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Modulating ZEB1 activity in stem cells

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This thesis is dedicated to my parents, whose love and encouragement shaped the person I am today. Although they are no longer with me, their support, wisdom, strength and passion for education continue to guide my every step. To my brothers, both of whom played vital roles in my journey. Your support and belief in me have been invaluable. Although, now I have been deprived to have one of you, But dearest Saeed, I cherish your presence and your lovely family— Elham and Hannah—in my life.

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Kurzzusammenfassung

Neuroinflammation gilt als ein wesentlicher Faktor bei der Entstehung und Progression neurodegenerativer Erkrankungen wie der Multiplen Sklerose (MS). Unter den beteiligten pathophysiologischen Mechanismen sticht der epithelial-mesenchymale Übergang (EMT) hervor, da er die normale neuronale Funktion erheblich beeinträchtigt. Der Transkriptionsfaktor ZEB1 (Zinc finger E-box-binding homeobox 1) nimmt als zentraler Regulator des EMT eine Schlüsselrolle ein und rückt zunehmend in den Fokus der Forschung, da ihm eine bedeutende Rolle in der Steuerung inflammatorischer Signalwege sowie der neuronalen Entwicklung zugeschrieben wird. Obwohl die vollständige Charakterisierung der Funktion von ZEB1 im Rahmen zentralnervöser Dysfunktionen noch andauert, deuten aktuelle Studien darauf hin, dass ZEB1 maßgeblich an der Regulation inflammatorischer Prozesse und zellulärer Übergänge beteiligt ist, die für die Pathogenese neurologischer Erkrankungen relevant sind. ZEB1 interagiert mit immunologischen Komponenten sowie inflammatorischen Zytokinen und trägt über die Modulation intrazellulärer Signaltransduktionswege zur Aufrechterhaltung neuroinflammatorischer Prozesse bei.

In den letzten Jahren wurde der Einsatz neuraler Stammzellen (NSCs) als innovativer Therapieansatz für neurodegenerative Erkrankungen intensiv erforscht. Allerdings stellen Immunabstoßungsreaktionen und eine bestehende Entzündung bedeutende Hürden für den Erfolg solcher Zelltransplantationen dar. Ein vertieftes Verständnis der Rolle von ZEB1 in diesen Zusammenhängen ist entscheidend, um stammzellbasierte Therapiestrategien zu optimieren.

Im Rahmen der vorliegenden Untersuchung wurde der Einfluss von ZEB1 auf NSCs, die aus der Subventrikulärzone von Rattenhirnen isoliert wurden, näher analysiert. Dabei wurde die Expression von ZEB1 sowohl vor als auch nach der Differenzierung der NSCs zu Oligodendrozyten – Zellen, die für Remyelinisierung und neuronale Reparatur essentiell sind – untersucht. Anschließend erfolgte eine gezielte Suppression der ZEB1-Expression mittels siRNA-Technologie. Um ein inflammatorisches Milieu zu simulieren, wurden die Zellen anschließend einem konditionierten Medium aus mit Lipopolysaccharid aktivierten Mikrogliazellen ausgesetzt. Die Auswirkungen der ZEB1-Downregulation wurden hinsichtlich Zellmigration, Koloniebildungskapazität, oxidativem Stress (gemessen über reaktive Sauerstoffspezies) sowie der Expression proinflammatorischer Zytokine wie IL-6, IL-1β, TNF-α und IL-17 untersucht. Zur Analyse der genannten Parameter kamen Methoden wie Immunzytochemie, quantitative PCR, ROS-Assays, Wundheilungstests und Koloniebildungsassays zum Einsatz. Die gewonnenen Ergebnisse liefern neue Erkenntnisse über die

funktionelle Bedeutung von ZEB1 unter neuroinflammatorischen Bedingungen und schaffen eine Grundlage für die Weiterentwicklung stammzellbasierter Therapiekonzepte bei Erkrankungen des zentralen Nervensystems.

Abstract

Neuroinflammation is known as a major contributor to the onset and progression of neurodegenerative conditions such as multiple sclerosis (MS). Among the mechanisms implicated in these disorders, the epithelial-mesenchymal transition (EMT) significantly disrupts normal neural function. The transcription factor ZEB1 (Zinc finger E-box-binding homeobox 1), as a central regulator of EMT, has been considered a molecule of interest due to its influence on inflammatory signaling and neural development. Although the complete recognition of ZEB1 activity in central nervous system dysfunction remains under investigation, accumulating evidence suggests it is a main regulator in mediating inflammatory responses and cellular transitions relevant to neurological pathology. ZEB1 interacts with immune components and inflammatory cytokines, contributing to neuroinflammatory processes by altering intracellular signaling pathways.

In recent years, the use of neural stem cells (NSCs) has been investigated as a novel therapeutic avenue for neurodegenerative diseases. However, immune rejection and existing inflammation often restrict the success of stem cell transplantation. Understanding how ZEB1 influences these interactions is crucial for optimizing stem cell-based therapeutic strategies.

In this research, we focused on examining the role of ZEB1 in NSCs derived from the subventricular zone of rat brains. The study assessed ZEB1 expression before and after the differentiation of NSCs into oligodendrocytes, which are essential for remyelination and neural repair. Then, ZEB1 expression was suppressed using siRNA techniques, and cells were subsequently exposed to an inflammatory environment created using a conditioned medium from lipopolysaccharide-activated microglia. The effects of ZEB1 knockdown were evaluated in terms of cell migration, colony-forming capacity, oxidative stress (via reactive oxygen species), and the expression of pro-inflammatory cytokines such as IL-6, IL-1β, TNF-α, and IL-17. A combination of immunocytochemistry, quantitative PCR, ROS assays, wound healing, and colony formation methods was applied to assess these parameters. The findings offer new insights into the functional role of ZEB1 in neuroinflammatory conditions and provide a foundation for enhancing the effectiveness of stem cell-based interventions in the treatment of CNS diseases.

List of Abbreviations

AD	Alzheimer's disease
ASPP2	Apoptosis-stimulating protein P53
BBB	blood-brain-barrier
BCL6	B-cell CLL/lymphoma 6
BSA	bovine serum albumin
CB839	*N*-[5-[4-[6-[[2-[3-(trifluoromethoxy) phenyl]-acetyl]amino]-3-pyridazinyl]butyl]-
CNS	central nervous system
CSFs	colony-stimulating factors
CSF2	colony-stimulating factor 2
CtBP2	C-terminal binding protein-2
COX-2	cyclooxygenase-2
CXCL1	C-X-C Motif Chemokine Ligand 1
DAPI	4',6-Diamidino-2-phenylindole
DMEM/F12	Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
e.g.	exempli gratia, for example
EMT	epithelial-mesenchymal transition
ESE1	Epithelial-specific ETS transcription factor 1
et al.	et alia, and others
etc.	et cetera
FBS	fetal bovine serum

FUS	Fused in sarcoma
GBM	glioblastoma
GFAP	Glial Fibrillary Acidic Protein
GFP	green fluorescent protein
GSK-3β	glycogen synthase kinase-3β
HIF-1α	hypoxia-inducible factor 1α
Iba1	ionized calcium-binding adaptor molecule 1
IFN-γ	interferon-gamma
IL	Interleukin
JAK/STAT	Janus Kinase/Signal Transducer and Activator of Transcription
lncRNA	long non-coding RNA
LPS	Lipopolysaccharides
MAFB	musculoaponeurotic fibrosarcoma oncogene homolog B
miRNAs	microRNAs
MMPs	matrix metalloproteinases
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple Sclerosis
NPCs	neural progenitor cells
NSCs	Neural stem cells
OLs	oligodendrocytes
OPCs	Oligodendrocyte precursor cells
PBS	Phosphate buffer saline
PD-L1	programmed death-ligand 1
Pen/strep	penicillin/streptomycin
PI3K	phosphatidylinositol 3-kinase
PI	propidium iodide
PKB	Protein Kinase B

qPCR	Quantitative Polymerase Chain Reaction
ref.	reference
RNA	ribonucleic acid
ROS	reactive oxygen species
siRNA	short interfering RNA
Sox-2	Sex determining region Y-box 2
Sox-10	Sry-related HMg-Box gene 10
SVZ	subventricular zone
T3	tri-iodothyronine
TKB1	Tyrosine Kinase B1
TLRs	Toll-like receptors
TGF-β	transforming growth factor-beta
TAMs	tumor-associated macrophages
TNF-α	Tumor necrosis factor alpha
TAMs	tumor-associated macrophages
TWIST1	Twist-related protein 1
VSNL1	visinin-like 1
Wnt	wingless-related integration site
WST	Water Soluble Tetrazolium
ZEB1	zinc finger E-box binding homeobox-1

Physical quantities

°C	degree Celsius
g	gram
h	hour

min	minute (60 s)
µL	microliter (10^{-6} L)
mL	milliliter (10^{-3} L)
µm	micrometer (10^{-6} m)
µmol	micromol (10^{-6} mol)
mol	mole (amount of substance, $6.02 \cdot 10^{23}$ particles)
nm	nanometer (10^{-9} m)
ng	nanogram (10^{-12} kg)
pmol	Picomoles (10^{-9} mol)
rpm	revolutions per minute

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1. Introduction

1.1. ZEB1 and it role in EMT

ZEB1 (Zinc Finger E-box Binding Homeobox 1) is a key member of the zinc finger-homeodomain family of transcription factors. Originally identified in 1991 as δEF1 due to its interaction with the δ1-crystallin enhancer specific to the lens, ZEB1 has since emerged as a vital regulator of gene expression (Funahashi et al., 1991; Poonaki et al., 2022; Madany et al., 2018). Its pivotal role lies in orchestrating epithelial-mesenchymal transition (EMT)—a dynamic biological process central to embryonic development, tissue remodeling, and pathological states such as cancer and fibrosis (Perez-Oquendo and Gibbons**,** 2022).

EMT is essential during embryogenesis, supporting stem cell differentiation and the acquisition of pluripotency. Beyond development, EMT is also implicated in wound healing, fibrotic diseases, cancer progression, and metastasis (Wu et al., 2020). It is typically categorized into three types: Type I (embryonic development), Type II (tissue regeneration and fibrosis), and Type III (associated with malignancy) (Ashrafizadeh et al., 2020).

Inflammatory cues play a critical role in modulating EMT, particularly in disease states such as neuroinflammation and cancer. Chronic inflammation can drive EMT, contributing to the onset and progression of several neurological and oncogenic disorders (Kang, 2020; Ricciardi et al., 2015; Zhou et al., 2012).

Transcriptional control of EMT is executed by a core group of EMT-associated transcription factors (EMT-TFs), including the Snail family, Twist-related protein 1 (TWIST1), ZEB1, and ZEB2 (Fig. 1) (Feldker N et al., 2020). ZEB1 suppresses epithelial markers like E-cadherin, disrupts cell polarity, and facilitates mesenchymal transformation (Stemmler et al., 2019). When acting alone or alongside other EMT-TFs, ZEB1 contributes to numerous disease processes, including brain tumor development (Krebs et al., 2017; Wellner et al., 2009).

Numerous studies have demonstrated that suppression of the EMT can be accomplished through the knockdown of ZEB1, which can impede the progression of aggressive tumors. Conversely, the upregulation of ZEB1 has been associated with the development of

chemoresistance in various cancers, primarily through the downregulation of E-cadherin (Zhang et al., 2019; Zhang et al., 2016; Chava et al., 2019; Ren et al., 2013). ZEB1 also plays an essential role in embryogenesis and cellular differentiation, acting as a critical regulator in several developmental processes (Sanchez-Tillo, 2010) (Fig. 1).

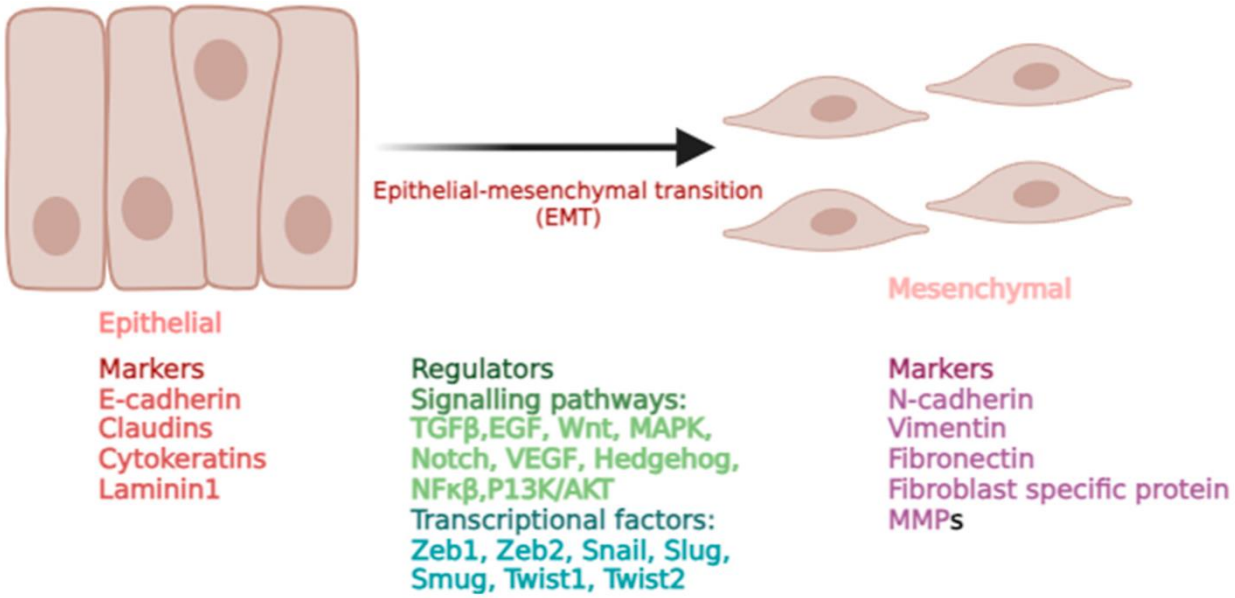

Fig.1 EMT-associated factors, Kanwal R et al., Cancers, 2025

Changes in ZEB1 levels have been shown to influence the self-renewal and lineage of neural stem cells (NSCs) in the brain, highlighting ZEB1's critical role in brain development (Gupta et al., 2021; Liu et al., 2008). ZEB1 helps maintain a delicate balance between epithelial and mesenchymal gene expression, and it also plays a part in controlling how progenitor cells grow and divide (Fratini et al., 2021). Beyond its involvement in the nervous system, ZEB1 has been linked to immune regulation, influencing both the development and activity of the immune response (Arnold et al., 2012; Robert et al., 2021).

1.2. Structural features and functional domains of ZEB1

ZEB1, also referred to as δEF-1 or TCF8, is encoded on chromosome 10p11.2 and belongs

to the ZEB transcription factor family (Williams et al., 1992; Madany et al., 2018). Structurally, the protein contains two C2H2-type zinc finger clusters and a central homeodomain, elements that are crucial for its function in gene regulation.

These domains facilitate its binding to E-box sequences (5'-CANNTG-3') in the promoters of target genes (Fontemaggi et al., 2005; Drapela et al., 2020) (Fig. 2). ZEB1 comprises 1,117 amino acids, and its homeodomain mediates interactions with cofactors such as the C-terminal binding protein (CtBP) and Smad-associated pathways (Chava et al., 2019; Perez-Oquendo and Gibbons, 2022).

In addition to its DNA-binding functions, ZEB1 contains Smad-interacting motifs that enable it to mediate the transforming growth factor-beta (TGF-β) signaling, thereby reinforcing its role in processes such as EMT and tumor progression.

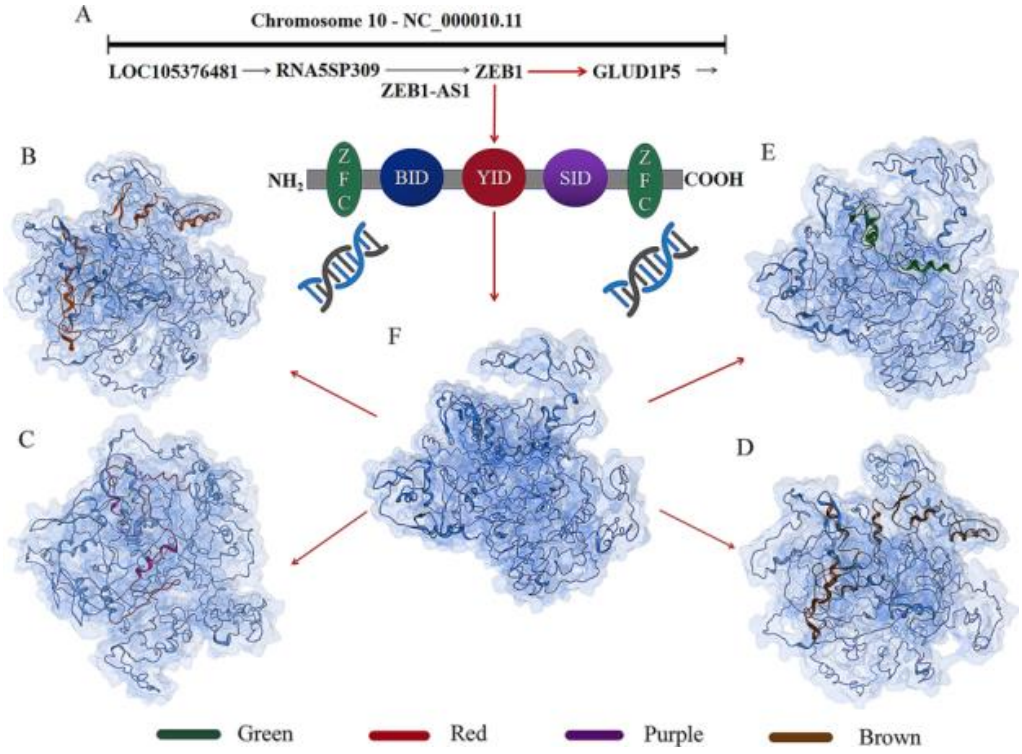

Fig. 2 Schematic representation of the chromosomal position and structural domains of human ZEB1. (A) Shows the genomic location and structural features of the ZEB1 protein. (B-E) Highlight specific interaction sites with molecular partners: SMAD (red), YAP (purple), DNA (brown), and BRG1 (green). (F) Depicts the predicted tertiary structure of ZEB1 in humans, generated using I-TASSER modeling. Abbreviations: ZEB1 – Zinc finger E-box-binding homeobox 1; SMAD – signaling molecule in the TGF-β

pathway; YAP – Yes-associated protein; DNA – Deoxyribonucleic acid; BRG1 – Brahma-related gene 1. (Li et al., Molecular and Cellular Biochemistry, 2020)

In addition to its homeodomain region, ZEB1 contains several key motifs within its central region that are essential for its role in gene regulation. These motifs interact with a variety of signaling proteins and transcription factors, enabling ZEB1 to modulate the activity of numerous genes (De Smedt et al., 2018).

A notable feature is the ability of ZEB1 to interact with Smad proteins through specific Smad-binding domains. This interaction links ZEB1 to the TGF-β signaling pathway (Derynck et al., 2003; Singh et al., 2022). By connecting with Smads, ZEB1 helps mediate signals received at the cell surface and regulates corresponding gene activity within the nucleus. Through these mechanisms, ZEB1 plays a central role in driving EMT and other processes involved in cancer development and progression.

1.3. The mechanism of ZEB1 on various signaling pathways

ZEB1 is a transcription factor involved in multiple signaling pathways that regulate critical cellular processes, including EMT, cell migration, and tumor progression. It plays a pivotal role in both physiological and pathological conditions, particularly in cancer metastasis and chemoresistance (Wu et al., 2020; Poonaki et al., 2022) (Fig. 3).

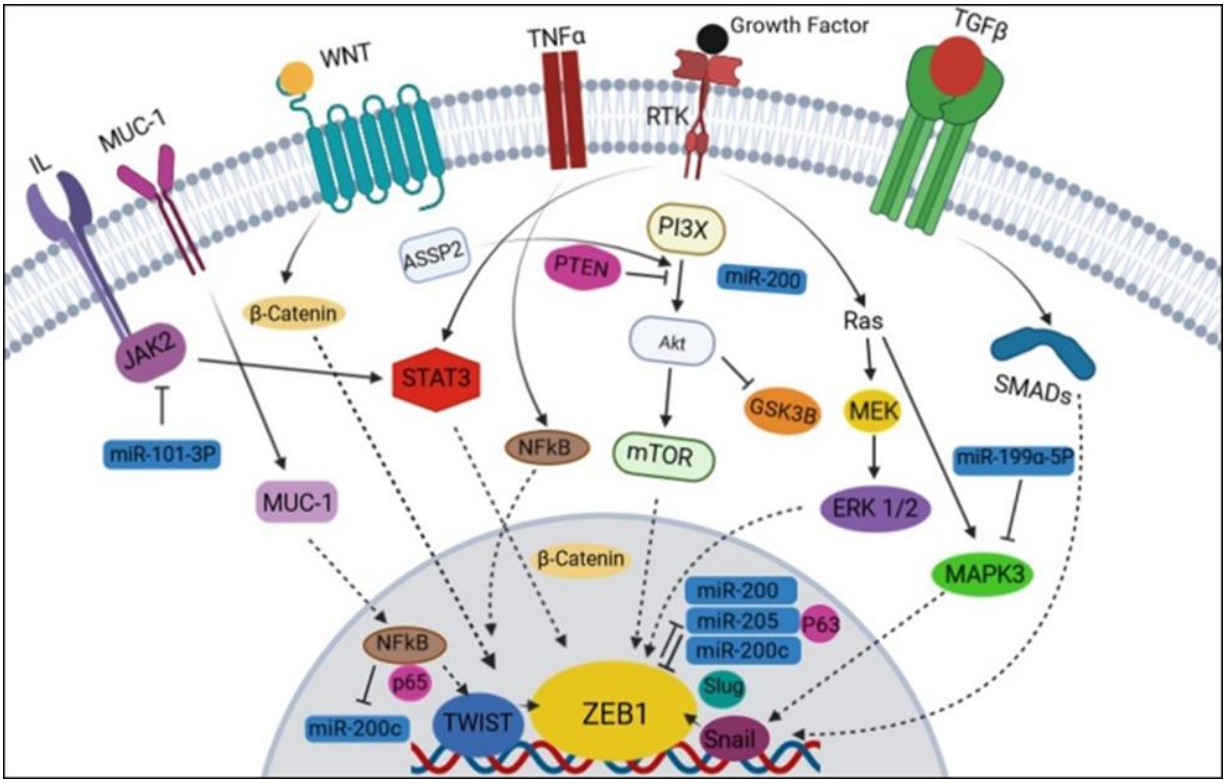

Fig. 3 Schematic figure of Zinc finger E-box binding homeobox 1 (ZEB1) cell signaling pathways: ZEB1 is a key transcription factor that regulates crucial cellular processes. Several upstream signaling pathways, including RAS/ERK, PI3K/AKT, NF-κB, JAK2/STAT3, Wnt/β-catenin, and Smad, promote the expression of ZEB1 either directly or through the mediation of factors such as TWIST, Snail, and Slug. ZEB1 expression is also modulated by miR-200, miR-200c, and miR-205. Additionally, NF-kB contributes to ZEB1 upregulation by inhibiting miR-200c. Factors like transforming growth factor β (TGF-β), tumor necrosis factor (TNFα), and Janus kinase 2 (JAK2) are also involved in this regulatory network. (Poonaki et al., Journal of Neuroinflammation**,** 2022)

A variety of signaling pathways work in concert to regulate the expression of ZEB1. These include TGF-β, Wnt, NF-κB, HIF-1α, COX-2, AKT/mTOR, RAS/ERK, Wnt/β-catenin, PI3K/AKT, and several microRNAs (Zhang P et al., 2015; Brabletz S et al., 2011). These pathways play critical roles in shaping cellular behavior, and ZEB1 often functions as a downstream effector, amplifying their impact, particularly in the induction of EMT.

ZEB1 activity is closely linked to TGF-β signaling, a key driver of EMT that is heavily implicated in fibrosis, tumor metastasis, and tissue repair (Zhang Y et al., 2019). Additionally, pathways such as RAS/ERK and PI3K/AKT, both responsive to extracellular

cues, also influence ZEB1 function (Zhang et al., 2019; Poonaki et al., 2022). Through its interaction with these pathways, ZEB1 plays a multifaceted role in regulating cellular dynamics, especially in the context of cancer progression.

ZEB1 promotes EMT primarily by binding to the promoter region of the E-cadherin gene and suppressing its transcription. E-cadherin is a critical adhesion molecule responsible for maintaining epithelial integrity, and its downregulation is a hallmark of EMT (Vannier et al., 2013). By repressing E-cadherin, ZEB1 disrupts epithelial cell-cell junctions, facilitating the transition to a more migratory and invasive mesenchymal phenotype (Škovierová et al., 2018).

Beyond promoting migration and invasion, ZEB1-mediated repression of E-cadherin also contributes to apoptotic resistance, thereby enhancing chemoresistance (Pan et al., 2021; Duan et al., 2022). Consequently, ZEB1 is regarded as a central regulator of EMT in cancer progression, and targeting ZEB1 has been proposed as a potential strategy to overcome chemoresistance in certain cancer types.

In addition to its role in cancer, ZEB1 is implicated in various pathological conditions of the CNS. By regulating EMT and other cellular processes, ZEB1 influences the progression of neurodegenerative diseases, brain tumors, MS, and other CNS disorders (Poonaki et al., 2022)

1.3.1. ZEB1 and its regulation of the TGF-β pathway

The TGF-β signaling pathway plays a crucial role in regulating ZEB1, particularly in its involvement in EMT. While ZEB1 can act as a downstream effector of TGF-β signaling, it also exhibits antagonistic functions, modulating the extent to which TGF-β promotes EMT.

TGF-β initiates EMT by forming a receptor-activated complex with Smad proteins. This complex then recruits co-activators such as P/CAF and P300 to the promoter regions of target genes. This mechanism is essential for a range of biological processes, including cellular differentiation, fibrosis, wound healing, and cancer metastasis (Postigo et al., 2003; Van

Grunsven et al., 2006). TGF-β is widely recognized as a central driver of EMT and often acts in concert with other signaling pathways, such as Wnt, RAS, and Notch as many of which also regulate ZEB1(Fuxe et al., 2010).

ZEB1 and Smad2 activities are typically upregulated in response to TGF-β stimulation, and together they contribute to the induction of EMT, tumor growth, and recurrence. Interestingly, some newer findings show that high levels of both Smad2 and ZEB1 can persist even in the absence of TGF-β signaling. This suggests the presence of an alternative regulatory mechanism influencing ZEB1 activity (Joseph et al., 2014; Hua et al., 2020).

Moreover, a tightly regulated feedback loop involving TGF-β, ZEB1, and the miR-200 family has been identified, which plays a crucial role in sustaining EMT once it has been initiated (Fig. 4).

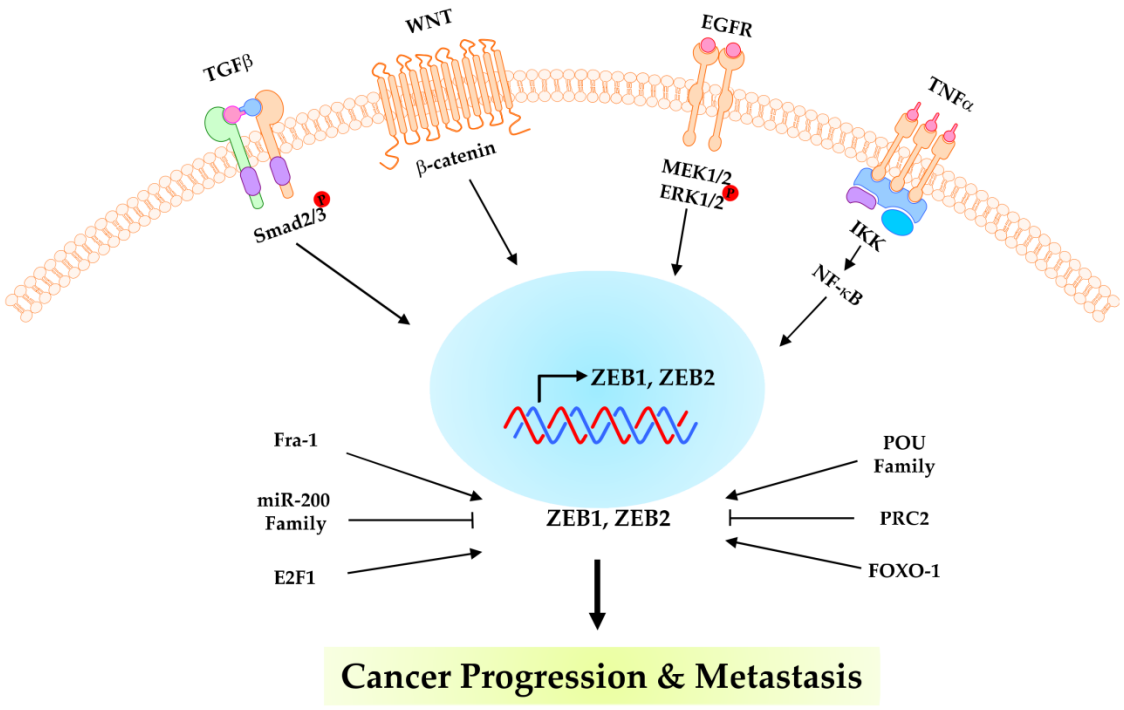

Fig. 4 A diagram illustrating how TGF-β promotes mesenchymal transition during cancer progression and metastasis, with pSMAD2 and ZEB1 playing central roles. TGF-β may originate from tumor cells themselves, surrounding stromal cells, or microglia, and it initiates the development of mesenchymal characteristics within glioblastoma (GBM). This shift adds to the cellular diversity commonly seen among different GBM subtypes (Joseph et al., Cell death & disease, 2014).

The miR-200 family plays a critical role in maintaining ZEB1 expression at controlled levels, thereby influencing the TGF-β/ZEB1 signaling axis and modulating the EMT process (Gregory et al., 2011). In addition to microRNA regulation, ZEB1 activity is also affected by BCL6, a transcriptional repressor known to indirectly suppress E-cadherin expression—an essential marker of the epithelial phenotype and a key target in EMT regulation (Yu et al., 2015; Li et al., 2016). The interplay between BCL6 and ZEB1 highlights the multilayered complexity of EMT regulation and its involvement in cancer progression.

1.3.2. ZEB1's role in E-Cadherin suppression and chromatin remodeling

One of the key mechanisms ZEB1 drives EMT is by suppressing the expression of E-cadherin, an essential adhesion protein that is responsible for maintaining the epithelial phenotype. ZEB1 mediates this suppression through both direct and indirect mechanisms. In one mechanism, ZEB1 interacts with chromatin-remodeling proteins like BRG1, a component of the SWI/SNF complex. This interaction enables ZEB1 to repress E-cadherin expression independently of CtBP, which promotes the mesenchymal characteristics associated with EMT (Sanchez-Tillo et al., 2010). Interestingly, blocking the interaction between ZEB1 and BRG1 can bring back E-cadherin expression, effectively slowing down EMT and reducing cell migration. This highlights just how vital chromatin remodeling is in controlling EMT.

Additionally, CtBP has been recognized as a major co-repressor for BCL6, and it also plays a role in regulating EMT by affecting E-cadherin levels. The dynamic between ZEB1, BCL6, and CtBP forms a complex network that carefully balances the switch between epithelial and mesenchymal states (Mendez et al., 2008; Papadopoulou et al., 2010).

1.3.3. ZEB1 and its modulation of NF-κB, TrkB, and other pathways

ZEB1 has been shown to interact with multiple signaling pathways, including the NF-κB, a

transcription factor essential for regulating inflammation, immune function, and cancer progression. Specifically, ZEB1 can form a complex with MUC1, leading to the activation of NF-κB and its p65 subunit. This activation contributes to the suppression of miR-200c and facilitates the induction of EMT (Rajabi et al., 2014).

Another important function of ZEB1 is its role in inhibiting anoikis, a form of programmed cell death triggered when cells detach from the extracellular matrix. This ability is particularly relevant in cancer metastasis, where tumor cells evade anoikis, survive in circulation, and establish secondary tumors. ZEB1 supports this process by modulating the activity of the receptor tyrosine kinase TrkB (Smit et al., 2011; Kupferman et al., 2010). ZEB1 promotes EMT by stimulating TrkB signaling, enhancing both tumor cell survival and mobility.

In addition, ZEB1 contributes to the regulation of the Twist-Snail axis, a network of EMT-related transcription factors. By modulating this axis, ZEB1 further accelerates EMT progression, thereby promoting increased cancer cell motility and invasiveness (Smit et al., 2009). Conversely, TKB1 has been identified as a factor that can suppress ZEB1 expression through the activation of glycogen synthase kinase-3β (GSK-3β), a crucial enzyme in the Wnt/β-catenin signalling pathway. GSK-3β inhibits EMT via decreasing ZEB1 expression, which is involved in different complex mechanisms that regulate EMT (Liu et al., 2014). Moreover, GSK-3β also operates in coordination with the PI3K/AKT pathway, further influencing ZEB1 expression and its role in EMT regulation (Wu et al., 2012)

1.3.4. ZEB1 and the PI3K/AKT pathway

The PI3K/AKT signaling pathway plays a crucial role in regulating ZEB1 expression. AKT, also known as Protein Kinase B (PKB), facilitates ZEB1 activity by modulating this pathway, thereby promoting EMT across various cancer types. Studies have consistently demonstrated that activation of the PI3K/AKT axis leads to increased ZEB1 expression, contributing to tumor growth and therapeutic resistance (Chen et al., 2013; Zhao et al., 2018).

Conversely, microRNAs such as miR-708 and miR-199a-5p have been identified as negative regulators of ZEB1, acting through the PI3K/AKT/mTOR signaling cascade. These miRNAs represent promising therapeutic targets for suppressing EMT and limiting cancer progression (Sun et al., 2019; Liu et al., 2020).

Additionally, the apoptosis-stimulating protein of p53 (ASPP2), which interacts with the PI3K/AKT pathway, forms a complex with β-catenin. This interaction supports EMT by enabling ZEB1 to repress E-cadherin, a critical mediator of cell-cell adhesion (Wu et al., 2018; Wang et al., 2014).

1.4. ZEB1 and immune modulation

ZEB1 plays a pivotal role in promoting cell migration, partly through its regulation of the miR-200 family and the PI3K signaling pathway (Yang et al., 2014). Beyond its role in cellular motility, ZEB1 also influences immune cell behavior, particularly by modulating macrophage polarization and T-cell migration. It has been shown to direct tumor-associated macrophages (TAMs) toward the M2 phenotype as a state associated with immune suppression and enhanced tumor survival, thereby facilitating tumor progression and metastasis (Guo et al., 2021). These findings underscore ZEB1's broader role in shaping the tumor microenvironment. Moreover, ZEB1 affects T-cell migration, a process essential for generating effective anti-tumor immune responses (Guo et al., 2021).

The tumor suppressor FLF3, which antagonizes oncogenic signaling pathways and negatively regulates EMT, has been shown to inhibit ZEB1 transcription, affecting both the Wnt and RAS pathways (Liu et al., 2019). Further regulation of ZEB1 occurs via the Wnt/β-catenin signaling pathway, where miR-33b has been identified as a suppressor of ZEB1 expression. Downregulation of ZEB1 through miR-33b can silence EMT, offering a potential therapeutic avenue to inhibit tumor progression (Zhang et al., 2015; Qu et al., 2015).

Additionally, the epithelial-specific ETS transcription factor 1 (ESE1) serves as a key regulator of EMT in breast cancer. ESE1 is downregulated by the MEK–ERK pathway, which in turn leads to ZEB1 upregulation and promotion of EMT (Sinh ND et al., 2017). The

ERK1/2-mediated signaling axis further contributes to EMT by regulating ZEB1 expression, supporting increased cell migration and resistance to apoptosis (Chiu et al., 2017).

1.5. Effects of ZEB1 on various cell types of the CNS

ZEB1 is essential for the development of the CNS, playing an important role in processes including cell differentiation, migration, and determining cell fate (Wang, 2019). It has an impact at various stages of neurodevelopment, from the regulation of NSCs to the maturation of neurons and glial cells. Notably, ZEB1's function is highly context-dependent; depending on the cellular environment and the signaling pathways involved, it may act either as a transcriptional repressor or activator, thereby directing the development and maturation of diverse CNS cell types (Poonaki et al., 2022).

1.5.1. NSCs and progenitor cells

ZEB1 plays a key role in controlling the NSCs' proliferation and migration during embryonic development. It functions as a repressive factor, helping to restrict NSC proliferation and migration at critical stages of embryogenesis. This regulatory action involves its interaction with the co-repressor CTBP2, which modulates the activity of important genes like Neurod1 and Pard6b, involved both in neuronal differentiation and polarity (Wang et al., 2019).

ZEB1 helps maintain tight control over the proliferation and movement of NSCs, which is crucial for healthy brain development. At the same time, it plays an important role in guiding human embryonic stem cells (hESCs) to become neurons. In this regard, ZEB1 supports neurogenesis by influencing gene expression patterns that drive the maturation and proper integration of new neurons into the nervous system (Jiang et al., 2018).

1.5.2. Radial glial cells and glial differentiation

The protein ZEB1 is vital for the differentiation of radial glia-like stem cells in the adult hippocampus. Its absence results in a significant decrease in the migration and differentiation of neurons and radial glial cells, which are both crucial for neurodevelopment. This deficiency is attributed to the activity of Pak3, a serine/threonine-protein kinase that plays a role in various cell migration and differentiation mechanisms. When ZEB1 is lacking, the activity of Pak3 leads to inappropriate migration of neural progenitors and disrupts the normal developmental pathways of neurons and glial cells (Liu et al., 2019).

Radial glial cells function as versatile stem cells in the adult brain, with the capacity to generate both neurons and astrocytes. ZEB1 has a critical activity in managing these progenitors, particularly in the hippocampus, where it supports neurogenesis as an essential process for memory, learning, and neural adaptability. ZEB1 helps stem cell development into appropriate neural cell types and maintains a stable environment for neurogenesis by regulating the expression of cell adhesion molecules and proteins that control cellular polarity. In addition, it also affects astrocyte precursor movement from the ventricular zone by modulating Cadherin-1, a key adhesion protein necessary for their migration and subsequent differentiation (Ohayon et al., 2016).

1.5.3. Stem cells and neuronal differentiation

NSCs have the potential to differentiate into the lineages, including neurons, astrocytes, and oligodendrocytes (Hong et al., 2014). The basic helix-loop-helix transcription factor Olig2 plays a significant role in the regulation of the oligodendrocyte lineage. Studies indicated that ZEB1 promotes the expression of Olig2, which facilitates the differentiation of oligodendrocyte precursor cells (OPCs) (Singh et al., 2016).

In addition, the transcriptional activity of ZEB1 suppresses the expression of genes, which are essential for neuronal differentiation, along with those associated with cell polarity and adhesion (Singh et al., 2016). ZEB1 exhibits its regulatory effects not only during the embryonic phase of development but also in the regulation of neuronal polarization,

maturation, and differentiation.

The interaction between ZEB1 and the hypoxia-inducible factor 1 alpha (HIF-1α) pathway is critical for cellular responses to oxygen availability and plays an important role in modulating neuronal polarization and maturation. This interaction emphasizes the important function of ZEB1 in adapting the surrounding microenvironment and ensuring the proper maturation of neurons during processes of development (Kullmann et al., 2020). Additionally, ZEB1 has also been shown to facilitate the transdifferentiation of mouse embryonic fibroblasts into functional neurons, a process with promising implications for regenerative medicine and the treatment of neurodegenerative diseases (Yan et al., 2017).

1.6. Polarity and migratory behavior of neural cells

ZEB1, as a regulator of polarity genes, plays a critical role in neuronal differentiation and migration. This transcription factor helps maintain the balance between self-renewal and differentiation as a key process for ensuring proper neuronal arrangement and connectivity within the CNS by repressing specific polarity genes in NSCs (Singh et al., 2016).

Moreover, ZEB1 is involved in NSC migration as an essential step in establishing neuronal networks during development and facilitating tissue repair in the adult brain. ZEB1 directs the proper positioning of progenitor cells by regulating their migration. This function is vital for tissue development and the regenerative processes (Kahlert et al., 2015).

Recent studies have also revealed the involvement of ZEB1 in retinal development, particularly in its regulation of retinal organoid formation. These organoids serve as models for understanding retinal development and related diseases. Through its influence on retinal organoid development, ZEB1 contributes to the promising therapeutic strategies for treating retinal degenerative diseases (Godidi et al., 2011). Although the precise molecular pathways by which ZEB1 exerts these effects are still under investigation, its impact on cell fate determination and differentiation within the retina is increasingly evident.

1.7. ZEB1's role in inflammatory responses and neurodegeneration

Numerous studies have highlighted the significant role of ZEB1 in promoting inflammatory responses (Fig. 5). ZEB1 is crucial for the development and function of both T cells and B cells (Arnold et al., 2012), and mutations in ZEB1 have been linked to T-cell immunodeficiency (Dean et al., 2015). Beyond its role in immune cell development, ZEB1 is essential for maintaining immune cell viability, mobility, and cytokine expression.

ZEB1 regulates the production of various pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, and TNF-α, primarily through modulation of TGF-β-related STAT3 signaling pathways and NF-κB (Li et al., 2019; Chen et al., 2018; Vandewalle et al., 2009; Kim et al., 2016; Liang et al., 2022).

Interestingly, the relationship is bidirectional: several pro-inflammatory cytokines, such as TGF-β, can also upregulate ZEB1 expression through activation of Smad proteins, tyrosine kinase receptors, NF-κB, and the JAK1-STAT3 signaling pathways (Tang et al., 2017). This feedback loop highlights the complex and dynamic role of ZEB1 in regulating immune and inflammatory responses.

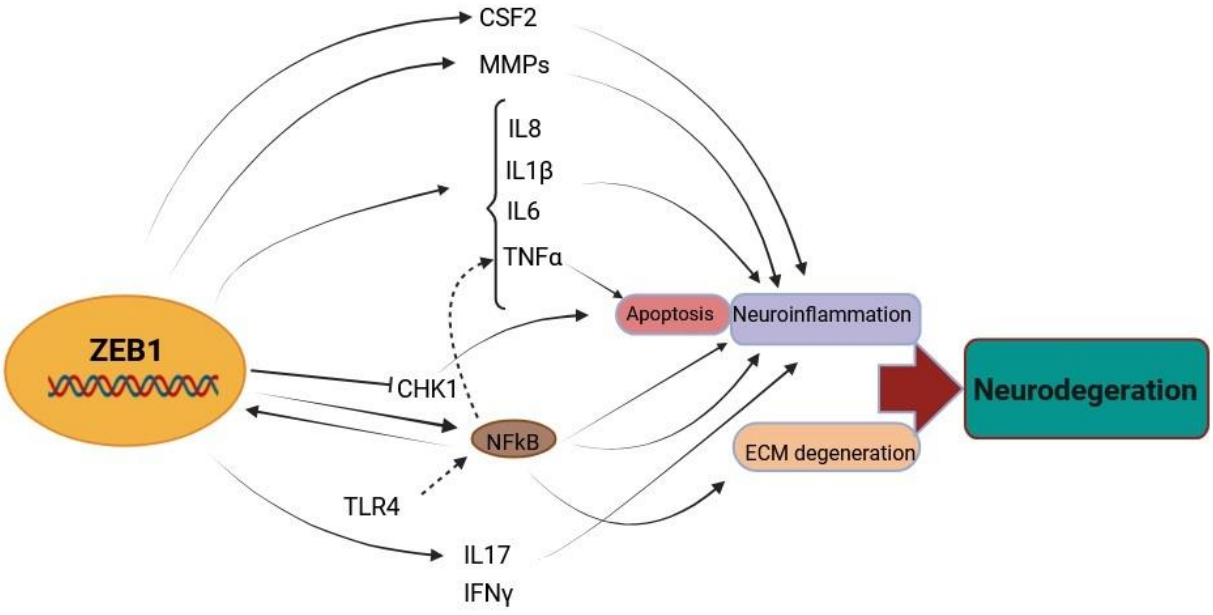

Fig. 5 The diagram illustrates the involvement of ZEB1 in interacting with various inflammatory mediators to promote neuroinflammation. These mediators include interleukin (IL), matrix metalloproteinases (MMPs), nuclear factor kappa B (NF-κB), Type 1 T helper cells (Th1), T helper 17 cells (Th17), colony-stimulating

factor 2 (CSF2), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β). The figure was illustrated using BioRender.

IL-1β has shown the ability to promote inflammation by upregulating ZEB1 expression (Dohadwala et al., 2010). ZEB1 directly enhances the production of inflammatory cytokines, such as IL-6 and IL-8, initiating inflammatory processes that also facilitate tumor growth (Katsura et al., 2017). ZEB1-mediated immune responses play a role in inflammation within the tumor microenvironment, partly through its direct regulation of IL-6 expression (Block et al., 2019). Furthermore, ZEB1 induction related to PD-L1 and CD47 has been shown to promote a proinflammatory microenvironment in the tumor vicinity, contributing to a hostile environment conducive to tumor progression (Guo et al., 2021).

ZEB1 further promotes inflammatory responses by suppressing N-methylpurine DNA glycosylase, a DNA glycosylase, in epithelial cells, which triggers the induction of inflammatory mediators like IL-1β and the generation of reactive oxygen species (de Barrios et al., 2019). Furthermore, IL-17 upregulates the ZEB1-mediated NF-κB pathway and enhances tumor cell migration by stimulating EMT (Gu et al., 2015). Sodium tanshinone IIA sulfunate, an antioxidant and anti-inflammatory compound, can inhibit EMT by targeting ZEB1, Snail1, and the Smad signaling pathway (Zhou et al., 2019).

Induction of local inflammation in the lungs through lipopolysaccharides administration enhances tumor cell migration via a ZEB1-dependent mechanism (De Cock et al., 2016). Increased TNF-α level elevates ZEB1, which is regulated by miR-200c and miR-141, leading to suppression of E-cadherin and modulation of EMT progression (Chakraborty et al., 2015). MiR-9 also directly targets NF-κB, triggering inflammatory responses in lymphatic endothelial cells by promoting EMT-related genes, including ZEB1 (Chakraborty et al., 2015).

In the CNS, microglia serve as the first line of innate immune defense (Streit et al., 2004), while astrocytes are key regulators of both innate and adaptive immune responses (Colombo et al., 2016). ZEB1 expression in both microglia and astrocytes plays a critical role in neuroinflammation (Li et al., 2018). ZEB1 regulates microglial immune responses to CNS insults and reduces the production of astrocytic CXCL1 through the TGF-β-dependent

signaling pathway (Ricciardi et al., 2015). ZEB1 also promotes the neuronal output of neural stem cells in the hippocampus at the expense of glial cells (Gupta et al., 2021).

Moreover, ZEB1 has been implicated in cognitive impairment in humans (Chen et al., 2020). The reduction of ZEB1/2 and lncRNA-1604 in the neocortex and striatum can trigger neurodegenerative processes in a mouse model of Huntington's disease (Langfelder et al., 2016).

Dysregulation of the lncRNA-1604/miR-200c/ZEB axis during neural differentiation may also contribute to neurodegenerative diseases (Weng et al., 2018). Fused in sarcoma (FUS), an RNA-binding protein linked to neurodegenerative diseases, affects ZEB1 expression through miR-200c and its target transcript (Zhang et al., 2018). Moreover, the loss of ubiquilin1, a protein essential for addressing neurological disorders related to protein aggregation, results in significantly increased ZEB1 expression (Shah et al., 2015).

1.8. ZEB1 in neurodegenerative diseases

Neurodegenerative diseases are a group of disorders that progressively impair the central nervous system, leading to the deterioration of nerve cells. This results in difficulties with movement, memory, and cognitive abilities, eventually affecting a person's ability to live independently (Lamptey et al., 2022). Conditions like Alzheimer’s, Parkinson’s, and Multiple Sclerosis are among the most famous disorders, and their increasing evidence, along with the lack of effective treatments, presents them as major challenges for patients and healthcare providers alike (Gadhave et al., 2024).

ZEB1 serves as a pivotal transcription factor in EMT, a biological process where epithelial cells shift toward a mesenchymal phenotype. This transformation is increasingly linked to several pathological states, including cancer progression, fibrotic diseases, and neurodegenerative conditions (Yang et al., 2022). ZEB1 has a wide-ranging impact on the CNS via shaping cellular adaptability, guiding tissue restructuring, and affecting immune signaling, neuronal longevity, and glial cell function. Its control over critical molecular pathways ties it to neuroinflammation as an underlying mechanism in multiple sclerosis and

various neurodegenerative disorders (Poonaki et al., 2022).

In MS, the immune system mistakenly attacks the myelin sheath, leading to demyelination, axonal injury, and progressive disability in patients. Central to the pathogenesis of MS is the activation of microglia and astrocytes, the resident immune cells of the brain, which secrete pro-inflammatory cytokines and chemokines that exacerbate tissue damage. These cells interact in response to various signaling molecules, such as cytokines and growth factors, that cause their activation, migration, and the extent of the inflammatory response (Baecher-Allan et al., 2018).

The role of ZEB1 in modulating the signaling pathways involved in neuroinflammation highlights its potential contribution to the regulation of immune responses in MS (Poonaki et al., 2022). ZEB1 has been shown to influence the expression of several pro-inflammatory mediators by interacting with key cell surface receptors, such as Toll-like receptors (TLRs), IL-1β, IL-6, TNF-α, and IL-8 receptors, which are critical in initiating immune responses during neuroinflammation (Fig. 6) (Liang et al., 2022).

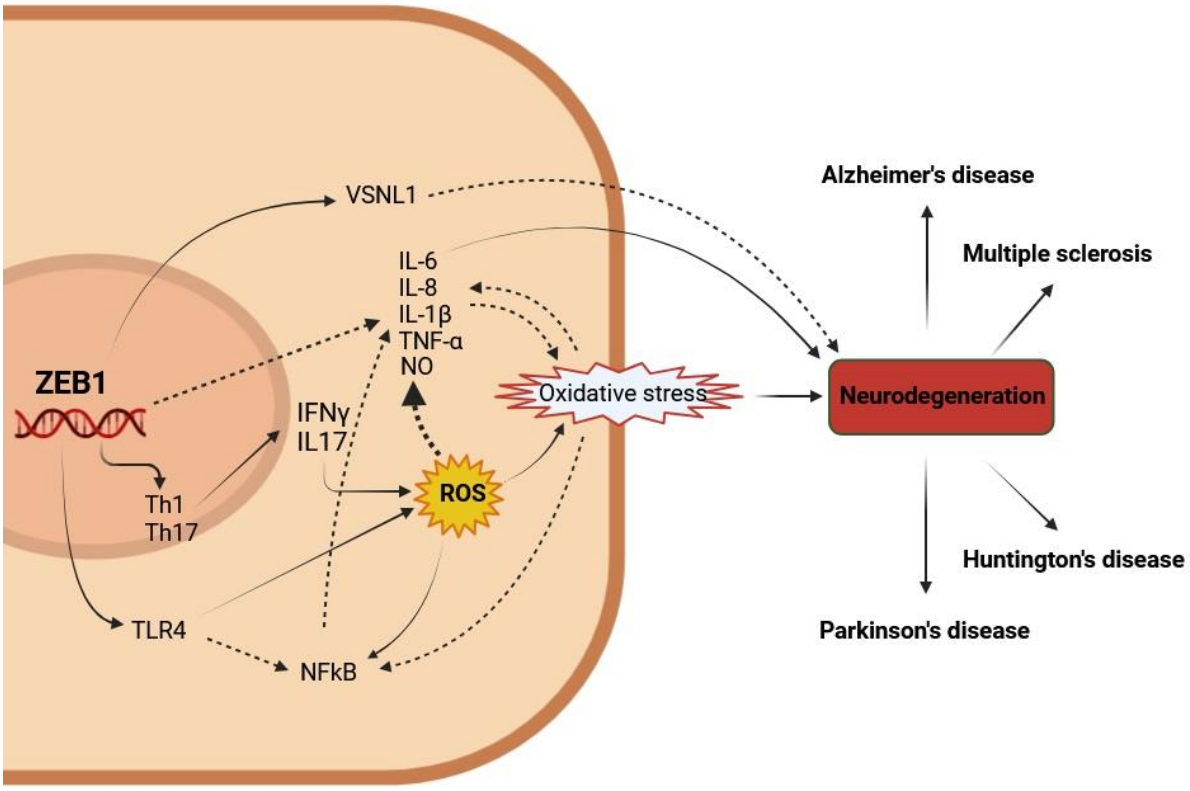

Fig.6 ZEB1 in Neurodegenerative Disease: ZEB1 is a crucial transcription factor that participates in various cellular processes, including EMT, which has been implicated in diseases like cancer, fibrosis, and neurodegenerative disorders. In the CNS, ZEB1 plays a role in regulating immune responses, neuronal survival, glial cell activity, and tissue remodeling. It is particularly involved in the neuroinflammation seen in conditions such as Alzheimer's disease (AD) and Multiple Sclerosis (MS). ZEB1 regulates signaling pathways that affect the activation of immune cells, leading to the production of pro-inflammatory cytokines, which can worsen tissue damage. In MS, ZEB1 contributes to the activation of microglia and astrocytes, which are central to myelin damage and disease progression. In AD, ZEB1 also influences genes involved in microglial function and neuronal signaling, such as VSNL1, a gene related to amyloid plaques and neurofibrillary tangles. The figure was illustrated using BioRender.

ZEB1 and MAFB were recognized as potential transcription factors crucial for regulating AD-related genes in neurons and microglia, respectively. Additionally, the microglial associations showed a significant enrichment for the heritability of AD risk, along with previously identified active regulatory regions (Anderson et al., 2023). VSNL1, as a gene regulated by ZEB1 and responsible for encoding a neuronal Ca2+ sensor, was downregulated in inhibitory neurons in AD. This decrease in expression has been associated with the

presence of amyloid plaques and neurofibrillary tangles (Braunewell, 2012).

1.8.1. ZEB1 and MS

Although the precise function of ZEB1 in MS remains to be fully understood, various studies have revealed its potential roles in the pathology of the disease. One of the key aspects is the dysfunction of brain endothelial cells, which contributes to the initiation of neuroinflammation and cell injury in MS. Research has suggested a connection between damage to the blood-brain barrier (BBB) and the EMT process (Troletti et al., 2016). Furthermore, there is increasing evidence pointing to the involvement of ZEB1 in the disruption of the BBB in pathological states (Leduc-Galindo et al., 2019).

In MS, inappropriate activation of interferon-gamma (IFN-γ)-producing Th1 and Th17 cells is a well-established hallmark of the disease (Arellano et al., 2017). ZEB1 plays a significant role in the differentiation of these pathogenic Th1 and Th17 cells in MS. A mutation in ZEB1 reduces the expression of miR-101-3p, leading to the inhibition of the JAK/STAT signaling pathway, which in turn results in the excessive secretion of IL-17 and IFN-γ (Qian et al., 2021) (Fig. 7).

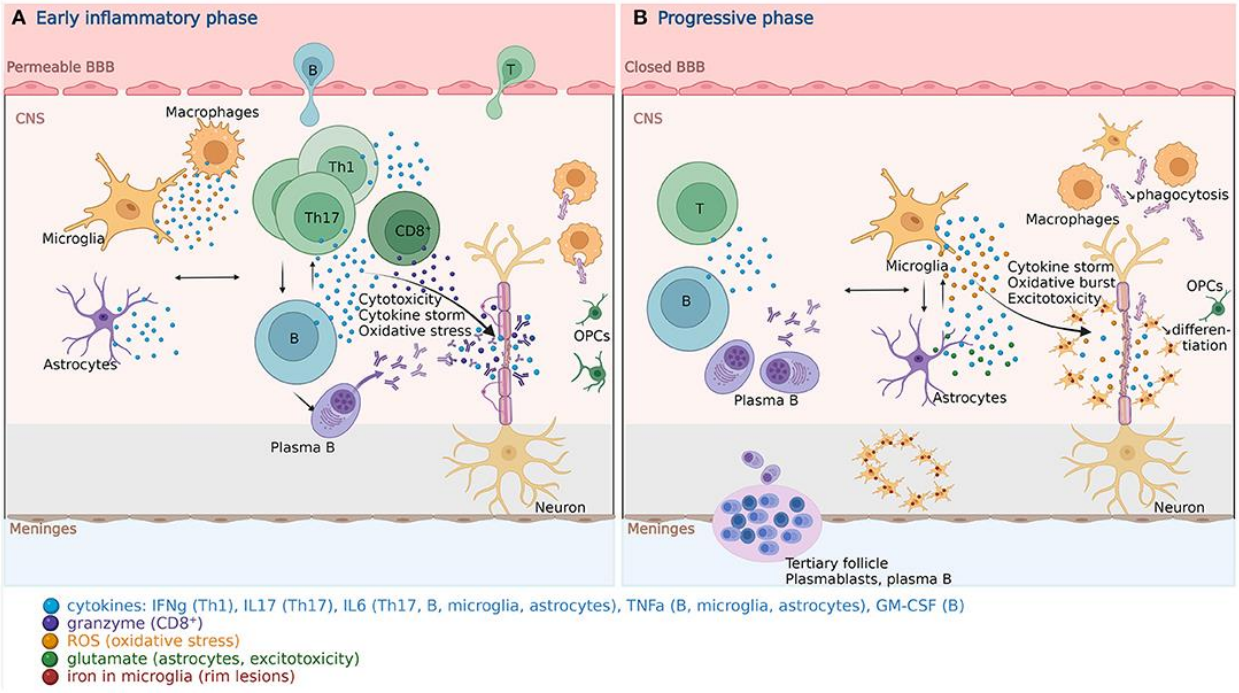

Fig. 7 Cellular Aging and Chronic Inflammation: Implications for Immune Dysfunction and Therapeutic Strategies in Multiple Sclerosis. Overview of the molecular pathways involved in immunosenescence and inflammation, and their contribution to the pathogenesis and therapeutic targeting of multiple sclerosis. Adapted from Perdaens and Van Pesch (2022).

Dysregulation of the JAK/STAT signaling pathway is a well-established contributor to the pathophysiology of various autoimmune disorders, including MS (Benveniste et al., 2014). Additionally, ZEB1 influences IL-2 expression and is implicated in T-cell development by modulating the balance between the splice variants Zfhep1 and Zfhep2 of ZEB1 in an experimental MS model (Stridh et al., 2010; Shuttleworth et al., 2010; George et al., 2016).

Silencing ZEB1 in dendritic cells has been shown to reduce IL-12 production and promote Th2 differentiation (Jalaiei et al., 2021). Another factor that potentially contributes to MS progression is the highly upregulated liver cancer (HULC) long non-coding RNA (lncRNA), which is elevated in MS patients. HULC is involved in the activation of the miR-200a-3p/ZEB1 signaling pathway and the regulation of EMT (Bao et al., 2018).

1.9. Aims of thesis

The central focus of this thesis is to investigate the regulatory role of ZEB1 in the behavior of NSCs under inflammatory conditions; with a specific emphasis on its impact on neurodegenerative disorders such as MS. Neuroinflammation is a key pathological aspect of several CNS diseases, including neurodegenerative diseases and traumatic brain injuries. In the context of these disorders, chronic inflammation can interfere with neurogenesis and have a negative effect on NSCs in supporting tissue repair and regeneration. Given the potential of NSC transplantation as a therapeutic strategy for neuroinflammatory diseases like MS, understanding the molecular mechanisms that modulate NSC behavior in the presence of neuroinflammation is crucial for developing therapeutic approaches.

ZEB1 is a transcription factor that is involved in cellular plasticity as well as having a main role in various aspects of neurogenesis, including NSC maintenance, proliferation, and differentiation. However, the specific mechanisms of ZEB1 in NSC behavior, particularly in inflammatory conditions, remain poorly understood. The goal of this thesis is to address the existing knowledge gap by investigating how the knockdown of ZEB1 impacts NSC behavior in inflammatory conditions. To achieve this, NSCs will be isolated from the SVZ of the rat brain, a key area known for its role in neurogenesis.

Silencing of ZEB1 was performed using small interfering RNA (siRNA) targeting ZEB1, allowing for precise knockdown of the gene. To mimic the inflammatory condition, a conditioned medium was derived from LPS-activated microglia. The effects of ZEB1 on NSCs in the presence and absence of inflammation have been evaluated, including colony formation, cytokine expression levels, cell migration, and ROS production.

The findings presented in this thesis illuminate the role of ZEB1 in regulating the behavior of NSCs, thereby providing a new understanding of the molecular pathways involved in neurogenesis. Furthermore, it identifies ZEB1 as a potential therapeutic target for developing novel strategies aimed at enhancing NSC transplantation in neuroinflammatory disorders of the central nervous system, such as MS.

2. Methods and Materials

2.1. Study design

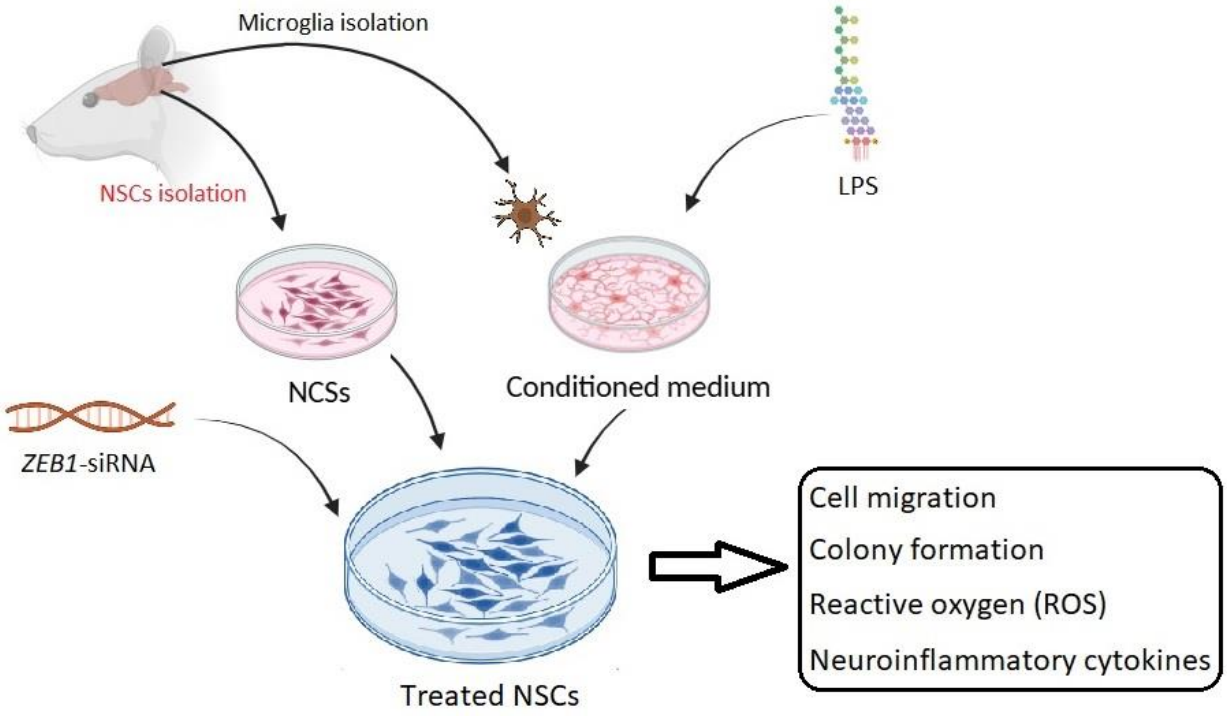

Fig. 8 Schematic study design of the thesis (Created by BioRender)

2.2. Isolation and culturing of neural stem cells from SVZ of rat brains

2.2.1. Ethical approval

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and the institutional guidelines of the University of Münster, Germany. Ethical approval was granted by the State Office for Nature, Environment and Consumer Protection of North Rhine-Westphalia (LANUV NRW). The approval number is 81-02.05.50.21.016, and the approval was granted in November 2021. All procedures involving animals adhered strictly to the approved protocols and ethical standards.

2.2.2. NSCs Cultivation

NSCs were isolated from the subventricular zone of the Rat brains. Adult male and female Norwegian rats (average age: 16 weeks; weight range: 290-650 g) were utilized in this study.

The isolated tissue was washed three times with phosphate-buffered saline (PBS) containing 1% penicillin/streptomycin (Sigma-Aldrich, Germany) to remove any contaminants. The tissue was then mechanically dissociated to break it into smaller fragments. It was then enzymatically digested using 0.25% trypsin/EDTA (Sigma-Aldrich, Germany). The enzymatic reaction was stopped by adding fetal bovine serum (FBS; Gibco, Germany), which neutralized the trypsin. The cell suspension was centrifuged at 900 rpm for 5 minutes, after which the supernatant was discarded. To remove any remaining clumps or debris, the suspension was passed through a 70 μm cell strainer. Following this, the cells were centrifuged again at 1100 rpm for 5 minutes to collect the pellet.

The resulting cell pellet was resuspended and cultured under non-adherent conditions in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12; Sigma-Aldrich, Germany). The culture medium was supplemented with various factors to promote stem cell maintenance and growth: 0.5% N2 supplement (Gibco, Germany), 2 μg/ml heparin (Ratiopharm, Germany), 1% L-glutamine (Sigma-Aldrich, Germany), 2% B27 supplement (Gibco, Germany), 20 ng/ml epidermal growth factor (EGF; Stemcell Technologies, Germany), 10 ng/ml basic fibroblast growth factor (bFGF; Stemcell Technologies, Germany), and 1% penicillin/streptomycin (Sigma-Aldrich, Germany). After 15-20 days of cultivation with regular medium change, the enriched population of NSCs was ready for seeding.

2.3. Differentiation of NSCs to mature myelinating oligodendrocytes

NSCs were seeded in 24-well plates under adherent conditions using Neurobasal™ Plus Medium (Gibco, Germany) as the base culture medium. The medium was supplemented with specific factors essential for supporting differentiation along the oligodendrocyte lineage. These included 20 ng/ml of bFGF (Stemcell Technologies, Germany) and 10 ng/ml of epidermal growth factor EGF (Stemcell Technologies, Germany), which are key mitogens promoting progenitor cell proliferation and expansion. Additionally, the medium was enriched with 1% of T3 hormone (Sigma-Aldrich, Germany) to support OPC maturation and

1% of glutamine (Invitrogen, Germany), a vital amino acid for cellular metabolism and survival. The medium also contained 2% B27 supplement (Gibco, Germany) to provide neurotrophic factors necessary for differentiation, along with 1% penicillin/streptomycin (Sigma-Aldrich, Germany) to prevent bacterial contamination. After five days, the concentrations of EGF and bFGF were reduced by half. EGF was then removed completely, and only 5 ng/ml bFGF was maintained for three additional days before being removed entirely.

The differentiation protocol was designed to follow a multi-step process: initially, NSCs were induced to differentiate into OPCs for approximately 5 days. Subsequently, further differentiation was continued to achieve mature oligodendrocytes (OLs) for 15 to 20 days. This multiphase process is essential for the full maturation of oligodendrocytes capable of myelination. Cells were characterized at different stages of differentiation using specific markers (Fig. 9). Markers included Nestin and SOX2 for NSCs, SOX10 and O4 for OPCs, and MOG (myelin oligodendrocyte glycoprotein) for mature oligodendrocytes.

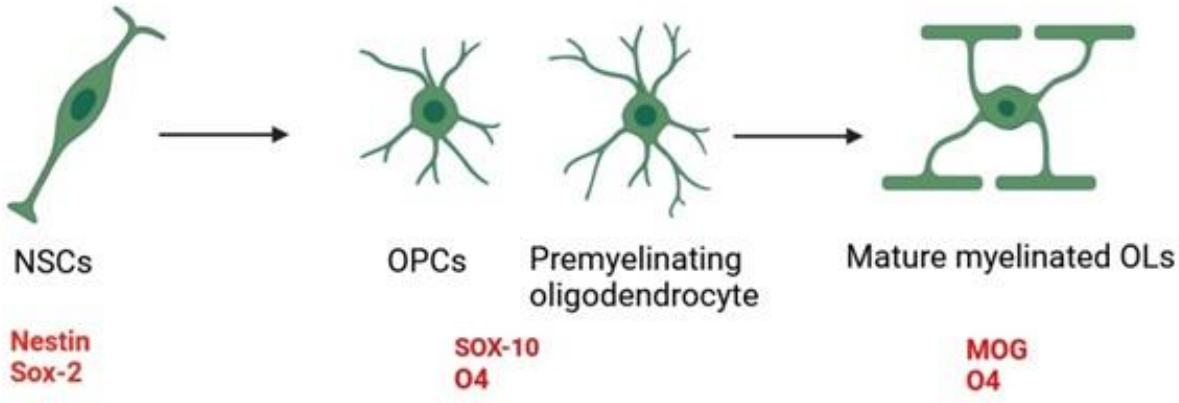

Fig. 9 NSCs differentiate into mature myelinating oligodendrocytes through several distinct stages, each of which can be characterized by specific molecular markers. In the first stage, NSCs express Nestin and SOX2 as key markers. As differentiation begins, SOX10 becomes overexpressed, indicating the transition to oligodendrocyte precursor cells (OPCs). During the OPC phase, O4 is expressed, marking the early stage of the oligodendrocyte lineage. In the final stage, mature oligodendrocytes, which are capable of myelinating axons, express MOG (myelin oligodendrocyte glycoprotein), a hallmark of myelination. These markers track the progression of NSCs through differentiation and their eventual maturation into myelinating oligodendrocytes.

2.4. Microglia isolation and cultivation

The tissue sample was taken under a biological hood, and the PBS in the Falcon tube was discarded. The tissue was transferred to a Petri dish, where it was washed two to three times with 2-5 ml of PBS to remove blood and debris. Large blood vessels were removed using the tips of two pipettes. The tissue was then cut into small pieces and minced with a scalpel to increase the surface area for trypsinization, a process that took between one to three minutes, depending on the tissue size. The tissue was treated with 3-4 ml of pre-warmed 0.25% Trypsin-EDTA (Sigma, Germany) and placed in a Falcon tube. This tube was incubated in a 37°C water bath for 5–10 minutes (or 15 minutes for larger pieces).

Every three minutes, the tube was gently tapped until a homogeneous mixture was formed. Following incubation, an equal volume of fetal bovine serum (Gibco, Germany) was added to neutralize the enzymatic action of trypsin. The suspension was pipetted multiple times to ensure proper inactivation of the enzyme. The resulting cell suspension was centrifuged at 900 rpm for five minutes at 22°C, and the supernatant was discarded. The suspension was resuspended in 1-2 mL of pre-warmed 37°C DMEM/F12 medium and gently pipetted to obtain a smooth, milky, single-cell suspension.

To eliminate undissociated tissue fragments and debris, 10-15 ml of DMEM/F12 medium was added, and the mixture was filtered through a 70µm strainer into a 50 ml tube, using an insulin syringe plunger to ensure the filter remained clear. The filtered suspension was then centrifuged at 1100 rpm for five minutes. After discarding the supernatant, the pellet was resuspended in 2 ml of NSC medium, which consisted of DMEM/F12 (Sigma-Aldrich, Germany), including 0.5% N2 supplement (Gibco, Germany), 2 µg/ml heparin (Ratiopharm, Germany), 1% glutamine (Sigma-Aldrich, Germany), 2% B27 supplement (Gibco, Germany), 20 ng/ml EGF (Stemcell Technologies, Germany), 10 ng/ml bFGF (Stemcell Technologies, Germany), and 1% penicillin/streptomycin (Sigma-Aldrich, Germany) for cultivating in the 24 adherent well plate. The medium was changed after 24 hours with a new medium containing DMEM/F12 (Sigma-Aldrich, Germany), 10% fetal bovine serum (FBS) (Sigma-Aldrich, Germany), and 1% L-glutamine (Sigma-Aldrich, Germany).

2.5. Cytotoxicity assay using WST-1 reagent

After 10 days in culture, microglial cells were treated with 100 ng/ml of lipopolysaccharides (LPS) from Escherichia coli O111: B4 (Sigma-Aldrich, Germany) for 16 h. When this treatment was complete, the LPS-containing medium was removed and replaced with NSC medium (penicillin/streptomycin free) for another 24 h to 48 h. This allowed the microglial cells to release inflammatory cytokines into the medium, creating an inflammatory medium as a conditioned medium (CM). This CM was then collected for use in our experiments. Utilizing all media free of antibiotics was to achieve a higher efficiency of siRNA transfection with Lipofectamine® 2000 in the next steps regarding the manufacturer's protocol (2.7, M+M section). The solution was developed and monitored with precision to confirm the correct concentration of LPS and to ensure sample consistency. The CM was stored at -20°C for a maximum of one month or at -80°C for longer-term storage, with a limit of six months.

To determine the effectiveness of the CM and its influence on the survival and cytotoxicity of NSCs, the WST-1 assay was executed in accordance with the manufacturer's guidelines (Sigma-Aldrich, Germany). NSCs were plated at a density of 2 × 104 cells per well in 96-well plates and incubated at 37°C with 5% CO_2. The cells were allowed to grow for 72 hours, reaching around 70–80% confluency.

NSCs were exposed to a 50% concentration of CM for either 24 or 48 hours to examine its impact on inflammation. After the designated treatment period, 100 µL of 10% WST-1 solution, diluted in serum-free medium, was added to each well. The WST-1 reagent interacts with mitochondrial enzymes in live cells, targeting a reaction that leads to the formation of formazan, a colored compound. The intensity of this color reflects the number of metabolically active cells, providing insight into their viability.

The plates were then incubated at 37°C for 3 h to ensure the reaction proceeded completely. Since the amount of formazan produced corresponds to cellular activity, higher levels indicate a greater number of viable cells. To ensure reliable results, all wells were treated under the same conditions. When the incubation period ended, the absorbance of the formazan product was measured at 440 nm using a TECAN® Sunrise microplate reader. The

absorbance values were displayed as the number of viable cells in the medium. Therefore, the impact of the inflammatory environment on NSC viability was assessed by evaluating the absorbance readings of CM-treated cells compared to those of untreated controls.

2.6. Flow cytometry assay for CM evaluation

The role of CM in inducing apoptosis of NSCs was investigated through flow cytometry, utilizing the Annexin V-DY-634/PI assay kit (Abcam, Germany). NSCs isolated from three individual rats were cultured in a 24-well plate at a density of 8×10^4 cells per well and exposed to CM treatment for 24 and 48 h. Following the treatment, the cells were thoroughly rinsed twice with PBS. They were then detached using 0.25% Trypsin-EDTA (Sigma, Germany) and centrifuged at 1100 rpm for 5 min. The harvested cells were combined for a comprehensive toxicity evaluation. For staining preparation, 100 µL of 1× binding buffer was added to the cell suspension.

Subsequently, 5µL of Annexin V-DY634 conjugate and 5 µL of propidium iodide staining solution were added to the mixture. To allow optimal staining, the samples were incubated in a dark environment at room temperature for 15 minutes. Following this incubation, an additional 400 µL of 1× binding buffer solution was incorporated into each sample before analysis. Flow cytometric assessment was conducted using a CytoFLEX™ flow cytometer, with fluorescence detection settings specifically configured for DY-634 and PI signals. Calibration was performed with unstained samples and compensation controls to ensure precise definition of gating parameters before initiating sample analysis.

The resulting data were analyzed using FlowJo software (version 10), which allowed for the evaluation of apoptotic and necrotic cell populations based on fluorescence intensity. Cells were classified into viable (Annexin V-negative, PI-negative), early apoptotic (Annexin V-positive, PI-negative), and late apoptotic or necrotic (Annexin V-positive, PI-positive) populations using quadrant-based gating. Statistical analyses were subsequently employed to identify significant differences between the CM-treated and untreated control groups.

2.7. Transfection of siRNA

To silence the expression of ZEB1 in cultured cells, siRNA transfection was performed using Lipofectamine® 2000, according to the manufacturer's instructions (Thermo Fisher Scientific, n.d.).

The siRNA sequence specifically targeting ZEB1 was obtained from the Silencer Select siRNA library (s130529, Ambion) with the following sequences:

Sense strand: 5′-GGCUGUAGAUGGUAACAUATT-3′

Antisense: 3′-UAUGUUACCAUCUACAGCCAC-5′.

For the experimental setup, cells were seeded into culture plates at predetermined densities depending on the assay requirements. Specifically, a density of 8×10^4 cells per well was used for 24-well plates, while 2×10^4 cells per well were plated in 96-well plates. The cells were then incubated at 37°C with 5% CO_2 for 72 h to allow them to reach an optimal confluency level of approximately 60–70% before transfection.

The transfection was performed under sterile conditions; antibiotics (penicillin and streptomycin) were excluded to avoid interference with transfection efficiency. For each well in a 96-well plate, 15 pmol of *ZEB1*-siRNA was used, whereas 30 pmol was applied to each well in a 24-well plate. A non-targeting siRNA, Silencer Select Negative Control siRNA (#4390843, Ambion), was included as a negative control to validate the specificity of *ZEB1* silencing.

To prepare the transfection reagent mixture, the siRNA was first diluted in 50 µL of Gibco™ Opti-MEM™ and incubated at room temperature for 5 minutes. In parallel, 1 µL of Lipofectamine® 2000 (Invitrogen, Germany) was diluted separately in 50 µL of Opti-MEM and incubated for the same duration. Afterward, the two diluted solutions were combined and incubated together for an additional 20 minutes to facilitate the formation of siRNA-Lipofectamine complexes (Table 1).

Table 1: Reagent Volumes for Different Culture Vessel Sizes

Culture Vessel	Surface Area (cm^2)	Medium Volume	RNA	Lipofectamine 2000
96-well	0.3	100 µL	5 pmol	0.25 µL
24-well	2	500 µL	20 pmol	1.0 µL
12-well	4	1 mL	40 pmol	2.0 µL
6-well	10	2 mL	100 pmol	5 µL

Following this, the transfection mixture was carefully added into the culture medium of each well, with gentle shaking of the plates to promote uniform distribution. The cells were kept at 37°C under standard culture conditions for the subsequent 48 hours before conducting downstream analyses.

ZEB1 knockdown efficiency was assessed at 24, 48, and 72 hours post-transfection using immunocytochemistry (ICC) and fluorescence microscopy (Keyence, Japan). The quantification of transfection efficiency was performed with ImageJ software, which calculated the ratio of fluorescent cells to their non-fluorescent counterparts.

2.8. ICC assay

To analyze cellular characteristics at different stages of NSC differentiation and to assess the impact of ZEB1 silencing under inflammatory conditions, an ICC assay was conducted using an established methodology. This approach enabled the identification of specific cellular markers associated with NSCs and microglial cells.

NSCs were cultured in laminin-coated 24-well plates, with 8×10^4 cells seeded per well. The cells underwent transfection with *ZEB1*-siRNA using Lipofectamine® 2000, as previously described. Additionally, two experimental groups were exposed to CM for 48 h to investigate

the inflammatory response in NSCs.

After the treatment period, cells were fixed using 4% paraformaldehyde (Sigma-Aldrich, Germany) to preserve cellular structures and antigen integrity. To enhance membrane permeability, cells were incubated with 0.2% Triton X-100 at room temperature for 15 min. Following this step, a blocking buffer containing 900 µl PBS,1 µl Tween® 20 (Applichem, Germany), 1% bovine serum albumin (BSA), and 100 µl goat serum (both Sigma, Germany) was applied for 30 minutes in the dark at room temperature to minimize non-specific antibody binding.

To detect specific cellular markers in our different stages of experiments, primary antibodies were applied overnight at 4°C. These included nestin (1:500, Abcam, Germany), as well as GFAP (1:500, Abcam, Germany), and SOX2 (1:500, Abcam, Germany) to identify NSCs originating from the SVZ. Additionally, anti-ZEB1 (Sigma-Aldrich, Germany) for all cell cultures to evaluate the expression of ZEB1 in our neuronal cells as well as SOX10 (1:500, Abcam, Germany), O4 (1:300, Sigma-Aldrich, Germany), and MOG (1:500, Abcam, Germany) were utilized to characterize the differentiation process of our cells, including NSCs, OPCs and OLs. Moreover, IBA1 (1:500, FUJIFILM Wako Pure Chemical Corp., Japan) was used for labeling microglial cells.

After primary antibody incubation, cells were thoroughly washed with PBS to remove any unbound antibodies. They were then incubated with secondary antibodies for 2 h at room temperature. The appropriate secondary antibodies were selected according to the species of the primary antibodies: either goat anti-mouse IgG (Abcam, Germany), which was used for GFAP, nestin, O4 and SOX2, or goat anti-rabbit IgG (Abcam, Germany) for detection of MOG, ZEB1, SOX10 and IBA1, both conjugated with fluorescein isothiocyanate (FITC), to allow fluorescence detection.

Cell imaging was performed using fluorescence microscopy (Keyence, Japan) to visualize protein expression and cellular morphology. The images obtained provided insights into the differentiation status of NSCs and the influence of ZEB1 knockdown in inflammatory conditions.

2.9. Colony formation assay

NSCs were seeded into laminin-coated 96-well plates for the colony formation assay to evaluate the role of ZEB1 in regulating stem cell proliferation under inflammatory conditions. This method allowed us to assess the stem cell potential in proliferation in response to the presence or absence of inflammatory conditions and ZEB1 silencing.

Single cells were seeded at a density of 2×10^4 cells per well in laminin-coated 96-well plates to ensure optimal attachment and growth. The cells were then subjected to different treatment conditions, including ZEB1 siRNA transfection and/or exposure to CM, depending on the experimental group. These conditions were maintained to analyze how ZEB1 suppression affects colony formation in the presence and absence of inflammation.

Cell growth and colony formation were recorded at predetermined time points on days 1, 3, 7, 10, and 14 to monitor changes in proliferation and morphology over time. At each interval, images were captured using an inverted microscope (Keyence, Japan), enabling a detailed visualization of colony formation and cellular dynamics. To assess the results, the captured images were analyzed using ImageJ software, which measured the colony size and number of spheres in the culture medium.

This assay provided valuable insights into the effects of ZEB1 on stem cell behavior under inflammatory conditions, helping to determine its role in colony-forming potential and self-renewal capacity in a challenged microenvironment.

2.10. Wound healing assay for migration assessment

A wound healing assay was conducted to assess the migratory ability of NSCs following ZEB1 knockdown, under both inflammatory and non-inflammatory conditions. NSCs were initially seeded into 24-well plates at a density of 8×10^4 cells per well and cultured until they reached approximately 85–90% confluence. Once confluence was achieved, a scratch was made across each well using a sterile 100 µL pipette tip.

Following the scratch, the wells were gently washed with phosphate-buffered saline (PBS; Sigma-Aldrich) to remove detached cells and debris, ensuring that only adherent NSCs remained in the culture.

Two groups of cells were then subjected to treatment conditions as previously described: exposure to CM to simulate an inflammatory environment and/or transfection with ZEB1 siRNA to downregulate ZEB1 expression and examine its role in cell migration.

Cell migration into the wound area was monitored at three time points: immediately after scratching (0 h), and then at 24 h and 48 h. At each time point, images were captured using an inverted microscope to document the extent of wound closure. These images were analyzed using ImageJ software to quantify the wound area and evaluate the migratory behavior of NSCs across different experimental conditions.

2.11. Quantitative polymerase chain reaction (qPCR) assay

Total RNA was extracted from treated cells that had been treated for 48 h in 24-well plates (8×10^4 cells per well) using the QIAGEN RNA Purification Kit, based on the manufacturer's guidelines (Fig. 10). Following this, the QIAGEN high capacity cDNA Reverse Transcription Kit was utilized to transcribe the isolated RNA into complementary DNA (cDNA), following the provided protocols.

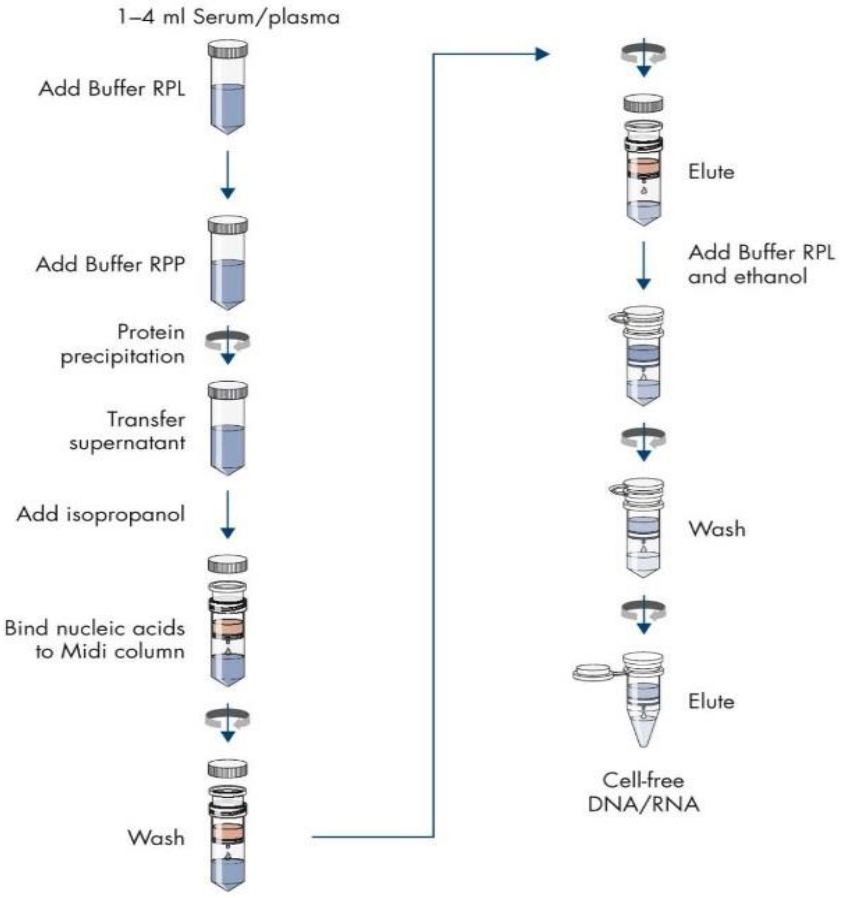

Fig. 10 QIAGEN kit RNAextraction (Fig. adapted from QIAGEN (n.d.)

The CFX Connect® Real-Time PCR detection system (BioRAD, Germany) was utilized to evaluate gene expression levels with specific oligonucleotide primers targeting rat ZEB1, IL6, IL-1β, IL17, TNF-α, and NF-kB synthesized by Eurofins Genetics Europe Company (Germany) (Table 2).

GAPDH and β-Actin were utilized as housekeeping genes for normalization. The relative expression of the target genes was calculated using the delta-delta Ct method ($2^{-\Delta\Delta Ct}$).

Table 2: Primer Sequences for rat genes utilized in qPCR analysis.

Gene	Stream	Sequence (5'-3')
ZEB1	Forward	GCCCATCGAGCTTCCTGTAA
	Reverse	TGGAAACATGGCTCCTGTGC
IL-1β	Forward	GGGTGGTTCAAGGCATAACA
	Reverse	GTCGAGATGCTGCTGTGAGA
IL6	Forward	TCCTACCCCAACTTCCAATGCTC
	Reverse	TTGGATGGTCTTGGTCCTTAGCC
IL17	Forward	GGGAAGTTGGACCACCACAT
	Reverse	CGCCTTCTTTTCAGGGTGGA
NFkB	Forward	ACACAGGACCAGGGACAG
	Reverse	AGGGGTTGTTGTTGGTCTGG
TNF-α	Forward	AAATGGGCTCCCTCTCATCAGTTC
	Reverse	TCTGCTTGGTGGTTTGCTACGAC

2.12. Reactive oxygen species (ROS)

To assess the ROS production in the stem cells, three different cells were cultured in 96-well plates pre-coated with laminin to promote proper adhesion. Following cell attachment, the wells were treated with a combination of *ZEB1*-siRNA and CM to induce cellular responses for 24 and 48 h. After treatment, the cells were thoroughly washed with PBS to remove any excess reagents and then incubated in the dark at 37°C for 30 minutes. During this incubation period, 100 µl of a 25 µM H2DCFDA solution (Abcam, Germany) was added to each well, following the manufacturer's protocol for ROS detection.

After the incubation, ROS production was visualized and analyzed using a fluorescent microscope (Keyence, Japan). The fluorescence emitted by the H2DCFDA-stained cells was captured to assess ROS levels. The images were further analyzed using ImageJ software to quantify the fluorescence intensity, reflecting intracellular ROS levels. This process provided insights into the oxidative stress induced by the treatments and the role of ZEB1 in

modulating ROS production.

2.13. Statistical analysis

All experimental data were analyzed and visualized using GraphPad Prism software (GraphPad, USA). Results are reported as the mean ± standard error of the mean (SEM). For statistical evaluation, a one-way analysis of variance (ANOVA) was employed to assess outcomes from ICC and qPCR assays. In contrast, a two-way ANOVA was applied for experiments related to colony formation, ROS levels, and wound healing, followed by Tukey's post-hoc test to identify significant differences among groups. In cases where comparisons between two groups were needed, a two-tailed Student's t-test was used. A significance threshold was set at $p < 0.05$. Levels of statistical significance were denoted as follows: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

To evaluate the relationships between variables, Pearson's correlation coefficient (r) was calculated using a two-tailed p-value. This coefficient, which ranges from -1 to +1, was interpreted according to the scale presented in Appendix Section [6.1].

3. Results

3.1. NSCs culturing and characterization

Neurospheres formed in the culture medium were subsequently seeded into 24-well plates. In this study, NSCs were cultured for 10-20 days, during which neurospheres began to form (Fig. 11). The formation of these spherical clusters of undifferentiated cells is a well-established indicator of the successful culture and self-renewing ability of NSCs.

A key achievement of this three-year study was the establishment of a robust stem cell bank, with some neurospheres exceeding 600 μm in diameter in certain experiments.

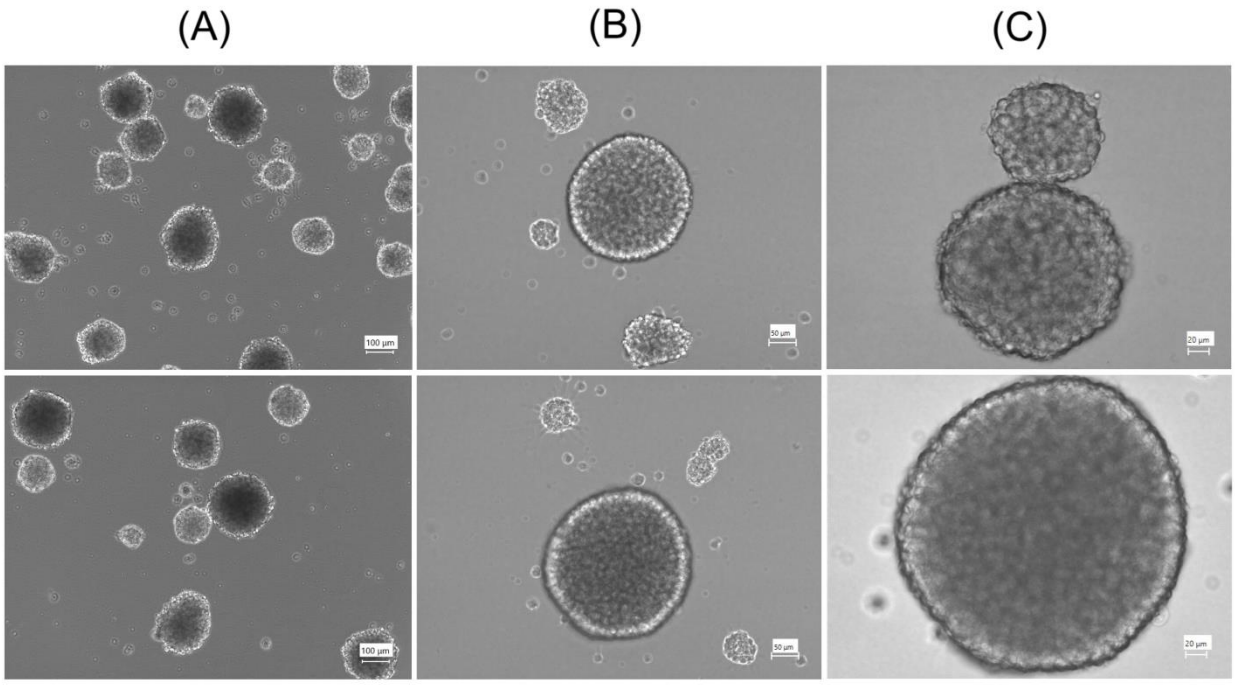

Fig. 11 (A) Morphology of NSC Spheres at Various Sizes. The size distribution of NSC spheres is shown at three different magnifications: 100 μm (A), 50 μm (B), and 20 μm (C). These images represent the typical sphere formation of NSCs in the culture medium at different scales.

To confirm the presence of NSCs in the culture, ICC was conducted using antibodies against two well-known NSC markers: nestin and SOX2. Nestin, a protein expressed in neural progenitors, is widely recognized as a marker for NSCs, while SOX2 is a transcription factor

that plays a critical role in maintaining the pluripotent state of stem cells.

The results from ICC showed that the cultured cells were positive for both nestin and SOX2, which confirmed their identity as NSCs (Figs. 12A and 12B). Additionally, to verify that these NSCs originated from the SVZ, the expression of GFAP as a marker used to identify SVZ-derived stem cells was assessed (Butruille et al., 2024). Immunocytochemical analysis revealed the expression of GFAP (Fig. 12C).

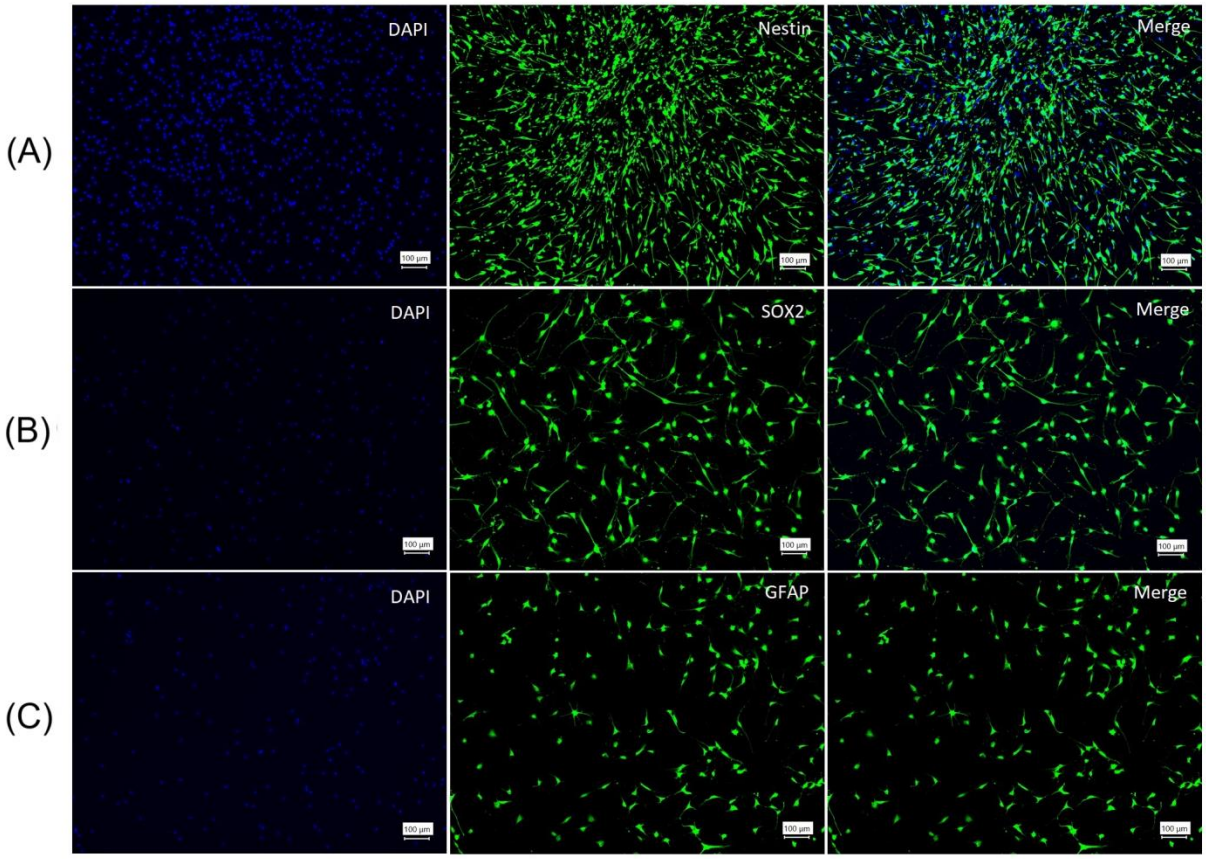

Fig.12 Characterization of NSCs derived from SVZ. The presence of NSCs in the culture was confirmed through the expression of specific markers. Nestin (B1), a marker of undifferentiated neural progenitors; SOX2 (B2), a transcription factor associated with self-renewal; and GFAP (B3), indicative of astrocytes' lineage, were all expressed in the cultured NSCs from SVZ. Nuclear staining was performed using DAPI (Blue), while the expression of antibodies was visualized with GFP (Green), demonstrating the successful labeling of NSCs. The figures are presented with a magnification of 100 µm.

3.2. Differentiation of NSCs to mature myelinating oligodendrocytes

NSCs can differentiate into OLs, which are the primary cells responsible for creating myelin in the brain. The loss or malfunction of OLs is strongly associated with demyelinating diseases like multiple sclerosis. To better understand how NSCs progress into OLs, we analyzed how ZEB1 expression changes at different stages of this process, especially as the cells mature into myelinating oligodendrocytes. This approach gave us a clearer picture of what role ZEB1 plays throughout this transformation.

To ensure the NSCs only differentiate into oligodendrocytes and no other cell types like neurones or astrocytes, we followed a carefully planned culture protocol. The differentiation process was broken down into two main stages. First, we guided NSCs to become OPCs and pro-oligodendrocytes, and then in the second stage, these precursor cells were further matured into myelinating oligodendrocytes.

To make sure the process worked as efficiently as possible, we optimized the culture media by adjusting the concentrations of various factors. This allowed us to create a media composition that supported the production of more than 70% mature oligodendrocytes. We could then track the maturation of these OLs based on their ability to form myelin sheaths, with a focus on myelin MOG as a marker. For the earlier stages, we monitored the presence of markers like SOX10 and O4, which are specific to OPCs, to confirm that the differentiation was progressing as expected (Fig. 13).

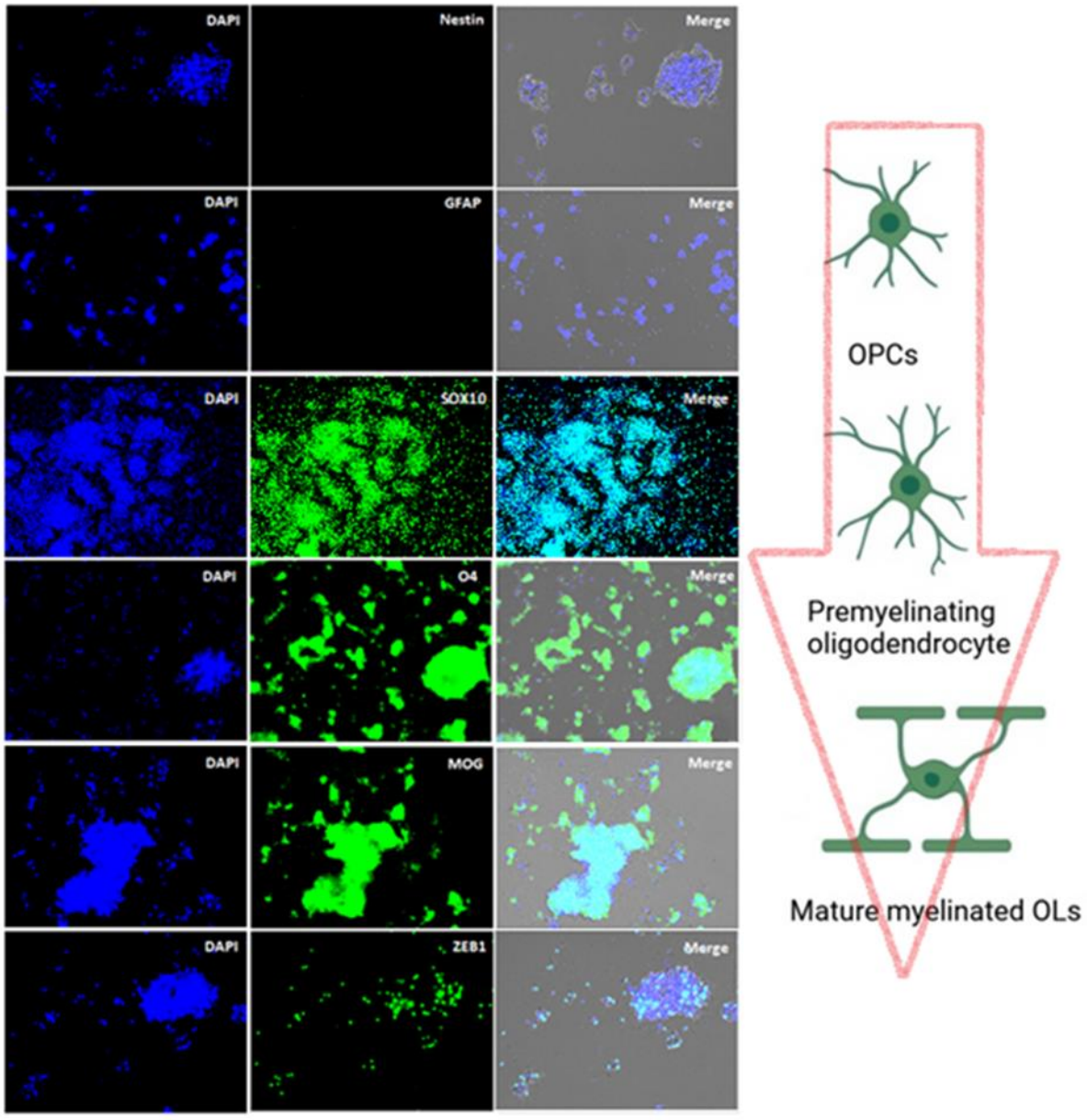

Fig. 13 Differentiation of NSCs into mature myelinating oligodendrocytes: This figure demonstrates the progressive differentiation of neural stem cells (NSCs) into mature oligodendrocytes. Initially, NSCs do not express nestin, indicating the loss of their undifferentiated state. During the intermediate stage of differentiation, the cells begin to express SOX10 and O4, markers associated with oligodendrocyte precursor cells (OPCs). In the final stage, mature oligodendrocytes are identified by the expression of MOG (Myelin oligodendrocyte glycoprotein), a key marker of myelination. The figure highlights the transition of NSCs through these stages of differentiation, providing insight into the development of myelinating oligodendrocytes. Scale bar: 100 μm for all figures.

ICC was used to assess the expression of these markers at different points in the differentiation process (Figs. 13 and 14). Co-expression of MOG and O4 in the later stages confirmed the successful maturation of oligodendrocytes. In contrast, nestin, as a marker for undifferentiated NSCs, was no longer detected in mature cells, further validating the completion of differentiation.

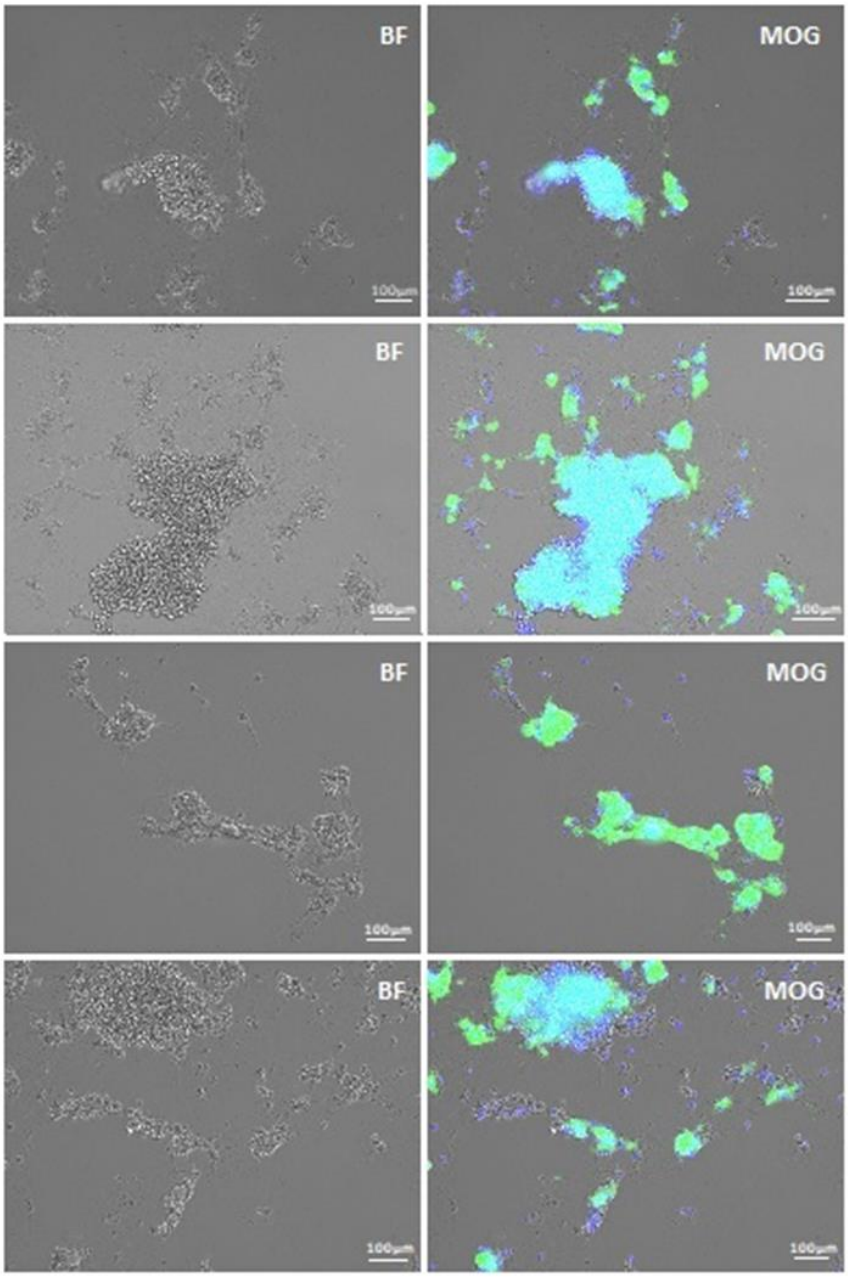

Fig. 14 Differentiation of NSCs into mature myelinating oligodendrocytes: This figure illustrates the progression of neural stem cells (NSCs) differentiating into mature oligodendrocytes capable of myelination. The cells were characterized by the expression of MOG (Myelin oligodendrocyte glycoprotein), a key marker of mature oligodendrocytes. The figure includes two forms of analysis: Bright Field (BF) images showing the overall morphology of the cells and MOG expression images highlighting the presence of MOG in mature oligodendrocytes. These markers confirm the successful differentiation of NSCs into myelinating oligodendrocytes. Figures presented at a scale of 100 µm.

In this regard, approximately 60% of the cells in our cultures strongly expressed MOG, indicating a highly efficient differentiation process. ZEB1 expression was observed to decrease by approximately 50% as the cells matured into OLs. This reduction in ZEB1 was confirmed via ImageJ-based fluorescence quantification, and both the graphical analysis and ICC data showed a significant decline in ZEB1 expression as the NSCs turned into myelinating oligodendrocytes (Figs. 13, 14, and 15).

These findings suggest that ZEB1 might play a role in regulating the maturation and differentiation of oligodendrocytes, particularly during the formation of myelin.

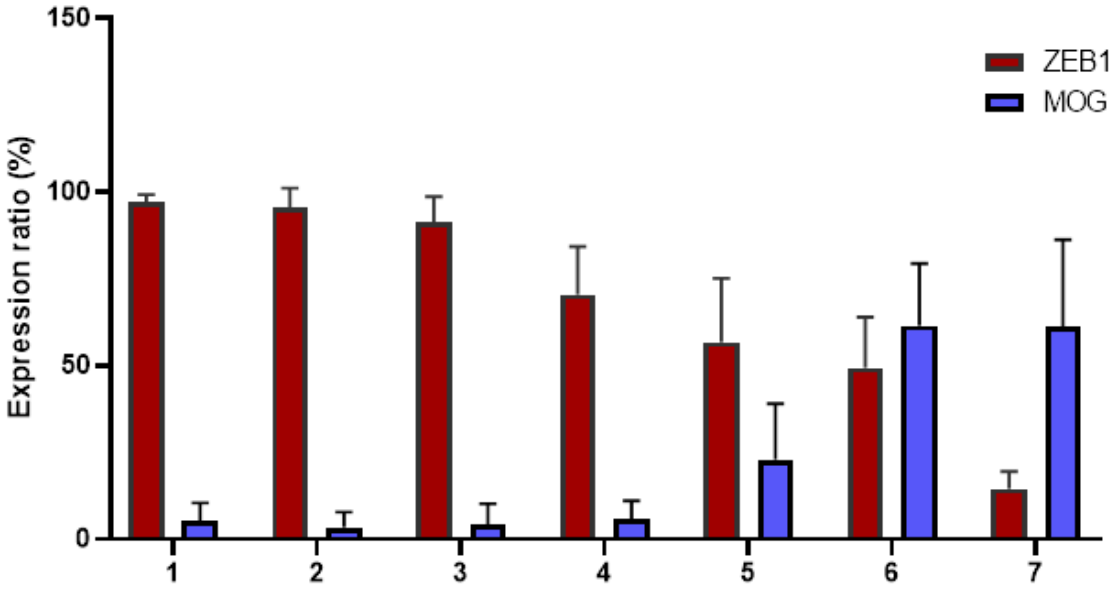

Fig.15 Gradual decrease of ZEB1 expression during NSC differentiation to mature myelinating oligodendrocytes. This graph illustrates the progressive reduction of ZEB1 expression as neural stem cells

(NSCs) differentiate into mature oligodendrocytes (OLs). The data shows that ZEB1 levels gradually decrease in correlation with the increasing expression of MOG (myelin oligodendrocyte glycoprotein) across seven distinct stages of differentiation, from NSCs to mature myelinating OLs. This trend suggests that ZEB1 may play a role in regulating the transition from NSCs to mature myelinating oligodendrocytes, with its downregulation accompanying the upregulation of myelination markers such as MOG. This graph presents the results based on the ICC assay using ImageJ software.

3.2.1. Correlation analysis between ZEB1 and MOG expression

A correlation study was performed to examine the association between the expression levels of ZEB1 and MOG. As illustrated in fig. 15, a scatter plot presents the distribution of data, with ZEB1 values along the horizontal axis and MOG values on the vertical axis. The analysis indicates a clear inverse relationship, as shown by the downward slope of the regression line, suggesting that lower ZEB1 expression is linked to higher MOG expression.

The Pearson correlation coefficient (r) was calculated as -0.8846, which indicates a strong negative correlation between ZEB1 and MOG. The 95% confidence interval ranged from -0.9829 to -0.3939, which supports that our correlation was statistically significant. The distribution of the data points reflects a stable inverse relationship, indicating that increased ZEB1 expression is typically linked to decreased MOG levels. Since MOG serves as a significant marker of myelination, its increased expression in conditions of lowered ZEB1 suggests that ZEB1 may influence pathways involved in myelin formation. This pattern supports the idea that suppressing ZEB1 could lead to greater MOG expression, potentially enhancing oligodendrocyte activity and myelin production (Fig. 16).

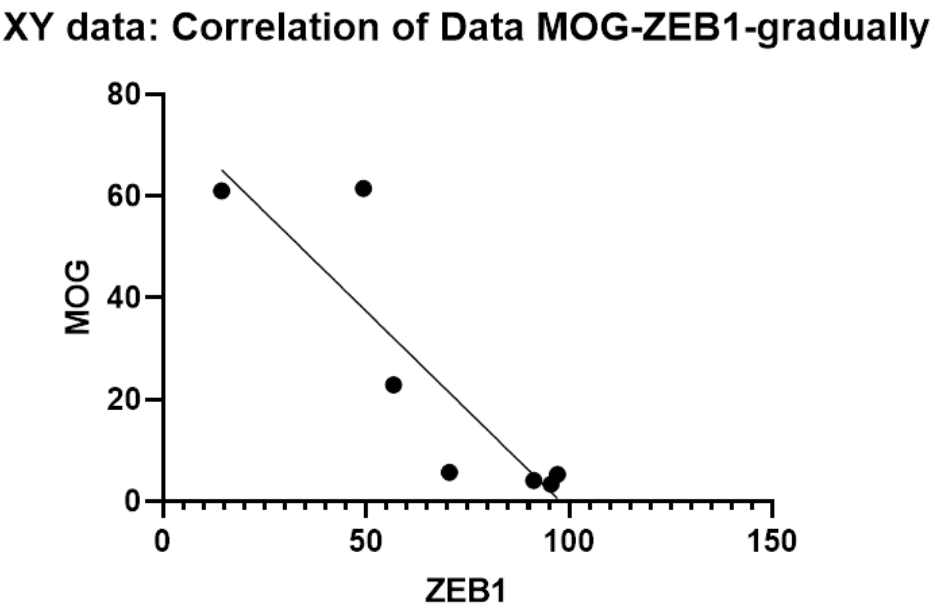

Fig. 16 Negative correlation between ZEB1 and MOG expression. The scatter plot illustrates an inverse relationship between ZEB1 and MOG expression, suggesting that reduced ZEB1 levels are associated with increased MOG expression, potentially promoting myelination. Significance was assessed using the Pearson correlation (two-tailed, $p < 0.05$).

3.3. Evaluation of CM obtained from microglial cells

To induce an inflammatory response, LPS was used to activate rat microglial cells (n=3) by applying an LPS concentration of 100 ng/ml for 16 h, following protocols established in previous literature (*M+M section*) (LIU et al., 2013; NAKAO et al., 2022; STÖBERL et al., 2023). After 16 h LPS treatment of microglial cells, LPS-containing medium was discarded, and cells were treated for 48 h with NSC medium without penicillin/streptomycin. Then the supernatant, containing the inflammatory factors, was collected from the microglial cells to serve as the CM. To assess the morphological changes in the microglial cells post-treatment with LPS, an ICC assay was conducted using the IBA1 antibody, a specific marker for microglial cells. The analysis revealed that LPS exposure led to a shift in the microglial morphology to form an amoeboid, rounded shape, accompanied by cell migration or "scratching" behaviors. The observed changes imply that exposure to LPS activated microglia, prompting the release of inflammatory cytokines, consistent with findings reported

in previous reports (Cunha et al., 2016; Jung et al., 2023) (Fig. 17).

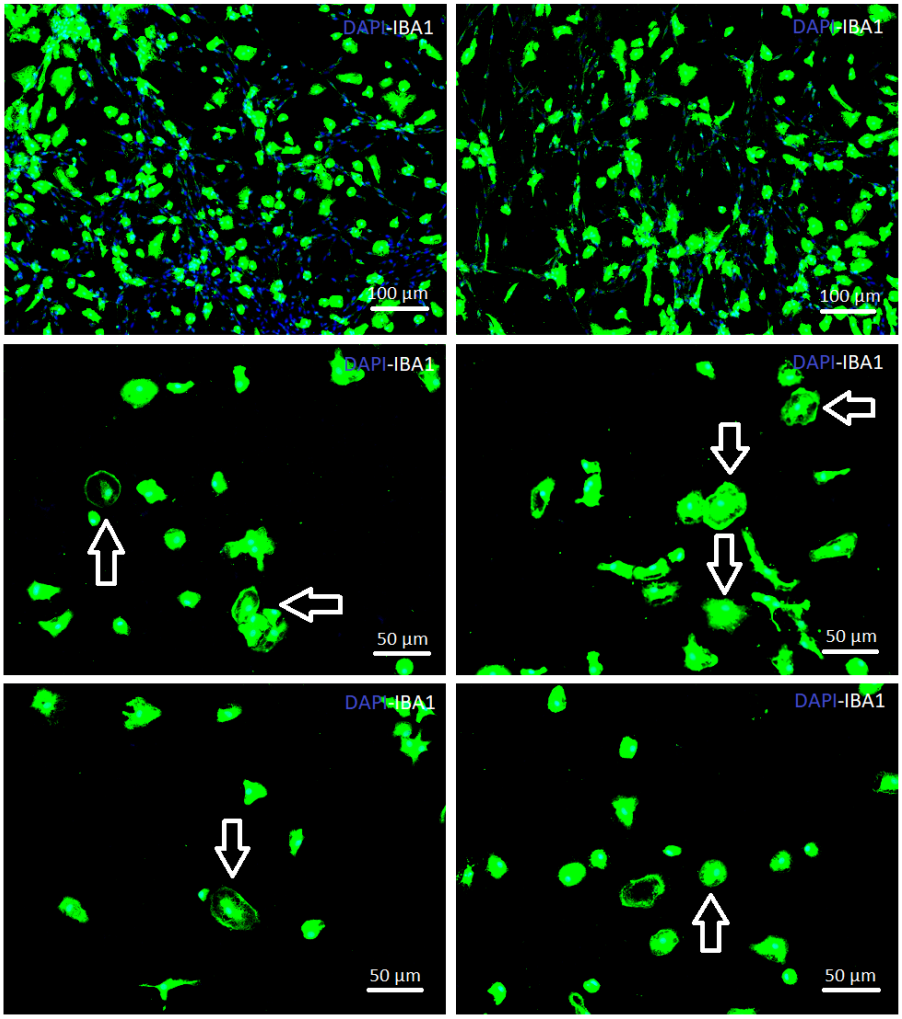

Fig. 17 Morphology and characterization of activated primary microglial cells: Primary microglial cells were isolated from rat brains and treated with 100 ng/mL LPS for 48 hours. Microglial activation was assessed using the IBA1 antibody in an ICC assay. The microglia exhibited amoeboid morphology, with notably round and pointed shapes, indicating activation following LPS treatment. These amoeboid shapes were consistently observed at both 100 µm and 50 µm magnifications.

The potential cytotoxicity and effectiveness of CM treatment on NSCs were evaluated through Annexin V/propidium iodide (PI) double staining for early and late apoptosis, as well as through cell viability assays using flow cytometry and WST assays, respectively. Flow cytometry analysis, including a review of the Annexin V/PI staining over three different NSCs, was performed for CM derived from LPS-activated microglia. This

experiment showed no signs of early apoptosis or necrosis in the CM-treated cells during the first 24 h. However, by the 48-hour mark, an increase in early apoptotic cells was observed, though necrosis remained absent (Fig. 18).

These findings suggest that CM induces a mild inflammatory response, triggering early apoptotic pathways without causing widespread cytotoxicity or necrosis. The absence of necrosis and the presence of early apoptosis indicate that CM effectively activated NSCs in a controlled manner, without compromising overall cell viability. These results indicate that CM can initiate an inflammatory signaling cascade in NSCs, possibly mimicking conditions of neural injury or disease (Fig. 18).

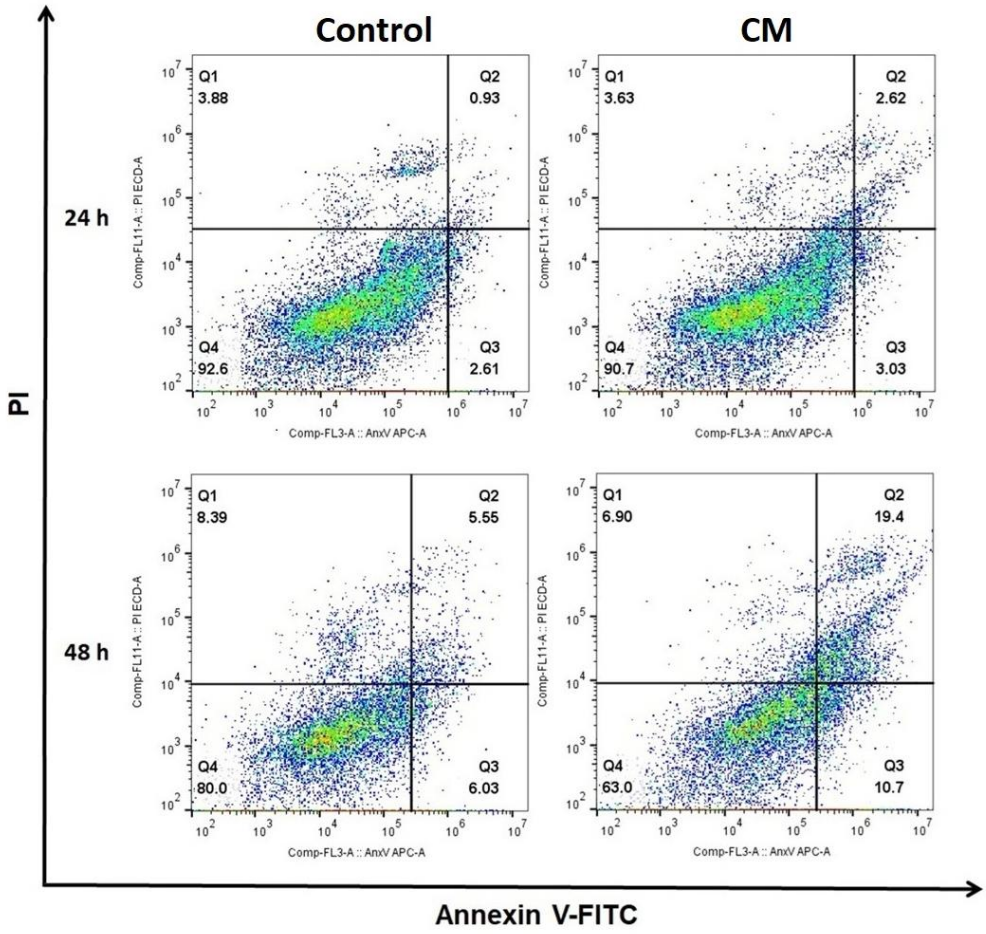

Fig. 18 Annexin PI Staining for apoptosis analysis: The apoptosis of neural stem cells (NSCs) treated with a CM for 24 and 48 h was evaluated using Annexin PI double staining. The control group consisted of untreated

cells. The results showed no evidence of early apoptosis or necrosis at 24 h, while early apoptosis increased, and no necrosis was observed at 48 hours when compared to the control.

In addition, the WST-1 assay, used to assess cell viability, showed that the viability of NSCs treated with CM for both 24 and 48 h remained comparable to that of the untreated control group. These results further confirm that CM exposure did not induce significant cytotoxicity in NSCs, supporting its suitability for mimicking inflammatory conditions without compromising NSC viability (Fig. 19).

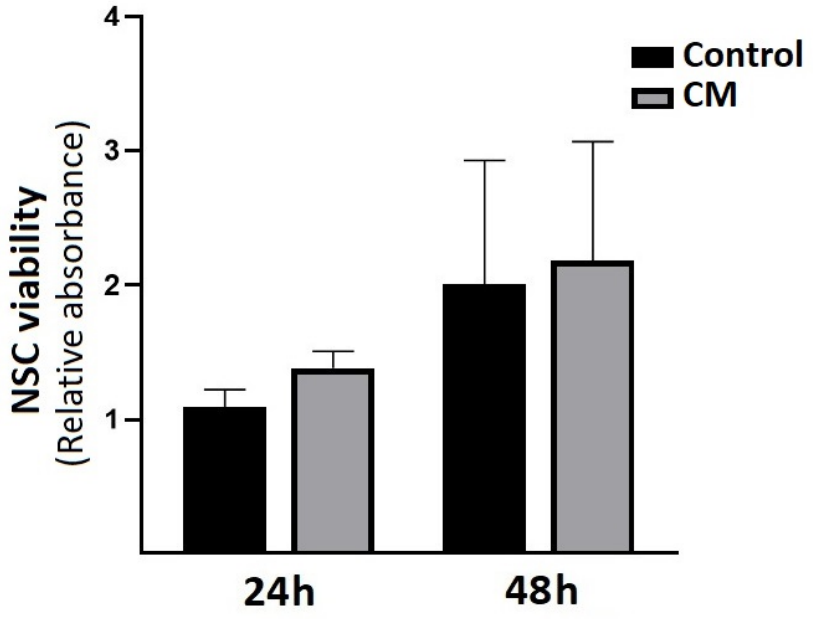

Fig. 19 WST viability assay for CM treatment. The cell viability of NSCs treated with the CM for 24 and 48 h was assessed using the WST assay. The data indicated that CM treatment did not cause significant toxicity compared to the control group at either time point.

3.4. Modulation of ZEB1 in NSCs

NSCs were isolated from the sub-ventricular zone of rat brains (n=5). To explore the role of ZEB1 in these cells, a gene knockdown approach was employed using *ZEB1*-siRNA, which was delivered through Lipofectamine® 2000. The siRNA transfection protocol adhered to the guidelines provided by Thermo Fisher Scientific, as outlined in the methodology section.

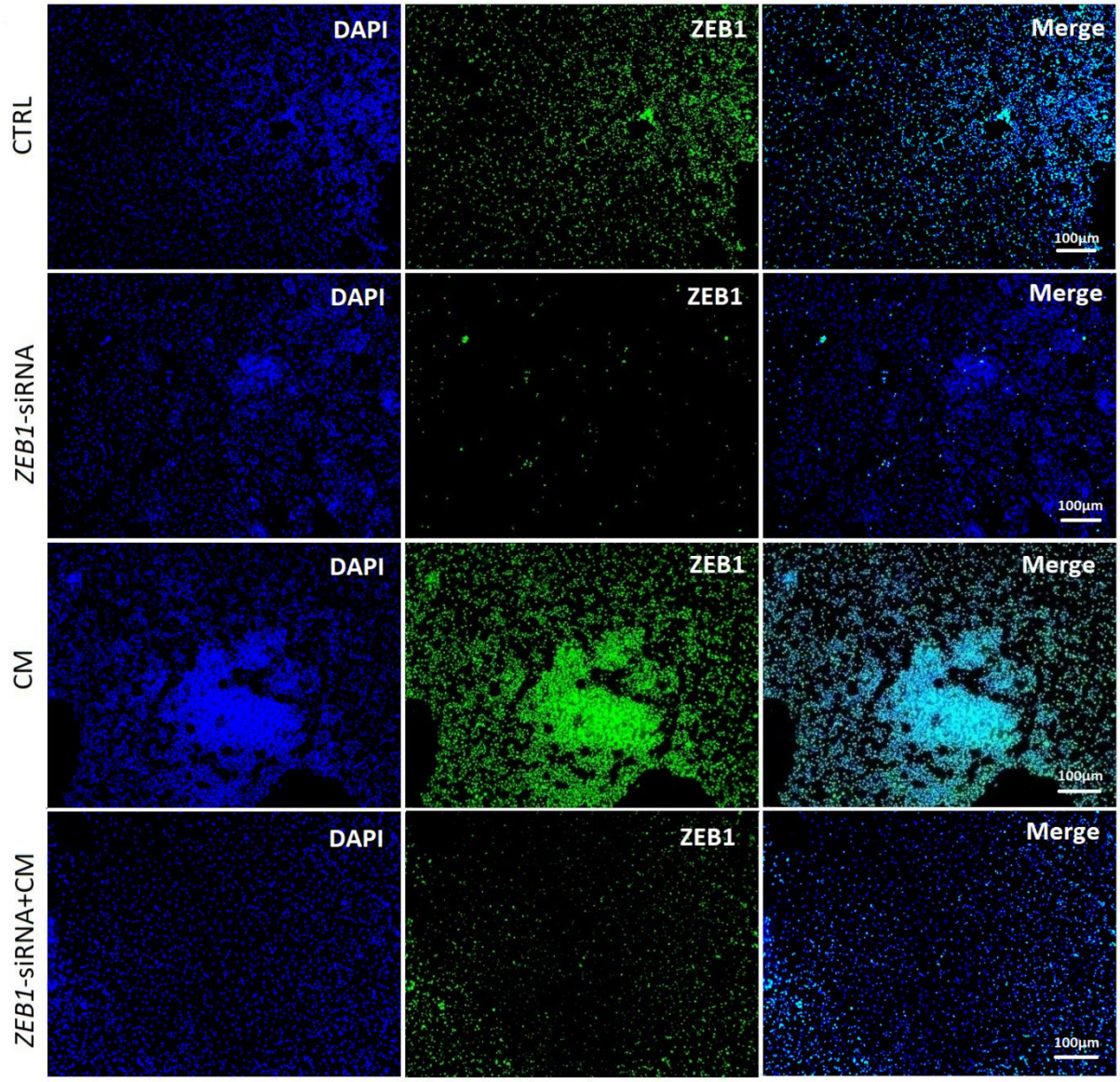

Fig. 20 Immunocytochemistry analysis of ZEB1 expression in NSCs following siRNA-mediated gene silencing and/or CM treatment. DAPI (blue) stained nuclei; ZEB1 (green) was visualized using a ZEB1-specific antibody. Reduced fluorescence indicates successful knockdown. Scale bar: 100 µm.

To ensure optimal delivery and transfection efficiency, all media, including those containing the inflammatory medium, were prepared without Pen/Strep, as the presence of Pen/Strep could interfere with the transfection process (Thermo Fisher Scientific, no date).

The cells were evaluated 48 hours post-treatment to assess the effectiveness of the transient transfection with *ZEB1*-siRNA. The experiment was designed with four distinct groups:

(i) Untreated control

(ii) *ZEB1*-siRNA-transfected cells

(iii) Cells exposed to CM

(iv) Cells receiving both *ZEB1*-siRNA and CM

These groups were compared to determine the effects of ZEB1 silencing both in the context of normal conditions and under inflammatory conditions induced by CM (Fig. 20).

ZEB1 expression levels were analyzed across different treatment conditions using both ICC and qPCR after 48-hour exposure to CM and/or ZEB1-targeted gene silencing. ICC results demonstrated a marked decrease in ZEB1 expression in the group treated with ZEB1-siRNA when compared to the untreated control, confirming that the silencing approach was successful ($P \leq 0.001$; Figs. 20 and 21).

In contrast, NSCs treated solely with CM showed a notable increase in ZEB1 expression relative to the control group ($P \leq 0.01$) and also when compared to both the *ZEB1*-siRNA and the combined *ZEB1*-siRNA + CM groups ($P \leq 0.001$) (Figs. 19 and 20). Interestingly, ZEB1 expression in the *ZEB1*-siRNA + CM group was still significantly higher than in the *ZEB1*-siRNA group alone ($P \leq 0.01$; Figs. 20 and 21).

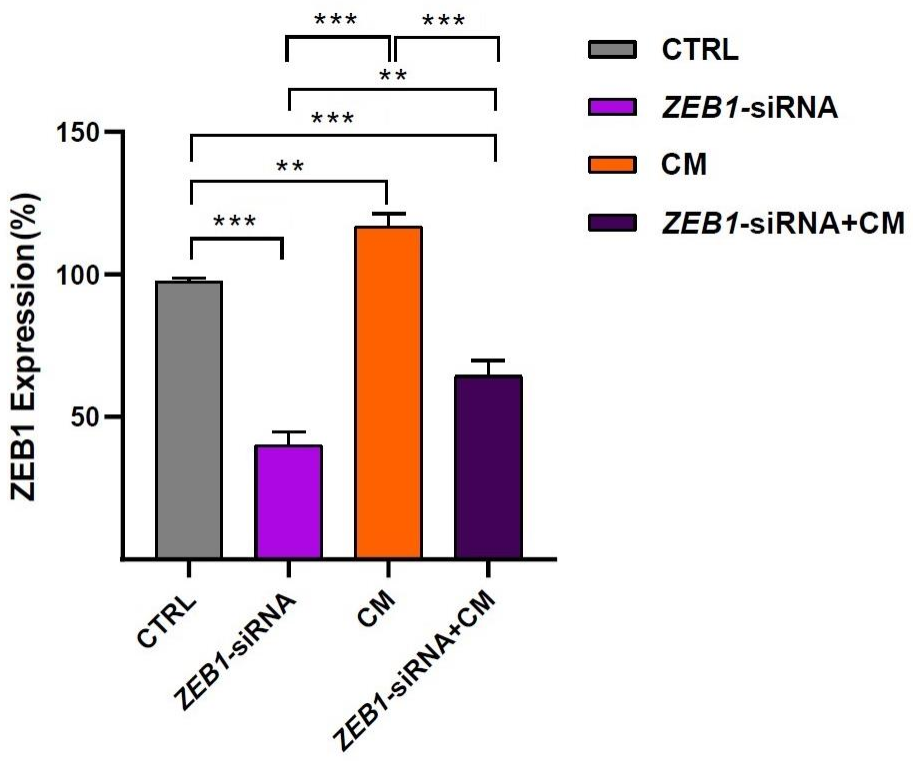

Fig. 21 Immunocytochemistry assay (ICC) of ZEB1 expression in NSCs across treatment groups (control, ZEB1-siRNA, CM, and ZEB1-siRNA + CM). ICC results confirm successful knockdown and CM-induced upregulation of ZEB1. Data analyzed via one-way ANOVA; *, **, and *** indicated significance as $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

Complementary PCR data supported these observations, revealing a significant upregulation of ZEB1-siRNA in NSCs exposed to CM in comparison to all other experimental groups ($P \leq 0.001$; Figs. 20 and 22).

Interestingly, when the cells were treated with both CM and ZEB1-siRNA, a significant reduction in ZEB1 expression was observed compared to the CM-only group. Both ICC and qPCR analyzes showed that the combined treatment resulted in a notable decrease in ZEB1 expression ($P \leq 0.001$ for both ICC and qPCR) compared to the CM group (Figs. 21 and 22). This suggests that the silencing of ZEB1 may mitigate the inflammatory effects induced by CM, as evidenced by the reduced expression of ZEB1 in the combined treatment group.

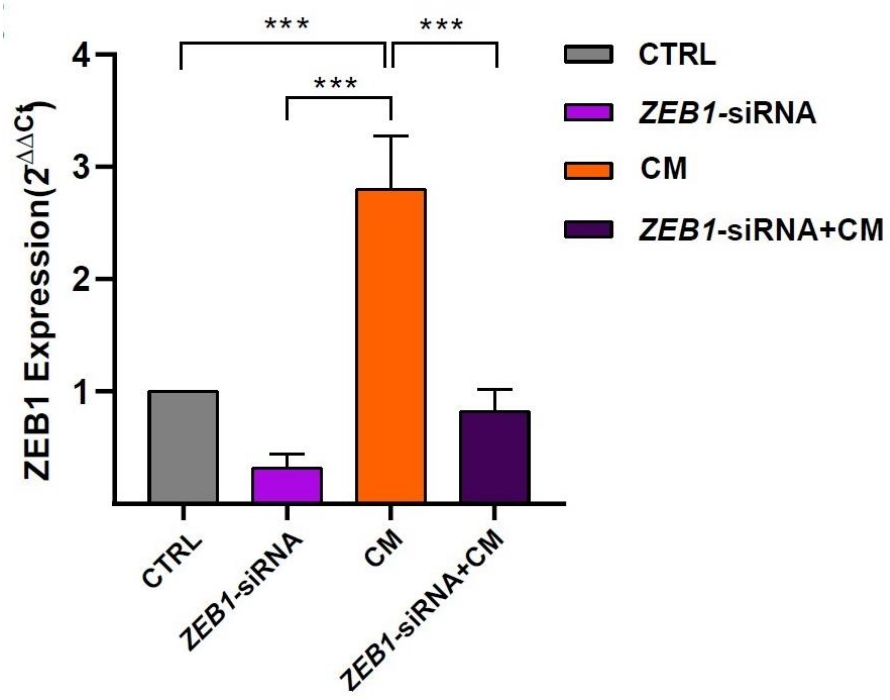

Fig. 22 Quantitative PCR Confirmation of ZEB1 gene silencing: qPCR results confirmed the successful silencing of the ZEB1 gene. Statistical significance is denoted by *, **, and ***, where *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$, respectively.

Conversely, the CM-only treatment group exhibited higher levels of ZEB1 expression compared to both the siRNA-treated groups ($P \leq 0.001$) and the control group in the qPCR analysis ($P \leq 0.001$), highlighting the inflammatory upregulation of ZEB1 in response to CM treatment.

Collectively, these findings suggest that siRNA-mediated knockdown of ZEB1 can effectively counteract the CM-induced elevation of ZEB1 expression in neural stem cells (Figs. 20, 21, and 22).

3.5. Colony formation assay

A colony formation assay was performed across various experimental groups (n = 6) to investigate the effects of different treatments on the clonogenic potential of neural stem cells (NSCs). Cells were seeded at a density of 8×10^4 cells per well in laminin-coated 24-well plates and cultured until approximately 70% confluence was achieved. Colony formation was monitored over 14 days. Data were collected at days 1, 3, 7, 10, and 14 (Fig. 23). To maintain transfection efficiency, treatment media were refreshed, and siRNA transfection was repeated every 72 h.

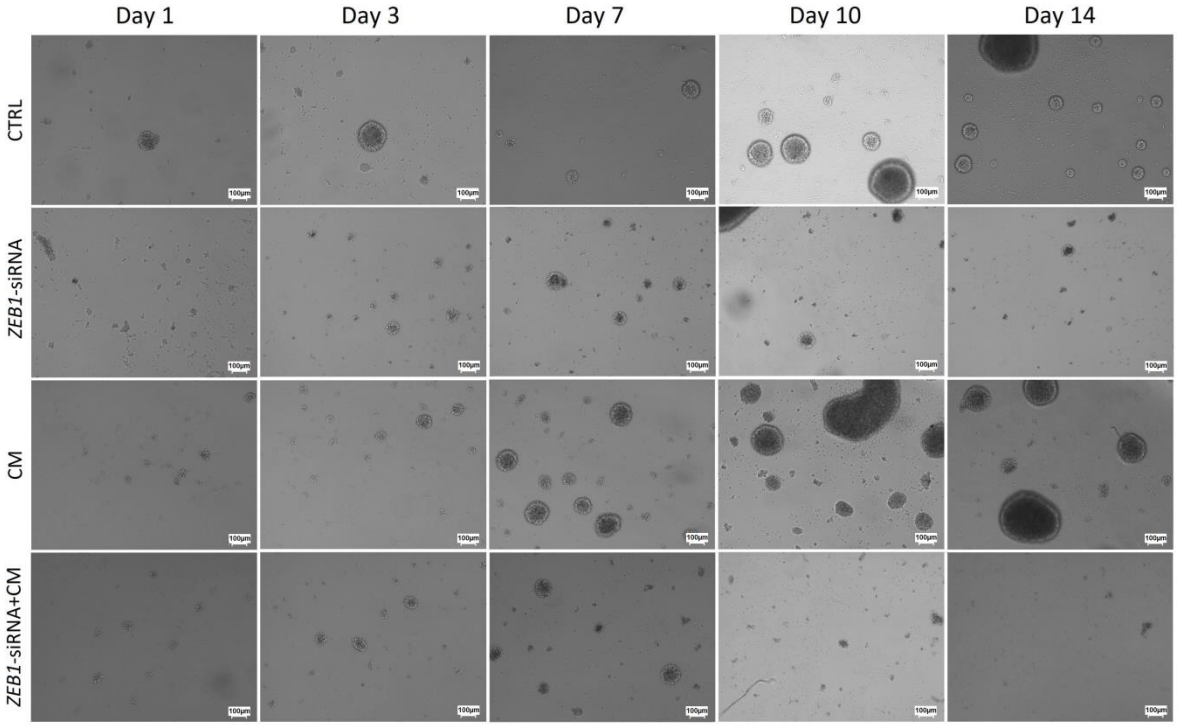

Fig. 23 Colony formation analysis. All figures are presented with a scale of 100 μm.

During the observation period, cells subjected to ZEB1 knockdown exhibited a pronounced reduction in both colony size and number, indicating impaired self-renewal capacity. In contrast, the CM-treated group displayed a progressive increase in colony number, peaking at day 7, significantly exceeding that of all other groups.

At day 3, the CM-only group had a significantly greater number of colonies than the group treated with both ZEB1-siRNA and CM ($P \leq 0.05$). Furthermore, the average colony size in the CM group was markedly larger than in both the ZEB1-siRNA + CM group ($P \leq 0.01$) and the ZEB1-siRNA group alone ($P \leq 0.001$), suggesting an early inhibitory effect of ZEB1 suppression on colony morphology and expansion.

By day 7, colony numbers continued to increase in the CM group, which remained significantly higher than in the ZEB1-siRNA group ($P \leq 0.001$). This trend persisted beyond day 7, where colony sizes in the CM group were significantly larger than in the ZEB1-siRNA + CM group ($P \leq 0.001$), highlighting a cumulative suppressive effect of ZEB1 knockdown under inflammatory conditions. Similarly, the untreated control group also exhibited significantly higher colony counts and larger sizes than either ZEB1-siRNA or ZEB1-siRNA + CM groups ($P \leq 0.001$).

At day 10, a notable shift occurred: colonies in the ZEB1-siRNA group became significantly larger than those in the ZEB1-siRNA + CM group ($P \leq 0.05$), suggesting a delayed impact of ZEB1 silencing on colony expansion.

By day 14, the CM-treated group demonstrated a significant increase in colony size compared to the control group ($P \leq 0.05$). In contrast, the combined ZEB1-siRNA + CM group showed a sustained decline in both colony number and size throughout the entire 14-day period. Additionally, by day 14, the CM-only group exhibited a significantly greater colony size than the ZEB1-siRNA group observed at both day 3 and after day 7 ($P \leq 0.001$) (Figs. 23 and 24).

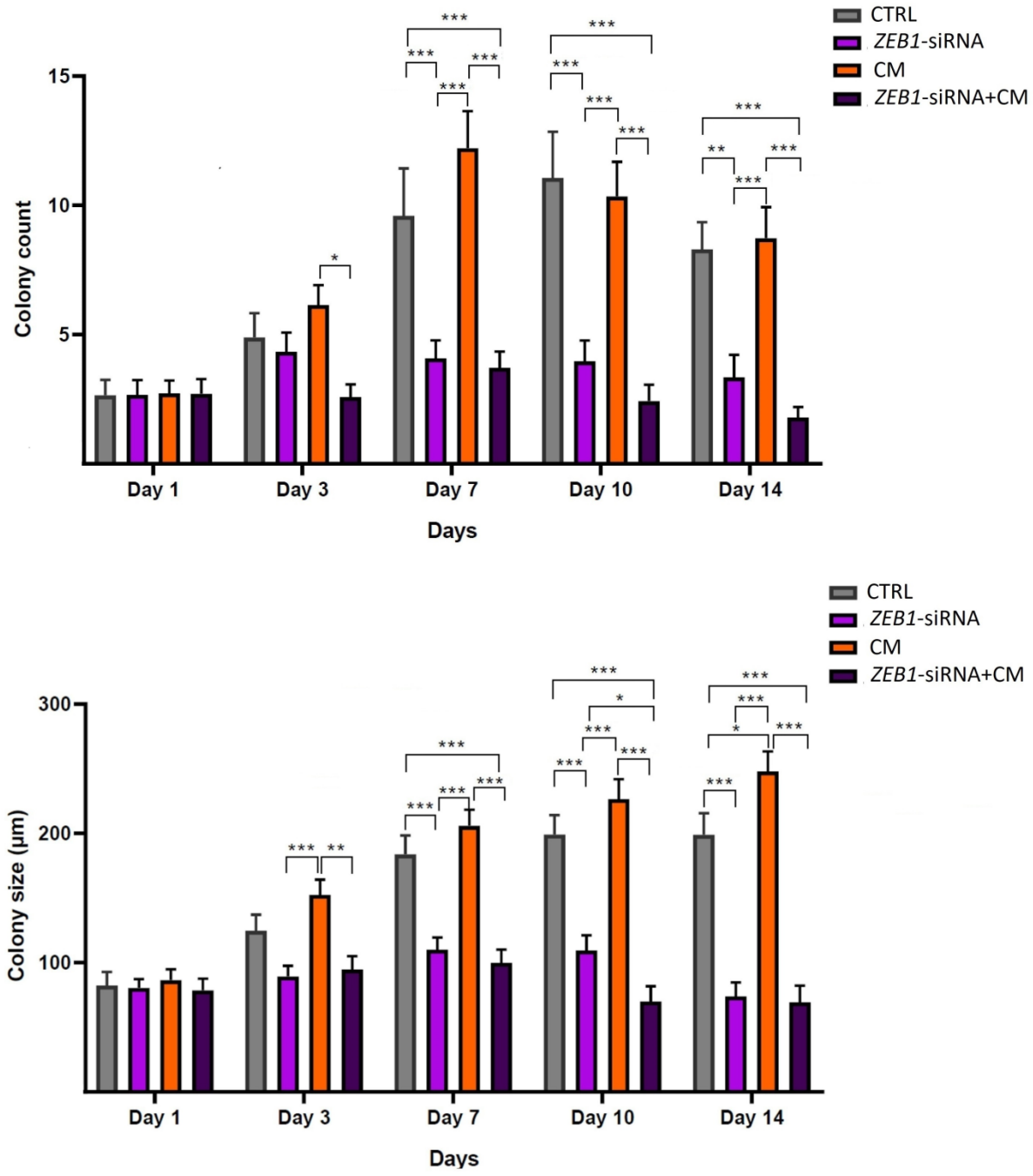

Fig.24 Analysis of colony number and size was performed over a 14-day period, with the following at days 1, 3, 7, 10, and 14. A significant decrease in both colony size and number was observed in the *ZEB1* knockdown groups. In contrast, the CM group showed a gradual increase in colony number up to day 7, with significant differences compared to the ZEB1 knockdown groups at day 7 ($P \leq 0.001$). The combined siRNA and CM groups also showed noticeable differences in colony formation at day 3 ($P \leq 0.05$) and day 7 ($P \leq 0.001$). However, the groups treated with both siRNA and CM demonstrated a notable decrease in colony size and number by day 14. On the other hand, the CM group exhibited a significant increase in colony size compared to

the *ZEB1*-siRNA-treated group at day 3 ($P \leq 0.001$), day 7 ($P \leq 0.001$), and the *ZEB1*-siRNA + CM group at day 3 ($P \leq 0.001$) and day 7 ($P \leq 0.001$). The CM group also showed significant enhancement in colony size compared to the control group at day 14 ($P \leq 0.05$). Statistical significance is indicated by $*p \leq 0.05$; $**p \leq 0.01$; and $***p \leq 0.001$.

Taken together, these findings underscore the time-dependent and differential effects of ZEB1 suppression and CM exposure on colony formation dynamics, suggesting that ZEB1 knockdown plays a critical regulatory role in modulating NSC colony growth under both physiological and inflammatory conditions (Figs. 23 and 24).

3.6. Wound healing assay

The wound healing assay was conducted to evaluate how ZEB1 influences cell migration in response to inflammatory stimuli, using six experimental groups. Stem cells were treated with CM and *ZEB1*-siRNA, and the migration of cells was tracked at two time points, 24 h and 48 h. This experimental setup allowed for the observation of the effects of both ZEB1 silencing and inflammatory conditions on wound closure (Fig. 25).

Using the Keyence microscope, specific observation points were consistently marked at identical locations for all time intervals. Initial images were captured within the first hours to establish a baseline. Subsequent observations and image acquisitions were carried out at 24 and 48 h to monitor any changes or developments over time.

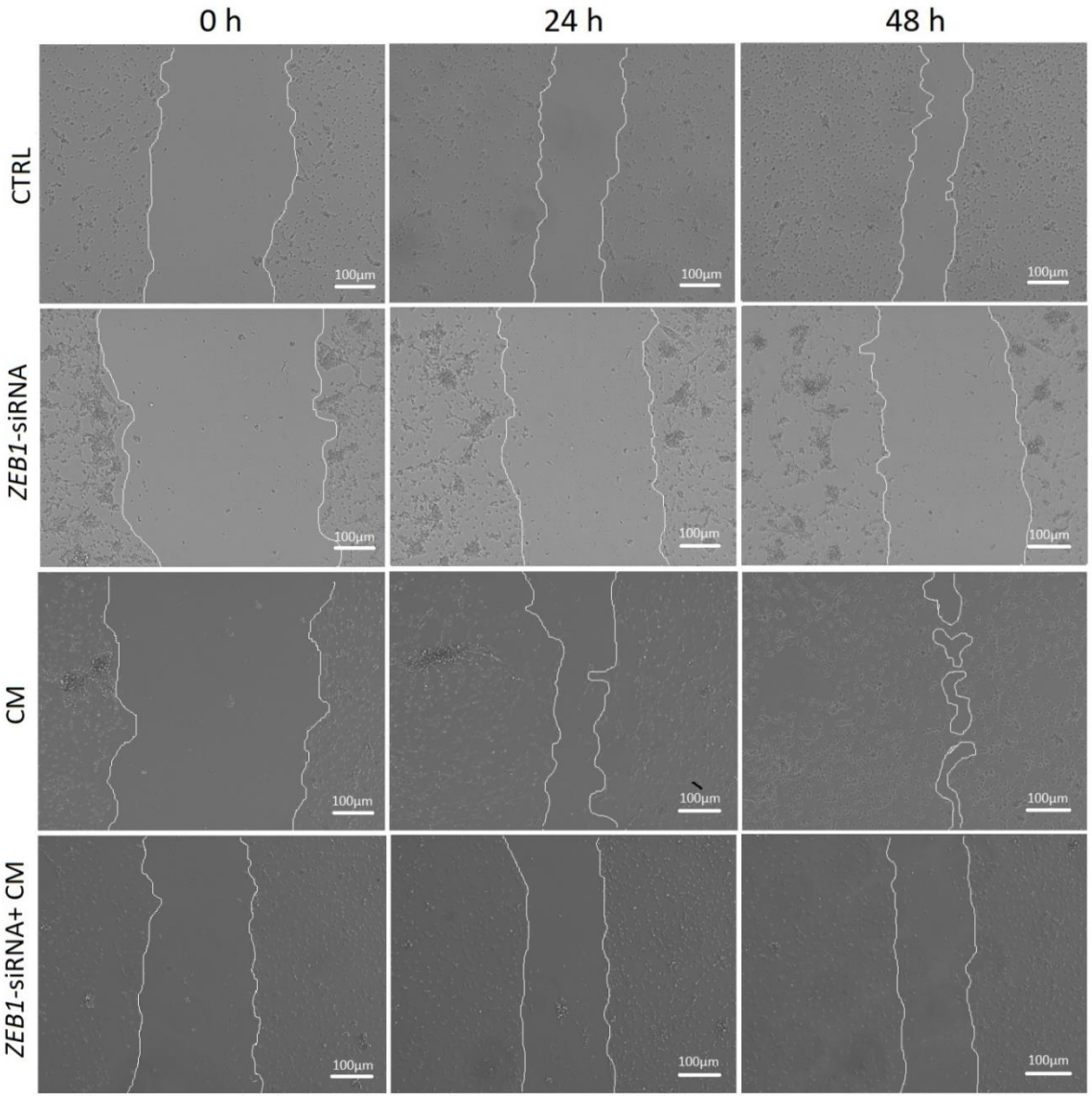

Fig.25 Wound healing analysis after 24 and 48h of treatment of the cells. All figures are presented with a scale of 100 µm.

In the present study, cells treated solely with CM demonstrated a significantly enhanced rate of wound closure at both 24 and 48 h when compared to all other experimental groups ($P \leq 0.001$) (Figs. 25 and 26). This suggests that CM enhances migration, likely through its inflammatory effects, which could promote wound closure and tissue repair. At the 24-hour time point, cells treated with *ZEB1*-siRNA showed a notable reduction in migration compared to the untreated control group, with a significant statistical difference observed ($P \leq 0.05$). By the 48-hour mark, this migratory inhibition was even more pronounced, with a

greater reduction in movement in the ZEB1 knockdown group, which reached a statistical significance of $P \leq 0.001$. These results indicate that ZEB1 plays an important role in facilitating cell migration, and silencing this gene significantly hinders the cells' ability to migrate (Figs. 25 and 26).

Notably, the group receiving combined treatment with ZEB1-siRNA and CM exhibited a significantly diminished wound closure rate compared to the control group, but only at the 48-hour mark ($P \leq 0.01$; Figs. 25 and 26).

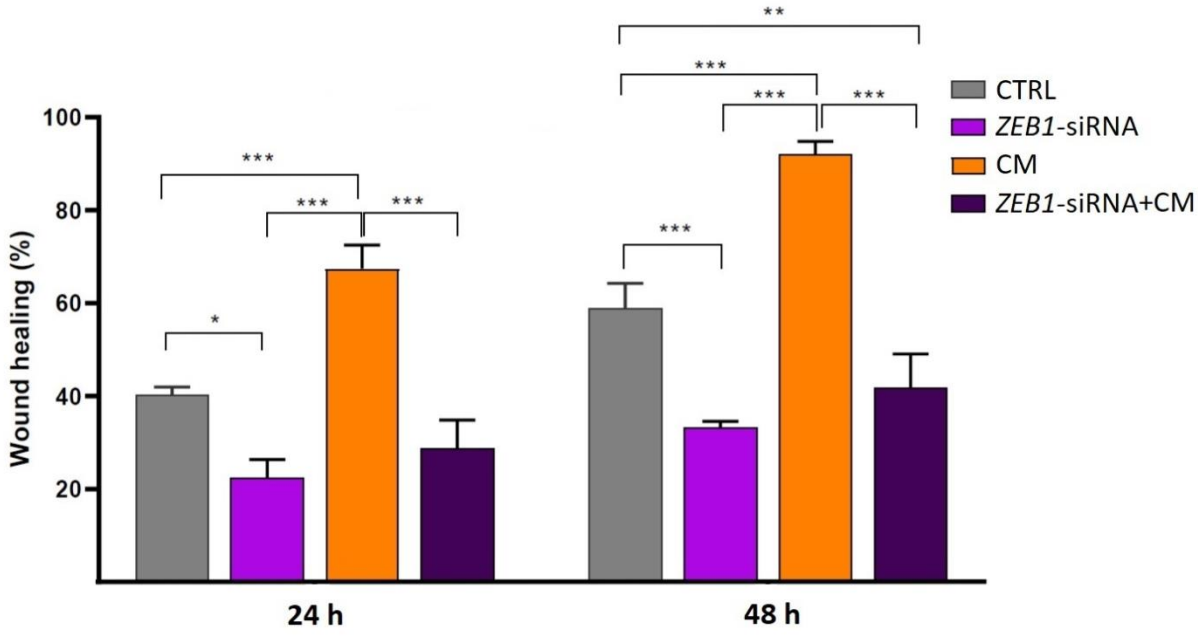

Fig.26 The analysis of the wound healing assay at 24 and 48 h demonstrated a significant impact of *ZEB1* silencing, even in the presence of CM. There was a clear reduction in cell migration observed in the *ZEB1* Knockdown groups at both 24 and 48 h following treatment. This indicates that silencing ZEB1 notably hindered the healing process, as migration was impaired compared to the control groups. Data presented as the mean ± SEM Statistical significance is indicated by $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

This trend was also apparent at the 24 h observation point, though it was slightly less noticeable. However, the most remarkable finding came from the group treated with both CM and *ZEB1*-siRNA, which exhibited a substantial decrease in wound healing compared to the CM groups. The group displayed statistically significant differences with the CM-treated group, observed at both 24 h ($P \leq 0.001$) and 48 h ($P \leq 0.001$). These results suggest that the combined treatment of CM and *ZEB1*-siRNA has a more pronounced inhibitory effect on

migration, indicating a potential interaction between ZEB1 silencing and the inflammatory response induced by CM. These results support the hypothesis that ZEB1 plays an essential role in modulating NSC motility, underscoring its potential contribution to mechanisms underlying neural regeneration and tissue repair (Figs. 25 and 26). Therefore, the absence of ZEB1 seems to inhibit cell movement, particularly when inflammatory signals like CM are present, highlighting its effects in the regulation of inflammatory responses and tissue repair mechanisms (Figs. 25 and 26).

3.7. Quantitative PCR analysis of neuroinflammatory cytokines

To examine the levels of neuroinflammatory cytokines, qPCR was employed following RNA extraction and cDNA synthesis. The qPCR assays were performed with a sample size of N=5 for each experimental group. The key cytokines selected for analysis were interleukin-6 (IL-6), interleukin-1β (IL-1β), Tumor necrosis factor-alpha (TNF-α), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and interleukin-17 (IL-17), as these are crucial markers in inflammatory diseases like MS.

The experimental data revealed a marked and statistically significant reduction in the expression levels of key neuroinflammatory cytokines following treatment with ZEB1-siRNA. Specifically, quantitative analysis demonstrated that the expression of IL-6, IL-1β, TNF-α, NF-Kb, and IL-17 was significantly diminished in the *ZEB1*-siRNA treated groups when compared to the CM-only treated groups ($P \leq 0.001$ for all comparisons; Fig. 27A–E).

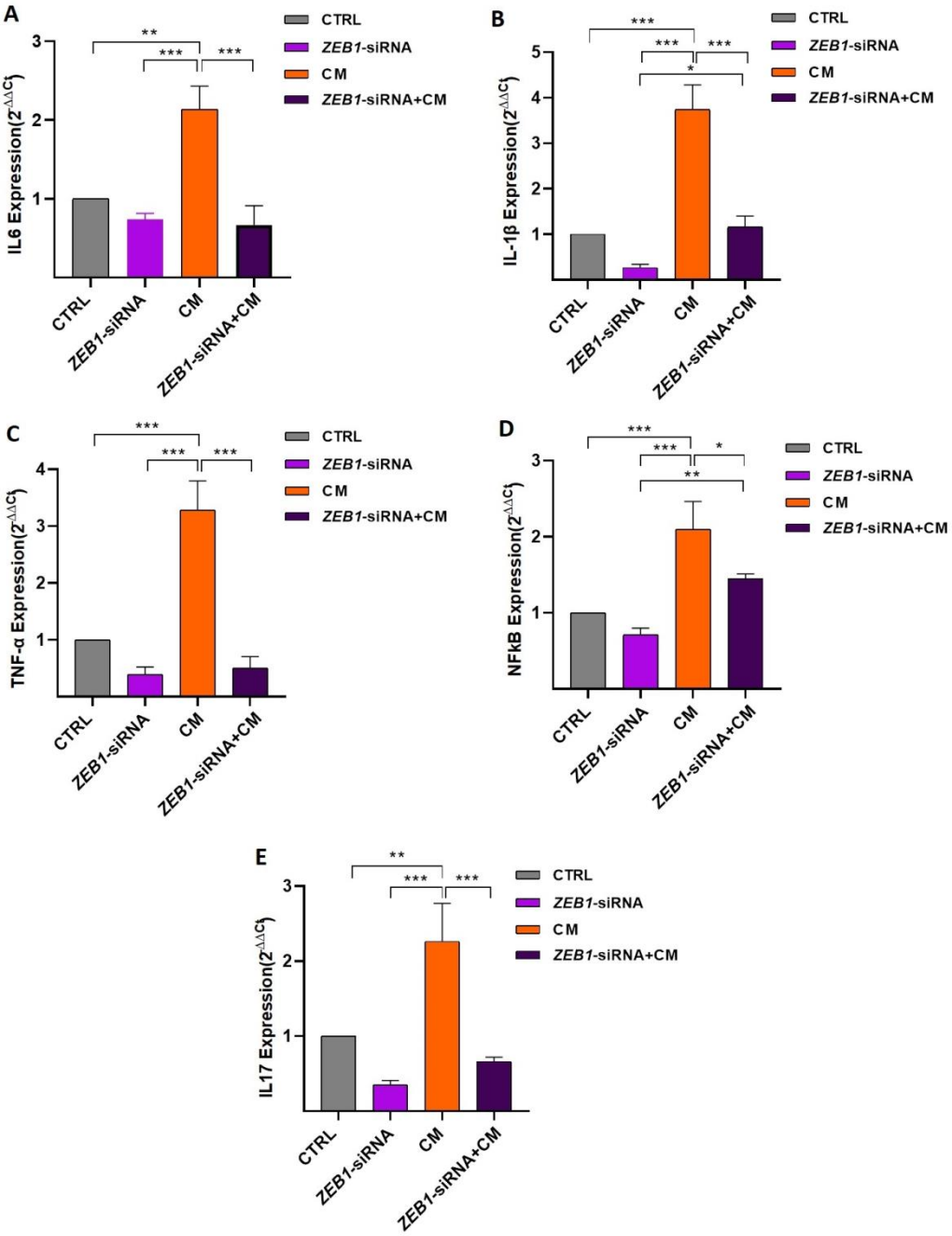

Fig. 27 Expression of pro-inflammatory markers: The levels of IL-6 (A), IL-1β (B), TNF-α (C), NFκB (D), and IL-17 (E) were assessed using real-time PCR after RNA extraction from cells treated for 48 h, followed by cDNA synthesis. The results showed that silencing ZEB1 led to a significant decrease in the expression of these pro-inflammatory cytokines across all experimental groups. Additionally, ZEB1 knockdown in cells treated with CM notably reduced the CM-induced inflammatory response, emphasizing the role of ZEB1 in regulating inflammation triggered by CM. Data expressed as the mean ± SEM. Statistical significance is indicated by $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

Combined treatment with CM and ZEB1-siRNA revealed a significant decrease in the CM-induced inflammatory response. Notably, the levels of key pro-inflammatory cytokines, including IL-6, IL-1β, TNF-α, NF-κB, and IL-17, were markedly reduced in the co-treatment group compared to CM alone. This suppression was particularly significant for IL-6, IL-1β, TNF-α, and IL-17 ($P \leq 0.001$), while NF-κB also showed a notable but less pronounced decrease ($P \leq 0.05$). These findings indicate that silencing ZEB1 can effectively downregulate inflammatory signaling triggered by CM exposure.

Compared to the control group, exposure to CM alone resulted in a significant upregulation of all five measured cytokines, reflecting a strong pro-inflammatory response. Specifically, CM treatment significantly increased the expression levels of IL-6 ($P \leq 0.01$; Fig. 27A), IL-1β ($P \leq 0.001$; Fig. 27B), TNF-α ($P \leq 0.001$; Fig. 27C), NF-κB ($P \leq 0.001$; Fig. 27D), and IL-17 ($P \leq 0.01$; Fig. 27E). These results emphasize the inflammatory potency of CM when applied independently.

Overall, these results indicate that the silencing of ZEB1 effectively attenuates neuroinflammatory signalling pathways, thereby contributing to a downregulation of pro-inflammatory cytokine production. These findings strongly support the hypothesis that ZEB1 plays a regulatory role in the modulation of inflammatory signaling within the neural microenvironment. Its knockdown not only influences the inflammatory effects of the CM, but may also represent a potential therapeutic strategy for regulating immune responses in neuroinflammatory conditions such as multiple sclerosis (Fig. 27).

3.8. ROS assay

To evaluate the level of oxidative stress in NSCs derived from rat brains (n=3), a ROS assay was conducted. This assessment was performed on cells with downregulation of ZEB1, examining conditions both with and without inflammation induced by CM. The evaluation took place at 24 and 48 h following treatment. The results at 24 h revealed a notable decrease in fluorescence intensity, which is indicative of reduced oxidative stress, in both *ZEB1*-siRNA ($P \leq 0.01$) and *ZEB1*-siRNA + CM ($P \leq 0.01$), when compared to the CM-only group (Fig. 28).

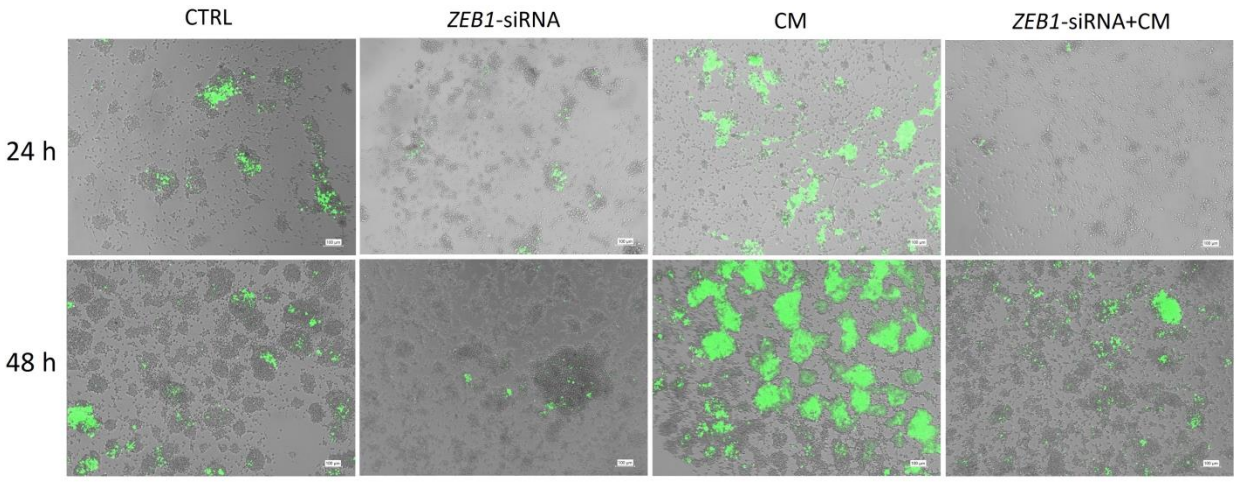

Fig. 28 Assessment of oxidative stress was performed using a Reactive oxygen species (ROS) assay in neural stem cells with ZEB1 silencing. ROS levels were detected using the fluorescent marker H2DCFDA at 24 and 48 h post-treatment.

At 48 h, however, a substantial increase in fluorescence intensity was observed in the CM group, relative to cells treated with siRNA targeting ZEB1 ($P \leq 0.001$) and a combination of ZEB1-siRNA and CM treatment ($P \leq 0.001$), and the untreated control group ($P \leq 0.001$). Moreover, the data showed a significant reduction in oxidative stress in the ZEB1 knockdown cells at 48 hours when compared to both the CM-treated group ($P \leq 0.001$) and the untreated control group ($P \leq 0.05$). These findings suggest that ZEB1 silencing effectively mitigates oxidative stress, even in the presence of CM, indicating a protective role of ZEB1 modulation against inflammation-induced oxidative damage (Figs. 28 and 29).

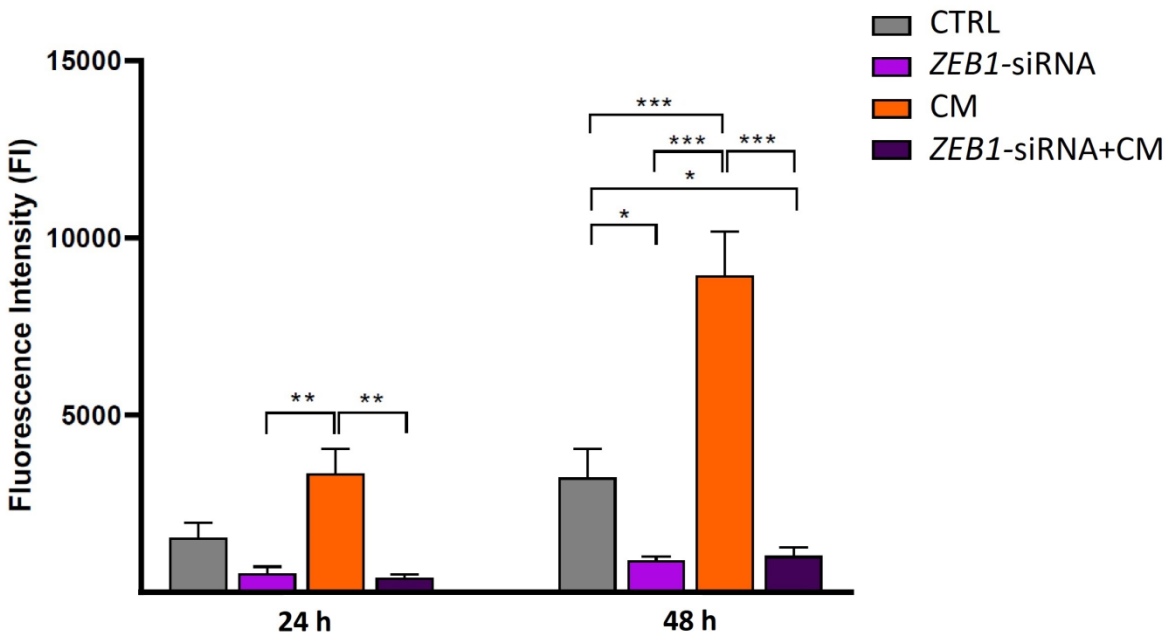

Fig. 29 ROS assay results: The data from the ROS assay after 24 and 48 h show that the CM-treated group resulted in a substantial increase in oxidative stress. However, silencing ZEB1 significantly reduced ROS levels, even in the presence of inflammation, with a more pronounced effect at the 48 h time point. These results suggest that ZEB1 inhibition may alleviate oxidative stress induced by inflammatory stimuli. Data expressed as the mean ± SEM. Statistical significance is indicated by $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

4. Discussion

4.1. Overview of key findings

This study investigated the role of ZEB1 in differentiation and regulating neuroinflammatory responses in NSCs exposed to CM from LPS-activated microglia as a stimulation condition of MS disease. In the initial phase of our study, we discovered a previously unrecognized role of ZEB1 in promoting the differentiation of NSCs into oligodendrocytes. This finding contributes new insight into the mechanism of oligodendrocyte maturation and the myelination process. In addition to its role in differentiation, our findings further demonstrated that ZEB1 is also essential in modulating oxidative stress and maintaining NSC viability, migration, and colony formation under both physiological and inflammatory conditions. Additionally, ZEB1 regulates cytokine expression, emphasizing its crucial role in balancing the neuroinflammatory environment and supporting NSC homeostasis.

4.2. ZEB1 regulatory functions under inflammatory conditions

Regarding previous studies, exposure of cells to CM from LPS-activated microglia resulted in increased levels of pro-inflammatory mediators such as TNF-α, IL-1β, IL-6, IL-17, NFκB, along with promotion of ROS generation, enhancing apoptosis, and impairing cell viability, migration, and colony formation (Pang et al., 2010; Guadagno et al., 2013; Guadagno et al., 2015; Nakao et al., 2022; Zhang et al., 2024; Tawbeh et al., 2025).

In this context, ZEB1 knockdown led to a marked reduction in the secretion of inflammatory cytokines, further indicating its immunomodulatory capabilities. This observation aligns with earlier studies that suggested ZEB1 modulates cytokine production, including IL-1β, IL-6, IL-8, IL-17, and TNF-α by influencing key intracellular signaling cascades such as Smad, tyrosine kinase receptors, TGF-β-related STAT3 signaling, NF-κB activity, and JAK1–STAT3 (Tang et al., 2017, Li et al., 2012; Qian et al., 2021; Liang et al., 2022; Lamouille et al., 2014). ZEB1 has also been implicated in BBB disruption and Th17 cell regulation, both of which are critical to MS pathology (Qian et al., 2021; Leduc-Galindo et al., 2019).

Our study showed that ZEB1 suppression altered cytokine profiles in NSCs under CM-induced inflammation. Specifically, CM treatment was associated with elevated IL-17 levels and also upregulated IL-6 expression, supporting previous research on the inflammatory pathways involved in MS pathology (Moser et al., 2020). To stimulate the MS-like inflammatory conditions, microglial cells were treated with 100 ng/ml LPS for 48 h to obtain CM, based on established protocols that replicate MS-like conditions in vitro (Lively et al., 2021; Li et al., 2021). The observation of early-stage apoptosis in NSCs aligns with current literature, indicating that dysregulated apoptosis contributes to neurodegenerative diseases like MS, where neurons reliant on mitochondrial function show increased susceptibility to cell death (EREKAT et al., 2021; Barcelos et al., 2019). CM triggers the release of various pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, MCP-1, TNF-α, and TGF-β, which play a crucial role in neuroinflammation via their interaction with TLR4 receptors (Syal et al., 2012). The inflammatory environment also activates transcription factors that enhance ZEB1 expression, promoting the release of inflammatory cytokines (Liang et al., 2022). Our findings suggest that CM-induced inflammation provides a suitable model for exploring the pathology of MS.

4.3. Molecular intractions involving ZEB1 in NSCs differentiation

Several studies have highlighted ZEB1 expression under physiological conditions, particularly in NSCs, where it is essential for maintaining self-renewal, stemness, differentiation potential, and coordinated migration during neurodevelopment (Jiang et al., 2018; Wang et al., 2019; Singh et al., 2023; Gupta et al., 2021). While further research is necessary to determine the precise molecular mechanism by which ZEB1 regulates NSC differentiation and its potential role in myelination, our findings revealed that ZEB1 influences the expression of MOG, a dominant and late-expressed marker of myelination on the surface of myelin sheaths (Iorio et al., 2015; Tea et al., 2019; Solly et al., 1996). This suggests that ZEB1 may govern genetic networks essential for oligodendrocyte maturation, potentially in cooperation with other transcriptional regulators such as SOX10, which is

known to orchestrate myelin gene expression (Vannier et al., 2013; Feldker et al., 2020; Durand et al., 2024).

Moreover, ZEB1 has been implicated in the maintenance of cellular plasticity in ventricular zone progenitors by repressing polarity genes, thus preserving cells in an undifferentiated and proliferative state (Liu et al., 2008; Singh et al., 2016). The loss of ZEB1 has been associated with impaired differentiation and migration of cortical neural progenitors, highlighting its indispensable regulatory role (Wang et al., 2019). ZEB1-mediated suppression of polarity genes in neuronal progenitors inhibits early polarization and maintains their retention within the germinal zone. Increased ZEB1 expression in SHH-subtype medulloblastoma reflects its role in polarity suppression and differentiation blockade, while restoration of polarity-related signaling facilitates proper neuronal differentiation and migration (Singh et al., 2016).

4.4. ZEB1 in migration and colony formation

In addition to its regulatory role in inflammation and differentiation, ZEB1 significantly influences NSC migration and colony-forming capacity. In our experimental model, the absence of ZEB1 led to a marked reduction in both cell migration and colony formation, even under inflammatory conditions. Interestingly, although ZEB1 deficiency compromised these cellular functions, CM treatment still promoted colony formation, possibly due to the upregulation of colony-stimulating factors in response to inflammation (Honda et al., 2023; Moore et al., 1980; Chen et al., 2018). This suggests a compensatory mechanism in which inflammation may, to some extent, counterbalance the loss of ZEB1 by promoting pathways that support cell proliferation.

Moreover, the impact of ZEB1 on migration was evident in our wound-healing assay, where CM treatment failed to promote efficient closure in ZEB1-deficient cells, thereby affirming the critical involvement of ZEB1 in regulating stem cell motility via pathways associated with EMT (Wang et al., 2019; Drake et al., 2009). Proper neurogenesis requires a finely tuned balance between NSC proliferation and differentiation, ensuring that newly generated cells are integrated at the correct developmental stage for central CNS functionality

(Gonçalves et al., 2016). ZEB1 contributes to this process by modulating the transition of neural progenitors from the SVZ and regulating their differentiation and migration through transcriptional control of essential gene networks (Wang et al., 2019). ZEB1 depletion disrupts the delicate balance between radial glial cell proliferation and differentiation, resulting in defective glial maturation and aberrant neuronal migration (Liu et al., 2019). Moreover, ZEB1 is indispensable for the tangential migration of cerebellar granule neuron progenitors. Its downregulation is crucial for their transition into mature, polarized neurons, a process vital for proper cerebellar development (Singh et al., 2016).

4.5. Implications of ZEB1 for MS pathology and therapy

NSCs and OPCs are considered promising candidates for treating MS due to their role in remyelination and reparative processes. However, challenges persist regarding the migration, localization, and differentiation potential of these stem cells within MS lesions, which can affect the success of stem cell therapies (Gruchot et al., 2019). ZEB1 and inflammation are reciprocally linked in a regulatory loop within epithelial and tumor cells, where pro-inflammatory cytokines like TGF-β drive ZEB1 expression during inflammation (Zhang et al., 2021). Previous studies indicate that ZEB1 dynamically regulates gene expression by either activating or repressing inflammatory mediators like IL-1β and TNF-α, which drive tissue inflammation and contribute to cell death (Do et al., 2024).

In MS, both Th17 and Th1 cells are instrumental in the progression of the disease through the production of IL-17 and interferon-γ, key cytokines that worsen inflammation (Arellano et al., 2017; Passos et al., 2016; Moser et al., 2020). ZEB1 suppression in T-cells mitigates autoimmune inflammatory disease symptoms in animal models, while downregulation of ZEB1 reduces pathogenic cytokine expression in T-cells from MS patients (Qian et al., 2021). Regarding these findings, recent research has also emphasized the role of ZEB1 in the condition of neuroinflammation, particularly in MS. ZEB1 regulates key cellular processes such as migration, differentiation, and inflammation, all of which are essential for the progression and treatment of MS (Poonaki et al., 2022).

Oxidative stress also plays a significant role in the pathogenesis of MS, with ROS implicated in oligodendrocyte death and myelin damage in experimental autoimmune encephalomyelitis (EAE) models (Padureanu et al., 2019; Honorat et al., 2013). In our study, CM-induced inflammation elevated ROS production, which was significantly reduced by ZEB1 suppression. This suggests that ZEB1 inhibition may protect cells from oxidative stress, offering potential therapeutic benefits for inflammatory conditions such as MS (Nikic I et al., 2010; Hsu et al., 2002).

In addition, we observed that inflammatory cytokines such as IL-6 and TNF-α, along with nitric oxide production, contributed to microglial migration in the CNS under inflammatory conditions, further intensifying neuroinflammation and progression of MS (Cen et al., 2022; Cheng et al., 2016). This finding is consistent with earlier studies indicating that these mediators exacerbate MS by promoting cell migration and neuroinflammation (Nguyen et al., 2021).

In summary, the ZEB1 inflammation axis is vital for the progression of neuroinflammatory disorders, such as MS, by pushing cell proliferation and migration, promoting a pro-inflammatory microenvironment, and impairing microglial and astrocyte function, thereby compromising the integrity of the blood-brain barrier (Poonaki et al., 2022; Zhang et al., 2021). Given the potential of NSCs for self-renewal, multi-lineage differentiation, and modulation of the inflammatory conditions, they present a promising therapeutic approach for MS and other neuroinflammatory diseases (Tang et al., 2023). Furthermore, modulation of ZEB1 expression in NSCs can improve their stability in pro-inflammatory conditions, thereby optimizing their therapeutic potential for tissue repair (Yan et al., 2018). Consequently, targeting ZEB1 in NSC therapy may enhance therapeutic outcomes by balancing neuroprotection, inflammation, and tissue regeneration.

4.6. Limitations and Directions for Future Research

While our findings provide novel insights into the regulatory functions of ZEB1 in NSC biology and neuroinflammation, certain limitations should be acknowledged to provide

perspective on the findings and guide future investigations. First, all experiments were conducted in vitro, which, despite offering controlled conditions, cannot fully capture the dynamic interactions and complexity of the in vivo environment within the central nervous system. Consequently, the translational relevance of these findings to clinical contexts requires careful interpretation.

Additionally, siRNA delivery was performed using Lipofectamine 2000 as a widely accepted non-viral transfection reagent known for its efficiency in *in vitro* applications. However, this method presents certain limitations, particularly regarding its transient gene silencing effect. Therefore, viral vectors such as lentiviral vectors, which enable stable and long-term gene expression or silencing, can offer a reliable platform for exploring the acute responses to ZEB1 suppression in NSCs.

Future research should include in vivo studies using relevant animal models of multiple sclerosis, such as experimental EAE, to validate the therapeutic effects of ZEB1 modulation under physiological conditions. This includes assessing the long-term impact of ZEB1 modulation on remyelination, neuroregeneration, and immune cell behavior in a living organism.

In conclusion, although these limitations do not diminish the value of our findings, they highlight important possibilities for deeper investigation. By identifying ZEB1 as a key regulator of NSC function under inflammatory conditions, this study provides a critical stepping stone for the discovery of targeted therapeutic agents appropriate for neuroinflammatory conditions, including MS.

4.7. Conclusion

To conclude, this study identifies ZEB1 as a significant regulator involved in neuroinflammatory signaling, NSC migration, and colony formation under inflammatory conditions. Down-regulation of ZEB1 appeared to influence both immune responses and oxidative stress, indicating its potential targeting role in stem cell-based therapies for MS and similar neuroinflammatory diseases. Further research is necessary to elucidate the molecular pathways through which ZEB1 mediates these effects and to evaluate its viability as a target for therapeutic intervention in neuroinflammation.

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6. Appendix

6.1. Explanation of the correlation coefficient

Table 4: Interpretation of Pearson's correlation coefficient (r)

Value of r	Interpretation
1.0	Indicates a perfect positive correlation
0.0 to 1.0	As r increases, the two variables tend to increase and r decreases together.
0.0	No correlation; the variables do not vary together
-1.0 to 0.0	As r decreases, one variable tends to increase while the other decreases.
-1.0	Indicates a perfect negative (inverse) correlation

6.2. Main Instruments

Table 5: The list of main instruments

Instrument	BRAND	Model	Country
Real-time system	BioRAD	CFX Connect®	Germany
Mini centrifuge	VWR	Ministar	Germany
Elisa Reader	Sunrise	TECAM	Germany
Nano drop	Thermo scientific	NanoDrop ONE	Germany
Flow cytometry	Beckman Counter	CytoFLEX	Germany
Microscope	KYENCE	BZ-X810 Series	Japan
Microscope	Zeiss	Axiovert 40 CFL	Germany
Centrifuge	Hettich	Universal 320R	Germany
Sclae	ACCULAB	ALC-210.4	Germany
Hood for animal	Erlab	Captair®	Germany
Biological Hood	Clean Air	EF/S4	Germany
CO2 Incubtor	BINDER	Apt.line ^{TM}CB	Germany
CO2 Incubator	Thermo Scientific	Heracell 240i	Germany
Water Bath	Medingen	WB 5	Germany
Vortex	IKA®	Genius 3	Germany