery and selectivity at the tissue level. Systemic oligonucleotides often accumulate in liver and kidney, while many cancers require efficient uptake into poorly perfused tissue. Endosomal trapping reduces the fraction that reaches nuclear pre-mRNA. Conjugates, nanoparticles, or local delivery may improve exposure, but each platform adds toxicity and manufacturing considerations (43).

A splice-switching program should also prove functional causality. Rescue with an intronless MCL-1L construct can test whether loss of the long isoform mediates cell death. Selective suppression of MCL-1S can determine whether the short isoform contributes independently. Global RNA sequencing is needed to detect sequence-dependent off-target splicing, and studies in normal cells are essential because a highly efficient MCL1 splice switch may reproduce the normal-tissue liabilities of direct protein inhibition (39).

8. Indirect Strategies to Reduce MCL 1 Activity

The rapid turnover of MCL-1L creates opportunities to interfere with its expression before the protein reaches its functional state (2). One approach is to restrict transcription. CDK9 contributes to productive transcriptional elongation through regulation of RNA polymerase II, and its inhibition can disproportionately affect proteins whose continued expression depends on rapidly renewed transcripts. MCL-1 is among the proteins susceptible to this effect. Reduction of MCL-1 through CDK9 inhibition may consequently restore apoptotic sensitivity in some treatment-resistant settings, although the strategy is

inherently broader than direct MCL1 targeting because numerous genes depend on the same transcriptional machinery (44).

MCL-1 abundance can also be modified at the level of translation. Pathways involving PI3K, AKT, mTOR, and cap-dependent protein synthesis contribute to maintaining MCL-1 production, and interference with these pathways may lower the available survival protein and facilitate apoptosis. The difficulty is specificity: these signaling systems regulate many cellular proteins and contain multiple feedback mechanisms, making it necessary to distinguish an MCL-1-mediated response from wider effects on cellular physiology (45). RNA-binding proteins and regulatory microRNAs provide additional routes through which MCL1 transcript stability or translation may be altered, although their broader transcriptomic functions create similar concerns (46).

Protein turnover provides a third route. MCL-1 abundance is controlled by phosphorylation, ubiquitin ligases, deubiquitinases, and the proteasome. Therapeutic strategies can promote degradation or prevent stabilization (47). Targeted protein degradation is conceptually attractive because one degrader molecule may catalytically remove multiple MCL-1 molecules, but the E3 ligase distribution, mitochondrial localization, and normal-tissue dependence must be considered. Most MCL-1 degraders remain preclinical (48).

Indirect strategies can be effective even when they do not generate MCL-1S. They should be categorized according to mechanism: reduction of transcription, translation, stability, or binding function. This classification helps select appropriate pharmacodynamic markers and prevents an MCL1-associated phenotype from being misrepresented as a splice-specific effect (49).

9. Combination Therapy and Resistance

MCL-1 rarely operates alone. Tumor cells distribute pro-apoptotic proteins among MCL-1, BCL-2, and BCL-XL, and this distribution can change rapidly after treatment. Combining inhibitors can therefore produce synergy by preventing a BH3 protein released from one pro-survival partner from being captured by another (50).

The interaction between MCL-1 and BCL-2 illustrates why apoptotic dependencies need to be considered as a network rather than as isolated therapeutic targets. In acute myeloid leukemia, resistance to venetoclax may be accompanied by greater reliance on MCL-1. Experimental strategies that either inhibit MCL-1 directly or decrease its abundance through CDK9-dependent mechanisms have restored apoptotic responses in venetoclax-resistant models (51). Comparable principles are being investigated in other hematological malignancies and selected solid tumors, although simultaneous disruption of multiple survival proteins may also narrow the margin between antitumor activity and normal-tissue toxicity.

The order and duration of treatment may be as important as the choice of agents. A therapy that first increases mitochondrial priming can increase the amount of activated pro-apoptotic protein restrained by MCL-1. Subsequent,

transient disruption of MCL-1 may then be sufficient to cross the apoptotic threshold. In contrast, prolonged or poorly sequenced inhibition may permit adaptation through BCL-XL or other stress-response pathways (11). Combination design should therefore be based on changes in apoptotic dependence over time rather than on the assumption that simultaneous maximal inhibition will necessarily provide the greatest therapeutic benefit.

Resistance can arise through increased expression of another pro-survival BCL-2-family protein, reduced BAX or BAK function, altered mitochondrial priming, mutation or stabilization of MCL-1, and changes in drug transport. MCL-1 inhibitors can paradoxically increase measurable MCL-1 protein by stabilizing the bound protein even while blocking its function. Protein abundance during therapy must therefore be interpreted together with target occupancy and partner release (52).

10. Safety and Therapeutic Window

The central challenge in MCL-1 therapy is on-target toxicity. MCL-1 is required for survival and function in several normal tissues. Cardiac findings in early clinical programs drew particular attention, but hematologic and hepatic effects also require monitoring. The risk depends on the depth and duration of inhibition, the pharmacokinetic profile, combination partners, and the reserve capacity of the patient (17).

Cardiac troponin elevation does not by itself define the mechanism or reversibility of injury, but it is a clinically important warning. Preclinical models should assess cardiomyocyte survival, contractility, mitochondrial function, and recovery after drug withdrawal. Species differences in inhibitor binding can complicate toxicology, making humanized models and human induced-pluripotent-stem-cell-derived cardiomyocytes valuable additions (53).

Several design principles may widen the window. Intermittent dosing can exploit the rapid apoptotic response of highly primed tumor cells while limiting sustained exposure in normal tissue. Tumor-targeted delivery or an E3 ligase with restricted tissue expression could increase selectivity. Biomarker-based enrollment can avoid exposing patients whose tumors are not functionally MCL-1 dependent. Combinations should aim to reduce dose, not simply maximize pathway blockade (54).

Splice-switching approaches will not automatically eliminate safety concerns. A durable reduction of MCL-1L in normal cells could be more difficult to reverse than a short inhibitor infusion. Tissue distribution may nevertheless produce a different safety profile, especially if delivery is tumor-selective. Comparative studies should measure duration of target suppression and normal-tissue recovery rather than assuming one modality is intrinsically safer (41).

11. Biomarkers and Clinical Translation

Clinical application of an MCL-1-directed therapy requires more information than whether a tumor simply expresses the target. Amplification of MCL1 and

measurement of total RNA or protein may help characterize a tumor, but none of these measurements alone establishes that survival is functionally dependent on MCL-1 (17). Assessing the relative abundance of MCL-1L and MCL-1S adds information about RNA processing, although the isoform ratio still cannot reveal which apoptotic proteins are being restrained by MCL-1 or how readily mitochondrial apoptosis can be triggered (39).

Functional approaches can help address this limitation. BH3 profiling evaluates mitochondrial apoptotic dependence, while its dynamic form examines how treatment alters mitochondrial priming before extensive cell death has occurred (55). These measurements can be complemented by assays that examine MCL-1 interactions with proteins such as BIM or BAK. In hematological malignancies, serial analysis of accessible tumor-containing specimens may additionally provide a practical means of following pharmacodynamic responses during treatment (56).

Splice-directed intervention requires a more specific biomarker framework. Measurement should establish the extent of exon 2 inclusion or exclusion and quantify the individual MCL-1L and MCL-1S transcripts rather than relying exclusively on a ratio. Whenever technically feasible, these RNA measurements should be paired with isoform-resolved protein analysis and downstream indicators of mitochondrial apoptosis. This is particularly important because an altered transcript ratio does not necessarily establish increased production of MCL-1S protein, and changes in RNA processing may occur before existing MCL-1L protein has been removed (39).

Clinical development should also account for intratumoral heterogeneity. A minority subpopulation with strong MCL-1 dependence may determine relapse even when the average tumor shows only moderate expression. Single-cell RNA analysis, functional profiling, and spatial assessment of the microenvironment may reveal protected compartments that are missed by bulk measurements (57).

12. Experimental Standards for MCL1 Therapeutic Studies

Mechanistic studies should establish a linked sequence of events. For a direct inhibitor, this means target binding, displacement of pro-apoptotic partners, BAX/BAK activation, mitochondrial permeabilization, and cell death. For a splice-switching agent, it means altered exon 2 choice, isoform-resolved protein change, altered BCL-2-family interactions, and apoptosis. A viability assay alone cannot distinguish these routes (58).

Genetic controls are essential. MCL1 knockout or knockdown can demonstrate dependence, but acute pharmacological inhibition may differ from long-term gene loss. Rescue with inhibitor-resistant MCL-1 or intronless MCL-1L can test target specificity. BAX/BAK-deficient models determine whether death proceeds through the expected mitochondrial pathway (59).

Studies should report concentration and exposure time, free rather than nominal drug levels when possible, biological replicate definitions, and the timing of biomarker collection relative to cell death. For oligonucleotides,

complete sequence and chemical modification information are required, together with uptake and nuclear-delivery measurements. For combinations, dose-response matrices and models of additivity should replace conclusions based on one concentration pair.

Normal-cell comparators are necessary from the beginning. Cardiomyocytes, hematopoietic progenitors, hepatocytes, and other relevant cells should be tested under exposures that match antitumor experiments. Animal efficacy without matched pharmacokinetic and toxicity data provides limited information about the clinical window.

13. Future Directions

The future of MCL-1 therapy will likely depend on precision rather than stronger universal inhibition. Short, biomarker-guided exposure may be preferable to continuous dosing. Tumor-targeted delivery and tissue-selective degradation could separate malignant from physiological MCL-1 dependence. Functional biomarkers should be incorporated early rather than added after dose selection.

MCL1 splicing deserves further development as a therapeutic axis. Systematic oligonucleotide tiling across exon 2 and the flanking introns can define the most efficient regulatory sites. Patient-derived organoids and xenografts can test delivery and isoform response in heterogeneous tissue. Single-cell analysis can establish whether resistant cells fail to receive the oligonucleotide, fail to switch splicing, or remain protected by another BCL-2-family protein.

A second opportunity is rational combination of partial splice redirection with lower-dose direct inhibition or BCL-2 targeting. Reducing MCL-1L synthesis may decrease the exposure needed from a BH3 mimetic. However, combinations should be guided by mitochondrial dependence and normal-tissue recovery, not by the assumption that more complete pathway inhibition is always better.

Finally, isoform biology should remain central. MCL-1L and MCL-1S are not interchangeable readouts of total MCL1 expression. Future trials and translational studies should measure the relevant RNA and protein species separately and determine which change predicts clinical response.

14. Conclusion

MCL1 occupies an unusual position at the intersection of apoptosis regulation, RNA processing, and therapeutic resistance. Its importance in cancer cannot be explained solely by the amount of MCL-1 protein present in a cell. Alternative processing of MCL1 pre-mRNA adds another regulatory dimension by generating isoforms with contrasting biological properties, making exon 2 selection particularly relevant to the survival-versus-death decision.

The accumulated experimental evidence also indicates that MCL-1 can be approached therapeutically at several levels. Direct inhibitors demonstrate that MCL-1-dependent cancer cells can be pharmacologically challenged, while their clinical development has simultaneously highlighted the consequences of

disrupting an apoptotic regulator that is also required by normal tissues. RNA-directed strategies offer a different opportunity by attempting to reshape isoform production before the mature protein is generated. Approaches affecting transcription, translation, and protein stability further illustrate the number of regulatory points through which MCL-1 activity can potentially be modified.

For future therapeutic development, the critical question is therefore unlikely to be simply how strongly MCL-1 can be inhibited. Greater emphasis should be placed on identifying tumors in which MCL-1 represents a genuine survival dependency and determining how that dependency changes during treatment. In splice-directed studies in particular, demonstration of exon-specific RNA changes should be connected to corresponding protein alterations, mitochondrial responses, and ultimately selective cancer-cell death. Combining such mechanistic measurements with appropriately timed treatment and selective delivery may provide a more realistic route for exploiting MCL1 biology while limiting effects on normal tissues.

Acknowledgements

The author gratefully acknowledges Professor Cyril Dominguez and Professor Ian C. Eperon for their doctoral supervision and scientific guidance, which provided the academic foundation for this review. This chapter was developed in relation to the author's doctoral research on MCL1 alternative splicing. No original or unpublished data from the doctoral thesis are presented in this chapter.

Funding

No specific funding was received for the preparation of this chapter.

Conflict of Interest

The author declares no conflict of interest.

Data Availability

No original data were generated or analysed for this narrative review.

References:

1. Sharma A, Boise LH, Shanmugam M. Cancer metabolism and the evasion of apoptotic cell death. Cancers. MDPI AG; 2019. doi:10.3390/cancers11081144

2. Tantawy SI, Timofeeva N, Sarkar A, Gandhi V. Targeting MCL-1 protein to treat cancer: opportunities and challenges. Frontiers in Oncology. Frontiers Media SA; 2023. doi:10.3389/fonc.2023.1226289

3. Hartman ML. Tipping the balance of cell death: alternative splicing as a source of MCL-1S in cancer. Cell Death and Disease. Springer Nature; 2024. doi:10.1038/s41419-024-07307-z PubMed PMID: 39695189.

4. Wright T, Turnis ME, Grace CR, Li X, Brakefield LA, Wang YD, et al. Anti-apoptotic MCL-1 promotes long-chain fatty acid oxidation through interaction with ACSL1. Mol Cell. 2024 Apr 4;84(7):1338-1353.e8. doi:10.1016/j.molcel.2024.02.035 PubMed PMID: 38503284.

5. Sancho M, Leiva D, Lucendo E, Orzáez M. Understanding MCL1: from cellular function and regulation to pharmacological inhibition. FEBS Journal. John Wiley and Sons Inc; 2022. p. 6209–34. doi:10.1111/febs.16136 PubMed PMID: 34310025.

6. Wei AH, Roberts AW, Spencer A, Rosenberg AS, Siegel D, Walter RB, et al. Targeting MCL-1 in hematologic malignancies: rationale and progress. Blood Reviews. Elsevier Ltd; 2020. p. 100672. doi:10.1016/j.blre.2020.100672.

7. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, et al. Molecular mechanisms of cell death: Recommendations of the Nomenclature Committee on Cell Death 2018. Cell Death and Differentiation. Nature Publishing Group; 2018. p. 486–541. doi:10.1038/s41418-017-0012-4 PubMed PMID: 29362479.

8. Skliutė G, Kuklytė E, Žučenka A, Borutinskaitė VV, Navakauskienė R. Effects of BCL-2 and MCL-1 Inhibition on Apoptotic and Transcriptional Profiles in Acute Myeloid Leukemia. Medicina (Lithuania). 2026 Jul 1;62(7). doi:10.3390/medicina62071425

9. Senichkin V V., Streletskaia AY, Gorbunova AS, Zhivotovsky B, Kopeina GS. Saga of Mcl-1: regulation from transcription to degradation. Cell Death and Differentiation. Springer Nature; 2020. p. 405–19. doi:10.1038/s41418-019-0486-3 PubMed PMID: 31907390.

10. Chin HS, Fu NY. Physiological Functions of Mcl-1: Insights From Genetic Mouse Models. Frontiers in Cell and Developmental Biology. Frontiers Media S.A.; 2021. doi:10.3389/fcell.2021.704547

11. Mohiuddin M. Targeting MCL-1 to Overcome Therapeutic Resistance and Improve Cancer Mortality. Health Science Reports. John Wiley and Sons Inc; 2025. doi:10.1002/hsr2.71390

12. Duan L, Maki CG. Potential biomarkers for MCL1 inhibitor sensitivity. Cell Signal. 2024.

13. Deng J, Carlson N, Takeyama K, Dal Cin P, Shipp M, Letai A. BH3 Profiling Identifies Three Distinct Classes of Apoptotic Blocks to Predict Response to ABT-737 and Conventional Chemotherapeutic Agents. Cancer Cell. 2007 Aug 14;12(2):171–85. doi:10.1016/j.ccr.2007.07.001 PubMed PMID: 17692808.

14. Elshazly AM, Li J, Yang GY, Radhakrishnan SK. Mcl1 as a Molecular Switch Linking Inflammatory Bowel Diseases to Colorectal Tumorigenesis. Biomolecules. Multidisciplinary Digital Publishing Institute (MDPI); 2026. doi:10.3390/biom16050694 PubMed PMID: 42194042.

15. Kim JH, Bae J. MCL-1ES induces MCL-1L-dependent BAX- And BAK-independent mitochondrial apoptosis. PLoS One. 2013 Nov 18;8(11). doi:10.1371/journal.pone.0079626 PubMed PMID: 24260268.

16. Bae J, Leo CP, Sheau Yu Hsu, Hsueh AJW. MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain. Journal of Biological Chemistry. 2000 Aug 18;275(33):25255–61. doi:10.1074/jbc.M909826199 PubMed PMID: 10837489.

17. Wang H, Guo M, Wei H, Chen Y. Targeting MCL-1 in cancer: current status and perspectives. Journal of Hematology and Oncology. BioMed Central Ltd; 2021. doi:10.1186/s13045-021-01079-1 PubMed PMID: 33883020.

18. Warren CFA, Wong-Brown MW, Bowden NA. BCL-2 family isoforms in apoptosis and cancer. Cell Death and Disease. Nature Publishing Group; 2019. doi:10.1038/s41419-019-1407-6 PubMed PMID: 30792387.

19. Vandenbroucke II, Vandesompele J, De Paepe A, Messiaen L. Quantification of splice variants using real-time PCR. Nucleic Acids Research. 2001.

20. Tantawy SI, Sarkar A, Hubner S, Tan Z, Wierda WG, Eldeib A, et al. Mechanisms of MCL-1 Protein Stability Induced by MCL-1 Antagonists in B-Cell Malignancies. Clinical Cancer Research. 2023 Jan 15;29(2):446–57. doi:10.1158/1078-0432.CCR-22-2088 PubMed PMID: 36346691.

21. Zhu ZM, Wu XM, Hu Y, Bian XL, Wang YQ, Zhu QN. Alternative Splicing: Molecular Mechanisms, Biological Functions, Diseases, and Potential Therapeutic Targets. MedComm. John Wiley and Sons Inc; 2025. doi:10.1002/mco2.70545

22. Gautrey HL, Tyson-Capper AJ. Regulation of Mcl-1 by SRSF1 and SRSF5 in Cancer Cells. PLoS One. 2012 Dec 17;7(12). doi:10.1371/journal.pone.0051497 PubMed PMID: 23284704.

23. Tyson-Capper A, Gautrey H. Regulation of Mcl-1 alternative splicing by hnRNP F, H1 and K in breast cancer cells. RNA Biol. 2018 Dec 2;15(12):1448–57. doi:10.1080/15476286.2018.1551692 PubMed PMID: 30468106.

24. Darman RB, Seiler M, Agrawal AA, Lim KH, Peng S, Aird D, et al. Cancer-Associated SF3B1 Hotspot Mutations Induce Cryptic 3' Splice Site Selection through Use of a Different Branch Point. Cell Rep. 2015 Nov 3;13(5):1033–45. doi:10.1016/j.celrep.2015.09.053 PubMed PMID: 26565915.

25. Zhang J, Ali AM, Lieu YK, Liu Z, Gao J, Rabadan R, et al. Disease-Causing Mutations in SF3B1 Alter Splicing by Disrupting Interaction with SUGP1. Mol Cell. 2019 Oct 3;76(1):82-95.e7. doi:10.1016/j.molcel.2019.07.017 PubMed PMID: 31474574.

26. Khan DH, Gonzalez C, Cooper C, Sun JM, Chen HY, Healy S, et al. RNA-dependent dynamic histone acetylation regulates MCL1 alternative splicing. Nucleic Acids Res. 2014 Feb;42(3):1656–70. doi:10.1093/nar/gkt1134 PubMed PMID: 24234443.

27. Duan Y, Jia Y, Wang J, Liu T, Cheng Z, Sang M, et al. Long noncoding RNA DGCR5 involves in tumorigenesis of esophageal squamous cell carcinoma via SRSF1-mediated alternative splicing of Mcl-1. Cell Death Dis. 2021 Jun 1;12(6). doi:10.1038/s41419-021-03858-7 PubMed PMID: 34099633.

28. Widden H, Placzek WJ. The multiple mechanisms of MCL1 in the regulation of cell fate. Communications Biology. Nature Research; 2021. doi:10.1038/s42003-021-02564-6 PubMed PMID: 34475520.

29. Mahendra Kadia T, Kantarjian HM, Konopleva M. Myeloid cell leukemia-1 dependence in acute myeloid leukemia: a novel approach to patient therapy [Internet]. 2019. Available from: www.oncotarget.com

30. Xiang W, Yang CY, Bai L. MCL-1 inhibition in cancer treatment. OncoTargets and Therapy. Dove Medical Press Ltd.; 2018. p. 7301–14. doi:10.2147/OTT.S146228

31. Palve V, Mallick N, Ghaisas G, Kannan S, Teni T. Overexpression of Mcl-1L splice variant is associated with poor and prognosis chemoresistance in oral cancers. PLoS One. 2014 Nov 19;9(11). doi:10.1371/journal.pone.0111927 PubMed PMID: 25409302.

32. Hagenbuchner J, Kiechl-Kohlendorfer U, Obexer P, Ausserlechner MJ. A novel Mcl1 variant inhibits apoptosis via increased Bim sequestration. Oncotarget [Internet]. 2013. Available from: www.impactjournals.com/oncotarget

33. Hagenbuchner J, Kiechl-Kohlendorfer U, Obexer P, Ausserlechner MJ. A novel Mcl1 variant inhibits apoptosis via increased Bim sequestration. Oncotarget [Internet]. 2013. Available from: www.impactjournals.com/oncotarget

34. Kotschy A, Szlavik Z, Murray J, Davidson J, Maragno AL, Le Toumelin-Braizat G, et al. The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models. Nature. 2016;538(7626):477–82. doi:10.1038/nature19830 PubMed PMID: 27760111.

35. Tron AE, Belmonte MA, Adam A, Aquila BM, Boise LH, Chiarparin E, et al. Discovery of Mcl-1-specific inhibitor AZD5991 and preclinical activity in multiple myeloma and acute myeloid leukemia. Nat Commun. 2018 Dec 1;9(1). doi:10.1038/s41467-018-07551-w PubMed PMID: 30559424.

36. Pal P, Zhang P, Poddar SK, Zheng G. Patent landscape of inhibitors and PROTACs of the anti-apoptotic BCL-2 family proteins. Expert Opinion on Therapeutic Patents. Taylor and Francis Ltd.; 2022. p. 1003–26. doi:10.1080/13543776.2022.2116311 PubMed PMID: 35993382.

37. Desai P, Lonial S, Cashen A, Kamdar M, Flinn I, O'Brien S, et al. A Phase 1 First-in-Human Study of the MCL-1 Inhibitor AZD5991 in Patients with Relapsed/Refractory Hematologic Malignancies. Clinical Cancer Research. 2024 Nov 1;30(21):4844–55. doi:10.1158/1078-0432.CCR-24-0028 PubMed PMID: 39167622.

38. Yuda J, Will C, Phillips DC, Abraham L, Alvey C, Avigdor A, et al. Selective MCL-1 inhibitor ABBV-467 is efficacious in tumor models but is associated with cardiac troponin increases in patients. Communications Medicine. 2023 Dec 1;3(1). doi:10.1038/s43856-023-00380-z

39. Esenkaya H. Investigation of Mcl-1 alternative splicing regulation by GQC-05, a putative G-quadruplex ligand. 2021. doi.org:10.25392/leicester.data.19144559

40. Shieh JJ, Liu KT, Huang SW, Chen YJ, Hsieh TY. Modification of alternative splicing of Mcl-1 pre-mRNA using antisense morpholino oligonucleotides induces apoptosis in basal cell carcinoma cells. Journal of Investigative Dermatology. 2009;129(10):2497–506. doi:10.1038/jid.2009.83 PubMed PMID: 19369967.

41. Shieh JJ, Liu KT, Huang SW, Chen YJ, Hsieh TY. Modification of alternative splicing of Mcl-1 pre-mRNA using antisense morpholino oligonucleotides induces apoptosis in basal cell carcinoma cells. Journal of Investigative Dermatology. 2009;129(10):2497–506. doi:10.1038/jid.2009.83 PubMed PMID: 19369967.

42. Havens MA, Hastings ML. Splice-switching antisense oligonucleotides as therapeutic drugs. Nucleic Acids Research. Oxford University Press; 2016. p. 6549–63. doi:10.1093/nar/gkw533 PubMed PMID: 27288447.

43. Juliano RL. The delivery of therapeutic oligonucleotides. Nucleic Acids Research. Oxford University Press; 2016. p. 6518–48. doi:10.1093/nar/gkw236 PubMed PMID: 27084936.

44. Shen Y li, Wang Y mao, Zhang Y xin, Ma S jie, Yang L he, Zhao C guang, et al. Targeting cyclin-dependent kinase 9 in cancer therapy. Acta Pharmacologica Sinica. Springer Nature; 2022. p. 1633–45. doi:10.1038/s41401-021-00796-0 PubMed PMID: 34811514.

45. Lopiccolo J, Blumenthal GM, Bernstein WB, Dennis PA. Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations.

46. Li W, Deng X, Chen J. RNA-binding proteins in regulating mRNA stability and translation: roles and mechanisms in cancer. Seminars in Cancer Biology. Academic Press; 2022. p. 664–77. doi:10.1016/j.semcancer.2022.03.025 PubMed PMID: 35381329.

47. Stewart DP, Koss B, Bathina M, Perciavalle RM, Bisanz K, Opferman JT. Ubiquitin-Independent Degradation of Antiapoptotic MCL-1. Mol Cell Biol. 2010 Jun 1;30(12):3099–110. doi:10.1128/mcb.01266-09 PubMed PMID: 20385764.

48. Tao AJ, Gadbois GE, Buczynski SA, Ferguson FM. Targeted protein degradation: Emerging concepts and protein state-specific targeting principles. Current Opinion in Chemical Biology. Elsevier Ltd; 2022. doi:10.1016/j.cbpa.2021.102114 PubMed PMID: 35042023.

49. Giercuszkiewicz-Haśnik K, Morak-Młodawska B, Jeleń M. Molecularly Targeted Therapies in Oncology: Mechanisms, Resistance, and Combination Strategies. Molecules. Multidisciplinary Digital Publishing Institute (MDPI); 2026. doi:10.3390/molecules31071195 PubMed PMID: 41976235.

50. Al-Odat O, von Suskil M, Chitren R, Elbezanti W, Srivastava S, Budak-Alpddogan T, et al. Mcl-1 Inhibition: Managing Malignancy in Multiple Myeloma. Frontiers in Pharmacology. Frontiers Media S.A.; 2021. doi:10.3389/fphar.2021.699629

51. Ramsey HE, Fischer MA, Lee T, Gorska AE, Arrate MP, Fuller L, et al. A novel MCL1 inhibitor combined with venetoclax rescues venetoclax-resistant acute myelogenous Leukemia. Cancer Discov. 2018 Dec 1;8(12):1566–81. doi:10.1158/2159-8290.CD-18-0140 PubMed PMID: 30185627.

52. Progress in understanding the mechanisms of resistance to BCL-2 inhibitors.

53. Chauin A. The main causes and mechanisms of increase in cardiac troponin concentrations other than acute myocardial infarction (Part 1): Physical exertion, inflammatory heart disease, pulmonary embolism, renal failure, sepsis. Vascular Health and Risk Management. Dove Medical Press Ltd; 2021. p. 601–17. doi:10.2147/VHRM.S327661 PubMed PMID: 34584417.

54. Becker S, Momoh J, Biancacci I, Möckel D, Wang Q, May JN, et al. Intermittent Fasting Primes the Tumor Microenvironment and Improves Nanomedicine Delivery in Hepatocellular Carcinoma. Small. 2023 Oct 25;19(43). doi:10.1002/smll.202208042 PubMed PMID: 37376850.

55. Potter DS, Du R, Bohl SR, Chow KH, Ligon KL, Bueno R, et al. Dynamic BH3 profiling identifies pro-apoptotic drug combinations for the treatment of malignant pleural mesothelioma. Nature Communications . 2023 Dec 1;14(1). doi:10.1038/s41467-023-38552-z PubMed PMID: 37210412.

56. Sionov RV, Vlahopoulos SA, Granot Z. Regulation of Bim in Health and Disease. Oncotarget [Internet]. Available from: www.impactjournals.com/oncotarget/

57. Rybinski B, Yun K. Addressing intra-tumoral heterogeneity and therapy resistance. Oncotarget [Internet]. Available from: www.impactjournals.com/oncotarget/

58. Luo X, O'Neill KL, Huang K. The third model of Bax/Bak activation: A Bcl-2 family feud finally resolved? F1000Research. F1000 Research Ltd; 2020. doi:10.12688/f1000research.25607.1 PubMed PMID: 32802314.

59. Hanna MR, Yarbrough M, Costanzo J, Gil M, Murrow M, Gama V. Pharmacological MCL-1 inhibition disrupts fatty acid oxidation and depletes neural progenitor cells [Internet]. 2025. Available from: http://biorxiv.org/lookup/doi/10.64898/2025.12.12.694056 doi:10.64898/2025.12.12.694056

CHAPTER 3

Alpha-Interferons: Biology, Recombinant Production, and Delivery Systems

Ogün Morkoç[1]
Betül Uluca[2]

[1] Research Assistant, Department of Molecular Biotechnology, Faculty of Science, Turkish-German University, Istanbul, Türkiye, ORCID: 0000-0003-0881-8803
[2] Assistant Professor Dr., Department of Molecular Biotechnology, Faculty of Science, Turkish-German University, Istanbul, Türkiye, ORCID: :0000-0001-7139-9010

Introduction

Interferons (IFNs) are a family of signaling proteins that are naturally produced by the human body to defend against external agents such as viruses, microbes, and tumor cells. Interferons belong to the cytokine family of proteins. Cytokines are signaling molecules that mediate communication between cells and contribute to the activation and regulation of immune responses against pathogens. Interferons bind to specific cellular receptors, leading to the activation of signal transduction pathways and the expression of numerous genes (De Andrea, Ravera, Gioia, Gariglio, & Landolfo, 2002).

Interferons are known for their ability to interfere with viral infections. Accordingly, the term "interferon" was derived from their ability to interfere with viral replication. The concept of viral interference was first described in the mid-1930s by Hoskins. In his study, rabbits were infected with herpes simplex virus and subsequently challenged with the same virus. The animals were found to be protected against the subsequent viral infection (Hoskins, 1935). However, the identification of interferon as a protein was not achieved until 1957, when Isaacs and Lindenmann demonstrated its antiviral activity. They observed that the supernatant obtained from virus-infected cell cultures contained a factor capable of transferring resistance to viral infection to previously uninfected cells. In their experiments, chicken embryo cells were exposed to heat-inactivated influenza virus. They subsequently demonstrated that a factor produced by these cells could protect other cells against infection with the same or different viruses (Isaacs & Lindenmann, 1957).

Following this discovery, the properties of interferons began to be investigated. It was subsequently revealed that interferons are not virus-specific and can protect cells against a wide range of viral infections. Nevertheless, interferons exhibit different activity profiles depending on the cell type. For example, human leukocyte interferon shows strong antiviral activity in bovine and porcine cell cultures, whereas fibroblast interferon exhibits lower activity in these cell cultures and immune interferon shows no detectable activity (Pestka, 1983). In addition to their antiviral activity, interferons were found to possess antiproliferative, antitumor, and immunomodulatory activities (Stark, Kerr, Williams, Silverman, & Schreiber, 1998; Boehm, Klamp, Groot, & Howard, 1997). These diverse biological activities have contributed to the clinical importance of interferons. They have been used in the treatment of viral infections, including hepatitis B and hepatitis C, as well as malignancies such as melanoma, hairy cell leukemia, and non-Hodgkin's lymphoma, and autoimmune diseases such as multiple sclerosis. In addition to these therapeutic applications, interferons can modulate apoptosis and thereby influence cell survival (Maher, Romero-Weaver, Scarzello, & Gamero, 2007).

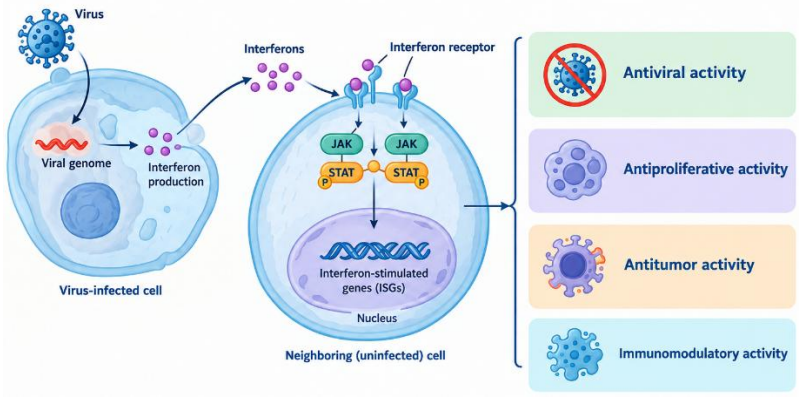


Figure 1 interferons prevent replication of viruses with different mechanisms.

Interferon activity differs from that of antibodies, which can bind to viral particles and promote their neutralization. In contrast, interferons act on cells by activating antiviral mechanisms that establish an intracellular state of resistance to viral infection. Interferons can thereby inhibit viral replication and, in some contexts, prevent virus-induced cell death (Pestka, 1983). When a virus infects a cell, its genome initiates a series of biochemical processes within the host cell, some of which can trigger the production of interferons. When interferon binds to its receptor on the producing cell, it activates signaling pathways that induce the expression of antiviral genes, thereby establishing an antiviral state. Similarly, interferon binding to receptors on neighboring cells activates antiviral defense mechanisms. As a result, cells can inhibit viral replication and limit the spread of infection (De Andrea et al., 2002) (Figure 1).

Although interferons comprise several structurally and functionally distinct groups, the present chapter focuses primarily on alpha-interferons (IFN-α) because of their historical importance as therapeutic proteins and their continued relevance to recombinant production, formulation, and advanced delivery-system development.

1. Interferon Classification

Interferons are broadly classified into three major groups type I, type II, and type III based primarily on their structural characteristics, receptor specificity, and biological functions. Type I interferons comprise several subtypes, including IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω, and are characterized by their interaction with the type I interferon receptor (IFNAR). Among these, IFN-α and IFN-β are the most extensively studied and clinically important members. Type II interferon is represented by a single member, IFN-γ, which signals through the interferon-γ receptor (IFNGR) and plays an important role in immune regulation. Type III interferons, also known as IFN-λs, include IFN-λ1, IFN-λ2, IFN-λ3, and IFN-λ4 and signal through a distinct receptor complex composed of IFNLR1 and IL-10R2. Although type I and type III interferons share several antiviral and immunomodulatory functions, they differ in their receptor specificity and tissue

distribution. Overall, these three interferon groups contribute to antiviral defense, immune regulation, and control of cellular proliferation through distinct but partially overlapping signaling mechanisms (Vorona et al., 2025; Hoffmann, Schneider, & Rice, 2015; Maher et al., 2007).

Among the type I IFNs, IFN-α is of particular therapeutic interest because of its extensive biological activity and established clinical applications. The multiple IFN-α subtypes exhibit overlapping but distinct biological properties, which have contributed to their use in the treatment of viral infections and malignancies. Consequently, IFN-α has also become an important target for recombinant protein production, formulation, and the development of advanced delivery strategies.

2. Mechanisms of Interferons Action

The binding of interferons to their cognate receptors initiates a series of intracellular signal transduction events that mediate their diverse biological activities. As illustrated in Figure 2, type I interferons bind to the IFN-α/β receptor (IFNAR), which consists of two receptor subunits, IFNAR1 and IFNAR2. In contrast, type II interferon (IFN-γ) binds to the interferon-γ receptor (IFNGR), a heterodimeric receptor composed of IFNGR1 and IFNGR2 subunits. Type III interferons (IFN-λs) signal through a receptor complex consisting of IFN-λ receptor 1 (IFNLR1) and interleukin-10 receptor 2 (IL-10R2) (Hoffmann et al.; 2015; Maher et al., 2007). All interferon receptors belong to the class II cytokine receptor family and share conserved structural features, including extracellular domains involved in ligand binding (Bazan, 1990) (Figure 2).

Interferon receptors are transmembrane proteins composed of extracellular and intracellular domains that contain specific structural and signaling motifs (De Andrea et al., 2002). Unlike receptors with intrinsic kinase activity, interferon receptors do not possess intrinsic protein kinase activity (Pestka, 1994). Instead, receptor-associated Janus kinases (JAKs) mediate the phosphorylation of specific tyrosine residues following receptor activation. Another important group of proteins involved in interferon signaling is the signal transducer and activator of transcription (STAT) family. STAT proteins play a dual role in this signaling pathway: they function as signal transducers by transmitting signals from activated receptors to the nucleus and subsequently act as transcriptional regulators. Following receptor activation and phosphorylation by JAKs, STAT proteins are phosphorylated, form functional complexes, and translocate to the nucleus, where they regulate the transcription of interferon-stimulated genes (ISGs) (De Andrea et al., 2002; Schindler & Darnell, 1995). In addition to JAKs and STATs, interferon regulatory factors (IRFs) constitute another important family of transcription factors involved in interferon signaling. IRFs can interact with one another and with other transcription factors to regulate the expression of genes involved in antiviral and immune responses. The IRF family comprises nine structurally related transcription factors that contribute to the regulation of interferon production and signaling (Taniguchi, Ogasawara, Takaoka, & Tanaka, 2001) (Figure 2).

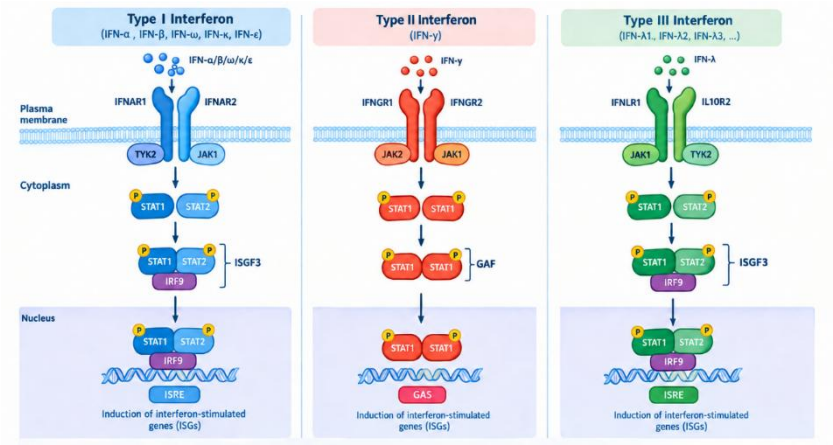


Figure 2 Type I and type III interferons activate JAK1 and TYK2, resulting in STAT1 and STAT2 phosphorylation and formation of the ISGF3 complex with IRF9. ISGF3 translocates to the nucleus and binds interferon-stimulated response elements (ISREs). Type II interferon activates JAK1 and JAK2, leading to STAT1 homodimerization and formation of the gamma-activated factor (GAF) complex, which binds gamma-activated sequences (GASs). Both pathways induce the transcription of interferon-stimulated genes (ISGs). (Hoffmann et al., 2015).

3. Therapeutic Alpha-Interferons

Since the discovery of interferons, their potential as therapeutic agents have been widely recognized. IFN-α exerts potent antiproliferative and immunomodulatory effects through multiple mechanisms, including the induction of apoptosis and cell-cycle arrest, upregulation of major histocompatibility complex (MHC) class I expression, activation of natural killer (NK) cells, enhancement of antigen presentation, and promotion of B- and T-lymphocyte differentiation (Ramos et al., 2021; Vorona et al., 2025). Despite this therapeutic potential, interferons were not approved for clinical use by regulatory agencies such as the U.S. Food and Drug Administration (FDA) until the mid-1980s (Maher et al., 2007). Subsequently, advances in modern biotechnology enabled the development of highly purified recombinant interferons suitable for clinical applications. Among the first recombinant interferon products to receive regulatory approval were human IFN-α2a (Hu-IFN-α2a) and human IFN-α2b (Hu-IFN-α2b), which were introduced for the treatment of hairy cell leukemia and Kaposi's sarcoma (Paredes & Krown, 1991).

IFN-α is one of the most widely used interferons in clinical practice. It has been used in the treatment of viral infections, including hepatitis B and hepatitis C (Brunetto et al., 2002; Gherlan et al., 2024), as well as malignant diseases such as chronic myelogenous leukemia, metastatic malignant melanoma, renal cell carcinoma, and basal cell carcinoma (Müller, 2006; Vorona et al., 2025). IFN-α has also been investigated as a potential treatment for SARS-CoV-2 because type I interferons possess broad antiviral activity and contribute to the early innate immune response. SARS-CoV-2 may impair type I interferon production and

signaling, while experimental studies indicate that exogenous interferons can inhibit viral replication, particularly when administered during the early stages of infection. Consequently, IFN-α remains of scientific interest in the context of COVID-19, but its therapeutic efficacy requires careful interpretation (Bastard, Zhang, Zhang, Jouanguy, & Casanova, 2022; Jhuti et al., 2022).

Therapeutic IFN-α products include conventional recombinant interferon alfa preparations and pegylated derivatives developed to improve pharmacokinetic properties and reduce dosing frequency. Representative products approved by the FDA or EMA are summarized in Table 1 (*Medicines | European Medicines Agency (EMA), 2023; Purple Book Search, 2026*; Castro et al., 2021). In addition to approved products, several clinical studies are currently investigating interferon-alpha formulations and interferon-based combination regimens in different therapeutic areas. The ClinicalTrials.gov database contains numerous records involving interferon-alpha products and interferon-based therapeutic regimens across a range of clinical indications. A search conducted on 9 September 2026 using the term "Interferon Alpha" identified 212 active interventional studies in phases 1–4. Of these, 28 studies were in phase 1, 119 in phase 2, 54 in phase 3, and 11 in phase 4. These findings indicate continued clinical investigation of interferon-alpha products and interferon-based therapeutics across a range of clinical indications (*ClinicalTrials.gov,* 2026.)

Table 1 Examples of IFN-α Products Approved by the FDA or EMA

IFN-α Subtype	Trade Name/Product	Marketing authorization holder	Principal approved indication(s)
IFN-α2a	Roferon A	Hoffmann–La Roche	Hairy-cell leukemia, AIDS-related Kaposi's sarcoma, and chronic myelogenous leukemia
PegIFN-α2a	Pegasys	Hoffman-La Roche	Chronic hepatitis B and chronic hepatitis C
IFN-αn3	Alferon N	AIM ImmunoTech	Condyloma acuminata
PegIFN-α2b	Besremi-ropeginterferon alfa-2b	AOP Orphan Pharmaceuticals	Polycythaemia vera in adults
IFN-α2b	Intron A	Merck Sharp & Dohme (MSD)	Hairy-cell leukemia, condyloma acuminata, AIDS-related Kaposi's sarcoma, chronic hepatitis B and C, malignant melanoma, follicular lymphoma, and chronic myelogenous leukemia

(adapted from (Medicines | European Medicines Agency (EMA), 2023;) Purple Book Search, 2026), (Castro et al., 2021).

3.1 Properties of Alpha-Interferons

Alpha-interferon (IFN-α), also known as leukocyte interferon, is a member of the type I interferon family. As mentioned previously, IFN-α does not represent a single unique protein; rather, it comprises a family of closely related proteins (De Andrea et al., 2002). This family consists of multiple IFN-α subtypes, which are designated as IFN-α1, IFN-α2, IFN-α3, IFN-α4, and so forth (Streuli, Nagata, & Weissmann, 1980.). The genes encoding human IFN-α are located as a cluster on chromosome 9 and are characterized by the absence of introns. The human IFN-α gene family consists of multiple non-allelic genes, including functional genes and pseudogenes; among these, the functional genes encode the different IFN-α protein subtypes (Nagata et al., 1980).

Human IFN-α subtypes generally consist of approximately 188–189 amino acid residues and have a molecular mass of approximately 18.5 kDa in their recombinant forms. However, naturally derived IFN-α2 has been reported to exhibit a slightly higher apparent molecular mass of approximately 19–20 kDa when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). This difference may be associated with post-translational modifications and variations in the amino acid sequence among IFN-α subtypes (Sen & Lengyel, 1992). Several amino acid residues are conserved among human IFN-α subtypes and contribute to their biological activity, including conserved cysteine residues that are important for disulfide bond formation (Wetzel, 1981).

The three-dimensional structure of human IFN-α has been investigated using nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography (Klaus, Gsell, Labhardt, Wipf, & Senn, 1997; Redwan, Matar, & Serour, 2006). These structural analyses have provided insights into the tertiary structure of the protein, which consists predominantly of five α-helices connected by loops. In addition, a disulfide bond connects the N-terminal region of the protein to helix C (Radhakrishnan et al., 1996). Proper protein folding is essential for the formation and maintenance of the biologically active conformation of IFN-α and also contributes to the prevention of protein aggregation. Therefore, for therapeutic applications, IFN-α must maintain its correct three-dimensional structure and remain in its biologically active state (Redwan et al., 2006).

Another important structural characteristic of IFN-α is its glycosylation pattern, which varies among different IFN-α subtypes. Although glycosylation is not essential for the biological activity of IFN-α, it can influence important protein properties, including structural stability, resistance to proteolytic degradation, and pharmacokinetic behavior. Some human IFN-α subtypes undergo glycosylation, whereas others are reported to be non-glycosylated (Adolf et al., 1991; Nyman et al., 1998). These differences in glycosylation may therefore contribute to variations in the stability and biological properties of individual IFN-α subtypes.

4. Recombinant Production of Alpha-Interferons

Alpha-interferon (IFN-α), are clinically important proteins for treatment of disease especially for viral infections and malignant diseases. However, natural production of IFN-α is very cumbersome. Because natural production enables only production of too small quantities which cannot fulfill market demand. Furthermore, purity of product is another important step to consider. Therefore until 1980 interferon production was very expensive. When DNA recombinant technologies became prevalent, new era of therapeutic IFN production has begun and mass production through cultivation of prokaryotic or eukaryotic organism and later downstream purification process become possible (Nagata et al., 1980; Weissmann et al., 1982). The manufacturing process of recombinant therapeutic proteins is typically comprised of four primary stages: the development of the expression system, upstream production, downstream recovery and purification, and fill-and-finish. The development stage encompasses the construction of the recombinant DNA and the establishment of a suitable host cell, while the upstream processing focuses on the expression of proteins under optimized culture conditions. Downstream processing is employed for the purpose of recovering and purifying the target protein from the production matrix, frequently through chromatographic methods. The final fill-and-finish stage incorporates formulation and packaging that are designed to preserve the product's stability, quality, and biological activity during storage and transportation. In the production of recombinant alpha-interferons, the quality and therapeutic performance of the final product depend not only on the expression host but also on the subsequent manufacturing and formulation steps. Therapeutic protein formulations are designed to maintain protein stability, biological activity, and effective delivery. Therefore, the development of a safe and effective IFN-alpha product requires coordinated optimization of the expression system, cell culture, protein synthesis, purification, analytical characterization, formulation, storage, and handling (Castro et al., 2021).

Two recombinant forms of IFN-α, which differ in their amino acid sequences, are widely used in today's clinical practice: IFN-α2a and IFN-α2b. Both are available in conventional, short-acting formulations, as well as in pegylated preparations, which provide therapeutic options with distinct pharmacokinetic characteristics (Vorona et al., 2025; Castro et al., 2021).

Furthermore, recombinant DNA technology provides opportunities for the protein engineering of IFN-α to generate molecules with tailored biological properties. Through site-directed mutagenesis, sequence recombination, and fusion-protein design, specific amino acid residues, functional regions, or targeting sequences can be modified or combined to enhance selected activities of the cytokine (Blatt, Davis, Klein, & Taylor, 1996; Karakoese et al., 2023; Li et al., 2017; Talebi, Saeedinia, Zeinoddini, Ahmadpour, & Sadeghizadeh, 2019). Recombinant IFN-α hybrids and mutants have demonstrated that alterations in specific residues can modulate receptor interactions and increase antiproliferative activity. In addition, fusion of targeting peptides to IFN-α has been investigated to improve its antitumor activity and cellular targeting. These findings highlight

protein engineering and hybrid production as promising strategies for developing next-generation IFN-α molecules with enhanced or tailored therapeutic properties (Blatt et al., 1996; Li et al., 2017; Pan et al., 2026).

Recombinant IFN-α proteins can be produced using a range of prokaryotic and eukaryotic expression systems. Among prokaryotic hosts, *Escherichia coli* is one of the most widely used organisms for recombinant interferon production. The selection of an appropriate host depends on several factors, including growth characteristics, expression capacity, protein folding, post-translational modification, product quality, and manufacturing cost. *E. coli* is frequently used because it grows rapidly on relatively inexpensive culture media and is supported by well-established genetic and fermentation technologies. It can also be cultivated to high cell densities, which may facilitate the production of substantial quantities of recombinant protein. These characteristics make *E. coli* an attractive and economically practical host for recombinant IFN-α production (Castro et al., 2021; El-Baky & Redwan, 2015). Its suitability for this purpose is further supported by the relatively small molecular size of IFN-α and the fact that glycosylation is not essential for its principal biological activity (Castro et al., 2021). Nevertheless, the absence of an essential requirement for glycosylation does not mean that glycosylation is biologically irrelevant. Naturally occurring human IFN-α2 has been reported to undergo O-glycosylation (Adolf, Kalsner, Ahorn, Maurer-Fogy, & Cantell, 1991), suggesting that glycan modification may influence selected physicochemical and biological properties of the protein. Studies of glycosylated IFN-α have indicated that glycosylation may modestly reduce in vitro antiproliferative activity while considerably increasing resistance to proteolytic degradation. The resulting improvement in proteolytic stability may contribute to enhanced biological activity in vivo (Prabhu, Yang, Tong, & Wang, 2021). In addition, modulation of host–glycan interactions may represent a potential strategy for optimizing the therapeutic properties of IFN-α by promoting beneficial activities while reducing undesirable effects (Giron et al., 2020).

Despite these advantages, *E. coli* presents several limitations for recombinant IFN-α production. High-level expression can result in the accumulation of the target protein as inclusion bodies, in which the protein is frequently misfolded and biologically inactive (Babu, Swaminathan, Marten, Khanna, & Rinas, 2000; Villaverde & Mar Carrió, 2003). Recovery of active protein therefore often requires solubilization and subsequent refolding, processes that can be technically demanding and inefficient (Redwan et al., 2006). Another limitation is codon bias. Differences in codon usage between prokaryotic hosts and eukaryotic genes may affect the quantity and quality of the expressed protein. Codon optimization or other strategies for reducing codon bias can improve the expression of eukaryotic genes in *E. coli* (Kane, 1995).

To address the limitations associated with *E. coli*, alternative expression platforms such as yeasts Pichia *pastoris, Saccharomyces cerevisiae*, and mammalian cell lines have also been investigated. These systems may provide advantages in protein folding, secretion, post-translational modification, or process flexibility, depending on the characteristics of the IFN-α product (Castro

et al., 2021; S. Katla et al., 2019; Loignon et al., 2008; Piggott, Watson, Doel, Goodey, & Carter, 1987). Cell-free protein synthesis has also been explored as a flexible production approach (EL-Baky, Omar, & Redwan, 2011) although its application to recombinant IFN-α remains less extensively investigated. The principal advantages and disadvantages of the selected IFN-α expression systems are summarized in Table 2.

Table 2 Comparison of different expression host for recombinant alpha interferon production**.**

Expression system	Main advantages	Main limitations	Source
Escherichia coli	Rapid growth; inexpensive media; well-established genetic tools; high volumetric productivity; easy scale-up	Inclusion-body formation; protein misfolding; endotoxin contamination; limited post-translational modification; codon-usage differences	(Babu et al., 2000; Kane, 1995; Lin et al., 2020; Villaverde & Mar Carrió, 2003)
Pichia pastoris	High cell density; strong promoters; efficient secretion; eukaryotic folding machinery; relatively inexpensive scale-up	Non-human glycosylation; methanol-related process and safety considerations when methanol-inducible systems are used; proteolysis	(Castro et al., 2021; S. Katla et al., 2019; Shi, Wang, Chan, & Cheng, 2007)
Saccharomyces cerevisiae	Well-characterized eukaryotic host; established fermentation technology; relatively safe and scalable; secretion possible	Lower secretion efficiency for some proteins, possible proteolysis	(Srikanth Katla & Sivaprakasam, 2019; Piggott et al., 1987)
Mammalian cells	High-quality protein folding; complex post-translational processing; more human-like glycosylation; strong regulatory familiarity for many biologics	Slow growth; expensive media and equipment; lower volumetric productivity; greater contamination-control requirements	(Attallah et al., 2020; Loignon et al., 2008; Song et al., 2023)
Cell-free protein synthesis	Rapid production; no living host; easy control of reaction conditions; useful for toxic or difficult proteins; potentially simplified screening	High reagent cost; limited reaction scale; lower productivity for some proteins; downstream purification still required	(EL-Baky et al., 2011; Hunt et al., 2025)

Among the eukaryotic expression systems, *P. pastoris* is an attractive host for recombinant IFN-α production because it can reach high cell densities and support relatively high levels of heterologous protein expression. In addition, this yeast provides selected eukaryotic post-translational modifications that are not available in *E. coli*. Compared with mammalian cell-based systems, *P. pastoris* is generally faster to cultivate, easier to handle, and less expensive. These characteristics make it a useful intermediate platform when both production efficiency and selected eukaryotic processing capabilities are required (Castro et al., 2021). Mammalian cells are particularly useful for producing correctly folded IFN-α with physiologically relevant post-translational modifications, including glycosylation, which may improve protein stability and serum half-life. However, mammalian systems generally have lower volumetric productivity and higher production costs than bacterial or yeast platforms. Cell lines such as HEK293, Chinese hamster ovary and NS0 cells have nevertheless been used to produce biologically active, glycosylated recombinant IFN-α2b (Attallah et al., 2020; Loignon et al., 2008).

The yield of recombinant IFN-α can be substantially influenced by the optimization of upstream culture conditions. Key parameters include the composition of the culture medium, inducer concentration, cell density at the time of induction, and the duration of the post-induction period. Careful adjustment of these parameters can improve protein productivity and provide a more favorable starting material for subsequent downstream processing (Castro et al., 2021; Pedra et al., 2026).

Downstream processing is essential for recovering and purifying recombinant IFN-α after production. The process should be reliable, robust, suitable for industrial-scale operation, and economically feasible, while providing high product yield and purity without compromising the protein's structural integrity or biological activity. Depending on the expression system and the localization of the recombinant protein, initial recovery may involve cell removal, cell disruption, or extraction of intracellular products, whereas proteins secreted into the culture medium can be recovered by centrifugation, ultrafiltration, or precipitation. Subsequent purification commonly relies on chromatographic techniques, including affinity chromatography, ion-exchange chromatography, size-exclusion chromatography, reverse-phase chromatography, and hydrophobic-interaction chromatography. These operations are designed to remove host-cell proteins, endotoxins, and other process-related contaminants while maintaining the quality and activity of the therapeutic interferon (Castro et al., 2021; Pedra et al., 2026; Thatcher & Panayotatos, 1986).

In *E. coli*, recombinant IFN-α may be directed to the periplasm through an appropriate signal peptide or retained in the cytoplasm, where it can occur either in a soluble form or as inclusion bodies. Periplasmic localization may facilitate recovery because selective disruption of the outer membrane can release the target protein while limiting contamination with soluble cytoplasmic proteins. By contrast, cytoplasmic production generally requires cell disruption, followed by separation, solubilization, and, when necessary, refolding of inclusion-body-

associated protein (Castro et al., 2021). When IFN-α is secreted into the culture medium, the absence of a cellular fraction simplifies the initial recovery step, which may involve dialysis, concentration, dilution with a suitable chromatographic binding buffer, or clarification by microfiltration. Thus, downstream processing must be designed in accordance with the upstream production strategy and the localization, solubility, and structural state of the recombinant protein (Castro et al., 2021; Pedra et al., 2026).

5. Alpha-Interferons Delivery Systems

Although recombinant production and molecular modification have improved the availability and pharmacokinetic properties of alpha-interferons, their therapeutic performance also depends on the method used to deliver the protein to the target site. As protein-based therapeutics, alpha-interferons may be susceptible to denaturation, aggregation, proteolytic degradation, and loss of biological activity during formulation, storage, and administration. Therefore, delivery systems are being investigated to improve protein stability, prolong residence time, control drug release, and reduce the frequency of administration (Castro et al., 2021; Ramos et al., 2021).

Several delivery and encapsulation strategies have been investigated for IFN-α, including PEGylation, liposomes, polymeric micelles, and micro-/nanoencapsulation. The principal mechanisms, advantages, and limitations of these approaches are summarized in Table 3. Overall, PEGylation has the greatest clinical experience, whereas liposomal, micellar, and micro-/nanoencapsulation approaches remain promising strategies for improving the stability, pharmacokinetics, and sustained delivery of IFN-α, but require further optimization and clinical evaluation. (Ramos et al., 2021).

5.1 Pegylation of Alpha-Interferons

To improve the pharmacokinetic properties of IFN-α, PEGylation can be employed as a protein modification strategy. PEGylation involves the covalent attachment of one or more polyethylene glycol (PEG) chains to IFN-α. The attached PEG chains can be linear or branched and increase the effective molecular size of the protein. Because PEG is generally non-toxic and chemically inert, its conjugation to IFN-α can reduce renal clearance and prolong its circulating half-life. In addition, PEGylation can improve protein solubility and stability while protecting IFN-α from proteolytic degradation. These effects can contribute to improved pharmacokinetic properties, therapeutic efficacy, and potentially the safety profile of IFN-α (Harris, Martin, & Modi, 2001; Maher et al., 2007; El-Baky & Redwan, 2015a; Peleg-Shulman et al., 2004). Apart from those advantages pegylation of IFN-α can reduce the antigenicity and immunogenicity of protein. Therefore, potency of drug decrease. Which is due to steric hindrance caused by PEG chains prevent interaction of IFN molecule with its target cell surface receptor (Peleg-Shulman et al., 2004).

Pegylated interferons are currently commercially available. Although it has some disadvantages clinical trials revealed that pegylated IFN-α show sustainable

drug response compared with unmodified IFN-α (Zeuzem et al., 2000). Thus, pegylated IFN are useful for clinical application. Some of the pegylated commercial products are summarized in Table 1.

5.2 Encapsulation of Alpha-Interferons

Encapsulation is one of the principal strategies investigated for improving the delivery of alpha-interferons. In this approach, the protein is incorporated into or associated with a carrier system that may protect it from environmental degradation and provide controlled or sustained release. Depending on the carrier composition, encapsulation may also influence tissue distribution, cellular uptake, and the duration of exposure to the therapeutic protein. Liposomes, polymeric micelles, microparticles, hydrogels and nanoparticles have been explored as carriers for alpha-interferons (Castro et al., 2021; Pedra et al., 2026; Ramos et al., 2021).

Despite these potential advantages, the encapsulation of IFN-α presents several formulation challenges. Interactions between the protein and the encapsulating matrix, as well as formulation pH, can affect protein solubility, stability, aggregation, and release behavior. The composition of the carrier and the use of suitable stabilizers are also important for controlling particle characteristics and achieving predictable release and biological activity. In addition, successful encapsulation requires consideration of the physicochemical properties of IFN-α and their compatibility with the selected carrier system. Therefore, the development of effective encapsulated IFN-α formulations requires careful optimization of both the protein and the delivery matrix (Pedra et al., 2026; Ramos et al., 2021)

Table 3. Comparison of Delivery and Encapsulation Strategies Investigated for IFN-α.

Delivery strategy	Basic principle	Main advantages	Main limitations
PEGylation	Covalent attachment of PEG chains to IFN-α to increase molecular size and reduce renal and plasma clearance.	Prolonged circulation and half-life; improved stability; reduced dosing frequency; comparatively advanced clinical experience.	Can reduce biological activity; PEG size and structure influence activity; toxicity may remain a concern.
Liposomes	Encapsulation of IFN-α within lipid bilayer vesicles containing aqueous compartments.	Biocompatibility; potential protection from degradation; prolonged residence and improved pharmacokinetics; possibility of site-specific delivery.	Encapsulation efficiency can be variable; instability, aggregation and fusion; possible immune-system interactions; relatively high production cost.

Polymeric micelles	Self-assembly of amphiphilic block copolymers into structures with a hydrophobic core and hydrophilic corona that can associate with IFN-α.	Structural flexibility; potential for improved stability and circulation; potential reduction in dose and toxicity.	Limited IFN-α studies; relatively low/uncertain encapsulation efficiency; stability and release-control issues; formulation complexity.
Micro-/nanoencapsulation	Entrapment of IFN-α within or surrounded by polymeric matrices to protect the protein and provide controlled or sustained release.	Protection from degradation; sustained release; prolonged pharmacokinetics; improved bioavailability; potential reduction in dosing frequency and systemic exposure.	Possible loss of biological activity; encapsulation efficiency can be limited; manufacturing and scale-up challenges.

(Ramos et al., 2021).

6. Conclusion and Future Prospects

Interferons are cytokine molecules that are well known for their antiviral, antiproliferative, antitumor, and immunomodulatory activities. Their evolutionary appearance dates back to the emergence of multicellular organisms, and they have subsequently diversified and specialized for different biological functions. These properties have established interferons as important therapeutic molecules. Among the different interferon subtypes, IFN-alpha has been particularly valuable in the treatment of viral infections, including hepatitis B and hepatitis C, and in the management of several malignancies, such as Kaposi's sarcoma, ovarian carcinoma, and hairy-cell leukemia. The development of recombinant DNA technology has enabled the production of relatively large quantities of highly purified IFN-alpha using different expression systems, including bacteria, yeast, mammalian cell lines and cell free systems. Each system offers specific advantages and limitations with respect to productivity, protein folding, post-translational processing, purification, manufacturing cost, and product quality. Over the years, pharmaceutical development has focused on improving the pharmacokinetic and therapeutic properties of IFN-alpha. Pegylation has been one of the most important approaches used to extend the serum half-life, reduce dosing frequency, and provide more sustained drug exposure. The development of IFN-alpha products has also increased interest in improving product accessibility and reducing treatment costs. Nevertheless, the quality, safety, efficacy, and similarity of these products must be demonstrated through appropriate analytical, functional, pharmacokinetic, and clinical evaluations. Future progress in IFN-alpha therapy will likely depend on the combined development of protein engineering, recombinant production,

formulation and delivery technology. Improvements in expression systems and downstream processing may increase production efficiency and product consistency, while advances in protein modification may help generate interferon variants with improved stability, prolonged half-lives, and reduced adverse effects. Overall, IFN-alpha remains an important example of the successful translation of cytokine biology into biopharmaceutical development. Continued integration of molecular research, manufacturing technology, and clinical investigation may lead to IFN-alpha-based treatments that are more effective, better tolerated, and more precisely targeted to patients who are most likely to benefit.

Statement on the Use of Artificial Intelligence Artificial intelligence tools were used to assist in the preparation of illustrative figure included in the chapter, AI-based tools were also used for grammatical editing, and improvement of sentence clarity and flow. The authors reviewed, revised, and verified the generated material and remain fully responsible for the scientific content of the chapter.

References

Adolf, G. R., Kalsner, I., Ahorn, H., Maurer-Fogy, I., & Cantell, K. (1991). Natural human interferon-alpha 2 is O-glycosylated. *Biochemical Journal*, *276*(2), 511–518. https://doi.org/10.1042/bj2760511

Attallah, C., Aguilar, M. F., Forno, G., Etcheverrigaray, M., Brigido, M. D. M., Maranhão, A. Q., & Oggero, M. (2020). The glycosylation of anti-rhIFN-α2b recombinant antibodies influences the antigen-neutralizing activity. *Biotechnology Letters*, *42*(8), 1369–1381. https://doi.org/10.1007/s10529-020-02879-0

Babu, K. R., Swaminathan, S., Marten, S., Khanna, N., & Rinas, U. (2000). Production of interferon-α in high cell density cultures of recombinant Escherichia coli and its single step purification from refolded inclusion body proteins. *Applied Microbiology and Biotechnology*, *53*(6), 655–660. https://doi.org/10.1007/s002530000318

Bastard, P., Zhang, Q., Zhang, S. Y., Jouanguy, E., & Casanova, J. L. (2022, February 1). Type I interferons and SARS-CoV-2: from cells to organisms. *Current Opinion in Immunology*, Vol. 74, pp. 172–182. Elsevier Ltd. https://doi.org/10.1016/j.coi.2022.01.003

Bazan, J. F. (1990). Structural design and molecular evolution of a cytokine receptor superfamily. *Proceedings of the National Academy of Sciences*, *87*(18), 6934–6938. https://doi.org/10.1073/pnas.87.18.6934

Blatt, L. M., Davis, J. M., Klein, S. B., & Taylor, M. W. (1996). The biologic activity and molecular characterization of a novel synthetic interferon-alpha species, consensus interferon. *Journal of Interferon & Cytokine Research : The Official Journal of the International Society for Interferon and Cytokine Research*, *16*(7), 489–499. https://doi.org/10.1089/JIR.1996.16.489

Boehm, U., Klamp, T., Groot, M., & Howard, J. C. (1997). CELLULAR RESPONSES TO INTERFERON-γ. *Annual Review of Immunology*, *15*(1), 749–795. https://doi.org/10.1146/annurev.immunol.15.1.749

Brunetto, M. R., Oliveri, F., Coco, B., Leandro, G., Colombatto, P., Gorin, J. M., & Bonino, F. (2002). Outcome of anti-HBe positive chronic hepatitis B in alpha-interferon treated and untreated patients: a long term cohort study. *Journal of Hepatology*, *36*(2), 263–270.

Castro, L. S., Lobo, G. S., Pereira, P., Freire, M. G., Neves, M. C., & Pedro, A. Q. (2021). Interferon-based biopharmaceuticals: Overview on the production, purification, and formulation. *Vaccines*, *9*(4). https://doi.org/10.3390/vaccines9040328

ClinicalTrials.gov. (2026.). Retrieved September 8, 2026, from https://clinicaltrials.gov/

De Andrea, M., Ravera, R., Gioia, D., Gariglio, M., & Landolfo, S. (2002). The interferon system: An overview. *European Journal of Paediatric Neurology*, *6*(SUPPL. 1). https://doi.org/10.1053/ejpn.2002.0573

El-Baky, N. A., & Redwan, E. M. (2015). Therapeutic alpha-interferons protein: Structure, production, and biosimilar. *Preparative Biochemistry and Biotechnology*, *45*(2), 109–127. https://doi.org/10.1080/10826068.2014.907175

EL-Baky, N. A., Omar, S. H., & Redwan, E. M. (2011). The anti-cancer activity of human consensus interferon-alpha synthesized in cell-free system. *Protein Expression and Purification*, *80*(1), 61–67. https://doi.org/10.1016/j.pep.2011.07.003

Gherlan, G. S., Lazar, S. D., Culinescu, A., Smadu, D., Vatafu, A. R., Popescu, C. P., … Calistru, P. I. (2024). Results of Response-Guided Therapy with Pegylated Interferon Alpha 2a in Chronic Hepatitis B and D. *Tropical Medicine and Infectious Disease*, *9*(4). https://doi.org/10.3390/tropicalmed9040073

Giron, L. B., Colomb, F., Papasavvas, E., Azzoni, L., Yin, X., Fair, M., … Abdel-Mohsen, M. (2020). Interferon-α alters host glycosylation machinery during treated HIV infection. *EBioMedicine*, *59*, 102945. https://doi.org/10.1016/j.ebiom.2020.102945

Harris, J. M., Martin, N. E., & Modi, M. (2001). Pegylation. *Clinical Pharmacokinetics*, *40*(7), 539–551. https://doi.org/10.2165/00003088-200140070-00005

Hoffmann, H. H., Schneider, W. M., & Rice, C. M. (2015). Interferons and viruses: An evolutionary arms race of molecular interactions. *Trends in Immunology*, *36*(3), 124–138. https://doi.org/10.1016/j.it.2015.01.004

Hoskins, M. (1935). A Protective Action of Neurotropic Against Viscerotropic Yellow Fever Virus in Macacus Rhesus. *The American Journal of Tropical Medicine and Hygiene*, *s1-15*(6), 675–680. https://doi.org/10.4269/ajtmh.1935.s1-15.675

Hunt, A. C., Rasor, B. J., Seki, K., Ekas, H. M., Warfel, K. F., Karim, A. S., & Jewett, M. C. (2025). Cell-Free Gene Expression: Methods and Applications. *Chemical Reviews*, *125*(1), 91–149. https://doi.org/10.1021/acs.chemrev.4c00116

Isaacs, A., & Lindenmann, J. (1957). Pillars Article : Virus Interference . I . The. *Proc R Soc Lond B Biol Sci*, *147*, 258–267.

Jhuti, D., Rawat, A., Guo, C. M., Wilson, L. A., Mills, E. J., & Forrest, J. I. (2022). Interferon Treatments for SARS-CoV-2: Challenges and Opportunities. *Infectious Diseases and Therapy*, *11*(3), 953–972. https://doi.org/10.1007/s40121-022-00633-9

Kane, J. F. (1995). Effects of rare codon clusters on high-level expression of heterologous proteins in Escherichia coli. *Current Opinion in Biotechnology*, *6*(5), 494–500. https://doi.org/10.1016/0958-1669(95)80082-4

Karakoese, Z., Le-Trilling, V. T. K., Schuhenn, J., Francois, S., Lu, M., Liu, J., … Sutter, K. (2023). Targeted mutations in IFNα2 improve its antiviral activity against various viruses. *MBio*, *14*(6). https://doi.org/10.1128/mbio.02357-23

Katla, S., Karmakar, B., Tadi, S. R. R., Mohan, N., Anand, B., Pal, U., & Sivaprakasam, S. (2019). High level extracellular production of recombinant human interferon alpha 2b in glycoengineered Pichia pastoris: culture medium optimization, high cell density cultivation and biological characterization. *Journal of Applied Microbiology*, *126*(5), 1438–1453. https://doi.org/10.1111/jam.14227

Katla, Srikanth, & Sivaprakasam, S. (2019). *Yeasts as Microbial Factories for Production of Recombinant Human Interferon Alpha 2b of Therapeutic Importance*. https://doi.org/10.1021/bk-2019-1329.ch003

Klaus, W., Gsell, B., Labhardt, A. M., Wipf, B., & Senn, H. (1997). The three-dimensional high resolution structure of human interferon α-2a determined by heteronuclear NMR spectroscopy in solution. *Journal of Molecular Biology*, *274*(4), 661–675. https://doi.org/10.1006/jmbi.1997.1396

Li, S. J., Liu, C. S., Li, H. J., Li, Y., Zhou, L., Li, J. C., … Yu, D. H. (2017). A novel engineered interferon-α hybrid molecule increases anticancer efficacy of doxorubicin in breast cancer chemotherapy. *International Journal of Oncology*, *51*(3), 949–958. https://doi.org/10.3892/ijo.2017.4062

Lin, H.-J., Xiao Joe, J. T., Lu, W.-J., Huang, M.-Y., Sun, T.-H., Lin, S.-P., … Victor Lin, H.-T. (2020). Secretory Production of Functional Grouper Type I Interferon from Epinephelus septemfasciatus in Escherichia coli and Bacillus subtilis. *International Journal of Molecular Sciences*, *21*(4), 1465. https://doi.org/10.3390/ijms21041465

Loignon, M., Perret, S., Kelly, J., Boulais, D., Cass, B., Bisson, L., Durocher, Y. (2008). Stable high volumetric production of glycosylated human recombinant IFNalpha2b in HEK293 cells. *BMC Biotechnology*, *8*. https://doi.org/10.1186/1472-6750-8-65

Maher, S., Romero-Weaver, A., Scarzello, A., & Gamero, A. (2007). Interferon: Cellular Executioner or White Knight? *Current Medicinal Chemistry*, *14*(12), 1279–1289. https://doi.org/10.2174/092986707780597907

Medicines | European Medicines Agency (EMA). (2023, October 11). European Medicines Agency (EMA). https://www.ema.europa.eu/en/medicines

Müller, F. (2006). Overview of Clinical Applications of Type I Interferons. In *The Interferons* (pp. 275–308). Wiley. https://doi.org/10.1002/3527608206.ch10

Nagata, S., Taira, H., Hall, A., Johnsrud, L., Streuli, M., Ecsödi, J., … Weissmann, C. (1980). Synthesis in E. coli of a polypeptide with human leukocyte interferon activity. *Nature*, *284*(5754), 316–320. https://doi.org/10.1038/284316a0

Nyman, T. A., Kalkkinen, N., Tölö, H., & Helin, J. (1998). Structural characterisation of N-linked and O-linked oligosaccharides derived from interferon-alpha2b and interferon-alpha14c produced by Sendai-virus-induced human peripheral blood leukocytes. *European Journal of Biochemistry*, *253*(2), 485–493.

Pan, Y., Tang, Y., Chen, L., Xu, Y., Hu, X., Zhang, J., … Xu, X. (2026). Modularly designed hybrid vesicles as dual-adjuvant nanovaccines for cancer immunotherapy. *Biomaterials*, *335*. https://doi.org/10.1016/j.biomaterials.2026.124320

Paredes, J., & Krown, S. E. (1991). Interferon-alpha therapy in patients with Kaposi's sarcoma and the acquired immunodeficiency syndrome. *International Journal of Immunopharmacology*, *13 Suppl 1*, 77–81.

Pedra, A. C. K., de Oliveira, N. R., Maia, M. A. C., Madruga, A. B., Santos, B. C. M., Dellagostin, O. A., & Bohn, T. L. O. (2026, December 1). Recombinant cytokines produced in Komagataella phaffii: advances in design, production, and purification strategies. *Molecular Biology Reports*, Vol. 53. Springer Science and Business Media B.V. https://doi.org/10.1007/s11033-026-11829-4

Peleg-Shulman, T., Tsubery, H., Mironchik, M., Fridkin, M., Schreiber, G., & Shechter, Y. (2004). Reversible PEGylation: A Novel Technology To Release Native Interferon α2 over a Prolonged Time Period. *Journal of Medicinal Chemistry*, *47*(20), 4897–4904. https://doi.org/10.1021/jm0497693

Pestka, S. (1994). Identification of functional type I and type II interferon receptors. *[Hokkaido Igaku Zasshi] The Hokkaido Journal of Medical Science*, *69*(6), 1301–1319.

Pestka, Sidney, Krause, C. D., & Walter, M. R. (2004). Interferons, interferon-like cytokines, and their receptors. *Immunological Reviews*, *202*, 8–32. https://doi.org/10.1111/j.0105-2896.2004.00204.x

Pestka, Sidney. (1983). Invited Paper. *Archives of Biochemistry and Biophysics*, *221*(1), 1–37.

Piggott, J. R., Watson, M. E. E., Doel, S. M., Goodey, A. R., & Carter, B. L. A. (1987). The secretion and post translational modification of interferons from Saccharomyces cerevisiae. *Current Genetics*, *12*(8), 561–567. https://doi.org/10.1007/BF00368057

Prabhu, S. K., Yang, Q., Tong, X., & Wang, L.-X. (2021). Exploring a combined Escherichia coli-based glycosylation and in vitro transglycosylation

approach for expression of glycosylated interferon alpha. *Bioorganic & Medicinal Chemistry*, *33*, 116037. https://doi.org/10.1016/j.bmc.2021.116037

Purple Book Search. (2026.). Retrieved September 8, 2026, from https://purplebooksearch.fda.gov/index.cfm?event=about

Radhakrishnan, R., Walter, L. J., Hruza, A., Reichert, P., Trotta, P. P., Nagabhushan, T. L., & Walter, M. R. (1996). Zinc mediated dimer of human interferon-alpha 2b revealed by X-ray crystallography. *Structure (London, England : 1993)*, *4*(12), 1453–1463.

Ramos, T. I., Villacis-Aguirre, C. A., Santiago Vispo, N., Santiago Padilla, L., Santana, S. P., Parra, N. C., & Toledo Alonso, J. R. (2021, October 1). Forms and methods for interferon's encapsulation. *Pharmaceutics*, Vol. 13. MDPI. https://doi.org/10.3390/pharmaceutics13101533

Redwan, E.-R. M., Matar, S. M., & Serour, I. A. (2006). Recombinant human J-chain: fix the protein aggregations and yield maximize. *Human Antibodies*, *15*(3), 95–102.

Schindler, C., & Darnell, J. E. (1995). Transcriptional Responses to Polypeptide Ligands: The JAK-STAT Pathway. *Annual Review of Biochemistry*, *64*(1), 621–652. https://doi.org/10.1146/annurev.bi.64.070195.003201

Sen, G. C., & Lengyel, P. (1992). The interferon system. A bird's eye view of its biochemistry. *The Journal of Biological Chemistry*, *267*(8), 5017–5020.

Shi, L., Wang, D., Chan, W., & Cheng, L. (2007). Efficient expression and purification of human interferon alpha2b in the methylotrophic yeast, Pichia pastoris. *Protein Expression and Purification*, *54*(2), 220–226. https://doi.org/10.1016/j.pep.2007.03.005

Song, B., Wei, W., Liu, X., Huang, Y., Zhu, S., Yi, L., … Chen, J. (2023). Recombinant Porcine Interferon-α Decreases Pseudorabies Virus Infection. *Vaccines*, *11*(10), 1587. https://doi.org/10.3390/vaccines11101587

Stark, G. R., Kerr, I. M., Williams, B. R. G., Silverman, R. H., & Schreiber, R. D. (1998). HOW CELLS RESPOND TO INTERFERONS. *Annual Review of Biochemistry*, *67*(1), 227–264. https://doi.org/10.1146/annurev.biochem.67.1.227

Streuli, M., Nagata, S., & Weissmann, C. (1980). At Least three Human Type α Interferons: Structure of α 2. *Science*, Vol. 209, pp. 1343–1347. American Association for the Advancement of Science. https://doi.org/10.2307/1685192

Talebi, S., Saeedinia, A., Zeinoddini, M., Ahmadpour, F., & Sadeghizadeh, M. (2019). Evaluation of a single amino acid substitution at position 79 of human IFN-α2b in interferon-receptor assembly and activity. *Preparative*

Biochemistry and Biotechnology, *49*(8), 735–743. https://doi.org/10.1080/10826068.2019.1566143

Taniguchi, T., Ogasawara, K., Takaoka, A., & Tanaka, N. (2001). IRF family of transcription factors as regulators of host defense. *Annual Review of Immunology*, *19*(1), 623–655. https://doi.org/10.1146/annurev.immunol.19.1.623

Thatcher, D. R., & Panayotatos, N. (1986). [24] Purification of recombinant human IFN-α2. *Methods in Enzymology*, *119*(C), 166–177. https://doi.org/10.1016/0076-6879(86)19026-4

Villaverde, A., & Mar Carrió, M. (2003). Protein aggregation in recombinant bacteria: biological role of inclusion bodies. *Biotechnology Letters*, *25*(17), 1385–1395. https://doi.org/10.1023/A:1025024104862

Vorona, K., Ryapolova, A., Sokolova, O., Karabelsky, A., Ivanov, R., Reshetnikov, V., & Minskaia, E. (2025, December 1). Interferon-Based Therapeutics in Cancer Therapy: Past, Present, and Future. *International Journal of Molecular Sciences*, Vol. 26. Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/ijms262311679

Weissmann, C., Nagata, S., Boll, W., Fountoulakis, M., Fujisawa, A., Fujisawa, J.-I., … Weidle, U. (1982). Structure and Expression of Human IFN-alpha genes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *299*(1094), 7–28. https://doi.org/10.1098/rstb.1982.0102

Wetzel, R. (1981). Assignment of the disulphide bonds of leukocyte interferon. *Nature*, *289*(5798), 606–607. https://doi.org/10.1038/289606a0

Zeuzem, S., Feinman, S. V., Rasenack, J., Heathcote, E. J., Lai, M.-Y., Gane, E., … Brunda, M. J. (2000). Peginterferon Alfa-2a in Patients with Chronic Hepatitis C. *New England Journal of Medicine*, *343*(23), 1666–1672. https://doi.org/10.1056/NEJM200012073432301