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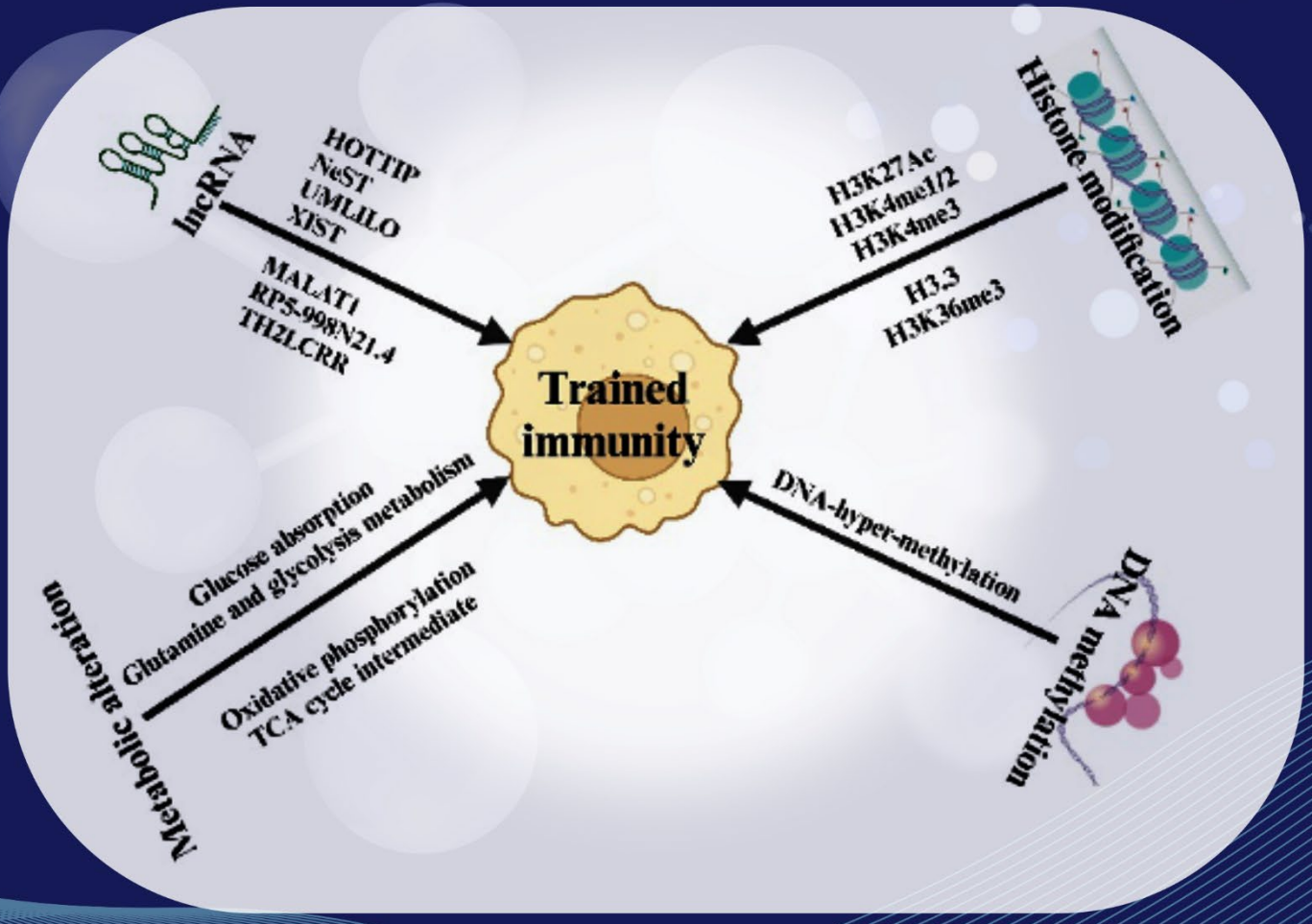

Long non-coding RNAs: A trained immunity perspective

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A graphic illustration of double-stranded DNA

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REVIEW ARTICLE

Long non-coding RNAs: A trained immunity perspective

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Abstract

The conventional understanding of immunological memory within adaptive immunity has faced recent challenges, paving the way for a novel concept known as "trained immunity." This phenomenon revolves around the epigenetic and metabolic reprogramming of cells as its central components. A growing body of evidence suggests that long non-coding RNAs (lncRNAs) play a crucial role in regulating immune cell development, function, and response to various diseases. Through intricate protein-protein interactions and interactions with DNA and RNA, lncRNAs significantly contribute to the modulation of immune processes. However, our comprehension of the involvement of lncRNAs in trained immunity is still in its early stages. This review delves into the recent advancements in lncRNA research, focusing on their diverse functions in immune cell development, host-pathogen interactions, potential processes, and their biological significance in trained immunity. Special attention is given to the role of lncRNAs in altering chromatin structure, orchestrating chromosomal looping, and driving metabolic reprogramming within cells.

Keywords: Long non-coding RNA; Trained immunity; Metabolic reprogramming; Epigenetics

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1. Introduction

Only a small fraction, approximately 2%, of genes undergo transcription to generate mRNAs that encode functional proteins and peptides. In stark contrast, the majority of the genome is transcribed into non-coding RNA (ncRNA), often termed "dark matter," lacking protein translation.[1-4] The Human Encyclopedia of DNA Elements (ENCODE) study revealed that around 80% of the genome is functional, with 62 – 75% undergoing transcription, primarily into ncRNA.[5,6] Analysis of RNA-seq data from human cells highlights the prevalence of ncRNAs, surpassing non-ribosomal and non-mitochondrial RNAs.[5] ncRNAs exhibit a dual classification based on their functions: Housekeeping RNAs, crucial for fundamental cellular processes, encompass transfer RNAs, small

nuclear RNAs, small nucleolar RNAs, and ribosomal RNAs. In contrast, regulatory RNAs, which govern gene expression and cellular activities, include small ncRNAs such as miRNAs, siRNAs, piRNAs, and long ncRNAs (lncRNAs).[3,6,7] The lncRNAs, a subset of endogenous RNAs, have a length exceeding 200 nucleotides and do not possess the capacity to translate into proteins. Alternatively, some lncRNAs may only encode very short peptides.[8-10] In Figure 1, various types of RNA are illustrated. It is noteworthy that in the past, ncRNAs were commonly perceived as biologically insignificant and often dismissed as mere "transcriptional noise."[5,11] These seemingly insignificant ncRNAs unravel the mystery of DNA's "dark energy."[5] Despite their former reputation as "junk," ncRNAs have recently been shown to play vital biological functions, especially in disease initiation and progression, and have proven helpful in novel drug development.[7] Unlike mRNA, lncRNAs are poorly preserved and expressed in lower quantities.[12] Moreover, lncRNAs share several similarities with mRNAs, such as being modified with a 3'-polyadenylated tail, 5'-capped, and transcribed by RNA polymerase II.[1] In the human genome dataset (GENCODE; version 29), 16,066 lncRNA genes and 29,566 lncRNA transcripts are reported, in contrast to the 19,940 protein-coding genes.[13]

The classification of lncRNAs into five distinct groups is based on their chromosomal location and relationship to protein-coding genes. These include: (i) long intergenic ncRNA (lincRNA), transcribed between known protein-coding genes; (ii) intronic lncRNA, transcribed within a protein-coding gene's introns; (iii) sense or pseudogene. lncRNA, transcribed from a gene but incapable of producing protein; (iv) natural antisense transcription, transcribed across the exons of a protein-coding gene in the opposite direction; and (v) bidirectional transcription, transcribed in opposite directions to the promoter of a protein-coding gene.[5,14] A schematic diagram illustrating the different types of lncRNA is presented in Figure 2.

Indeed, a substantial and increasing body of evidence highlights the importance of lncRNAs in a diverse array of biological processes. These processes encompass transcription, splicing, translation, protein localization, maintenance of cellular structure integrity, imprinting, regulation of the cell cycle, apoptosis, modulation of stem cell pluripotency, reprogramming, response to heat shock,

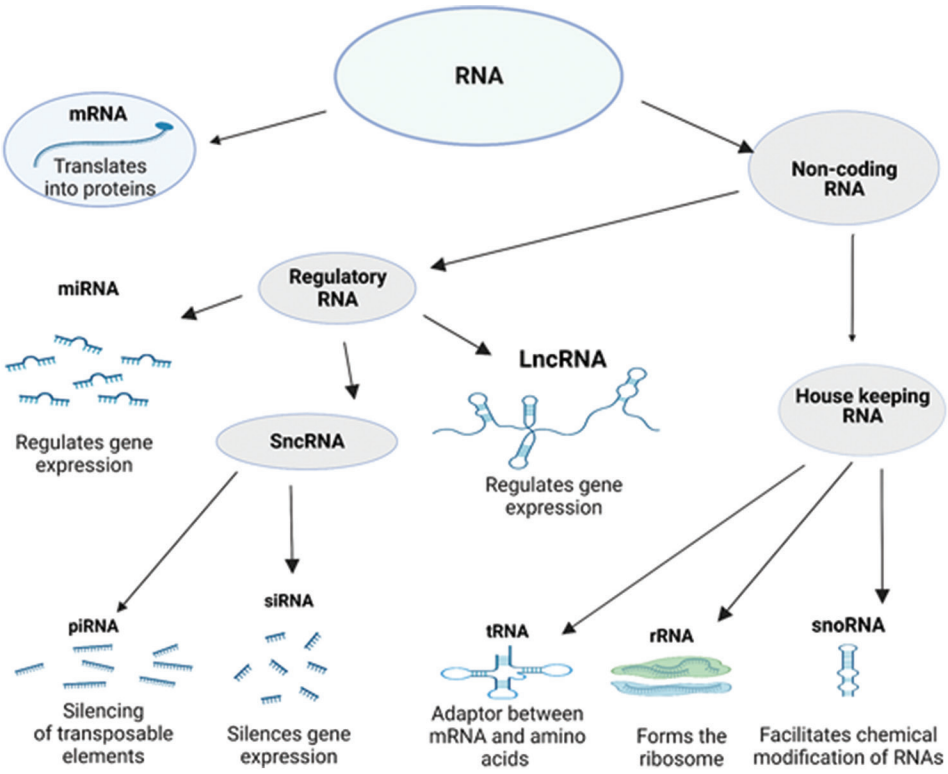

Figure 1. Different types of RNA. This diagram illustrates the multitude of RNA varieties integral to cellular functions. It encompasses coding messenger RNA (mRNA), vital for protein synthesis, alongside non-coding RNA variants, including housekeeping and regulatory RNA. Within the regulatory and housekeeping RNA classifications, it delineates subclasses such as transfer RNA (tRNA), ribosomal RNA (rRNA), small nucleolar RNAs (snoRNA), microRNA (miRNA), lncRNA, Piwi-interacting RNAs (piRNA), and small interfering RNA (siRNA).

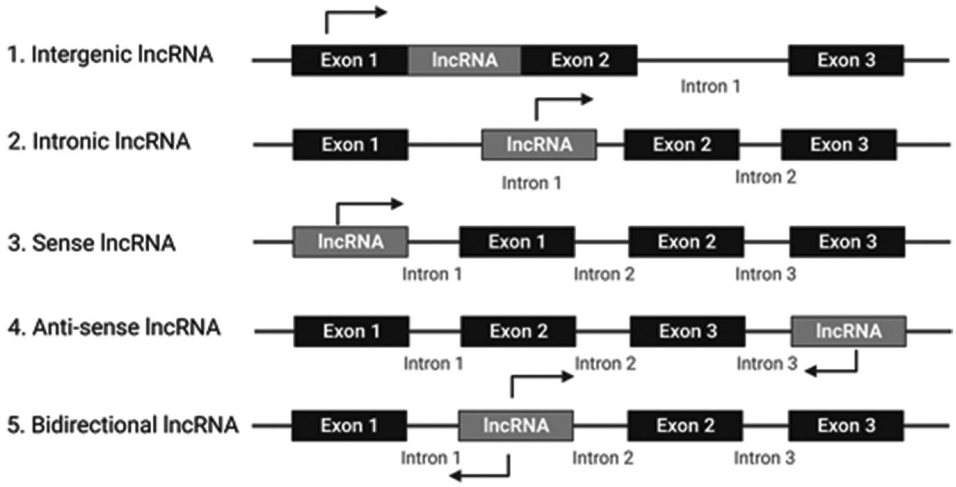

Figure 2. Different types of long non-coding RNA (lncRNA). This diagram depicts various subclasses of lncRNA molecules, encompassing intergenic lncRNAs, intronic lncRNAs, sense lncRNAs, antisense RNAs, and bidirectional lncRNAs.

progression of cancer, modulation of the innate immune response, and the development of various other human diseases. The multifaceted roles of lncRNAs underscore their significance in orchestrating and influencing fundamental cellular functions and disease pathways.[1,8] The lncRNAs regulate these functions through interactions with DNA, RNA, and protein.[15] Despite their widespread presence and abundance, only a small percentage of these molecules have a known function, and their production varies between healthy and pathological conditions.[8] The functions of lncRNAs are extremely lineage-specific, with growing evidence showing their involvement in the development and function of innate and adaptive cell types and fine-tuning their functional responses to diseased conditions. In addition, lncRNAs have been reported to play a role in chromatin remodeling, leading to the regulation of chromatin shape and gene expression.[16,17]

There are two kinds of host immunity: innate, also called in-born immunity, and adaptive or acquired immunity. The innate and adaptive immune cells work closely together to defend against pathogens and tumors. Innate immune cells respond quickly and in a non-specific manner, while adaptive immune cells respond slowly and with high-ordered specificity against invaders. Initially, innate immunity was thought to be a primordial type of immune response, while adaptive immunity was thought to be more sophisticated. However, it has lately been questioned whether immunological memory is specific to only adaptive immunity, particularly with the memory T and B cells, or if it is also associated with innate immune cells. The concept of "trained immunity" or "innate immunological memory" arises from evidence of the development of resistance to reinfection in organisms lacking adaptive immunity, such as plants, invertebrates, and SCID mice.[18,19] The notion of trained immunity is based on the finding that following an initial illness, secondary infection, or stimulus triggers an increased innate immune response.[20] In contrast to adaptive immunological memory, trained innate immunity cells' heightened secondary reaction is not only specific for the antigen that elicited the first response but also non-specific for heterologous stimuli. For example, Bacillus Calmette-Guerin (BCG) and the nucleotide-binding oligomerization domain 2 ligand muramyl dipeptide have been shown to have non-specific anti-infection properties.[21,22] This suggests that vaccinations may have a long-lasting heterologous effect through epigenetic reprogramming of genes.[23] The induction of trained immunity has been experimentally demonstrated in a range of immune cells, encompassing mononuclear myeloid cells such as monocytes, macrophages, dendritic cells (DCs), and neutrophils. In addition, lymphoid cells, including natural killer (NK) cells and innate lymphoid cells (ILCs), have also exhibited the capability for trained immunity induction. This broad involvement across different immune cell types underscores the diverse impact of trained immunity on both myeloid and lymphoid components of the immune system.[24]

This review focuses on the recent advancement of the role of lncRNAs in immunity, particularly trained immunity and putative processes. In conclusion, it sheds light on the newly predicted functional role of lncRNAs in epigenetic and metabolic reprogramming in connection with trained immunity.

2. lncRNAs in immune cell development

Hematopoietic stem cells (HSCs) represent multipotent cells with the remarkable ability to differentiate into various

types of blood cells. This includes cells belonging to both myeloid and lymphoid lineages. Within the lymphoid lineage, HSCs can give rise to T cells, B cells, and NK cells. On the other hand, myeloid lineage differentiation leads to the formation of diverse cell types, such as monocytes, macrophages, erythrocytes, basophils, neutrophils, eosinophils, and megakaryocytes. The differentiation potential of HSCs plays a pivotal role in maintaining the balance and functionality of the entire blood cell repertoire. DCs have a heterogeneous hematopoietic lineage and can be generated from both lymphoid and myeloid lineage cells. Recently, the role of lncRNAs has been reported in regulating immune cell growth and functions, including their formation, differentiation, activation, and polarization.[25] Table 1 below summarizes the role of lncRNAs in the development, polarization, and activation of both myeloid and lymphoid cells within the immune response.

2.1. Role of lncRNAs in the development and polarization of macrophage

Macrophages, expansive phagocytic myeloid cells found in various tissues, hold a crucial position in innate immunity and actively participate in a range of biological processes. These include the engulfment of dying cells, processing and presenting antigens, facilitating wound repair, and releasing both pro- and anti-inflammatory mediators. In the intricate landscape of macrophage development and polarization, a multitude of lncRNAs have been recognized for their substantial roles. One noteworthy lncRNA in this context is lncMorrbid, a conserved element observed in species such as humans and mice. Initially identified in murine myeloid cells, including eosinophils, neutrophils, and Ly6C hi-monocytes, lncMorrbid assumes a critical role in regulating myeloid cell lifespan by modulating the pro-apoptotic gene *Bim* (*Bcl2l11*). The bivalent promoter of the *BCL2L11* gene undergoes regulation through histone marks, with Histone K27 methylation (H3K27me3) serving to repress its expression, a process counteracted by Histone K4 methylation (H3K4me3). LncMorrbid orchestrates this regulatory mechanism by recruiting the polycomb repressive complex 2 (PRC2), which deposits H3K27me3 histone marks at the *BCL2L11* locus. This concerted action prevents the expression of the *BCL2L11* gene, thereby exerting influence over the lifespan and fate of myeloid cells.[8,25,26,37]

The non-coding transcript in T cells (*NTT*), a 17-kb unspliced polyadenylated lncRNA located on chromosome 6q23 – q24, was initially identified in human CD4+ T cells and peripheral blood mononuclear cells. Interestingly, it is

Table 1. LncRNAs in myeloid cell development and functions

LncRNA	Cell type	Type	Function	References
MORRBID (Gm14005)	Myeloid cells (granulocyte)	Intergenic	It regulates the survival of short-lived myeloid cells by cis-regulating the expression of *Bcl2l11*.	26
NTT	Monocytes	Intergenic	It complexes with hnRNP-U, binding to the *PBOV1* gene promoter and inducing cell cycle arrest. This event initiates differentiation into M0-M2, elevates *IL-10* and *CXCL10* mRNA levels, and upregulates costimulatory molecules.	27
PACER (COX2-lncRNA)	Monocytes	Antisense	It displaces the NF-κB1 homodimer from the *COX2* promoter, facilitating the binding of the activating RELA/NF-κB1 heterodimer for transcription initiation.	28
Lnc-MC	Monocytes	Intergenic	It enhances monocyte differentiation by amplifying the effects of PU.1, sequestering miR-199a-5p, and upregulating *ACVR1B* expression.	29
TCONS_00019715	Monocytes	Intergenic	It promotes macrophage polarization towards the pro-inflammatory (M1) phenotype.	30
HOTAIR	Monocytes	Antisense	Facilitates the breakdown of IkBα, leading to an increase in pro-inflammatory NF-κB signaling.	31
Lnc-DC	DCs	Intergenic	Enhances STAT3 signaling by binding to the STAT3 C-terminus, inhibiting SHP1-mediated dephosphorylation of STAT3 at Y705.	32
HOTAIRM1	DCs	Intergenic	Promotes monocyte/DC differentiation by competitively binding to endogenous miR-3960.	33
NEAT	DCs	Antisense	It promotes a tolerogenic phenotype in DCs by targeting miR-3076-3p.	34,35
MALAT1 (NEAT2)	DCs	Intergenic	Serving as a miR-155 sponge and boosting PU.1 expression, it induces a tolerogenic phenotype in DCs, fostering the polarization of regulatory T cells (Treg) through increased PU.1 expression.	36

Abbreviations: LncRNAs: Long non-coding RNAs; DC: Dendritic cells; IL-10: Interleukin-10.

also present in the nuclei of myeloid-origin cells including monocytes, monocyte-derived macrophages, and the monocytic cell line THP-1. Studies have revealed that the monocytic transcription factor CCAAT-enhancer-binding proteins, binding to the promoter region of the *PBOV1* gene via hnRNP-U, regulate the expression of *NTT*. In THP-1 cells, P*BOV1* overexpression resulted in G1 stage cell cycle arrest, decreased CD14 expression, and increased CD68 expression, indicating a differentiation process favoring macrophages. Furthermore, *PBOV1* overexpression led to a significant increase in interleukin (IL)-10 and CXCL10 mRNA levels, along with an upregulation of costimulatory molecules. These findings provide compelling evidence of *NTT*'s pivotal role in lineage commitment and the activation of macrophage cells, underscoring its significance in the intricate regulatory network governing cellular differentiation and immune response activation.[8,27]

An additional antisense nuclear lncRNA, recognized as *COX-2*-lncRNA or *PACER* (P50-associated COX-2 extragenic RNA), is positioned upstream of the COX-2 promoter. In a PMA-driven human monocyte-macrophage differentiation system, when stimulated with LPS, PACER expedites the expression of the *COX-2* gene. The evidence suggests that *PACER* accomplishes this by directly sequestering the repressive NF-κB p50 subunit from the COX-2 promoter. This action enables the formation of the active p50 – p65 form of NF-κB in the COX-2 promoter region. Moreover, PACER enhances the recruitment of the p300 histone acetyltransferase (HAT) and the RNAP II pre-initiation complex. This enhancement leads to increased histone acetylation, ultimately inducing COX-2 transcription. In summary, PACER assumes a crucial role in modulating *COX-2* gene expression by orchestrating the dynamics of NF-κB subunits and facilitating the recruitment of key components involved in the transcriptional process.[8,28]

During the differentiation of monocytes into macrophages, a complex series of transcriptional events highlights the crucial role of long non-coding monocytic RNA (lnc-*MC*) and its interaction with the master regulator PU.1. As lnc-MC levels increase in THP-1 cells, HL-60 cells, and CD34+ hematopoietic stem/progenitor cells (HSPCs), PU.1 assumes control. It activates lnc-MC production, counteracting miR-199a-5p's suppression of the lnc-MC promoter and enhancing overall lnc-MC expression. This partnership extends its influence to activin A receptor type 1B (ACVR1B), a crucial protein in monocyte/macrophage development. In this intricate molecular interplay, the collaboration between PU.1 and lnc-*MC* serves as a guiding force, orchestrating the transition of monocytes into macrophages. This coordination between transcription factors and lncRNA elucidates the regulatory mechanisms governing myeloid cell development.[8,29] Macrophages display versatility and can undergo polarization into distinct subpopulations in response to environmental cues: the classically activated macrophages are also called M1-macrophage and alternatively activated macrophages or M2-macrophage. IFN-γ or LPS lipopolysaccharide (LPS) stimulation typically causes M1-like-phenotype, while cytokines IL-4, IL-10, or IL-13 induce M2-like phenotypes. Several lncRNAs involved in macrophage polarization have been identified. The lncRNA-*Cox2*[38] and lncRNA *TCONS 00019715* favor M1 polarization, whereas lncRNA *LINC00662* favors M2 macrophage polarization in hepatocellular carcinoma (HCC) through Wnt/- catenin pathways.[39] In breast cancer, the lncRNA *BCRT1* and nuclear paraspeckle assembly transcript 1 *(NEAT1)* enhance the M2-polarization of macrophages and astrocyte activation, whereas the lncRNA-MM2P was found essential for macrophage M2-polarization.[40-42]

2.2. lncRNAs-DC developments

lncRNAs play a crucial role in the development and functioning of DCs, specialized antigen-presenting cells that connect innate and adaptive immune responses in vertebrates. The activities of DCs are intricately regulated by various transcription factors, anti-inflammatory cytokines, and ncRNAs. Noteworthy lncRNAs such as lnc-DC, HOX antisense intergenic RNA myeloid 1 (*HOTAIRM1*), *NEAT 1*, and *MALAT1* have been identified as key regulators of human DC differentiation.[32]

Long non-coding-DCs are particularly essential in governing the STAT3 pathway, a critical element in differentiating human monocytes into DCs. Positioned between the *HOXA1* and *HOXA2* genes, *HOTAIRM1* undergoes significant histone modifications (H3K4me3 and H3K27me3) in its promoter during the transition from monocytes to DCs. An association is suspected between *HOTAIRM1*, *miR-3960*, and *HOXA* genes in the development of monocyte-dendritic (Mo-DC) cells. The downregulation of *HOTAIRM1* and *HOXA1* expression facilitates the transformation of monocytes into DCs.[8,33]

The NEAT1, localized in the nucleus, has two isoforms: *NEAT1-1 (*3.7-kb) and *NEAT1-2 (*23-kb). *NEAT1-1* significantly contributes to the tolerogenic phenotype of DCs, which are crucial in immunological disorders as they shape T-cell responses and promote immune tolerance. In addition, *NEAT1* in DCs exhibits similarities with the *MALAT1*-mediated tolerogenic phenotype, suggesting collaborative actions among multiple lncRNAs to achieve

specific functional states in immune cells.[34,35] Recent findings indicate that the *MALAT1* lncRNA is implicated in the innate immune response. Elevated levels of MALAT1 are observed in the invading cells of tolerized mice, and *MALAT1* overexpression favors the transition of DCs toward a tolerant phenotype.[36]

2.3. lncRNAs' impact on NK cell development and activation

NK cells are categorized as ILCs and, alongside monocytes, macrophages, and DCs, constitute the frontline defense of the innate immune system. Their primary functions involve detecting and eliminating infected or abnormal cells, thereby playing pivotal roles in the immediate and rapid responses of the innate immune system against diverse threats.[43] NK cells operate autonomously of the antigen processing and presentation pathways. Instead, they carry out their functions by releasing pro-inflammatory cytokines, such as interferon-gamma (IFN-γ), and deploying cytotoxic granules containing perforin and granzymes, leading to the lysis of target cells. This immediate and non-specific response is a characteristic feature of the innate immune system. Recent evidence further indicates that innate immune memory can be transferred through progenitor and HSCs, highlighting the ability of specific immune cells to retain a memory-like response to past encounters with pathogens, thereby contributing to a more effective and rapid immune response on subsequent exposures.[18] Building on this notion, NK cell memory has been acknowledged independently of T and B cells. Observations indicate that following exposure to cytokine combinations (e.g., IL-12, IL-15, and IL-18)[44] or hapten sensitization,[45] NK cells exhibit a heightened and more robust response on encountering a previous challenge. This underscores a form of memory-like behavior in the innate immune system, where NK cells display an enhanced capability to respond effectively to familiar threats. After cytomegalovirus (CMV) infection, the activation of NK cells assumes a critical role in offering T cell-independent protection against reinfection. This protective mechanism involves the swift degranulation of cytotoxic granules and cytokine production, underscoring the remarkable capacity of NK cells to orchestrate an accelerated and effective immune response when encountering a familiar pathogen for a second time.[46]

Emerging evidence strongly supports the participation of lncRNAs in both the development and activation of NK cells. Specifically, lnc-*CD56* has been identified as a key regulator influencing NK cell differentiation. In addition, the lncRNA Rroid has been shown to exert regulatory control over the expression of the transcriptional regulator *ID2*, thereby influencing the function and lineage identity of group 1 ILCs, a category encompassing NK cells. These discoveries emphasize the integral role played by lncRNAs in shaping the development and function of NK cells, providing insights into the regulatory mechanisms within the immune system.[47]

The lncRNA *GAS5* has emerged as a crucial regulator of the cytotoxic function of NK cells, particularly in the context of hepatocellular carcinoma. The GAS5 exercises control over the expression of IFN-γ in NK cells. On activation of *GAS5*, NK cells exhibit elevated levels of IFN-γ. Conversely, when *GAS5* is knocked down, the secretion of IFN-γ is significantly reduced. This decrease in IFN-γ secretion is correlated with a decline in NK cell cytotoxicity, diminished levels of CD107a+ NK cells, and a reduction in apoptosis of HepG2 and Huh7 cells. These findings underscore the pivotal role of *GAS5* in modulating the cytotoxic function of NK cells and its potential impact on the immune response against hepatocellular carcinoma.[48]

2.4. The function of Lnc-RNA in host-pathogen interaction

Numerous lncRNA species play crucial roles in modulating immune responses against microbial components, thereby significantly contributing to the coordination of the host's defense mechanisms. One notable example is lincRNA-*Cox2*, located near the cyclooxygenase 2 (*Cox2*) gene. Initially discovered in murine DCs exposed to LPS, a bacterial cell wall component, the presence of lincRNA-*Cox2* implies its potential role in orchestrating immune responses to microbial stimuli. This is particularly relevant within DCs, which are central players in coordinating various aspects of the immune system.[49] The expression of the *lincRNA-Cox2* gene markedly increased when bone marrow-derived mouse macrophages (BMDMs) were stimulated with LPS or Pam3CSK4. Similarly, macrophages infected with Listeria monocytogenes exhibited heightened expression of lincRNA-*Cox2*.[50] Another lncRNA, *NEAT1*, is implicated in regulating the immune response to microbial components. It serves as a crucial component of the *HEXIM1-DNA-PK*-paraspeckle and ribonucleoprotein complex (HDP-RNP). The knockdown of *NEAT1* resulted in the loss of *IFN*-β mRNA expression over DNA (ISD)-mediated stimulation.[51] Conversely, lncRNA-*GM* has been identified as a suppressor of viral replication and an enhancer of type I interferon (IFN-I) generation. In mice lacking the lncRNAs lncRNA-GM, there was an observed increase in sensitivity to viral infection, coupled with a reduction in interferon-I (IFN-I) production. Notably, LncRNA-*GM* was found to interact with glutathione S-transferase M1 (GSTM1), preventing GSTM1 from engaging with the kinase TBK1. This interaction resulted

in the attenuation of GSTM1-mediated S-glutathionylation of TBK1. The subsequent decrease in S-glutathionylation led to heightened TBK1 activity, thereby amplifying the downstream production of antiviral mediators. These findings highlight the regulatory role of lncRNA-*GM* in the antiviral immune response by modulating the activity of key signaling molecules integral to the innate immune system.[52]

2.5. Epigenetically modified lncRNAs: their impact on immunity

Epigenetic alterations on lncRNAs play a crucial role in modulating their expression levels and functional outcomes. Notably, the hypomethylation of *MALAT1* has been linked to increased expression, correlating with elevated production of inflammatory cytokines.[53] Similarly, the presence of H3K27 acetylation in the promoter region of *NEAT1* facilitates inflammasome assembly and activation, underscoring the intricate regulatory influence of epigenetic modifications on immune responses.[54]

2.6. lncRNAs' confirmations and implications in immunity

LncRNAs, although unable to encode proteins, assume distinct structural configurations that facilitate interactions with various molecules. For example, the presence of N6-methyladenosine modifications within lncRNA hairpins predisposes them to protein binding.[55] Consider *MALAT1*, which undergoes reversible methylation at position A2577, thereby promoting HNRNPC protein binding.[56] Furthermore, m6A modifications stabilize *MALAT1* and activate the NF-κB pathway.[57] The incorporation of multivalent structural motifs and long-stem structures is crucial for lnc-Lsm3b's interaction with the innate RNA sensor RIG-I, leading to inhibition.[58] These findings collectively suggest a significant role for lncRNA conformation in immune responses.

3. lncRNAs' function in trained immunity

The concept of "trained immunity" was introduced by Netea *et al.*[18] to elucidate the phenomenon wherein innate immune cells, notably macrophages, monocytes, and NK cells, display heightened reactivity on reencountering infections, even in the absence of adaptive immunity. This enhanced immune response is linked to a notable shift in cellular metabolism and epigenetic reprogramming, particularly at the level of histone modifications.[18,59] Epigenetic reprogramming induced alterations in gene expression without affecting the underlying DNA sequences. Notably, these modifications exhibited a lasting impact that could endure across numerous generations.[60] Furthermore, studies have indicated that macrophages may demonstrate memory-like characteristics following LPS induction.[61] Recent studies have unveiled that trained immunity responses are not exclusive to macrophages; monocytes have also been proven to exhibit such responses after infection by parasitic pathoigens[62] and viral agent.[63] Monocytes typically have a short half-life in circulation, lasting up to 1 day.[64] However, it is intriguing to note that trained monocytes have been identified in the bloodstream of individuals vaccinated with BCG for at least 3-month post-vaccination. This observation suggests that the reprogramming leading to trained immunity occurs at the progenitor cell level in the bone marrow. The prolonged presence of trained monocytes in circulation underscores the enduring impact of BCG vaccination on the immune system, emphasizing the potential establishment of innate immune memory at the level of progenitor cells in the bone marrow.[65]

Although the mechanism of trained immunity is still being unraveled,[18] there is compelling evidence indicating that epigenetic reprogramming, including histone modification, DNA methylation, and ncRNA, plays a substantial role in this process (Figure 3). Histone alterations and chromatin reconfiguration have been identified as important processes in the development of learned (trained) immunity. H3K27Ac, H3K4me1/2, H3K4me3, histone H3.3, and H3K36me3 have all been found to be upregulated by reinfection.[66-68] When challenged by the human CMV (HCMV), NK cells displayed trained immunity through DNA methylation.[69]

3.1. lncRNAs: orchestrators of epigenetic regulation in trained immunity

Beyond immune cell development, lncRNAs play a critical role in epigenetically modulating the human genome. Operating through direct interactions with histone writers, readers, and erasers, lncRNAs significantly impact chromatin structure and gene expression by influencing histone modifications. The intricate involvement of lncRNAs in epigenetic processes underscores their multifaceted role in shaping the epigenome and influencing diverse cellular functions.[70] Indeed, lncRNAs play a significant role in human diseases, including cancer, inflammation, and schizophrenia, by interacting with epigenetic factors. In the context of glioma, the lncRNA *MIR155HG* has been observed to undergo epigenetic activation. This activation is mediated by promoter hypomethylation and involves the transcription factor SP1. Importantly, the epigenetic changes in *MIR155HG* have been correlated with immune infiltration in glioma. This suggests a link between epigenetic regulation of specific lncRNAs and the modulation of immune responses in the context of certain diseases, such as glioma.[71] The study by

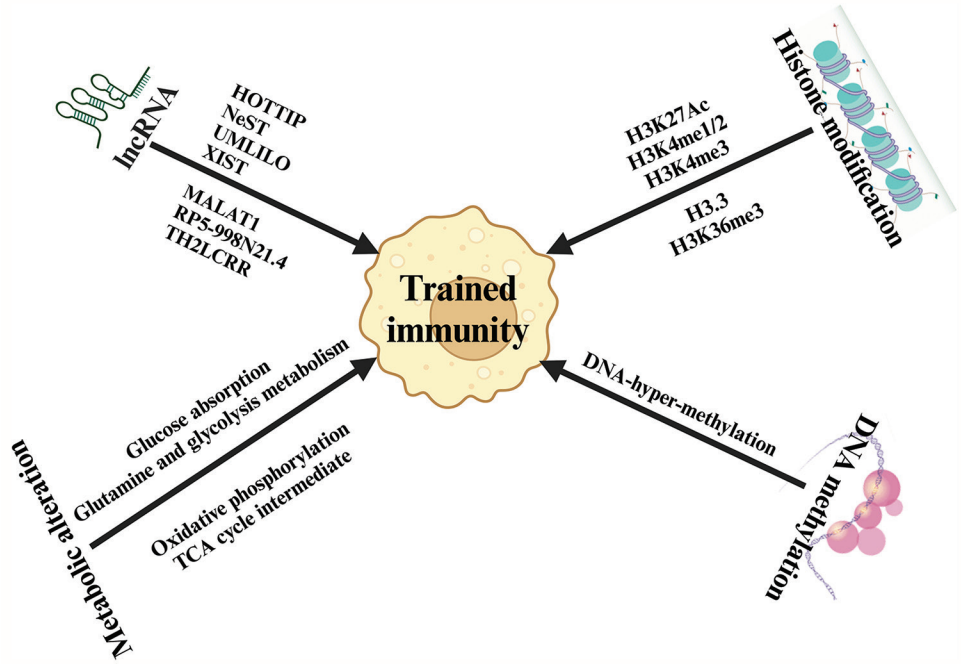

Figure 3. Schematic diagram illustrating the probable mechanisms of trained immunity modulation
Abbreviation: TCA: Tricarboxylic acid.

Guo *et al.*[72] recently demonstrated that the lncRNA *RP5-998N21.4* plays a role in promoting immune defense in the context of schizophrenia. The researchers found that *RP5-998N21.4* upregulates the expression of interferon-induced protein with tetratricopeptide repeats *IFIT2* and *IFIT3*. Furthermore, the authors observed that *RP5-998N21.4* positively regulates the transcription of *IFIT2* and *IFIT3* by binding to their promoter regions and influencing histone modifications. This research sheds light on the involvement of specific lncRNAs in the immune response and their potential implications in the context of schizophrenia.[72] Targeting the epigenetic regulation of myeloid-derived suppressor cells (MDSCs) in human sepsis is considered imperative. Addressing the epigenetic factors associated with MDSCs in the context of sepsis could potentially contribute to resolving post-sepsis immunosuppression and improving sepsis survival. By modulating the epigenetic landscape of MDSCs, there is potential for interventions that could enhance the immune response and mitigate the adverse effects of sepsis, leading to improved outcomes for individuals affected by this condition.[73] These studies highlight the importance of lncRNAs as epigenetic functions. Thus, further understanding the molecular mechanisms behind trained immunity would help explore the lncRNAs as epigenetic targets for therapeutic purposes. The *XIST* is indeed one of the most extensively studied lncRNAs and represents a prime example of a lncRNA directly involved in the formation of repressive chromatin. It plays a crucial role in the process of X-chromosome inactivation. Moreover, several other lncRNAs are implicated in the regulation of DNA methylation status in human cells. They can either recruit or inhibit the action of DNA methyltransferases and demethylases, influencing the epigenetic modifications of DNA. This involvement in the regulation of DNA methylation highlights the diverse and intricate roles that lncRNAs play in modulating the epigenetic landscape of the genome.[74] The observation that CD8+ T cells can differentiate into short-lived effector cells and memory cells, providing sustained protection during immune responses to microbial infection, is well-established. In the context of this differentiation process, a study has suggested the potential involvement of the lncRNA *MALAT1*. This lncRNA may play a role in the regulation of CD8+ T cell differentiation by mediating epigenetic repression in response to acute infection. The study implies that *MALAT1* could contribute to the epigenetic control of CD8+ T cell fate during immune responses.[75] The regulation of histone modifications plays a crucial role in the establishment of epigenetic memory in the context of trained immunity. Specifically, lysine 27 of histone 3 (H3K27) and lysine 4 of histone 3 (H3K4) at the promoters of trained immune genes undergo rapid acetylation and trimethylation, respectively, during the initial phase of training. Interestingly, upon the removal of the training stimulus, it has been observed that H3K4me3 occupancy remains accumulated on the chromatin, while

H3K27Ac is gradually lost over time. This long-lived accumulation of H3K4me3 is essential for the establishment of epigenetic memory, contributing to a sustained and effective immunological response upon reexposure to the same pathogen. In a related context, the lncRNA *HOTTIP* has been noted to function epigenetically, regulating the expression of homeotic genes during development. This highlights the diverse roles of lncRNAs in epigenetic regulation across different biological processes.[76] In a separate study, the expression of the lncRNA *UMLILO* was found to exhibit a positive correlation with the accumulation of histone modification H3K4me3 on the promoter of the *CXCL* gene. This observation suggests a potential role for *UMLILO* in mediating the regulation of the H3K4me3 chromatin establishment mechanism during trained immunity. The interplay between lncRNAs and histone modifications further emphasizes the intricate regulatory networks involved in shaping the epigenetic landscape during immune responses and trained immunity.[77] The emerging role of lncRNAs in acquiring epigenetic memory underscores their potential as novel drug targets for the development of the next generation of immunotherapies. The intricate involvement of lncRNAs in epigenetic regulation presents an opportunity to manipulate these molecules for therapeutic purposes, especially in the context of immune memory and responses. Figure 3 and Table 2 summarize lncRNAs with their epigenetic functions.

3.2. lncRNAs' target chromatin structure

The regulation of gene expression is a highly intricate and compartmentalized process in eukaryotes. lncRNAs play a pivotal role in influencing numerous essential processes. These include chromatin remodeling, recruitment of the transcription machinery, modulation of mRNA processing and cytoplasmic distribution, regulation of mRNA stability, control of translation, and involvement in posttranslational activities, among others. The multifaceted impact of lncRNAs underscores their significance in orchestrating the complexity of gene regulation within eukaryotic cells.[78] Eukaryotic gene expression is regulated by the positioning of nucleosomes, which are fundamental units of chromatin consisting of DNA wrapped around four core histone proteins (H2A, H2B, H3, and H4). The interaction between histone cores and DNA significantly influences DNA accessibility. Chromatins exhibit distinct modifications to histones post-translationally, depending on whether they are transcriptionally active or silent. In actively transcribed genes, specific modifications are commonly found. These include high levels of lysine acetylation on the tails of H3 and H4, trimethylation of H3 lysine 4, trimethylation of H3 lysine 79, ubiquitylation of H2B, and trimethylation of H3 lysine 36. Conversely, repressed genes are associated with modifications such as trimethylation of lysine 27, ubiquitylation of H2A on lysine 119, and trimethylation of H3 lysine 9. These post-translational modifications to histones contribute to the dynamic regulation of chromatin structure, reflecting the transcriptional status of genes in eukaryotic cells.[79,80] Growing evidence suggests that lncRNA can control the genome activity at the chromatin level.[80]

It has long been assumed that lncRNAs interact with RNA, DNA, and proteins to modulate gene expression.[64] In addition, depending on the spatial organization of chromatin, lncRNA activity can be directed to certain loci.[31,65] As a result, the combination of nuclear architecture and lncRNA activity can modulate immune gene transcription in a spatially dependent manner.

3.3. lncRNAs-guided genome targeting through chromosome looping

In eukaryotes, the folding of DNA into chromosomal loops plays a pivotal role. These loops, formed within the nucleus, facilitate transcription by bringing co-regulated genes and enhancer-promoter elements into close spatial proximity, both in cis and trans configurations. Cis-acting

Table 2. LncRNAs in epigenetic regulation

lncRNA	Function	References
HOTTIP	Bind with *WDR5* and regulate the H3K4 methylation at the *IFN* gene locus.	84
NeST	Bind with *WDR5* and regulate the H3K4 methylation at the *IFN* gene locus.	85
UMLILO	Directly interacting with the WDR5/MLL complex regulates the H3K4 methylation at the *IFN* gene locus.	76
XIST	Formation of repressive chromatin.	74
MALAT1	Associated with an augmented deposition of H3K27me3 at genes linked to memory cells, facilitated through direct interaction with EZH2.	75
RP5-998N21.4	Boosts immune defense by binding to promoter regions and exerting influence on histone modifications.	72
TH2LCRR	H3K4me3 is added to the promoters of T helper-2 cell cytokines, such as IL-4, IL-5, and IL-13.	77

Abbreviations: IL-5: Interleukin-5; LncRNAs: Long non-coding RNAs.

lncRNAs exert their influence at the site of transcription, impacting the expression of nearby genes. Conversely, trans-acting lncRNAs operate at a distance from the synthesis site. The chromatin organization extends to domains enriched in chromosomal loops, known as topologically associating domains (TADs), contributing significantly to the three-dimensional architecture of the genome. The TADs play a crucial role in shaping the spatial arrangement of genes and regulatory elements within the eukaryotic nucleus, ensuring proper gene regulation and coordination of transcriptional activities.[81] Indeed, evidence has shown that lncRNAs play a role in mediating the assembly of TADs. Enhancer RNAs (eRNAs), a specific class of lncRNAs, are synthesized from active enhancer regions and contribute to the regulation of gene expression. One of the functions of eRNAs is to facilitate chromatin looping between enhancers and promoters during the transcription process. This dynamic interplay involving lncRNAs, particularly eRNAs, adds another layer of complexity to the regulation of gene expression. By participating in the spatial organization of chromatin and the formation of chromosomal loops, lncRNAs contribute to the intricate orchestration of transcriptional activities within the eukaryotic genome.[16,82,83] In addition, certain lncRNAs are involved in remodeling the chromatin complex and catalyzing the trimethylation of histone H3 at lysine 4 (H3K4me3) on the promoters of target genes. For instance, *HOTTIP*, a lncRNA transcribed from the HOXA locus, and *NeST* lncRNA directly interact with WD repeat-containing protein 5 (WDR5), influencing the modulation of H3K4me at specific gene loci such as the *IFN* gene locus. This interaction highlights the role of lncRNAs in regulating histone modifications and chromatin structure, contributing to the precise control of gene expression.[84,85] In a study conducted by Fanucchi *et al.*,[81] the pivotal role of lncRNAs in trained immunity was first demonstrated. The researchers identified a specific group of lncRNAs referred to as immune gene priming lncRNAs (IPLs or IP-lncRNAs), which were found to be brought into proximity with immune genes before their activation, as revealed by chromatin 3D structure analysis. *UMLILO*, an upstream master lncRNA of the inflammatory chemokine locus, was employed as a representative IPL to validate the findings. This IPL established chromosomal contacts with the ELR+ CXCL chemokines (IL-8, CXCL1, CXCL2, and CXCL3) and acted in cis to guide the WDR5–mixed lineage leukemia protein 1 (MLL1) complex across the chemokine promoters. This facilitated the epigenetic priming of these promoters through H3K4me3 modification, preparing them for active transcription. Notably, the regulation of genes such as *IL-6* and *IL-1* followed a similar pattern. These findings highlight the critical role of lncRNAs, specifically IPLs, in orchestrating chromosomal contacts and facilitating epigenetic priming of immune genes as part of the mechanism underlying trained immunity.[81,82] lncRNAs have also been identified as key regulators of toll-like receptors (TLR) signaling and innate immunity.[86,87] For example, the LPS-sensitive lncRNA *Mirt2* is expressed in macrophages and inhibits TLR4 signaling by inhibiting NF-κB and MAPK activation and subsequent TNF generation[88] (Table 2).

In the field of trained immunity, a central question that remains to be answered is the mechanism by which epigenetic marks are precisely deposited at specific loci, particularly at the promoters of trained genes. Understanding the molecular processes and regulatory factors involved in the discrete deposition of epigenetic marks is crucial for unraveling the intricacies of trained immunity and its underlying mechanisms. This includes elucidating the roles of various molecular players, such as lncRNAs, chromatin-modifying complexes, and other epigenetic regulators, in orchestrating the targeted establishment of epigenetic marks at specific genomic loci. Further research in this area is essential for gaining comprehensive insights into the molecular basis of trained immunity and advancing our ability to modulate immune responses for therapeutic purposes.[81] Evidence suggests that metabolic changes serve as the principal initiators, supplying the necessary substrates and co-factors crucial for epigenetic reprogramming. However, despite the widespread presence of these accumulated substrates and co-factors throughout cells, evidence suggests the existence of specialized machinery within the nucleus that orchestrates targeted epigenetic changes, as depicted in Figure 3.

3.4. lncRNAs role in metabolic alterations

A growing body of research suggests that metabolic alterations contribute to myeloid cell epigenetic reprogramming, leading to a trained immunological phenotype and non-specific resistance to secondary infection. Recently, various lncRNAs such as lncRNA-*p23154*, lncRNA-*NEF*, *HOTAIR*, and *MACC1-AS1* have been identified in association with metabolic alterations, where they control GLUT1 for glucose absorption. In addition, lncRNAs *ftx* and *SNHG3* regulate the oxidative phosphorylation metabolic enzyme in cancer.[89] Pathogens employ diverse strategies to manipulate host cell metabolism, involving glutamine and glycolysis metabolism. Notably, lncRNAs like lncRNA-*ACOD1* and lncRNA-*HOTAIR* have been implicated in the metabolic regulation of virus infection. In colorectal cancer, *F. nucleatum* targets lncRNA enolase 1-intronic transcript 1 for glycolysis.[90] Inflammation triggers the dectin-1-Akt-mTOR-HIF-1 pathway to shift from oxidative phosphorylation to

aerobic glycolysis, forming the metabolic foundation of trained immunity.[19] Increased glycolysis and tricarboxylic acid cycle intermediates, such as fumarate and glutamate, regulate the methylation (H3K4me3) and acetylation (lysine 27 acetylation, H3K27ac) of histones, highlighting the metabolic basis of trained immunity.[91] BCG-induced trained immunity is also an example of metabolic reprogramming, in which a shift in glucose metabolism to glycolysis is required for histone modification to occur.[92]

4. Conclusion

Initially dismissed as "junk RNA," lncRNAs have garnered increasing significance in recent years, with numerous studies highlighting their crucial roles in various disease conditions. The relatively new phenomenon of "trained immunity," characterized by metabolic and epigenetic gene expression reprogramming, has demonstrated a positive impact on host immunity. Emerging evidence points toward a critical role for lncRNAs in pathogen recognition and the modulation of immune cell fate, affecting macrophages, DCs, and NK cells through metabolic and epigenetic reprogramming. While the direct link between lncRNAs and trained immunity is still under investigation, current review articles may offer valuable insights into this relationship between trained immunity and lncRNAs. The use of the trained immunity-based BCG vaccine against COVID-19 suggests potential positive effects on the burden of COVID-19.[93] As research progresses, delving into the intricate interplay between lncRNAs and trained immunity could shape future treatments for pandemics. Harnessing the functions of lncRNAs offers a promising avenue for finely tuning host immunity by modulating the mechanisms of trained immunity. This exploration may lead to the development of targeted interventions and therapeutic strategies aimed at bolstering the host's defense mechanisms during pandemics and infectious diseases.

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Author contributions

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

REVIEW ARTICLE

Application and research progress of CAR-T cell therapy in autoimmune diseases

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Abstract

The conventional clinical therapies for autoimmune diseases (ADs) lack specificity, necessitating long-term medication that can lead to serious side effects. In contrast, chimeric antigen receptor (CAR) T cell therapy for ADs, characterized by fewer side effects and longer-lasting therapeutic effects, represents a new direction for the specific treatment of ADs. T cells modified with CAR genes possess the ability to not only secrete perforin, granzymes, and other molecules that target autoreactive immune cells but also to lead effector and regulatory T cells into autoimmune environments, thereby exerting transport, proliferation, and immune regulatory functions. Chimeric autoantibody receptor T cells can recognize and kill autoreactive cells expressing target autoantibodies through their specific antigens. In this article, we comprehensively expound on the application of CAR-T cell therapy in different ADs and summarize the current research progress in this regard. This review aims to enhance the application of CAR-T therapy in AD treatment and facilitate further studies aimed at addressing the existing gaps in CAR-T therapy for ADs.

Keywords: Autoimmune diseases; Chimeric antigen receptor T cells; T cells

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1. Introduction

Autoimmune diseases (ADs) are characterized by the loss of immune tolerance to autoantigens in the body, leading to attacks by the autoimmune system.[1] Examples of ADs include systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and myasthenia gravis (MG).[2] At present, the worldwide prevalence of ADs, which is on the rise, is approximately 5 – 20%,[3] with women showing a higher incidence rate.[4,5] Furthermore, AD-associated complications often affect multiple organs, including the kidneys, lungs, heart, and brain, as well as the joints, muscles, bones, and surrounding soft tissues, resulting in inflammation and tissue damage that can cause pain, joint deformity, weakness, and mortality.[6] The treatment of ADs is challenging, and once diagnosed, patients typically require long-term or lifelong medication.[7] Complications arising from specific diseases, such as SLE complicated by lupus

nephritis, can greatly affect the quality of life and safety of affected individuals.[8,9]

ADs can be categorized into organ-specific ADs and systemic ADs, depending on the extent of the associated tissue damage.[10] Organ-specific ADs involve the immune system attacking specific organs or tissues in the body,[11] for example, Type 1 diabetes (T1D), MG, RA, inflammatory bowel disease (IBD), and multiple sclerosis (MS).[12] In contrast, systemic ADs affect multiple areas of the body and include conditions such as SLE and Sjögren's syndrome.[13]

The development of ADs is driven by autoreactive T cells and B lymphocytes that secrete autoantibodies.[14] During AD development, B lymphocytes generate autoantibodies that target their tissues and organs, leading to inflammation, tissue damage, and organ dysfunction. For instance, in SLE, autoantibodies produced by B lymphocytes cause harm to various tissues and organs in the body, including the skin, joints, and kidneys.[15]

Another significant factor in the development of ADs is the involvement of autoreactive T cells.[16] Specifically, T lymphocytes, a type of immune cell, typically regulate and coordinate immune responses. However, in ADs, autoreactive T lymphocytes may malfunction, mistakenly recognizing and attacking their tissues, leading to damage and inflammation.[17] An example of self-reactive T lymphocyte-mediated AD is RA, in which T lymphocytes attack joint tissue, causing inflammation and pain.[18]

The abnormal activity and dysfunction of B lymphocytes secreting autoantibodies, along with autoreactive T lymphocytes, are significant contributors to the development of ADs.[19] At present, treatment methods targeting B and T lymphocytes, including the use of immunosuppressants, immunomodulators, and therapies targeting specific cell surface markers, are being extensively researched.

Several mechanisms could be related to the development of ADs: (i) Epitope spreading, wherein the immune response switches from targeting primary epitopes to targeting secondary epitopes or creating new epitopes on antigen-presenting cells (APCs); (ii) bystander activation, characterized by T cell receptor (TCR)-independent activation of autoreactive T cells; (iii) persistent viral infections, in which the immune system is stimulated by the continuous presence of viral antigens; (iv) molecular mimicry, which occurs because of shared immune epitopes or sequence similarity, leading to cross-reactivity between host and pathogen; and (v) genetic factors.

The purpose of this study is to provide a foundation for treatment improvements by conducting a systematic review of advancements in the field of chimeric antigen receptor (CAR)-T therapy as a therapeutic strategy for ADs.

2. CAR-T cell therapy molecular mechanism and development

The conventional clinical therapies for ADs include glucocorticoids, non-steroidal anti-inflammatory drugs, broad-spectrum immunosuppressants, and biological agents.[6] However, as these common treatments lack specificity, patients often experience disease relapses and require lifelong medication, which can lead to systemic immunosuppression and an increased risk of infection and cancer. Recently, novel medications such as TNF inhibitors, B cell-depleting agents, T cell co-stimulatory blockade inhibitors, anti-interleukin-6 (IL-6) agents, anti-IL-1 agents, and protein kinase inhibitors have been developed for localized treatment (biologics). Notably, monoclonal antibodies targeting various B cell subtypes and other abnormal cells in ADs have also been developed.[20,21] For example, rituximab, targeting CD20, has demonstrated efficacy across various ADs. The half-life of monoclonal antibodies varies depending on the dosage and frequency of administration. Typically, this half-life falls within the range of 1.6 – 20 days and may require multiple doses during treatment to achieve the desired therapeutic effect,[22,23] which greatly limits the application of monoclonal antibodies in ADs. In contrast, CAR-T cell therapy recognizes target antigens within the patient's body, triggering their activation and subsequent amplification, thereby enhancing their anti-disease efficacy. Importantly, CAR-T cells exhibit good tissue penetration and a longer therapeutic effect, making them a promising avenue for the management of ADs.

2.1. Composition of CAR-T cells

CAR-T cells have CAR antigen binding selectivity and T cell cytotoxicity. They can grow extensively when coupled with target cells and exhibit cytotoxic effects through the release of granzymes and perforins. CAR T-cells can also trigger apoptosis within targeted cells by activating apoptotic signaling pathways. Furthermore, cytokines from CAR-T cells can stimulate various immune cells, resulting in synergistic effects that speed up the clearance of harmful cells.[24]

The CAR comprises four parts: (i) the extracellular antigen-binding domain, (ii) the hinge region, (iii) the transmembrane domain, and (iv) the intracellular co-stimulatory domain. The extracellular antigen binding domain is a single-chain fragment variable (scFv) generated by a peptide link between the variable heavy and variable light chains. This domain binds to the target

antigen with high affinity and is used to stimulate CAR signaling and T-cell activation.[25] Hinges derived from CD8, CD28, IgG1, or IgG4 are flexible segments that overcome spatial hindrance, allowing CAR to transmit signals and recognize T cell activation.[26] Transmembrane domains from CD3ζ, CD28, CD4, and CD8α not only serve to anchor the antigen-binding domain to the cell membrane but also play a crucial role in the activity of CAR.[27] The intracellular co-stimulatory domain includes the activation and co-stimulatory domains, and common co-stimulatory domains include CD28 and 4-1BB (CD137) (Figure 1).[28,29]

2.2. Development of CAR

To enhance the antitumor efficacy of CAR-T cells while mitigating T-cell activation-associated cytotoxicity, CAR technology targeting intracellular signaling pathways has been developed. The activation of T cells requires two signaling pathways. First, the TCR-CD3 complex recognizes the MHC/antigenic peptide complex and transmits the specific recognition signal. Second, non-specific co-stimulatory signals are provided by APC co-stimulatory molecules. The first generation of CAR contained solely the CD3ζ intracellular domain, limiting CAR-T cell activation and proliferation *in vivo*. Second-generation CARs feature two co-stimulatory domains, CD3ζ, along with either CD28 or 4-1BB (CD137), enhancing stimulation signals. These dual co-stimulatory domains enable the CAR-T cells to proliferate extensively and sufficiently produce cytokines. The increased expression of anti-apoptotic factors leads to the CAR-T cells exhibiting a longer survival time and a more durable therapeutic effect.[30] Third-generation CARs, in addition to CD3ζ, have two co-stimulatory domains, such as CD28, CD4, ICOS, 1-137BB (CD40), or OX134 (CD3), which provide T cells with more effective anti-tumor effects while increasing their cytokine production ability and improving their proliferation and persistence. Fourth-generation CARs, compared with third-generation CARs, integrate an activated T cell-nuclear factor transcription response element. After CAR-T cells recognize target antigens, they activate downstream transcription factors and stimulate the production of cytokines, which improve T cell survival rates and recruit and activate other immune cells. Fifth-generation CARs are created by combining an IL-2 receptor chain fragment (IL-2R) with a second-generation CAR. The JAK-STAT signaling pathway is activated by the downstream signaling pathway of IL-2R. Furthermore, when CAR-T cells detect antigens, the antigen-specific activation of receptors activates all downstream signaling pathways, resulting in full T cell activation and increased therapeutic agent persistence.[31] Fifth-generation CARs are also classified as universal CARs (UCARs), as their fabrication is based on gene editing technology to prevent human rejection. They can also be pre-prepared for allogeneic T cells before administration but may still face clearance due to immune rejection and may induce graft-versus-host disease.[32] Third- and fourth-generation CAR-T cells exhibit increased tumor-killing capacities and cytotoxicity but are presently in the research and development stage. Second-generation CARs, characterized by their milder approach, are widely employed in tumor therapy, representing the majority of listed CAR-T products[33] (Figure 2).

3. Research progress of CAR-T in ADs

The use of CAR-T cell therapy for ADs primarily involves three different mechanisms: (i) CAR-T cells recognize specific antigens in target cells and initiate cytotoxic activity against those cells. For example, CAR-T cell therapy targeting B cell CD19 is used in the treatment of SLE. After infusion into the body of the patient, the CD19 CAR-T cells undergo expansion and specifically target and eliminate CD19-expressing B cells, thereby reducing

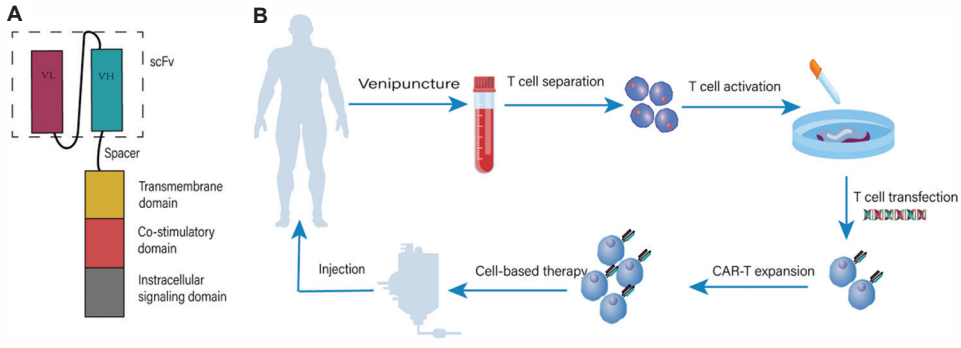

Figure 1. Flowchart of CAR T cell preparation (A) chimeric antigen receptor (CAR) comprising an antigen-binding region (scFv region) made up of heavy and light variable chains, a hinge region, a transmembrane domain, and an intracellular co-stimulatory domain. (B) CAR-T cell production process involves the extraction of peripheral blood from a patient, isolation, and activation of T cells, transfection of T cells with the CAR gene, expansion of CAR-T cells *in vitro*, and infusion back into the body of the patient.

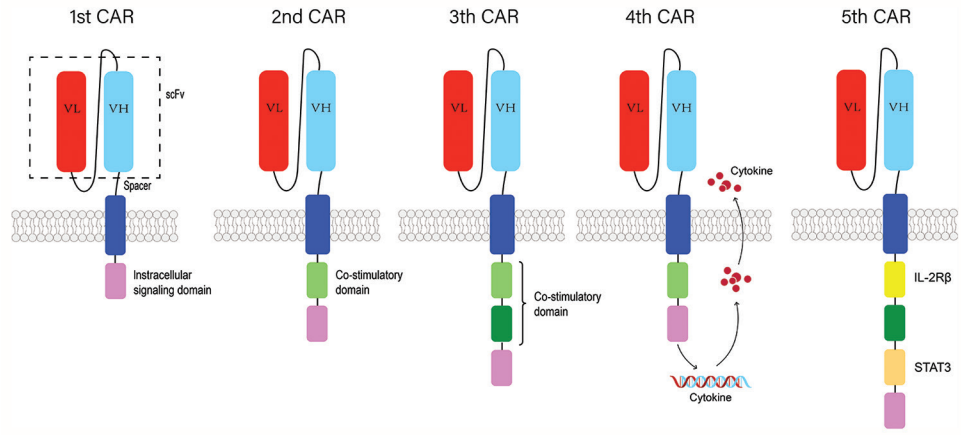

Figure 2. Development of chimeric antigen receptor (CAR). The first-generation CAR contains only the CD3ζ co-stimulatory domain. The second-generation CAR contains two co-stimulatory domains, CD3ζ and one of CD28 or 4-1BB (CD137). The third-generation CAR contains two co-stimulatory domains, such as CD28, CD4, ICOS, 1-137BB (CD40), or OX134 (CD3) in addition to CD3ζ. The fourth-generation CAR contains CD3ζ and activated T cytokine transcription elements, which enable CAR-T cells to secrete cytokines. The fifth-generation CAR is a general-purpose CAR based on gene editing technology and gene designs that can prevent rejection reactions in the body.

antibody production.[34] (ii) Chimeric autoantibody receptor T (CAAR-T) cells exhibit high affinity for the TCR on the surface of B cells that secrete specific autoantibodies and then exert cytotoxic effects on these cells. For instance, muscle-specific tyrosine kinase (MuSK) CAAR-T cell therapy is used to treat MG. Through genetic engineering, the extracellular domain of the MuSK antigen gene was integrated into CARs, enabling MuSK CAAR-T cells to specifically target B cells that secrete MuSK antibodies, thereby selectively reducing the number of pathogenic B cells.[35] (3) CAR-regulatory T lymphocytes (CAR-Tregs) bind to specific antigens in target cells and activate and regulate Treg function. In T1D, CD28/CD3 second-generation CAR constructs containing insulin-specific scFvs and the sequence of the main Treg cell marker Foxp3 are transduced into CD4+ T effector cells, resulting in the reprogramming of CD4+ T cells into insulin-specific Tregs (CAR-cTregs), which play regulatory roles in the immune system[36] (Figure 3).

3.1. Application of CAR-T cells in the treatment of SLE

SLE is a chronic multisystem AD characterized by the presence of autoantibodies, immune complex-mediated inflammation, and organ damage.[37] Common SLE symptoms include fatigue, joint pain, skin lesions, and renal dysfunction, with the potential for severe impairment of the heart, lungs, and central nervous system (CNS).[38] Autoantibodies in SLE include Smith, antinuclear, and DNA antibodies, often accompanied by complement activation.[39] Basta *et al.*[40] applied anti-CD19 CAR-T to SLE animal models, effectively clearing B cells and preventing the development of SLE; the therapeutic effect was more significant than that of antibody therapy.[40]

Kretschmann *et al.*[7] demonstrated successful autologous CD19 CAR-T cell administration in six patients with stable blood separation products, suggesting its suitability for clinical CAR-T cell manufacturing in SLE and other B-cell-driven ADs.[7] In addition, Mougiakakos *et al.*[34] reported a case of a patient with severe refractory SLE. Despite exhibiting no response to B-cell-targeted therapy, the patient experienced rapid symptom relief following treatment with CD19 CAR-T cells. This treatment resulted in the disappearance of dsDNA autoantibodies, normalization of C3 and C4 complement levels, and a decrease in SLE scores to 0 without observed adverse symptoms during follow-up.[34]

Zhang *et al.*[41] documented the case of a patient with SLE and stage IV diffuse large B-cell lymphoma (DLBCL) who received B-cell maturation antigen (BCMA)-CD19 dual-target CAR-T therapy. The symptoms of SLE stabilized after 7 weeks of therapy, and B cell depletion was achieved after 6 months. Furthermore, the patient exhibited durable remission for both SLE and DLBCL after 23 months of therapy.[41] Similarly, Mackensen *et al.*[42] reported on five patients with SLE who received CD19 dual-target CAR-T cell infusion treatment. All five patients achieved SLE remission after 3 months, which was maintained even after B-cell recurrence, with no medication required throughout extended follow-up periods. The presence of immature B cells and non-switched B cell receptors defined the return of B cells in this case.[42] These reports indicate that CD19 CAR-T cell transplantation in the treatment of SLE is feasible, tolerable, and effective (Table 1).

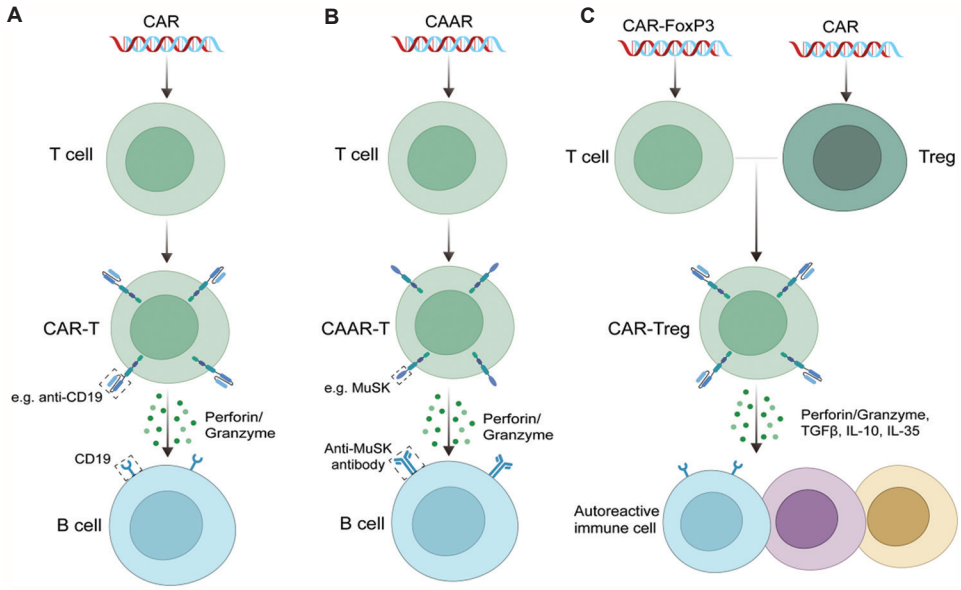

Figure 3. Application of chimeric antigen receptor-T cell (CAR-T cell) therapy in ADs. (A) CAR-T cells produce cytotoxic perforin/granzyme, targeting memory B cells or long-lived plasma cells, and T cells of antigen-presenting cell (APC) receptors through major histocompatibility complexes (MHCs). (B) Chimeric autoantibody receptor T cells targeting autoreactive B and T cells carrying autoantigens and autoreactive APC receptors through the MHC. An immune attack was performed based on cytotoxic perforin/granzyme. (C) CAR-regulatory T lymphocytes (CAR-Tregs) produce cytokines that target receptors on autoreactive T and B lymphocytes that express autoantigens and autoreactive APCs through MHC.
Abbreviation: MuSK: Muscle-specific tyrosine kinase.

3.2. Application of CAR-T cells in the treatment of T1D

T1Ds are characterized by the autoimmune destruction of beta cells, resulting in the absolute loss of insulin production due to pancreas-specific autoimmunity. In a spontaneous non-obese diabetic (NOD) mouse model, the disease can be prevented by genetically manipulating and removing key individual insulin epitopes. Therefore, selectively eliminating APCs carrying these pathogenic epitopes is a strategy for inhibiting the development of T1D.[43] In a pilot study utilizing the NOD mouse model, Zhang *et al.* demonstrated that CAR-T cells can selectively target APCs presenting pathogenic T cell epitopes associated with autoimmunity. This study aided in the development of a successful antigen-specific adoptive cell treatment for T1D based on disease-relevant epitope presentation. Furthermore, this therapy may elicit fewer adverse effects compared to alternative medications currently undergoing clinical trials.[44] Tenspolde *et al.*[45] employed a phage display library to select the strongest insulin-binding scFvs specific to insulin. They constructed a CD28/CD3 second-generation CAR with insulin-specific scFvs and transduced CD4+ T effector cells with the Treg cell marker Foxp3 sequence to reprogram the CD4+ T cells into CAR-cTregs.[45] These CAR-cTregs exhibited phenotypes and functions comparable to those of normal Tregs, proliferating and remaining active in the spleen to achieve long-lasting therapeutic effects.[46] Treating animal models of autoimmune diabetes (diabetic NOD mice) with monoclonal BDC2.5 TCR transgenic Tregs or genetically engineered NOD T cells expressing Foxp3 and BDC2.5 TCR can suppress the development of diabetes.[36] The IAg7, a subtype of major histocompatibility complex (MHC) II molecules, is strongly associated with NOD mice.[47] Spanier *et al.*[48] developed a CAR specific to the insulin B chain (InsB) 10-23 peptide presented by IAg7 MHC II molecules in NOD mice. This CAR, referred to as the InsB-g7 CAR, was designed to generate Tregs that can recognize pancreatic antigens. Thus, it was observed that the InsB-g7 CARs redirected the specificity of NOD Tregs, enhancing their suppressive function in response to stimulation with the InsB 10-23 peptide. This improved function was accomplished by lowering the proliferation of BDC2.5 T and dendritic cells, as well as their IL-2 production and expression of CD80 and CD86. InsB-g7 CAR Treg co-transfer successfully averted the development of transferable diabetes in immune-deficient NOD mice. It was also discovered that InsB-g7 CAR-Tregs stably expressed Foxp3 in wild-type NOD mice and prevented the development of spontaneous diabetes. These findings imply that employing CARs, similar to TCR, to change the specificity of Tregs for pancreatic antigens can prevent the development of autoimmune diabetes.[48]

Table 1. Ongoing clinical trials of CAR-T cell therapy in SLE

Trial registration number	Target	Start date	Study design	Overview of the study	Clinical phases	Sample size	Clinical progress
NCT03030976	CD19	March 2017	Non-randomized controlled trials	To evaluate cellular immunotherapy using CD19-targeting CAR-engineered T cells in patients with CD19+B-cell SLE	I	5	Unknown
NCT05765006	CD19	February 2023	Non-randomized controlled trials	A phase I dose-escalation study evaluating the safety, tolerability, pharmacokinetics (PK), and pharmacodynamics (PD) of relma-cel in subjects with moderately to severely active SLE	I	24	Recruit patients
NCT05030779	CD19-BCMA	September 2021	Non-randomized controlled trials	A clinical trial of the safety and efficacy of CD19/BCMA CAR-T cells in patients with refractory SLE	I	9	Recruit patients
NCT05869955	CD19	July 2023	Non-randomized controlled trials	A Phase 1, multicenter, open-label study of CC-97540 (BMS-986353) targeting CD19's next-T CAR T cells in patients with severe refractory SLE	I	43	Recruit patients
NCT05474885	CD19-BCMA	April 2022	Non-randomized controlled trials	BCMA-CD19 cCAR T-cell therapy for relapsed/refractory SLE	I	15	Recruit patients
NCT05798117	CD19	February 2023	Non-randomized controlled trials	An open-label, multicenter, phase 1/2 study to evaluate the safety, efficacy, and cytokinetics of YTB323 in patients with severe refractory SLE	I	27	Recruit patients
NCT05858684	CD19-BCMA	May 2023	Non-randomized controlled trials	Dual-target CAR-T cell therapy in patients with refractory SLE	I	18	Recruit patients
NCT05930314	CD19	June 2023	Non-randomized controlled trials	Dual-targeted CAR-T cell therapy for patients with refractory SLE	I	12	Recruit patients
NCT05846347	CD19-BCMA	May 2023	Non-randomized controlled trials	Phase I clinical study of GC012F injection in the treatment of refractory SLE	I	15	Recruit patients

Abbreviations: SLE: Systemic lupus erythematosus; CAR: Chimeric antigen receptor.

3.3. Application of CAR-T in the treatment of pemphigus vulgaris (PV)

PV is an autoimmune blistering disorder that can prove fatal in the presence of autoantibodies targeting the cell adhesion protein Dsg3.[49] Studies indicate that short-lived plasma cells are the source of autoantibodies in PV.[50] Therefore, targeting and eliminating anti-Dsg3-specific antibody-secreting B cells offers a promising avenue for treating PV without causing overall immunosuppression.[51]

PV and CAAR-T cells in antibody-mediated ADs can be created to selectively drive T cells to destroy self-reactive B lymphocytes, guided by B cell receptor specificity. Ellebrecht *et al.*[52] engineered human T cells to produce CAAR comprising the PV self-antigen desmoglein 3 linked to CD137-CD3 signaling domains. They discovered that Dsg3 CAAR-T cells demonstrated selective cytotoxicity *in vitro* against cells expressing the anti-Dsg3 B cell receptor. They also discovered that in animal models, Dsg3 CAAR-T cells could multiply, survive, and precisely destroy Dsg3-specific B cells. At present, a phase 1 open-label dosage and safety trial utilizing Dsg3-CAAR-T cells is underway in patients with active anti-DSG3, mucosal-dominant PV (NCT04422912). These findings highlight the potential of CAAR-T cells as an effective and adaptable method for specifically targeting self-reactive B cells in antibody-mediated ADs.[53]

3.4. Application of CAR-T in the treatment of MG

In MG, self-antibodies target proteins at the neuromuscular junction, resulting in persistent, variable, and sometimes debilitating weakness and muscle exhaustion.[54] Prevalent pathogenic antibodies linked with MG include anti-AChR, anti-MuSK, and anti-LRP4 antibodies. However, there remains a considerable unmet medical need for MG patients who are currently unresponsive to standard immunosuppressive therapy or experience severe adverse effects.[55]

The autoantibodies in MG are produced by plasma cells expressing an antigen called BCMA on their surface. Thus, an mRNA-modified rCAR-T cell therapeutic agent, DesCAR-Tes-08, has been developed to target BCMA.[56] This agent has shown superior safety and lower toxicity compared with traditional CAR-T cell therapy, as mRNA does not replicate and has fewer side effects. In a clinical trial (MG-001) evaluating the safety and clinical activity of autologous RNA-CAR-T cells for MG, DesCAR-Tes-08 significantly improved clinical symptoms and demonstrated safety in patients with MG. Notably, two patients achieved independence from intravenous immunoglobulin administration, whereas three patients exhibited minimal clinical symptoms. These gains were sustained across a 6 – 12-month follow-up period. Our previous study reported the first successful clinical trial of mRNA-based CAR-T cell therapy for Alzheimer's disease, implying that CAR-T immunotherapy may become a new therapeutic option for Alzheimer's disease.[57]

MuSK MG is a life-threatening condition characterized by severe muscular weakness. This weakness stems from the destruction of neuromuscular junction signal transmission caused by autoantibodies targeting MuSK. Oh *et al.*[35] designed a CAR for MuSK (MuSK-CAART) that includes the extracellular domain of MuSK and the CD137-CD3ζ signaling domain, to precisely target B cells expressing autoantibodies against MuSK. The MuSK-CAART cells demonstrated therapeutic effects in terms of killing anti-MuSK B cells and preserving cellular cytotoxic activity. The MuSK-CAART decreased anti-MuSK IgG without affecting total B cell count or total IgG levels in EAMG, indicating the depletion of MuSK-specific B cells. A clinical trial is currently underway to explore the safety and dosing of MuSK-CAART cells in patients with MuSK-MG (NCT05451212) (Table 2).[35]

3.5. Application of CAR-T cells in the treatment of neuromyelitis optica spectrum disease (NMOSD)

Neuromyelitis optica (NMO) was originally described as a form of MS characterized by both optic neuritis and myelitis.[58] Antibodies against aquaporin-4 (AQP 4) were found in 2004, allowing NMO to be distinguished from MS.[59-61] NMO can cause ocular neuritis or transverse myelitis, as well as a variety of clinical symptoms, including posterior brain, diencephalon, brainstem, and symptomatic brain syndromes caused by AQP 4-IgG antibodies. Thus, NMO was renamed NMOSD.[62]

A clinical trial investigating CD19 and CD20 CAR-T cells (NCT03605238) for NMOSD was halted because of recruiting issues.[63] However, a Phase I clinical study (NCT04561557) examining BCMA CAR-T cell therapy in patients with refractory AQP4-IgG seropositive NMOSD successfully enrolled 12 patients and observed only mild cytokine release syndrome (CRS) during treatment. During a median follow-up of 5½ months, serum AQP-4 antibody levels decreased in 11 patients without recurrence. CAR-T cell growth in two patients lasted longer than 6 months. Therefore, BCMA CAR-T cell therapy in NMOSD has a durable therapeutic effect and a tolerable safety profile.[64] A clinical trial of BCMA CAR-T cells for the treatment of other neuroinflammatory diseases is underway.

3.6. Application of CAR-T cells in the treatment of RA

RA, a common systemic AD characterized by self-antibodies targeting citrullinated antigens, often results in persistent inflammation and synovial joint degeneration.[65] At present, the pathogenesis and etiology of RA remain unclear. However, protein citrullination has long been implicated as a primary trigger for the immune response in RA.[66,67] Notably, serum anti-citrullinated protein antibodies detected in RA patients are strongly linked to disease development and progression. Moreover, several target proteins of anti-citrullinated protein antibodies have been identified.[61,68]

Zhang *et al.*[69] utilized universal anti-fluorescein isothiocyanate (FITC) CAR-T cells to target FITC-labeled antigenic peptide epitopes and eliminate the autoreactive B cell subpopulation recognizing these antigens in RA to solve the challenges of selectivity and durability associated with RA therapy.[69] Four guanine peptide epitopes derived from guanine autoantigens were selected as ligands for targeting autoreactive B cells: guanine-modified vimentin, guanine-modified Type II collagen, guanine fibronectin, myosin-C, and cyclic guanine peptide-1. T cells expressing a fixed anti-FITC CAR were generated and utilized as universal CAR-T cells to precisely eliminate protein-specific autoreactive B cells by recognizing FITC-labeled autoantigenic peptide epitopes.[70] Their findings indicated that anti-FITC CAR-T cells can be precisely guided to destroy hybridoma cells created through peptide vaccination and autoreactive B cell subsets from RA patients. This method provides a precise treatment strategy for RA and may be applicable to other systemic ADs.[28]

The HLA-II-encoded MHCII molecules can transmit extracellular antigens to CD4+ T cells, initiating their activation and proliferation. Upon activation, CD4+ T cells further stimulate B cells to produce antigen-specific antibodies. Notably, the HLA-DR1 variant is associated with susceptibility to RA.[71] Therefore, the targeted recognition of RA-associated HLA-DR CD4+ T cells is a feasible method to treat RA. Whittington *et al.* developed HLA-DR1 CARs to precisely target CD4+ T

Table 2. Ongoing clinical trials of CAR-T cell therapy for MG

Trial registration number	Target	Start date	Study design	Overview of the study	Clinical phases	Sample size	Clinical progress
NCT05828225	CD19	April 30, 2023	Non-randomized controlled trials	The primary objective is to evaluate the safety of CD19 CAR-T in patients with refractory MG and to evaluate the pharmacokinetics of CD19 CAR-T in patients.	I	9	Recruit patients
NCT04146051	BCMA	December 4, 2019	Randomized controlled trials	Application of DesCAR-Tes-08 CAR-T cells in generalized MG	IIb	30	Recruit patients
NCT05451212	MuSK-specific B cells	November 23, 2022	Non-randomized controlled trials	A phase 1, open-label, safety and dose-finding study of autologous muscle-specific tyrosine kinase chimeric autoantibody receptor T cells (MuSK-CAART) in subjects with anti-MuSK antibody-positive MG	I	24	Recruit patients
NCT04561557	BCMA	September 22, 2020	Non-randomized controlled trials	An open-label clinical trial evaluating the safety and efficacy of CT103A cells in the treatment of relapsed/refractory antibody-related idiopathic inflammatory diseases of the nervous system	I	18	Recruit patients

Abbreviations: MG: Myasthenia gravis; CAR: Chimeric antigen receptor.

cells, and *in vitro* cytotoxicity tests using cloned CD4+ T cells as target cells revealed that DR1-CII CAR-T cells effectively detect and kill CII-specific autoreactive CD4+ T cells. Treatment with DR1-CII CAR-T cells considerably reduced the CD4+ T cell response specific to CII, blocked autoantibody formation, and reduced disease severity in B6 DR1 mice with induced autoimmune arthritis. These findings suggest that HLA-DR CAR-T cells hold promise as highly targeted therapeutic options for ADs.[72]

3.7. Application of CAR-T cells in the treatment of ulcerative colitis

The significance of Tregs in maintaining the integrity of the intestinal mucosa in ulcerative colitis, an IBD, has been extensively demonstrated.[73] Human Foxp3 is one of the key transcription factors controlling the development and function of Tregs, and mutations in the Foxp3 gene can lead to severe ADs, including IBD.[74] Similarly, mice with deficient Treg activity are prone to severe colitis.[75] To alleviate colitis, Tregs may reduce the proliferation of effector T cells and the release of pro-inflammatory cytokines and block components of the innate immune system.[76]

Elinav *et al.*[77] successfully generated transgenic mice with T cells expressing chimeric receptors (CR). These receptors, comprising antibody-variable regions for recognition along with T-cell stimulation and co-stimulation domains, are designed to specifically target the predetermined model antigen, 2,4,6-trinitrophenol (TNP). Furthermore, TNP-specific CR-Tregs can be activated through external TNP stimulation, enabling them to suppress effector T cells even in the absence of the co-stimulatory factor B7-CD28. When Tregs carrying CRs were transplanted into wild-type mice with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, a significant increase in their survival rate was observed. Interestingly, although TNP-specific CR-Tregs were unable to suppress dextran sulfate sodium (DSS)-induced colitis, administering a small amount of TNBS led to the cure of the DSS colitis mouse model. Within a few hours of colitis induction, *in vivo* imaging of transplanted CR-carrying Tregs revealed their tendency to migrate to locations of TNBS-induced colonic mucosal injury. This evidence suggests that Tregs accumulate at the sites of colitis inflammation owing to a specific redirecting mechanism that differs from that of pathogenic lymphocytes. These Tregs were able to suppress effector T cells in a non-MHC-restricted and non-co-stimulatory-dependent manner. Thus, they significantly alleviated colitis.[77]

Carcinoembryonic antigen (CEA) is overexpressed in human colitis and colorectal cancer. Blat *et al.*[78] utilized CEA-specific CAR-Tregs to treat a CEA-colitis animal model. Following systemic treatment, CEA-CAR-Tregs aggregated in the colons of afflicted mice and demonstrated a reduction in colitis symptoms compared to control Tregs. Furthermore, the CEA-CAR-Tregs hindered the development of colorectal cancers in an azoxymethane-DSS model. These results suggest a high potential for CEA-CAR-Tregs in the treatment of both ulcerative colitis and colorectal cancer.[78]

3.8. Application of CAR-T cells in the treatment of MS

MS, a demyelinating disease of the CNS,[79] results from the migration of self-reactive T cells into the CNS, where they become activated by APCs, leading to inflammatory demyelinating disease. At present, MS is mainly treated with non-specific immunosuppressive medications.[80]

Fransson *et al.*[81] transferred myelin cell glycoprotein (MOG)-CAR into CD4+ T cells along with the mouse Foxp3 gene, which drives Treg differentiation. These modified Treg cells inhibited T cell activation *in vitro* and were subsequently employed to treat experimental autoimmune encephalomyelitis (EAE) mice. Administering the modified Tregs through the intranasal route facilitated their entry into various brain regions, resulting in the alleviation of disease symptoms and a reduction in IL-12 and interferon-gamma mRNA levels in brain tissue. Immunohistochemical analysis revealed restoration of myelin basic protein (MBP) and glial fibrillary acidic protein, which indicated the restoration of myelin formation and reactive astrocyte proliferation in mice treated with modified Tregs compared with the control treatment group. On re-exposure to the EAE-inducing inoculum, symptom-free mice remained mitigated, demonstrating the sustained therapeutic effects of the modified Tregs.[81] Furthermore, De Paula Pohl *et al.*[81,82] engineered Tregs expressing a TCR specific for the MBP peptide, which decreased MBP-reactive T effector cell growth and alleviated MOG-induced EAE symptoms. These findings suggest that CAR-Tregs hold promise for cellular therapy in MS patients.[81,82]

4. Challenges associated with CAR-T cell therapy and possible solutions

While CAR-T cell therapy has demonstrated preliminary efficacy in treating ADs, its widespread application in the field of autoimmunity is hindered by several challenges. For example, CAR-T cell treatment can easily trigger CRS, with severe CRS occurring in 27 – 53% of cases. This condition typically manifests 6 – 20 days post-therapy, presenting symptoms such as fever, weariness, muscle soreness, and even organ malfunction, sometimes progressing to life-threatening complications.[27] Therefore, to increase the safety of CAR-T cell treatment, we must better understand the pathophysiology of CRS and establish predictive and management strategies for its side effects. Specifically, the mechanism underlying CRS involves the rapid clearance of immune cells and the generation of numerous inflammatory factors, such as cytokines and chemokines, inducing inflammation, tissue damage, and potentially fatal outcomes.[83]

CAR T cell-induced cytokine release syndrome is mediated by macrophages and can be blocked using cytokine IL-1 and IL-6 inhibitors.[84,85] These findings set the foundation for improving the safety of CAR-T treatment and developing new intervention therapies.[86] At present, the primary strategies to overcome the adverse reactions of cytokine storms include the administration of glucocorticoid drugs such as hydrocortisone, suppression of cytokine secretion, utilization of drugs that target and block cytokine signaling pathways (such as IL-6 receptor inhibitors),[87-89] introduction of controllable suicide genes (such as caspase 9 or HSV-TK) as safety switches[90] that can induce T cell apoptosis and reverse graft-versus-host disease in cases of acute toxicity, and development of "on switch" CAR-T cells that are closed by default and only open under the action of regulating drugs to control the timing and dosage of CAR-T therapy and improve safety.[91] In addition, the severity of CRS is related to disease progression and disease burden, with CRS being more severe in patients with a higher disease burden.[92] Therefore, administering CAR-T cell therapy before disease deterioration can reduce the risk of CRS occurrence.

Furthermore, the development of non-invasive imaging and monitoring platforms provides support for monitoring and evaluating CAR-T cell transport, toxicity, and expansion in real time. This technology facilitates the rapid implementation of techniques aimed at enhancing CAR-T cell transport and minimizing toxicity, particularly in Phase I clinical trials.[93] Another typical adverse effect associated with CAR-T cell treatment is neurotoxicity. Following CAR-T therapy, transgenic T cells can be detected in the cerebrospinal fluid, accompanied by the presence of cytokines in the brain. Elevated cytokine levels in the brain can lead to brain CRS, leading to neurotoxicity characterized by symptoms such as expressive aphasia, seizures, and syncope.[94] To manage neurotoxicity resulting from CAR-T therapy, corticosteroids are frequently employed due to their ability to penetrate the blood–brain barrier effectively, a feat often unattainable by many monoclonal antibodies.[95]

The production and preparation of CAR-T cells pose significant challenges due to their complexity and difficulty. Mass production is hindered by these complexities, rendering it both challenging and expensive. After infusion, ensuring the expansion and subsequent safety of CAR-T cells requires long-term monitoring and evaluation. This process is highly intensive in terms of human and financial resources, often surpassing the capacity of most patients.[96] Furthermore, CAR-T cells are generally modified from autologous T cells, and those derived from allogeneic sources are prone to immune rejection, posing challenges to their widespread adoption.[97] However, these restrictions on CAR-T cell treatment can be overcome by developing

universal CARs, such as uniCAR T and CAR NK cells, and streamlining production procedures.[98] The development of CAR-T cells that can be rapidly mass-produced, such as FasTCAR and TCharge systems, can also reduce treatment time and costs. Specifically, the TCharge method has been developed for the rapid expansion of CAR-T cells *in vivo*.[99] The application of this method reduces the *ex vivo* expansion time of CAR-T cells and significantly shortens the preparation time, enabling rapid attainment of the desired therapeutic effect. Furthermore, non-integrative and non-viral microcarrier systems, known as DNA nanocarriers, can reproduce chromosomes in the nuclei of dividing cells,[100] enabling the efficient creation of modified human T cells. This technique was originally developed for CAR-T cell therapy and has recently emerged as a dependable, safe, and efficient way to produce modified T cells, thereby reducing the overall cost of CAR-T cell therapy.[101,102]

5. Conclusion

Although the application of CAR-T cell therapy in cancer treatment has been extensively studied and the preliminary therapeutic effects of this treatment strategy in ADs have been shown, extensive high-quality clinical trials involving large sample sizes and longer treatment durations are lacking. Thus, there remains a need for long-term studies to assess both the efficacy and safety of this treatment strategy. Current clinical trials of CAR-T therapy in ADs have mainly extended from clinical trials conducted in cancer treatments. These trials primarily focused on universal targets such as CD19, CD20, BCMA, and BAFF on B cells, resulting in general immunosuppressive action and associated side effects. However, many ADs are caused by autoantibodies targeting their antigens. Therefore, CAR-T cell therapies targeting AD-specific antigens offer more defined therapeutic efficacy and demonstrate higher safety profiles. For instance, CAART therapies used in PV and MG introduce antigens into CARs and specifically target B cells secreting autoantibodies without affecting normal B cells or the overall B cell population, providing enhanced specificity and targeting. Similarly, based on the regulatory role of Tregs, CAR-Treg therapies represent a promising immunomodulatory approach for AD treatment. These therapies not only alleviate clinical symptoms but also exhibit lower cytotoxicity, offering significant potential for the management of ADs. Overall, there remains ample scope for further research into the development and application of CAR-T cell therapy in the treatment of ADs.

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Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

Not applicable.

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Not applicable.

Data availability

The data supporting this systematic review were derived from previously reported studies, which were collected from the PubMed database and had been cited. The processed data are available from the corresponding author upon request.

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

REVIEW ARTICLE

Decoding and understanding molecular mechanisms: Cell signaling pathways, pancreatic β-cell regeneration, and stem cell niche engineering for diabetes

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Abstract

Stem cell bioengineering addresses regenerative medicine and cellular therapies by applying advanced techniques to stem-cell-derived systems. Despite their promise, stem cell applications are limited by incomplete knowledge. Stem cells and phytochemicals show potential in treating diabetes by halting β-cell degeneration and promoting endogenous islet regeneration. Current diabetes cell therapies include stem cells, mature pancreatic cells, endocrine progenitors, and β-cells, with researchers actively seeking new cell sources for clinically relevant β-cells. Stem cell-derived pancreatic cells are particularly promising for pancreatic islet regeneration. Diabetes mellitus results from cell loss or malfunction: Type 1 diabetes stems from autoimmune damage, whereas Type 2 diabetes is largely attributed to cell malfunction or insulin resistance. The only operative therapy, islet transplantation, necessitates lifelong immune suppression. Significant progress has been made in strategies for therapeutic adult β-cell regeneration. This review assesses studies on cellular signaling pathways linked to β-cell survival and proliferation, exploring regenerative medicine methodologies for pancreatic islet replacement or regeneration. While the "replacement" technique involves cell transplantation, the "regeneration" strategy preserves cell populations through replication. Moreover, artemether and gamma-aminobutyric acid induce pancreatic cells to adopt β-cell-like phenotypes, potentially aiding in the development of new β-cell-like cells for treating severe diabetes in rats. Understanding G-protein-coupled receptor activation pathways is crucial, as new treatment strategies for insulin-dependent diabetic mellitus may emerge from this knowledge.

Keywords: Stem cell bioengineering; Cell signaling pathways; Stem cell niche; Pancreatic β cell regeneration; Clinical and preclinical agents

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1. Introduction

Diabetes type 2 (T2D) is characterized by insulin resistance and dysfunctional pancreatic beta cells, which play a crucial role in glucose regulation. This dysfunction reduces β-cell mass and function.[1] Recent advancements have enabled the creation of glucose-

responsive pancreatic islet cells from pancreatic stem cells. These advancements have revealed new signaling pathways and molecules involved in lineage commitment during pancreatic differentiation and maturation processes, enhancing *in vitro* pancreatic maturation methods.[2] Regenerative treatments made possible by stem cell biology hold revolutionary potential; yet, the regulatory networks that control the formation of complex tissues and organs are still poorly understood, limiting these therapies' applications. Stem cell engineering addresses these complexities by exploring gene regulatory networks in individual stem cells and systemic relationships across organs and tissues.[3] Single-cell sequencing technology, which analyzes cell heterogeneity at the single-cell level, has advanced significantly due to advancements in cell sorting and nucleic acid extraction. Recent findings in stem cell research, such as pluripotent stem cells (PSCs) and tissue-specific stem cells, have been encouraging. While the potential of stem cells in regenerative medicine is well-discussed, the development of actual medicines has been slow. The goal is to construct a biofunctional artificial niche for multipotency, differentiation, and proliferation, allowing for more definitive experiments by pharmaceutical specialists, biologists, and tissue engineers. The therapeutic potential of pancreatic islet cells derived from pancreatic stem cells is being explored through gene-editing techniques and cell transplantation into diabetic animal models.[4] These cells have potential use in drug testing and disease modeling research. Artemether and gamma-aminobutyric acid (GABA) can induce pancreatic cells to adopt a β-cell-like phenotype, potentially promoting the development of new β-cell-like cells for treating severe diabetes in rats.

Understanding the signaling pathways linked to G-protein-coupled receptor (GPCR) activation and their interactions within cells is crucial for developing therapeutic methods to regulate insulin secretion and maintain cell mass. Apoptosis induced by diabetogenic stresses results in a reduction in functioning cells as diabetes progresses. It is essential to prevent the loss of cell molecular characteristics, as this loss results in decreased cell mass and impaired function. This review focuses on the causes, consequences, and potential reversibility of cell failure as a treatment approach for T2D.[5] Dedifferentiated cells have the ability to redifferentiate into mature, functioning cells, indicating that dedifferentiation is not an irreversible process. Thus, therapeutic approaches that prevent cell dedifferentiation and promote cell regeneration hold promise for treating T2D.[6] The pancreas comprises the exocrine pancreas, which stores digestive enzymes, and the endocrine islets, which produce the essential metabolic hormone — insulin. Treatment is crucial for disorders like Type 1 diabetes (T1D) that cause islet cell loss.[7] Additional techniques include inducing natural β-cell proliferation, transforming non-β-cells into β-like cells, and isolating islets from genetically altered animals. Recent technological developments and analytical techniques for genome-wide research at the single-cell level can help identify disease-specific cell subpopulations and relate them to genetic risk factors, allowing for personalized precision-based therapy. Enhanced resolution and specificity afforded by these technologies, combined with the interdisciplinary convergence of engineering and biology, will enable the development of therapeutically exploitable niches.[8] Decoding and understanding molecular mechanisms in cell signaling pathways, pancreatic cell regeneration, and the engineering of stem cell niches will open new avenues for treating and regenerating pancreatic cells in the near future.

2. Cell signaling pathways and pancreatic β-cell regeneration

The loss or dysfunction of pancreatic insulin-producing cells leads to diabetes, a global health concern of paramount importance. It is essential to recognize the inherent capacity of diabetic patients' cells to proliferate under both normal and pathological conditions, as this capacity is integral to restoring functional cell mass. Recent advancements in understanding the mechanisms underlying the differentiation of various pancreatic cell lineages into insulin-producing cells have been facilitated by developments in cell regeneration *in vivo*. Reactivation of the gene encoding the transcription factor neurogenin-3, which regulates pancreatic endocrine cells, is a key component of these pathways. The pancreas, composed of the exocrine pancreas and endocrine islets, functions in enzyme storage and insulin production. Islet cell loss occurring in conditions such as T1D requires therapeutic intervention due to the limited regeneration capacity of the cells. The most effective methods involve creating and transplanting fresh cells from human PSCs, inducing endogenous β-cell proliferation, transforming non-β-cells into β-like cells, and extracting islets from genetically engineered animals (Figure 1). Pancreatic regeneration emerges as a potential therapeutic method for the recovery of cell loss.

The ability of endocrine islets to regenerate is limited, especially in adults. Most hypoglycemic medications can preserve cells by reducing oxidative stress and inflammation caused by hyperglycemia, and by inhibiting cell death and dedifferentiation. Compounds such as glucagon-like peptide-1 and GABA increase cell proliferation believed to be the primary source of regenerated cells in adult rats,

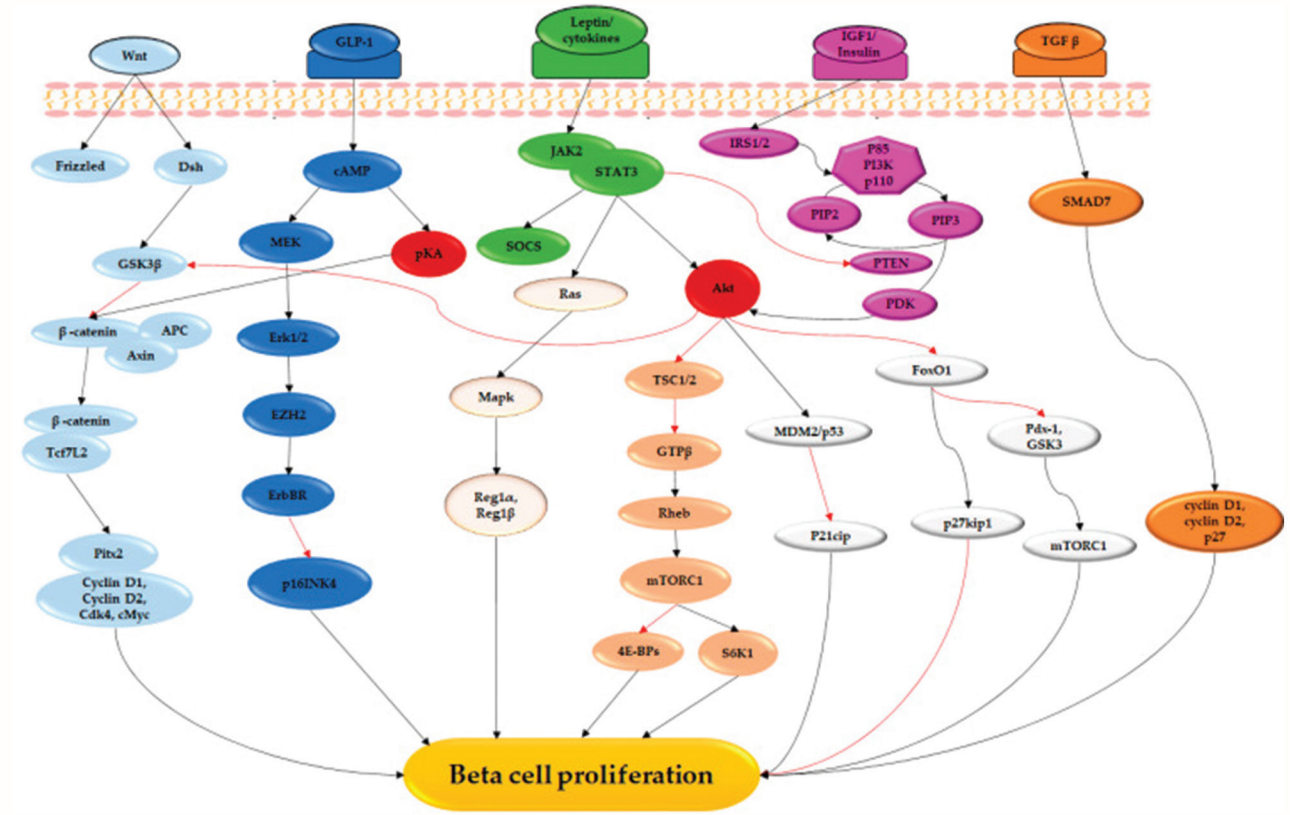

Figure 1. Mechanisms of signaling pathways controlling β-cell proliferation induced by phytochemicals. Upregulation/activation is represented by black arrows, while inhibition is indicated by red arrows. Reprinted from Kimani *et al.*[9]

although this is less certain in humans. Under specific conditions, pancreatic progenitor cells may become active. Although there are disagreements, artemisinins and GABA can stimulate cell-to-cell conversion (Figure 2). Ongoing research on FOXO1 inhibition shows promise, as deletion of this gene allows intestinal endocrine progenitors to transform into insulin-producing cells in the gut. Small-molecule inhibitors of non-canonical IB kinases, such as TKB1 and IKK, have been found to induce cell regeneration in various species. Mammalian islets primarily include β-cells that express TBK1, with expression levels elevated during exposure to diabetogenic insults, such as T2D. In streptozotocin-induced diabetic mice, PIAA expedited the restoration of functioning β-cells and increased expression of cell cycle regulatory molecules and cell differentiation markers in response to diabetogenic stimuli.

Pancreatic cells play a crucial role in glucose homeostasis through the timely release of the insulin hormone. *In vitro* proliferation of human PSC-derived islets remains an effective therapy, and a deeper comprehension of regeneration mechanics holds the potential for significant advancements in diabetes care. Both T1D and T2D are characterized by decreased cell numbers and declined cellular function. Current research aims to enhance cell efficiency to delay or reverse diabetes onset. Therapeutic techniques include direct islet transplantation, implantation of progenitors/stem cells into β-cells, replication of pre-existing β-cells, and stimulation of endogenous β-cell progenitors. Attention has been drawn to the discovery of cellular signaling networks linked to genes or proteins playing integral roles in diabetes (Figure 3). However, unresolved pathways and molecules associated with β-cells, particularly in humans, contribute to a lack of understanding of their specialized functions, underscoring the need for further research. The majority of cell pathways and chemicals, as well as their specialized roles, are still elusive, especially in humans. Therefore, additional studies are warranted to further understand the cellular processes governing human β-cell proliferation and its underlying mechanisms and roles.

3. Pancreatic β cell regeneration and stem cell

Diabetes mellitus, a prominent pancreatic disorder, arises from metabolic dysfunction due to a lack of β-cells that produce insulin. Replenishing β-cell populations through

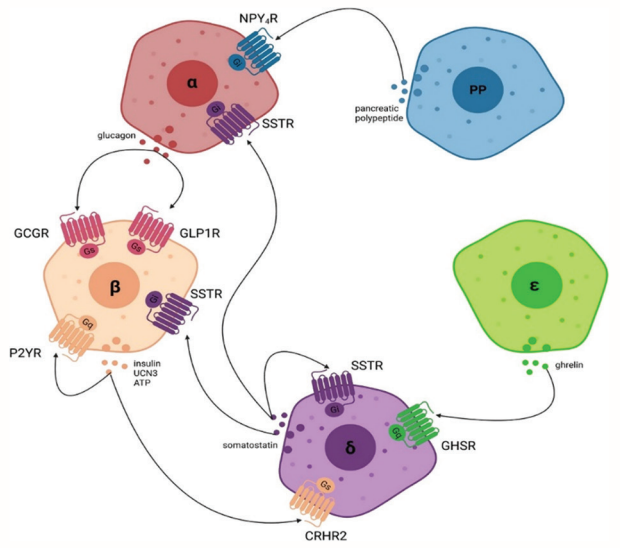

Figure 2. G protein-coupled receptors (GPCRs) control the function of pancreatic islets through paracrine signaling. Endocrine pancreas cells develop a communication network to react to nutrition levels. Cells release ATP and recombinant urocortin 3 (UCN3), which activate corticotropin-releasing factor (CRHR2) and purinergic receptors. This signaling promotes the production of glucagon, which, in turn, boosts the production of insulin. Somatostatin decreases the secretion of both insulin and glucagon, while pancreatic polypeptides produced by pancreatic polypeptide (PP) cells inhibit glucagon production. Reprinted from Thor.[10]

Abbreviations: GCGR: Glucagon receptor; GHSR: Ghrelin receptor; GLP1R: Glucagon-like peptide-1 receptor; NPY4R: Neuropeptide Y receptor Y4; P2YR: P2Y purine nucleotide receptor; SSTR: Somatostatin receptor.

cell transplantation offers a viable solution to restoring normal metabolic function. However, the scarcity of donor pancreata has underscored the urgent need for alternative β-cell sources, driving exploration into regenerative avenues such as *in vivo* β-cell regeneration and cellular reprogramming.[12] The pancreas comprises two distinct components: the exocrine pancreas, which is a source of digestive enzymes, and the endocrine islets, which are responsible for insulin production. Human islets exhibit limited regenerative capacity. Consequently, islet β-cell loss in conditions like T1D necessitates therapeutic intervention.[13] The primary technique for regaining cell mass involves the generation and implantation of new cells derived from human PSCs. Other methods include promoting the growth of endogenous β-cells, converting non-β-cells into β-cells, and extracting islets from animals that have undergone genetic engineering.[14] The endocrine pancreas offers a promising landscape for cell therapies and regenerative medicine.

In the foreseeable future, PSCs emerge as an ideal choice for regenerative β-cell therapies, offering an abundant source of healthy β-cells. Glucose, through unfolded proteins and metabolic pathways, significantly influences quiescent β-cells' entry into the cell cycle.[15] Hormones, growth factors, and signaling pathways, including the calcium-calcineurin nuclear factor of activated T lymphocytes, exert varying impacts on β-cell replication. Therefore, a deeper comprehension of the molecular mechanisms underpinning pancreatic β-cell regeneration and protection holds promise for the discovery and development of novel therapeutic strategies. The integration of stem cells and diverse phytochemicals has opened up new avenues for arresting β-cell senescence and fostering islet regeneration. Given that diabetes results from insufficient pancreatic β-cell mass, the replacement of functional β-cells stands out as a promising treatment (Figure 4).

To increase β-cell mass, stimulating the replication of surviving β-cells and fostering new islet formation from pancreatic progenitors through neogenesis is crucial.[14] While rodent studies have showcased the stimulation of neogenesis and replication, demonstrating that their clinical relevance remains a challenge. Stem cells are being explored as a replacement for β-cells in human islet transplantation due to donor scarcity and graft failure. Pancreatic epithelial cells, which are capable of differentiating into β-cells, present an appealing alternative. However, despite extensive *in vitro* expansion, this capability has yet to be translated to human cells.

Addressing the immune system's destruction of transplanted cells through allo or autoimmunity is a critical consideration in cell therapy.[17] In this review, recent advancements in encapsulation and immunomodulation approaches are discussed, focusing on β-cell replacement therapies, existing immune evasion methods, and essential procedures for translating novel techniques from the laboratory to the clinic (Figure 5). Moreover, β-cell regeneration occurs *in vivo* through three mechanisms: (i) proliferation of existing β-cells, (ii) transdifferentiation of other cell types into β-cells, and (iii) neogenesis of β-cells from adult ductal progenitors.[18] Although differentiated pancreatic duct and acinar cells can dedifferentiate into progenitor-like states in response to damage, their contribution to rebuilding pancreatic β-cell mass remains unclear despite their function as facultative progenitor cells.

4. Engineering the stem cell niche and stem cell

Investigating the biology and engineering of stem cell niches involves a broad range of research. Current findings on adult and embryonic stem cell (ESC) niches

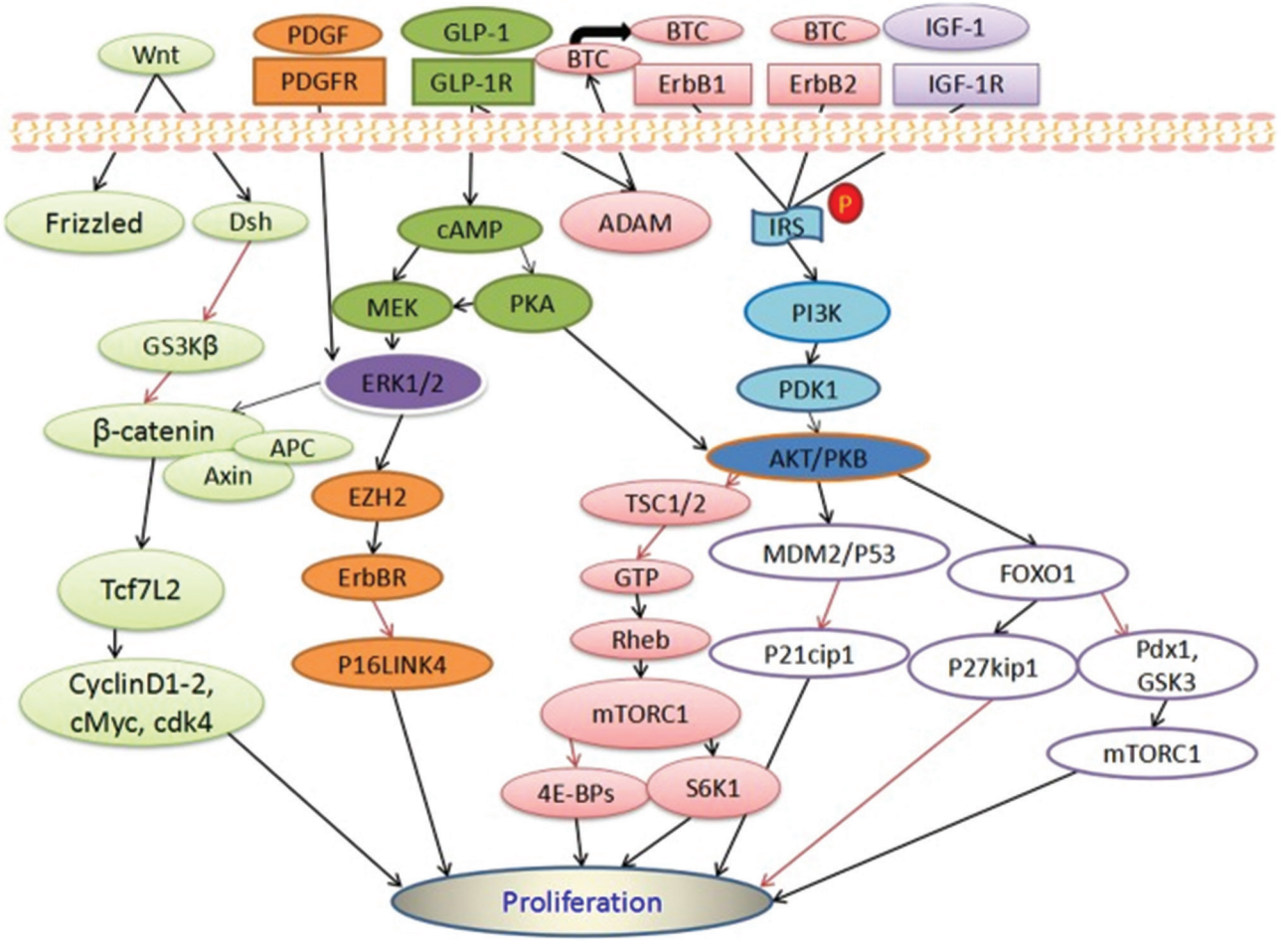

Figure 3. The binding of PDGF, BTC, GLP-1, and IGF-1 to their respective receptors is crucial for the proliferation of β-cells. The Wnt pathway regulates GSK3 to prevent β-catenin phosphorylation, which in turn controls the production of Tcf7L2, cMyc, cdk4, and Cyclin D1-2, which in turn controls proliferation. When PDGF binds to PDGFR, the ERK1/2 pathway is activated, boosting the production of EZH2. The IRS/PI3K pathway is activated by IGF-1 binding to IGF-1R and by BTC binding to ErbB1 and ErbB2. GLP-1 activates the cAMP-PKA pathway by binding to GLP-1R and also works through ADAM proteins to release BTC, which then affects ErbB1/2. Pathways with the same signaling route are represented by circles of the same color. Inhibition and promotion are indicated by red and black lines, respectively. Reprinted from Jiang *et al.*[11]

Abbreviations: 4E-BP: Eukaryotic translation initiation factor 4E-binding protein 1; APC: Adenomatous polyposis coli; BTC: β-catenin; cdk: Cyclin-dependent kinase; Dsh: disheveled; EGFR: Epidermal growth factor receptor; EZH2: Enhancer of zeste homolog 2; FOXO1: Forkhead box protein O1; GLP-1: Glucagon-like peptide 1; GLP-1R: Glucagon-like peptide 1 receptor; GSK3β: Glycogen synthase kinase-3β; IGF-1: Insulin-like growth factor 1; IGF-1R: Insulin-like growth factor 1 receptor; IRS: Insulin receptor substrate; MDM2: Mouse double minute 2 homolog; MEK: Mitogen-activated protein kinase; mTORC: Mechanistic target of rapamycin complex; P: Phosphorylated; PDGF: platelet-derived growth factor; PDGFR: Platelet-derived growth factor receptor; PDK1: Phosphoinositide-dependent kinase 1; Pdx1: Pancreatic and duodenal homeobox 1; PI3K: Phosphoinositide 3-kinase; PK: Protein kinase; Rheb: Ras homolog enriched in brain; S6K1: Ribosomal S6 kinase 1; TSC1/2: Tuberous sclerosis complex 1/2.

focus on the roles of molecules present in stem cell niches and their signaling pathways. Cell-cell/cell-matrix interactions are exemplary fields of study in this context. The variety of biochemical and biophysical components that constitute the local microenvironment is extremely important to stem cells.[19] One of biomedicine's objectives is to create synthetic yet biologically functional niches to promote multipotency, differentiation, and proliferation. By developing such tools, biologists, pharmaceutical researchers, and tissue engineers can conduct more thorough experiments.[20] At present, there exist many platforms and methods for bioengineering, and technological advancements are available to explore biological transformations with high precision and resolution. The goal is to create niches at a therapeutically exploitable scale, achievable through the multidisciplinary combination of engineering and biology. Stem cell-based tissue engineering aims to mimic the natural stem cell niche and deliver sufficient microenvironmental signals in a systematic and repeatable manner to regulate stem

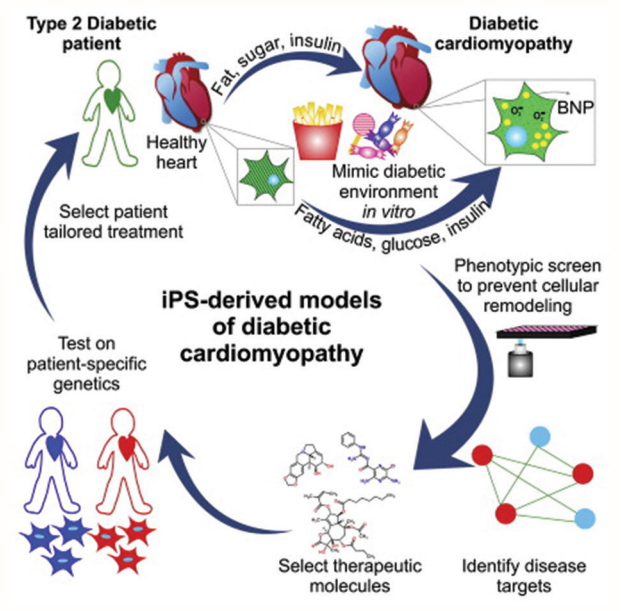

Figure 4. An organoid kidney was created using a human pluripotent stem cell line. Immunofluorescence allows for the observation of differentiated nephrons. Reprinted with permission from Drawnel *et al.*[16]
Abbreviations: BNP: Brain natriuretic peptide; iPS: induced pluripotent stem cell.

cellular metabolism within the graft.[21] Niche control is vital for processes that result in organogenesis and tissue homeostasis. The application of engineering principles has significantly improved the general success of stem cell research. However, numerous significant challenges persist, including understanding the long-term impacts concerning systems biology (Figure 6). Understanding biomimetics has substantially facilitated the development of designs for materials and devices, refining the architecture of matrices and scaffolds, and integrating compatible biological and physical constituents.[22]

Regenerative medicine encompasses developments in stem cell biology, molecular biology, engineering, and clinical methodologies. Stem cells are crucial for therapeutic use in regenerative medicine, with engineering concepts driving stem cell-based applications aimed at improving human health. Various engineering methodologies are geared toward the development of regenerative and preventative medicine to address diverse illnesses and disorders. The rapid expansion of stem cell research and its clinical applications calls for novel engineering ideas and biomaterials.[24] The use of stem cells in regenerative medicine primarily relies on engineering concepts such as biomaterials, microfluidics, and nanotechnology.[25] Technological advancements focus on creating functional niches for cells, with stem cells emerging as promising candidates in tissue engineering and regeneration. In coculture settings, stem cells exhibit the ability to support tissue homeostasis, repair, metabolism, and growth, while also serving as targets for differentiation into various cell types.[26] The development of complex tissues or organs hinges on their integration into coculture systems, employing both direct and indirect coculture approaches to elucidate cellular events in tissue engineering.[27] This review discusses the benefits of stem cell co-culture techniques in tissue engineering and regenerative medicine, with a particular focus on various tissues, including orthopedic soft tissues, vasculature, bone, heart, lung, nerve, kidney, and liver. Despite recent exciting discoveries, a decade of experimental and clinical studies has shown that the routine use of stem cells to repair solid organs is not imminent amidst the promise of their medicinal uses in cell-based tissue regeneration, drug testing, and basic research.[28] Biologists only partially comprehend the mechanisms governing cell differentiation, with only certain extrinsic and intrinsic components mapped, and less known about the intricate interactions of these elements in various differentiation processes.[29] Current research focuses on discovering soluble ligands that regulate and modulate signaling pathways, despite limited knowledge about the impact of the physical and structural microenvironment (Figure 7). Offering effective ways to examine organoids at the single-cell level is a way to advance the genesis of organoids with substantially improved accuracy.

The emerging techniques in organoid engineering represent a significant stride forward in our comprehension of the stem cell niche.[31] Employing organoids to answer important issues about developmental and regenerative biology necessitates their integration into investigative endeavors. These novel tools streamline the exploration of fundamental ideas in stem cell biology, including the influence of the stem cell niche on cellular plasticity and how its physical characteristics govern stem cell self-organization, subsequently affecting stem cell fate. Scaffolds designed for tissue engineering modify the physical, chemical, and biological milieu surrounding a cell population. Moreover, novel biological and chemical biology tools facilitate the examination of stem cell populations. The publication *Genomics and Proteomics: Principles, Technologies, and Applications* by Apple Academic Press discusses the use of genomes and proteomics in stem cell studies.[32] These technologies furnish a precise diagnostic fingerprint of the cellular state. High-throughput molecular profiling, primarily employing RNA sequencing (RNA-seq) and metabolomics, has led to the development of complex analytic algorithms, thereby enabling fundamental discoveries in stem cell biology

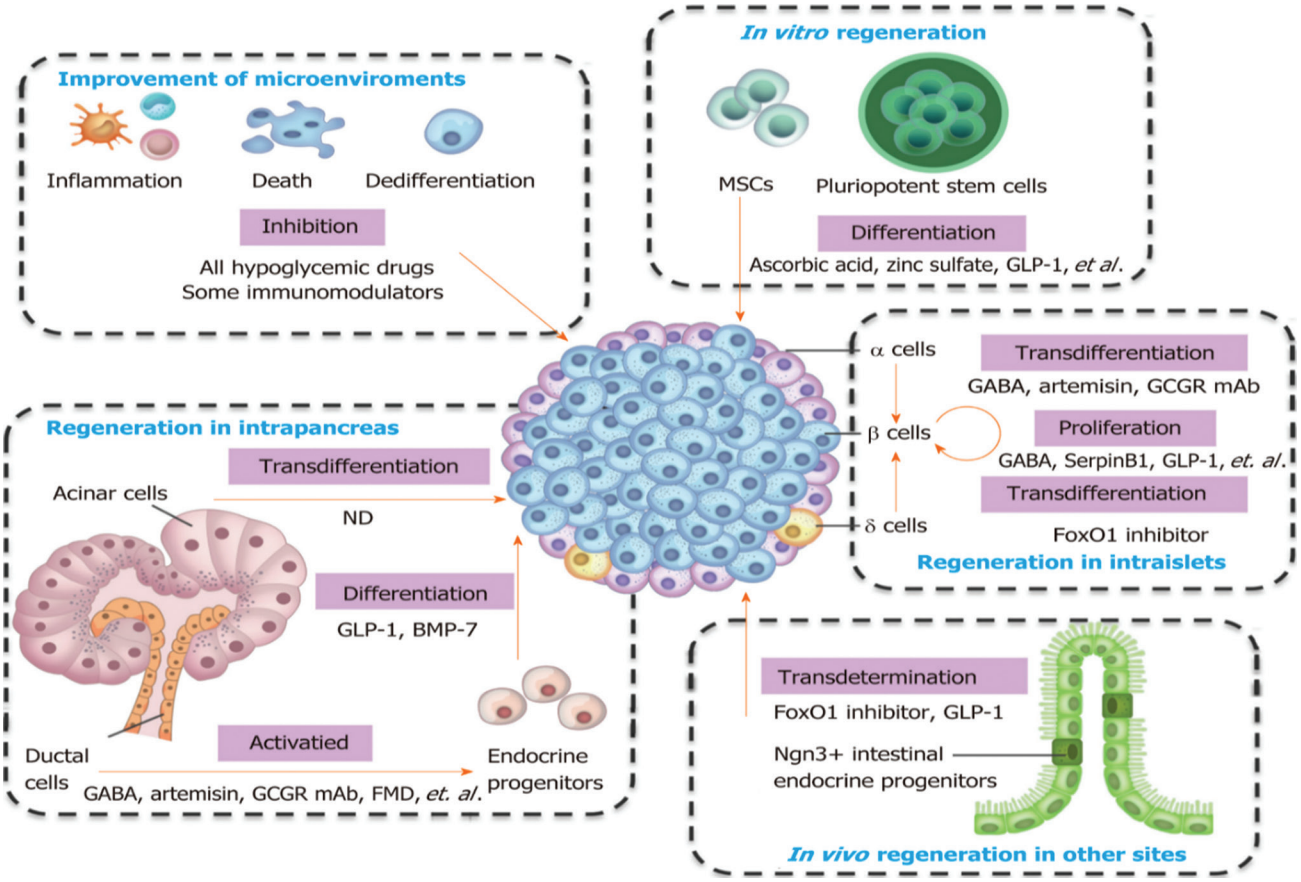

Figure 5. Clinical and preclinical medications for cell regeneration viewed from an integrated perspective involving various cell types
Abbreviations: FMD: Fasting-mimicking diet; GABA: Gamma-aminobutyric acid; GCGR mAb: Glucagon receptor monoclonal antibody; GLP-1: Glucagon-like peptide-1; ND: Not determined. Reprinted (adapted) with permission from Wang *et al.*[14]

through omics and focused analytics.[33] In addition, they can help in the investigation of important genetic and communication networks that regulate crucial stem cell functions. The foremost biological application of this field is the potential utilization of stem cell research in tissue engineering projects, wherein cells directly address illnesses or injuries (Figure 8). As Cesar *et al.*[34] pointed out, stem cells can also be used to investigate and improve the therapeutic potential of cutting-edge therapies.[35]

The failure of numerous small-molecule chemical compounds is often ascribed to toxicological concerns, emphasizing the imperative for improved preclinical models to assess cytotoxicity (especially concerning the liver and heart), thereby significantly expediting pharmaceutical drug development.[36] Human stem cells hold promise for application in high-throughput screening to evaluate drug pharmacology and toxicology, theoretically capable of producing an infinite number of distinct, differentiated human cells. Nonetheless, the extent to which these significant utilities will translate into short-term benefits for human healthcare through stem cell utilization remains a subject of debate. This review underscores several notable engineering-based advancements in stem cell biology. It encompasses a thorough examination of the mechanisms influencing stem cell behavior and the corresponding methodologies for determining how the provision of appropriate matrix molecules, mechanical cues, and/or chemical cues ultimately affects them.[37] The review discusses various tools and techniques for devising biomaterial-based approaches to enhance and manipulate stem cell fate *in vitro* or simulate synthetic stem cell niches *in vivo*.

5. G protein-coupled receptor signaling and pancreatic β-cell

Recent research has provided insights into the mechanisms by which mechanotransduction influences stem cell fate and the intrinsic mechanical properties of stem cells, revealing new perspectives on the role of biomechanics in stem cell fate determination.[38] Significant advancements in GPCR biology have recently emerged, providing valuable knowledge that greatly informs current and

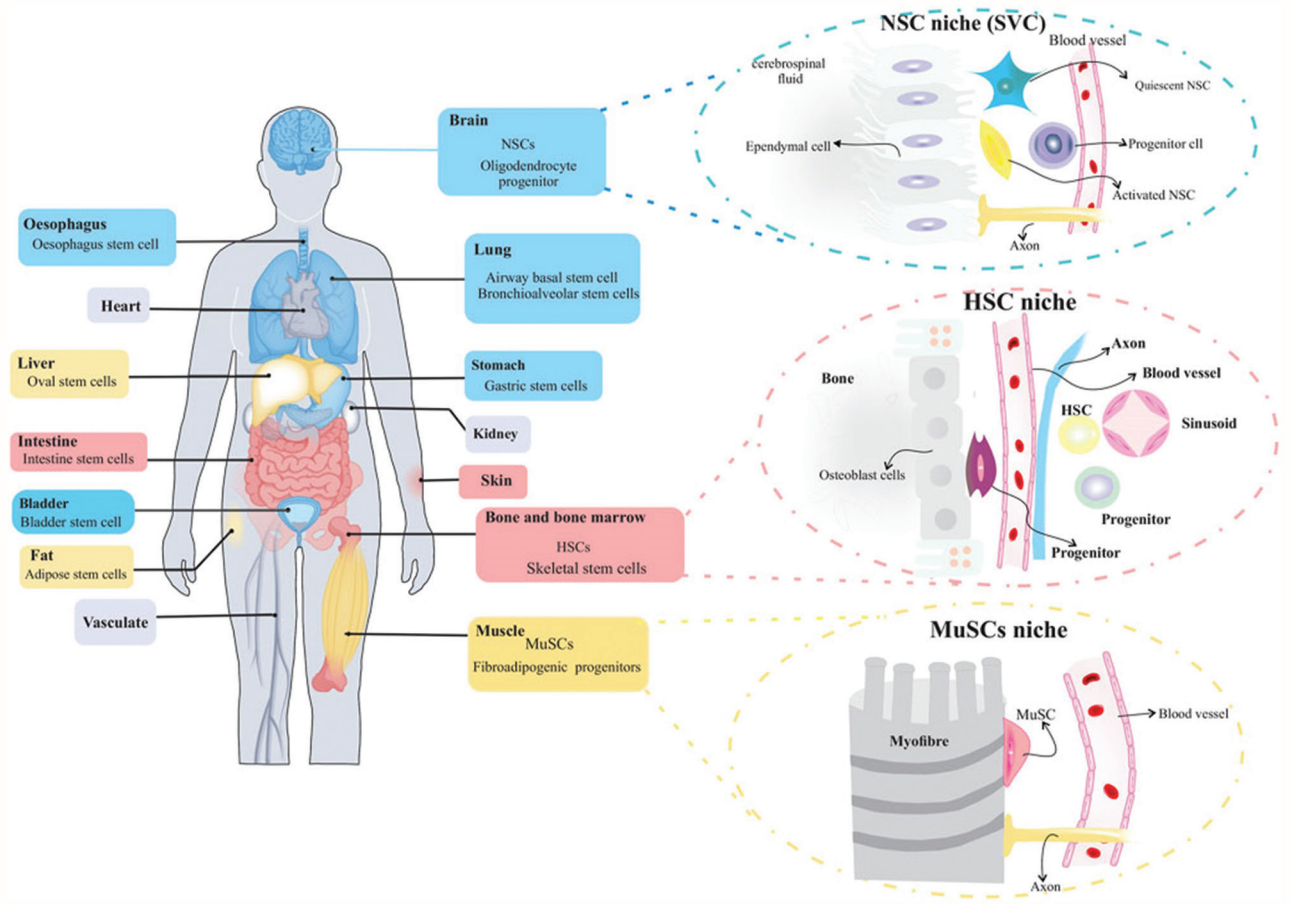

Figure 6. Stem cells and their niches in various organs of humans and other mammals. The arrangement of adult stem cell niches in the muscle, blood, and brain is depicted. Reprinted from Farahzadi *et al.*[23]

future research in rational drug strategies. This interplay of mechanosensing, intracellular signaling, and cell surface mechanics has exerted a significant influence on biological processes.[20] Wojciech Zakrzewski *et al.* present a comprehensive analysis of how these components interact to regulate stem cell fate and formation.[39] Consequently, mechanical signaling impacts various biological functions during development and in adult organisms, such as cell fate transitions, immunological responses, cell migration, and morphogenesis. Investigations are underway to understand the mechanisms and roles of two primary mechanical signaling pathways: outside-in mechanical signaling, which involves mechanosensing substrate properties or shear stresses, and inside-out mechanical signaling, which is regulated by the physical characteristics of the cell surface.[40] Ning Ren *et al.* answered how these two forms of mechanical signaling control *in vivo* developmental processes and stem cell activity.[34] Moreover, the review appropriately addresses how intracellular signaling influences cell surface mechanics and, in turn, how cell surface mechanics regulate intracellular signaling, providing input into the programming of mechanosensing. Further research is necessary to fully comprehend the significant impact of mechanosensing, cell surface dynamics, and intracellular signaling on biological processes.[41]

Christopher S. Chen *et al.* discuss the three factors governing interactions with one another to control stem cell destiny and development, based on current research findings.[42] Through the integration and improvement of massive amounts of data by artificial intelligence, effective pharmaceutical compounds with new scaffolds and distinct chemotypes for all classes of GPCRs will be virtually and experimentally screened. However, as stated in this section, numerous variables, such as chemical diversity, polypharmacology, clinical indication, therapeutic target, ligand-binding site, and signaling mechanisms that affect the development of GPCR-based drugs are also discussed.[43] Future prospects include de-organizing orphan GPCRs to identify novel targets, discovering new therapeutic

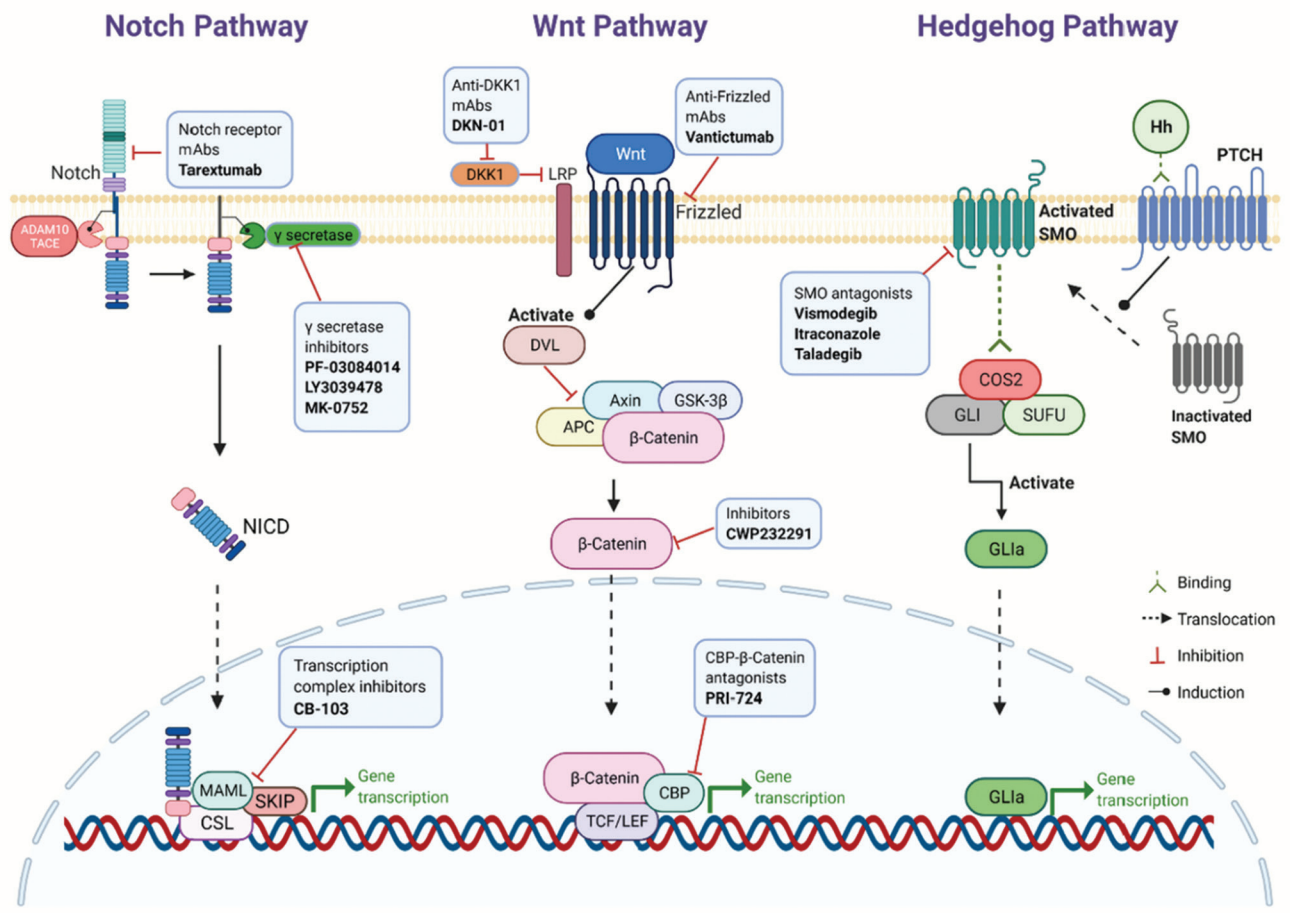

Figure 7. Targeted inhibitors and important signaling pathways that regulate cancer stem cells. The Notch intracellular domain (NICD), which interacts with the transcription complex to promote gene expression, is released as part of the Notch pathway. The Dishevelled protein (DVL) is activated by the Wnt pathway, leading to the breakdown and accumulation of β-catenin in the cytoplasm. The Hedgehog (Hh) pathway entails the binding of Hh to patched (PTCH), the translocation of smoothened (SMO) to the cell membrane, and the release of GLIa, which triggers the transcription of downstream target genes. Reprinted from Ju *et al.*[30]
Abbreviations: APC: Adenomatous polyposis coli; CBP: CREB-binding protein; COS2: Costal2; CSL: CBF1, suppressor of hairless, Lag-1; DKK1:; GLI: Glioma-associated oncogene; GLIa: Glioma-associated oncogene a; MAML: *Mastermind-like proteins*; SKIP: ski-interacting protein; SUFU: Suppressor of Fused homolog; TCF/LEF: T-cell factor/lymphoid enhancer factor.

interventions through both discovery and repurposing initiatives, developing chemical compounds targeting classes B2 and F GPCRs to address unmet medical needs, and validating polypharmacology for enhanced drug treatments.

According to contemporary studies in stem cell biology, the topography and viscoelasticity of the extracellular matrix, in particular, exert a substantial influence on various aspects of stem cell behavior, such as self-renewal capacity and differentiation (Figure 9). The mechanical characteristics of stem cells play a crucial role in maintaining their attachment to the physical milieu, thereby preserving their quiescence and hematopoietic regeneration.[44] Both intrinsic and extrinsic mechanical features are pivotal in regulating stem cell activity and fate. A ubiquitous biological mechanism known as protein phosphorylation enables the regulation of protein activity in a complex and reversible manner. Protein phosphatase 2A (PP2A), a heterotrimeric serine-threonine phosphatase comprising structural, catalytic, and regulatory subunits, regulates several cellular processes by dephosphorylating proteins.[45] While the fundamental biochemistry of PP2A is well understood, its precise function is challenging to discern due to the diversity of its components, particularly the multitude of regulatory subunits. This complexity leads to PP2A's dual regulation of signaling networks, such as the Wnt pathway, which exhibits both positive and negative effects.[27] Wnt signaling is crucial in determining stem cell fate, self-renewal, and the formation of cancer stem cells. Research in this area is advancing to deepen

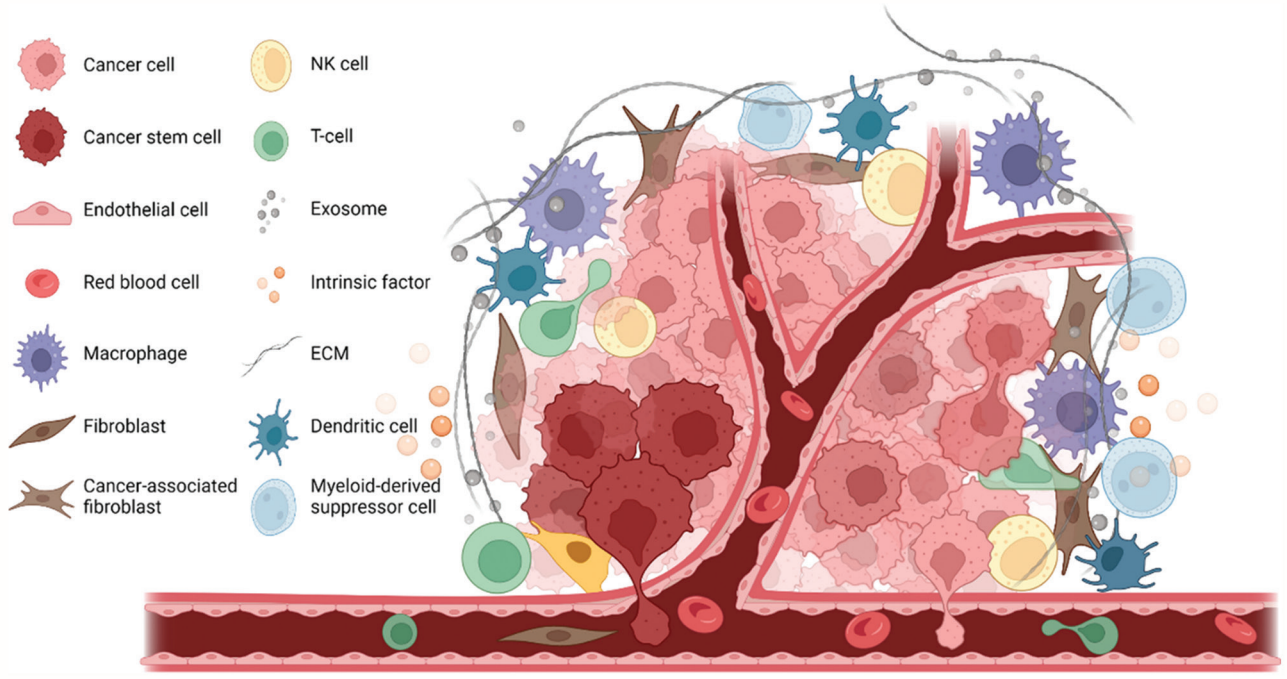

Figure 8. The cancer stem cell niche in solid tumors is composed of a complex milieu that includes cancer cells, stem cells, stromal cells, endothelial cells, fibroblasts, extracellular matrix (ECM), exosomes, and intrinsic factors. This niche contributes to the recurrence of tumor malignancy. Reprinted from Ju *et al.*[30]
Abbreviation: NK cell: Natural killer cell.

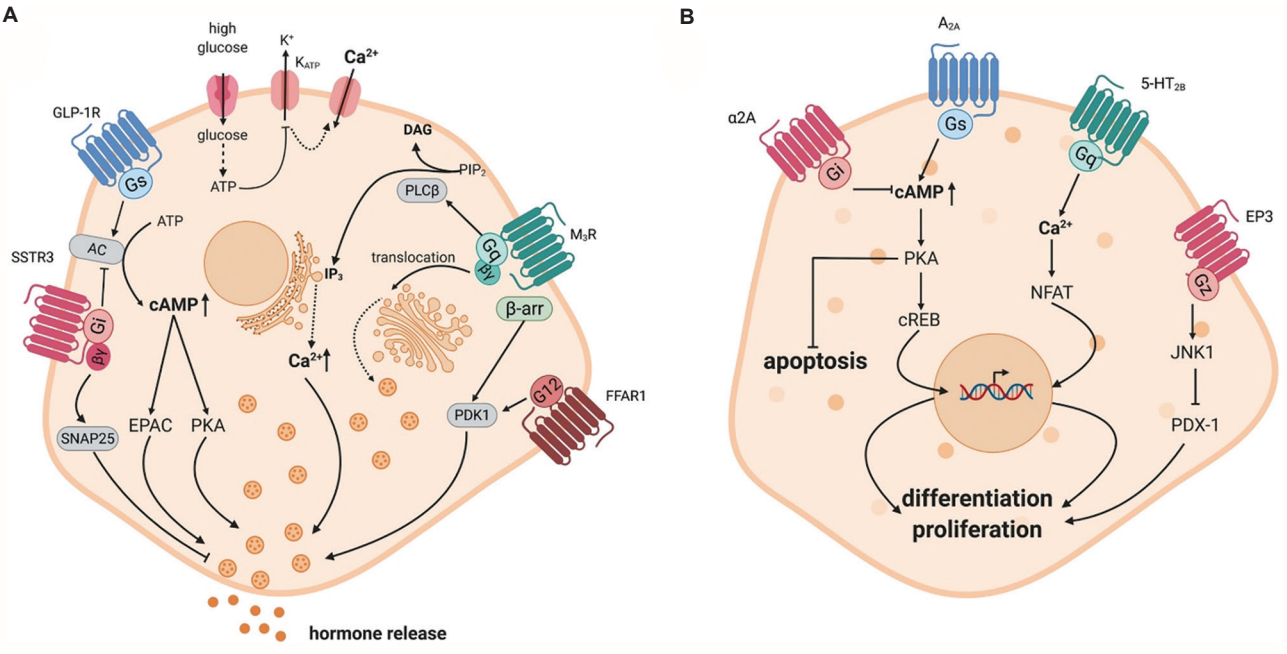

Figure 9. A and B Islet hormone secretion and development are regulated by G protein-coupled receptors (GPCRs). Glucose metabolism leads to increased ATP production in mitochondria, which closes ATP-regulated potassium channels (K_{ATP}), depolarizes β-cells, and opens voltage-dependent Ca^{2+} channels. Reprinted from Thor.[10]

our understanding of stem cell biology and propose novel treatments for malignancies driven by changes in Wnt signaling, as PP2A modulates Wnt signaling at various levels. Protein dephosphorylation is a complicated and

subtle process, and the serine-threonine phosphatases of the PP2A family serve as crucial regulators of numerous signaling pathways associated with cancer, self-renewal, and stem cell viability.[46] Given the high specificity of each regulatory component, along with their high degree of similarity, studies involving knockdown and overexpression must carefully consider compensatory mechanisms.

Biomechanics emerges as a novel regulator of stem cell fate, offering a simplified approach to identifying molecular mechanisms and interactions between biomechanics and stem cells.[47] In addition, this advancement not only sheds light on stem cell biology but also holds promise for driving progress in regenerative medicine by influencing the biomechanical regulation of stem cell fate. GPCRs utilize multiple signaling pathways to communicate from the plasma membrane. The GPCR organization at the cell surface is essential for controlling the signaling effects of receptor activation, as it regulates the interaction of specific effector proteins with particular receptors. Furthermore, the dynamic interaction with proteins and transmembrane microdomains influences GPCR organization.[48] In addition, the endolytic rate of GPCRs, which impacts receptor signaling, is ligand- and receptor-dependent. This understanding of the fundamental structure of GPCRs in the plasma membrane, along with how endocytosis and signaling are affected by this structure, is essential. For appropriate tissue development and homeostasis, precise regulation of cell division and fate decisions is necessary. Epithelial stem cells play a crucial role in maintaining the highly organized tissue structure of the mammalian epidermis. Epithelial stem cells strike a balance between cell fate and self-renewal decisions to build a protective barrier.[49] Recent research has revealed new information on the regulatory systems that govern the specification, self-renewal, and maintenance of epithelial stem cells. These discoveries deepen our understanding of the essential mechanisms enabling epithelial stem cells to establish a functional barrier during formation and preserve tissue homeostasis in adults, thereby enhancing the understanding of the role of epithelial stem cells in organisms.

The continuous integration of information from both the micro- and macro-environment is crucial for adapting to changing circumstances, preserving tissue homeostasis, and triggering repair mechanisms in somatic stem cells.[50] GPCRs enable this integration by affecting a complex network of pathways that regulate stem cell fate through their affinity for various hormones, metabolites, and inflammatory mediators. This role is particularly vital in tissues such as the skin, which are susceptible to environmental damage. In the skin, basal keratinocyte stems and progenitor cells are intricately regulated by various GPCRs and their signaling partners within the intermolecular epidermis and hair follicles.[51] GPCRs translate extracellular signals into intracellular molecular cascades that govern the activation of networks involved in keratinocyte proliferation and differentiation, such as WNT/β-catenin, HH/GLI, and Hedgehog/Yes-associated protein 1. These pathways collectively affect stem cell characteristics, their activation being both dependent on and independent of heterotrimeric G proteins. A classic example of a GPCR-associated cancer is smoothened-driven basal cell carcinoma, underscoring the significant role of GPCRs in pathological conditions.

Dysregulation of GPCR signaling underlies various inflammatory skin conditions and skin cancers.[52] Consequently, extensive explorations have been conducted to elucidate how GPCRs and their signaling partners affect the biology of skin keratinocytes, especially concerning the regulation of the niche that contains ESCs. In general, GPCRs play a crucial role in every aspect of ESC biology, including the maintenance of the adult epithelial stem cell characteristics, facilitation of communication between stem cells and their niche, and integration of extrinsic signals to support stem cells' capacity for contextual adaptation. While attempts have been made to correlate the functions of GPCRs and their ligands with the control of stem cells, more specialized models that study the role of receptors in stem cell destiny must be utilized, such as those involving the overexpression or knockout of GPCRs in certain stem cell niches.[51]

Furthermore, single-cell analysis techniques with enhanced sensitivity for detecting under expressed genes could provide a better understanding of the GPCRs regulated in each niche of ESCs. In addition, given the species-specific difference in skin structure, immunological modulators, and GPCR genes, further research is warranted to validate findings from mouse models in human skin biology.[52] While this review concentrated on the functions of nonsensory GPCRs, it is noteworthy that keratinocytes possess sensory receptors such as light-activated opsins (melanopsin and neuropsin). GPCRs activated by various nutrients and microbial compounds may establish a link between nutrition, the microbiome, and somatic stem cells, suggesting a potential connection between these factors.[53] A fascinating yet understudied topic is the specific function of GPCRs in translating information from the environment, food, and microbiome to determine epithelial stem cell fate.

The role of GPCR signaling in regulating tumor development and suppression represents a remarkable

aspect of this signaling system. For instance, many GPCRs and heterotrimeric G proteins, in addition to pathways such as hedgehog, hippo, and prostaglandins signaling, interact with tumor progression and are frequently implicated in cancer. Despite this understanding, the precise role of the GPCRs in interpreting environmental, dietary, and microbiome signals to regulate epithelial stem cell fate is an enthralling yet understudied topic. A deeper understanding of the specific GPCRs, their signaling partners, and the cellular mechanisms involved will be crucial for developing therapeutic strategies targeting intrinsic stemness pathways associated with malignant transformation and tumor growth. GPCR signals exert a long-lasting influence on the fate of epithelial stem cells, transiently and simultaneously activating multiple pathways that desensitize receptors and halt intracellular cascades.[54] Therefore, a comprehensive comprehension of the interactions between GPCR signaling, transcriptional regulation, and cellular differentiation will shed light on the processes that maintain stem cell characteristics and the strategies employed by stem cells to adapt to microenvironmental alterations.[55] In the body, the epidermis serves as a protective barrier against external environmental aggressors, microbial invasion, and other threats. The basal layer of the epidermis harbors proliferating, immature ESCs, while multiple layers of differentiated, non-proliferating suprafacial cells constitute the upper layers.

Cornified cells from the outer layer are continually replenished through the mitotic activity of ECSs, requiring a delicate balance between self-renewal and differentiation in the adult epidermis to maintain epidermal homeostasis and a functional barrier. Extensive research has been dedicated to understanding how ESCs respond to the demands of tissue growth and homeostasis, as well as the regulatory mechanisms orchestrating these changes.[49] This section discusses recent research on the regulation of the ESC during development and their dynamics in adult homeostasis, arguing that the microenvironment solely dictates the fate of hematopoietic stem cells (HSCs). Instead, intrinsic and environmental signals collaboratively affect HSC behavior. The concept of the stem cell niche integrates these aspects into a more comprehensive and holistic framework. For patients in need of blood and marrow transplants, the search for donors with appropriate histocompatibility antigens can frequently pose significant challenges.[56] Cord blood (CB) cells, now widely available and containing various histocompatibility gene haplotypes, are increasingly being recognized as a valuable source of human stem cells.[57]

Since there are only a limited number of HSCs in each CB sample, the capacity to grow CB stem cells *ex vivo* before transplantation would considerably broaden the utility of this stem cell source. While stem cell expansion can also be achieved *in vivo* following the revascularization of the transplanted cells, this strategy may present greater risks than *ex vivo* expansion unless the growth stimuli employed *in vivo* are carefully controlled.[58] The attractiveness of *ex vivo* stem cell expansion lies in the precise definition of culture conditions and the ease with which transitory modification of regulatory pathways can be exploited *in vitro*. However, this strategy has proven to be quite difficult because complete stem cell proliferation requires symmetric self-renewal divisions of HSCs, where both daughter cells retain HSC characteristics. *In vitro*-grown HSCs typically divide asymmetrically, producing one HSC and one more differentiated progenitor cell, or symmetrically, producing two progeny cells that are no longer capable of becoming HSCs. The equilibrium between HSC proliferation and quiescence is ultimately maintained by positive and negative regulators.[59]

Activating pathways that promote HSC self-renewal and/or inhibit those that lead to HSC quiescence, differentiation, or apoptosis should be a key component of stem cell expansion efforts. Recent advancements in understanding stem cell self-renewal mechanisms have enabled the development of new techniques for expanding stem cells, some of which require viral vector-mediated gene transfer for effective growth.[60] For safety, it is ideal that the vectors induce transitory gene expression and are non-integrating. Secure methods for stem cell expansion often use soluble components such as cytokines, developmental cues, or angiopoietin-like (Angptl) proteins. Jagged, a soluble version of the Notch ligand, promotes the expansion of severe combined immunodeficient repopulating cells, making it a potential tool for *ex vivo* stem cell expansion. However, the most promising soluble factors for murine HSC growth identified to date are the Angptl proteins. Angptl proteins may prove useful for future cell and gene therapy techniques if they can increase human HSCs as effectively as they do in the mouse system. Fibroblast growth factors are also particularly important to highlight because they have been proven to support and preserve the primitive phenotype of murine HSCs in culture.[61]

It should be underlined that studies in mice have provided most of our knowledge concerning stem cell growth. Mouse and human stem cells differ in cytokine receptors, proliferative potential, and telomere biology, leading to both differences and parallels in strategies for increasing HSCs. Nephroblastoma overexpressed should be considered for clinical stem cell growth techniques, as it can expand primitive HSCs. Small molecule medications

may be used to modify pathways, such as manipulating Wnt signaling, p21, and homeobox protein Hox-B4.[58]

Membrane proteins known as GPCRs can detect a wide range of signals, including photons, ions, proteins, neurotransmitters, and hormones. The GPCR superfamily is divided into five subfamilies: glutamate, frizzled/taste2, rhodopsin, adhesion, and secretin receptors. Abnormalities in ligand concentration, GPCR protein expression, or mutations are implicated in numerous pathophysiological conditions, including diseases of the gastrointestinal system, central nervous system, respiratory system, musculoskeletal pathologies, cardiovascular and metabolic systems, immune diseases, and eye disorders. GPCRs account for 30% of all recognized pharmacological targets, making them central to the development of innovative drugs and a common therapeutic intervention strategy.[62] However, a significant challenge persists: integrating extrinsic and intrinsic regulatory fate determinants through the main signaling channels into cohesive networks of regulatory systems.[46] This understanding could lead to novel methods for preserving and boosting HSCs *in vitro*. Interestingly, the molecular mechanisms for managing and maintaining plant stem cells show similarities to those in animals.

Furthermore, this section covers the most recent research on the genetic control of signaling in plant meristems. It begins with providing guidance on the regulation of the shoot and root apical meristems. The borders between plant stem cells and their developing progeny are maintained through transcriptional and posttranscriptional regulation. A graphic representation of the roles of signaling molecules, such as the hormones auxin and cytokines, is provided (Figure 10). Another aspect of control is documented in lateral meristems, where numerous crucial regulators of stem cell maintenance show unexpected parallels to those in apical meristems. The regulation of grass rhizomes is also discussed, as many agricultural plants fall into this category. The reproductive success of these plants, and consequently their agronomic yield, depends greatly on shoot architecture. Therefore, topics such as phyllotaxy, leaf initiation patterning, and floral induction are covered, given their importance for members of the grass family.[63] A brief presentation on the relevant genetic and hormonal regulation is presented.

The study investigated second messenger activation in mouse pancreatic islets, which regulate glucose-induced insulin release. It was found that activating Gq/11-coupled receptors in primary cells increased second messenger IP1, but this signal was eliminated when a Gq/11 protein inhibitor was used. Activation of V1 vasopressin and ghrelin receptors did not significantly increase second messengers. However, fura-2-based fluorescence imaging revealed calcium signals within intact pancreatic islets when arginine vasopressin or ghrelin was applied.[64] The methodology enabled the measurement of intracellular cyclic adenosine monophosphate (cAMP) levels caused by receptors linked to Gs and Gi/o proteins, allowing for an accurate assessment of GPCR activity in intact islets. The second messengers IP1, cAMP, and calcium can be detected, thus enhancing our understanding of their diverse effects on cellular function. Continuous investigation into ligand-receptor interactions, conformational analyses, effector-receptor engagement, and signaling patterns should lead to new approaches for developing novel therapeutics. The G protein-coupled estrogen receptor (GPER) and its potential involvement in illness development and disease treatment are currently under research. GPER binding mediates a number of activities, including metabolic flaws and insulin resistance, as suggested by *in vivo* research.

The treatment of T2D and obesity often involves the use of glucagon-like peptide-1 receptor (GLP-1R) agonists, which enhance pancreatic cell activity and increase insulin sensitivity by promoting weight loss.[65] These agonists are synthetic analogs of naturally occurring agonists such as GLP-1(7-36) NH_2 or its paralogue exendin-4, modified to possess longer pharmacokinetic half-lives. Current research has demonstrated that altering the amino acid sequence of orthosteric GLP-1R peptide agonists can significantly change their intracellular signaling profiles, resulting in decreased coupling to intracellular effectors. This imbalance, favoring G protein-dependent signaling, is frequently referred to as "bias." In the initial stages of diabetes, there is a disruption in the initial phase of insulin production, followed by a gradual decline in the glucose's capacity to stimulate insulin secretion. To develop therapeutic interventions aimed at altering insulin secretory activity and/or cell mass, a better knowledge of the signaling pathways associated with the activation of GPCRs and their interactions within cells is essential. Gi-GPCRs, with interact with G proteins of the i/o class, play a role in regulating a variety of cellular activities in mammalian tissues, but their influence on tissue development and expansion is less apparent.[66] This article covers recent findings on GPCRs functionally expressed in β-cells, which have important properties for the development of T2D therapies. While pancreatic islet cells express GPCRs, their specific roles remain unclear. A study identified specific GPCRs, such as free fatty acid receptor 4 (FFAR4), prostaglandin E receptor 4 (PTGER4), purinergic receptor (P2RY14), adrenoceptor beta 2 (ADRB2), and G-protein coupled receptor 54 (GPR54, also identified as KISS1R), located in primary cilia of both mouse and human islet cells.[67] These GPCRs stimulate ciliary cAMP

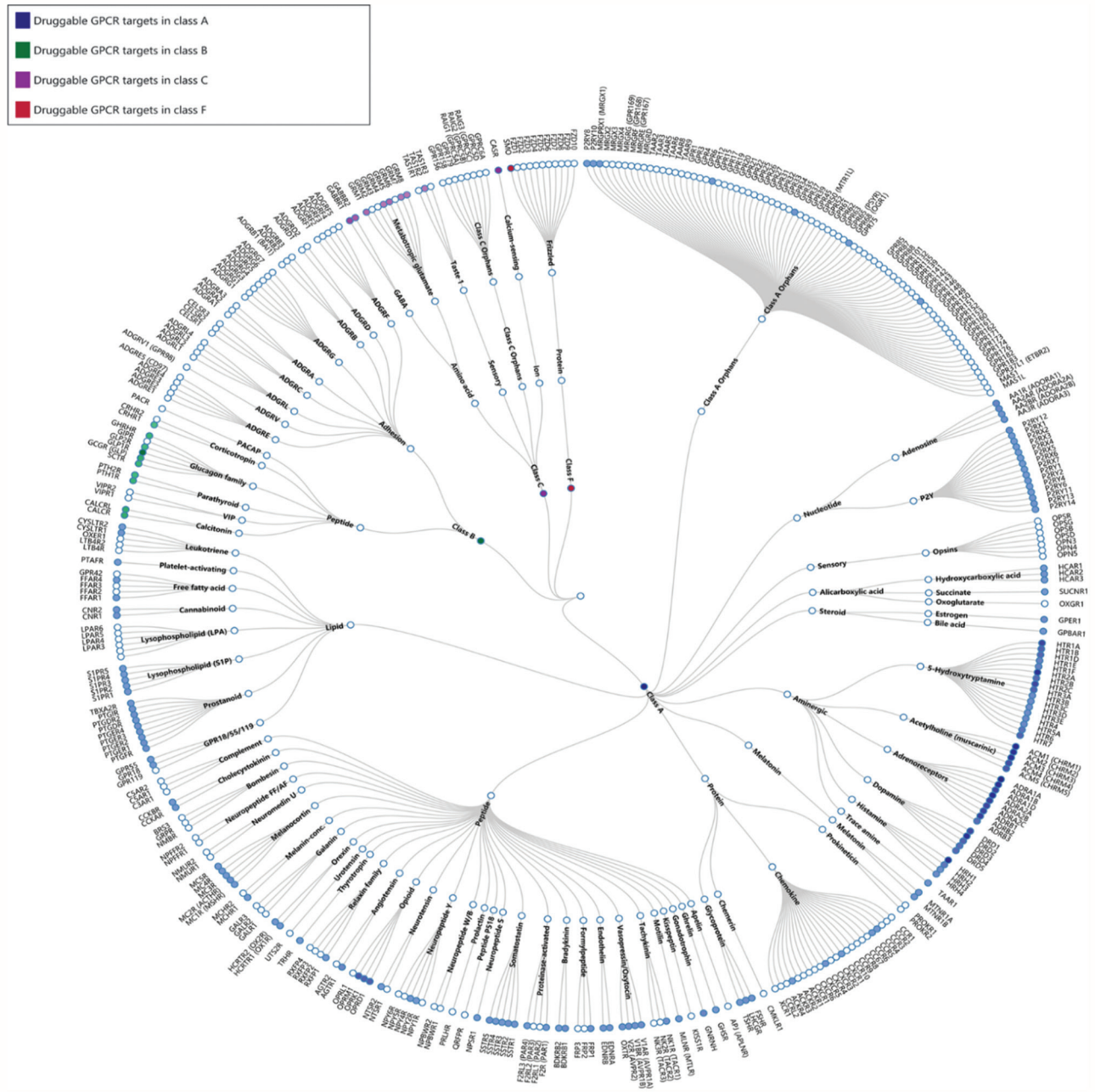

Figure 10. The G protein-coupled receptor (GPCR) phylogenetic tree is utilized to identify pharmacological targets, with each GPCR represented as a node with its gene name. Receptors with commercially available medicines are highlighted in color. Reprinted (adapted) with permission from Yang *et al.*[68]

signaling, enhancing glucagon and insulin production in cell lines as well as in mouse and human islets. Tubby family protein (TULP3) facilitates the transportation of GPCRs to primary cilia, and its knockdown impairs controlled glucagon or insulin production without altering the ciliary structure.

Both the gastric inhibitory polypeptide receptor (GIPR) and the glucagon-like peptide-1 receptor (GLP1R) belong to the class B1 GPCRs, playing pivotal roles in regulating insulin secretion and glucose homeostasis. GLP-1R agonists, such as dulaglutide, liraglutide, exenatide, and semaglutide, represent a burgeoning class of medications beneficial for managing T2D and obesity. A novel pharmacological family, GLP-1R-GIPR co-agonists, including the peptide-based co-agonist tirzepatide, is a potential treatment for T2D by improving glycemic

control and weight loss.[69] GPCRs regulate metabolic functions such as glucose and energy balance, insulin production, and glucose homeostasis. Given their role in insulin sensitivity, they are popular therapeutic targets for medications targeting T2D. Abdelaziz Ghanemi examines GPCRs and their associated signaling pathways, including both G protein-dependent and arrestin-dependent pathways.[70] Arrestin1 and arrestin2 are cytosolic adaptor proteins with widespread expression, and their role in T2D treatment is underscored.[71] GPCR adapter proteins, characterized by high structural conservation and 80% amino acid similarity, can initiate clathrin-coated pits-mediated removal of activated GPCRs from cell surfaces. In addition, arrestins have been shown to mediate the G protein-independent signaling pathway of GPCRs, demonstrating a pharmacological separation between these two signaling pathways.[68 Nov]el medications targeting the GLP-1R are currently underway for the treatment of T2D. These medications stimulate insulin secretion, reduce glucagon secretion, reduce appetite, and promote early satiety, leading to weight reduction in many patients. Furthermore, researchers have developed drugs that block the enzyme dipeptidyl peptidase IV (DPP-IV), thereby raising GLP-1 levels and prolonging its action.

6. Future prospective and outlook

A computer model has been developed to understand the intricate interactions between messengers and signaling pathways in pancreatic β-cells, incorporating data on glucose metabolism, plasma membrane potential, GPCRs, calcium dynamics, and cAMP and phospholipase C pathways that regulate second messenger interactions.[72] This model provides a framework for examining how changes in metabolism, hormones, and neurotransmitters affect insulin secretion. The study suggests that the actions of catecholamines, GLP-1, and GIP are significantly influenced by the activation of Ca^{2+}-dependent adenylyl cyclases. Furthermore, it reveals that a combination of GPCR agonists can enhance insulin secretion more effectively than a single pathway, emphasizing the need to understand connections across second messenger pathways for a better understanding of regulatory sites and pharmaceutical targets in T2D.

Advancements in single-cell multimodal assay development, computational methodologies, and the convergence between biology and engineering have improved our understanding of biological processes and algorithm design. Organoid stages enable *in vitro* organogenesis and clinical diagnostics, paving the way for the development of novel treatments. Techniques in genome editing and genetic circuitry refine organoids, although challenges remain in regulating them at microscopic levels and maintaining them in culture. Regenerative medicine is revolutionizing disease modeling and drug screening through kidney cell cultures and organoids. Decellularized scaffolds infused with iPSC-derived kidney cells hold the potential to create functional replacement organs, enabling organ-on-a-chip technology based on microfluidics and kidney cell types.[73] Hydrogen materials can regulate stem cell fate, enabling the development of biomimetic tissue structures and therapeutic stem cells. Single-cell transcriptomics is expected to advance our knowledge of cell expansion and design.

Human PSCs offer an *in vitro* platform for studying cardiovascular disorders. A comprehensive understanding of stem cell biology and their derivatives is essential for assessing their advantages and disadvantages. GPCRs are crucial regulators of stem cell maintenance and development, playing a key role in cardiovascular cell signaling.[74] Gabor Földes *et al.* explore the role of GPCRs in the development and functionality of cardiomyocytes, endothelial cells, and vascular smooth muscle cells derived from PSCs.[75] It suggests that these models could be used to unravel disease mechanisms and formulate treatment plans. The secretion of hormones from pancreatic islets is a complex process tightly controlled by GPCRs, suggesting that targeting GPCRs could represent a promising strategy for regulating islet functionality. Fabian J. Theis *et al.* draw on RNA-seq datasets from both human and mouse islets to support its insights.[76]

Future options for diabetes therapy may benefit from targeting cellular signaling networks, as they can promote the growth of β-cells. These pathways entail interactions with the proliferative machinery of β-cells, as well as various ligands and receptors. Uddin *et al.* comprehensively assess the potential underlying mechanisms of signaling pathways, including TLR4, Wnt, JAK-STAT, insulin, and growth factor.[77] The application of cellular signals in β-cells poses challenges due to differences in expression across species, age groups, and tissue types, compounded by a lack of thorough investigation into their specific functions. It is important to note that excessive activation of these signaling pathways may negatively impact β-cells. Various therapeutic strategies, including stem cell differentiation induction and islet transplantation, could be used for diabetes treatment. Identifying molecular targets is crucial for developing novel strategies and improving patient outcomes. The regeneration approach seeks to maintain a population of preserved β-cells through *in situ* exposure to agents that enhance cell survival, replication, and insulin secretion. In addition, it entails stimulating the spontaneous conversion of

non-β- to β-cell by activating innate adaptive pathways. Paracrine and immunomodulatory pathways play a critical role in the therapeutic effect of transplantation. Including undifferentiated stem or progenitor cells in the transplant strategy is essential. The replacement technique entails transplanting β-cells, or β-cell-like cells, either naturally matured from cadaveric or xenogeneic islets or from a donor pancreas, along with a strict immunosuppression regimen, following several *ex vivo* pretreatments. For more sophisticated options, artificial pancreas transplants offer a solution by shielding donor islets from the recipient's immune system. However, creating β-cell-like cells *ex vivo* from progenitors or differentiated somatic cells through exposure to small-molecule inducers or genetic alterations poses greater challenges. Reprogrammed cells, while sharing many characteristics with other cells, partially retain some of their original characteristics, requiring further research on their safety.

7. Conclusion

Systems biology combines existing treatments to predict reactions and improve drug effectiveness. While computational biology faces challenges, researchers strive for publications that comprehensively cover all aspects, provide new insights into biological processes, and optimize computational approaches. Data must be meticulously characterized and stored for re-analysis, and collaboration between computational and evolutionary biologists can lead to more fruitful investigations. Combining computational and predictive models with control rules can enhance therapeutic performance. Regenerative medicine, which focuses on promoting the regeneration of injured tissues and organs, has gained momentum alongside biotechnological advancements. Our understanding of reparative regeneration, which restores the shape and functionality of tissues and organs affected by disease or trauma, continues to expand, enriching available treatment modalities. PSCs serve as invaluable tools for modeling disease populations, elucidating pathophysiological mechanisms, predicting survival rates, identifying biomarkers and therapies, detecting haploinsufficient genes, and conducting random drug testing. Human PSC-derived organoids offer robust tools for drug development and cancer research. Computer-based cell labeling can be achieved through annotation, comparison with benchmark datasets, and interpretation. Recent advancements in understanding the structure–function correlations of GPCRs have accelerated drug development, particularly in preventing disorders such as diabetes mellitus by preserving glucose homeostasis. GPCRs regulate hormone production from pancreatic islets, which are crucial for islet development. Targeting GPCRs shows potential in modulating pancreatic islet function by promoting cell replication and proliferation. RNA-seq datasets from human and mouse islets have been instrumental in determining GPCR expression profiles and their effect on islet function.

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Gene & Protein in Disease

REVIEW ARTICLE

TOPK: A noteworthy target for lung cancer treatment

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Abstract

T-LAK cell-originated protein kinase (TOPK) is a serine/threonine protein kinase that is specifically expressed in actively proliferating cells, such as normal testicular germ cells, lymphocytes, and various tumor cells. It plays a key role in multiple biological processes, such as cell growth, metastasis, drug resistance, angiogenesis, and inflammation, and is a promising therapeutic target for tumors. Aberrant TOPK overexpression or activation has been observed in lung cancer and is related to lung cancer occurrence and development, clinical outcome, and poor prognosis. The inhibition of TOPK has demonstrated significant therapeutic potential for reducing tumor growth and can even be used in combination with chemotherapy or radiotherapy. Thus, targeting TOPK provides a promising avenue for the prevention and treatment of lung cancer. This article reviews the role of TOPK in the occurrence, development, and drug resistance of lung cancer; summarizes the main signaling pathways affected by TOPK in lung cancer; and analyzes its therapeutic value. The role and potential of TOPK in targeted therapy, chemotherapy, radiotherapy, and immunotherapy for lung cancer are also discussed. In addition, the latest progress in the use of TOPK inhibitors for lung cancer treatment is summarized, and their future clinical application is discussed. Overall, TOPK is a valuable target for the treatment of lung cancer, and further development of specific TOPK inhibitors is indispensable for the comprehensive treatment of lung cancer.

Keywords: TOPK; Lung cancer; Cancer treatment; Inhibitors; Target

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1. Introduction

T-LAK cell-originated protein kinase (TOPK) is increasingly known as a mitogenic protein kinase, which is specifically expressed in many highly proliferative cells.[1,2] It is located in both the cytoplasm and nucleus. In normal adult tissues, the expression of TOPK is mainly limited to lymphoid tissues and testis and is involved in the activation of lymphoid cells and spermatogenesis.[1] In numerous cancers, TOPK is overexpressed and can directly phosphorylate ERK2,[3] PRPK,[4] c-Jun,[5] histone H3,[6] H_2AX,[7] Prx-1,[8] ULK1[9] and NF-κB,[10] thus participating in the regulation of tumor cell cycle progression, transformation, proliferation, metastasis and drug resistance. Recent studies have

indicated that TOPK is one of the most valuable tumor markers, and upregulation of TOPK expression is demonstrated to be correlated with tumor diagnosis and unfavorable prognosis.[11,12]

Lung cancer is known to cause the highest mortality among all tumors, and it is also one of the most important factors leading to the increase in the global cancer burden.[13] Surgery, radiotherapy, and drug therapy are the standard clinical treatments for lung cancer. During the past few years, there has been a remarkable advancement in targeted therapies and immunotherapies, which effectively improve patient survival rates.[14]

Here, we summarize the roles and mechanisms of TOPK in lung cancer and analyze the efficacy of TOPK inhibitors in various therapeutic treatments for lung cancer. Existing findings have substantiated TOPK as a valuable target for the treatment of lung cancer; therefore, further research efforts to develop specific inhibitors for comprehensive lung cancer therapies are warranted.

2. The role of TOPK in lung cancer

TOPK is considered a diagnostic/prognostic marker and therapeutic target for lung cancer. As shown in Figure 1, the expression of TOPK was significantly greater in both lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) tissues than in normal tissues, according to The Cancer Genome Atlas database (http://gepia.cancer-pku.cn/; accessed on October 25, 2023). High expression of TOPK promotes tumorigenesis, progression, and drug resistance of lung cancer.

2.1. TOPK promotes the tumorigenesis and progression of lung cancer

TOPK is a MAPK-like protein kinase that is known to be involved in tumorigenesis, and its expression is closely related to the malignant transformation of cells and the malignant potential of tumor cells. According to reports, TOPK overexpression actuates JB6 epidermal cell transformation and proliferation both *in vitro* and *in vivo*.[15,16] Consistent with these results, in lung cancer cell lines, TOPK knockdown leads to tumor growth inhibition, which may help with improving the treatment efficacy and long-term survival rate of lung cancer patients. Our previous study revealed that TOPK was highly expressed in anaplastic lymphoma kinase (ALK)-positive non-small cell lung cancer (NSCLC) and that the inhibition of TOPK attenuated cell growth and promoted cell apoptosis.[17] In addition; inhibiting TOPK could also effectively cause cell morphological changes and inhibit the proliferation and survival of small cell lung cancer (SCLC) cells. The TOPK inhibitor OTS514 suppressed the stemness of cancer stem cells (CSCs) by inhibiting forkhead box protein M1 (FOXM1) activity, thereby exerting greater cytotoxic effects on pulmonary spheroid CSC-like SCLC cells.[18] Moreover, TOPK expression was found to be upregulated in NSCLC patients with *KRAS* mutations. TOPK promoted the growth and proliferation of A549 cells bearing *KRAS* G12C mutations by activating the MAPK/ERK signaling pathway. Aside from these, TOPK can also promote the activation of NF-κB signaling in A549 cells carrying *KRAS* G12C mutations by promoting the phosphorylation of TAK1.[19]

Additionally, TOPK is related to the metastasis of lung cancer cells. Shih *et al.* demonstrated that TOPK promoted the invasion and migration of lung cancer cells by inhibiting the expression of PTEN, thus alleviating the negative regulatory effect of PTEN on the PI3K/Akt signaling pathway.[20] Moreover, hypoxia-inducible factor-1α (HIF-1α) can effectively activate and promote epithelial-mesenchymal transition under hypoxic conditions. Another study showed that TOPK could upregulate HIF-1α levels through hypoxic signaling, thus promoting the expression of Snail, which led to epithelial-mesenchymal transition in NSCLC cells. As a novel hypoxia signal regulator, TOPK plays a key role in the migration of NSCLC cells.[21]

These studies showed that TOPK could promote the tumorigenesis and progression of lung cancer through multiple pathways and could be a potential therapeutic target for lung cancer.

2.2. TOPK promotes drug resistance in lung cancer

Drug resistance poses a huge challenge in the treatment of lung cancer. Many researchers have found that TOPK is an important molecule leading to drug resistance in lung cancer. TOPK is substantially expressed in NSCLC cells that are resistant to epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs). It has also been demonstrated that TOPK can phosphorylate c-Jun and subsequently activate CCND1 and CDC2 due to the resistance of lung cancer cells to EGFR-TKIs. Silencing TOPK can enhance the sensitivity of EGFR-TKI-resistant lung cancer cells to gefitinib.[5] Furthermore, our previous study revealed high expression of TOPK in NSCLC cells with MET amplification-induced resistance to gefitinib. As an upstream molecule, MET regulates the activity of TOPK. The COX2-TXA2 signaling pathway regulates MET through AP-1, which is then phosphorylated at Tyr74 to activate TOPK, leading to NSCLC resistance. Another study showed that the combination of three U.S. Food and Drug Administration (FDA)-approved drugs (celecoxib, pantoprazole, and gefitinib) promoted apoptosis in

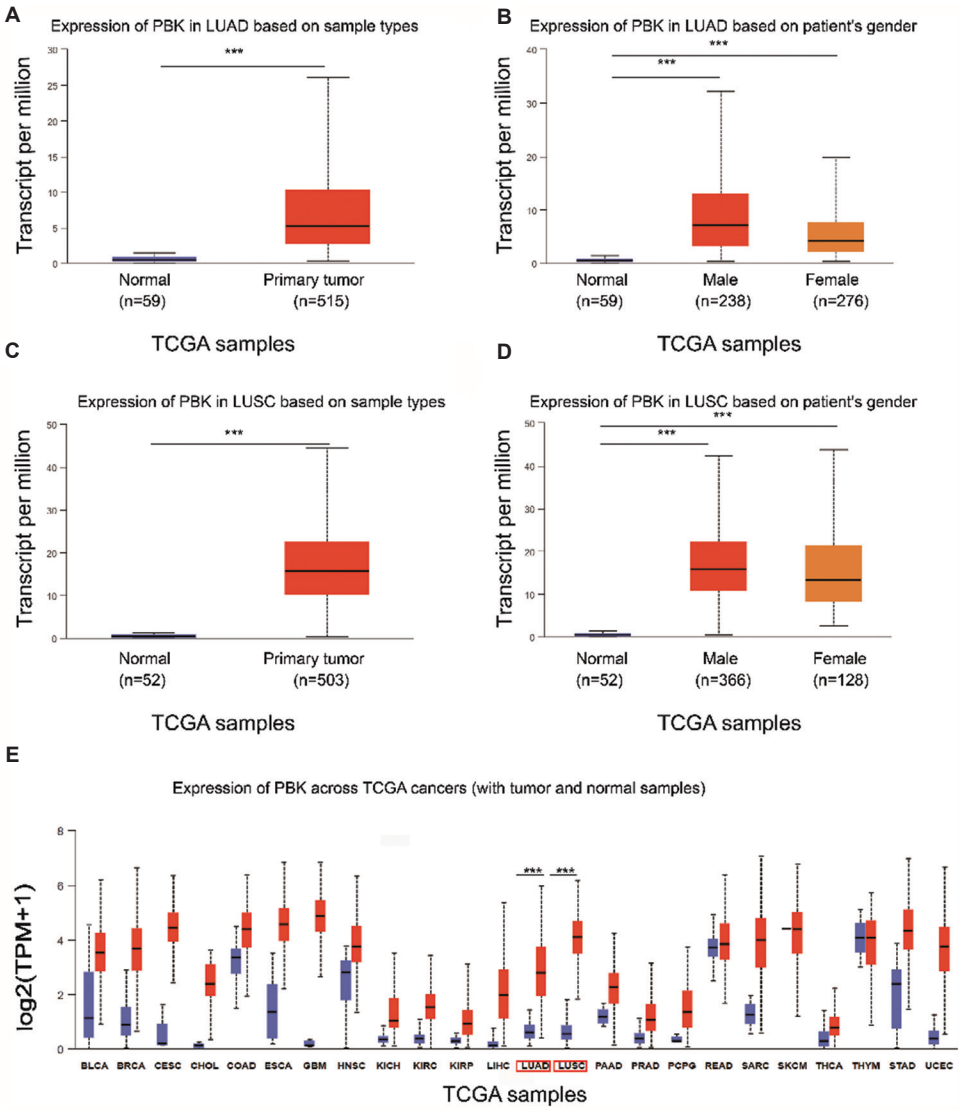


Figure 1. PBK expression in LUAD and LUSC. (A and B) TOPK expression in LUAD based on sample types (A) and gender of patients (B); data obtained from The Cancer Genome Atlas database. (C and D) TOPK expression in LUSC based on sample types (C) and gender of patients (D). (E) Gene expression profiles in all tumor samples paired with normal tissue. The height of the bars indicates the median expression of a certain tumor type or normal tissue. ***$P < 0.001$. Source: Diagram made by the authors.
Abbreviations: TOPK: T-LAK cell-originated protein kinase; LUAD: Lung adenocarcinoma; LUSC: Lung squamous cell carcinoma.

NSCLC cells with MET amplification-induced gefitinib resistance.[22] Thus, TOPK may serve as a novel target for overcoming resistance to gefitinib.

2.3. TOPK as a prognostic indicator of lung cancer

Accumulating evidence indicates that TOPK is a marker of poor prognosis in lung cancer patients. Early immunohistochemical analysis revealed that patients with high expression of TOPK in stage I NSCLC were more likely to experience recurrence and metastasis, and their overall survival (OS) rate was lower than that of patients with low expression of TOPK; moreover, high expression of TOPK predicted poor prognosis.[20,23,24] Similarly, after analyzing 127 patients with LUAD, it was found that high expression of TOPK and mutant P53, as well as lymph node metastasis and distant metastasis, were found to be independent predictors of poor prognosis in patients with LUAD.[25] Recently, one study based on the Oncomine database showed that TOPK levels were negatively correlated with the OS of lung cancer patients, particularly those with LUAD. Subgroup analysis suggested that overexpression of TOPK was related with a significantly shorter survival period in patients classified by sex, grade, and clinical stage, with the exception of Stage 4 lung cancer patients

whose prognostic data were unavailable.[26] In addition, in a pan cancer analysis, Ma *et al.*[27] indicated that TOPK was upregulated significantly in LUAD patients, indicating a poor prognosis. Taken together, TOPK is a potential prognostic marker for lung cancer, especially LUAD.

3. TOPK signaling pathway in lung cancer

TOPK is involved in many biological functions of cells. It also promotes the occurrence, metastasis, metastasis development, and drug resistance of lung cancer cells and serves as a marker for the diagnosis and prognosis of lung cancer. Research has shown that its function is regulated by a variety of upstream and downstream molecules, making it a linchpin in the tumor signaling network.[12] In lung cancer, TOPK is directly or indirectly regulated by important targets such as MET,[22] ALK,[17] and EGFR,[5] and the phosphorylation of TOPK activates downstream signaling pathways, leading to the occurrence, progression, or drug resistance of lung cancer (Figure 2). These studies suggest that as a kinase in the signaling network, TOPK is a promising therapeutic target for lung cancer.

4. TOPK and treatment of lung cancer

Surgery is the predominant treatment choice for early-stage lung cancer patients, significantly raising their survival rate. Patients in the middle to late stages of the disease require comprehensive treatment. Traditional treatment for advanced lung cancer mainly relies on radiotherapy and chemotherapy, which have poor efficacy.[28] Approximately 65% of patients are diagnosed with locally advanced lung cancer, and the 5-year survival rate is approximately 20%. Thus, designing effective strategies for preventing and treating advanced lung cancers that are associated with low patient survival rates is the major focus of research.[29]

TOPK has attracted the interest of many researchers and clinicians as a potential therapeutic target, and the investigations on TOPK inhibitors have been ongoing. It has been reported that some small molecule compounds and FDA-approved drugs, such as HI-032,[17] OTS964,[30] OTS514,[18] SKLB-C05,[31] 3-deoxysappanchalcone,[32,33] ADA-07,[34] eupafolin,[34] acetylshikonin,[35] glycycoumarin,[36] pantoprazole,[37] ilaprazole,[38] fucoidan,[39] baicalin,[40] cefradine,[41] sulfasalazine,[42] coffee phenolic

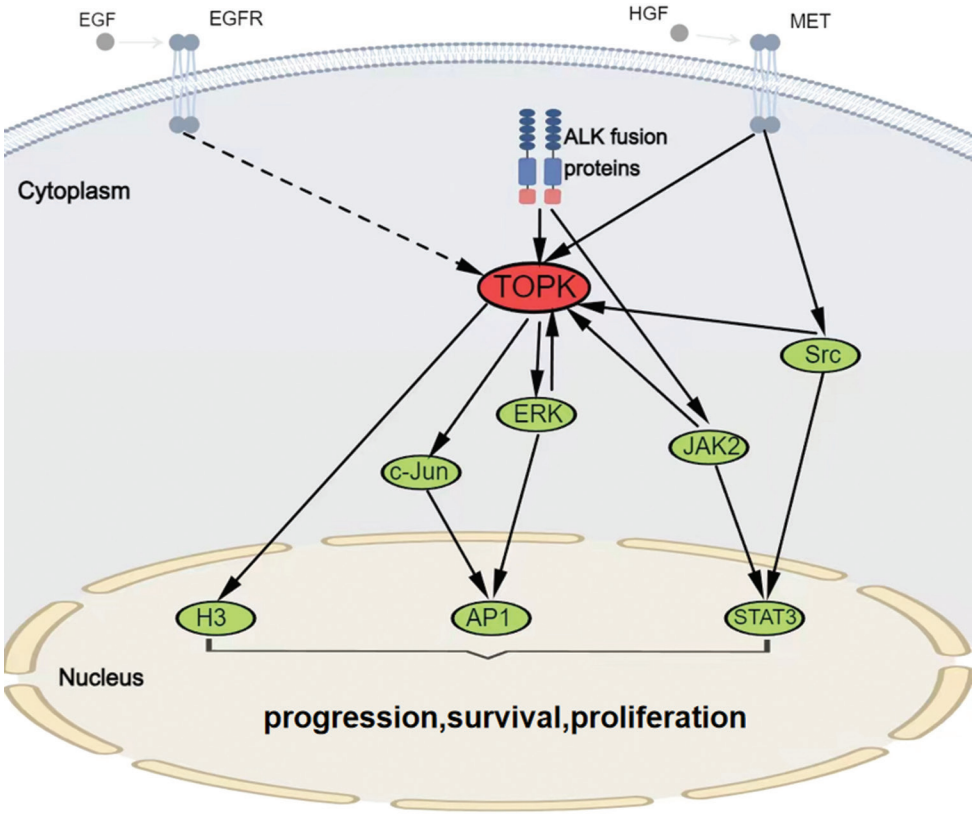


Figure 2. TOPK signaling pathway in lung cancer. Source: Schematic diagram made by the authors.
Abbreviation: TOPK: T-LAK cell-originated protein kinase.

phytochemicals,[43] the ginsenoside Rh2,[44] paeonol,[45] xanthohumol,[46] glycyrol,[47] and worenine[48] can target TOPK to inhibit its activity.[12] Multiple treatment options available for lung cancer nowadays contain TOPK inhibitors. Table 1 lists the inhibitors that are effective against lung cancer.

4.1. TOPK and targeted therapies for lung cancer

Gefitinib was the first targeted drug for lung cancer approved by the FDA for the treatment of NSCLC in 2003. Subsequently, other driver gene alterations for lung cancer, including *ALK* rearrangement, *RET* rearrangement, *ROS1* gene fusion, neurotrophic tyrosine receptor kinase 1/2/3 gene fusion, *BRAF* (v-Raf sarcoma viral oncogene homolog B) V600E mutation, *HER2* mutation, *KRAS* G12C mutation, and *MET* exon 14 skipping were also identified and discovered. After proving that these genetic alterations were therapeutic targets, corresponding targeted drugs were subsequently developed as the first-line treatment for patients with advanced NSCLC, greatly improving the prognosis of these patients.[49,50] At present, there are more than 20 targeted drugs approved by the FDA for the first-line clinical treatment of lung cancer.[51] Targeted drugs have greatly improved outcomes for advanced NSCLC patients carrying the driver gene alterations.[49]

On the one hand, TOPK — a protein kinase — is a promising drug target. The small molecule compound inhibitors OTS514 and OTS964 have been proven to inhibit the growth of lung cancer cells by targeting TOPK.[30] Three traditional Chinese medicine monomer compounds (baicalin, glycerol, and xanthohumol) can also effectively decrease TOPK activity and inhibit lung cancer growth both *in vivo* and *in vitro*.[17] On the other hand, TOPK inhibitors combined with other targeted drugs can also enhance sensitivity to targeted drugs and delay the development of drug resistance. Xiao *et al.*[22] reported that the TOPK inhibitor pantoprazole combined with celecoxib and gefitinib led to apoptosis in gefitinib-resistant lung cancer cells and inhibited tumor growth. HI-032 (another TOPK inhibitor) in combination with alectinib (a first-line targeted drug for patients with ALK-positive lung cancer) may be a viable treatment approach for increasing patient sensitivity to targeted therapy.[17] In addition, a recent report showed that *TOPK* transcription can be promoted by UPF1, which can subsequently block its anticancer function by phosphorylating FOXO1 protein in lung cancer cells. The protein phosphorylation level of FOXO1 decreased in a dose-dependent manner when the kinase activity of TOPK was inhibited by OTS514. Thus, the UPF1-TOPK-FOXO1 axis may be a potential therapeutic target for lung cancer.[52]

Taken together, TOPK inhibitors emerge as a class of targeted therapies for lung cancer and are expected to overcome drug resistance.

4.2. TOPK and chemotherapies for lung cancer

Chemotherapy is still an important part of targeted therapy, immunotherapy, or other new treatments for lung cancer, and it is a guaranteed salvage therapy after the occurrence of resistance to other therapies. It has been reported that TOPK regulates paclitaxel-induced cancer cell death and inhibits paclitaxel-induced autophagy in H460 cells by inhibiting p53.[53] The TOPK inhibitor OTS514 could enhance the anticancer effect of fluorouracil (5-FU), and the combination of 5-FU, OTS514 and the *KRAS* G12C inhibitor AMG510 showed synergistic antitumor effects.[19] Therefore, the inhibition of TOPK combined with chemotherapy presents an effective strategy for treating lung cancer.

4.3. TOPK and radiotherapy for lung cancer

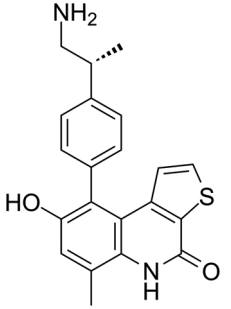
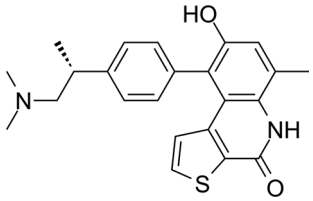
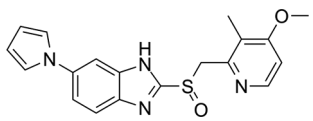
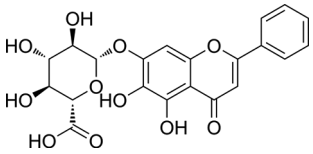
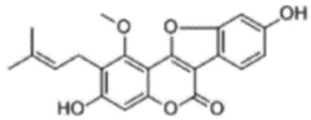
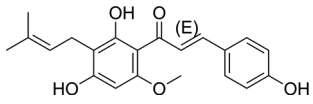
Approximately 60–70% of lung cancer patients require radiotherapy at various stages of the disease. For patients with locally advanced NSCLC, thoracic radiotherapy should be administered as soon as possible, and simultaneous radiochemotherapy followed by immunotherapy is the preferred treatment.[54] It has been shown that the tumor-suppressive E3 ubiquitin ligase CHIP could inhibit the TOPK-ERK pathway, and subsequently inhibit the stem cell properties and induce radioresistance of NSCLC cells, providing a promising therapeutic approach for enhancing the efficacy of radiotherapy.[55] In lung cancer xenograft models, TOPK inhibition with OTS964 was shown to potentiate fractionated radiotherapy *in vivo*.[56] Therefore, the inhibition of TOPK can improve the sensitivity of lung cancer cells to radiotherapy.

4.4. TOPK and immunotherapy for lung cancer

Immunotherapy for tumors can be divided into four categories: Immune checkpoint inhibitors (PD-1/L1), cellular immune cell therapy (CAR-T), tumor vaccines, and nonspecific immunomodulators.[57] Through the analysis of lung cancer microarray data in the GEO database and weighted gene co-expression network analysis, *TOPK* was identified as an immune cell infiltration (ICI)-related hub gene. It is a potential lung cancer-specific marker and immunotherapy target and thus, more in-depth studies are warranted.[58]

Another study suggested that TOPK is a promising prognostic marker for immune escape in patients with LUAD. Across bioinformatics analysis, the researchers found that patients with overexpression of TOPK tended to have an immune rejection phenotype, accompanied by an elevated level of antitumor ICI. Moreover, immune infiltration analysis revealed that the immune escape triggered by the highly expressed TOPK group might be related to antigen presentation

Table 1. Information of a selection of TOPK inhibitors and their efficacy against lung cancer

Name	Chemical structure	*In vitro*	Concentration	*In vivo*	Dose	Efficacy	Treatment
OTS514		A549 and LU-99 cell lines	N/A	Human lung cancer xenograft model	1,2.5 and 5 mg/kg	TGIs of 90.3% (2.5 mg/kg) and 99.8% (5 mg/kg)	Targeted treatment[30]
		A549 cell lines	OTS514 (20 nM) 5-FU (25 μM)	Human lung cancer xenograft model	OTS514 (2.5 mg/kg) 5-FU (100 mg/kg)	Inducing apoptosis synergistically	Combined with 5-FU[19]
OTS964		A549 cell lines	IC_{50}=31 nM	Human lung cancer xenograft model	40 mg/kg	TGIs of 110% (40 mg/kg)	Targeted treatment[30]
		LU-99 cell lines	IC_{50}=7.6 nM				
		H460 cell lines Calu-6 cell lines	10 or 20 nM	Human lung cancer xenograft models	OTS964 (20 mg/kg) Gulmay (2 Gy/min)	Enhancing tumor sensitivity to fractionated irradiation	Combined with radiotherapy[56]
Ilaprazole		Virtual ligand screening	ICM scores = −31.88 Kd = 111.0 ± 2.3 μM	N/A	N/A	N/A	Targeted treatment[38]
		A549 cell lines	IC_{50}=30.10 – 148.21 μM				
Baicalin		Kinase assay	10, 20, and 50 μM	Human lung cancer xenograft model	20 or 50 mg/kg	Growing remarkably; more slowly with 50-mg/kg baicalin	Targeted treatment[40]
Glycerol		Kinase assay	5,7.5,10 μM	Human lung cancer xenograft model	20 mg/kg	Significant inhibition	Targeted treatment[47]
		A549 cell lines	IC_{50}=4.823 μM				
Xanthohumol		Kinase assay	0, 5, 10, 15, 20 μM	Human lung cancer xenograft model	25 mg/kg	Suppressing tumor growth	Targeted treatment[46]

Abbreviations: 5-FU: Fluorouracil; TGI: Tumor growth inhibition; TOPK: T-LAK cell-originated protein kinase.

and infiltration by dendritic cells and $CD8^+$ T cells. However, the immune spectrum of patients was poorly characterized, and the role of immunosuppressive cells in the tumor microenvironment remains unclear.[27] An experimental study showed that inhibition of TOPK significantly enhanced $CD8^+$ T cell infiltration and activated $CD8^+$ T cells and enhanced the effect of anti-PD-L1 therapy, thereby synergistically enhancing the immune response against renal cell carcinoma.[59]

In addition, mRNA vaccines are a novel immunotherapy strategy for lung cancer. Across antigen-presenting cell-associated cancer-associated antigen screening and prognosis analysis, Zhou *et al.*[60] identified TOPK as a potential candidate antigen for mRNA vaccines. These predictive analyses need to be further verified in lung cancer in a well-designed experimental scheme.

5. Conclusions and perspectives

The discovery of EGFR, ALK, MET, and other targets has profoundly prompted the development of targeted drugs, which improve the prognosis of advanced NSCLC patients carrying the corresponding driver gene alterations.[49,51] Unfortunately, only approximately 25% of patients can benefit from targeted therapy.[61] There are two major challenges in the development of targeted therapies for lung cancer: (1) The known targets and drugs available are limited. Thirty-one percent of the unknown tumor driver genes have potential therapeutic targets for lung cancer, which is worthy of further study and[50] (2) drug resistance hinders targeted therapy. The molecular mechanism of resistance is related not only to genetic changes, such as site mutations, deletions, and gene amplifications but also to the activation of bypass pathways or phenotypic changes in tumor cells. The mechanism of drug resistance in more than 10% of drug-resistant patients remains unclear.[62] Overall, it is necessary to explore novel targets for lung cancer treatment and develop corresponding inhibitors for targeted therapy. Of note, protein kinases are the main targets of NSCLC-targeted drugs. The targets of the first-line targeted drugs for advanced NSCLC approved by the FDA are all tumor-associated protein kinases.[63]

Similar to classical targets such as ALK and MET, TOPK is involved in a wide variety of biological functions in lung cancer, such as tumorigenesis, progression, metastasis, and drug resistance, through the activation of diverse downstream signaling pathways. Based on the research by Illuminating the Druggable Genome (IDG), initiated by the United States National Institutes of Health, TOPK could bind to small drug-like molecules with high affinity and specificity. Thus, it has the potential to be a "druggable" kinase.[64] TOPK, an oncokinase that inhibits mitosis by attenuating its activity. The inhibition of TOPK does not undermine the function of non-proliferative cells and has lesser off-target effects. Therefore, TOPK would serve as an ideal target. However, the three-dimensional structure of TOPK remains uncharacterized, and the development of TOPK inhibitors is progressing slowly. The efficacy of inhibitors such as OTS964 in lung cancer has yet to be confirmed in preclinical and clinical studies. Overall, TOPK is a noteworthy kinase for targeted therapy in lung cancer, but in-depth research is required to explore its inhibitors.

In addition, TOPK is regarded as a promising immunotherapy target for lung cancer. Because of its highly specific expression in normal testes and tumors, TOPK has also been identified as a cancer/testis antigen (CTA) and is included in the CT database (http://www.cta.lncc.br/gene_annotations.html). Lung cancer vaccines and other immunotherapies target CTAs because of their high antigenicity and tumor selectivity. Vaccines, chimeric antigen receptor-modified T cells (CAR-T cells), and small molecule inhibitors targeting CTAs have been used in preclinical and early-stage clinical trials for lung cancer treatment.[65] TOPK has also been claimed as an immunotherapy target and a potential candidate antigen for mRNA vaccines. An experimental study showed that inhibition of TOPK significantly enhanced $CD8^+$ T-cell infiltration activated $CD8^+$ T cells and potentiated the effect of anti-PD-L1 therapy, thereby synergistically strengthening the immune response against renal cell carcinoma.[59] Therefore, the role of TOPK in lung cancer immunotherapy is worthy of further exploration.

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Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

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Gene & Protein in Disease

REVIEW ARTICLE

Opportunities and challenges of integrating HIF-1 into clinical practice for cancer treatment

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Abstract

The Warburg effect is one of the most studied mechanisms involved in cancer progression. It refers to the increased glucose uptake by cancer cells through aerobic glycolysis, instead of the Krebs cycle that takes place under normal conditions, followed by lactic acid fermentation. This mechanism is regulated by hypoxia-inducible factor-1 (HIF-1), a transcription factor that regulates the expression of genes responsible for the synthesis of proteins involved in glucose metabolism. Overexpression of HIF-1 has been linked to the Warburg effect. While several HIF-1-targeted strategies have been investigated, the majority have proven to be unsuccessful, especially in cases of aggressive tumors with hypoxic tumor microenvironments. Current strategies expand beyond conventional chemotherapeutic agents and include chemodynamic therapy, radiation therapy, and immune checkpoint molecules. The aim of this literature review is to highlight the implication of HIF-1 in the Warburg effect and the limitations that render cancer treatment less effective.

Keywords: HIF-1; Warburg effect; Cancer metabolism; Hypoxia; Angiogenesis; Drug resistance

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1. Introduction

1.1. Overview of Warburg effect

Malignancies pose a major public health threat, being ranked as the most burdensome condition as measured in disability-adjusted life years.[1] According to the American Cancer Society, the most prominent cancers in each gender are prostate cancer and breast cancer, with 268,490 and 287,850 cases, respectively. Lung cancer is also prevalent in both males and females, claiming the lives of 130,180 Americans and accounting for 18.85% of the total 690,360 cancer-related deaths.[2] According to the World Health Organization (WHO), the most prevalent cancer type in 2020 was breast cancer, followed by lung, colon, and rectal cancers. The cancers with the highest mortality rates were lung, colon, rectum cancer, and hepatocellular carcinoma.[3] Future projections indicate that, under current trends, cancer will remain a significant threat in the upcoming years.[4] According

to the literature, the majority of cancer types exhibit the Warburg effect, making it crucial to understand its nature and underlying mechanisms to develop novel treatments aimed at improving the overall survival and quality of life for cancer patients.

One of the most studied mechanisms in cancer progression is the Warburg effect. This term, named after the scientist Otto Warburg, describes the abnormal and unprecedented increase in glucose uptake by cancer cells, which is, in turn, utilized for glycolysis under aerobic conditions. Glycolysis is a significantly less efficient metabolic process in contrast to aerobic respiration but sustains energy for intensive metabolic processes.[5] However, when high adenosine triphosphate (ATP) levels are present, mitochondrial function is suppressed due to limited glycolysis.[6] These cancer cells may also trigger neighboring stromal cells to undergo aerobic glycolysis and transfer metabolites back to the originating cancer cells through a process known as the reverse Warburg effect.[7]

The metabolic reprogramming associated with the Warburg effect enables cancer cells to enhance their survival under unfavorable conditions, evade the immune system, and resist treatment. Metabolites, such as lactate, and the resulting acidic environment contribute to immune evasion, as analyzed below.[6] In addition, cancer cells can acquire chemoresistance through several mechanisms, such as the regulation of apoptosis, glucose uptake in glycolysis, stress response, and detoxification, particularly in cancer stem cells (CSCs).[8]

1.2. The human hypoxia-inducible factor (HIF) family and the link between HIF-1 and the Warburg Effect

The HIF family consists of transcription factors that regulate genes commonly associated with hypoxic conditions. These genes are involved in angiogenesis, cell growth and cycle, and cellular and systemic metabolism. The primary members of the HIF family are HIF-1α, HIF-2α, and HIF-3α, all of which heterodimerize with HIF-1β, as further analyzed in the subsequent sections. Each member has distinct roles and distribution. HIF-1 is the most widely distributed and plays a significant role in processes related to acute hypoxia, such as sugar metabolism, vascularization, and erythropoiesis. In contrast, HIF-2 and HIF-3 have more limited distributions and are located in specific tissues. HIF-2 is involved in chronic hypoxia, while HIF-3 acts as an antagonist of the other HIFs by competing for the HIF-1β binding site. Although this article primarily focuses on the role and function of HIF-1α in cancer, it is important to note that both HIF-2 and HIF-3 also significantly impact neoplastic diseases.[9-12]

HIF-1 is recognized as one of the most important proteins related to the Warburg effect. Structurally, HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits. The relatively unstable HIF-1α subunit is heavily regulated by oxygen levels; it is hydroxylated by oxygen-dependent prolyl hydroxylase domain proteins (PHDs), which triggers a sequence of reactions that lead to its degradation. In hypoxic environments, HIF-1α cannot be hydroxylated; thus, it is stabilized and transported to the nucleus, where it binds with HIF-1β subunits.[1] HIF-1β, commonly referred to as aryl hydrocarbon receptor nuclear translocators, is expressed constantly and independently of oxygen concentration.[13] Given the regulated expression of HIF-1α, our literature review focuses on this subunit.

Functionally, HIF-1 acts as a transcription factor and is one of the key moderators of genes related to hypoxia metabolic pathways, angiogenesis, inflammation, tumor development, and proliferation in physiologically unfavorable conditions.[14] Several metabolic pathways lead to the activation and/or upregulation of HIF-1 in cancer cells, such as the phosphoinositide 3-kinase (PI3K)/Akt/protein kinase C (PKC)/histone deacetylase (HDAC) pathway.[15] Once activated, the HIF-1 complex assembles and translocates to the cell nucleus to trigger Warburg-related pathways, as further analyzed in this literature review.

1.3. The significance of the HIF-1-dependent Warburg effect

Glucose is the main energy source for both normal and cancer cells. Normal cells metabolize glucose through glycolysis, followed by oxidative phosphorylation through the Krebs cycle, a process that occurs exclusively under limited oxygen supply in normal cells. However, the tumor microenvironment (TME) is hypoxic due to the inability of existing blood vessels to supply tumor cells with sufficient oxygen and nutrients. Consequently, cancer cells adapt by reprogramming their metabolism to favor glycolysis even in the presence of abundant oxygen, a phenomenon known as aerobic glycolysis or the Warburg effect.[16]

Many factors contribute to the metabolic reprogramming of cancer cells, such as oncogene activation, underexpression of tumor suppressor genes, and overexpression of growth factor receptor genes, occasionally combined with epigenetic changes. These changes result from irregular cell signaling.[16,17] Specifically, the overexpression of HIF-1, a transcription factor that regulates the expression of genes responsible for the synthesis of proteins partaking in glucose metabolism, contributes to the reprogramming of tumor cell metabolism from a state of oxidative phosphorylation to aerobic

glycolysis. HIF-1 enhances glucose transporters, mainly glucose transporters 1 and 4, and activates genes coding for glycolytic enzymes.[18] The HIF-1-dependent Warburg effect is critically important, as increased expression of HIF-1α is prevalent in solid tumors such as colon, prostate, lung, breast, and stomach cancer.[16] In addition, HIF-1 can induce angiogenesis when activated by lactate uptake in the cells and activate the pentose phosphate pathway (PPP). Specific biochemical and molecular mechanisms by which HIF-1 influences cancer cell metabolism, and behavior are analyzed in the subsequent subsections.

1.3.1. Effect of HIF-1 on angiogenesis

Cells produce and secrete lactate as the final product of lactic acid fermentation, which occurs after glycolysis. Cancer cells absorb lactate and convert it to pyruvate, thus increasing its intracellular concentration. Increased pyruvate levels lead to reduced HIF-1 degradation and, consequently, enhanced HIF-1 activity, promoting tumor angiogenesis.[15]

1.3.2. Effect of HIF-1 on the PPP

Pyruvate kinase M2 (PKM2) is a HIF-1-dependent glycolytic enzyme that regulates PPP. In comparison to other pyruvate kinase (PK) complexes, PKM2 is less effective at converting phosphoenolpyruvate to pyruvate. Therefore, glucose-6-phosphate, an intermediate glycolysis metabolite, accumulates and is supplied to PPP. This pathway produces pentoses, which are used as raw material to create nucleotides and NADPH. NADPH can then be utilized for the production of anti-oxidant glutathione-SH (GSH), thereby granting cancer cells antioxidant capabilities and resistance to radiotherapy.[15]

1.3.3. HIF-1-dependent reprogramming of the glucose metabolic pathway

For glycolysis to occur, glucose must be taken up by glucose transporters (GLUTs). HIF-1 regulates the expression of GLUT1 and, combined with the hypoxic environment of the tumor, enhances glucose uptake, thus reinforcing aerobic glycolysis. The pyruvate derived from glycolysis is downstream processed into lactate through lactic fermentation, instead of acetyl coenzyme A (CoA), with the help of lactate dehydrogenase A (LDH-A), whose expression is also affected by HIF-1. Lactic acid fermentation is essential for glycolysis as it produces NAD^+, a necessary coenzyme for a glycolytic reaction.[15]

1.3.4. HIF-1-induced decline in mitochondrial function

Activation of HIF-1 results in decreased acetyl-CoA levels since pyruvate partakes in aerobic glycolysis instead of pyruvate decarboxylation, which produces acetyl-CoA. HIF-1 also suppresses the enzyme responsible for pyruvate decarboxylation through a series of reactions. Consequently, there is insufficient acetyl-CoA to participate in the Krebs cycle within the mitochondria.[15] HIF-1, further, hinders mitochondrial functions through the elimination of proteins associated with mitochondrial activity. This impairment is achieved through the regulation of certain microRNAs, which targets and suppresses the expression of the mRNAs encoding these proteins. In addition, HIF-1 can affect the mitochondrial population by hindering the biogenesis of new organelles and initiating mitochondrial autophagy.[15] Figure 1 summarizes mitochondrial metabolic pathways associated with HIF-1.

1.3.5. HIF-1-induced overexpression of programmed death ligand-1 (PD-L1)

HIF-1 is responsible for the increased expression of PD-L1 on the surface of cancer cells. PDL-1 binds to its receptor, programmed cell death protein 1 (PD-1), which is expressed by T-cells. This interaction enables cancer cells to evade immune system surveillance and promotes cancer cell proliferation by hindering cytotoxic T-cell infiltration.[19]

2. Overview of HIF-1

2.1. Biological role and functions of HIF-1

Cancer progression is driven by complex intercellular signaling networks between tumor and stromal cells. The TME is specific in terms of its internal conditions, with hypoxia being one of the most common and contributing significantly to tumor aggressiveness.[20] Cells adapt to the hypoxic environment through a family of transcription factors known as HIFs.[21] One particular member with a well-established role in cancer progression is HIF-1.[22] This protein synchronizes the activities of many transcription factors and signaling molecules that collectively influence tumorigenesis.

HIF-1 is a heterodimer, consisting of an oxygen-regulated α subunit and an oxygen-independent β subunit (also called aryl hydrocarbon receptor nuclear translocator). It binds to the promoter regions of target genes containing hypoxia-responsive elements (HREs; 5'-RCGTG-3', where R = A or G), further stimulating the expression of different genes.[23] Together with its partners, HIF-1 is involved in various cancer hallmarks, such as angiogenesis, migration, invasion, generation of CSCs, pH regulation, and glucose metabolism.[24]

Moreover, HIF-1 plays an essential role in the inflammatory process of immune cells. During extravasation from the bloodstream to the inflammation site, immune cells are suddenly exposed to hypoxic

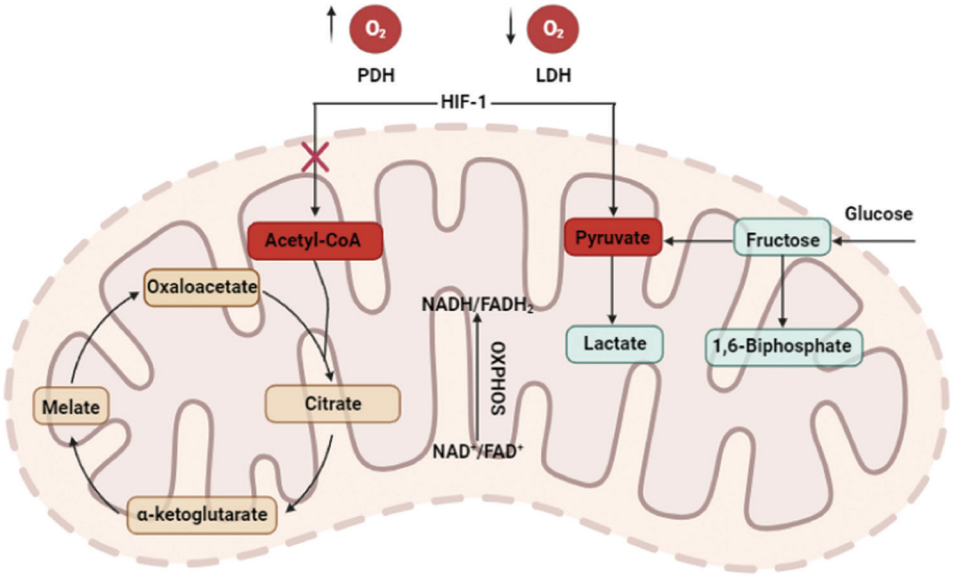

Figure 1. Overview of hypoxia-inducible factor-1 targeting aerobic and anaerobic mitochondrial pathways
Abbreviations: CoA: Coenzyme A; $FADH_2$: Flavin adenine dinucleotide; LDH: Lactate dehydrogenase; NADPH: Nicotinamide adenine dinucleotide phosphate; OXPHOS: Oxidative phosphorylation; PDH: Pyruvate dehydrogenase.

conditions, activating HIF-1.[25] The importance of HIF-1 is illustrated by the fact that its deletion in myeloid cells reduces cellular ATP levels, leading to dysfunctions in aggregation, motility, antibacterial activity, and survival.[26] In addition, HIF-1 supports the maturation of dendritic cells, regulates lymphatic regeneration during wound repair, and can exacerbate experimental colitis with its influence on the macrophage migration inhibitory factor.[27]

During glycolysis, glucose is converted to pyruvate and ATP through a series of oxygen-independent enzymatic reactions. GLUT1 is one of 12 glucose transporters whose expression is increased by HIF-1, leading to a greater glucose uptake. Subsequently, pyruvate is preferentially metabolized to lactate through lactic acid fermentation, mediated by LDH-A, an enzyme highly expressed in cancer cells in a HIF-1-dependent manner.[15] This metabolic pathway in tumors, known as the Warburg effect, operates at the expense of a suppressed mitochondrial function through several mechanisms involving HIF-1. Inactivation of the Krebs cycle is followed by reduced acetyl-CoA levels,[28] reduced protein levels necessary for mitochondrial function,[29] and a reduced number of mitochondria due to inhibited biogenesis and increased autophagy, as illustrated in Figure 2.[30] Altogether, these metabolic effects provide cell protection against oxidative stress and may operate as a selective advantage during metastasis.[31]

The molecular mechanisms underlying breast cancer cell metastasis to specific organs and tissues, such as bones and lungs, remain to be clarified. However, it has been shown that increased HIF-1 expression in cases of primary and metastatic breast cancer is linked to disease progression with severe cases of metastasis.[32] This finding is based on the HIF-1-assisted expression of lysyl oxidase (LOX) 2 and 4 in hypoxic breast cancer cells within primary breast tumors.[33] LOX causes extracellular matrix remodeling, aiding in the formation of pre-metastatic niches at distant sites such as the lungs.[34] Breast cancer and stromal cells produce different growth factors and cytokines, including stromal cell-derived factor 1, transforming growth factor-β1 (TGF-β1), and bone morphogenetic proteins. Combined with increased expression of HIF-1, nuclear factor-kappa B (NF-κB), vascular cell adhesion molecule-1, and Notch, these factors assist in the development of bone metastasis.[35]

Pancreatic ductal adenocarcinomas (PDACs) are considerably hypoxic solid tumors, containing dense stromal fibrosis with poor vascularization. The overexpression of HIF-1 in PDACs appears crucial for the adaptation of pancreatic cancer cells and stromal cells to hypoxic conditions, contributing to invasiveness, metastasis formation, and treatment resistance.[36] Analyses of HIF-1 expression levels in 48 pancreatic cancer tissues from patients on adjuvant gemcitabine treatment after pancreatectomy have correlated HIF-1 expression with increased microvascularization and gemcitabine resistance.[37] Patients with intense HIF-1 expression had sooner disease recurrence compared to those with weak HIF-1 expression.[37]

The heterogeneous involvement of HIF-1 in various cancer stages and in the regulation of the inflammatory

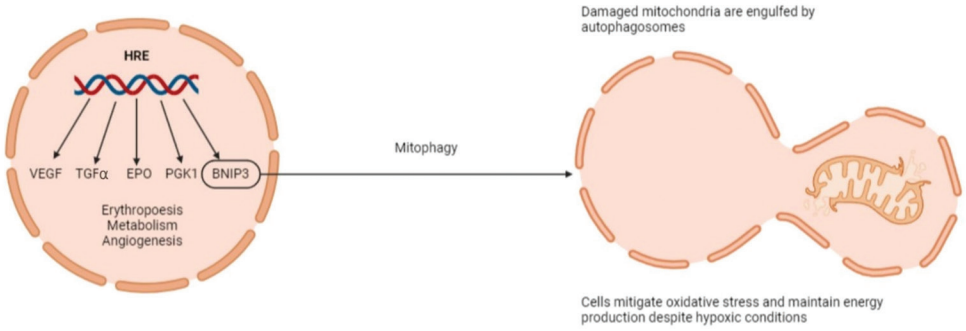

Figure 2. Under hypoxic conditions, hypoxia-inducible factor-1 (HIF-1) translocates to the nucleus, where it targets genes encoding vascular endothelial growth factor (VEGF), transforming growth factor-alpha (TGF-α), erythropoietin (EPO), phosphoglycerate kinase 1 (PGK1), and BCL2 interacting protein 3 (BNIP3). These genes affect erythropoiesis, angiogenesis, and metabolism through the hypoxia response element (HRE). The expression of BNIP3 induces mitophagy, where mitochondria are engulfed by autophagosomes, mitigating stress and maintaining energy production despite hypoxic conditions.

response highlights the need for further research, especially in mitigating cancer resistance and developing effective anticancer therapies.

2.2. HIF-1 interactions

Due to the rapid growth of solid tumors, oxygen and nutrients are often depleted, leading to the development of hypoxia and necrosis. This depletion triggers the production of proinflammatory mediators, recruitment of immune cells, tumor cell proliferation, angiogenesis, and metastasis.[38]

HIF-1 contributes to the maintenance of hypoxia and inflammation through the regulation of gene expression of c-Jun and AP-1 transcription factors. This regulation provides an additional mechanism for tumor growth, which involves the induction of vascular endothelial growth factor (VEGF), tyrosine hydroxylase, and endothelin-1 in hypoxic areas.[39] NF-κB and signal transducer and activator of transcription 3 (STAT3) are transcription factors that contribute to inflammatory signaling.[40,41] Inhibitory IκB proteins dissociate from NF-κB, which, further, translocates to the nucleus and activates pro-survival genes such as *BCL2*, *CXCR1*, and *CXCR2*, as well as tumor-promoting genes encoding interleukin-6, cyclooxygenase 2, inducible nitric oxide synthase, platelet endothelial cell adhesion molecule-1, and matrix metalloproteinase-9 (MMP-9), as part of an orchestrated response to the inflammatory process, as illustrated in Figure 3.[40,42] The interaction between HIF-1 and STAT3 regulates the activity of MCL1, c-Myc, cyclin D1, MMP-2, and VEGF, which are responsible for survival, proliferation, invasion, and angiogenesis, respectively.[43] STAT3 also physically interacts with HIF-1α, which is vital for the activation of HIF-1 target genes under hypoxic conditions, as illustrated in MDA-MB-231 human breast cancer and RCC4 renal carcinoma cells.[44] Significantly, the HIF-1/STAT3 interaction is responsible for the development of immunoresistance in lung cancer cells.[45]

Toll-like receptor 4 (TLR4) is a member of the pattern recognition receptor family of proteins and an HIF-1 target. Its interaction with HIF-1 contributes to the inflammatory process in glioblastoma tumorigenesis[46] and leads to the development of pancreatic adenocarcinoma.[47]

The preservation of the Warburg effect heavily depends on the expression and activity of several glycolytic enzymes activated by HIF-1. Hexokinases, aldolases, 6-phosphofructokinase liver type, enolase alpha, PKM2, and LDH-A interact with HIF-1 to ensure uninterrupted energy production and macromolecular biosynthesis in malignant cells.[48] Collaboration between the oncogenic MYC transcription factor, a mammalian target of rapamycin, and HIF-1 results in a high glycolytic flux. As a consequence of enhanced glycolysis, there is an increase in lactate and H^+ levels, leading to an acidic TME. The resulting acidic TME, in turn, attenuates the antitumor immunity and reduces drug uptake by tumors.[49] Moreover, the hypoxic conditions in pancreatic cancer stimulate HIF-1-dependent tumor–stromal interactions, creating a fibroinflammatory microenvironment with decreased perfusion and limited cancer drug delivery.[50]

One study highlighted the important role of the HIF-1/carbonic anhydrase IX system in the production of soluble mediators, such as granulocyte colony-stimulating factor (G-CSF), which are necessary for the recruitment of myeloid-derived suppressor cells to the lungs, triggering the generation of premetastatic niches.[51]

The process in which epithelial cells lose their polarity and switch to an invasive mesenchymal phenotype, known as epithelial-to-mesenchymal transition (EMT), significantly impacts tumor metastasis.[52] HIF-1 and TGF-β mutually enhance each other's expression and provoke

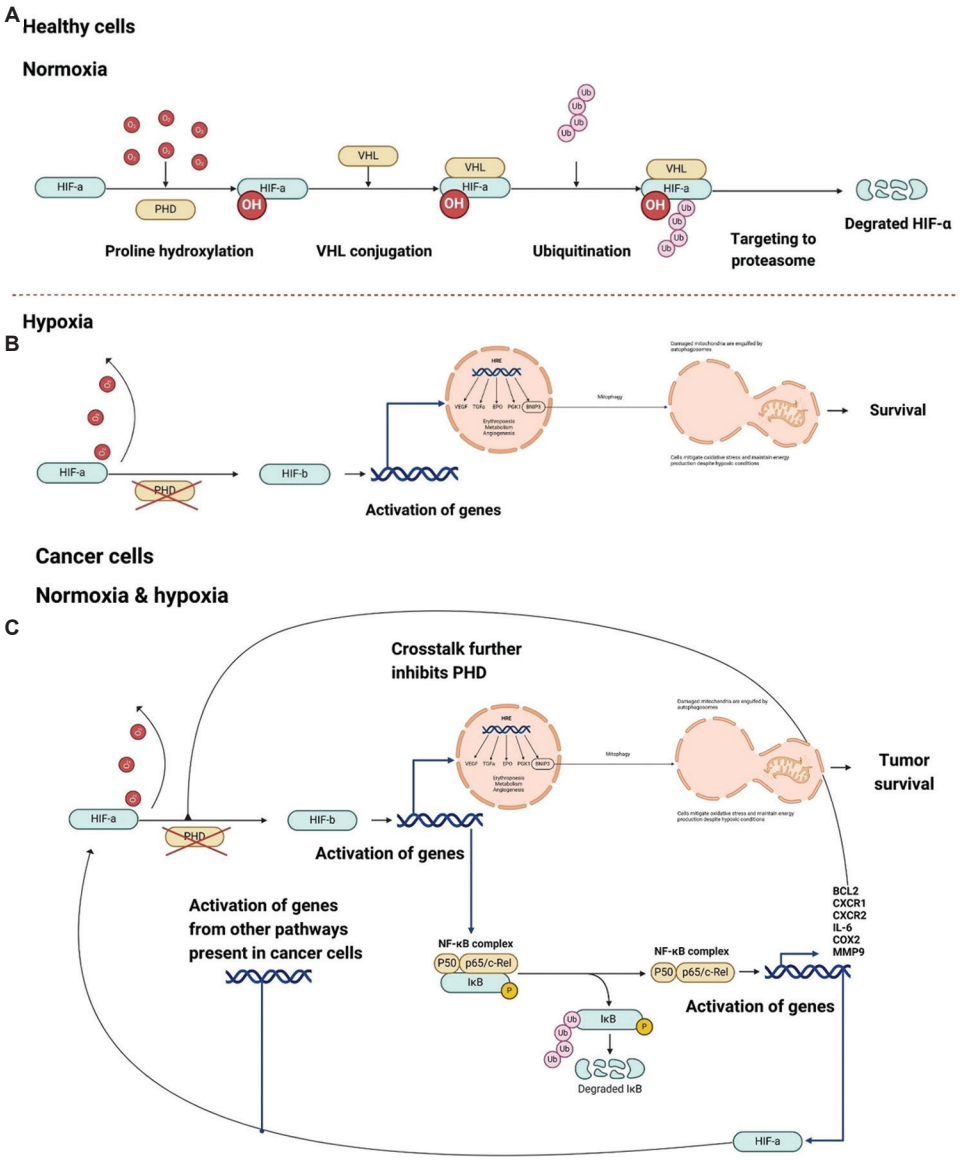

Figure 3. Summary of hypoxia-inducible factor-1 interactions. (A) Normoxia in healthy cells: Under normoxic conditions, prolyl hydroxylase domain protein (PHD) induces hydroxylation of HIF-α, leading to its subsequent ubiquitination. (B) Hypoxia in healthy cells: Under hypoxic conditions, hydroxylation and ubiquitination of HIF-α do not occur. Instead, HIF-α translocates to the nucleus, where it targets genes through the hypoxia response element (HRE), affecting erythropoiesis, angiogenesis, and metabolism, ultimately leading to cell survival. (C) Normoxia and hypoxia in cancer cells: both under normoxic and hypoxic conditions in cancer cells, inhibitory IκB proteins dissociate from the NF-κB complex, leading to activation of pro-survival genes such as *BCL2*, *CXCR1*, and *CXCR2*, as well as tumor-promoting genes including *IL-6*, *COX-2*, and *MMP-9*. This process inhibits PHD activity, thereby promoting cancer cell survival.
Abbreviations: NF-κB: Nuclear factor-kappa B; *IL-6*: Interleukin 6; *COX-2:* Cyclooxygenase-2*MMP-9*: Matrix metalloproteinase-9.

EMT in many cancer cell types.[53] Studies also demonstrated the important role of the Wnt/β-catenin signaling in HIF-1-induced EMT in human prostate[54] and hepatocellular carcinoma cell lines.[55]

In addition, it has been discovered that overexpression in combination with activation of Rho GTPases, including Cdc42, RhoC, RhoA, and Rac1, dramatically enhances EMT and, consequently, the invasiveness and the metastatic capabilities of solid tumors such as pancreatic adenocarcinoma, liver, ovarian, and breast cancers. This enhancement of EMT and invasiveness appears to be reasonable since RhoA plays a crucial role in several key events of the cell cycle, such as cytokinesis, and existing data on the physiology, pathology, and alteration of related

pathways support this observation.[56,57] Significant metrics include the presence and levels of RhoA and RhoC and their relationship during the cell cycle and the lifecycle of the neoplasm. Complementary to the above stands, HIF-1α alters the architecture of the cytoskeleton through the regulation of Rho kinase, which, in turn, further stabilizes HIF-1α in a positive feedback loop, as evidenced by the accumulation of the latter.[57,58] Concerning blood supply, HIF-1α reorganizes the cytoskeletal structure of endothelial, parenchymal, and immune cells, resulting in morphologies that favor intracellular adhesion and increase vascularization, which leads to proliferation and higher metastatic potential.[59] Considering the above, it is unsurprising that elevated RhoC levels serve as an unfavorable prognostic factor.[57]

Another aspect of EMT is the emergence of stem-like characteristics in a subset of cancer cells, which promotes tumorigenesis, facilitates metastasis, and leads to poor treatment outcomes.[60] HIF-1, as a mediator in EMT, increases the population of stem-like cells in thyroid[61] and prostate cancer,[62] and plays a key role in promoting mammary tumor growth and metastasis.[63]

Pancreatic cancer metastasis is facilitated by EMT, and one particular study examined the influence of intermittent hypoxia on the invasiveness of human pancreatic cancer cell lines (Panc-1 and BxPC-3). Western blotting and flow cytometry analysis were used to quantify stem-like cells in the migratory cells during intermittent hypoxia in these human pancreatic cancer cells. Under normoxia or intermittent hypoxia, the expression of autophagy-related proteins (LC3-II and Beclin), HIF-1, and EMT-related markers (E-cadherin, vimentin, and N-cadherin) was examined using Western blotting. The study showed that under intermittent hypoxia, pancreatic cancer cells demonstrated enhanced invasiveness and enriched stem-like cells. Increased levels of HIF-1 upregulated autophagy and both were associated with the metastatic ability and EMT of pancreatic CSCs.[64]

Chronic hypoxia raises the levels of intracellular reactive oxygen species (ROS), which are crucial for tumorigenesis and metastasis.[65] ROS stabilize HIF-1 through a redox-mediated inhibition of its proteolysis and triggers its downstream pathways.[66] The signaling cascade ROS/STAT3/HIF-1α/TWIST1/N-cadherin is implicated in prostate cancer progression.[67] In addition, ROS and HIF-1 induce resistance to doxorubicin and etoposide in lung, cervical carcinoma, and melanoma cell lines.[68]

As a key contributor in cell adaptation to hypoxia, HIF-1 influences target gene transcription through RNA polymerase II (Pol II), but this enzyme stalls after transcribing approximately 30 – 60 nucleotides and requires HIF-1 binding for its release. One particular study on breast cancer cells used ChIP-qPCR and RNA-sequencing to demonstrate that HIF-1 mobilizes TRIM28 and DNA-dependent protein kinase (DNA-PK) to HREs to release the stalled Pol II and enable effective transcription. The increased expression of HIF-1, DNA-PKcs, or TRIM28 in different types of human cancer is associated with increased patient mortality.[69] Thus, inhibition of HIF-dependent transcription may partially contribute to the anticancer effects of drugs targeting DNA-PK in breast cancer.

A recent study demonstrated that hypoxia-inducible gene expression in estrogen receptor (ER)-positive MCF7 and ER-negative SUM159 human breast cancer cells requires the presence of the histone H2A/H2B chaperone facilitates chromatin transcription (FACT), and the H2B ubiquitin ligase RING finger protein 20/40 (RNF20/40). Hypoxic conditions provoke the accumulation of HIF-1α, its translocation to the nucleus, subsequent heterodimerization with HIF-1β, binding to HREs in target genes, and recruitment of FACT and RNF20/40. The creation of this HIF-1-FACT-RNF20/40 multiprotein complex produces several consequences. Due to protein-protein interactions, each protein contributes to a stabilized occupancy of the HRE by the other two. H2B-K120 ubiquitination by RNF20/40 facilitates the FACT-dependent displacement of an H2A-H2B dimer from nucleosomes, promoting the release of stalled RNA Pol II and enhancing transcriptional elongation. RNA-sequencing data showed that FACT and RNF20/40 are required to regulate the expression of virtually all hypoxia-responsive RNAs in MCF7 breast cancer cells, providing the basis for increased rates of transcription initiation and elongation.[70] HIF-1, FACT, and RNF20/40 are all elevated in breast cancer and are linked with increased patient mortality.[71] Therefore, suppressing their activity could be a potential therapeutic approach in cases of triple-negative breast cancers, for which targeted therapy is currently unavailable.

2.3. Factors regulating HIF-1

During normoxia, specific proline residues of HIF-1α, namely, P402 and P564, are hydroxylated by PHDs. This modification causes the ubiquitination of HIF-1α by the von Hippel-Lindau (VHL)-containing E3 ubiquitin ligase and subsequent HIF-1α degradation. Under hypoxic conditions, these oxygen-dependent hydroxylases become inactive, allowing the formation of HIF-1 heterodimer through the interaction between HIF-1α and HIF-1β.[72] This heterodimer binds to HRE, triggering the expression of many genes, including *VEGF* and platelet-derived growth factor-B (*PDGFB*). Clinical research has shown a

correlation between HIF-1 and different cancer features such as angiogenesis, metabolic reprogramming, invasion, metastasis, and poor patient outcomes. Therefore, targeting HIF-1 and its upstream regulators is considered a valuable strategy for cancer therapy.[73]

Apart from hypoxia, different factors may influence HIF-1 overexpression, including insulin, insulin-like growth factor, v-Src, lactate, pyruvate, ROS levels, and genetic alterations such as activation of oncogene signaling or inactivation of tumor suppressor genes.[74] There is growing evidence that numerous microRNAs are involved in HIF-1-mediated regulation of the Warburg effect.[15] One example is miR-31-5p, a microRNA upregulated in lung adenocarcinoma, which has been found to amplify the Warburg effect and encourage cell proliferation by inhibiting FIH-1 and enhancing HIF-1 activity.[75] Another example is miR-150, which is abnormally expressed in various cancers. This microRNA targets and inhibits the pVHL tumor suppressor gene, the E3 ligase responsible for HIF-1 degradation, in glioma cells. In the presence of miR-150, HIF-1 is stabilized, leading to increased glucose uptake, lactate production, and cell proliferation.[76]

Specific genetic screening experiments have identified several novel activators of HIF-1, including ubiquitin C-terminal hydrolase-L1 (UCHL1),[77] isocitrate dehydrogenase 3α (IDH3α),[78] and lymphocyte antigen 6 locus E (LY6E).[64] *In vitro* experiments have shown that UCHL1 stabilizes and stimulates HIF-1 through its deubiquitination activity.[77]

The expression levels of UCHL1 correlated well with those of HIF-1, and UCHL1 expression levels in tumors were associated with poor prognosis in both breast and lung cancer patients. It is of note, however, that the influence of UCHL1 on HIF-1 expression was higher in clinical tumor tissues compared to cancer cells cultured under low-oxygen conditions. The particular reason for this difference is currently unclear, but it is possible that other conditions besides hypoxia, including glucose or oxygen deficiency, may influence the activity of the UCHL1–HIF-1 axis, as demonstrated by the *in vitro* experiments. Specifically, under hypoxic conditions and decreased glucose concentrations, the impact of UCHL1 on HIF-1 stability was expedited.[77] Following UCHL1 overexpression, quantitative analysis of intermediary metabolites showed that these genes induced a switch in the glucose metabolic pathway from oxidative phosphorylation to aerobic glycolysis. In parallel, the PPP was activated, allowing increased production of NADPH and reduced GSH. The elevated GSH levels due to an enhanced UHCL1 expression promoted the radioresistance of cancer cells, according to *in vitro* colony formation assays.[79] Numerous studies have implicated UCHL1 in tumorigenesis, metastasis, and invasiveness of cancers, providing a rationale for using it as a prognostic marker and treatment target for cancers. To do so, the following aspects must be taken into consideration: the influence of UCHL1 on HIF-2α, the detection of downstream substrates by UCHL1, the expression levels of UCHL1 in normal tissues, and the impact of epigenetic alterations.[77]

The abnormal expression of IDH3α, a subunit of the IDH3 heterotetramer, has been shown to intensify HIF-1 stability and activity, as opposed to the decrease of α-ketoglutarate levels in cancer cells. On the other hand, silencing of IDH3α interrupted the Warburg effect and angiogenesis, leading to significantly slower tumor growth. In lung and breast cancer patients, IDH3α expression was associated with poor post-operative OS.[78]

In vitro studies suggest that *LY6E* overexpression enhanced the transcription of HIF-1, which subsequently increases the expression of proangiogenic factors, VEGF and PDGFB. This effect occurs through the decreased levels of both endogenously and exogenously expressed *PTEN* mRNA and activation of the PI3K/Akt pathway. The expression levels of *LY6E* were significantly higher in human breast cancers in comparison with normal breast tissues, contributing to poor prognoses in patients with lung, bladder, brain, and skin cancers. These results justify the use of LY6E as a prognostic marker as well as a therapeutic target for cancers. Importantly, the basal expression levels of *LY6E* were shown to be high in normal T-cells, which should be carefully considered when developing LY6E-targeted cancer therapies.[80]

Histone deacetylase inhibitors (HDAIs) have shown promising anticancer and antiangiogenic potential in clinical trials. HDAIs have been demonstrated to possess destabilizing and repressive effects on HIF-1 function, an essential pharmacological mechanism supporting their ability to suppress tumor growth and angiogenesis. However, it must be taken into consideration that HDAIs may cause significant adverse effects due to off-target hyperacetylation.[79]

Hypoxia-associated factor (HAF) is an E3 ligase that regulates the stability and functionality of HIF-1. Elevated HAF levels in tumor cells compared to normal cells under similar conditions demonstrate its effect on cancer progression. In addition, HAF operates as a neoplastic antigen, also known as squamous cell carcinoma antigen recognized by T-cells (SART1800), indicating tumor aggressiveness and poor prognosis.[81] HAF has been reported to bind to HIF-1 and trigger its degradation in an oxygen-independent manner. Although this action might be expected to inhibit tumor proliferation and expansion, it

is considered a double-edged sword. Some reports indicate that HAF-mediated degradation of HIF-1α can limit tumor growth. However, the increased ratio of HIF-2α to HIF-1α, along with the upregulation of HIF-2α by HAF, leads to significantly less favorable outcomes for the patient. These adverse outcomes are due to enhanced migration and invasion through the activation of different sets of genes, as demonstrated in T24 cells.[82] Consequently, an expanded stem cell population, enhanced tumor development and invasion, increased progression, and morphological and biochemical changes that favor proliferation are expected. Complimenting these effects is the activation of the NF-κB pathway by interfering with the degradation of the IκB molecule, which inhibits the NF-κB, as well as the induction of *VEGF* transcription.[83]

3. Challenges and limitations of HIF-1 integration in clinical practice

The hypoxic conditions of the TME increase the potency of the neoplasm by rendering several therapeutic approaches ineffective and promoting the survival of the fittest and most aggressive cells in the tumor population.[84] Granted the significance of HIF-1 in the survivability of cancer cells in the hypoxic TME and its multifactorial function, several therapeutic approaches involving the dimer have been investigated and implemented. These interventions include the use of substances that regulate the HIF-1 activity, such as flavonoids,[85] and molecules that act as inhibitors at various stages of the protein's lifecycle. These stages include mRNA expression, protein translation, protein degradation, DNA binding, and transcriptional activity.[86] Novel therapies, such as gene therapy, have also been explored.[87] It is evident that HIF-1 plays a significant role in situations where cancer becomes more aggressive and clinically challenging. The impact is observed across the therapeutic spectrum, as indicated by elevated HIF-1α serum levels in a study from Brazil.[88] It shall be noted that a significant majority of the supporting results are derived from *in vivo* and *in vitro* studies.

3.1. Chemotherapy

Beyond radioresistance, HIF-1 has been established as a trigger for pathways involved in the resistance of cancer cells to chemotherapeutic agents. This etiology is related to the mechanism of radioresistance, as HIF-1 protects cancer cells from damage caused by these agents.[89] In addition, the relatively limited blood flow, which results in hypoxia and thereby initiates the action of the dimer, poses a challenge to the efficient and effective delivery of chemotherapeutic molecules. The following are examples of the effects of HIF-1 on the action of some of the most widely used small oncotherapeutic molecules.

3.1.1. Cisplatin

The efficacy of cisplatin, one of the most well-known small molecules used against neoplasms, appears to be influenced by hypoxic conditions. In fact, the effectiveness of cisplatin was hindered under hypoxic conditions, particularly when investigated in HepG2/DDP-resistant cells compared to HepG2.[90] New drug delivery systems for cisplatin are being investigated and developed to address issues such as limited access to the tumor site and the elevated production of ROS associated with the action of this molecule and other obstacles.[87,91]

3.1.2. Docetaxel

Docetaxel is an interesting case as it can limit the expression and accumulation of HIF-1a, resulting in improved clinical outcomes.[92] In addition, due to its effectiveness on cancer cells even under hypoxic conditions, docetaxel is used as an alternative to paclitaxel when the latter demonstrates no significant effect.[93] Combining docetaxel therapy with nitroglycerin to increase blood flow to the tumor site is controversial, as there are reports with contradicting results and suggestions, although positive outcomes slightly outnumber negative ones.[94-96] HIF-1 activity may also reduce the effectiveness of docetaxel, as observed in the study by Li *et al.*[97] on triple-negative breast cancer.

3.1.3. Doxorubicin

A bilateral relationship between doxorubicin and HIF-1 has been observed, with HIF-1 inducing resistance to doxorubicin,[98] which, in turn, promotes autophagy under hypoxic conditions.[99] In addition, doxorubicin, when assessed *in vitro* and *in vivo* through animal models, was found to inhibit the expression of HIF-1 and related genes.[100] However, significant cardiovascular damage, one of the most prevalent side effects of doxorubicin, should be taken into consideration.[101] To combat the effects of HIF-1, coencapsulation with inhibitors has been suggested.[102]

3.1.4. Gemcitabine

Acquired resistance to gemcitabine is induced through increased catalase presence induced through the HIF-1/ABCB6 pathway, resulting in decreased levels of ROS.[103] Hypoxic conditions have also been reported to be related to higher chemoresistance and metastatic ability in cholangiocarcinoma,[104] and the inhibition of HIF-1 increases the effectiveness of therapy for PDACs.[105] However, another study indicates that hypoxia does not influence the cytotoxic and radio-sensitizing effect of gemcitabine and its metabolites.[106] Novel drug delivery solutions, such as the use of vectors, can assist in more targeted and effective treatment using gemcitabine.[107]

3.1.5. Chemodynamic therapy

Chemodynamic therapy relies on hydrogen peroxide for the production of ROS, which induces oxidative stress and cancer cell death.[108] HIF-1 has a protective effect against radicals through the expression of GSH, which, in turn, inhibits oxidation and the production of ROS.[109] In chemodynamic therapy, catalytic molecules can be combined with anti-HIF factors in a common carrier, such as liposomes or other delivery systems.[108-110]

3.2. Radiation therapy

As mentioned earlier, HIF-1 can be activated by methods under the umbrella of radiation therapy due to the generation of ROS and concurrent vascular damage. These conditions physiologically mimic a state of hypoxia, thus activating HIF-1α and conferring new properties to the neoplastic cells.[111] For instance, acquired radioresistance of cancer cells is linked to HIF-1-induced hypoxia,[112] which leads to limited production of ROS and DNA damage that can be easily countered by the oxidation of free sulfhydryl groups on biomolecules such as proteins.[113] The effects of the dimer are evident in various *in vitro* studies. Experiments conducted on the MS5 cell line of mouse mesenchymal stromal cells cultured under hypoxic conditions have demonstrated that these cells have increased genetic stability and, thus, survivability when exposed to ionizing radiation.[114] In terms of *in vivo* studies, relevant neoplasms include prostate and cervical cancer, glioblastoma, squamous cell carcinoma, and osteosarcoma.[115] It is noted that post-radiation exposure can help prostate cancer cells evade apoptosis and maintain stemness, as observed in squamous cell carcinoma cells.[116,117] HIF-1 plays a crucial role in the progression of glioblastoma along with radioresistance due to lactate accumulation.[118] Osteosarcoma is particularly interesting because, in addition to the mechanisms mentioned above, hypoxia-induced autophagy contributes to its high potency and radioresistance.[119] Overcoming radioresistance is a promising venture, as several molecules have been reported to increase radiosensitivity by targeting HIF-1. For instance, Guo *et al.*[120] observed that baicalein, among other compounds, intercepts pathways that lower radioresistance in KYSE150 squamous cell carcinoma cell lines. Another promising finding is the significant damage observed in HeLa cervical cancer cell lines when placed in a low-oxygen, low-glucose environment, implying lower radioresistance.[121]

3.3. Immunotherapy

Immunotherapy is a broad term encompassing some of the most recent and effective strategies against cancer. As this therapeutic approach is based on the immune system, its effectiveness is largely influenced by the capabilities and limitations of the immune response. A characteristic example is glioblastoma, which presents challenges due to the complexity of its environment, limited access, and heterogeneity.[122]

3.3.1. TME

The TME is immunosuppressive and, in general, hostile toward somatic and immune cells, primarily due to its hypoxic state driven by HIF-1. Therefore, interference with HIF-1 formation and function can make the tumor more susceptible to immune system attacks.[123] An effective strategy may involve the oxygenation of the tumor to counteract the effects of the dimer, thus enhancing immune response.[124] In addition, T-cells are disadvantaged in hypoxic conditions due to metabolic stress,[125] and acidosis reduces the formation and secretion of interferon-γ and tumor necrosis factor-α.[109]

3.3.2. Checkpoint molecules

HIF-1 promotes the expression of immunosuppressive factors and immune checkpoint molecules such as PD-L1, further dampening the immune response and reducing the chance of success for the immunotherapeutic interventions.[126,127] The impact of this immunosuppressive mechanism is evident in the results of Bailey *et al.*,[128] which reported that anti-CTLA-4 therapy is optimized when combined with anti-HIF-1 therapy, yielding results comparable to those of PD-1/PD-L1 coupling in rodent models. Therefore, the optimal strategy would be to couple PD-L1 inhibitors with anti-HIF-1 factors. HIF-1 also promotes the expression of CD47, human leukocyte antigen-G checkpoint molecule, and V-domain immunoglobin, all of which enhance the immune evasive characteristics of neoplastic cells in a hypoxic environment.[129-131] In addition, two HIF-1 subunit inhibitors have recently been approved by the United States Food and Drug Administration: daprodustat (Jesduvroq, GSK), approved in 2023 for treating anemia in adults with chronic kidney disease on dialysis,[132-134] and belzutifan (Welireg, Merck), approved in 2021 for adult patients with VHL disease requiring therapy for associated renal cell carcinoma, central nervous system hemangioblastomas, or pancreatic neuroendocrine tumors that do not require immediate surgery.[135,136]

3.3.3. Immune cell interaction and cytotoxic activity

In addition to the expression of PD-L1 on dendritic cells, which inhibits T-cells, HF-1 also increases adenosine levels in the tumor, which negatively affect CD8 T lymphocytes, and the resulting lactate accumulation from the hypoxic conditions promotes M2 macrophage polarization,[137]

favoring further tumor development.[138] HIF-1 activity also hampers the formation of MHC-I molecules necessary for proper cancer-related antigen presentation, thus assisting in immune evasion. Furthermore, HIF-1 mediates the formation of myeloid-derived suppressor cells and tumor-associated macrophages,[125] enhancing immune suppression.[139] In addition, the phenomenon of induced autophagy, as mentioned in **Sections 3.3.1.3 and 3.3.2**, presents an obstacle to cell-mediated death by natural killer cells and cytotoxic T-cells.[140]

4. Conclusion

HIF-1, a major factor in the Warburg effect, plays a significant role in the development and outcome of neoplastic diseases. The significance of HIF-1 is evident from the numerous interactions that it has with molecules associated with tumor formation, progression, and treatment resistance. Therefore, further investigation is proposed to gain a better understanding of these interactions, which will facilitate the development of more effective disease prevention and treatment strategies.

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

REVIEW ARTICLE

Tiny messengers, big results: A review of exosome-mediated treatments and considerations in dermatology

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Abstract

Exosomes are small extracellular vesicles that play an important role in intercellular communication by transporting proteins, lipids, and nucleic acids between cells. They have emerged as relevant research areas due to their involvement in regulating various physiological and pathological processes. This review explores the potential applications of exosome-based therapies in dermatology and examines the safety aspects of these treatments. Past research has demonstrated that exosomes may effectively treat conditions such as alopecia and accelerate wound healing by stimulating hair follicle growth and enhancing tissue regeneration. Studies have shown that exosomes can promote the proliferation of dermal papilla cells and hair follicle growth in cases of alopecia. They also accelerate wound healing by modulating processes involved in inflammation, cell migration, and tissue remodeling. However, more research is needed to fully characterize the long-term safety profile of exosomes and establish standardized clinical protocols. Both human-derived and plant-derived exosomes appear to have favorable safety profiles based on current evidence, though plant sources may offer advantages in terms of production and biocompatibility. Continued exploration of exosomes' mechanisms and potential risks will optimize these innovations and offer safe, effective exosome treatments to patients. While further research is warranted, current findings provide valuable insights into the applications of exosome therapy for dermatological conditions and its emerging role in precision medicine.

Keywords: Exosomes; Alopecia; Wound healing; Tissue regeneration; Regenerative medicine

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1. Introduction

1.1. Defining exosomes and their modes of action

Exosomes, which are small extracellular vesicles, have become a focal point of scientific exploration due to their importance in intercellular communication.[1] These minuscule entities, secreted by a variety of cell types, play a crucial role in orchestrating

the exchange of information among cells. What makes exosomes particularly intriguing is their composition, which includes a diverse array of bioactive molecules such as proteins, lipids, and nucleic acids.

The key function of exosomes lies in their capacity to serve as messengers, facilitating the intricate transfer of their molecular cargo from one cell to another. This process is integral to influencing cellular processes and contributes significantly to the regulation of various physiological and pathological conditions. Essentially, exosomes act as specialized vehicles that deliver specific payloads to recipient cells in a targeted manner, as shown in Figure 1.[1]

By transferring these bioactive molecules, exosomes actively participate in modulating cellular behavior, signaling pathways, and gene expression. This targeted delivery system enables them to influence a wide range of cellular activities, contributing to the finely tuned regulation of biological processes. As a result, exosomes have emerged as essential mediators in maintaining homeostasis and responding to changes in the cellular microenvironment.

Understanding the mechanisms by which exosomes operate facilitates their potential therapeutic applications. Researchers are exploring how manipulating exosomal content or utilizing their natural ability to transport specific molecules could be harnessed to develop innovative treatments for various diseases. The growing field of exosome research holds promise for advancing our understanding of intercellular communication and potentially revolutionizing approaches to medical interventions in the future.

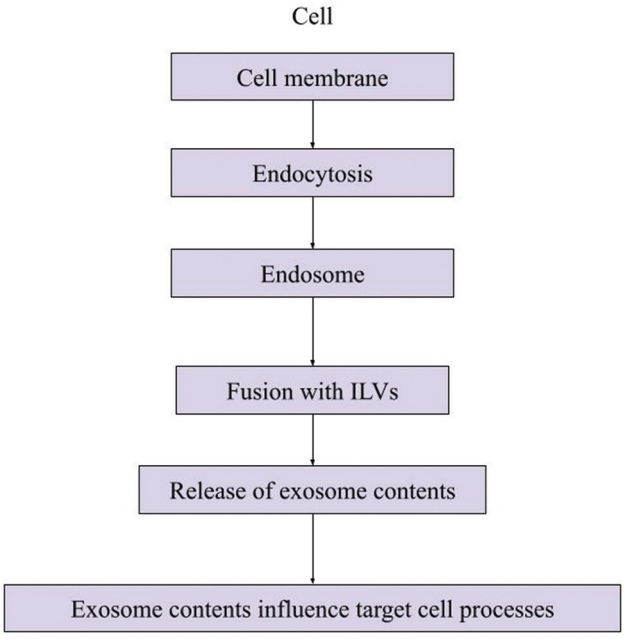

Figure 1. Mode of action of exosomes inside the cell
Abbreviation: ILVs: Intraluminal vesicles.

1.2. Exosomes in dermatological treatments

The dynamic role of exosomes in dermatological treatments has proven effective in therapeutic approaches within the field of skincare. These tiny extracellular vesicles showcase immense potential due to their inherent capability to transport crucial signaling molecules, which can profoundly influence cellular behavior, tissue regeneration, and repair processes.

In the context of addressing dermatological conditions, researchers and clinicians are actively exploring the versatile applications of exosomes.[1] One prominent area of focus is in combating the effects of skin aging. Exosomes play a pivotal role in this regard by promoting the production of collagen, a fundamental protein that contributes to skin elasticity and firmness. In addition, exosomes help mitigate oxidative stress, a significant factor in aging, thereby rejuvenating aging skin cells and contributing to an overall improvement in skin texture. The use of exosomes in anti-aging treatments represents a promising avenue for promoting skin health and combating the visible signs of aging.[2,3]

Furthermore, in the realm of acne treatment, exosomes derived from specific cell types exhibit the ability to regulate sebum production and modulate inflammatory processes.[4] This capacity of exosomes introduces novel possibilities for the development of effective and targeted acne treatments. By influencing the underlying factors contributing to acne, exosomes offer a unique therapeutic approach that could revolutionize the management of this common skin condition.[4]

Individuals with pigmentation disorders, such as vitiligo or hyperpigmentation, can also benefit from the application of exosomes in dermatology. Since exosomes play a role in influencing the activity of melanocytes – the cells responsible for pigment production – this influence introduces an avenue for developing innovative therapies aimed at correcting pigmentation irregularities. By precisely modulating melanocyte function, exosomes can contribute to the restoration of skin color and address conditions characterized by uneven pigmentation.[5]

The integration of exosomes into dermatological treatments represents a paradigm shift in skincare strategies. Their ability to influence key cellular processes offers a targeted and personalized approach to address a spectrum of skin conditions, from aging-related concerns to acne and pigmentation disorders. As research in this field progresses, exosomes are likely to play an increasingly

significant role in shaping the future of dermatological therapeutics.

1.3. Efficacy in alopecias and wound healing

Alopecia, characterized by the distressing phenomenon of hair loss, has long been a problem in dermatology. Current treatments for alopecias often fall short of delivering satisfactory results, necessitating the exploration of alternative approaches.[6] In this context, exosomes have garnered attention due to their potential to stimulate hair follicle growth and enhance the process of hair regeneration.[7] Several studies have suggested that exosomes can play a crucial role in modulating the microenvironment of hair follicles, promoting the proliferation of hair cells, and contributing to the restoration of hair density.[7] The regenerative properties of exosomes, particularly their ability to influence signaling pathways involved in hair growth, make them a promising candidate for developing more effective treatments for various forms of alopecia.

Exosomes also play a significant role in terms of wound healing. The ability of exosomes to accelerate tissue repair and mitigate scarring is a subject of increasing interest. Exosomes contribute to wound healing by orchestrating complex cellular responses involved in inflammation, cell migration, and tissue remodeling. Their role in promoting angiogenesis, the formation of new blood vessels, is particularly noteworthy, as it enhances the blood supply to the injured area and accelerates the overall healing process. Furthermore, exosomes can modulate the activity of fibroblasts, the cells responsible for collagen production, thereby influencing scar formation. Leveraging the increased organization and control of this tissue repair process, exosomes offer a promising strategy for minimizing scarring and optimizing wound healing outcomes.[8]

Exosome-based therapies are important, specifically with their potential efficacy in alopecias and enhancing wound healing. The regenerative properties of exosomes, their ability to stimulate hair follicle growth, and their impact on tissue repair processes signify a transformative shift in dermatological interventions. Section 2 will address current findings and future directions of exosomes in alopecias and wound healing in more depth.

1.4. Significance of investigating the safety of exosome injections in the skin

While the therapeutic potential of exosomes in dermatology is promising, it is crucial to thoroughly investigate the safety aspects of exosome injections in the skin.[9] A 2019 study explored the usage of needle-free injections of exosomes derived from human dermal fibroblast spheroids and their role in ameliorating skin photoaging.[10] As exosomes exert influence on cell behavior, understanding their impact on local and systemic physiology is essential to ensure patient safety. Section 3 addresses current research findings regarding the safety profile of exosome-based dermatological treatments, shedding light on potential adverse effects and considerations for clinical applications.

1.5. Relevance for clinicians and researchers in the field of dermatology

The exploration of exosome-based therapies is expected to make a significant impact in dermatology, offering a wealth of opportunities for both clinicians and researchers alike. This field also holds immense promise for transforming patient care by introducing novel and effective treatments for challenging conditions.

For clinicians, the potential integration of exosome-based therapies into dermatological practice represents a shift in treatment options. The prospect of leveraging exosomes for conditions such as alopecia and wound care opens avenues for more efficacious and targeted interventions. Should the promising findings in research translate into clinical applications, clinicians stand to witness a revolution in their approach to patient care. Exosome-based therapies could offer solutions where traditional treatments have been limited, providing patients with more effective, less invasive, and potentially safer options. The ability to harness the regenerative capabilities of exosomes could lead to improved outcomes, enhanced patient satisfaction, and an overall advancement in the field of dermatology.

With this development, the emergence of exosomes as a potential therapeutic poses a myriad of ethical challenges. Ethical considerations include the sourcing of human stem cells, equitable access and distribution of exosome therapies, and the long-term effects on patients. Another important consideration is regarding exosome dosing. Exosome dosing is complex in that it involves balancing the intended therapeutic effect, parental cell type, and biological properties.[11] As such, the most efficacious delivery method has yet to be confirmed, and optimal dosing will depend on the delivery method.[11] Further investigations of varied methods of delivery across different dosages with an assessment of therapeutic effect and risk profile are needed.

Simultaneously, researchers are presented with a unique opportunity to contribute to the expanding knowledge base surrounding exosomes. As investigations into exosome-based therapies progress, researchers can unravel the intricate mechanisms underlying the therapeutic effects of exosomes in dermatological contexts. Insights gained from

this review can contribute to a deeper understanding of the molecular and cellular processes involved, paving the way for further innovations in dermatological therapeutics. By elucidating the nuances of exosome-mediated signaling and regenerative properties, researchers can refine and optimize treatment strategies, ultimately shaping the future of dermatology.

This review serves as a crucial bridge between research findings and clinical applications, aiming to synthesize and disseminate knowledge to both clinicians and researchers. By providing a comprehensive overview of the current landscape and future directions in utilizing exosomes for dermatological treatments, it seeks to facilitate the seamless integration of cutting-edge research into practical clinical applications. Bridging this gap ensures that the transformative potential of exosome-based therapies is realized in the clinic, offering tangible benefits to patients, and pushing the boundaries of what is achievable in the realm of dermatology. As the synergy between research and clinical practice strengthens, exosome-based therapies may emerge as key interventions in the field, transforming the way dermatological conditions are managed and treated.

1.6. Methodology

For this narrative review, a comprehensive search strategy across various databases, including PubMed, Science Direct, and Google Scholar, was utilized. The search focused on a combination of keywords such as exosomes, alopecia, wound healing, tissue regeneration, and regenerative medicine. Articles were screened without time restrictions to ensure a thorough examination of the relevant literature pertaining to the use of exosomes in alopecia. This approach aimed to provide a comprehensive overview and synthesis of existing evidence in the field while also guiding the identification of future research directions necessary to advance knowledge in this domain.

2. Exosomes in dermatological treatments

2.1. Efficacy in alopecias

2.1.1. Review of current research and case studies

The exploration of exosome efficacy in addressing alopecias has become a focal point of dermatological research. Numerous studies and case reports have provided valuable insights into the potential of exosomes for hair regeneration.[7,12] Current research indicates that exosomes when applied to the scalp or hair follicles, may stimulate the proliferation of dermal papilla cells (DPCs) and promote hair follicle growth.[13] Case studies have reported positive outcomes, with visible improvements in hair density and thickness following exosome-based interventions. Moreover, investigations into specific types of exosomes, such as those derived from mesenchymal stem cells (MSCs) or adipose-derived sources, have shown distinct mechanisms underlying their effectiveness.[4,14]

In terms of the selection of topical or injectable exosome treatments, both have shown promising results in utilizing exosomes for treating alopecia. One study demonstrated that exosomes derived from sources such as adipose-derived stem cells (ADSCs) and DPCs have demonstrated promising results in pre-clinical studies across different model systems.[15] Specifically, the topical application of exosomes isolated from ADSCs (ADSC-Exos) was successfully implemented in 39 androgenetic alopecia patients, resulting in significant increases in hair density and thickness.[15] It was noted that both injection and topical administration routes offered potential avenues for using exosomes in therapeutic interventions, with demonstrated efficacy and safety profiles. Another study investigated the therapeutic potential of ADSC-Exos in promoting hair regrowth and demonstrated that subcutaneous injection of ADSC-Exos significantly enhanced DPC proliferation and migration while also reducing apoptosis.[16] Subcutaneous injection of ADSC-Exos in mice led to improved hair growth, increased hair follicles, and a thicker dermis compared to controls. These findings highlight the promising role of ADSC-Exos as a cell-free therapeutic strategy for immune-mediated alopecia, both via topic use and injections.

Primary sources for commercial exosome products include bone marrow-derived MSCs (BM-MSCs) and placenta tissue-derived MSCs (PD-MSCs).[17] An analysis of these exosome sources concluded that BM-MSCs exhibit a superior safety profile and efficacy compared to PD-MSCs.[17] Human-derived exosomes are considered conventional sources, whereas non-conventional sources include animal, plant, and microbes.[18] Sources of exosomes used in dermatology have included plasma-derived exosomes, oral squamous cell carcinoma, myeloid-derived suppressor cells, blister fluid-derived NK-92 cells, and ADSCs, among others.[18] Exosomes have also been derived from plants and snake venom, and exosome-like vesicles have been acquired from bee glandular secretion products such as honey.[18]

Furthermore, combining exosomes with platelet-rich plasma (PRP) presents a promising approach for addressing alopecia, offering the potential for enhanced hair restoration outcomes. Exosomes, derived from cell cultures typically sourced from MSCs, contain a combination of signaling molecules and growth factors known to stimulate tissue regeneration and cell proliferation. Similarly, PRP, prepared from the patient's

own blood, is rich in platelets and growth factors that promote wound healing and tissue repair.[19] When combined with exosomes, which have shown the ability to stimulate DSC proliferation and promote hair follicle growth, the combined effects can potentially enhance hair regeneration outcomes.[20] Injection techniques are used, such as microneedling or direct injection, to facilitate precise delivery to the scalp. Due to limitations in current medicinal therapies for hair loss (e.g., variability, ineffectiveness, non-compliance, and adverse effects), regenerative strategies such as PRP and cell-based therapies, including those employing exosomes, offer a comprehensive approach to conditions such as alopecia.[20] Further research and clinical trials are needed to fully elucidate the efficacy and optimal protocols for this combination therapy. That being said, understanding the nuances of these mechanisms is critical for improving exosome therapies for different types of alopecias to enhance and optimize treatment precision.

2.1.2. Potential applications and benefits

The potential applications of exosomes in treating alopecias extend beyond traditional approaches. Exosomes offer a non-invasive and targeted therapeutic option, allowing us to circumvent the limitations associated with current treatments, such as topical minoxidil or oral medications, which can accompanied by side effects, systemic impact, and the need for long-term adherence.[21] In addition, the regenerative potential of exosomes can not only halt hair loss but also stimulate the growth of new, healthy hair.[22] Side effects of exosome use in the treatment of androgenetic alopecia have included minimal pain at the injection site in the scalp for up to 2 days following treatment.[21]

Furthermore, the versatility of exosomes allows for combination therapies, such as co-administration with growth factors or other regenerative agents, potentially amplifying their efficacy.[2,23] As research progresses, the identification of optimal dosage, frequency, and delivery methods will refine the application of exosomes in alopecia management, ushering in a new era of personalized and effective treatments.

The potential advantages of exosome-based therapies over currently available dermatological treatments, such as their ability to address multiple aspects of skin health and regeneration, are presented in Table 1. This table also underscores the need for more comparative clinical studies to fully evaluate the efficacy and safety of exosome-based interventions in relation to standard-of-care options. Addressing these knowledge gaps will be crucial for the successful integration of exosome-based therapies into dermatological practice. The various applications are also demonstrated in Figure 2.

2.2. Efficacy in wound healing

2.2.1. Examination of studies showcasing exosome effectiveness

Studies investigating the role of exosomes in wound healing have demonstrated their remarkable effectiveness in accelerating the regenerative processes of the skin.[24] These studies often utilize both *in vitro* and *in vivo*

Table 1. Comparative analysis of exosome-based therapies and current dermatological treatments

Condition	Exosome-based therapy	Current treatments	Comparative outcomes
Alopecia	(i) Stimulated hair follicle growth and proliferation of dermal papilla cells (ii) Increased hair density and thickness in clinical studies	(i) Topical minoxidil (ii) Oral finasteride (iii) Low-level light therapy	(i) Exosomes showed promising hair regenerative effects, potentially overcoming limitations of existing treatments (e.g., side effects, long-term adherence)
Wound healing	(i) Accelerated wound closure and re-epithelialization (ii) Enhanced angiogenesis and tissue remodeling (iii) Modulated inflammatory response	(i) Topical wound dressings (ii) Growth factor therapies Negative pressure wound therapy	(i) Exosomes demonstrated the ability to orchestrate multiple stages of wound healing, offering a more comprehensive therapeutic approach
Skin aging	(i) Promoted collagen production and reduced oxidative stress (ii) Improved skin texture and elasticity	(i) Topical retinoids (ii) Antioxidant serums (iii) Laser treatments	(i) Exosomes exhibited rejuvenating effects on aging skin, potentially providing a more targeted and natural alternative to current anti-aging interventions
Acne	(i) Reduced inflammation and sebum production (ii) Improved appearance of acne scars	(i) Topical retinoids (ii) Oral antibiotics (iii) Hormonal therapies	(i) Exosomes showed promise as an adjunct therapy to enhance the outcomes of existing acne treatments, particularly in addressing scarring
Pigmentation disorders	(i) Modulated melanocyte function and melanin synthesis	(i) Topical depigmenting agents (ii) Chemical peels (iii) Laser treatments	(i) Exosomes offer a novel approach to targeting the underlying mechanisms of pigmentation disorders, potentially leading to more effective and personalized treatments

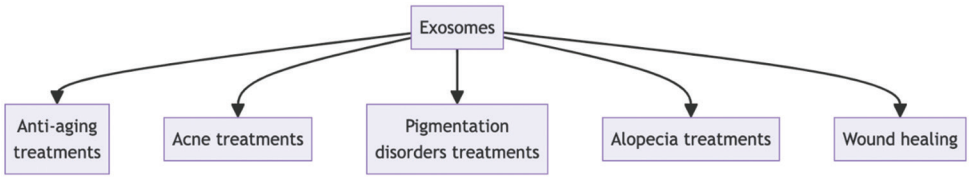

Figure 2. Diverse dermatological applications of exosomes

models to assess the impact of exosome administration on various aspects of wound healing. A significant advantage of using exosomes in wound healing is their diversity and the various sources from which they can be derived. The type of parent cells impacts the exosome's effects, determining its specific use in certain dermatological wound conditions. Furthermore, various sources of exosomes have been studied and reported in the literature, including oral mucosal lamina propria progenitor cells, epidermal stem cells, human amnion MSCs, menstrual blood-derived MSCs, macrophages, induced pluripotent stem cells, and human embryonic stem cells.[25] Promising findings highlight that exosomes derived from different cell sources, such as MSCs or endothelial cells, exhibit distinct regenerative properties.[26] Examining these studies allows us to discern the specific contributions of exosomes to the various stages of wound healing, providing a foundation for designing targeted therapeutic interventions for various disease models, including diabetic wound healing, burn-induced inflammation, and scarring.

One significant field of research that is currently expanding is the study of stem cells as a source of exosomes. Stem cells were previously studied for their use in promoting wound healing; however, a group of studies reported that therapeutic effects persisted even after the cessation of stem cell therapy, suggesting the role of released secretory mediators. Further investigations have found evidence that exosomes derived from stem cells augment therapeutic effects throughout all stages of wound healing, including inflammation, cell migration, proliferation, and extracellular matrix (ECM) remodeling.[27,28]

Exosomes, containing various growth factors, cytokines, and ECM components, influence the wound-healing process through various mechanisms of action. Their multi-target sites are important for their potential use in various dermatology conditions. First, studies have shown that exosomes facilitate the migration, activation, and proliferation of the different cell types involved in wound healing, including keratinocytes, fibroblasts, and endothelial cells.[29-31] Reports have also shown that the use of exosomes accelerates the closure of acute and chronic wounds, including diabetic wounds and pressure ulcers.[32,33] They facilitate the tissue repair process by promoting the formation of granulation tissue, re-epithelization, angiogenesis, and increased vascularity, leading to faster wound healing, including in cases of burn-induced inflammation.[34-36] Furthermore, studies have highlighted that exosomes enhance ECM formation, leading to increased tensile strength, improved tissue structure in the wound bed, and reduced scar formation.[37] In addition, their efficacy has been supported by their use in reducing wound-related keloid formation, decreasing the amount of scar tissue.[25,38]

However, their optimal efficacy has been illustrated in multiple studies on their effect on different inflammatory signaling pathways to modulate inflammation, a key process in wound healing, as highlighted in the next section.

2.2.2. Discussion on mechanisms and outcomes

The discussion on the mechanisms of exosome-mediated wound healing encompasses their ability to modulate inflammatory responses, enhance angiogenesis, and stimulate the proliferation of fibroblasts and keratinocytes.[39-41] These cellular processes collectively contribute to expedited tissue repair, reduced scarring, and improved overall wound healing outcomes. A key characteristic of exosomes in wound healing is their role in facilitating a favorable, healthy microenvironment for tissue regeneration and decreased inflammation.

Understanding the intricate interplay between exosomes and the cellular components involved in wound healing is crucial for optimizing their therapeutic potential. The current literature highlights common pathways that are involved in inflammatory processes and the stages of wound healing. First, studies have found that the use of exosomes decreases the inflammatory response by inhibiting the secretion of pro-inflammatory cytokines, such as TNF-α and IL-6, which are key mediators of the inflamed state and apoptosis.[33,42] In addition, they can increase the production of anti-inflammatory cytokines, such as VEGF, to prevent secondary injuries and more inflammation.[33] Exosomes also affect the expression of enzymes that play an important role in inflammation, such as metalloproteinases (MMP). Studies have shown that

the over-activation of MMP-9 led to the degradation of collagen and elastin, hindering diabetic foot ulcer healing.[43] However, exosomes can alter the ratio of MMP to its inhibitors, thereby promoting a more balanced and healing environment.[44] Furthermore, by regulating oxidative stress and encouraging tissue regrowth in hypoxic conditions, exosomes further promote wound healing. They have also been shown to induce the M2 macrophage phenotype, a change that naturally occurs at a later stage of the healing process, which promotes an immunosuppressive and anti-inflammatory environment.[45] Reports have also shown that exosomes operate on certain inflammatory signaling pathways, including TLR4, AKT/β-catenin, and transforming growth factor-β/SMAD signaling.[46,47] These effects are primarily mediated through exosomes' paracrine signaling.

Ultimately, the comprehensive examination of exosome efficacy in alopecia and wound healing not only highlights the current state of research but also underscores the potential transformative impact of exosome-based therapies on dermatological treatments. This multifaceted approach paves the way for innovative and effective interventions in clinical dermatology. In addition, it is important to address that current research regarding the role of exosomes in wound repair is still evolving, with most studies focused on the cellular level and on animal models. While medical applications show great promise, more clinical interventions and trials must be conducted to fully understand and utilize exosomes' therapeutic potential. In addition, the exploration of potential challenges, such as optimal dosages and timing of exosome administration, will contribute to the development of standardized protocols for clinical applications.

2.2.3. Efficacy in other dermatological conditions

Exosomes have shown great promise as an alternative therapeutic agent for alopecias and wound healing, and their use is being investigated in a variety of other dermatological conditions, including skin photoaging, acne, and pigmentation disorders (Figure 2). Current research illustrates the anti-senescence effect of exosomes in rejuvenating aging skin by delivering growth factors, microRNA, and other bioactive molecules to promote dermal fibroblast activity, collagen production, and skin elasticity.[48-50] In addition, studies have demonstrated the efficacy of bovine milk-derived exosomes in maintaining the skin barrier, preserving moisture, and reducing the appearance of wrinkles.[51] The efficacy of exosomes has also been studied in acne patients. ADSC-Exos was used in patients as an adjunct therapy after carbon dioxide laser therapy, which is most commonly used for severe or persistent acne that has not responded well to other treatments. Using stem cell-derived exosomes improved the appearance of acne scars and reduced the post-treatment recovery period in patients treated with the combination therapy.[2] In addition, exosomes' anti-inflammatory effects may foster a microenvironment that decreases acne flare-ups, and their role in regulating skin homeostasis through the PI3K/Akt pathway is currently under investigation.[52,53] Exosomes are also being explored for their application in pigmentation disorders, a common type of dermatologic condition. The current literature supports the therapeutic efficacy of exosomes in enhancing the epidermal-melanin unit, which describes the relationship between melanocytes (cells that produce melanin) and keratinocytes (the predominant cells in the epidermis). Keratinocytes naturally secrete exosomes to enhance melanin synthesis by increasing the signaling of melanosome proteins.[54] Certain microRNAs, such as MiR203 and MiR145, have been identified to play a role in regulating pigmentation by targeting tyrosinase expression and melanogenesis.[48]

Currently an expanding field of research, exosomes present an exciting, novel therapeutic approach for common dermatological conditions. Beyond the conditions discussed in this article, further applications are being studied, including the role of exosomes in treating other inflammatory conditions, such as psoriasis and atopic dermatitis, as well as their potential in diagnosing and treating skin cancer.[55-57]

3. Safety assessment of exosome injections

3.1. Risk of adverse events

3.1.1. In-depth analysis of potential concerns

As highlighted in previous sections, exosomes function by delivering biomolecules derived from an original donor cell, including proteins and RNA, to recipient cells to modulate gene expression and cell signaling, thereby affecting the overall function of the recipient cell. In the healthy human body, this process is tightly regulated. Thus, there are concerns revolving around the efficacy and safety of exosomes as a treatment modality, given their effects on cellular behavior. Current concerns include the potential of exosomes to cause genomic instability and cancer. In addition, the delivery method of exosomes, including dermal injections, raises questions about their local and systemic effects, as well as any off-target impacts.

Exosomes have gained attention over the past decade due to the great promise held by this novel modality in regenerative medicine; however, the United States food

and drug administration (FDA) has yet to approve their use for the treatment or diagnosis of any conditions. As with any investigational new drug, extensive clinical studies must be conducted to determine the safety and efficacy of exosomes. There is no substantial evidence to suggest that exosomes directly alter the DNA composition of recipient cells, and they are, therefore, generally considered safe.[58,59] Multiple studies evaluating the adverse effects of exosome therapy report no harmful effects at the local, systemic, and organ levels.[60,61] Local site reactions, such as redness, swelling, and congestion, were not observed in tissue subjugated to exosomal injection.[62-64] Other studies have evaluated the cytotoxic effect of exosomes *in vitro* and found no adverse effects on hemolysis, cell membranes, DNA, or cell proliferation.[60] Furthermore, studies have not shown a correlation between exosomes and organ damage, as assessed through various methods, such as measuring liver and renal function markers, as well as pathological examination of abnormalities or collections of inflammatory cell infiltrates in tissue sections of the body, including the heart, liver, lungs, kidney, and brain. Another investigation assessing the toxicological characteristics of ADSC-Exos determined that these exosomes are safe for topical application. They exhibited no indications of oral toxicity, had minimal impact on skin sensation, and caused no irritation to the skin or eyes.[65]

It is imperative to comprehend potential safety concerns to ensure that patients and providers are well-informed and equipped to address potential complications. While the majority of preclinical studies suggest that exosomes may represent a safe avenue for alternative treatment, it is important to recognize the current absence of standardized regulations. This lack of regulation can jeopardize patient safety, particularly due to the ethical dilemmas associated with the sourcing of parent cells used to derive exosomes. In addition, the cultivation of exosomes must adhere to proper sterile protocols. Reports have emerged regarding the utilization of unapproved exosomes derived from stem cell, placental, and umbilical cord blood products. Instances have been documented where individuals who received exosomes derived from C-section placentas contracted infections, prompting the FDA to issue warnings regarding the use of unapproved stem cell-derived products.[66,67]

3.1.2. The risk of cancer and other adverse reactions

Currently, cell therapy is limited, and ongoing studies are being conducted to enhance the understanding of the associated risks, including tumorigenicity. In the context of exosome therapy, it is important to discuss the risk of cancer, as exosomes can be derived from human induced pluripotent stem cells (hiPSCs). HiPSCs are currently limited in clinical trials due to their intrinsic risk of tumorigenicity. Being undifferentiated cells, they possess a high tumorigenic potential and have been linked to the formation of tumors and ectopic tissue, chromosomal abnormalities, and mutations in cancer-related genes.[68-71] However, while exosomes derived from hiPSCs still offer the benefits of pluripotency, they are not associated with tumorigenicity; while exosomes may affect cell behavior, they do not alter the base DNA sequence in cells. Furthermore, other additional side effects associated with the use of stem cells, such as infusion toxicity, cellular rejection, and microvasculature trapping affecting distribution, are not observed with exosome use. The inherent characteristics of exosomes include their inability to form tumors, their smaller nanometer particle size, and their reduced probability of eliciting an immune response from the host.[71-74] The latter property stems from the feature of exosomes being hypo-immunogenic as they are cell-free, have low expression of MHC-I, and show no expression of MHC-II.[75-77]

3.2. Comparative safety profiles

3.2.1. Evaluation of evidence for exosomes derived from human, plant, and animal cells

Based on existing literature, exosomes are generally categorized into two groups: Those derived from animals and those from plants. However, among animal-derived exosomes, the majority originate from human sources, including both allogenic and autologous exosomes, such as those derived from stem cells and bone marrow, rather than from other species, which are referred to as xenogeneic exosomes.[78-80] While current studies are being conducted, including on exosomes derived from snakes and pigs, this section mainly discusses the safety profiles of plant-derived exosomes and human-derived exosomes, as current research is limited regarding other species.

The safety and quality of exosomes greatly depend on the source cell type and the process by which they are obtained. Exosomes are cultivated through a multistep process that begins with the culturing of the source cells, either cell lines or tissue isolates, in a conditioned culture media. Isolation of exosomes can be achieved through a variety of different processes to separate the extracellular particles from other components, including ultracentrifugation, density gradient centrifugation, ultrafiltration, precipitation, or affinity-based isolation techniques.[81,82] Further purification occurs to remove any contaminants. These isolated exosomes are then characterized and monitored through a variety of quantitative and qualitative tests to analyze

and confirm the morphology, concentration, and surface markers of the exosomes.[83] Finally, exosome products are stored at −80°C to maintain their stability and biological activity.[84] The process of obtaining plant-derived exosomes is more limited and is based on the standard techniques described above for animal-derived exosomes.[85] However, modified techniques and additional considerations are required, as both types of cells have a different chemical composition while sharing a similar structure. Certain techniques that have been successfully applied in animal-derived cells do not work for plant-derived cells, such as immunoaffinity capture, as there is a limitation in labeling plant proteins and identifying common plant antibodies, which are important in the characterization phase of cultivation. There is also a need for additional steps, such as multiple rounds of centrifugation to separate contaminates and certain plant components, such as cellulose and starch.[85,86]

In summary, the process of obtaining exosomes involves careful isolation, purification, and characterization to ensure the quality and integrity of preparation, which is crucial to the downstream applications and safety of exosomes in research and the clinical setting.

Overall, both plant-derived and human-derived exosomes exhibit significant promise in terms of efficacy and safety, sharing a similar structure. The safety and biocompatibility of plant-derived exosomes have been investigated through *in vitro* and *in vivo* models.[87-92] These studies reveal a low risk and minimal systemic drug toxicity associated with the use of these exosomes. Notably, there were no observed instances of tissue damage, alterations in immunity, pathological or histological changes in organs, or increases in inflammatory markers or liver enzymes.[93-96] Moreover, the literature demonstrates the effective application of plant-derived nanovesicles across various disease models, including inflammation, tumors, and infections, as well as tissue regeneration, cell proliferation, and wound healing.[97-101]

However, it is essential to note that the use of plant-derived exosomes is less prevalent and less extensively researched compared to their human-derived counterparts, resulting in limitations in current studies. Nevertheless, the existing literature suggests several advantages of using plant-derived exosomes over human-derived ones. These advantages include easier mass production, ethical sourcing, reduced immunogenicity, and a lower risk of rejection reactions as allografts.[102-106] Human-derived exosomes, sourced from components such as bone marrow and stem cells, involve more time-consuming, expensive, and limited production processes compared to sourcing from plants. Consequently, exosome yield is more favorable when derived from plants.[107] Plant-derived exosomes are also proposed to carry a lower risk of eliciting an immune response and exhibit greater biocompatibility as they originate from a non-animal source.[92,108] Given the natural incorporation of agricultural products into the human diet, plant-derived exosomes may offer enhanced compatibility. In contrast, animal-derived exosomes bear closer resemblance to human proteins, which increases their risk for rejection.

Addressing the risk of allogeneic reactions is crucial when considering the application of various exosome types. Allogeneic reactions occur when the recipient's immune system identifies transplanted tissues or cells from a donor as foreign, eliciting an immune response against them. Currently, the literature showcases mixed results on the immunogenicity of exosomes. Although limited, some laboratory and clinical studies indicate that exosomes derived from allogeneic stem cells have demonstrated a favorable safety profile with minimal immediate and delayed adverse effects. For instance, their use in mitigating inflammation in COVID-19 patients has shown promising results.[109,110] Moreover, compared to conventional cell therapy, exosome administration has been associated with a lower incidence of immune rejection.[111] However, as exosomes carry a small amount of allogenic protein, which can stimulate an autoimmune response, their exact potential to interact with the body's immune system still requires better understanding. One study investigated the induction of antigen-specific naïve CD4+ T cell activation *in viv*o through the injection of antigen-bearing exosomes and found that antigen-dependent T cell stimulation occurred only when mature CD8α and dendritic cells present in the cultures.[112] Therefore, it seems many factors are at play when assessing the risk of allogeneic reactions with exosome use. From the types of cell surface markers present on the exosome to the potential environment the exosome will be interacting with, further emphasizing the importance of ensuring the cultivation process has strict measures. Through the establishment of standardized cultivation methods, studies can focus on enhancing the purification process of exosomes to minimize the risk of allogeneic reactions. One prevalent approach involves conducting host cell protein and DNA analyses during the characterization phase. By identifying the presence of parent cell components, this method offers insight into how process-related impurities may affect the product's safety by potentially inducing undesired immunogenic responses.[83]

It is also important to acknowledge the disadvantages of plant-derived exosomes, including their challenges

with biosafety and toxicity. Plants may contain unknown bioactive components or toxic elements from pesticides. Certain plants may also face seasonal and geographic limitations, complicating the cultivation of exosomes. In addition, studies on plant exosomes have primarily focused on oral administration, with limited research on local injection.[85] Moreover, plant-derived exosomes tend to have a larger size than MSC-derived exosomes, potentially reducing their bioavailability and cellular uptake rate.[113,114]

3.2.2. Insights into potential long-term implications

While plant-derived exosomes show significant promise, additional studies comparing them with animal-derived exosomes are necessary to fully understand their safety and efficacy. Currently, there are no FDA regulations or standard protocols in place for cultivation. However, the approval of exosome-based therapeutics will need further long-term studies to investigate the genotoxicity, off-target effects, biodistribution, and safety profiles of both plant-derived and animal-derived exosomes before regulatory approval. Furthermore, the therapeutic effect of exosomes is regulated by various factors revolving around their creation, such as the parent cell source, culturing methods, and isolation technique. It is recommended that the production of both animal-derived and plant-derived exosomes be tightly regulated to ensure pure sourcing and minimize the risk of contamination, as the activity of nanovesicles is impacted by their processing method. Contaminants can result in adverse effects, with exosomes mimicking the properties of the original parent cell source.[75] For instance, exosomes derived from human colorectal cancer cells have been found to induce oncogenic behavior in colonic mesenchymal stromal cells.[115] In addition, the environmental impact of exosome use must be considered, especially in the large-scale production and disposal of exosomes derived from humans, animals, and plants.

4. Balanced approach to exosome safety

4.1. Examination of both benefits and risks

When considering exosome-based technologies, it is important to weigh both their benefits and risks. As discussed in the previous sections, current studies have indicated a favorable opinion of their use, as they have shown potential for stimulating hair growth, possess an encouraging safety profile with minimal toxicity, and offer a promising alternative to existing therapies. In particular, plant-based exosomes may also provide additional advantages in terms of ethical sourcing, mass production, and enhanced biocompatibility due to their natural incorporation into the human diet. However, given the current limitations in research, especially in regard to exosomes derived from plants, there remains uncharted territory that future research must address to better understand the risks associated with the use of exosomes. Current complications and risks include ethical sourcing, long-term effects, lack of regulatory approval, challenges with biosafety, and environmental impact. In the case of plant-based exosomes, while the safety regarding systemic toxicity has been established with oral or intravenous administration, other methods of administration, such as local injections, must be further studied.

4.2. Importance of a balanced perspective for clinicians and researchers

While exosome-based therapy emerges as a promising novel treatment option for alopecia, a balanced approach must be adopted, as with any therapeutic intervention. First, clinicians and researchers must thoroughly understand how exosomes function, their role in regenerative medicine, and the associated challenges. It is also important to compare the benefits against the risks associated with using exosome-based technologies to ensure that patients will overall have positive outcomes while minimizing negative effects. In addition, adopting an unbiased approach will not only guide clinical decision-making but also aid in garnering trust from the public, especially from patients who may be hesitant to try out a novel treatment. Finally, an objective outlook will facilitate informative discussions between both clinicians and researchers to ensure that no aspects are overlooked when addressing questions about the use of exosomes. This approach will guide future studies to test the therapeutic potential and limitations of exosomes, driving further research in the avenues of optimizing treatment protocol, addressing safety concerns, and expanding the field of exosome-based therapies.

4.3. Implications for the growing interest in exosome research

The growing interest in exosome research carries various implications across multiple fields, including biomedicine, drug delivery, regenerative medicine, and diagnostics. Therefore, it is crucial to further explore and conduct studies to evaluate treatments utilizing exosomes in regard to their safety, long-term effects, efficacy, and therapeutic potential. Further research in this field will facilitate the development of improved biomedical therapies and fine-tuning of the cultivation process, leading to the development of superior exosome products. With a better understanding, both researchers and clinicians can make informed decisions regarding their use in certain conditions, aiding in the recommendation and treatment of patients. Furthermore,

ongoing surveillance and monitoring of exosome-based therapies are imperative for regulatory approval before they can be offered to consumers in the marketplace. This practice will also foster greater trust and confidence in exosome-based technology among the public. In summary, exosome research is a rapidly evolving field that shows great promise not only in dermatological treatments but in medicine as a whole.

5. Conclusion

5.1. Summary of key findings

This review highlights several key findings regarding the potential applications and safety considerations of exosome-based therapies in dermatology. Research demonstrates that exosomes show promise for effectively treating conditions such as alopecia and accelerating wound healing through their ability to stimulate hair follicle growth, enhance tissue regeneration, and modulate processes involved in repair. However, further studies are still needed to fully characterize the long-term safety profile of exosomes and establish standardized protocols for clinical use. Both human-derived and plant-derived exosomes exhibit favorable safety profiles based on current evidence, although plant sources may offer advantages in production and biocompatibility that human-derived sources do not. Moving forward, continued exploration of exosomes' therapeutic mechanisms and potential risks will be important for optimizing these innovations and providing safe, effective exosome-based treatments to patients. While more research is still warranted, the findings presented provide a comprehensive overview of the burgeoning applications of exosome therapy within dermatology.

5.2. Relevance for the readership, including those interested in protein and exosome therapies

A rapidly advancing field in therapeutic intervention, exosome-based therapies extend beyond the scope of dermatology. Exosomes exhibit promising potential in diverse medical applications, including drug delivery, molecular tagging, and their effects across various disease models, such as inflammation, tumors, cell regeneration, and infections. Insights into protein and exosome therapies will not only foster a better understanding across the field but also allow for further advancements and address the current challenges. This new avenue of patient care, though only scratching the surface, will captivate those eager to push the current limits of management and interventions. Encouraging future research, collaboration, and communication is essential to unraveling more about exosomes and their novel applications in medicine. Given the growing interest in the interplay between biomedicine and innovation, this article's review of concepts related to exosomes and presentation of the recent studies in dermatology are relevant to our readers, particularly those exploring protein and exosome therapies in human diseases.

5.3. Encouragement for further exploration and research in the field of dermatology and exosome applications

Exosome-based therapies present a promising avenue in medicine, particularly for conditions with limited treatment options, such as alopecia. Recent studies have highlighted their potential in stimulating DSCs, promoting hair follicle growth, accelerating wound healing, and facilitating tissue restoration. The growing body of evidence highlights the importance of further investigating the role of exosomes in dermatology and their safety profile. Future research to advance our understanding of exosome-based technology will not only deepen our knowledge of skin health and regeneration but also facilitate the development of safe innovations and novel treatments. While the current field of dermatology offers various treatment avenues, there is always a need for additional exploration to assist patients who may not tolerate or respond favorably to existing treatment options. By advancing the frontiers of biomedicine in dermatology, exosome-based therapies may enhance patient outcomes and improve the quality of life in dermatological care.

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Gene & Protein in Disease

ORIGINAL RESEARCH ARTICLE

Rational virtual screening, ADME, and molecular simulation studies of potential inhibitors of human superoxide dismutase 1 in a dysfunctional antioxidant system

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Abstract

Superoxide dismutase 1 (SOD1), a copper-dependent enzyme, facilitates the conversion of superoxide anions into hydrogen peroxide and oxygen, thereby regulating superoxide levels. Dysfunctions in SOD1 have been linked to neurodegenerative disorders such as amyotrophic lateral sclerosis, as well as liver and lung cancers. This study aimed to identify SOD1 modulators using *in silico* rational virtual enrichment screening, pharmacokinetics, docking, and molecular dynamic simulation (MDS). The findings yielded 38 compounds, predominantly exhibiting high gastrointestinal absorption but mostly non-permeable across the blood–brain barrier, with few exhibiting inhibitory effects on selected cytochrome P450s. Molecular docking revealed that compound 1 (PubChem CID: 36791369) exhibited the highest binding affinity (–6.771 kcal·mol^{-1}), followed by compound 19 (PubChem CID: 30935) with –6.468 kcal·mol^{-1}, and compound 20 (PubChem CID: 135744521) with –5.978 kcal·mol^{-1}. MDS and molecular mechanics/generalized Born surface area analysis indicated that the compound CID 36791369 – SOD1 complex and compound CID 30935 – SOD1 complex remained stable and energetically favorable under simulated physiological conditions at 0 ns and 100 ns. In conclusion, this study identified 38 compounds, among which compounds SN5, SN6, SN7, SN12, and SN25 emerged as potential inhibitors of SOD1 based on overall analyses. Further, research will be necessary to investigate the therapeutic effectiveness of these top five compounds *in vitro* and *in vivo* against SOD1.

Keywords: SOD1; Cancer; Amyotrophic lateral sclerosis; Novel inhibitors; Pharmacokinetics; Molecular docking; Molecular dynamics simulation

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1. Introduction

The copper-dependent enzyme superoxide dismutase 1 (SOD1) belongs to the SOD enzyme family, which is distributed across various cell organelles.[1] SOD1 facilitates the conversion of superoxide anions into hydrogen peroxide and oxygen, thus maintaining low levels of superoxide. This function is crucial due to the toxicity and poor membrane permeability of superoxide.[1,2] SOD1, a copper/zinc-containing enzyme, is found in the cytosol, nucleus, and intermembrane space of mitochondria. In contrast, SOD2, a manganese-containing enzyme, resides in the mitochondrial matrix, and extracellular SOD (ecSOD or SOD3), a secreted copper-containing protein, is located in the extracellular matrix.[1,2]

Mutations in SOD1 are found in approximately 20% of amyotrophic lateral sclerosis (ALS)-affected families and can be inherited in autosomal dominant or recessive patterns.[3,4] Over 160 mutations in SOD1 have been linked to ALS, primarily comprising missense mutations along with a minority of small deletions and truncations.[5] Notably, misfolding and aggregation of mutant SOD1, rather than loss of enzymatic function, are central to ALS pathology.[6] Mutant SOD1 interacts with the dynein-dynactin complex in motor neurons, leading to impaired axonal transport and correlating with disease progression in SOD1 transgenic mice.[7,8]

In addition, SOD1 plays a crucial role in growth factor signaling in endothelial and tumor cells. SOD1-deficient mice have been demonstrated to exhibit increased susceptibility to liver tumors due to elevated DNA mutation rates.[9,10] Knockdown of SOD1 induces senescence in fibroblasts and decreases fertility in female mice.[11,12] Conversely, overexpression of SOD1 in lung cancer cells promotes growth by enhancing survival mechanisms, suggesting modulation of cellular redox status, including SOD1 inhibition, as a potential therapeutic strategy for cancer treatment.[13] In light of these considerations, this study aimed to identify potential SOD1 modulators using *in silico* rational virtual enrichment screening, pharmacokinetics, docking, and molecular dynamic simulations (MDSs).

2. Materials and methods

2.1. Protein-protein interaction (PPI) analysis

The 3D structure of the human SOD1 protein (UniProt ID: P00441, gene ID: SOD1), retrieved from the UniProt database (www.uniprot.org), served as the basis for this study. Its UniProt ID was subjected to PPI analysis using the STRING webserver (https://string-db.org/).[14]

2.2. Rational virtual screening

The UniProt ID of the protein was employed for rational enrichment virtual screening using the LIGQ webserver (www.ligq.qb.fcen.uba.ar), utilizing the full pipeline setting. LIGQ constructs a compound set with documented binding evidence to the input protein or similar ones, leveraging prior biological assays.

2.3. Clustering analysis

The obtained sets of ligands in SMILES format underwent scrutiny to eliminate any non-drug-like molecules. Subsequently, clustering analysis with multidimensional scaling was conducted on the ChemMine server (http://chemmine.ucr.edu/)[15] utilizing the ligands' SMILES representations.

2.4. *In silico* absorption, distribution, metabolism, and excretion (ADME) prediction

The compounds underwent *in silico* ADME screening using the SwissADME server (www.swissadme.ch),[16] employing default parameters and utilizing the SMILES format. Duplicated ligands and those with low gastrointestinal absorption (GIA) were subsequently excluded from further analyses.

2.5. Molecular docking

Molecular docking studies followed the protocol outlined by Fatoki *et al.*[17] Initially, the ligands' SMILES underwent 3D structure optimization using ACDLab/Chemsketch software and were saved in.mol format. Subsequently, PyMol software facilitated the conversion of ligand files from. mol to. pdb format. The 3D structure of human SOD1 was retrieved in AlphFold pdb format from the Uni Prot data base. Before docking, both the ligands and target protein structures in pdb format were converted to pdbqt format using AutoDock Tools v1.5.6.[18] Following this, ligand-protein molecular docking was performed utilizing AutoDock Vina v1.2.3.[19,20] Post-docking, binding affinity was determined, and close interactions between the ligands and targets were analyzed and visualized using ezLigPlot on the ezCADD web server available at http://dxulab.org/software.[21]

2.6. Molecular dynamics simulation

Molecular dynamics simulations were conducted for 100 ns using Desmond, a package of Schrödinger LLC.[22-24] The initial configurations of the protein and ligand complexes for molecular dynamics simulations were obtained from docking studies. Preprocessing of the protein-ligand complexes was performed using Maestro's protein preparation wizard, which included optimization and minimization of the complexes. All systems were prepared using the System Builder tool with the solvent model employing an orthorhombic box with TIP3P water molecules. The Optimized Potential for Liquid

Simulations 2005 (OPLS-2005) force field was utilized for the simulations, and the models were neutralized by adding 0.15 M NaCl counter ions to mimic physiological conditions.[25] The constant-temperature, constant-pressure (NPT) ensemble was selected with a temperature of 310 K and pressure of 1 atm for the complete simulation. Before the simulation, the models were relaxed. Trajectories were saved after every 100 ps during the simulation, and post-simulation analysis of the trajectories was conducted to determine root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), and protein-ligand interaction profiles. In addition, prime molecular mechanics/generalized Born surface area (MMGBSA) calculations were performed to evaluate the binding free energy (ΔG^{bind}) of the complexes,[23,24,26] as shown in Equation I:

$$\text{MMGBSA } \Delta G^{bind} = \Delta G^{Coulomb} + \Delta G^{Covalent} + \Delta G^{Hbond} + \Delta G^{Lipo} + \Delta G^{Packing} + \Delta G^{SolvGB} + \Delta G^{vdW} \quad \text{(I)}$$

3. Results

In this study, the PPI analysis of human SOD1 predicted its primary interacting proteins, including SOD2, CCS (copper chaperone for SOD), BCL2 (apoptosis regulator Bcl-2), PARK7 (Parkinson's protein 7 or protein/nucleic acid deglycase DJ-1), VDAC (voltage-dependent anion-selective channel protein 1), FUS (RNA-binding protein FUS), TARDBP (TAR DNA-binding protein 43), NEFL (neurofilament light polypeptide), HSPA5 (endoplasmic reticulum chaperone BiP), and DERL1 (Derlin-1), as depicted in Figure 1.

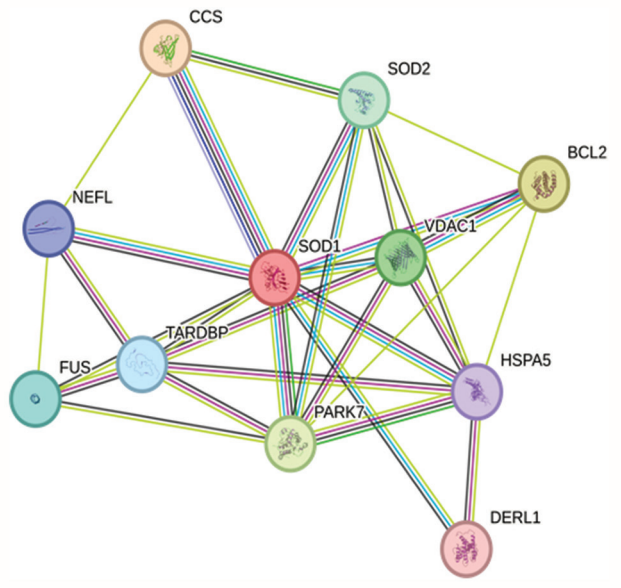

Figure 1. Protein-protein interaction profile of superoxide dismutase 1

Enrichment virtual screening was conducted on human SOD1 to identify compounds with potential modulatory effects. The virtual screening yielded 72 compounds in SMILES format (Supplementary File S1). Hierarchical clustering of these compounds revealed their structural similarities, as illustrated in Figure 2. In addition, pharmacokinetic prediction was performed on the 72 compounds, with results presented in Supplementary File S2. Following manual inspection of the pharmacokinetic results, duplicated compounds and those with low GIA were excluded from the study. This process yielded 38 chemical

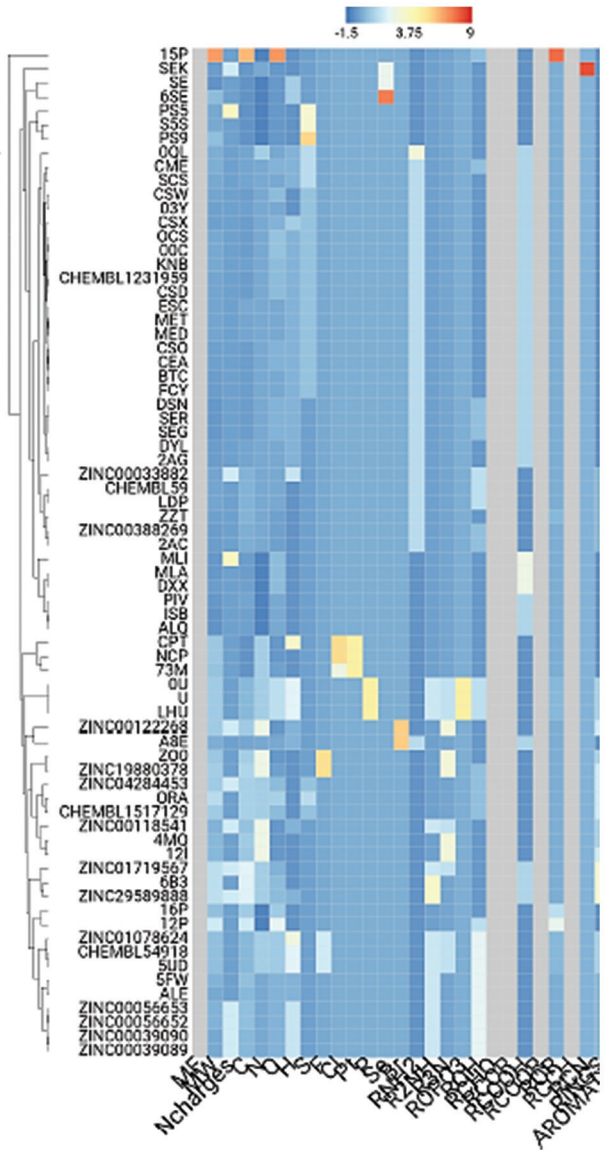

Figure 2. Hierarchical clustering results. Parameter options used are Heatmap (distance matrix), Linkage Method (single), Physicochemical Properties Heatmap (ChemmineR Properties), and Properties Color and Display Values (Z-scores).

compounds with high GIA, predominantly impermeable to the blood-brain barrier (BBB), possessing good solubility, and exhibiting minimal inhibitory effects on selected cytochrome P450s, as summarized in Table 1.

The SMILES of the 38 compounds were cross-checked on the PubChem database, and their corresponding PubChem CIDs were obtained. Subsequently, molecular docking analyses were conducted on these compounds. The results of the molecular docking revealed the binding affinities of human SOD1 for 38 analyzable compounds, as presented in Table 2. Compound 1 (PubChem CID: 36791369) exhibited the highest binding affinity of -6.771 kcal·mol^{-1}, followed by compound 19 (PubChem CID: 30935) with -6.468 kcal·mol^{-1}, compound 20 (PubChem CID: 135744521) with -5.978 kcal·mol^{-1}, and others. The IUPAC names of the top three compounds are 2-[2-[(6-oxo-5H-phenanthridin-3-yl) carbamoyl]phenyl]benzoate, 3-[(2-hydroxynaphthalen-1-yl) diazenyl]benzenesulfonic acid, and 3-[(2-hydroxynaphthalen-1-yl)diazenyl]benzenesulfonate, respectively. The binding poses of the complexes with high binding affinities are depicted in Figure 3, illustrating the involvement of specific

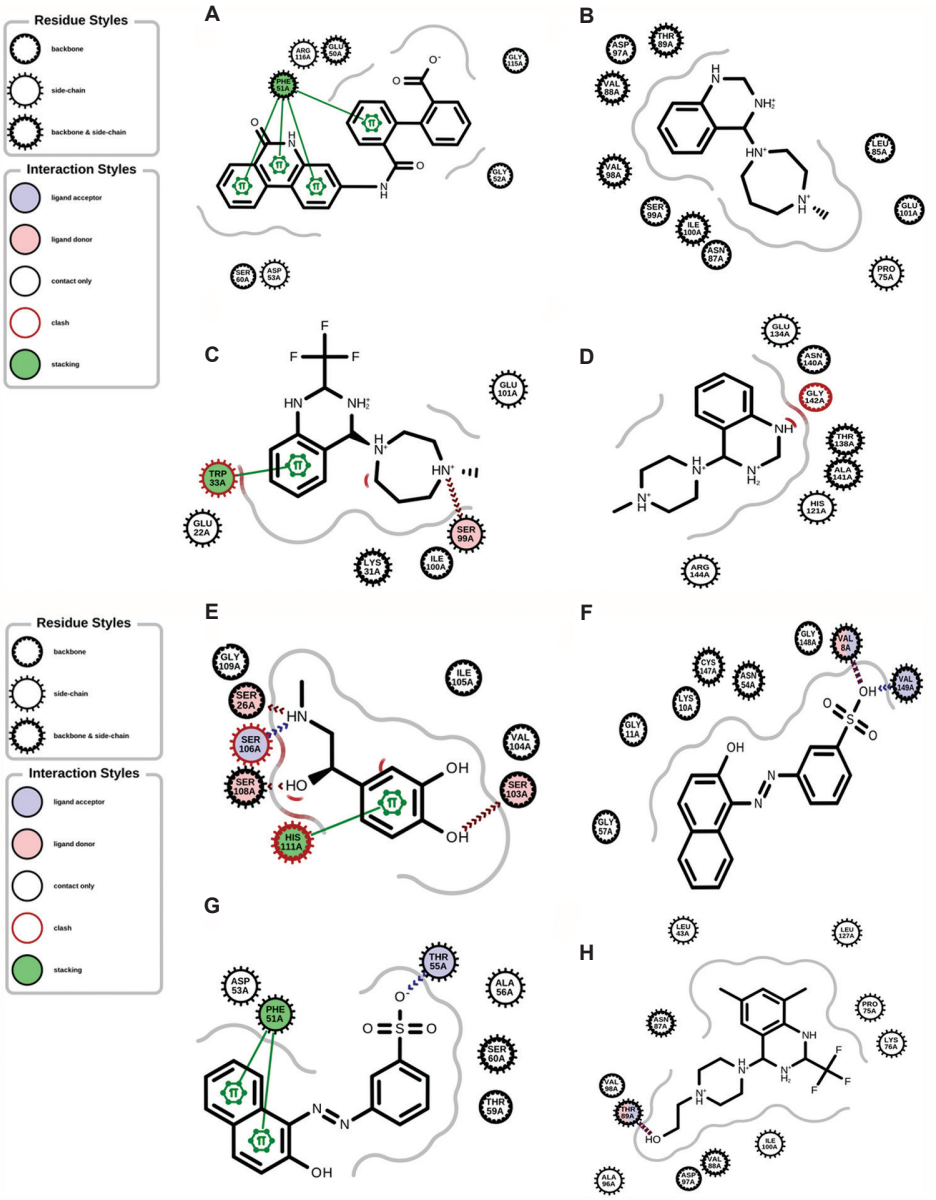

Figure 3. Interaction of the binding poses of soluble epoxide hydrolase with (A) PubChem CID: 36791369, (B) PubChem CID: 47121004, (C) PubChem CID: 17445796, (D) PubChem CID: 21029584, (E) PubChem CID: 5816, (F) PubChem CID: 30935, (G) PubChem CID: 135744521, and (H) PubChem CID: 993363.

Table 1. Predicted pharmacokinetics of 38 compounds with high low gastrointestinal absorption GIA

S. No.	Ligands code	Predicted ADME parameter from SWISSADME												
		MW	MR	TPSA (Å²)	Log P	ESOL Log S	ESOL class	GIA	BBB	P-gp	CYPs	Lip	BS	SA
1	ZINC29589888	433.43	126.74	102.09	4.02	−5.58	Moderately soluble	High	No	No	2C9, 2C19	0	0.56	2.76
2	ZINC00388269	123.15	37.84	46.25	1.18	−1.83	Very soluble	High	Yes	No	3A4	0	0.55	1
3	CHEMBL1231959	152.15	28.92	129.55	−2.9	1.73	Highly soluble	High	No	No	-	0	0.55	2.28
4	KNB	167.18	34.79	105.84	−1.94	1.84	Highly soluble	High	No	No	-	0	0.55	2.61
5	4MQ	242.32	80.61	32.26	1.89	−3.1	Soluble	High	Yes	Yes	1A2, 2D6	0	0.55	2.24
6	ZO0	310.32	85.61	32.26	2.99	−3.92	Soluble	High	Yes	Yes	1A2, 2C19, 2D6	0	0.55	2.5
7	12I	228.29	75.8	32.26	1.64	−2.82	Soluble	High	Yes	Yes	1A2	0	0.55	2.03
8	ZINC00056653	212.27	59.9	77.3	−0.89	1.01	Highly soluble	High	No	No	-	0	0.55	2.08
9	MET	149.21	38.22	88.62	−0.59	0.68	Highly soluble	High	No	No	-	0	0.55	2.43
10	ESC	163.24	43.03	88.62	−0.23	0.42	Highly soluble	High	No	No	-	0	0.55	2.66
11	ZINC00039090	184.21	50.29	77.3	−0.81	−0.26	Very soluble	High	No	No	-	0	0.55	1.82
12	ALE	183.2	49.03	72.72	0.1	−0.26	Very soluble	High	No	No	-	0	0.55	1.79
13	PS5	160.32	37.5	75.9	0.1	−0.26	Very soluble	High	No	No	-	0	0.55	1.79
14	S5S	163.35	42.14	167	0.1	−0.26	Very soluble	High	No	No	-	0	0.55	1.79
15	PS9	256.52	60.73	202.4	0.1	−0.26	Very soluble	High	No	No	-	0	0.55	1.79
16	CSO	137.16	30.18	108.85	−1.82	1.78	Highly soluble	High	No	No	-	0	0.55	2.45
17	CSD	153.16	30.86	119.83	−2.2	2.05	Highly soluble	High	No	No	-	0	0.56	3.05
18	PIV	102.13	27.66	37.3	0.99	−1.33	Very soluble	High	Yes	No	-	0	0.85	1
19	CHEMBL1517129	328.34	86.45	107.7	3.09	−4.3	Moderately soluble	High	No	No	-	0	0.56	3.01
20	ZINC04284453	327.33	84.6	110.53	3.02	−4.29	Moderately soluble	High	No	No	2C19	0	0.56	2.98
21	16P	294.38	75.92	55.38	1.62	−0.51	Very soluble	High	Yes	No	-	0	0.55	3.15
22	SER	105.09	22.18	83.55	−1.97	1.57	Highly soluble	High	No	No	-	0	0.55	1.51
23	OCS	169.16	31.55	126.07	−2.43	2.13	Highly soluble	High	No	No	-	0	0.56	2.82
24	CSW	153.16	30.86	119.83	−2.24	2.05	Highly soluble	High	No	No	-	0	0.56	3.05
25	ZINC19880378	354.37	96.7	52.49	2.83	−3.81	Soluble	High	Yes	Yes	1A2, 2D6	0	0.55	2.66
26	CHEMBL59	153.18	42.97	66.48	0.46	−0.44	Very soluble	High	No	No	-	0	0.55	1.01
27	ZINC00033882	154.19	44.23	68.1	−0.42	−0.45	Very soluble	High	No	No	-	0	0.55	1.04
28	MLA	104.06	20.08	74.6	−0.65	0.16	Highly soluble	High	No	No	-	0	0.85	1
29	DXX	118.09	24.89	74.6	−0.23	−0.48	Very soluble	High	No	No	-	0	0.85	1.08
30	CME	197.28	46.97	134.15	−0.99	0.7	Highly soluble	High	No	No	-	0	0.55	3.09
31	SCS	181.28	45.81	113.92	−0.27	0.07	Highly soluble	High	No	No	-	0	0.55	2.97
32	FCY	121.16	28.94	102.12	−1.31	1.11	Highly soluble	High	No	No	-	0	0.55	1.75
33	03Y	135.18	33.79	102.12	−1.12	1.27	Highly soluble	High	No	No	-	0	0.55	1.84
34	6SE	238.9	19.49	0	−1.12	1.27	Highly soluble	High	No	No	-	0	0.55	1.84
35	ZINC00118541	217.29	67.42	42.25	0.81	−2.93	Soluble	High	No	No	-	0	0.55	2.01
36	ZINC00122268	322.22	89.27	33.46	1.55	−3.9	Soluble	High	No	Yes	1A2	0	0.55	2.31
37	A8E	194.03	38.02	63.32	−0.34	0.28	Highly soluble	High	No	No	-	0	0.55	2.64
38	DYL	115.13	30.15	63.32	−0.83	1.07	Highly soluble	High	No	No	-	0	0.55	1.87

Notes: Physicochemical properties: Molecular weight (MW), molar refractivity (MR), total polar surface area (TPSA). Lipophilicity: Consensus Log P. Water Solubility: ESOL Log S, ESOL Class. Pharmacokinetics: gastrointestinal absorption (GIA), blood-brain barrier (BBB), P-glycoprotein (P-gp) substrate, inhibition of cytochrome P450 (CYPs) type CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4. Druglikeness: Lipinski (Lip), bioavailability score (BS). Medicinal chemistry: synthetic accessibility (SA).

Table 2. Molecular docking results

S. No.	Phytochemicals	PubChem CID	SOD1 (Alpha-Fold ID: AF-P00441) binding affinity ΔG (kcal·mol^{-1})
1	ZINC29589888	36791369	−6.771*
2	ZINC00388269	7264	−4.106
3	CHEMBL1231959	6398956	−4.136
4	KNB	11283261	−3.991
5	4MQ	47121004	−5.796*
6	ZO0	17445796	−5.739*
7	12I	21029584	−5.549*
8	ZINC00056653	6921600	−4.952
9	MET	6137	−3.966
10	ESC	25674	−3.895
11	ZINC00039090	6920144	−4.781
12	ALE	5816	−5.057*
13	PS5	5289210	−2.104
14	S5S	NA	−1.935
15	PS9	66348	−2.459
16	CSO	165339	−3.569
17	CSD	1549098	−4.164
18	PIV	6417	−3.507
19	CHEMBL1517129	30935	−6.468*
20	ZINC04284453	135744521	−5.978*
21	16P	90206	−3.058
22	SER	5951	−3.927
23	OCS	72886	−3.979
24	CSW	109	−4.008
25	ZINC19880378	993363	−5.756*
26	CHEMBL59	681	−4.923
27	ZINC00033882	3713609	−4.255
28	MLA	867	−3.818
29	DXX	487	−4.022
30	CME	170018	−4.239
31	SCS	480046	−3.937
32	FCY	5862	−3.529
33	03Y	9989321	−3.695
34	6SE	137348516	−1.49
35	ZINC00118541	5078469	−4.663
36	ZINC00122268	3449846	−5.053*
37	A8E	2762282	−3.938
38	DYL	14044	−3.845

Notes: NA: Not available. Docking parameter: SOD1 (spacing: 0.375, NTPS: 106×106×106, center: 1.984×2.466×3.811). *Suitable score.

amino acid residues in the binding interactions. SOD1 amino acid residues PHE51, TRP33, and HIS111 participate in pi-stacking interactions, while SER99, SER103, SER26, and SER108 act as the main ligand hydrogen donors. In addition, THR55 and VAL149 serve as the primary ligand hydrogen acceptors.

MDSs were conducted to evaluate the structural stability of both the protein and the binding status of the ligand in a physiologically relevant environment. The binding complexes of human SOD1 with the two compounds exhibiting the best binding affinities (PubChem CID 36791369 and PubChem CID 30935) were utilized for MDS analysis to facilitate comparison. The outcomes of these MDS analyses are depicted in Figure 4A-F, providing valuable insights into the dynamic behavior and interactions of the protein-ligand complexes under realistic conditions.

The binding complex of SOD1 with compound CID 36791369 exhibited an RMSD of the protein ranging from 0 to 100 ns, with a value of 2.25 Å. Conversely, for the ligand, the RMSD was 19 Å during the same period (Figure 4A). The RMSF analysis of SOD1 revealed maximal fluctuations at amino acid residues 50 – 55 and 125 – 135 (Figure 4B). Protein-ligand interactions included hydrophobic interactions, hydrogen bonds, and water bridges involving amino acid residues such as HIS49, THR59, HIS64, GLY142, and ARG144 (Figure 4C). Similarly, for the binding complex of SOD1 with compound CID 30935, the RMSD of the protein was 2.25 Å, and that of the ligand was 15 Å from 0 to 100 ns (Figure 4D). The RMSF showed maximum fluctuation at amino acid residues 125 – 135 (Figure 4E). The protein-ligand interactions comprised hydrophobic interactions, hydrogen bonds, water bridges, and ionic interactions involving amino acid residues such as VAL8, LYS10, ASN54, and CYS147 (Figure 4F).

The results of MDS demonstrated suitable stability and interactions between compound CID 36791369 and SOD1, with major amino acid residues including HIS49, HIS64, HIS121, GLY142, and ARG144. Similarly, compound CID 30935 exhibited interactions with SOD1 involving LYS10, ASN54, and CYS147 as major amino acid residues. A schematic detailing the interactions between the ligand atoms and protein residues is presented in Figure 5. Overall, the protein-ligand interaction profiles validated the amino acid residues identified in the docking interactions. The binding free energies for all complexes were computed using MMGBSA at both 0 and 100 ns time points. The MMGBSA results (Table 3) revealed a binding energy ΔGbind (Total) of -32.867 and -56.110 kcal·mol^{-1} for the compound CID 36791369 – SOD1 complex at 0 ns and 100 ns, respectively. For the compound CID 30935 – SOD1 complex, the values were -28.518 and -39.790 kcal·mol^{-1} at 0 ns and 100 ns, respectively. These calculations indicate

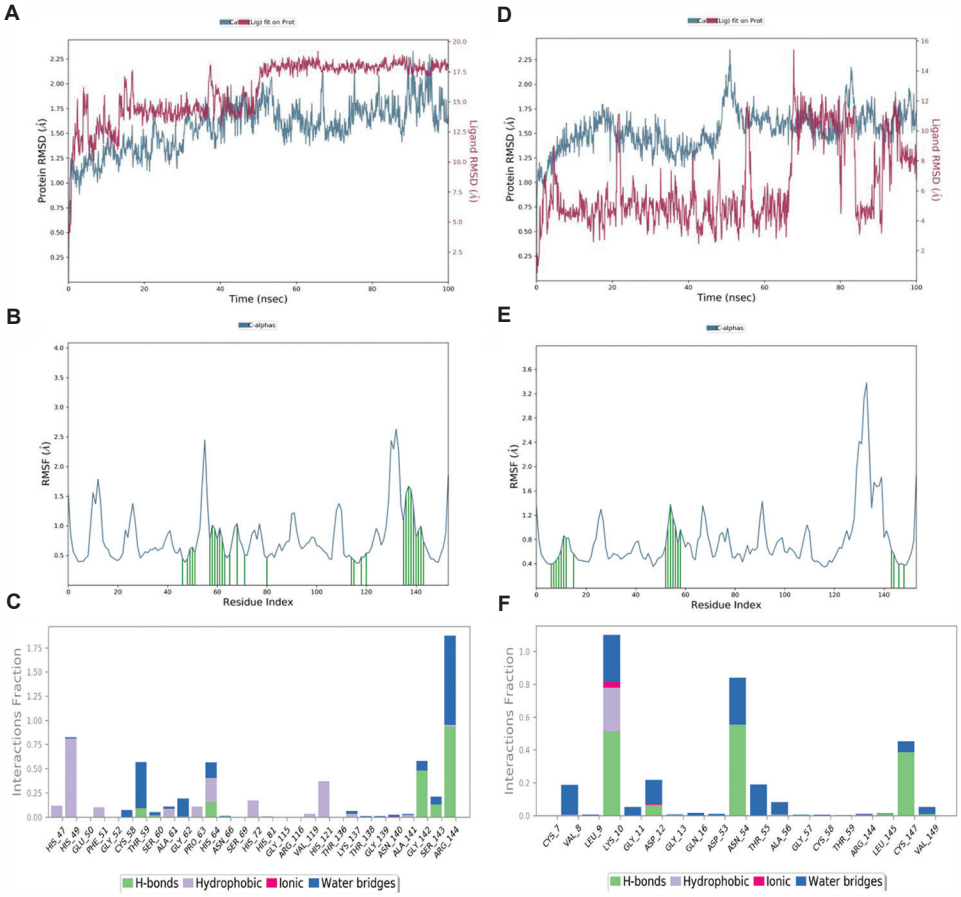

Figure 4. Protein-ligand complex simulation results. (A) RMSD of compound CID 36791369 – SOD1. (B) RMSF of SOD1. (C) Interaction profile of the contact between compound CID 36791369 – SOD1. (D) RMSD of compound CID 30935 – SOD1. (E) RMSF of SOD1. (F) Interaction profile of the contact between compound CID 30935 – SOD1.
Abbreviations: RMSD: Root-mean-square deviation; RMSF: Root-mean-square fluctuation; SOD1: Superoxide dismutase 1.

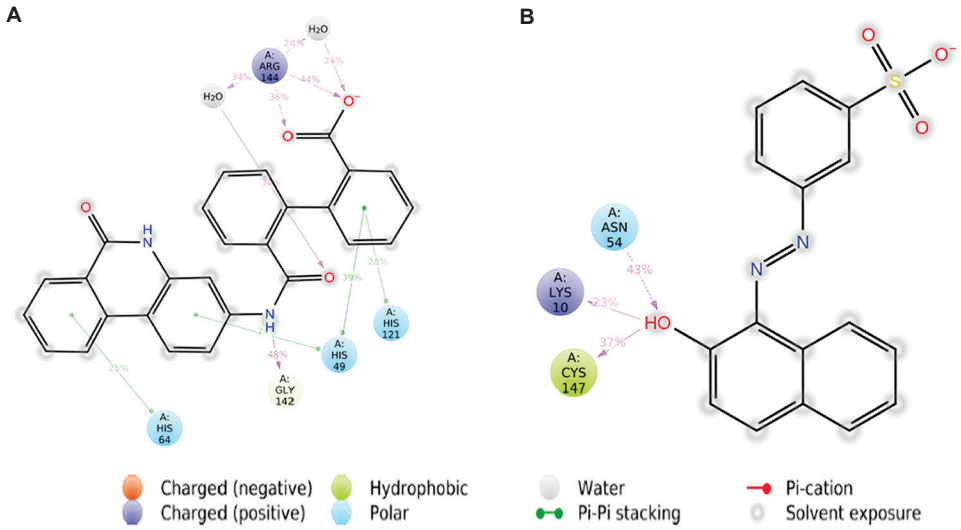

Figure 5. A schematic of detailed ligand-protein interactions. (A) SOD1 with compound CID 36791369. (B) SOD1 with compound CID 30935.
Abbreviation: SOD1: Superoxide dismutase 1.

Table 3. Prime MMGBSA binding energy of interaction of SOD1 with compound CID 36791369 and compound CID 30935, respectively, before and after molecular dynamics simulation

Complex	Simulation time (ns)	MMGBSA ΔG^{bind} (kcal·mol^{-1})							
		Coulomb	Covalent	Hbond	Lipo	Packing	Solv_GB	vdW	G^{bind} (Total)
CID 36791369 – SOD1	0	26.424	8.668	−0.000	−17.151	−5.576	−18.209	−27.025	−32.867
	100	13.001	2.805	−0.611	−15.064	−8.597	−1.900	−45.746	−56.110
CID 30935 – SOD1	0	−3.166	0.816	−1.223	−6.874	−1.900	12.937	−29.108	−28.518
	100	−4.042	4.773	−1.955	−11.130	−1.686	13.991	−39.742	−39.790

Notes: Coulomb: Coulomb energy; Covalent: Covalent binding energy; Hbond: Hydrogen bonding energy; Lipo: Lipophilic energy; Packing: Pi-pi packing correction; Solv GB: Generalized Born electrostatic solvation energy; vdW: van der Waals energy; Total: Total energy (Prime energy).

that both complexes were stable and energetically favorable under simulated physiological conditions.

4. Discussion

SOD1 serves as a therapeutic target for cancer treatment, as inhibiting its activity leads to the downregulation of multiple signaling pathways crucial for endothelial and tumor cell function.[2] PPI analysis of SOD1 in this study revealed its association with other antioxidant genes, the dysfunction or deregulation of which have been implicated in various cancers (e.g., breast cancer and chronic lymphatic leukemia) and neurodegenerative diseases (e.g., ALS and Parkinson's).[27-31]

SOD1 is centrally connected to other proteins encoded by disease-associated genes, which often become prominent components of ubiquitin-positive inclusions found in carriers of corresponding mutations. These proteins include TDP-43, fused in sarcoma, optineurin, ubiquilin-2, ataxin-2, and C9ORF72 (encoded by *TARDBP*, *FUS*, *OPTN*, *UBQLN*, *ATXN2*, and *C9ORF72* genes, respectively).[31,32]

A potential mechanism underlying SOD1 pathogenesis in ALS involves gain-of-interaction, where mis-folded soluble SOD1 forms abnormal PPIs with various cellular proteins, including other SOD1 molecules, thereby disrupting their function. These aberrant PPIs are associated with numerous human diseases, such as cancer, infectious diseases, and neurodegenerative disorders.[33] Identifying and studying PPI networks in disease states is crucial for developing therapies targeting interactions that play functional roles in disease progression and patient outcomes.[34]

Compound SN1 (PubChem CID: 36791369), with the IUPAC name 2-[2-[(6-oxo-5H-phenanthridin-3-yl) carbamoyl]phenyl]benzoate, is a patented compound known to differentially modulate connexin and/or pannexin hemichannels in astrocytes without affecting gap junctions. It finds applications in the treatment of psychiatric disorders.[35] Inhibition of SOD1 protects protein tyrosine phosphatases from oxidation by preventing the formation of hydrogen peroxide, thereby inhibiting ERK phosphorylation in cells stimulated with EGF, FGF-2, or IGF-1.[2] The activity of growth factors such as EGF, IGF-1, PDGF, and VEGF is redox-regulated. When a growth factor binds to its receptor tyrosine kinase, it activates the production of reactive oxygen species, leading to phosphatase inactivation and favoring phosphorylation within cells, thus allowing kinase cascades to propagate.[2] In addition, inhibition of SOD1 by tetrathiomolybdate increases the steady-state levels of superoxide in human umbilical vein endothelial cells, inhibiting their proliferation and subsequently abolishing FGF-2- and VEGF-mediated ERK1/2 phosphorylation.[36]

The compounds obtained in this study were mostly not BBB permeants and not P-gp substrates, exhibited good solubility, and only a few exhibited inhibitory effects on selected cytochrome P450s (CYP2C9, CYP2C19, CYP3A4, CYP1A2, and CYP2D6). This result suggests that their bioavailability is affected by CYPs through the first-pass effect rather than P-gp. Specifically, compounds SN5, SN6, SN7, SN12, and SN25 exhibited BBB permeant features and had moderate to no inhibitory effects on CYPs. P-gp can influence drug bioavailability, and its modulation is considered in drug development to enhance efficacy.[37] Modulation of CYP activities can alter drug metabolism profiles, affecting bioavailability or efficacy.[38]

In this study, compounds SN1, SN5, SN6, SN7, SN12, SN19, SN20, SN25, and SN36 exhibited sufficient binding affinity, with values less than -5.0 kcal·mol^{-1}. Previous research has identified specific SOD1 amino acid residues involved in hydrogen bond formation with various compounds, including L-methionine, aniline (2-methoxy-5-methylaninline), and 2-trifluoromethyl-4-aminoquinazoline.[39] Notably, compounds SN5, SN6, SN7, and SN25 in this study were quinazoline-based compounds. Another study proposed SOD1 as a target of the LCS-1 compound, with the IUPAC name 4,5-dichloro-2-m-tolylpyridazin-3(2H)-one.[13]

Disrupting SOD1 activity has been shown to inhibit cell growth and enhance lipid accumulation in nasopharyngeal carcinoma, a commonly occurring cancer with the highest incidence of malignant proliferation among head and neck cancers.[40] SOD1 mRNA has been reported to be significantly increased in head and neck cancer tissues, according to data from the Oncomine microarray database (https://www.oncomine.org). Multiple signaling pathways, including ERK, NF-κB, and PI3K-Akt, which are essential for the onset and development of cancer, can be activated by intracellularly sustained high levels of hydrogen peroxide provided by elevated activity and expression of copper/zink-containing SOD1. The use of SOD1 inhibitors, including LD100, U0126, LY294002, and BAY117082, has been reported to attenuate some of these signaling pathways.[41]

Molecular docking was utilized to assess the binding affinity of the protein with the ligand, facilitating the determination of the binding site and type of inhibitions. A binding affinity score of ≤ -5.00 kcal·mol^{-1} indicates a good ligand-protein interaction.[42] A previous computational study utilizing molecular docking and MDS has demonstrated that 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside, hesperidin, and hyperoside have high affinity to mutant SOD1, which is relevant to ALS.[43]

MDSs were then conducted to evaluate atomic-level variations in the protein-ligand system and assess the stability of the protein-ligand complex in a dynamic environment.[44] RMSD values less than 4 Å suggest relatively small conformational changes in the complexes during the simulation, indicating their stability.[45] In addition, the RMSF is useful for characterizing local changes along the protein chain. Prime MMGBSA provided various energy properties, reporting energies for the ligand, receptor, and complex structures, as well as energy differences related to strain and binding.[26] The more negative the score, the higher the free energy released in complex formation. The total binding free energy confirmed the stability of the complexes under physiological conditions, indicating their reasonable stability. A high total energy indicates a strong binding affinity between the molecules. However, the interpretation depends on the specific contributions of individual energy terms and the context of the study.

In this study, the MMGBSA binding energy of the compound CID 30935 – SOD1 complex is influenced by several contributing energies, such as Coulomb, covalent, and hydrogen bonding. A high Coulomb energy indicates robust electrostatic interactions between charged groups, suggesting either strong attraction or repulsion between the binding partners. Understanding these interactions is significant for elucidating the stability or specificity of the binding interaction. Meanwhile, a high covalent binding energy suggests the formation of strong covalent bonds between the binding partners, which may indicate a stable binding complex. However, it is important to note that in drug design, covalent binding can sometimes lead to undesirable side effects, necessitating careful consideration. Moreover, a high hydrogen-bonding correction suggests the formation of strong hydrogen bonds between the binding partners, contributing to the specificity and stability of the binding interaction.

Regarding the compound CID 36791369 – SOD1 complex, MMGBSA binding energy relies on various contributory energies, such as lipophilic, pi-pi, generalized Born electrostatic solvation, and van der Waals interactions. High lipophilic energy suggests robust hydrophobic interactions, which are important for stabilizing binding complexes, especially within hydrophobic pockets of proteins. However, excessively high lipophilic energy might indicate a propensity for non-specific binding to hydrophobic regions. Similarly, high pi-pi packing correction indicates strong interactions between aromatic rings, which are essential for stabilizing binding complexes, especially in protein-ligand interactions. Moreover, high solvation energy may suggest strong interactions between the solute and solvent molecules, potentially affecting the overall stability of the binding complex. However, excessively high solvation energy could imply poor solubility or unfavorable interactions with the solvent. High van der Waals energy indicates robust, attractive interactions between non-polar groups, contributing to the stability of the binding complex. However, excessively high van der Waals energy might lead to non-specific binding or aggregation.

5. Conclusion

This study explored several inhibitors of human SOD1 using enrichment virtual screening, docking, ADME prediction, and molecular dynamics simulation. Among the 42 compounds selected for thorough analyses, five demonstrated favorable ADME properties, promoting BBB permeability and high GIA. In addition, seven compounds exhibited binding affinity values of less than -5.00 kcal·mol^{-1} for SOD1, indicating robust interaction. Specifically, compounds SN5, SN6, SN7, SN12, and SN25 emerged as potential SOD1inhibitors. Further research will be necessary to investigate the therapeutic effectiveness of these top five compounds in both *in vitro* and *in vivo* settings against SOD1.

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Conflict of interest

No conflicts of interest to declare.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data used in this study are available on request from the corresponding author.

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

ORIGINAL RESEARCH ARTICLE

Establishment of a myostatin gene-knockout C2C12 cell line and evaluation of related microRNA expression

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Abstract

The strategy of blocking myostatin (MSTN) signal transduction has long been regarded as a promising approach in the treatment of patients with muscle loss. However, individuals taking blocking agents often encounter issues such as lack of strength, fatigue, and poor muscle proliferation due to muscle hypertrophy and the involvement of multiple receptors. To address these challenges, a series of experiments were conducted on a C2C12 cell line in this study. First, the pX601-SaCas9-sgRNA/puro vector carrying a Cas9-encoded gene was constructed and subsequently used to produce *Mstn*-knockout (*Mstn*-KO) C2C12 cell lines. The expression level of the MSTN protein and the growth characteristics of the cell lines were verified. Moreover, the expression of muscle growth-related microRNAs in the cell lines was analyzed through real-time polymerase chain reaction (PCR). The results indicate that we have successfully established a method for constructing *Mstn*-KO cell lines with stable passage. No expression of the MSTN protein and strong cell proliferation were observed in the cell lines. Moreover, real-time PCR experiments showed that the expression levels of miR-1, miR-431, miR-206, and miR-133a were significantly increased ($P < 0.01$), the expression level of miR-23a was significantly increased ($P < 0.05$), and the expression level of miR-486 was significantly decreased ($P < 0.05$). These findings indicate that multiple miRNAs are closely associated with MSTN regulation. This study lays the foundation for further investigation into the effects of the *Mstn* gene on the physiological function of myoblasts and the development of drugs that block the MSTN signaling pathway.

Keywords: Myostatin; Gene knockout; C2C12 cell line; MicroRNA; Muscle growth

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1. Introduction

Myostatin (MSTN), a member of the transforming growth factor-β (TGF-β) superfamily, exerts a negative regulatory effect on skeletal muscle growth and development.[1] Gene-

targeting studies have shown that MSTN-deficient mice exhibit a dramatic increase in overall skeletal muscle mass, with individual muscle sizes approximately twice their normal size.[2] Subsequent studies have shown that the *MSTN* gene is highly conserved throughout evolution, with the mature MSTN amino acid sequences remaining identical across different species.[3] Targeting or naturally mutating MSTN in cattle,[4] sheep,[5] dogs,[6] pigs,[7] and humans[8] leads to significant muscle gain. In addition to its role in muscle growth regulation, MSTN also plays an important role in bone development, fat browning, and metabolism.[9-11]

Therefore, understanding the molecular mechanism of the *MSTN* gene is paramount for identifying active molecules associated with MSTN regulation and for developing drugs that target this pathway. Previous studies have primarily focused on cytokines regulated by MSTN,[12-14] with fewer studies reporting on the effect of microRNA (miRNA) variation on muscle growth and development. MiRNAs are evolutionarily conserved molecules widely present in various organisms and play roles in fundamental biological processes such as cell proliferation, differentiation, apoptosis, and tumorigenesis.[15,16] Notably, a subclass of miRNAs specifically exists in muscle tissues and plays an important role in muscle development.[17,18] Rachagani *et al.*[19] demonstrated that MSTN might regulate the expression of miR-133a/b, miR-1, and miR-206 in skeletal muscle, with these miRNAs being significantly upregulated in *Mstn*-knockout (*Mstn*-KO) mice compared with wild-type and heterozygous mice. In addition, the findings of Wada *et al.*[20] demonstrated that miR-23a is associated with atrophy and hypertrophy processes, inhibiting the expression of ubiquitin protease (*Murf-1* and *Atrogin-1* mRNA) to prevent atrophy in skeletal muscle. This inhibition is consistent with the signaling pathway effects observed after *Mstn* knockout, suggesting a potential interaction.[20,21] The regulatory effects of MSTN on miRNA expression related to muscle growth and development and the specific signal transduction pathways involved remain unclear.

This study aims to establish a *Mstn*-KO C2C12 cell line and verify whether MSTN regulates skeletal muscle growth and development through associated miRNA changes. The results are intended to lay the foundation for further research on the active molecules and cellular mechanisms of MSTN at the miRNA level, ultimately shedding light on the development of drugs that block MSTN signal transduction.

2. Materials and methods

2.1. Experimental materials

The experimental materials used in this study were purchased from various suppliers, with detailed related information as follows: C2C12 cell line from Wuhan Punosey Life Technology Co. Ltd (China); pX601 and pKO.1 plasmid carriers from Chengdu Chuanshikewei Biotechnology Co. Ltd (China); TRIzol reagent from Invitrogen (USA); DNA glue recovery kit and cell genome DNA extraction kit from Shanghai Shenggong Biotechnology Co. Ltd. (China); HiScript II 1st Strand cDNA Synthesis Kit from Nanjing Nuoweizan Biotechnology Co. Ltd (China); TurboFect transfection reagent from Promega (USA); DH5α competent cells from Takara Bio Co. Ltd (Japan); high glucose DMEM medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), and trypsin were purchased from Gibco Co. Ltd (USA); plasmid extraction kit from QIAGEN Corporation (Germany); Q5 DNA polymerase, Bsa I endonuclease, BamH I endonuclease, EcoR I endonuclease, 10 × NEBuffer 2, T4 DNA ligase, and T7E I endonuclease from NEB Co. Ltd (USA); DL2000 Marker, DL10000 Marker, 50 bp DNA Ladder, TB Green® Fast qPCR Mix from Takara Co. Ltd (Japan); Murine Anti-MSTN antibody, murine anti-GAPDH antibody, and horseradish peroxidase-coupled Goat anti-mouse IgG from Abcam Limited (UK); protease inhibitor, strong RIPA lysate, and bicinchoninic acid assay (BCA) protein quantitative detection kit from Biyuntian Biotechnology Co. Ltd. (China); CCK-8 kit from Dongren Chemical Technology (Shanghai) Co. Ltd. (China); and fluorescent dye propidium iodide (PI) from Beijing Zhuangmeng International Biological Gene Technology Co. Ltd (China).

2.2. Design of sgRNA targeting sites in the *Mstn* gene

The mice *Mstn* gene (NM_010834.3) was downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/). The *Mstn* exons sequence was uploaded into the sgRNA Design software (http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design). Based on the score and the 5' region of the exon, two pairs of suitable sgRNAs were selected and named sgRNA1-MSTN and sgRNA2-MSTN. Fragments of ACCT and AAAC were added to the 5' end of the sgRNA sense and antisense chain templates, respectively, to complement the adhesive ends of the pX601 carrier after Bsa I endonuclease digestion. The primers for detecting target site knockout were designed and identified (Table 1). The sgRNA single nucleotide chains and related primers were synthesized by Shenggong Biotechnology Co. Ltd. (China).

2.3. Vector construction

The puromycin (Puro) resistance gene was amplified from the pLKO.1 vector using the designed hPGK-puro upstream and downstream primers. The polymerase

Table 1. Oligonucleotide sequences in this study

Name	Oligonucleotide sequence (5' – 3')
sgRNA1-MSTN	F: acctCATGCTTTAACACTGCCTA
	R: aaacTAGGCAGTGTTAAAGCATG
sgRNA2-MSTN	F: acctGAGGAAATGAAAGCGATTCTCC
	R: aaacGGAGAATCGCTTTCATTTCCTC
sgRNA1-MSTN-test (520 bp-184/336)	F: AATGCATGTACTTGGAGACA
	R: AGTCCTTGCCTTGGTGGTAT
sgRNA2-MSTN-test (533 bp-175/358)	F: GTCACTTAAGCATAAGCTAC
	R: GTGATAAACCTGGTAGCCTC
hPGK-puro (BamHI/ EcoRI -1121bp)	F: ggatccATGTCGACAGGGACAGCAGAGATC
	R: gaattcATGTGCCTACAGCTGCCTTGTAAG

chain reaction (PCR)-amplified product of the puromycin resistance gene and the pX601 vector were then digested with BamH I and EcoR I endonucleases. The digestion products were ligated and subsequently transformed into DH5α competent cells, which were inoculated on an LB plate with ampicillin and cultured overnight at 37°C. Monoclonal bacteria were selected and sequenced, and the plasmid with the correct clone was extracted. The final plasmid was named pX601-puro.

For the sgRNA-MSTN construction, 100 μM sgRNA-MSTN-F/R were mixed in equal amounts for annealing. Two micrograms of pX601-puro were digested with Bsa I endonuclease at 37°C. The digested products were detected using agarose gel electrophoresis, and the linear carrier pX601-puro was recovered using a gel extraction kit. The recovered pX601-puro and the annealed sgRNAs were subsequently ligated by T4 DNA ligase. The ligation mixture was transformed into DH5α competent cells, inoculated onto an LB plate with ampicillin, and cultured overnight at 37°C. Five colonies were selected, colony PCR screened, and then cultured. The plasmid DNA was extracted and sequenced using pX601 universal primers. The sequencing results were compared with the designed sequences to confirm the correct insertion of sgRNA. Large-scale plasmid extraction was performed using the plasmid extraction kit. The concentration of the extracted plasmids pX601-puro-sgRNA-MSTN1 and pX601-puro-sgRNA-MSTN12 (abbreviated pX601-sgMSTN1 and pX601-sgMSTN2, respectively) was diluted to 1 μg/μL for later use.

2.3.1. Cell culture, transfection, and screening

The C2C12 cells were cultured in DMEM containing 10% FBS at 37°C with 5% CO_2. Before transfection, the C2C12 cells were seeded into 6-well plates at a concentration of 2×10^5/mL. Upon reaching 70% confluency, the cells were transfected with either pX601 empty vector, pX601-sgMSTN1, or pX601-sgMSTN2 utilizing TurboFect transfection reagent. These groups were designated as the control group (CN), the sgMSTN1 experimental group (sgMSTN1), and the sgMSTN2 experimental group (sgMSTN2), respectively. After 48 h, puromycin was added at a concentration of 2 μg/mL to completely eliminate cells in the control group. Following resistance screening, the surviving cells were transferred to 96-well plates to establish monoclonal cell lines. The growth characteristics of monoclonal cells in each well were observed for 7–14 days, and cells displaying desirable growth characteristics were selected and transferred to 24-well plates for further expansion. Genomic DNA was extracted from each monoclonal cell line using a genomic DNA extraction kit, followed by PCR amplification using the identification primers sgRNA1-MSTN-Test and sgRNA2-MSTN-Test. The gene-knockout C2C12 monoclonal cell lines were confirmed by sequencing the PCR products using upstream primers.

2.4. Identification of T7E I endonuclease

First, genome DNA was extracted from different groups of C2C12 cells 48 h post-transfection. Subsequently, PCR amplification of the intracellular *Mstn* gene was performed using identification primers sgRNA1-MSTN-Test and sgRNA2-MSTN-test. The resulting target fragments were recovered using a 1% agarose gel containing a nucleic acid dye after electrophoresis. The recovered DNA sequence was annealed at 95°C for five minutes, followed by gradual cooling from 95°C to 85°C at a rate of 2°C/s, then from 85°C to 25°C at a rate of 0.1°C/s, and finally, the reaction was stopped at 4°C. Following annealing, 0.5 μL of T7E I endonuclease was added, and the mixture was incubated at 37°C for 30 min. Subsequently, polyacrylamide gel electrophoresis was performed to examine the digestion. The gel was then stained with SYBR Green staining solution for 1 h, and images were captured using a gel imaging system.

Image J quantitative software was used to calculate the indel rate using Equation I:

$$\text{Indel rate} = 100\% \times -\left(\sqrt{1-(b+c)/(a+b+c)}\right) \quad \text{(I)}$$

Where a and b represent the gray values of the new strip generated by cutting, and c represents the gray value of the uncut strip.

2.5. Real-time PCR analysis

Different groups of C2C12 cells were collected, and total RNA was extracted using the TRIzol reagent. The RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer. Subsequently, miRNAs

were converted into cDNA from 1 μg of total RNA using the HiScript II 1st Strand cDNA Synthesis Kit according to the manufacturer's instructions. Real-time PCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR Cycler (Applied Biosystems, USA) and amplified using the TB Green® Fast qPCR Mix following the manufacturer's instructions. The thermocycling conditions were as follows: predenaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, and annealing at 60°C for 30 s.[22] The gene expression level was calculated using the formula $2^{-\Delta\Delta Ct}$ to determine the relative expression level of the target gene, with U6 snRNA serving as the internal reference gene. The primers for real-time PCR are listed in Table 2.

2.6. Western blot analysis

First, the total protein was extracted from C2C12 cells in different groups, and then, the protein concentration was determined using the BCA method. The specific protocol followed the instructions provided with the BCA protein quantitative detection kit. Samples with known protein concentrations were supplemented with 5× loading buffer and boiled for 10 min to ensure thorough denaturation of the protein samples. Subsequently, 30 μg of total protein samples were loaded into each well, and electrophoresis was performed at 110 V. Before protein transfer, the polyvinylidene fluoride (PVDF) membrane was pre-soaked in methanol, and then, the electrophoretically isolated proteins were transferred onto the PVDF membrane using an electric current. The membrane was then treated with 5% bovine serum albumin (BSA) at room temperature for 1 h, followed by overnight incubation with MSTN/GAPDH primary antibody diluted in 3% BSA (1:1000) at 4°C. Subsequently, the membrane was washed three times using tris-buffered saline with 0.1% Tween® 20 detergent (TBST). Subsequently, the membrane was exposed to HRP-coupled goat anti-mouse secondary antibody (1:2000) at room temperature for 1 h. Finally, the resulting image was captured after washing the membrane three times with TBST.

Table 2. List of primers for real-time PCR

Name	Oligonucleotide sequence (5' – 3')
miR-23a	F: TTGGCCGGCTGGGGTTCCTG
	R: AGGTCAGTTGGAAATCCCTG
miR-1	F: CGAACTACCTGCTTGGGGCA
	R: CTGGCCTGAAATACACACTT
miR-133a	F: CCCTGCTCTGGCTGGTCAAAC
	R: TTGCCAGCCCTGCTGTAGCTGG
miR-206	F: CCAGGCCACATGCTTCTTTA
	R: CCAAAACCACACACTTCCTTAC
miR-431	F: CGTCCTGCGAGGTGTCTTGC
	R: GATGTCGTCTTGCGAGAAGC
miR-486	F: CAGCCAGCTCTGATCTCGCC
	R: TGGCTTGTTCCCCGTTGTCTC
U6 snRNA	F: ATTGGAACGATACAGAGAAG
	R: GGAACGCTTCACGAATTTG

Abbreviation: PCR: Polymerase chain reaction.

2.7. Evaluation of proliferative characteristics of C2C12 cells using CCK-8

Suspensions of C2C12 cells from different groups were transferred into 96-well plates at a density of 5×10^3 cells/well. After 48 h of incubation, the culture medium was replaced. Subsequently, 10 μL of CCK-8 solution was added to each well, and the 96-well plates were further incubated for 2 h. The absorbance at 450 nm was then measured using an enzyme-linked immunoassay.

2.8. Measurement of the growth cycle of C2C12 cells using propidium iodide

Different groups of C2C12 cells (2×10^6 cells) were digested using the pancreatic enzyme (200 g) and centrifuged for 5 min to collect the cell pellet. The cells were then washed twice with pre-cooled PBS. The cell pellets were suspended in 0.5 mL of PBS, followed by the addition of 1.2 mL of pre-cooled pure ethanol. The suspended cells were gently mixed to prevent cell aggregation and then fixed at 4°C for 18 h. After fixation, the cells were washed once with 1 mL of PBS, centrifuged to obtain a cell pellet, and then, 100 μL of RNaseA and 400 μL of PI were added to the mixture. The samples were incubated at 37°C for 30 min in the dark. The stained samples were analyzed using flow cytometry, and the results were analyzed using the cell cycle software ModFit 3.

2.9. Statistical analysis

Statistical analysis and visualization of experimental results were performed using GraphPad Prism 6. Data from each group were collected from three independent replicates. Independent *t*-tests were used to compare significant differences between groups. A *P*-value between 0.01 and 0.05 was considered indicative of a significant difference, while a $P < 0.01$ was considered a highly significant difference.

3. Results

3.1. Construction of pX601-puro-sgRNA vectors

The vector construction method is illustrated in Figure 1A. First, the puro resistance gene containing the hPGK promoter was inserted into the pX601 linear vector to construct the pX601-puro plasmid. Subsequently, sgRNA1-MSTN and sgRNA2-MSTN were inserted

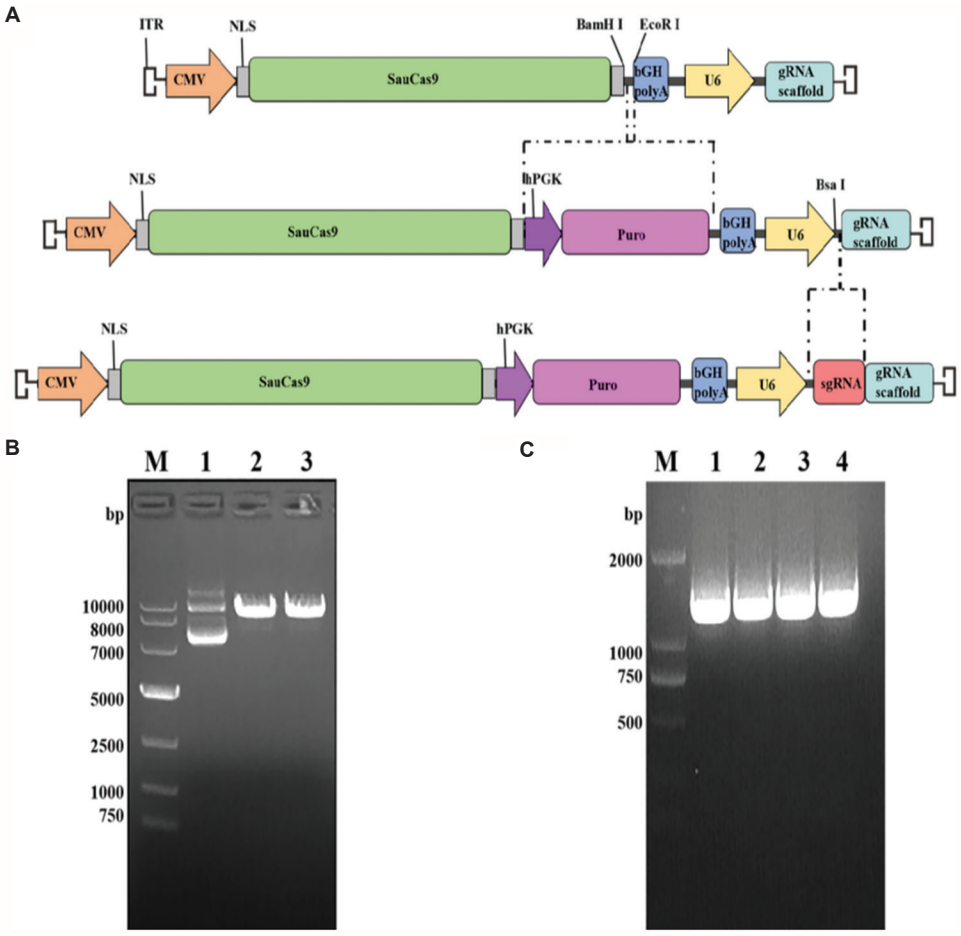

Figure 1. Structure diagram of the pX601-puro-sgRNA vectors and their identifications. (A) Structure diagram of the plasmids. (B) Agarose gel electrophoresis of the pX601-SaCas9 vector. Notes: M: DL10000 DNA Marker; 1: Expression vector of Cyclic pX601-SaCas9; 2: Expression vector of linear pX601-SaCas9 cut by BamHI endonuclease; 3: Expression vector of linear pX601-SaCas9 cut by EcoRI endonuclease. (C) Agarose gel electrophoresis of purinomycin resistance gene. Notes: M: DL2000 DNA Marker; 1 – 4: The hPGK promoter and purinomycin resistance gene.
Abbreviations: bGH polyA: Bovine growth hormone polyadenylation signal; CMV: Cytomegalovirus promoter; ITR: Inverted terminal repeat; NLS: Nuclear localization signal; Puro: Puromycin.

between the U6 promoter of the pX601-puro linear carrier and gRNA scaffold to construct the pX601-puro-sgRNA plasmid (Figure 1A). The position of the linear plasmid cut by BamHI and EcoRI endonucleases in the agarose gel was higher than that of the circular plasmid without the enzyme, with the fragment positioned at 7345 bp, which was consistent with the length of the pX601 expression vector (Figure 1B). PCR was used to amplify the hPGK promoter and purinomycin resistance gene, yielding an amplified product of 1121 bp, consistent with the combined size of the hPGK promoter and purinomycin resistance gene (Figure 1C).

3.2. Identification of the efficiency of enzyme digestion at sgRNA sites

The editing efficiency of the constructed vector at the target site was assessed using T7E I endonuclease, known for its ability to recognize and cleave imperfectly paired double-stranded DNA. In the CN cells transfected with the empty vector, a single band corresponding to the target site was observed. Conversely, in the sgMSTN1 and sgMSTN2 cells, two additional bands appeared below the target band, indicating the presence of base mutations in the edited genes. Analysis using Image J software revealed a mean editing indel of 30.7% for the sgMSTN1 cells and 19.1% for the sgMSTN2 cells (Figure 2A and B).

3.3. Sequencing and identification of the *Mstn*-KO C2C12 cell line

The sgMSTN1 and sgMSTN2 cells, selected using purinamycin screening, were subjected to limited dilution to obtain single-cell cultures. Monoclonal cell lines with mutations in the *Mstn* gene were identified through sequencing analysis. In the sgMSTN1 group, the 1# cell

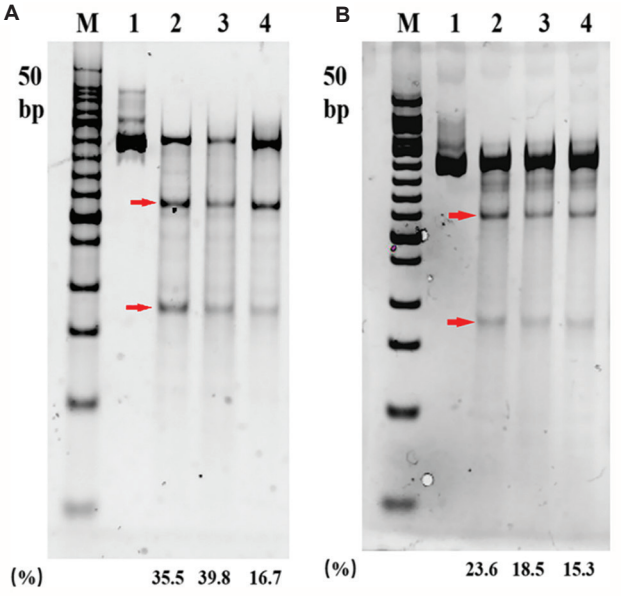

Figure 2. Assessment of knockout efficiency of pX601-SaCas9-sgRNA in C2C12 cells. (A) Agarose gel electrophoresis results demonstrating knockout efficiency at the sgRNA1 site. Notes: M: 50 bp DNA Ladder; 1: Control cells transfected with empty pX601-SaCas9 plasmid; 2 – 4: Gene-edited cells transfected with pX601-SaCas9-sgRNA1 plasmid. (B) Agarose gel electrophoresis results illustrating knockout efficiency at the sgRNA2 site. Notes: M: 50 bp DNA Ladder; 1: Control cells transfected with empty pX601-SaCas9 plasmid; 2 – 4: Gene-edited cells transfected with pX601-SaCas9-sgRNA2 plasmid. The estimated percentage of gene editing per sample is indicated at the bottom.

line exhibited a 20-base deletion at the target compared to the standard sequence. The 2# cell line demonstrated a minimum of 13 base mutations at the target, while the 3# cell line had a 4-base deletion and a 6-base mutation at the target site (Figure 3A). Similarly, in the sgMSTN2 group, the 1# cell line displayed a 4-base deletion at the target site compared to the standard sequence. The 2# cell line exhibited a 6-base deletion at the target site, and the 3# cell line had a 4-base deletion at the target site (Figure 3B).

3.4. Identification of MSTN protein expression and proliferative activity in the *Mstn*-KO C2C12 cell line

The expression levels of MSTN protein in each monoclonal cell line were determined using Western blotting. Monoclonal cell lines sgRNA1-KO and sgRNA2-KO, exhibiting complete non-expression of MSTN protein, were selected from the sgMSTN1 and sgMSTN2 groups for subsequent study. The expression levels of MSTN protein in the sgRNA1-KO, sgRNA2-KO, and CN groups are illustrated in Figure 4A. The proliferative characteristics of C2C12 cells following *Mstn* knockout were assessed using the CCK-8 method. Compared with the CN group, the optical density (OD) value at 450 nm was significantly increased in the sgRNA1-KO group ($P < 0.01$) and the

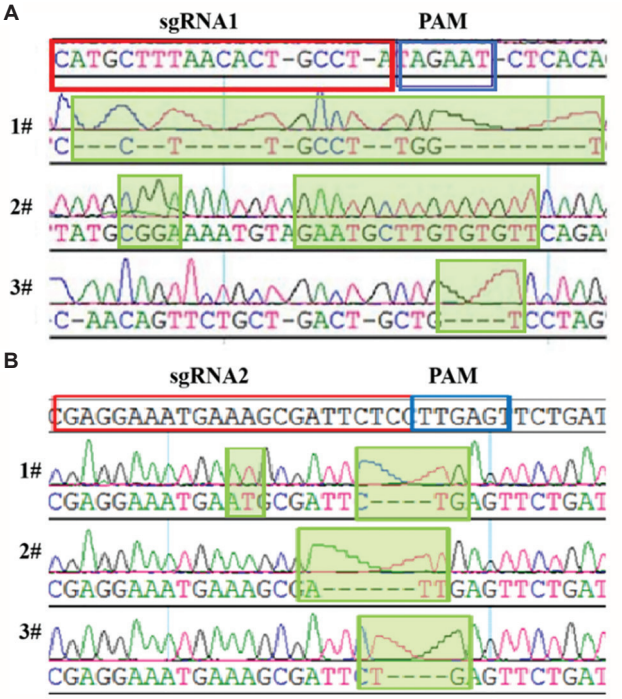

Figure 3. (A and B) Sequencing identification results for the C2C12 monoclonal cell
Abbreviation: PAM: Protospacer adjacent motif.

sgRNA2-KO group ($P < 0.01$). These results indicate that *Mstn* knockout upregulates the proliferative characteristics of C2C12 cells, leading to an increased cell number (Figure 4B). The cell cycle distribution of CN, sgRNA1-KO, and sgRNA2-KO cell lines at the G0/G1, G2/M, and S phases was analyzed through flow cytometry. The results indicate that the proportion of cells in the G0/G1 phase decreased from 81.19% in the CN group to 51.79% in the sgRNA1-KO group and 73.69% in the sgRNA2-KO group. Conversely, the proportion of the G2/M phase increased from 5.60% in the CN group to 10.83% in the sgRNA1-KO group and 9.12% in the sgRNA2-KO group. In addition, the proportion of cells in the S stage increased from 13.22% in the CN group to 37.37% in the sgRNA1-KO group and 17.20% in the sgRNA2-KO group. These results indicate that after *Mstn* knockout, C2C12 cells exhibit increased activity in the division phase and enhanced proliferative capacity (Figure 4C).

3.5. Expression of muscle-associated miRNA in the *Mstn*-KO C2C12 cell line

To verify the effect of MSTN on miRNA expression, we selected six miRNAs reported to influence or be related to muscle development for real-time PCR analysis. We observed that, compared with the CN group, the expression levels of miR-1, miR-431, miR-206, and miR-

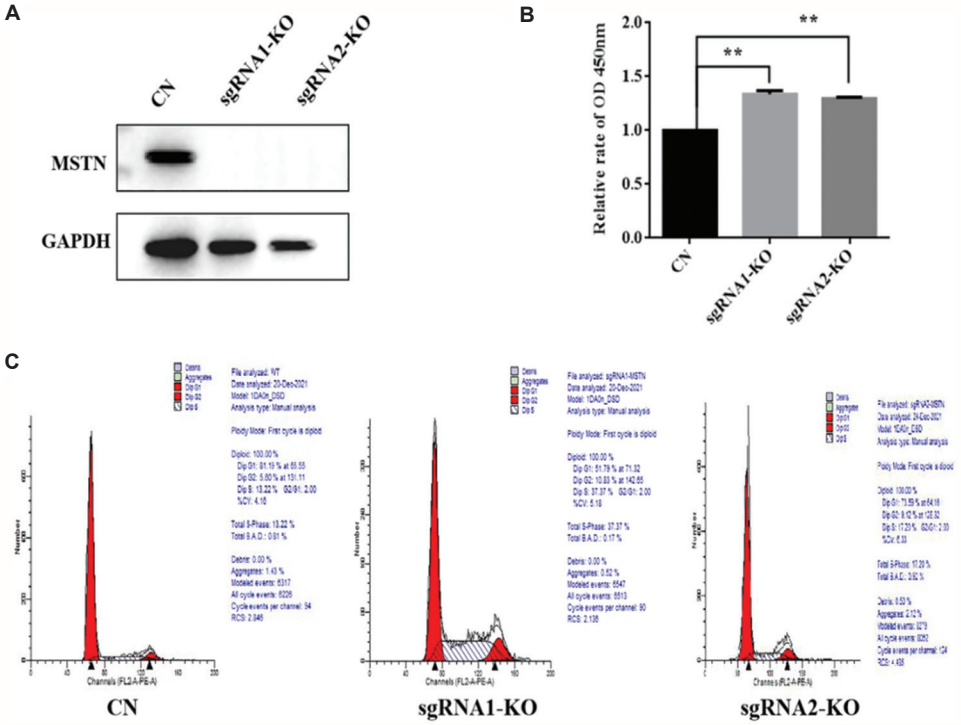

Figure 4. Change in MSTN protein expression and proliferative characteristics in the *Mstn*-KO C2C12 cell line. (A) Detection of MSTN protein expression in C2C12 monoclonal cell lines using Western blotting. (B) Evaluation of proliferative characteristics in the sgRNA1-KO, sgRNA2-KO, and CN groups using the CCK-8 assay. (C) Representative cell cycle analysis of the sgRNA1-KO, sgRNA2-KO, and CN groups was measured using flow cytometry.
Note: $^{**}P < 0.01$.

133a were significantly upregulated in both sgRNA1-KO and sgRNA2-KO groups ($P < 0.01$). The expression level of miR-23a was significantly upregulated ($P < 0.05$), while the expression level of miR-486 was significantly downregulated ($P < 0.05$). These results indicate that after *Mstn* knockout, the transcriptional levels of multiple miRNAs were changed in C2C12 cells (Figure 5).

4. Discussion

Studies on the regulation and function of MSTN in both normal and pathological states, along with numerous preclinical studies on the role of MSTN inhibition in mouse models of various human diseases, have supported the development of MSTN inhibitors for clinical applications.[11] However, all trials on muscular dystrophy patients have failed, mainly due to the failure of translating increased muscle mass into optimal muscle function after MSTN inhibition, such as force ratio[23] or fatigue resistance.[24] Changes in the function of other receptors may also affect muscle mass or strength.[25] In addition, inhibition of signaling in other cell types, such as those affecting fat content[26] and metabolism,[27] may interfere with muscle function. Due to these reasons, comprehensive studies on *Mstn* gene receptors, target genes, interaction factors, signaling pathways, satellite cells, and other aspects are necessary in both clinical trials and *in vitro* experiments to better apply MSTN inhibitors in the treatment of muscle degeneration diseases.

Gene-knockout cell lines play an important role in the study of cell regulatory mechanisms. Using CRISPR/Cas9-mediated gene editing technology, Wang *et al.*[28] obtained human embryonic stem cells (hESCs) with the knockout of RelA and IκBα. Multidimensional phenotypic evaluation and transcriptomic analysis demonstrated that RelA protects vascular cells against apoptosis and regulates the response of vascular inflammation to TNF-α stimulation, providing guidance for cardiovascular disease prevention and drug development. Pascucci *et al.*[29] used CRISPR/Cas9 technology to knock out the *MAGEC2* gene in the A375 melanoma cell line, demonstrating the role of the MAGEC2 protein in reducing p53 transcriptional activity in cells with overactive MEK/ERK signaling. These studies have revealed the integral role played by MAGEC2 in promoting tumor development, laying the foundation for the development of anti-tumor drugs. In this study, we successfully established and stably maintained an *Mstn*-KO C2C12 cell line, which exhibited abolished MSTN expression and enhanced cell growth ability. Notably, there was a significant difference

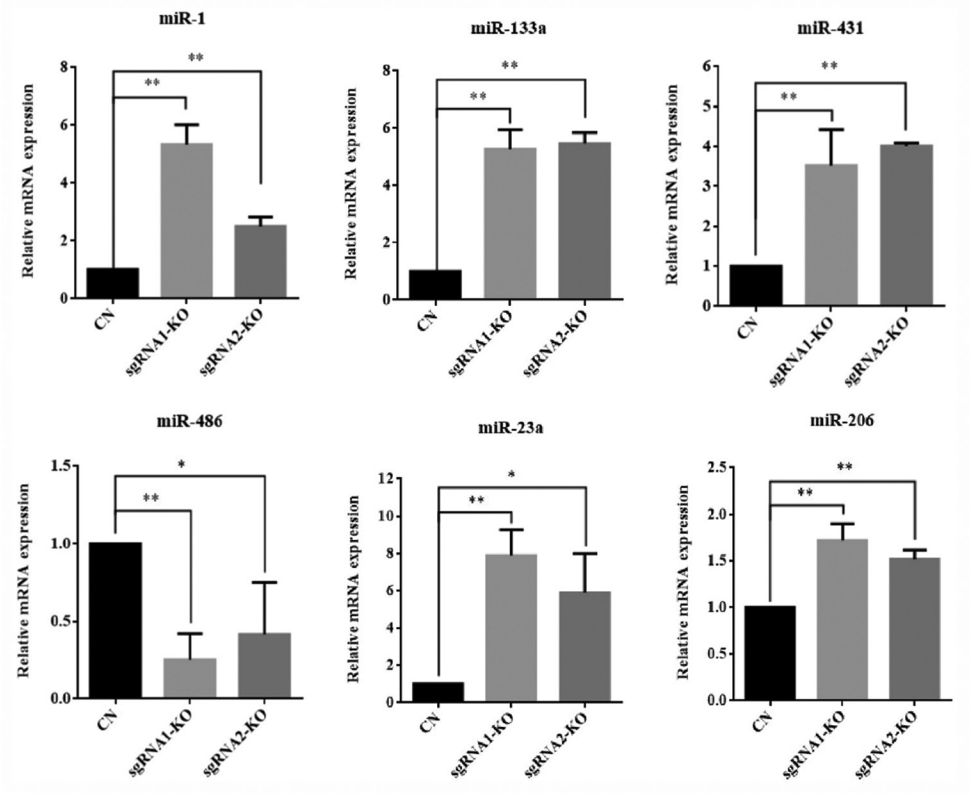

Figure 5. Expression of microRNAs in the *Mstn*-KO C2C12 cell lines. Notes: $^{*}P < 0.05$; $^{**}P < 0.01$.

in cell proliferative capacity between the wild-type and *Mstn*-KO groups, with the *Mstn*-KO groups exhibiting more than 1.75 times the cell volume of the wild-type group. In the cell cycle analysis, the proportion of cells in the G0/G1 phase was significantly reduced in the *Mstn*-KO group, while the proportion of cells in the G2 and S phases was increased, indicating that the proliferation activity of cells was enhanced after *Mstn* knockout.

MiRNA expression is essential for muscle formation and growth. Research by Hitachi *et al.*[30] has shown that overexpression of miR-486 induces myoblast hypertrophy *in vitro* and that miR-486 is essential for maintaining skeletal muscle size both *in vitro* and *in vivo*. In addition, miR-486 acts as an intermediate molecule connecting the MSTN signaling pathway and the Akt/mTOR pathway to regulate skeletal muscle size. Wu *et al.*[31] have shown that MSTN regulates miR-431 expression through the Ras-Mek-Erk signaling pathway, thereby affecting the proliferation and differentiation of C2C12 myoblasts. In this study, we selected six miRNAs related to muscle development to explore their role in MSTN regulation. Real-time PCR results showed that the expression levels of miR-133a, miR-23a, miR-1, miR-206, and miR-431 were significantly increased, while the expression level of miR-486 was significantly decreased in the *Mstn*-KO group. Therefore, we believe that multiple miRNAs related to muscle growth and development may be involved in the regulation of muscle cell growth and metabolism by MSTN.

5. Conclusion

In this study, the *Mstn*-KO C2C12 cell line was successfully established using pX601-SaCas9-sgRNA/puro, and knockout cells were identified through gene sequencing, nuclease identification, protein Western blotting assay, and flow cytometry. Analysis of the *Mstn*-KO C2C12 cell line revealed significantly increased expression levels of miR-1, miR-431, miR-206, miR-23a, and miR-133a, alongside significantly decreased expression levels of miR-486. These findings suggest that multiple miRNAs are closely involved in the regulation of MSTN. This study lays the foundation for further investigation of the effect of the *Mstn* gene on the physiological function of myoblasts and the development of pharmacological interventions targeting the MSTN signaling pathway.

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Conflict of interest

The authors declare no competing interests.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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ORIGINAL RESEARCH ARTICLE

Deciphering novel molecular gene expression signatures and pathways in cystic fibrosis through integrative bioinformatics strategies

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Abstract

Cystic fibrosis (CF), a fatal autosomal recessive disorder, is triggered by a genetic alteration of the CF transmembrane conductance regulator (*CFTR*) gene. On a global scale, around one in 3000 live births are affected with CF annually. While diagnosis and therapy are available for CF patients with non-specific and rare mutations, the current research is dedicated to exploring customized biomarkers, genes, signaling networks, and therapy for improving the management of CF. Although still in the early stages of development and validation, mRNA and gene-based treatment strategies are aimed to target patients who are resistant to *CFTR* gene restoration therapies. In this study, we utilized the systems biology approaches integrated with gene expression analysis to identify novel biomarkers and pathways for CF treatment. At first, out of 54,676 differentially expressed genes, we identified 104 upregulated and 107 downregulated genes. The upregulated genes were largely concentrated on Glutamatergic synapses, and the downregulated genes were enriched in ubiquitin-mediated proteolysis. Utilizing the enrichment analysis, we explored deeper into the pathways linked to these genes, with emphasis on relevant pathways involving bronchial epithelial cells. Following the enrichment analysis, we identified six essential genes: *WWP2*, *RNASEL*, *CUL1*, *CDC42*, *HDAC4*, and *UBA2*. Furthermore, the discovered genes were evaluated using expression profile analysis. Finally, our data indicate that the *WWP2* gene has a critical role in CF management. The current findings provide a coherent theoretical foundation for future experiments to further explore the *WWP2* gene as a unique and prognostic target for developing an effective CF therapeutic approach.

Keywords: Cystic fibrosis; Cystic fibrosis transmembrane conductance regulator; Differentially expressed genes; Upregulated gene; Downregulated gene; Biomarkers; Gene Expression Omnibus

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1. Introduction

Each year, thousands of new cystic fibrosis (CF) diagnosis in infants within one year of life are reported,[1] and approximately 162,428 individuals are living with CF worldwide.[2] In the USA, the leading cause of mortality for Caucasians is CF,[3] with an incidence rate of one in approximately 15,000–32,000 individuals within this specific ethnic group.[1] According to the European Cystic Fibrosis Society patient registry and analysis, it is predicted that by 2025, the percentage of individuals living with CF will increase by 75%.[4] The incidence rate of CF in Africa and Asia is thought to be very low.[5] Nevertheless, the true prevalence report of this disease is often underestimated due to a lack of awareness, inadequate healthcare benefits, and a high infant mortality rate.[1]

Franconi and Anderson were the first to characterize CF, an autosomal recessive disorder, in 1936 and 1938, respectively.[6,7] Knowledge about the CF transmembrane conductance regulator (*CFTR*) gene, which is responsible for the pathogenesis of CF, was first disclosed in 1989.[8] The CFTR is a transmembrane protein expressed at the outer surface of many epithelial cells and acts to control the flow of anions (Cl^- and HCO_3^-).[9,10] However, the mutation in the *CFTR* gene leads to dysfunctional transportation that impairs the function of a variety of organs.[10] According to the CF mutation database, more than 2000 mutations of the *CFTR* gene have been identified. Among them, defective protein folding is associated with the deletion of phenylalanine at the position of 508 (F508delCFTR), resulting in the incapability of peptide to cross the membrane of endoplasmic reticulum (ER) and the rapid ER-mediated degradation of entrapped protein.[7,11] The uses of small molecules as a corrector, as well as in gene therapy and low-temperature action, are promising strategies to restore the function of the aberrant *CFTR* gene.[12,13] For individuals who are resistant, gene- or mRNA-based therapy based on the *CFTR* gene restoration is the current mode of treatment. Moreover, at present, bacteriophages are given more considerations compared to traditional antibiotics in addressing the issue of multi-drug resistance of various bacteria.[14] Apart from the *CFTR* gene, several studies reported that epigenetically regulated genes can promote the severity of CF clinical symptoms.[15,16] Due to technological advancements in molecular biology, it is now possible to simultaneously analyze and measure relative expression of thousands of genes using DNA microarray technology. The pathogenesis of complex diseases can be explored using microarray-based gene expression profiling through establishing novel molecular gene signatures based on data available in Gene Expression Omnibus (GEO).[17-22]

To the best of our knowledge, the current work represents the first attempt to determine the specific pathways and gene signatures of CF using transcriptome profiling of human bronchial epithelial cells. In this study, we investigated the specific differentially expressed genes (DEGs), gene networks, pathways, and the interactions between proteins associated with CF. We utilized an integrative systems biology approach to identify DEGs in bronchial epithelial cells using data from the GEO database.

Combining different fields of study such as computer science, molecular biology, genetics, and statistics, bioinformatics provides a tool to solve issues involving molecular data by creating theoretical and computational models and tools. The escalating amount of biological and genetic data is managed using information technology approaches, which entail collection, storing, analysis, and integration of data. At present, bioinformatics is applied to a wide range of significant tasks, such as analysis and prediction of the regulatory network of genes, gene expression, protein, and gene structure, as well as functions and metabolic pathways, to understand specific gene-disease relation. Figure 1 shows a schematic layout of the full integrated bioinformatics analytical techniques for identifying unique gene signatures and associated pathways of CF.

2. Methods

2.1. Dataset collection

We performed the analysis using GEO dataset GSE70442. The transcriptomic profile (GSE70442) of bronchial epithelial

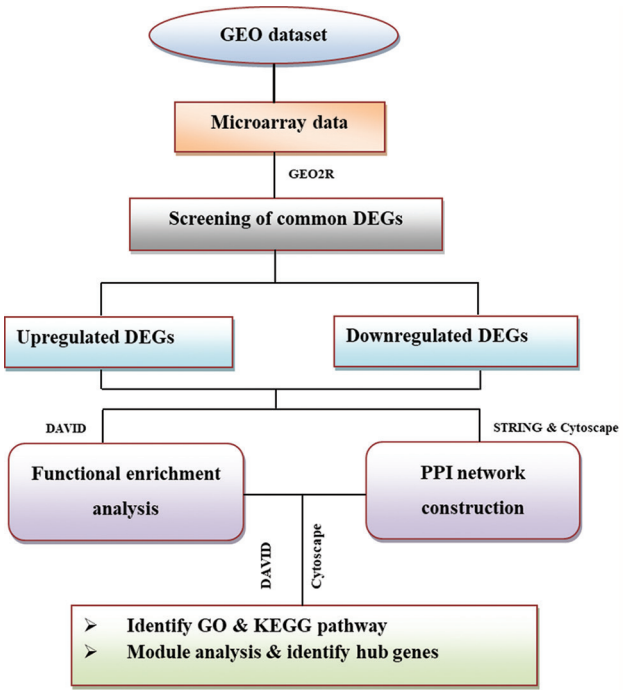

Figure 1. Flow diagram depicting the integrated bioinformatics techniques used in this work

cells from control versus treated CF patient samples based on the program of GPL570 (Affymetrix Human Genome U133 Plus 2.0 Array [HG-U133_Plus_[2]), which was handled by the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) in 2022, was assembled from the GEO database (https://www.ncbi.nlm.nih.gov/geo/).[23] The GSE70442 dataset encompasses eight samples in total. The GSM1754933, GSM1754934, GSM1754935, and GSM1754936 were used as control bronchial epithelial cells, while the GSM1754937, GSM1754938, GSM1754939, and GSM1754939 were used as treated bronchial epithelial cells (treated at 27°C).

2.2. Identification of DEGs

The statistical program GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/) was used to verify whether genes showed differential expression based on the comparison between control and treated cells of CF.[24] The false discovery rate by Benjamini and Hochberg and *t*-test procedures were applied with the GEO2R program to determine the DEGs and compute the FDR and *P*-values.[25] For the DEGs, we deemed a $P < 0.05$ and a logFC >1 (important fold changes) to be statistically significant. We created a volcano plot based on all the identified DEGs using the R language's pheatmap package. To identify the most significant DEGs, a $P < 0.05$ was employed as the cutoff value. LogFC ≥1 and logFC ≤−1 were deemed to represent upregulated and downregulated DEGs, respectively.[26-28] Afterward, the DEG dataset was gathered and used for further analysis.

2.3. Functional enrichment of gene sets

Using DAVID v6.8 (https://david.ncifcrf.gov/),[29] an online bioinformatics tool, the first gene ontology (GO) and KEGG pathway enhancement assessments of the DEGs were performed ($P < 0.05$). The NetworkAnalyst online tool[30] was used to crosscheck the enriched DEGs. The functional experiment is presently a widely utilized technique for analyzing gene expression function based on the genomic data collected.[31,32] To comprehend metabolic pathways, the Kyoto Encyclopedia of Genes and Genomes (KEGG) was employed for analyzing functional genomics,[33,34] using Zhang *et al.*'s ontological concepts as the foundation for the analysis.[35]

2.4. Network construction through protein–protein interaction

With the help of the STRING (v11.0, http://www.string-db.org/), the protein–protein interaction (PPI) network of DEG-encoded proteins was built. STRING is an extensive online archive containing 24,584,628 proteins from 5090 species, specifically designed to predict gene connections.[36] To be deemed significant, the total score has to be less than 0.75 (medium confidence level).

2.5. Selection of hub proteins from the PPI network

Cytoscape (http://www.cytoscape.org/) was used to visualize the derived PPI networks.[37] The molecular complex detection (MCODE) plug-in for Cytoscape[38] was employed to identify noteworthy modules that possessed an established score of higher than three and nodes with a larger than four. High-degree nodes were regarded as hub genes in the PPI network, where nodes' degree value was determined by the number of edges that they included. The PPI information of the hub genes was measured by mapping them. Hub genes from the built PPI network were assessed using cytoHubba,[39] a Cytoscape plugin. The degree score was utilized in this research to discover hub genes using the cytoHubba program, which calculates hub genes from the PPI network using 11 distinct approaches.

3. Results

3.1. DEG identification

The GSE70442 dataset comprises eight samples obtained from four CF patients, including four samples maintained at 37°C as controls and four samples maintained at 27°C as treated samples (Table 1). We used GEO2R to determine the DEGs from the patients and control groups and to obtain the log2FC and *P*-values. DEGs were defined as the resultant genes that satisfied the threshold values, which were logFC ≥ 1, logFC ≤ −1, and $P < 0.05$. With the help of the GEO2R tool, a total of 4229 genes from the GEO dataset were found. Using RStudio's Shiny Volcano Plot, we created a volcano plot to compare the patients and control groups (Figure 2). Subsequently, 211 DEGs

Table 1. Essential information of GSE70442 dataset obtained from the GEO database

Group	Accession	Organism	Disease state	Cell Type
Control	GSM1754933	*Homo sapiens*	Cystic fibrosis	Bronchial epithelial cells
	GSM1754934	*Homo sapiens*	Cystic fibrosis	Bronchial epithelial cells
	GSM1754935	*Homo sapiens*	Cystic fibrosis	Bronchial epithelial cells
	GSM1754936	*Homo sapiens*	Cystic fibrosis	Bronchial epithelial cells
Treated	GSM1754937	*Homo sapiens*	Cystic fibrosis	Bronchial epithelial cells
	GSM1754938	*Homo sapiens*	Cystic fibrosis	Bronchial epithelial cells
	GSM1754939	*Homo sapiens*	Cystic fibrosis	Bronchial epithelial cells
	GSM1754940	*Homo sapiens*	Cystic fibrosis	Bronchial epithelial cells

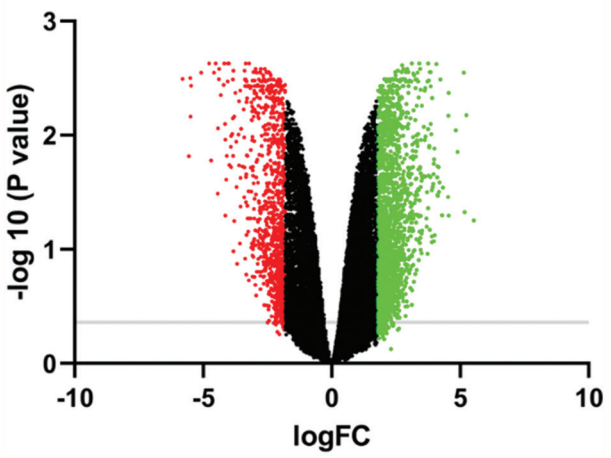

Figure 2. Volcano plot of differentially expressed genes from the GSE70442 dataset. The X-axis represents log2FC (large-scale fold changes), while the Y-axis denotes –log10 of the *P*-value, which indicates the level of statistical significance. The green points denote upregulated genes, whereas the red points represent downregulated genes.

were determined as highly significant, with 104 and 107 identified upregulated and downregulated, respectively, based on the threshold values (Tables S1 and S2).

3.2. Analysis of DEGs' functionality

The GO analysis of DEGs revealed analysis outputs concerning the top 10 enrichments utilizing the DAVID database. Figure 3A and B demonstrate the results of the analysis of enrichment for biological process, cellular component, and molecular function. N-acyl phosphatidylethanolamine-specific phospholipase D activity and protein kinase activity are the primary enrichments of the upregulated DEGs. In contrast, the downregulated DEGs were significant in protein polyubiquitination, ubiquitin-protein transferase action, and ubiquitin-mediated protein catabolic mechanism. The NetworkAnalyst analysis and DAVID analysis of KEGG pathways showed that the upregulated DEGs are primarily associated with pathways such as glutamatergic synapse

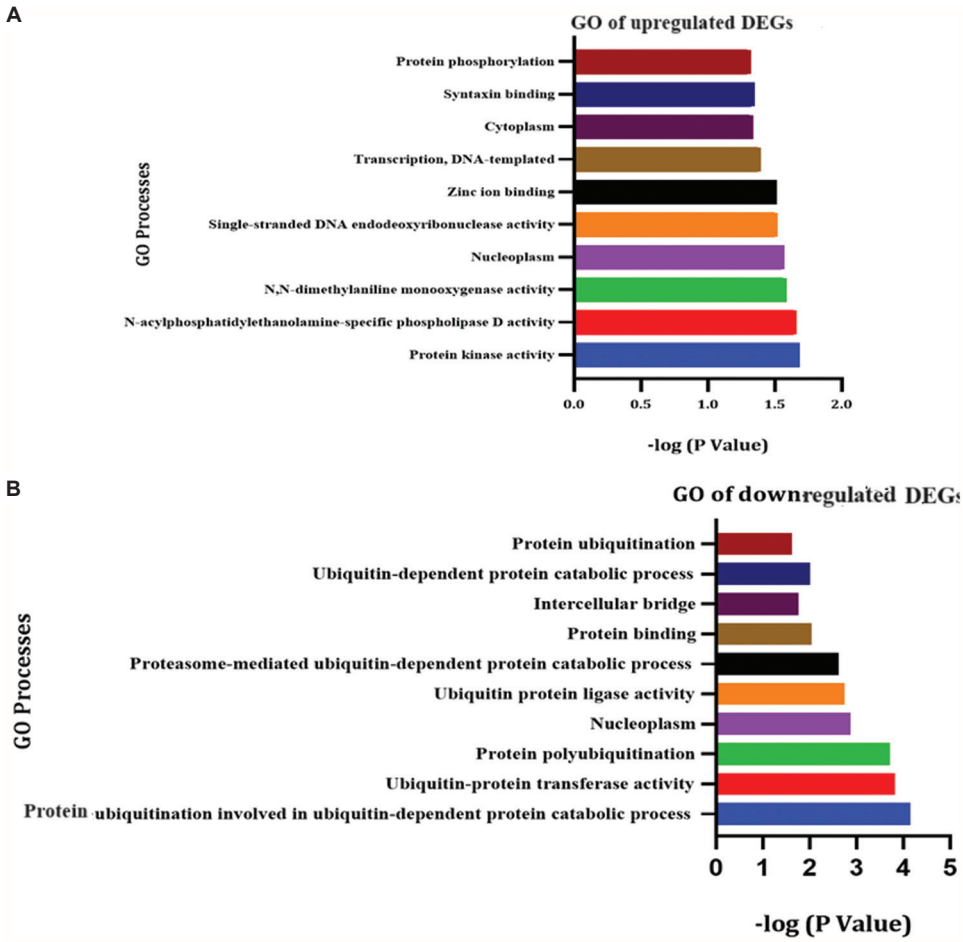

Figure 3. Gene ontology (GO) terms identified following enrichment with differentially expressed genes. (A) GO terms of upregulated genes. (B) GO terms of downregulated genes.

and basal transcription factors (Figure 4A). The primary pathways associated with the downregulated DEGs include proteolysis mediated by ubiquitin, nucleotide excision repair, and cell cycle (Figure 4B). Tables S3-S5 provide the outputs of GO and KEGG analysis of the DEGs.

3.3. PPI network construction

With the STRING, we were able to determine the PPI networks for both up and downregulated genes, which allowed us to assess the PPIs linked to the DEGs. For visualization, we loaded the generated PPI network as a ".csv" file and entered Cytoscape v3.8.0 after exporting it as a ".txt" file from STRING. For both up and downregulated DEGs, we separately enhanced the PPI networks. Once all 211 DEGs had been found, we combined the PPI networks. As shown in Figure 5B and C, the PPI networks for up- and down-regulated DEGs include 74 nodes and 191 connections, and 82 nodes and 282 connections, respectively. There were 515 edges overall for 167 nodes in the combined PPI network including all DEGs (Figure 5A).

4.4. Identification of gene signatures: Module analysis from PPI network

Using the Cytoscape plugin MCODE, we were able to identify two important modules from the combined PPI network. Figure 6A shows that Module 1 exhibited 13 nodes and 55 connections, while eight nodes and 12 connections occurred in Module 2 (Figure 6B). The DEGs of the modules had a vital impact in enriching critical

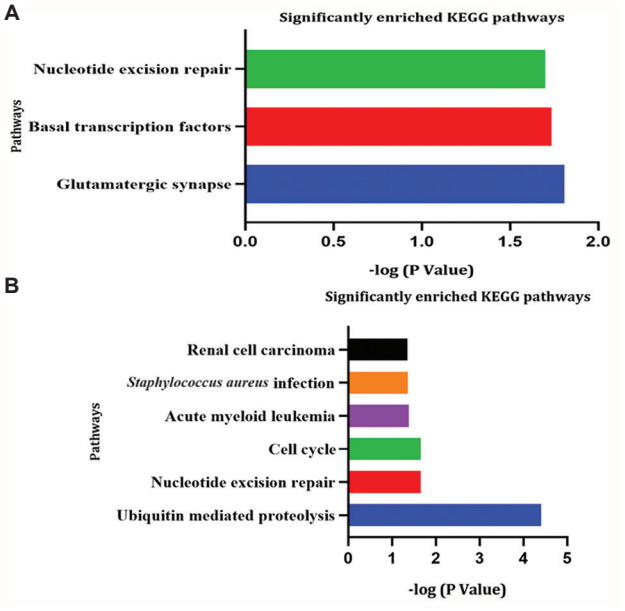

Figure 4. Identification of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways following enrichment with differentially expressed genes. (A) KEGG pathways enriched in upregulated genes. (B) KEGG pathways enriched in downregulated genes.

GO terms "protein binding" and "cytoplasm." In addition, the cytoHubba program enabled us to identify the top 6 hub genes (*CDC42*, *UBA2*, *HDAC4*, *CUL1*, *RNASEL*, and *WWP2*) from the PPI network (Figure 7). Table 2 displays the topological metrics for each of the five molecular hub genes in the PPI linkage, including each gene's degree, betweenness centrality, clustering coefficient, and closeness centrality.

5. Discussion

CF is a recessive inherited disorder that causes death in 90% of patients.[40] The major dysfunctions of CF include mucus-blocked airways leading to severe lung infections, massive neutrophil infiltration, and inflammation contributing to tissue damage.[41] Although the vast majority of morbidity and mortality is accounted for by chronic progressive lung disease in CF, it is important to note that CF is not confined to the lungs and may affect other major organs resulting in several comorbidities.[42] However, current treatment approaches are not effective in all patients.[43] For instance, highly effective modulator therapy is not efficacious to 10 – 20% of CF patients, and the multidrug bacterial resistance also limits the current antimicrobial choices.[14] On the other hand, it has recently been reported that many children with CF are born without any clinical symptoms, and the diagnosis was only confirmed their birth.[44] Thus, the search for early and precise detection of CF has become more intense in recent years, especially for techniques to identify gene signatures. In the present study, we evaluated the DEGs, gene networks, pathways, and protein–protein connections that are exclusive to CF. We used temperature as a means to manipulate gene expression from the GSE dataset. It has been reported that cell growth was nearly halted after moderate hypothermia and did not resume when temperatures returned to 37°C. The expression of cold shock genes, *CIRBP* and *RBM3*, was enhanced at 25°C and recovered to baseline levels following rewarming, whereas that of *HSP70* was inversely regulated. Our data also supported that the temperature can affect gene expression, a finding consistent with another published study.[45]

The volcano plot (Figure 2) in this article was used to visualize the patterns of the DEGs from both the control and treated groups. Upregulated genes are denoted by green points, while downregulated genes are represented by red points.

Following the enrichment, we identified a few important GO terms enriched in the upregulated genes, including: protein kinase activity, N-acyl phosphatidylethanolamine-specific phospholipase D activity, N,N-dimethylaniline monooxygenase activity, transcription, and protein phosphorylation in the biological process; nucleoplasm, cytoplasm, and zinc ion binding in the cellular component; and

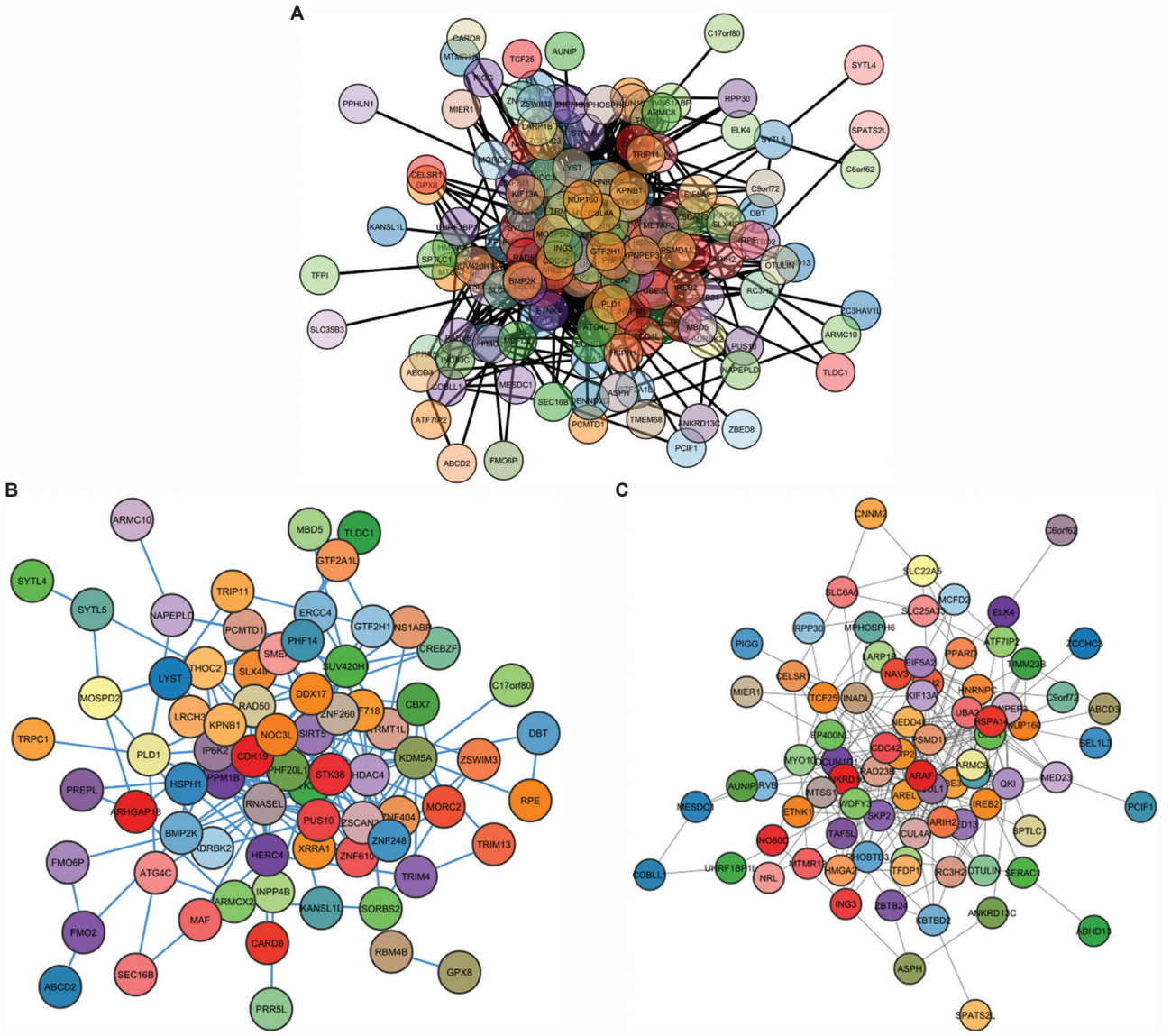

Figure 5. Visualization of the protein-protein interaction (PPI) network of cystic fibrosis. (A) Merged PPI network for all DEGs. This PPI network had 515 connections for 167 nodes. (B) PPI network for upregulated genes with 74 nodes and 191 edges. (C) PPI network for downregulated genes with 82 nodes and 282 edges.

protein kinase activity and N-acyl phosphatidylethanolamine-specific phospholipase D activity in the molecular function (Figure 3A).

On the contrary, the GO terms enriched in the downregulated genes include: (i) protein ubiquitination connected with ubiquitin-supported protein catabolic procedure, ubiquitin-protein transferase action, and protein polyubiquitination in the biological process; and (ii) protein binding and intracellular bridge in the cellular component (Figure 3B).

From the pathway analysis, we found that the most upregulated DEGs are mainly clustered in pathways including glutamatergic synapse and basal transcription factors (Figure 4A). Conversely, the downregulated DEGs are mainly involved in ubiquitin-mediated proteolysis, nucleotide elimination repair, and cell cycle (Figure 4B).

We identified six hub genes based on degree value, betweenness centrality, clustering coefficient, closeness centrality, and stress from the combined PPI analysis. These six genes — *CDC42*, *UBA2*, *HDAC4*, *CUL1*, *RNASEL*, and *WWP2* — may have a critical diagnostic value to differentiate CF patients from healthy individuals, since the latter do not exhibit higher expression of these genes (Table 2).

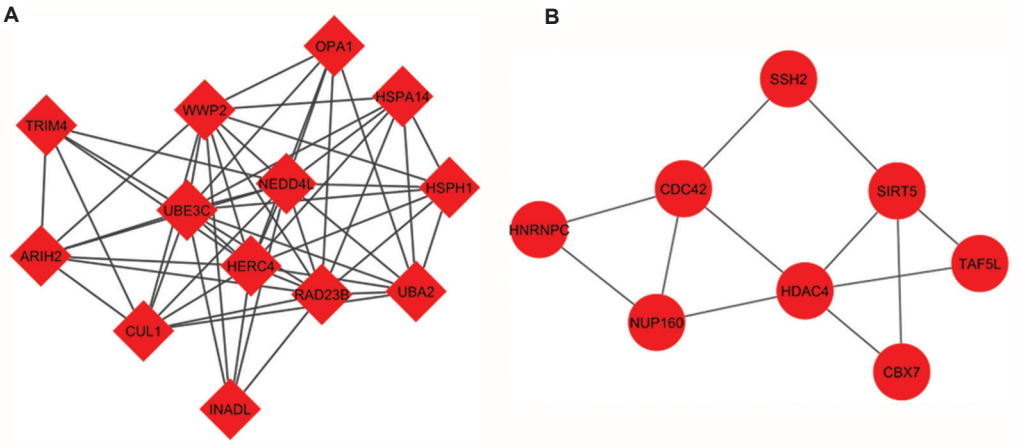

Figure 6. Module analysis of protein-protein interaction network. (A) Module 1 has 13 nodes with 55 edges. (B) Module 2 has eight nodes and 12 connections.

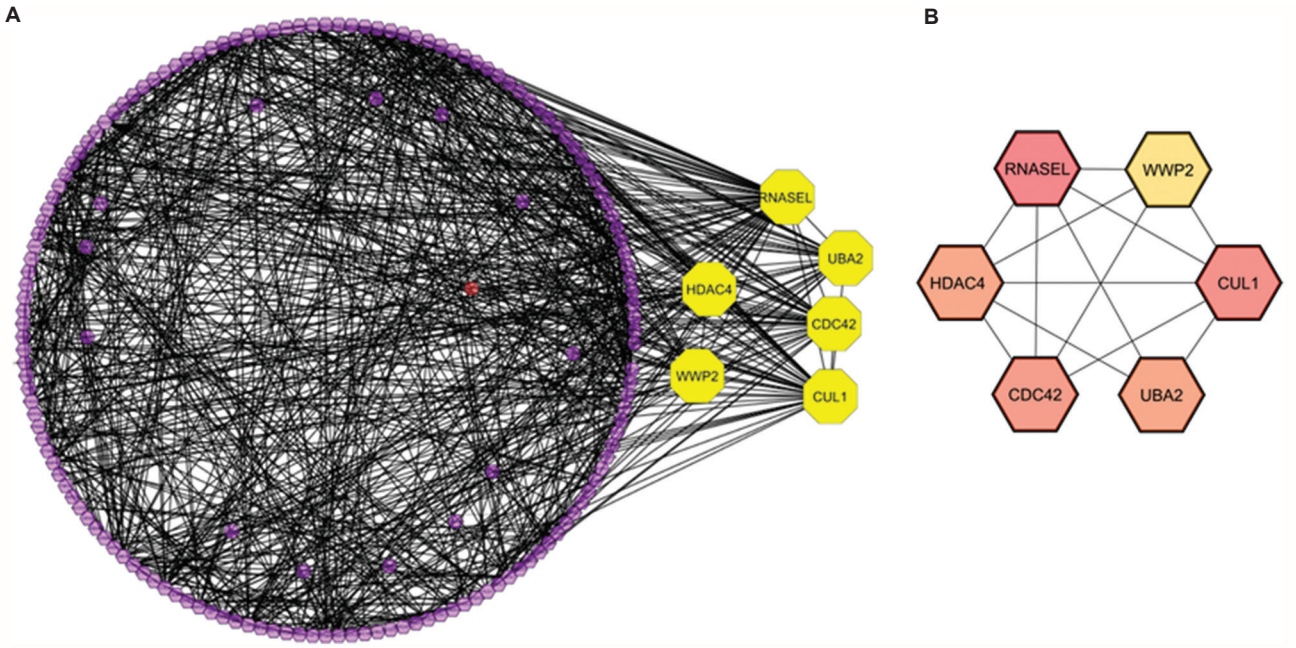

Figure 7. Protein-protein interaction (PPI) network used for discovering hub genes. The six highlighted nodes represent the hub genes. CytoHubba's degree score technique takes into account 94 nodes from the combined PPI network.

Table 2. Topological characteristics for the five molecular genes of the PPI network

Gene signature name	Degree	Betweenness centrality	Clustering coefficient	Closeness centrality	Stress
RNASEL	56.462	124,079	56	55	0.17013
CUL1	53.17	39,940	38	38	0.21195
CDC42	50.534	586	36	34	0.15873
HDAC4	48.957	678	30	30	0.2023
UBA2	50.98	68,772	30	30	0.3195
WWP2	23	350.8	39	86.5	0.3014

Abbreviation: PPI: Protein-protein interaction.

CDC42 belongs to the Rho family of small GTPases and has numerous cellular functions. One of the extensively characterized roles of CDC42 over the recent decades is actin cytoskeleton remodeling.[46] Among the plethora of cellular functions, CDC42 has been found to involve in the endocytosis and reutilizing of some plasma membrane proteins.[47,48] The transducer of the Cdc42-dependent actin assembly (Toca) family and the Wiskott–Aldrich syndrome protein (WASP) are two examples of downstream effectors that are bound and activated by GTP-bound CDC42. Through the Arp2/3 complex, they cause actin filaments to branch. It has been reported that the pharmacological inhibitor wiskostatin causes a decrease in CFTR protein in cell surface and an inhibition of the CFTR-guided chloride currents.[49] On the other hand, studies revealed that CDC42 regulates the initial steps of CFTR biogenesis and processing, which is crucial for the stability of CFTR in the plasma membrane.[47] Furthermore, CDC42 signaling regulates efferocytosis and Fcγ receptor-mediated phagocytosis in CF.[50] By adding the tiny protein SUMO, the ubiquitin-activating enzyme UBA2 modifies proteins post-translationally and controls their intracellular localization and structure. UBA2 is considered a candidate modifier of CF phenotype and found to be upregulated in individuals with severe CF.[51]

Researchers have explored the association of histone deacetylase (HDAC) isoforms with CF, and inhibitors have also been developed against HDACs.[52,53] HDAC4 is an HDAC isoform involved in the control of gene transcription, cell growth, survival, and proliferation.[54] A variety of cellular dysfunctions are caused by the aberrant expression of this enzyme.[53] CUL1 belongs to the CULLIN family and provides a scaffold for ubiquitin ligases that acts an important player in protein degradation and ubiquitination and regulates the equilibrium between normal cellular growth and uncontrolled proliferation.[55] Studies have identified *CUL1* as a validated target gene of respiratory diseases such as asthma and chronic obstructive pulmonary disease.[56-58]

RNASEL is a regulated endoribonuclease and mediates antiproliferative, antiviral, and apoptotic effects of the interferons.[59] Nonetheless, an immunogenicity test using clinical samples from the CF patients revealed that the RNASEL was expressed comparatively higher in the unmodified mRNA-treated samples compared to the chemical-induced samples.[60] WWP2 is an E3 ubiquitin ligase belonging to the NEDD4-like protein family that regulates the activation of T cells, transcription, cellular transport, and the fate of embryonic stem cells.[61,62] Numerous investigations have revealed WWP2 as an essential regulatory component in the formation of renal, pulmonary, and cardiac fibrosis.[63,64] Based on the overall analysis of the genes reported in this study, the *RNASEL* and *HDAC4* genes were upregulated, and the *WWP2*, *CUL1*, *CDC42*, and *UBA2* genes were downregulated. This indicates that CF patients boast elevated expression levels of the *RNASEL* and *HDAC4* genes and reduced expression levels of *WWP2*, *CUL1*, *CDC42*, and *UBA2* genes. However, it should be noted that the *HDAC4*, *CUL1*, *RNASEL*, and *WWP2* genes have never been studied for their individual involvement in CF development. Thus, independent validations should be performed to corroborate their utility in diagnosis and prognosis evaluation.

6. Conclusion

DEGs such as *CDC42*, *UBA2*, *HDAC4*, *CUL1*, *RNASEL*, and *WWP2*, as well as the pathways these genes are enriched in such as glutamatergic synapse, basal transcription factors, nucleotide elimination repair, ubiquitin-mediated proteolysis, cell cycle, and *Staphylococcus aureus* infection, might be involved in the pathogenesis of CF. Our findings also shed light on the significant roles of *CDC42*, *UBA2*, *HDAC4*, *CUL1*, *RNASEL*, and *WWP2* in the pathogenesis of CF. Among these genes, *WWP2* is a new potential genetic marker for CF that has never been disclosed in the past. The current set of results offers a theoretical direction for conducting the subsequent, more in-depth experiments to substantiate the newly identified markers as distinct targets for diagnosis, prognosis, and treatment. Moreover, larger sample sizes are needed to validate these findings and pinpoint possible targets for CF treatments.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Arafat Rahman Oany
Investigation: Mamun Mia, Arafat Rahman Oany
Writing – original draft: Mamun Mia, Arafat Rahman Oany, Tahmina Pervin
Writing – review & editing: All authors

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Dataset GSE70442 can be accessible through the GEO database, https://www.ncbi.nlm.nih.gov/geo

Further disclosure

A preprint version of this article is available at Research Square (https://doi.org/10.21203/rs.3.rs-3031398/v1).

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

ORIGINAL RESEARCH ARTICLE

Association between dietary soy prevention of fetal alcohol spectrum disorder and normalization of placental insulin and insulin-like growth factor signaling networks and downstream effector molecule expression

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Abstract

Chronic prenatal alcohol exposure causes fetal alcohol spectrum disorder (FASD), often associated with impaired placentation and intrauterine growth restriction. Ethanol's inhibition of insulin and insulin-like growth factor Type 1 (IGF-1) signaling compromises trophoblastic cell motility and maternal vascular transformation at the implantation site. Previous studies have demonstrated that dietary soy effectively normalizes placentation and fetal growth in an experimental model of FASD. The studies were extended to better understand the mechanisms underlying soy's beneficial effects. Pregnant Long Evans rats were pair-fed with isocaloric liquid diets containing either 0% or 36% caloric ethanol from gestation day (GD) 6. The protein source in the diets consisted of either casein (standard and control) or soy isolate. On GD19, placentas were harvested to measure mRNA levels corresponding to major components of the insulin/IGF-1 pathway, as well as aspartyl-asparaginyl-β-hydroxylase (ASPH), Notch, and HES, which play critical roles in placentation. Chronic gestational ethanol exposure in rats fed diets containing casein significantly reduced the expression of insulin, insulin receptor, *Igf1*, IGF-1 receptor (*Igf1r*), insulin receptor substrate Type 1 (*Irs1*), *Irs2*, *Asph*, and *Hes1*. In addition, ethanol significantly decreased ASPH protein expression. Dietary soy mitigated most of these effects and further enhanced signaling by upregulating *Igf2*, *Igf2r*, *Irs1*, *Irs2*, *Irs4*, *Notch*, and *Hes1* in rats chronically exposed to ethanol relative to corresponding control samples. The protective effects of dietary soy in FASD act at the mRNA level and positively impact pathways imperative for normal placentation and fetal development. Gestational dietary soy may provide an effective means of preventing FASD in vulnerable populations.

Keywords: Aspartyl-asparaginyl-β-hydroxylase; Notch; mRNA; Polymerase chain reaction

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1. Introduction

Fetal alcohol spectrum disorder (FASD) is a significant public health concern, driven by socioeconomic, mental health, and cultural disparities that contribute to heavy alcohol consumption during pregnancy. In addition to characteristic craniofacial abnormalities, FASD features birth and neurodevelopmental defects, intrauterine growth restrictions (IUGR), postnatal growth limitations, and increased fetal mortality rates. Placentation, encompassing placental maturation, growth, and implantation, is integral for optimizing the fetal environment, ensuring ample nutrient delivery and waste product removal. The occurrence and severity of FASD are partially rooted in placental dysfunction, mediated by impairments in placentation. Previous studies have established a link between FASD-mediated impairments in placentation and reduced signaling through insulin and insulin-like growth factor (IGF) pathways.[1] These pathways regulate the expression and function of aspartyl-asparaginyl-β-hydroxylase (ASPH), which plays critical roles in regulating cell motility and invasion necessary for effective placentation.[2-5] Heavy alcohol consumption during pregnancy compromises ASPH's functions, leading to disruptions in critical cellular processes.[6] Mechanistically, ASPH catalytically hydroxylates and activates Notch,[7,8] which, in turn, upregulates transcription of hairy and enhancer of split-1 (HES1).[7,9] Notch is pivotal in regulating a broad array of cellular functions during development, including growth, motility, and maturation.[10] The inhibitory effects of ethanol have been well-documented.[9]

Potential preventive and treatment measures for FASD should take into consideration the intricate interactions among maternal factors, placental reactions, and the diverse fetal responses.[11] Addressing problems arising from insulin/IGF resistance due to impaired signal transduction presents a promising avenue. Earlier research has demonstrated the efficacy of peroxisome-proliferator-activated receptor (PPAR) agonists in preventing or reducing the disease-related effects of dysregulated insulin/IGF signaling in experimental models of chronic alcohol exposure, obesity, or nitrosamine administration.[12-17] PPAR agonists act by bypassing poorly responsive cell surface receptors and stimulating gene expression at the level of transcription within the nucleus.[18,19] However, practical and health-related uncertainties regarding the administration of PPAR agonist medications to pregnant women warrant exploration into alternative approaches. Investigating whether the insulin-sensitizing effects of natural products, such as soy or bioactive isoflavones (daidzein and genistein),[20] could counteract the adverse effects of developmental alcohol exposures is of particular interest. Recent experiments along these lines have yielded promising results, suggesting the potential of soy-based interventions to mitigate neurodevelopmental deficits associated with FASD.[21-23] In a comprehensive model of FASD, dietary soy was found to effectively mitigate alcohol-mediated impairments in placentation and neurodevelopmental features.[1] However, further mechanistic research is warranted to elucidate the underlying mechanisms and inform future clinical and population-based applications. The present study builds on our earlier efforts by examining the effects of alcohol and dietary soy on the mRNA expression of upstream insulin/IGF signaling pathway molecules, including *Asph*, *Notch1*, and *Hes1*. This investigation aims to assess the degree to which dietary soy mediates its protective effects on placentation at the mRNA level by modulating gene expression.

2. Materials and methods

2.1. Materials

Qiazol reagent, EZ1 RNA universal tissue kit, QuantiTect SYBR Green polymerase chain reaction (PCR) master mix, and the BIO Robot Z1 were procured from Qiagen Sciences Inc (USA). The AMV first strand cDNA synthesis kit was obtained from Roche Diagnostics Corporation (USA). Enzyme-linked immunosorbent assay (ELISA) plates and the ELISA plate washer were sourced from Thermo Scientific Nunc (USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies and the soluble fluorophore (Amplex Red) were supplied by Invitrogen (USA).

2.2. *In utero* ethanol exposure model

The Lifespan Institutional Animal Care and Use Committee (IACUC) of Rhode Island Hospital approved the experimental use and treatment of Long Evans rats for this research. On gestation day (GD) 6, following timed mating as previously described,[1] we initiated feeding with isocaloric liquid diets (BioServ, USA). Control diets contained 0% ethanol. The experimental diets comprised 36% caloric ethanol or 8.2% vol/vol. The protein sources consisted of either 100% casein, which was considered a control due to its standard inclusion in rodent diets, or 100% organic soy protein isolate, which was considered an experimental. Consequently, four study groups were designated as follows: (i) CC for control-casein, wherein the rats were fed ethanol-free diets with casein as the protein source; (ii) CS, corresponding to ethanol-free diets with soy as the protein source; (iii) EC, in which the rats were fed ethanol-containing diets with casein as the protein source; and (iv) ES, corresponding to ethanol-containing diets with soy as the sole protein source. Eight placentas were randomly selected from each group for analysis. GD6 was selected as the start time for ethanol feeding because

earlier exposures cause excessive fetal loss and impair placentation.[24,25] The dams' food intake, behavior, and body weights were monitored daily, as previously reported.[1]

2.3. Quantitative reverse transcriptase-PCR analysis

Quantitative reverse transcriptase-PCR (qRT-PCR) analysis was employed to measure insulin (*Ins*), *Igf1*, *Igf2*, insulin receptor (*Insr*), *Igf1r*, *Igf2r*, insulin receptor substrate type 1 (*Irs1*), *Irs2*, *Irs4*, *Asph*, *Notch1*, and *Hes1* mRNA transcripts using gene-specific primer pairs through the methodology previously described.[7] The PCR primers were designed using MacVector 10 software (MacVector, Inc., USA), and their target specificities were confirmed through the NCBI-BLAST (National Center for Biotechnology Information-Basic Local Alignment Search Tool). Results were analyzed using the Mastercycler ep realplex instrument and software (Eppendorf AG, Germany). The relative abundance of each mRNA transcript was expressed as the calculated ratio of specific mRNA to 18S rRNA. All assays were performed in triplicate.

2.4. ELISAs

Placental tissue homogenates, prepared in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors,[26,27] were used to measure immunoreactivity to ASPH utilizing rabbit polyclonal antibodies. Protein concentrations were determined using the BCA assay. Direct binding ELISAs were conducted with 50 ng protein per sample.[9,21,28] Immunoreactivity was detected using HRP-conjugated secondary antibody and Amplex Red soluble fluorophore.[26,28] Fluorescence intensity was measured (Ex 530/Em 590) in a SpectraMax M5 microplate reader (Molecular Dynamics, USA). Negative control reactions were performed with primary, secondary, or both antibodies omitted. Between steps; the wells were rinsed three times with Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST) using a Nunc ELISA plate washer.[28]

2.5. Statistical analysis

Data corresponding to levels of gene expression or immunoreactivity are illustrated using boxplots and whiskers to depict the data distributions along with the mean (horizontal bar), 95% confidence interval limits (upper and lower limits of the boxplots), and range (upper and lower stems). Inter-group comparisons were conducted through one-way analysis of variance (ANOVA) followed by *post hoc* Tukey tests of significance. Statistical analysis was performed using the GraphPad Prism 10.2 software (USA). The threshold for statistical significance was set at $P \leq 0.05$. For calculated *P*-values falling between 0.05 and 0.10, the statistical outcome was interpreted as indicative of a trend.

3. Results

While it is established that soy offers antioxidant and insulin-sensitizing benefits, the mechanisms implicated in normalizing placentation and fetal development vis-à-vis continued chronic high-level ethanol exposures have not been thoroughly evaluated. Herein, we investigated the potential effects of ethanol and dietary soy on insulin/IGF pathway gene expression, as previous studies indicated that chronic ethanol exposures can significantly modulate the expression of mRNA transcripts encoding proteins critical to signal transduction.[7] Moreover, if the previously reported experimental responses to ethanol and dietary soy in placentas and fetuses were mediated by chronic alterations in gene expression, it would be necessary to ascertain measures that could ensure future therapeutic interventions favorably impact long-term pathophysiological processes responsible for sustained impairments in intracellular signaling. To achieve this, we measured mRNA levels corresponding to the insulin and IGF polypeptides and receptors, including *Ins*, *Igf1*, *Igf2*, *Insr*, *Igf1r*, *Igf2r*, *Irs1*, *Irs2*, *Irs4*, *Asph*, *Notch1*, and *Hes1*.

3.1. Insulin and insulin growth factor mRNA expression

One-way ANOVA revealed significant inter-group differences in the levels of insulin ($P < 0.0001$), *Igf1* ($P < 0.0001$), and *Igf2* ($P < 0.0001$) expression (Table 1).

Table 1. Summary of ethanol and dietary soy effects on placental expression of insulin, IGF, and IRS signaling pathway molecules

Variable (mRNA)	*F*-ratio	*P*-value
Hormone		
Ins	11.36	<0.0001
Igf1	10.27	<0.0001
Igf2	13.02	<0.0001
Receptor		
Insr	5.763	0.0015
Igf1r	3.775	0.0141
Igf2r	6.74	0.0006
Substrate		
Irs1	12.16	<0.0001
Irs2	9.356	<0.0001
Irs4	47.62	<0.0001

Notes: The mRNA transcripts were measured in CC, CS, EC, and ES placental tissue homogenates through qRT-PCR analysis. Results were normalized to 18S rRNA measured in the same samples. Inter-group comparisons (n=8/group) were conducted using one-way ANOVA. The *F*-ratios and *P*-values are tabulated. See Figures 1-3 for graphed data and *post hoc* Tukey multiple comparisons test results.

Notably, *Igf2* exhibited markedly higher expression levels compared to *Igf1*, with insulin ranking second across all groups (Figure 1). Ethanol exposure induced a significant reduction in insulin ($P < 0.0001$) (Figure 1A) and *Igf1* ($P < 0.0001$) (Figure 1B) mRNA expression when standard casein served as the dietary protein source. However, there was no significant impact observed on *Igf2* expression (Figure 1C). Substituting dietary soy for casein effectively mitigated ethanol's inhibitory effects on insulin (Figure 1A) and *Igf1* (Figure 1B) mRNA levels, normalizing them to comparable levels with the CC and CS groups while significantly surpassing expression in the ES group. Furthermore, dietary soy intake significantly elevated *Igf2* expression in the ES group compared to all other groups ($P < 0.0001$) (Figure 1C).

3.2. Insulin and insulin growth factor receptor expression

Insr ($P = 0.0015$), *Igf1r* ($P = 0.014$), and *Igf2r* ($P = 0.0006$) expression exhibited significant variations among the experimental groups, as revealed by one-way ANOVA (Table 1). Among these receptors, *Igf1r* demonstrated the highest abundance, followed by *Igf2r*, with *Insr* ranking third in expression levels (Figure 2). *Post-hoc* Tukey tests further elucidated significantly reduced *Insr* ($P = 0.0002$) (Figure 2A) and *Igf1r* ($P = 0.0168$) (Figure 2B) mRNA levels in the EC group compared to the CC group. Conversely, the expression of *Igf2r* mRNA remained consistent between EC and CC groups (Figure 2C). Replacing casein with dietary soy resulted in similar mean mRNA levels of *Insr* and *Igf1r* in both CS and ES groups. However, there was a notable elevation in *Igf2r* expression in the ES group relative to the other

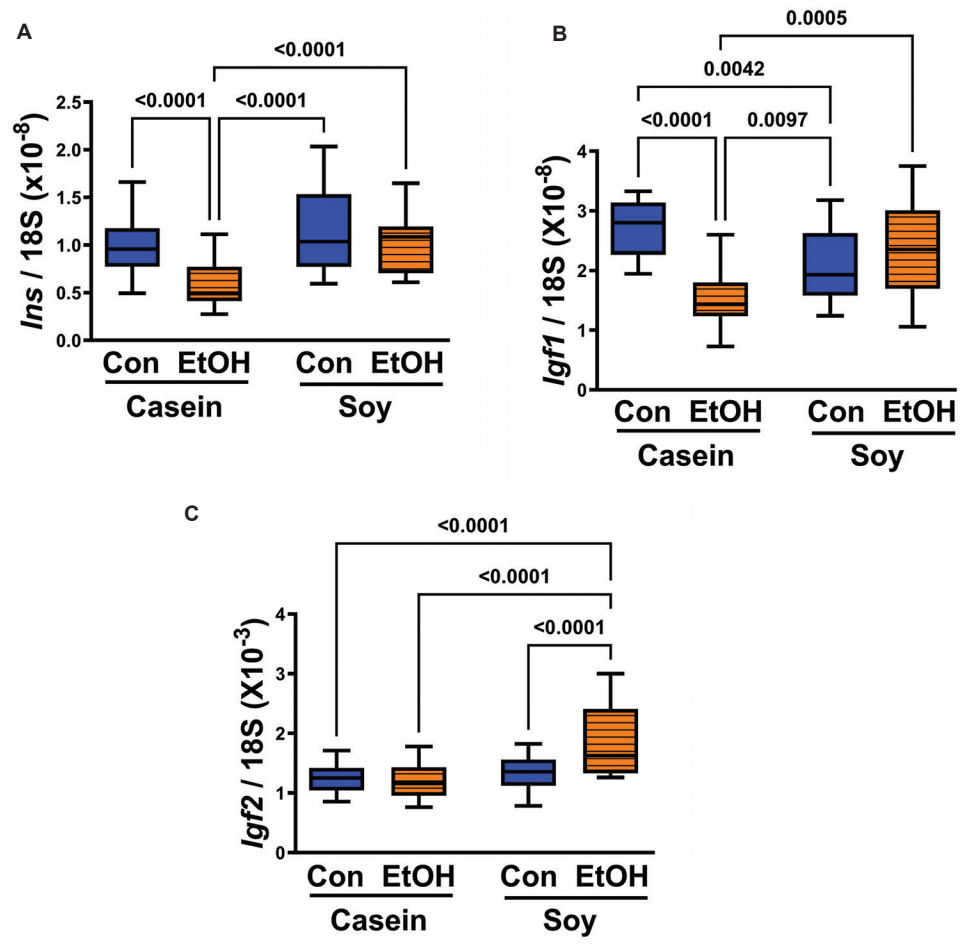

Figure 1. Effects of chronic gestational exposure to ethanol and dietary soy isolate on (A) *Ins*, (B) *Igf1*, and (C) *Igf2* mRNA expression in placental tissue. Reverse-transcribed RNA was polymerase chain reaction amplified using gene-specific primer pairs. Transcript abundance was calculated relative to 18S rRNA. Inter-group statistical comparisons were conducted using one-way analysis of variance (see Table 1) with *post hoc* Tukey tests. The $P \leq 0.05$ are considered statistically significant. Notes: Con: Control; EtOH: Ethanol.

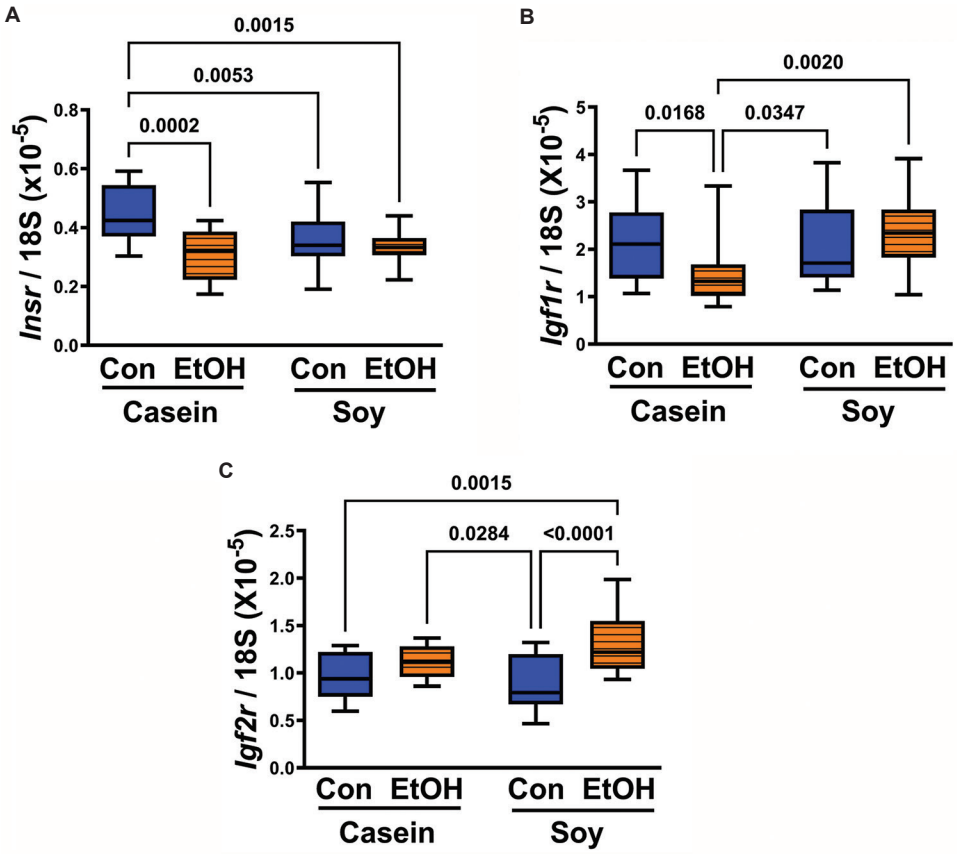

Figure 2. Comparisons of chronic gestational exposure to ethanol and dietary soy versus casein effects on (A) *Insr*, (B) *Igf1r*, and (C) *Igf2r* mRNA levels in placental tissue. Boxplots depict relative mRNA transcript abundance calculated from the ratio of specific polymerase chain reaction-amplified products (mRNAs) to 18S rRNA. Inter-group statistical comparisons were made by one-way analysis of variance (see Table 1) with *post hoc* Tukey tests. The $P \leq 0.05$ are considered statistically significant. Notes: Con: Control; EtOH: Ethanol.

three groups (Figure 2C), mirroring, in part, the response observed in IGF-2 hormone responses (Figure 1C). Dietary soy correlated with lower levels of *Insr* but comparable levels of *Igf1r* in the CS and ES groups compared to the CC group. In contrast, *Igf1r* expression levels were similar across CS, ES, and CC groups, all of which exhibited higher expression levels compared to the EC group (Figure 2B).

3.3. Insulin receptor substrate expression

One-way ANOVA analysis revealed significant inter-group differences in *Irs1*, *Irs2*, and *Irs4* expression (Table 1). Among these insulin receptor substrate proteins, *Irs2* emerged as the most abundant, followed by *Irs1*, with *Irs4* exhibiting the lowest expression levels (Figure 3). In rats subjected to chronic gestational exposure to ethanol while maintained on liquid diets containing casein as the primary protein source, a significant reduction in expression was observed for the two predominant insulin receptor substrate forms, *Irs1* (Figure 3A) and *Irs2* (Figure 3B). Conversely, control placentas from dams fed with soy isolate (CS) instead of casein exhibited comparable levels of *Irs1* (Figure 3A) and *Irs4* (Figure 3C), but demonstrated reduced expression of *Irs2* ($P = 0.0008$) (Figure 3B) relative to the CC group. The observed reduction in *Irs4* was modest and did not attain statistical significance (Figure 3C). Dietary soy intake did not exert significant effects on mRNA levels of *Irs1* or *Irs4* when comparing CS to CC groups. However, it significantly reduced the mean expression level of *Irs2* ($P = 0.0008$). In contrast, soy consumption prevented ethanol-induced reductions in insulin receptor substrate expression measured in the EC group, elevating *Irs1*, *Irs2*, and *Irs4* above levels measured in the CS and EC groups. In addition, *Irs1* and *Irs4* expression were significantly elevated in the ES group relative to the other three groups, while *Irs2* levels were normalized compared to the CC group.

3.4. Chronic gestational ethanol and dietary soy effects on placental *Asph*, *Notch1*, and *Hes1* expression

One-way ANOVA analysis indicated a statistical trend in *Asph* mRNA expression and a statistically significant

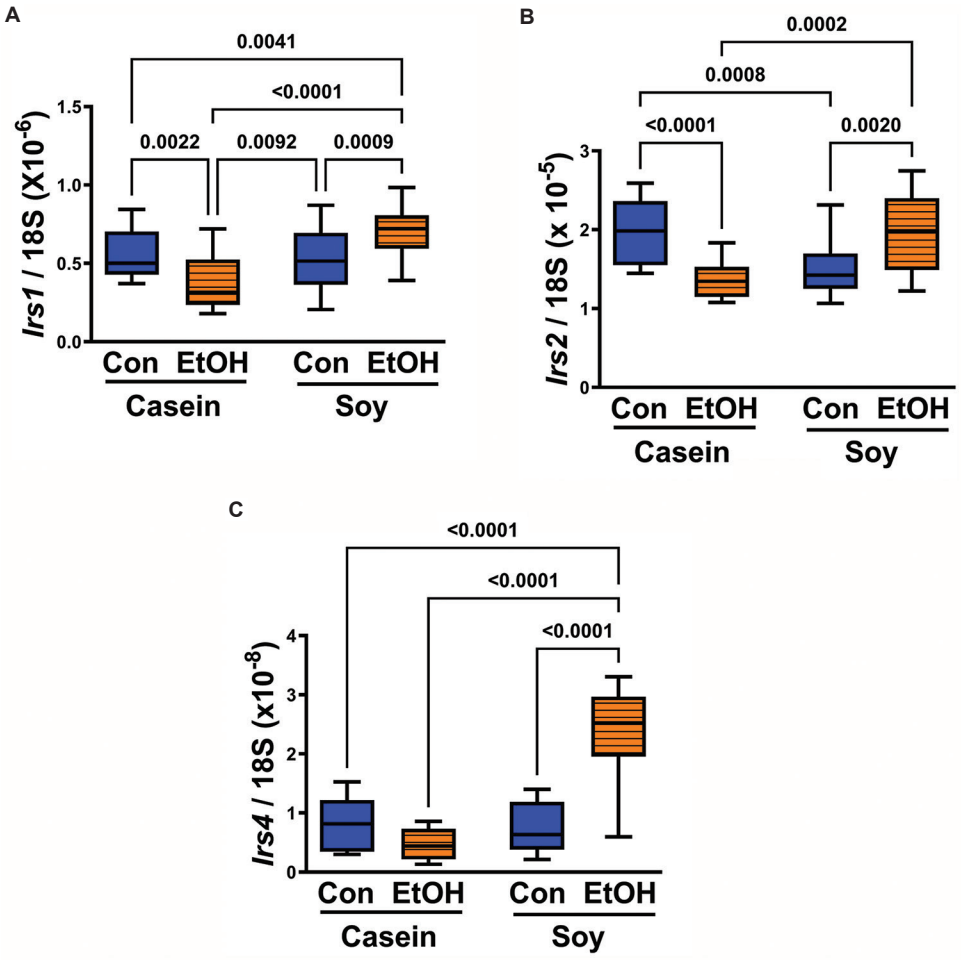

Figure 3. Effects of chronic gestational ethanol exposure in combination with dietary soy versus casein on the relative abundance of (A) *Irs1*, (B) *Irs2*, and (C) *Irs4* mRNA levels in placental tissue. Boxplots depict the calculated ratios of specific polymerase chain reaction-amplified products (mRNAs) to 18S rRNA. Inter-group statistical comparisons were conducted using one-way analysis of variance (see Table 1) followed by *post hoc* Tukey tests. The *P*-values ≤0.05 are considered statistically significant. Notes: Con: Control; EtOH: Ethanol.

difference in ASPH immunoreactivity (Table 2). *Post hoc* Tukey tests revealed that in the casein groups, ethanol significantly reduced *Asph* mRNA levels ($P = 0.0136$) (Figure 4A) and immunoreactivity ($P < 0.0001$) (Figure 4B) compared to corresponding controls. In contrast, in the soy groups, both control and ethanol-exposed groups exhibited similar levels of *Asph* mRNA (Figure 4A) and ASPH immunoreactivity (Figure 4B). Furthermore, *Asph* mRNA levels in the CS and ES groups were comparable to those in the CC group, whereas ASPH immunoreactivity levels, though elevated relative to the EC group, were reduced relative to the CC group (Figure 4B).

Notch1 mRNA expression varied significantly among the groups, as indicated by one-way ANOVA analysis ($P = 0.0013$) (Table 2). Ethanol did not significantly inhibit *Notch1* expression. Instead, the main response was a significant elevation of *Notch1* expression in the ES group compared to the other three groups (Figure 4C). *Hes1* mRNA levels also varied significantly among the groups ($P < 0.0001$) (Table 2). *Post hoc* Tukey tests revealed significantly lower levels of *Hes1* in the EC group compared to the CC group, and higher levels in the CS group compared to the other three groups (Figure 4D).

4. Discussion

In an earlier companion study, we demonstrated that experimental chronic gestational exposure to ethanol led to increased fetal loss, IUGR, placentation abnormalities, and fetal morphogenic attributes reminiscent of FASD.[1] These ethanol-induced effects were associated with altered expression of insulin, IGF, and downstream

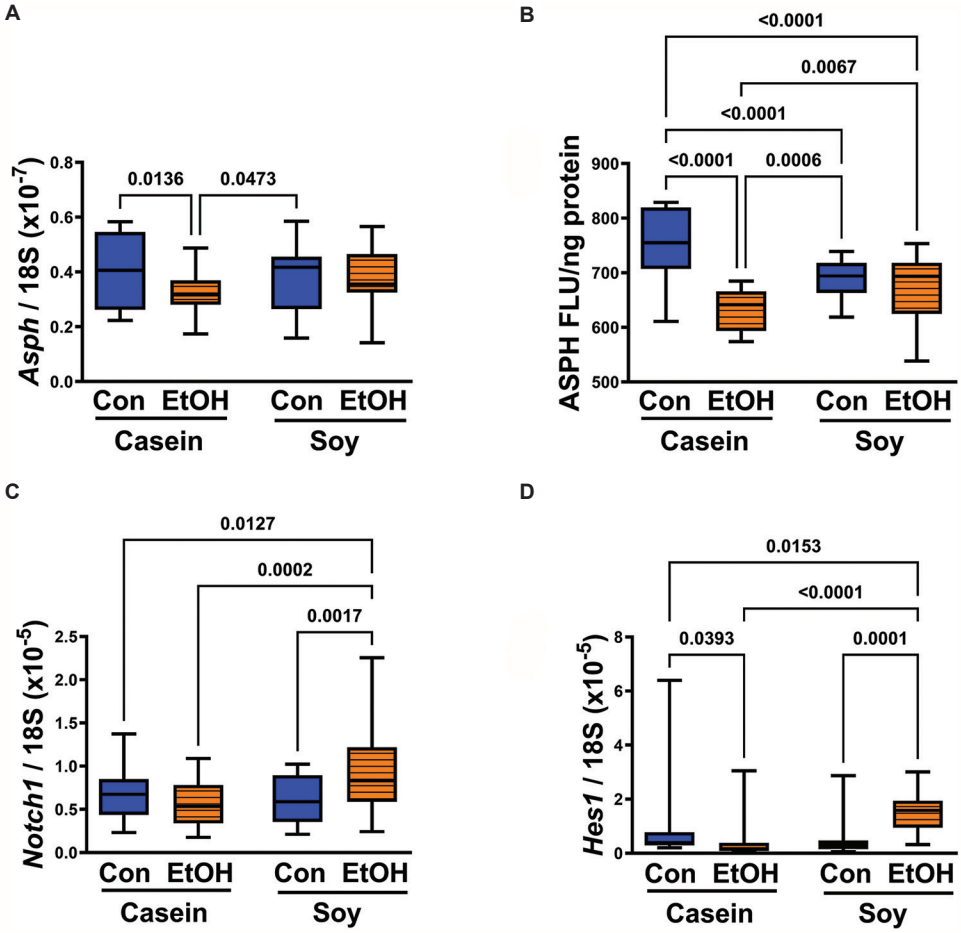

Figure 4. The inhibitory effects of ethanol on (A) *Asph* mRNA expression level and (B) ASPH immunoreactivity, as well as on (D) *HES1* mRNA expression level, and the stimulatory effects of dietary soy on (A) *Asph* mRNA expression level and (B) ASPH immunoreactivity, as well as on (C) *Notch1* and (D) *Hes1* mRNA expression levels. *Asph, Notch1*, and *Hes1* mRNA expression levels were measured using qRT-PCR analysis, with results normalized to 18S rRNA. ASPH immunoreactivity was measured using duplex ELISA, with immunoreactivity normalized to protein content. Inter-group statistical comparisons were conducted using one-way analysis of variance (Table 2) followed by *post hoc* Tukey tests. The $P \leq 0.05$ are considered statistically significant.
Notes: Con: Control; EtOH: Ethanol.
Abbreviations: ASPH: Aspartyl-asparaginyl-β-hydroxylase; ELISA: Enzyme-linked immunosorbent assay; qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction.

signaling proteins and phospho-proteins.[1] The shifts in phospho-protein levels correspond with the net dynamic results of kinase and phosphatase activities, along with the expression levels of corresponding signaling molecules. However, we observed that replacing casein with soy in the diets diminished or eliminated many of the alcohol-associated impairments in insulin/IGF signaling in the placenta, reduced the occurrences of fetal loss, and prevented characteristic FASD-related morphometric developmental abnormalities.[1] Importantly, the normalization of placentation and fetal growth was linked to the insulin-sensitizing and antioxidant effects of soy, which enhanced signaling through the insulin and IGF-1 receptors and downstream through Akt pathways in placental trophoblast.[1]

The overall goal of the present study was to investigate the mechanisms by which prenatal alcohol exposure impairs insulin and IGF signaling, and how soy mitigates these effects by characterizing the relevant effects on gene (mRNA) expression. Conceptually, ethanol could adversely impact intracellular signaling through insulin/IGF pathways by altering protein stability, turnover, and phosphorylation state through the regulation of kinase or phosphatase activities. Although we used the same previously described model, this study focused on insulin/IGF signaling through IRS pathways, primarily by measuring mRNA expression. In addition, since ASPH is a downstream target of insulin/IGF, plays a critical role in trophoblast motility and invasion required for implantation, and has been shown to be inhibited by

Table 2. Ethanol and dietary soy effects on placental expression of ASPH, Notch1, and HES1

Variable	*F*-ratio	*P*-value
mRNA		
Asph	2.431*	0.0726*
Notch1	5.692	0.0013
Hes1	8.342	<0.0001
Protein		
ASPH	21.63	<0.0001

Notes: The mRNA transcripts were measured in CC, CS, EC, and ES placental tissue homogenates through qRT-polymerase chain reaction analysis. Results were normalized to 18S rRNA measured in the same samples. ASPH protein was measured using ELISA, with results normalized to protein content. Inter-group comparisons (n=8/group) were conducted using one-way ANOVA. The *F*-ratios and *P*-values are tabulated. *Marks *P*-values with a statistical trend ($0.05 \leq P \leq 0.10$). See Figure 4 for graphed data and *post hoc* Tukey multiple comparisons test results.

ethanol in other FASD studies,[6] we examined ASPH's mRNA and protein expression to corroborate the ethanol-induced alterations in insulin/IGF signaling and further evaluate the insulin-sensitizing effects of dietary soy in our model.

Previous studies of rat placental tissue demonstrated the expression of insulin, *Igf1*, and *Igf2* trophic factors. The higher level of *Igf2* compared with *Igf1* mRNA is consistent with the concept that IGF-2 plays more important metabolic and mitogenic signaling roles during early development, whereas IGF-1 regulates similar functions later in development and life.[29] The current work suggests that ethanol-associated impairments in placental insulin signaling could be mediated by reduced insulin mRNA expression, akin to trophic factor withdrawal. The selective absence of an ethanol inhibitory effect on IGF-2 suggests that IGF networks may be less vulnerable than insulin pathways to the adverse effects of ethanol. The significant soy-mediated increases in both insulin and IGF-2 in ethanol-exposed placentas suggest that the insulin-sensitizing, anti-inflammatory, and antioxidant effects of soy enhance the availability of trophic factors and ligand regulation of pathways utilized for growth, metabolism, and placentation in the setting of chronic ethanol exposure. In addition, there is supportive evidence that plant-based phytonutrients in soy positively impact metabolism and cellular functions by influencing gene expression.[30]

The expression of insulin, IGF-1, and IGF-2 receptors in placental tissue has been previously reported.[24] The ethanol-associated inhibition of *Insr* mRNA is consistent with previous findings from ELISA analyses,[1] suggesting that its expression is regulated at the transcriptional level. However, the reduction in *Igf1r* mRNA level observed with ethanol exposure does not align with immunoreactivity results, which indicated no significant modulation by ethanol. There are no prior data on the impact of chronic ethanol exposure on IGF-2R immunoreactivity. Dietary soy differentially impacted insulin/IGF receptor expression, suppressing insulin receptor mRNA levels in control groups while elevating *Igf1r* and *Igf2r* levels in ethanol-exposed groups. The reduced insulin receptor mRNA levels in both control and ethanol soy groups, compared to casein controls, are consistent with multiplex ELISA findings. However, the comparable levels of *Insr* and *Igf1r* mRNA in the CS and ES groups contrast sharply with the significantly lower protein levels in the ES group.[1] Altogether, these findings suggest that the expression of placental insulin and IGF receptors is regulated at the mRNA level. However, in the setting of chronic ethanol exposure, post-transcriptional mechanisms, such as translation regulation or protein stability, negatively impact receptor protein expression.

The concurrent reductions in insulin polypeptide and receptor levels in the EC group indicate that ethanol impairs placental insulin signaling, consistent with previous reports in other cell types and tissues.[26,31] In contrast, the IGF-1 pathway appears to be moderately resistant to ethanol's inhibitory effects; despite reductions in the *Igf1r* mRNA level, *Igf1* mRNA and IGF-1R protein expressions were preserved. Similarly, the absence of effects on *Igf2* and *Igf2r* mRNA expression reflects the preservation of related pathways vis-à-vis chronic ethanol exposure. A puzzling observation was that despite dietary soy-associated reductions in INSR and IGF-1R proteins, and a lack of *Insr* mRNA stimulation, placentation normalized, and the phenotypic effects of FASD were abolished.[1] Therefore, while it is reasonable to attribute the ethanol effects to impairments in insulin signaling, the rescue effects of dietary soy were not mediated by the restoration of insulin pathway mediators. Similarly, the findings that *Igf1* mRNA level was unaffected, *Igf1r* was normalized, but IGF-1R protein was inhibited do not strongly support the notion that IGF-1 signaling was restored by dietary soy. Instead, the dominant positive responses were observed with respect to IGF-2, as both ligand and receptor mRNA levels were significantly increased by dietary soy in the ES group. IGF-2 can compensate for impaired insulin pathways by supporting mitogenesis and metabolic functions during development.[32,33] This study provides the first demonstration that the positive rescue effects of dietary soy during development are mediated, at least in part, through IGF-2-activated networks.

The present study included measurements of *Irs1*, *Irs2*, and *Irs4*, whereas the multiplex ELISAs evaluated in the

earlier publication only assessed IRS-1 protein expression.[1] In the casein groups, the inhibitory effects of ethanol on *Irs1* mRNA level corresponded with decreases in protein concentration. Along with the further demonstration of ethanol-reduced *Irs2* and *Irs4* mRNA levels, it is reasonable to conclude that chronic gestational exposure to ethanol broadly inhibits signaling through IRS molecules, thereby contributing to impairments in placentation and fetal development. Dietary soy's broad upregulation of *Irs1*, *Irs2*, and *Irs4* mRNA levels in ethanol-exposed placental tissue supports the notion that the corresponding normalization of impaired placental and fetal development was mediated by enhanced signaling through these docking proteins. However, the interpretation of the data is limited by the lack of information on IRS-2 and IRS-4 protein expression. For example, although dietary soy elevated *Irs1* mRNA to levels above those in the EC, CC, and CS groups, IRS-1 immunoreactivity was suppressed relative to the CC and EC groups, suggesting that the capacity to transmit downstream signals through IRS-1 may have remained compromised. Conversely, the lower levels of inhibitory S312 phosphorylation of IRS-1 would have supported IRS-1 signaling in ethanol-exposed placentas.[1] The lack of information about the abundances of IRS-2 and IRS4 limits further mechanistic interpretation. However, it can be speculated that the combined increases in *Irs1*, *Irs2*, and *Irs4* mRNA levels induced by dietary soy in ethanol-exposed samples contributed to the prevention of FASD and placentation impairments.

ASPH is a downstream target of insulin/IGF signaling.[34,35] Its stimulatory effects on cell motility and invasion, which are essential for placentation, are mediated by catalytic activation of Notch (hydroxylation) and the subsequent increase in *Hes1* transcription.[4,7,35,36] Previous experiments demonstrated that molecular silencing of ASPH in the placenta inhibits trophoblast motility, Notch signaling, and fetal growth.[37] Correspondingly, ethanol inhibition of ASPH expression in the placenta is associated with impaired placentation and IUGR, along with inhibition of Notch-1 signaling.[24] Therefore, it was of interest to determine if dietary soy positively impacted ASPH expression to mediate enhanced placentation in ethanol-fed dams.

The adverse effects of ethanol on insulin and IGF signaling can be detected by measuring the expression of downstream molecular targets such as ASPH.[2,21,28] Previous studies have demonstrated that *Asph* mRNA and protein levels are increased through insulin or IGF stimulation.[34] ASPH is abundantly expressed in normal placental trophoblasts and plays functional roles in placentation, including implantation,[37] which is crucial to fetal development. Consistent with previous reports, chronic ethanol exposure inhibited ASPH expression. In addition to reducing its mRNA levels, ethanol inhibits ASPH protein by increasing GSK-3β activity, leading to its phosphorylation and proteolytic degradation.[2,35] The modest but statistically significant increase in ASPH protein in the ES group compared with the placentas from the EC group corresponds with previously reported reductions in GSK-3β activity.[1] The increased ASPH expression in the placentas of ES dams likely contributed to the normalization of placentation and the prevention of FASD effects.

ASPH functions in part by hydroxylating and activating Notch[37] within its epidermal growth factor-like domain.[38] Activation of Notch results in cleavage and nuclear localization of the Notch intracellular domain,[10] followed by increased transcription of *HES* or *HEY* genes.[10] Although ethanol did not reduce Notch expression in the EC group, dietary soy significantly increased *Notch1* mRNA in the ES group, correspondingly increasing *Hes1* expression. In contrast, the EC group was associated with significantly reduced *Hes1* expression. Altogether, these findings suggest that dietary soy enhances insulin/IGF signaling through IRS molecules, thereby improving placentation by increasing ASPH expression and Notch activation of HES1.

5. Conclusion

The combined information from the earlier report and the present study provides a better mechanistic understanding of how alcohol exposures during pregnancy impair placentation and how dietary soy could ameliorate these adverse effects. Future studies should investigate the extent to which dietary soy interventions at different stages — early gestation, later gestation, or pre-gestation — prevent alcohol-related impairments in placentation and fetal development. Recent experimental evidence highlights the benefits of postnatal and adolescent-stage dietary soy in preventing long-term cognitive and motor deficits caused by chronic ethanol exposure.[21] The potential benefits of consuming lower levels of soy or other phytonutrient-rich legumes during pregnancy should be studied, as a 100% dietary soy protein regimen would be extremely challenging to maintain. Furthermore, evidence suggests that prenatal dietary soy has beneficial effects on normal brain development and function[1] and supports insulin resistance disease states[39] known to be linked to neurodegeneration.[40]

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

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Methodology: Fusun Gundogan, Suzanne M. de la Monte
Writing-original draft: Suzanne M. de la Monte
Writing-review & editing: All authors

Ethics approval and consent to participate

The Lifespan IACUC of Rhode Island Hospital approved the experimental use and treatment of Long Evans rats for this research.

Consent for publication

Not applicable.

Availability of data

Data can be shared on request from the corresponding author.

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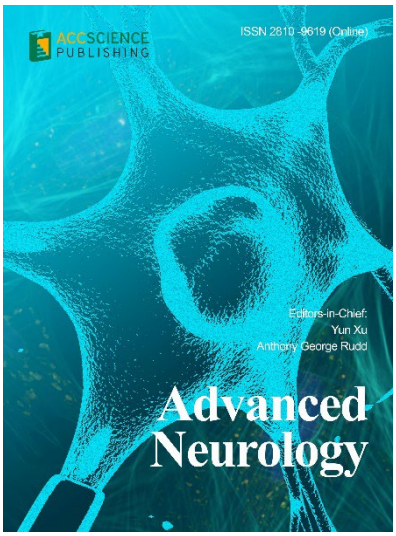

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