

NKX6.2 related autosomal recessive hypomyelinating leukodystrophy, is rescue therapy via homeoprotein transfer a possibility?

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Abstract

NKX6.2 is a homeodomain transcription factor whose CNS expression is enriched in mature myelinating oligodendrocytes. Loss-of-function mutations in NKX6.2 cause Spastic Ataxia type 8 (SPAX8), a rare hypomyelinating leukodystrophy for which no curative treatment currently exists. Homeoproteins (HPs) constitute a superfamily of transcription factors capable of unconventional intercellular transfer via the third helix of their homeodomain, raising the possibility that wild-type NKX6.2 could rescue NKX6.2-deficient oligodendrocytes in SPAX8 patients through paracrine transfer. The aim of this study was to determine whether NKX6.2 behaves as a secretory homeoprotein and to evaluate its intercellular transfer in oligodendrocyte-relevant experimental models. Using a bicistronic pCAGIG-V5-NKX6.2-IRES-EGFP construct that allows discrimination between donor cells (V5+/GFP+) and putative transfer recipients (V5+/GFP-) by immunofluorescence, transfer was assessed in HEK293 cells, Human Oligodendroglioma (HOG) cells, and chick embryo neural tubes via *in ovo* electroporation. NKX6.2 intercellular transfer was detected in HEK293 cells at a mean frequency of $1.5 \pm 1.3\%$ across five independent experiments (6,831 cells total), demonstrating that NKX6.2 fulfils the criteria for a secretory homeoprotein. Transfer in oligodendrocyte-relevant systems could not be conclusively assessed: HOG cells proved refractory to lipofection-mediated transfection, precluding generation of sufficient donor cells; a conditioned medium co-culture approach produced no detectable V5 signal in HOG recipient cells, consistent with possible HSPG-mediated sequestration of secreted protein; and *in ovo* electroporation confirmed plasmid expression in the chick neural tube but revealed no detectable intercellular transfer. These results establish that NKX6.2 is competent for transfer *in vitro*, while highlighting the technical obstacles that must be resolved before the HP-based rescue hypothesis can be directly tested in oligodendrocyte-relevant models of SPAX8.

Résumé

NKX6.2 est un facteur de transcription à homéodomaine dont l'expression dans le SNC est enrichie dans les oligodendrocytes myélinisants matures. Les mutations perte de fonction du gène NKX6.2 causent l'Ataxie Spastique de type 8 (SPAX8), une leucodystrophie hypomyélinisante rare pour laquelle il n'existe actuellement aucun traitement curatif. Les homéoprotéines (HP) constituent une superfamille de facteurs de transcription capables d'un transfert intercellulaire non conventionnel via la troisième hélice de leur homéodomaine, suggérant que la protéine NKX6.2 sauvage pourrait restaurer la fonction des oligodendrocytes déficients chez les patients SPAX8 par transfert paracrine. L'objectif de cette étude était de déterminer si NKX6.2 se comporte comme une homéoprotéine sécrétoire et d'évaluer son transfert intercellulaire dans des modèles expérimentaux pertinents pour les oligodendrocytes. À l'aide d'une construction bicistronique pCAGIG-V5-NKX6.2-IRES-EGFP permettant de discriminer les cellules donneuses (V5+/GFP+) des potentielles cellules réceptrices (V5+/GFP-) par immunofluorescence, le transfert a été évalué dans des cellules HEK293, des cellules HOG (Human Oligodendroglioma) et le tube neural d'embryons de poulet par électroporation *in ovo*. Le transfert intercellulaire de NKX6.2 a été détecté dans les cellules HEK293 à une fréquence moyenne de $1,5 \pm 1,3\%$ sur cinq expériences indépendantes (6 831 cellules au total), démontrant que NKX6.2 remplit les critères d'une homéoprotéine sécrétoire. Le transfert dans des systèmes pertinents pour les oligodendrocytes n'a pas pu être évalué de manière concluante : les cellules HOG se sont révélées réfractaires à la transfection par lipofection, empêchant la génération d'un nombre suffisant de cellules donneuses ; une approche de co-culture avec milieu conditionné n'a produit aucun signal V5 détectable dans les cellules HOG réceptrices, en accord avec une possible séquestration de la protéine sécrétée au niveau des HSPGs de surface ; et l'électroporation *in ovo* a confirmé l'expression du plasmide dans le tube neural sans révéler de transfert intercellulaire détectable. Ces résultats établissent que NKX6.2 est compétente pour le transfert *in vitro*, tout en soulignant les obstacles techniques qui devront être résolus avant de pouvoir tester directement l'hypothèse de sauvetage à base d'HP dans des modèles d'oligodendrocytes pertinents pour SPAX8.

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List of Abbreviations

AAV:	Adeno-Associated Virus
CNS:	Central Nervous System
DAPI:	4',6-diamidino-2-phenylindole
DMEM:	Dulbecco's Modified Eagle Medium
EGFP:	Enhanced Green Fluorescent Protein
EN1:	Engrailed 1
EN2:	Engrailed 2
FACS:	Fluorescence-Activated Cell Sorting
FBS:	Fetal Bovine Serum
GFP:	Green Fluorescent Protein
HEK293:	Human Embryonic Kidney 293
HH:	Hamburger–Hamilton (staging)
HOG:	Human Oligodendroglioma
HP:	Homeoprotein
HSPG:	Heparan Sulfate Proteoglycan
HSC-GT:	Hematopoietic Stem Cell Gene Therapy
HTH:	Helix-Turn-Helix
IRES:	Internal Ribosome Entry Site
LV:	Low Voltage
MBP:	Myelin Basic Protein
MLD:	Metachromatic Leukodystrophy
MRI:	Magnetic Resonance Imaging
NG2:	Neural/Glial Antigen 2 (CSPG4)
NLS:	Nuclear Localization Signal
nsHP:	Non-secretory Homeoprotein
OPC:	Oligodendrocyte Precursor Cell
OTX2:	Orthodenticle Homeobox 2
P/S:	Penicillin/Streptomycin
PBS:	Phosphate-Buffered Saline
PDGFRA:	Platelet-Derived Growth Factor Receptor Alpha

PFA:	Paraformaldehyde
PLP:	Proteolipid Protein
RA:	Retinoic Acid
SD:	Standard Deviation
sHP:	Secretory Homeoprotein
SPAX8:	Spastic Ataxia type 8
TBS:	Tris-Buffered Saline
TBS-T:	Tris-Buffered Saline + 0.1% Tween-20

Introduction

1. NKX6.2-related leukodystrophy: a rare hypomyelinating disease without curative treatment

Leukodystrophies are a heterogeneous group of rare inherited disorders defined by abnormalities in the formation, maintenance, or turnover of myelin in the central nervous system (CNS) [1,2]. Although individually rare, they collectively affect approximately 1 in 7,000 live births, placing them among the most prevalent groups of pediatric neurological disorders overall [2]. Most are present in infancy or early childhood with progressive neurological decline, and the majority currently lack any curative treatment. This therapeutic gap has driven interest in novel molecular strategies targeting the underlying genetic causes directly [2].

Among these conditions, a subset is classified as *hypomyelinating leukodystrophies*, in which myelin fails to form adequately from early development rather than being progressively lost. On MRI, this manifests as persistently abnormal white matter signal (hyperintense on T2-weighted sequences) without the age-related maturation expected in healthy individuals [2]. NKX6.2-related leukodystrophy, also designated Spastic Ataxia 8 (SPAX8), belongs to this subgroup and represents one of its most recently identified and least understood forms (Figure 1).

SPAX8 was first described in 2017 by Chelban and colleagues, who identified biallelic loss-of-function mutations in the *NKX6-2* gene in patients presenting with a consistent clinical triad:

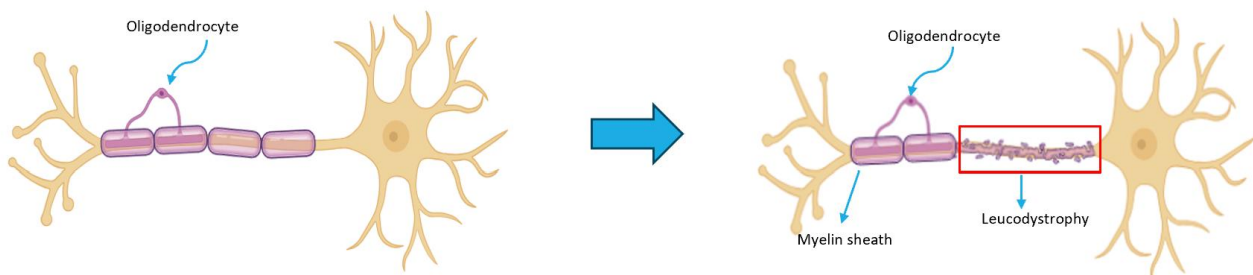


FIGURE 1 OLIGODENDROCYTE-MEDIATED MYELINATION AND ITS DISRUPTION IN LEUKODYSTROPHY. IN NORMAL CONDITIONS (LEFT), OLIGODENDROCYTES EXTEND PROCESSES THAT WRAP AROUND AXONS TO FORM COMPACT MYELIN SHEATHS, ENABLING RAPID SALTATORY CONDUCTION. IN LEUKODYSTROPHY (RIGHT), MYELIN SHEATHS ARE ABNORMAL OR ABSENT, COMPROMISING AXONAL SIGNAL TRANSMISSION.

spasticity (velocity-dependent increase in limb muscle tone resulting from upper motor neuron dysfunction), axial hypotonia (selective reduction of truncal muscle tone, contrasting with the appendicular hypertonicity), and nystagmus (involuntary rhythmic eye movements reflecting impaired brainstem or cerebellar oculomotor control), appearing before the age of one year, frequently accompanied by delayed motor development and cognitive impairment in approximately two thirds of cases [3,4]. MRI findings showed diffuse hypomyelination, often associated with cerebellar and brainstem abnormalities, and in some cases optic nerve involvement (a pattern reflecting the selective vulnerability of CNS myelin to NKX6.2 dysfunction). The disorder follows an autosomal recessive inheritance pattern, with causative mutations spanning the full spectrum of loss-of-function alleles: nonsense variants, frameshift deletions, and missense mutations, particularly those disrupting the homeodomain, the homeoprotein DNA-binding module [3].

The clinical and genetic scope of SPAX8 was subsequently expanded by Chelban *et al.* in 2020, through the analysis of 33 patients from 21 independent families, in whom 13 distinct pathogenic variants have been identified to date [4]. A 14th variant, p.Ala182Val (c.545C>T), located within

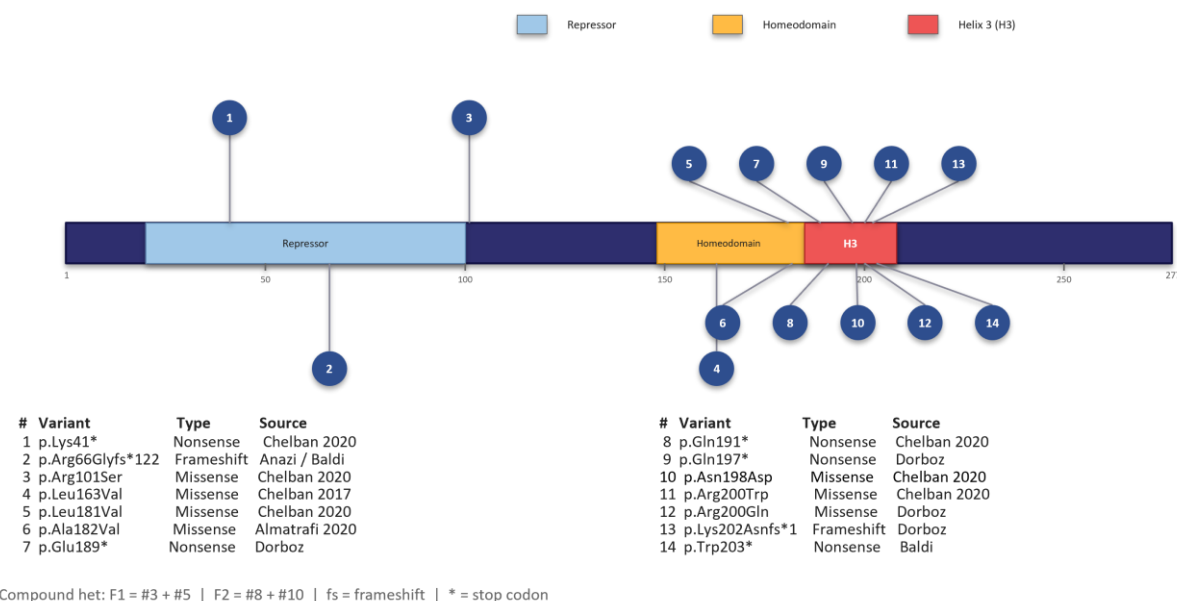


FIGURE 2 LINEAR DOMAIN MAP OF THE NKX6.2 PROTEIN (277 AMINO ACIDS). THE N-TERMINAL REPRESSOR DOMAIN (SKY BLUE), HOMEODOMAIN (YELLOW, AA 148–207), AND HELIX 3 (H3, ORANGE), THE SUBREGION MEDIATING INTERCELLULAR TRANSFER, ARE ANNOTATED. REPRESENTATIVE PATHOGENIC VARIANTS ASSOCIATED WITH SPAX8 ARE INDICATED.

the homeodomain, was subsequently reported in an independent consanguineous family [5]. This larger cohort revealed a clear genotype-phenotype correlation: truncating mutations are associated with the most severe presentations, characterized by near-complete absence of myelination and lack of speech acquisition, whereas certain missense mutations permit milder courses with preserved cognitive function [4]. This variability underlines that the degree of NKX6.2 loss of function directly determines clinical severity, a point with direct therapeutic implications, as even partial restoration of NKX6.2 activity could, in principle, shift the phenotype toward a milder form (Figure 2).

Despite these advances in molecular diagnosis, no disease-modifying or curative treatment exists for SPAX8 [3,4]. Current management remains strictly symptomatic, addressing spasticity and supporting motor development without rescuing the underlying hypomyelination. Given the monogenic nature of the disease and the well-defined role of NKX6.2 in oligodendrocyte biology, SPAX8 represents a compelling candidate for targeted molecular intervention, provided that an effective strategy for delivering functional NKX6.2 protein to affected cells can be identified. The present work investigates one such strategy, grounded in a well-characterized and conserved property of homeodomain-containing proteins: their ability to transfer spontaneously between cells.

2. NKX6.2: a homeodomain transcription factor essential for oligodendrocyte maturation

The homeodomain is a conserved ~60 amino acid DNA-binding motif first characterized in *Drosophila* homeotic selector genes and subsequently identified in hundreds of eukaryotic transcription factors across metazoan lineages [6]. Its canonical architecture consists of three alpha-helices: helix 1 packs against helix 2 through hydrophobic contacts to form the stable structural core of the fold, while helices 2 and 3 constitute a helix-turn-helix (HTH) motif in which helix 3 (the recognition helix) inserts into the major groove of DNA, where residues at positions 47, 50, 51, and 54 establish direct base-specific contacts that determine binding sequence preferences [6] (Figure 3). The N-terminal arm of the homeodomain projects into the adjacent minor groove, providing an additional layer of DNA-binding stability. Most homeodomain proteins recognize a TAAT core motif, but the precise flanking sequence selectivity is governed by variations at the critical contact residues of helix 3, accounting for the functional diversity observed across the homeodomain superfamily. However, helix 3 is not only the structural determinant of transcriptional specificity: as will be detailed in the following section, it also mediates a conserved capacity for intercellular protein transfer.

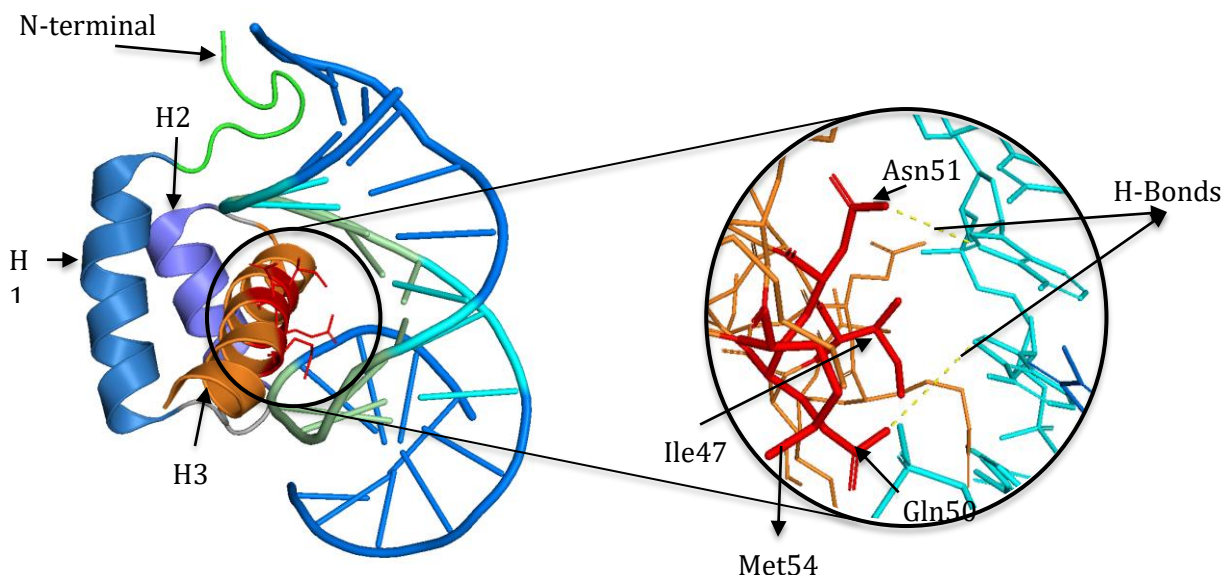


FIGURE 3 SCHEMATIC REPRESENTATION OF HOMEODOMAIN ARCHITECTURE AND INTERACTION WITH DNA. HELIX 3 (H3, ORANGE) INSERTS INTO THE MAJOR GROOVE, WITH RESIDUES AT POSITIONS 47, 50, 51, AND 54 MAKING BASE-SPECIFIC CONTACTS. THE N-TERMINAL ARM ENGAGES THE MINOR GROOVE. H3 ALSO MEDIATES UNCONVENTIONAL RECEPTOR-INDEPENDENT INTERCELLULAR TRANSFER, A DUAL FUNCTION CENTRAL TO THE RESCUE HYPOTHESIS EXPLORED IN THIS THESIS.

NKX6.2 (historically designated Gtx for Glial transcription factor x) belongs to the NKX6 subfamily of NK-class homeodomain transcription factors, which in vertebrates includes NKX6.1 and NKX6.3. The human NKX6-2 gene maps to chromosome 10q26, a region recurrently deleted in brain tumors, and encodes a 277 amino acid protein sharing 97% amino acid identity with its murine orthologue [7]. In the postnatal CNS, NKX6.2 expression is predominantly associated with mature, myelinating oligodendrocytes (although expression in progenitor cell populations of the developing spinal cord has also been documented [9]): immunofluorescence studies have demonstrated that NKX6.2 co-localizes with NKX2.2 in APC-positive oligodendrocytes, while

being absent from NG2-positive oligodendrocyte precursor cells (OPCs) [8] (Figure 4). This late-stage, differentiation-dependent expression profile situates NKX6.2 downstream of the lineage commitment steps governed by early specification factors such as OLIG2 and SOX10, placing it instead among the transcriptional effectors that sustain terminal maturation and myelin gene expression.

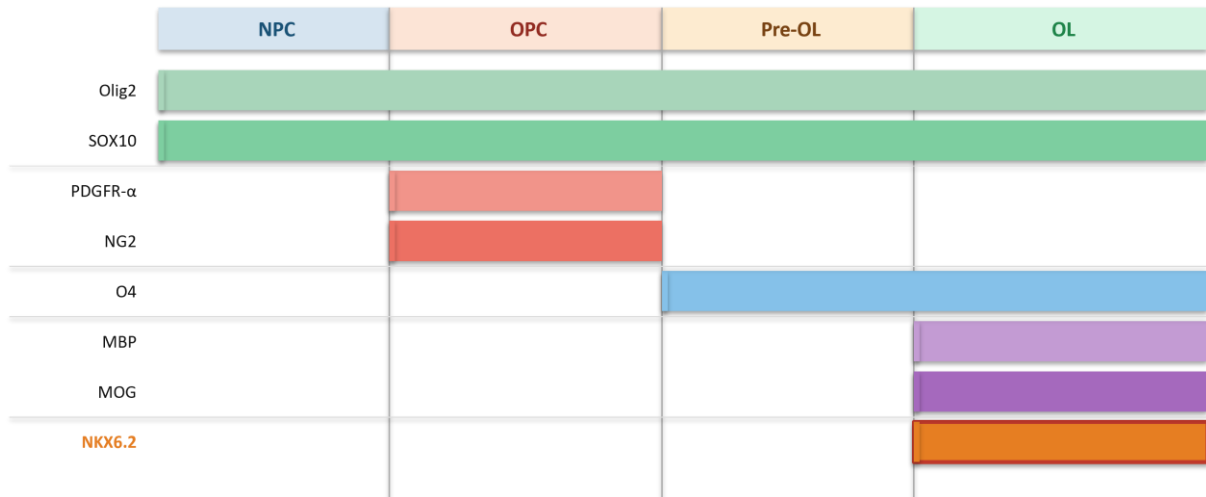


FIGURE 4 NKX6.2 EXPRESSION IS RESTRICTED TO THE MATURE MYELINATING STAGE OF THE OLIGODENDROCYTE LINEAGE. NKX6.2 IS DETECTED IN MBP/PLP-EXPRESSING MATURE OLIGODENDROCYTES AND IS ABSENT FROM NG2/PDGFR-POSITIVE PRECURSORS, CONSISTENT WITH A ROLE IN SUSTAINING TERMINAL DIFFERENTIATION AND MYELIN GENE PROGRAMS RATHER THAN IN INITIAL LINEAGE COMMITMENT. ADAPTED FROM [8] AND [10].

Mechanistically, NKX6.2 acts as a transcriptional repressor. Awatramani *et al.* identified a repressor domain located N-terminal to the homeodomain and determined the optimal NKX6.2 binding sequence as (A/T)TTAATGA [11]. Reporter assays in heterologous and oligodendrocyte-lineage cell lines demonstrated that NKX6.2 constitutively represses transcription from its consensus binding site; however, this activity is context-dependent, being modulated by cell-intrinsic co-factors in oligodendrocyte-lineage cells in a manner consistent with cooperative regulation within the broader myelination transcriptional network [11]. The downstream target genes whose repression is functionally required for proper myelination remain incompletely defined; nonetheless, the paranodal ultrastructural abnormalities observed in knock-out animals imply that NKX6.2-mediated repression contributes to the transcriptional control of genes encoding components of the axo-glial junction.

The *in vivo* requirement for NKX6.2 in CNS myelination was directly assessed in a knock-in/knock-out mouse model in which a lacZ reporter cassette was inserted into the *Nkx6-2* locus, simultaneously disrupting the endogenous gene (such that the allele is null while enabling lacZ-based fate mapping) [12]. *Nkx6-2*-null animals are viable and fertile, and gross histological examination reveals no overt dysmyelination [12]. However, electron microscopy discloses discrete paranodal ultrastructural defects: the paranodal axo-glial junctions — specialized adhesion complexes in which oligodendroglial neurofascin-155 (NF155) bridges to axonal contactin and Caspr, anchoring paranodal myelin loops to the axolemma and maintaining the

diffusion barrier required for proper segregation of nodal ion channels — are disrupted, and aberrant expression of the paranodal proteins neurofascin, contactin, and stathmin 1 is observed [12]. This relatively mild phenotype (markedly less severe than the clinical presentation of SPAX8 patients) is likely attributable to partial functional compensation by Nkx6.1, which shares expression domain overlap in mature oligodendrocytes, as well as to the purely loss-of-function nature of the knock-out model versus the potential dominant-negative effects conferred by certain human missense mutations within the homeodomain. Paradoxically, NKX6.2 has been characterized as a transcriptional repressor [11], raising the question of how silencing target genes could promote myelination. This apparent paradox is resolved by a de-repression mechanism: NKX6.2 binding sites within the M3 regulatory module of the MBP promoter are required for high-level MBP expression [13], indicating that NKX6.2 activates the myelin gene program indirectly, by repressing transcriptional inhibitors that would otherwise suppress myelin structural gene expression.

Beyond its role in developmental myelination, NKX6.2 is also re-recruited during myelin repair. In a rat model of experimental demyelination, NKX6.2 mRNA re-expression during the remyelination phase closely mirrors the recovery kinetics of the major myelin structural genes *Mbp* and *Plp*, suggesting that NKX6.2 participates in the transcriptional program activated when oligodendrocytes re-engage their myelination machinery [14]. Importantly, this regenerative NKX6.2 expression is markedly attenuated in aged animals, identifying NKX6.2 as one of the molecular correlates of the well-documented age-dependent decline in remyelination efficiency [14]. These findings extend the biological relevance of NKX6.2 beyond the developmental window, reinforcing the concept that its activity is continuously required to sustain the competence of oligodendrocytes to establish and re-establish axonal myelination in the CNS.

NKX6.2 thus emerges from these studies as a non-redundant effector of oligodendrocyte terminal differentiation and myelin maintenance, whose loss of function is sufficient to produce the hypomyelinating phenotype that causes SPAX8. In the context of a rescue strategy, the central question is whether functional NKX6.2 protein could be delivered to affected oligodendrocytes in a manner that bypasses the underlying genetic defect. A potential mechanism for such non-cell-autonomous delivery is provided by the intercellular transfer capacity shared by homeodomain-containing proteins, a property whose structural basis resides in helix 3 of the homeodomain, and which is examined in detail in the following section.

3. Homeoprotein intercellular transfer: a non-cell-autonomous signaling mechanism

The first evidence that a homeoprotein could cross biological membranes independently of classical secretory pathways was provided by Derossi *et al.* in a landmark study demonstrating the spontaneous translocation of the full *Drosophila Antennapedia* homeodomain into living cells [15]. Systematic deletion analysis revealed that this activity was abolished by mutations in the C-terminal region of the homeodomain, pointing to helix 3 as the critical structural determinant. Strikingly, a synthetic 16 amino acid peptide corresponding solely to helix 3 (subsequently designated penetratin) retained full internalization capacity and was recovered intact within the intracellular compartment, demonstrating that the translocating information is fully encoded in this short sequence [15]. Translocation occurred efficiently at 4°C, a temperature that essentially abolishes classical endocytic processes, an observation that ruled out energy-dependent vesicular uptake and pointed instead to a direct biophysical interaction between the peptide and the plasma membrane bilayer. These results had a broader implication: the classical assumption that proteins devoid of signal peptides are strictly confined to the intracellular compartment was thereby overturned, opening the possibility of non-classical intercellular transfer for transcription factors.

The molecular basis of helix 3 translocation was dissected further by demonstrating that both a retro analogue (in which the amino acid sequence is written from C- to N-terminus) and a D-amino

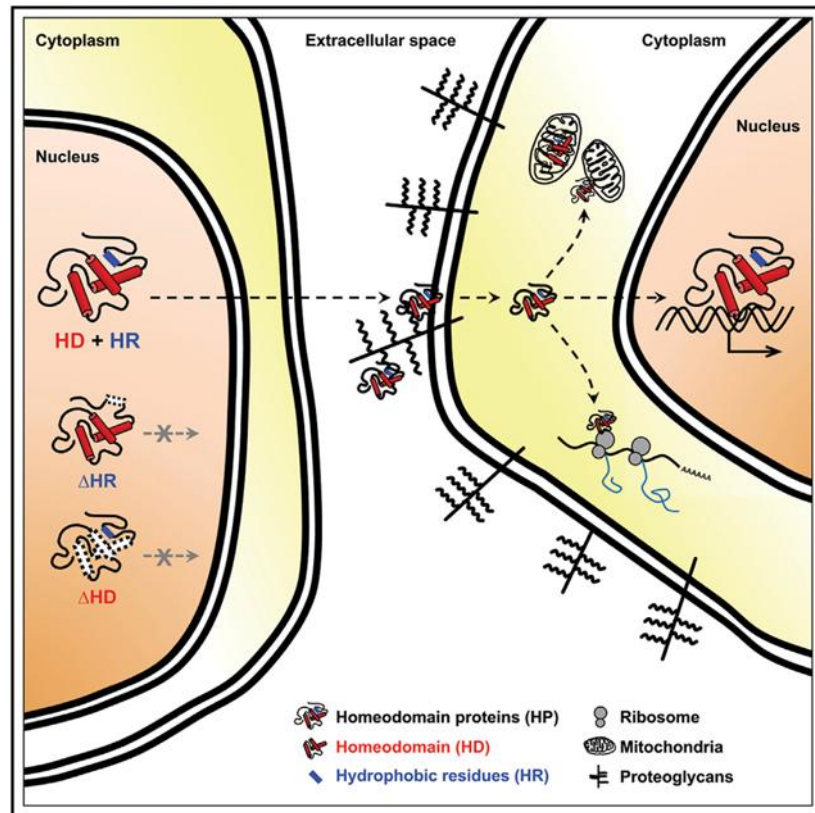


FIGURE 5 FOUR-STEP MODEL OF HOMEOPROTEIN INTERCELLULAR TRANSFER. (1) UNCONVENTIONAL, SIGNAL-PEPTIDE-INDEPENDENT SECRETION FROM THE PRODUCING CELL. (2) EXTRACELLULAR CAPTURE BY HEPARAN SULFATE PROTEOGLYCANS (HSPGs) AT THE RECIPIENT CELL SURFACE. (3) RECEPTOR-INDEPENDENT, ENERGY-INDEPENDENT TRANSLOCATION ACROSS THE PLASMA MEMBRANE MEDIATED BY HELIX 3-PHOSPHOLIPID INTERACTIONS. (4) INTRACELLULAR DELIVERY OF THE TRANSFERRED PROTEIN IN THE RECIPIENT CELL, ENABLING TRANSCRIPTIONAL AND/OR NON-TRANSCRIPTIONAL FUNCTIONS. ADAPTED FROM LEE ET AL., 2019 [18].

acid enantiomeric version of the penetratin peptide were internalized with equal efficiency [16]. Since biological receptors are strictly stereospecific and cannot accommodate D-amino acid ligands, this chirality-independence strongly argued against receptor-mediated uptake and supported the interpretation that translocation is a receptor-independent process [16]. Furthermore, insertion of proline residues, which would disrupt an alpha-helical conformation, did not prevent internalization, indicating that translocating capacity is an intrinsic property of the primary sequence rather than of secondary structure. Current mechanistic models propose that the positively charged and aromatic residues of helix 3, particularly tryptophans and lysines/arginines, establish electrostatic and hydrophobic contacts with the negatively charged phospholipid head groups of the inner membrane leaflet, enabling direct membrane perturbation and translocation. On the extracellular side, capture and surface presentation of secreted homeoproteins is facilitated by heparan sulfate proteoglycans (HSPGs) at the recipient cell surface, which act as low-affinity co-receptors that concentrate the protein near the plasma membrane prior to internalization, and whose disruption markedly reduces uptake efficiency (Figure 5) [17].

Intercellular transfer is not a peculiarity of *Antennapedia* but a conserved property of the homeoprotein superfamily. Systematic analysis across the more than 200 known human homeoproteins has identified a conserved transfer domain within helix 3 in virtually all family members [18,19]. Evidence for functional intercellular transfer has been documented for

homeoproteins as structurally and functionally diverse as Engrailed, OTX2, HOXB4, and PAX6, in organisms ranging from unicellular eukaryotes to mammals, and in both neuronal and non-neuronal cell types [18,19,20]. The evolutionary conservation of this transfer capacity across such divergent organisms (including in plants, where it has been proposed to contribute to morphogenetic field formation during sexual reproduction) argues strongly for selection-driven maintenance of a biologically meaningful mechanism rather than a non-specific membrane artifact [19].

Perhaps the most compelling argument for the physiological relevance of homeoprotein transfer comes from studies of OTX2 and Engrailed in the postnatal mammalian CNS. OTX2, produced in the choroid plexus, is actively transferred to parvalbumin-expressing inhibitory interneurons in the visual cortex, where it regulates the timing and closure of the critical period for visual plasticity [20,21]. Experimental interference with this transfer (either by sequestering extracellular OTX2 or by injecting recombinant OTX2 directly into the brain) shifts the critical period in predictable ways, demonstrating that the transferred protein is functionally active in recipient cells and causally involved in a complex neurodevelopmental process [21]. Similarly, Engrailed 1 and 2, secreted by isthmic organizer cells, regulate axon guidance through non-cell-autonomous signaling, and exert neuroprotective effects on mesencephalic dopaminergic neurons in mouse models of Parkinson's disease by enhancing local mitochondrial translation [21,22]. These *in vivo* examples strongly suggest that intercellular homeoprotein transfer is biologically meaningful and functionally significant in the recipient cell, with effects that cannot be replicated by a cell-autonomous mechanism.

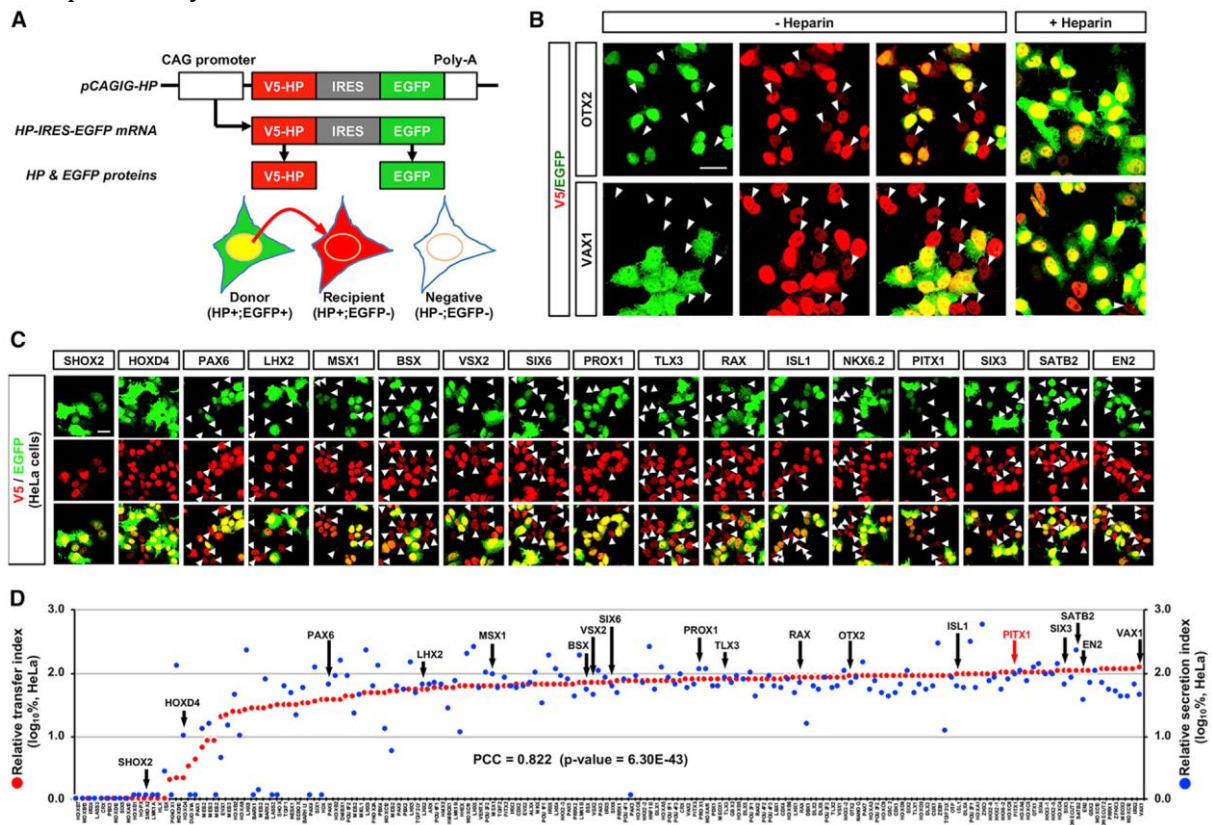


FIGURE 6 SCHEMATIC OF THE BICISTRONIC pCAGIG-V5-HP-IRES-EGFP EXPRESSION CONSTRUCT [18]. THE CAG PROMOTER (A COMPOSITE OF THE CMV ENHANCER AND THE CHICKEN B-ACTIN PROMOTER THAT DRIVES STRONG CONSTITUTIVE EXPRESSION IN MAMMALIAN CELLS) DRIVES TRANSCRIPTION OF A BICISTRONIC MRNA ENCODING A V5-TAGGED WILD-TYPE NKX6.2 HOMEOPROTEIN FOLLOWED BY AN IRES-EGFP CASSETTE. GFP FLUORESCENCE UNAMBIGUOUSLY IDENTIFIES TRANSFECTED DONOR CELLS; V5 IMMUNOREACTIVITY DETECTED IN GFP-NEGATIVE CELLS IN CO-CULTURE CONSTITUTES EVIDENCE OF INTERCELLULAR PROTEIN TRANSFER.

The generality of this phenomenon across multiple homeoproteins and cell types was further consolidated by Lee *et al.*, who demonstrated widespread intercellular transfer across 162 human homeodomain proteins in multiple cell systems, establishing that transfer is not restricted to specific cell types or to homeoproteins with known paracrine functions [18] (Figure 6).

In an oncological context, this principle has been extended to pathological settings: systematic analysis in medulloblastoma identified multiple homeoproteins capable of intercellular transfer capacity with altered expression, and proposed direct protein supplementation as a therapeutic avenue [23]. NKX6.2, as a member of the homeodomain superfamily possessing a conserved helix 3, is thus a structural and mechanistic candidate for intercellular transfer. Whether wild-type NKX6.2 produced by donor cells undergoes intercellular transfer, and whether this can be detected in oligodendrocyte-relevant cellular and *in ovo* experimental models, are the central questions addressed in this study.

4. Towards a protein-based rescue strategy for SPAX8: rationale and study aims

The development of disease-modifying therapies for leukodystrophies has advanced substantially in recent years, with hematopoietic stem cell gene therapy (HSC-GT) achieving clinical proof-of-concept for metachromatic leukodystrophy (MLD), where lentiviral delivery of a functional ARSA transgene into autologous hematopoietic progenitors results in the generation of cross-correcting microglia-like cells that supply functional arylsulfatase A to the CNS [24]. However, the cross-correction principle that underpins this strategy is mechanistically suited to enzyme deficiencies where secreted proteins can be recaptured by neighboring cells; it is fundamentally ill-adapted to transcription factor deficiencies such as SPAX8, where the missing activity is nuclear and cell-intrinsic, and cannot be supplemented by conventional secreted enzyme delivery [24] — unless the transcription factor itself is capable of crossing cell membranes autonomously, as is the case for homeodomain-containing proteins. Direct CNS gene delivery via adeno-associated viral (AAV) vectors represents an alternative avenue but faces its own limitations in hypomyelinating leukodystrophies: therapeutic windows are narrow, requiring intervention prior to the establishment of irreversible hypomyelination; vector tropism for mature myelinating oligodendrocytes *in vivo* remains suboptimal; and immune responses to viral capsids may limit repeated dosing. To date, no therapy targeting the molecular defect underlying SPAX8 has been described.

A fundamentally different approach is suggested by the biology of homeoprotein transfer. As reviewed above, exogenously administered Engrailed protein, delivered by stereotaxic injection into the substantia nigra, protects mesencephalic dopaminergic neurons against neurotoxin-induced degeneration in MPTP-induced mouse models of dopaminergic neurodegeneration [22], an effect that depends on direct uptake of the transferred protein by neurons that do not endogenously produce Engrailed, and that does not require any manipulation of the genome of the target cells. Similarly, exogenous OTX2 protein injected into the visual cortex is taken up by parvalbumin-expressing interneurons and functionally shifts the timing of the visual critical period in a manner indistinguishable from endogenous OTX2 transfer [21]. Of particular mechanistic relevance, Leboeuf *et al.* demonstrated that endogenous EN1 transfer from spinal V1 interneurons constitutes a genuine paracrine neurotrophic signal required for the survival of adult α -motor neurons: sequestration of extracellular EN1 by a secreted single-chain antibody caused progressive motor neuron loss and limb weakness, while intrathecal injection of recombinant EN1 protein had lasting neuroprotective effects against neurodegeneration [25].

This concept of protein-level rescue via homeoprotein transfer has independently received support from the oncological field: a recent study identified 11 travelling homeoproteins with

altered expression medulloblastoma (including NKX2.2, a close structural paralog of NKX6.2) and proposed direct cell penetration as a therapeutic modality [23]. We therefore propose the following working hypothesis (Figure 7): wild-type NKX6.2, produced by healthy donor cells expressing functional *NKX6.2*, can be transferred to NKX6.2-deficient recipient cells via the conserved homeodomain helix 3 mechanism, localize to their nucleus, and engage downstream transcriptional targets that are disrupted in SPAX8 oligodendrocytes. The plausibility of this approach rests on several converging observations. The genotype-phenotype correlation in SPAX8 implies that even partial restoration of NKX6.2 activity (as suggested by milder missense alleles) substantially ameliorates the clinical phenotype [4], lowering the threshold for therapeutically meaningful rescue. Moreover, oligodendrocyte cell bodies in white matter are embedded in a densely packed cellular environment, providing the spatial proximity required for cell-to-cell protein transfer; and transferred homeoproteins have been shown to undergo nuclear localization in recipient cells in multiple experimental systems [18,21], consistent with the possibility that transferred NKX6.2 could engage its native nuclear targets. Homeoprotein transfer thus represents a mechanistically grounded candidate strategy for SPAX8 rescue.

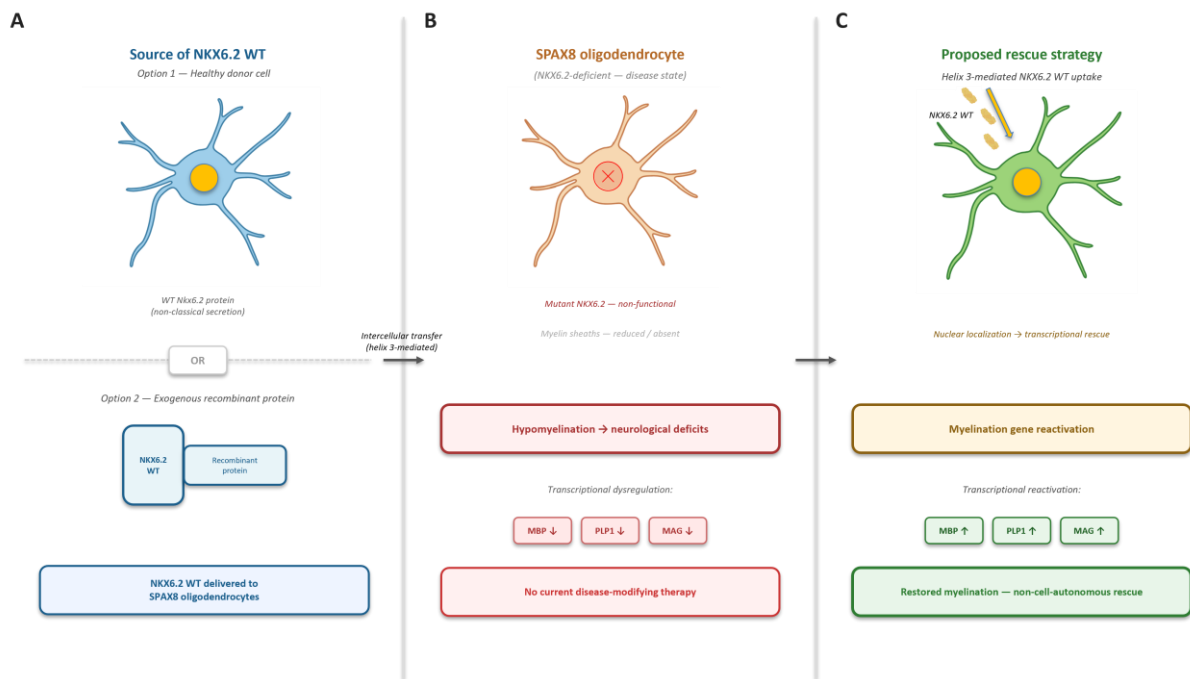


FIGURE 7 WORKING HYPOTHESIS. WILD-TYPE NKX6.2, PRODUCED BY DONOR CELLS, UNDERGOES UNCONVENTIONAL SECRETION AND CROSSES THE PLASMA MEMBRANE OF NKX6.2-DEFICIENT SPAX8 OLIGODENDROCYTES VIA THE HELIX 3 TRANSFER MECHANISM. FOLLOWING UPTAKE AND TRANSLOCATION TOWARD THE NUCLEUS IN THE RECIPIENT CELL, THE TRANSFERRED PROTEIN COULD RESTORE TRANSCRIPTIONAL ACTIVITY AT MYELINATION-RELATED TARGET GENES, PROVIDING A NON-CELL-AUTONOMOUS, GENOME-INDEPENDENT RESCUE OF OLIGODENDROCYTE FUNCTION.

To address this hypothesis experimentally, we evaluated whether wild-type NKX6.2, which carries a conserved helix 3, undergoes intercellular transfer in oligodendrocyte-relevant experimental models. The disease context motivating this study is SPAX8, caused by loss-of-function variants in *NKX6.2*, including missense mutations located within the homeodomain [3,4,5]. The pCAGIG-V5-HP-IRES-EGFP construct [18] was used to co-express a V5-tagged homeoprotein of interest and EGFP from a bicistronic transcript, enabling identification of transfected donor cells by GFP fluorescence and independent detection of protein transfer via V5 immunofluorescence in GFP-negative recipient cells (Figure 6). Human Oligodendrogloma (HOG) cells were selected as the primary CNS-relevant recipient model on the basis of their constitutive

expression of oligodendrocyte-lineage markers (see Section 2.2 and Results); P19 embryonal carcinoma cells were evaluated in parallel as a complementary oligodendrocyte-like model, given their capacity to differentiate toward glial lineages under defined conditions. HEK293 cells, previously shown to support NKX6.2 expression and secretion, served as donor cells in co-culture experiments with HOG recipients. *In ovo* electroporation in chick embryos was used in parallel to assess transfer in a neural tissue context. The central experimental question in all *in vitro* systems was whether NKX6.2 produced in donor cells could be detected in non-transfected recipient cells, providing direct evidence for intercellular transfer. In the *in ovo* model, where Hamburger–Hamilton stage 12 (HH12) chick embryo neuroepithelial cells are multipotent neural progenitors rather than committed oligodendrocyte-lineage cells, the question was whether helix 3-mediated transfer occurs in a physiological neural tissue context, acknowledging this as a limitation regarding oligodendrocyte specificity. Analysis of conditioned medium by western blot was additionally planned to directly assess NKX6.2 secretion from donor cells, but could not be completed within the experimental timeframe of this study.

The present study thus represents a first experimental attempt to establish cellular and *in ovo* models for NKX6.2 intercellular transfer in oligodendrocyte-relevant and neural tissue contexts, and to evaluate whether the conserved helix 3 mechanism supports transfer from donor cells to recipient cells. The findings obtained, together with the technical obstacles encountered, define the experimental foundations for future work aimed at validating homeoprotein transfer as a molecular basis for a non-cell-autonomous rescue strategy in NKX6.2-related hypomyelinating leukodystrophy.

Materials and Methods

1. Cell lines and culture conditions

Three mammalian cell lines were used in this study. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and were subcultured in T-75 flasks (Sarstedt; Fisher Scientific, NC0509296). Cells were routinely passaged every 2-3 days upon reaching approximately 80% confluence. Subculturing was performed by brief incubation with 0.05% Trypsin-EDTA (Gibco, #25300062) at 37°C until detachment, followed by neutralisation with complete medium, centrifugation at 300 × g for 5 min, and reseeding at the appropriate density. All media and supplements were pre-warmed to 37°C before use.

HEK293 cells were maintained in Dulbecco's Modified Eagle Medium supplemented with GlutaMAX (DMEM/GlutaMAX; Gibco, #61965026), 10% heat-inactivated Fetal Bovine Serum (FBS; Gibco, #10270106), and 1% Penicillin/Streptomycin (P/S; Gibco, #15140-122). HEK293 cells were used as the donor cell model in these experiments. These cells are known to be highly transfectable and have been used in previous studies to investigate homeoprotein secretion and intercellular transfer [18]. As they are non-oligodendrocytic, they also allow straightforward discrimination between donor and recipient populations in co-culture.

Human Oligodendrogloma (HOG) cells were maintained in high-glucose DMEM supplemented with GlutaMAX, 10% FBS and 1% P/S. HOG cells derive from a human oligodendrogloma and retain expression of oligodendrocyte lineage markers, including MBP and CNPase; they were used as the recipient cell model for NKX6.2 homeoprotein uptake experiments, providing a human oligodendrocyte-lineage context relevant to the myelination defects of SPAX8.

Murine P19 embryonal carcinoma cells were maintained in DMEM/GlutaMAX supplemented with 10% heat-inactivated FBS (Gibco, #10270106) and 1% P/S (Gibco, #15140-122). P19 cells are a multipotent line derived from a C3H/He mouse embryonic teratocarcinoma that retains the capacity to differentiate into neural and glial cell types under appropriate conditions. They were initially selected as a potential oligodendrocyte precursor model and processed in parallel before HOG cells were established as the primary recipient model (see Section 2.2).

2. P19 oligodendrocyte differentiation

P19 cells were induced to differentiate toward the oligodendrocyte lineage using a two-phase protocol. The first phase relies on retinoic acid (RA) treatment in non-adhesive conditions to drive embryoid body formation and neuroectodermal commitment; the second phase uses a defined serum-free growth factor regimen promoting oligodendrocyte precursor cell (OPC) specification. The growth factors — human basic Fibroblast Growth Factor (hbFGF) and PDGF-AA — were selected specifically to promote glial over neuronal fate. All steps involving RA were performed under subdued light conditions to prevent photodegradation. In the first phase, 1 mL of a cell suspension prepared from a 90% confluent maintenance culture was combined with 8 mL of complete maintenance medium in non-adhesive bacteriological-grade Petri dishes (total volume

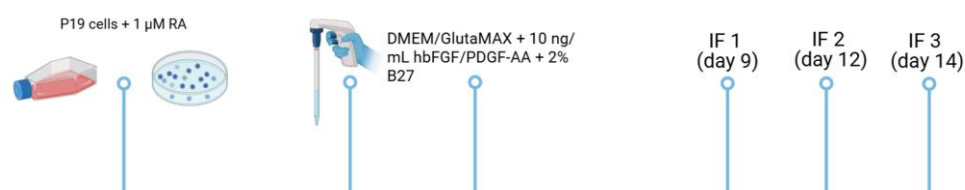


FIGURE 8 *TIMELINE OF THE DIFFERENTIATION PROTOCOL. P19 CELLS WERE TREATED WITH 1 μM RA FOR 4 DAYS, THEN DISSOCIATED AND REPLATED IN DIFFERENTIATION MEDIUM (DMEM/GLUTAMAX + 10 NG/ML HBFGF + 10 NG/ML PDGF-AA + 2% B27) FROM DAY 1. IMMUNOFLUORESCENCE FOR OLIG2 WAS PERFORMED AT DAYS 9, 12, AND 14.*

~9 mL). All-trans retinoic acid (RA; Sigma-Aldrich, #R2625) was added from a 1 mM stock in 100% ethanol (9 μ L per dish) to reach a final concentration of 1 μ M. Under these conditions, cells aggregated spontaneously into embryoid bodies over the following 48 h, a process that promotes commitment to a neuroectodermal fate. After 2 days, embryoid bodies were transferred to a 15 mL Falcon tube, allowed to sediment by gravity, and the conditioned medium was carefully aspirated to within 1-2 mm of the pellet using a Pasteur pipette. Fresh maintenance medium containing 1 μ M RA was added, embryoid bodies were resuspended by gentle pipetting, and returned to the non-adhesive dish for a further 2 days (total first phase: 4 days). At the end of the first phase, embryoid bodies were again collected by gravity sedimentation, washed once with PBS, and dissociated using Accumax cell dissociation solution (ThermoScientific, #00-4666-56) at 900 μ L per dish. After 5-10 min incubation at 37°C, cells were mechanically dissociated by vigorous up-and-down pipetting with a 2 mL pipette, then diluted in 15 mL of DMEM. The single-cell suspension was plated in 12-well plates on 12 mm glass coverslips pre-coated with 0.01 mg/mL poly-D-lysine in standard complete medium (without RA) for 24 h to allow cell adhesion. After the 24 h adhesion phase, medium was replaced with serum-free differentiation medium consisting of high-glucose DMEM/GlutaMAX supplemented with 1% P/S, 2% B27 supplement (Gibco, #A3582801), 10 ng/mL recombinant human basic Fibroblast Growth Factor (hbFGF; Sigma-Aldrich, #F0291), and 10 ng/mL recombinant human Platelet-Derived Growth Factor-AA (PDGF-AA; Miltenyi Biotec, #130-138-938). The absence of serum is important to avoid non-specific differentiation cues. Medium was renewed every 2–3 days. Oligodendrocyte lineage commitment was evaluated by immunofluorescence (Section 2.7) using an anti-OLIG2 antibody at days 9, 12, and 14 post-replating.

3. Plasmid preparation

All homeoprotein transfer experiments were performed using the bicistronic expression vector pCAGIG-V5-HP-IRES-EGFP, obtained without modification from the laboratory of Dr. Jin Woo Kim (Korea Advanced Institute of Science and Technology, KAIST, Daejeon, South Korea), as described in Lee et al. (2019) [18]. This construct drives constitutive co-expression of a V5-tagged homeoprotein and EGFP from a single CAG promoter-driven mRNA, with translation of the two proteins separated by an Internal Ribosome Entry Site (IRES). The bicistronic design is central to the transfer detection strategy: transfected cells co-express both the homeoprotein (V5+) and EGFP (GFP+), whereas cells that have internalized the homeoprotein exclusively via intercellular transfer are V5-positive but GFP-negative, allowing unambiguous single-cell identification of transfer events. For bacterial amplification, chemically competent *E. coli* OneShot TOP10 cells (Thermo Fisher Scientific) were transformed by heat shock: 1-2 μ L of plasmid DNA was added to 50 μ L of competent cells on ice for 30 min, heat-shocked at 42°C for exactly 30 s, then returned to ice for 2 min. After recovery in 250 μ L SOC medium at 37°C (1 h, 225 rpm), transformed bacteria were plated on LB agar containing 100 μ g/mL ampicillin and incubated overnight at 37°C. A single colony was expanded in 200 mL of LB broth with 100 μ g/mL ampicillin overnight at 37°C with vigorous shaking (225 rpm). Plasmid DNA was purified by alkaline lysis maxiprep using a NucleoBond Xtra Maxi Endo-free kit (Macherey-Nagel, #740424.50) according to the manufacturer's protocol. DNA was resuspended in endotoxin-free water and quantified using a NanoDrop One/OneC microvolume UV-Vis spectrophotometer (Thermo Scientific) at 230, 260 and 280 nm; samples with A260/A280 ratios above 1.8 were considered acceptable. Plasmid integrity was not formally assessed; functional quality was confirmed by GFP fluorescence following transfection into HEK293 cells. Stocks were stored at -20°C until use.

4. Cell transfection

Prior to seeding, glass coverslips (12 mm diameter) were coated with 0.01 mg/mL poly-D-lysine in sterile distilled water for at least 30 min at room temperature, then rinsed twice with sterile

water and air-dried under laminar airflow. This coating provides a positively charged surface promoting cell adhesion on glass. HEK293 transfections were performed using jetPRIME polyvalent transfection reagent (Polyplus, #114-07). For 24-well format, HEK293 cells were seeded on PDL-coated 12 mm coverslips 24 h before transfection without exact cell counting, targeting approximately 50–60% confluence at the time of transfection. For each well, 200 ng of plasmid DNA (1 μ L at 200 ng/ μ L) was mixed with 50 μ L of jetPRIME buffer. To this, 1 μ L of jetPRIME reagent was added, the mixture was vortexed for 10 s and incubated for 10 min at room temperature to allow transfection complex formation, then added dropwise to cells in 500 μ L of complete medium. Transfections were carried out with a constant jetPRIME:DNA volume ratio of 1:1 (μ L/ μ L), with buffer volume scaled according to well format (50 μ L for 24-well, 200 μ L for 6-well, giving buffer:jetPRIME:DNA ratios of 50:1:1 and 100:1:1 respectively). The jetPRIME:DNA ratio was occasionally increased empirically to improve transfection efficiency when required. Cells were analyzed 24 h post-transfection, a timepoint at which GFP expression and protein accumulation are robust while cell division-mediated signal dilution remains limited. Transfection of HOG cells was extensively attempted using the same jetPRIME-based protocol. Multiple optimization strategies were applied systematically: the plasmid quantity per well was progressively increased (by raising the DNA volume at constant concentration), the jetPRIME:DNA mass ratio was varied above the manufacturer-recommended range, the

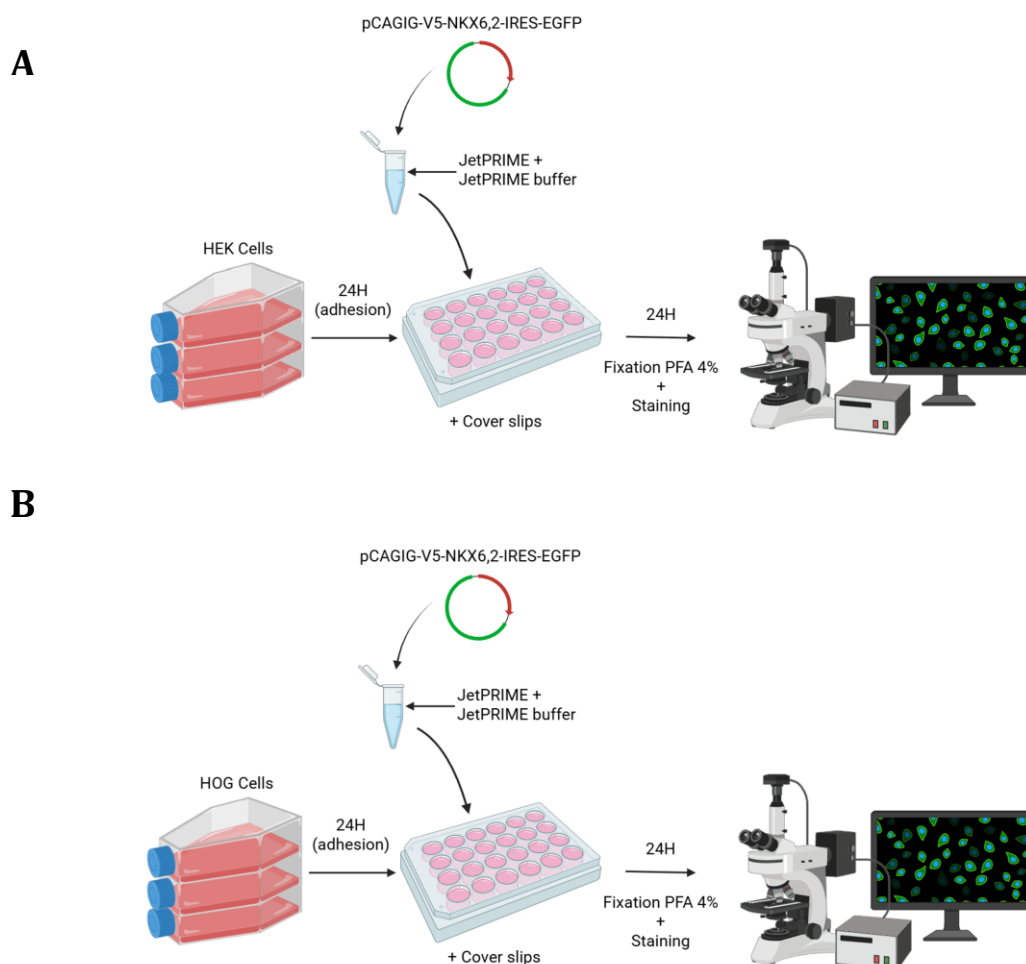


FIGURE 9 SCHEMATIC OF THE TRANSFECTION PROTOCOL FOR HEK293 (A) AND HOG (B) CELLS. BOTH CELL LINES WERE TRANSFECTED WITH pCAGIG-V5-NKX6.2-IRES-EGFP USING JETPRIME. AFTER 24 H, CELLS WERE FIXED IN 4% PFA AND PROCESSED FOR ANTI-V5 IMMUNOFLUORESCENCE.

incubation time before medium replacement was extended, and higher cell seeding densities were tested. Despite these modifications, GFP-positive cells were observed at only very low frequency in HOG cultures, insufficient to constitute a recipient population permissive to NKX6.2 uptake. The limitations of HOG transfection are discussed in Section 4.

5. HEK-HOG co-culture assay

To investigate intercellular transfer of NKX6.2 from transfected donor HEK293 cells to oligodendrocyte-like recipient HOG cells, an indirect co-culture assay based on shared conditioned medium was established. The bicistronic vector design (Section 2.3) is fundamental: because EGFP is produced only in transfected cells, any V5 immunoreactivity detected in GFP-negative HOG cells can be unambiguously attributed to intercellular protein transfer rather than transfection, mRNA transfer, or cell fusion. HEK293 cells were transfected with pCAGIG-V5-NKX6.2-IRES-EGFP in 6-well plates using jetPRIME (200 μ L buffer, 400 ng of plasmid DNA [2 μ L at 200 ng/ μ L], 2 μ L of jetPRIME reagent). The culture medium was replaced at 6 h post-transfection with fresh complete medium to remove residual jetPRIME reagent, which could

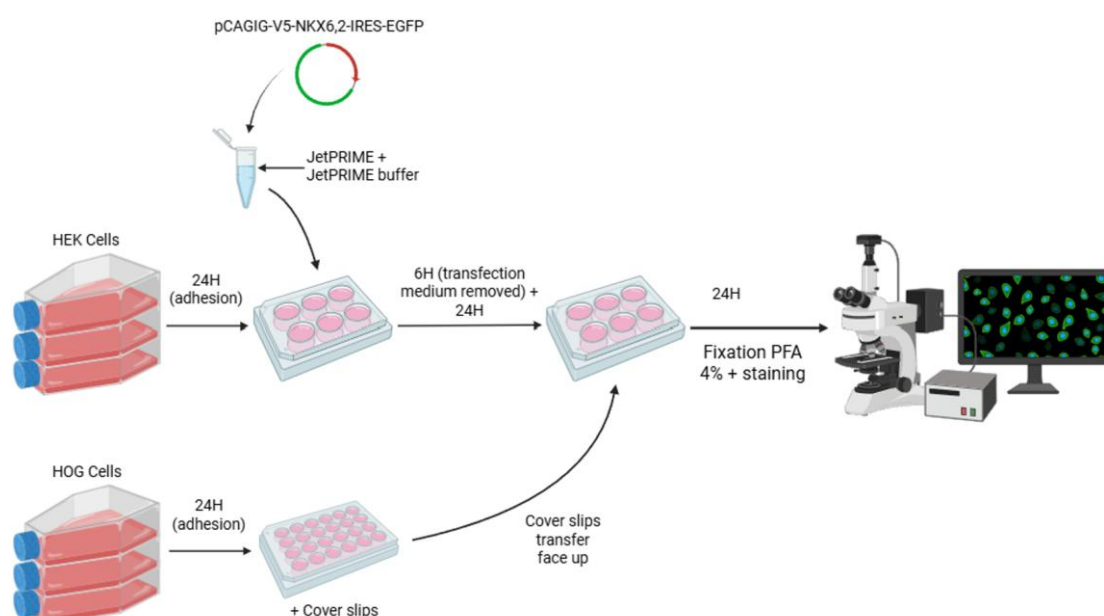


FIGURE 10 SCHEMATIC OF THE CO-CULTURE PROTOCOL. HEK293 CELLS WERE TRANSFECTED FOR 6 H, ALLOWED TO CONDITION THE MEDIUM FOR 24 H, THEN HOG CELLS ON COVERSLIPS WERE PLACED FACE-UP IN THE WELLS FOR A FURTHER 24 H BEFORE FIXATION AND ANTI-V5 IMMUNOSTAINING.

otherwise compromise HOG cell viability in co-culture. Successful transfection was verified by visual inspection of GFP fluorescence at this timepoint. At 24 h post-transfection, when NKX6.2 protein expression was well-established in donor HEK293 cells, 12 mm glass coverslips bearing confluent HOG cells were placed cell-side up directly into the wells containing the transfected HEK293 monolayer. The co-culture was maintained for 24 h under standard conditions (37°C, 5% CO₂), exposing HOG cells to the shared conditioned medium without direct cell-cell contact. After co-culture, HOG coverslips were carefully removed and both the HOG fraction and the HEK293 cells remaining on the plate surface were fixed separately and processed for immunofluorescence (Section 2.7). HEK293 cells served as a positive control for V5/GFP co-expression. HOG coverslips were screened for any V5-immunoreactive cells; the complete absence of both GFP and V5 signal on the HOG coverslips precludes any ambiguity regarding the origin of putative immunoreactive cells.

6. *In ovo* chick embryo electroporation

In ovo chick embryo electroporation was used as a complementary approach to assess NKX6.2 intercellular transfer in a tissue context closer to *in vivo* conditions than cell monolayers. Fertilised White Leghorn chicken eggs (Wijverkens, Halle) were incubated horizontally at 38°C in a humidified incubator from Monday of each experimental week. Electroporation was performed on Wednesday, at Hamburger-Hamilton stage 12 (HH12), corresponding to approximately 48 h of incubation at 38°C. The developmental stage of each embryo was verified prior to injection by counting somite pairs under a stereomicroscope. To access the embryo, approximately 5 mL of albumen was withdrawn from the blunt pole to detach the embryo from the inner shell membrane, and a rectangular window (~1.5 × 1.5 cm) was cut in the eggshell above the embryo. The injection mixture was prepared fresh before use: 1 µL pCAGIG-V5-NKX6.2-IRES-EGFP (8ug/µL) was added to 0.5 µL of 10% Fast Green solution and 8.5 µL sterile water (total 10 µL; plasmid concentration 0.8ug/µL). Fast Green served as an optical tracer to confirm correct injection into the neural tube lumen. The mixture was microinjected into the neural tube lumen using in-house pulled glass capillary needles connected to a microinjector. Electroporation was performed immediately after injection using a square pulse electroporator in Low Voltage (LV) mode: target voltage 24 V (device set to 27 V), 6 square pulses of 50 ms each separated by 400 ms intervals. Platinum plate electrodes were positioned flanking the embryo with correct polarity to drive the negatively charged DNA into the target tissue. Approximately 12 embryos were processed per experimental session. After electroporation, the

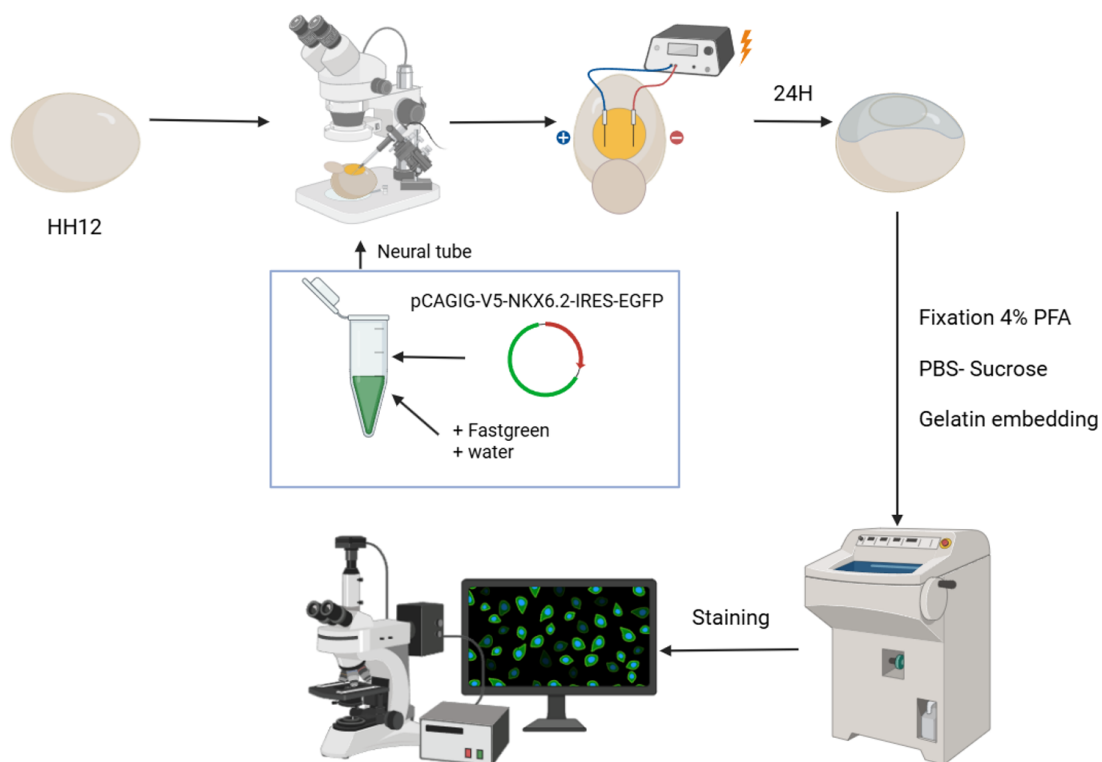


FIGURE 11 SCHEMATIC OF THE EXPERIMENTAL PROCEDURE. FERTILIZED CHICK EGGS AT HH12 STAGE WERE INJECTED WITH pCAGIG-V5-NKX6.2-IRES-EGFP AND FAST GREEN DYE INTO THE NEURAL TUBE, FOLLOWED BY ELECTROPORATION (6 × 50 MS PULSES, 24 V, LV MODE). AFTER 24 H INCUBATION, EMBRYOS WERE FIXED IN 4% PFA, CRYOPROTECTED IN PBS-SUCROSE, GELATIN-EMBEDDED, AND CRYOSECTIONED FOR IMMUNOFLUORESCENCE.

eggshell window was sealed with adhesive tape and eggs were returned to the incubator. Embryos were examined 24 h post-electroporation (Friday) by fluorescence inspection under a fluorescence microscope to assess survival and confirm GFP expression in the electroporated region. Electroporated embryos were harvested by removal from the yolk into cold PBS. Embryos were fixed in 4% PFA at 4°C (20 min), washed three times with PBS, cryoprotected in 30% (w/v) sucrose solution (in PBS) overnight to over-weekend at 4°C, then embedded in gelatin, snap-frozen in an isopentane bath at -55°C, and stored at -80°C. Cryosections of 10 µm were cut on a cryostat and collected on Superfrost Plus glass slides. Sections were stored at -20°C until immunostaining.

7. Immunofluorescence

Two distinct immunofluorescence protocols were applied depending on sample type: a coverslip-based protocol for cultured cells, and a cryosection protocol for chick embryo tissue.

Immunofluorescence on cultured cells

All steps were performed at room temperature unless otherwise specified, with 500 µL volumes per well in 24-well format. Cells were briefly rinsed with PBS and fixed with 4% paraformaldehyde (PFA; diluted from a 20% stock; Electron Microscopy Sciences) for 20 min. After three washes in TBS-T (Tris-Buffered Saline + 0.1% Tween-20; 5 min each), non-specific binding was blocked with TBS-T supplemented with 10% non-fat dry milk for 30-45 min. Primary antibodies were diluted in TBS-T + 1% milk and applied as 40 µL drops on Parafilm in a humidified chamber; coverslips were inverted cell-side down onto the drops and incubated overnight at 4°C. Primary antibodies used: polyclonal chicken anti-GFP (1:2000; Aves Lab, #GFP-1020) and monoclonal mouse anti-V5 (1:1000; Thermo Fisher, #MA5-15253). After three TBS-T washes (5 min each), secondary antibodies conjugated to Alexa Fluor fluorophores (anti-chicken Alexa Fluor 488 (Jackson ImmunoResearch, #703-485-155) and anti-mouse Alexa Fluor 594 (Invitrogen, #A21125)) were applied at 1:500 in 40 µL drops for 45 min to 1 h, protected from light. After three TBS-T washes, coverslips were incubated with DAPI in TBS for 10 min to label nuclei, then washed once in TBS-T for 10 min. Coverslips were mounted on glass slides with DAKO Fluorescence Mounting Medium and cured at room temperature in the dark before imaging. For assessment of oligodendrocyte lineage commitment in P19 cells (Section 2.2), the same coverslip-based protocol was applied using rabbit anti-OLIG2 (1:4000; Millipore, #AB9610) as primary antibody, revealed with a donkey anti-rabbit Alexa Fluor 488 secondary antibody (1:500; Jackson ImmunoResearch, #711-485-152).

Immunofluorescence on cryosections

Cryosections on Superfrost Plus slides were removed from -20°C storage and dried on the bench for a minimum of 30 min (up to 2 h). A hydrophobic barrier was drawn around each section with a DAKO pen. Sections were rehydrated with one PBS wash (5 min), then blocked with 300 µL of saturation buffer per section for 30-60 min at room temperature without coverslip. The saturation buffer consisted of: 5 mL heat-inactivated horse serum (Gibco, #26050-088), 5 mL 10× PBS, 500 µL Triton X-100 (10%), and 39.5 mL distilled water (total 50 mL), filtered through a 0.2 µm membrane. PBS was prepared from tablets (MP Biomedicals, #2810305). Primary antibodies (anti-GFP chicken 1:2000 and anti-V5 rat 1:1000; see above) were diluted in saturation buffer and applied as 300 µL per section. Sections were incubated for 24 h at room temperature followed by 24 h at 4°C in a PBS-humidified chamber. After two washes with PBS + 0.1% Triton X-100 (5 min each, with agitation) and one PBS wash (5 min), secondary antibodies (see above) were applied at 1:1000 in 300 µL saturation buffer for 2 h at room temperature, protected from light. DAPI was added to the secondary antibody solution or applied in a separate PBS wash step. Sections were

washed twice with PBS + 0.1% Triton X-100 (5 min each) and once with PBS (5 min). Slides were mounted with DAKO Fluorescence Mounting Medium and stored at 4°C, protected from light.

8. Image acquisition

Fluorescence images were acquired on a Zeiss Axio Observer 7 fluorescence microscope equipped with appropriate filter cubes for DAPI, Alexa Fluor 488, and Alexa Fluor 594. Images were captured with a ORCA-Flash 4.0 LT (Hamamatsu) camera controlled by ZEN software (Zeiss). For each coverslip or section, 3 to 5 representative fields of view per condition (qualitative analysis; additional fields included for quantitative cell counting) were acquired at random positions at 20× and 40× magnification. All images within a given experiment were acquired under identical exposure times and illumination intensities to allow direct qualitative comparison across conditions. Images were processed for display using Fiji/ImageJ (NIH); no non-linear adjustments were applied. Composite images were assembled with DAPI in blue, anti-GFP signal in green, and anti-V5 signal in red.

9. Image analysis and quantification

Fluorescence images were analyzed using Fiji/ImageJ (National Institutes of Health, USA). For quantification of intercellular NKX6.2 transfer in HEK293 cells, cells were classified manually on a cell-by-cell basis in each acquired field. Transfection efficiency was defined as the proportion of GFP-positive cells among total DAPI-positive nuclei (GFP+/DAPI+), and transfer frequency was defined as the ratio of V5-positive/GFP-negative cells to GFP-positive cells (V5+GFP-/GFP+) within the same field. Quantification was performed across five independent experiments, with data expressed as mean ± standard deviation (SD). No inferential statistical tests were applied, as the study was exploratory and descriptive in nature. For quantification of oligodendrocyte lineage commitment in P19 cells, the total number of DAPI-positive nuclei and OLIG2-positive nuclei per field were determined using a custom Python script (Python 3.x; libraries: NumPy, OpenCV, scikit-image). Briefly, the script performed automated nucleus detection based on fluorescence intensity thresholding in the DAPI channel, followed by identification of OLIG2-positive nuclei by spatial co-localisation with signal in the OLIG2 channel. The percentage of OLIG2+ cells was calculated per field and averaged across fields per time point.

Results

The present work aimed to characterize the capacity of NKX6.2 to undergo intercellular HP transfer, with potential relevance to a protein-based rescue strategy for SPAX8. We pursued a series of experiments in progressively more disease-relevant cellular contexts. We first validated the HP transfer paradigm using HEK293 cells as a donor-recipient system [Section 1]; then evaluated P19 embryonal carcinoma cells as an oligodendrocyte-like recipient model [Section 2]; and subsequently attempted to demonstrate transfer in HOG cells [Section 3] using a conditioned medium co-culture as a bridge [Section 4] and *in ovo* in chick neural tube [Section 5].

1. NKX6.2 undergoes intercellular transfer in HEK293 cells

We first sought to confirm that NKX6.2 can behave as a secretory homeoprotein capable of intercellular transfer under our experimental system. Although Lee *et al.* (2019) detected NKX6.2 in conditioned medium supplemented with competing heparin to displace HSPG-bound protein, direct evidence for intercellular protein transfer — defined by the uptake of extracellular NKX6.2 by non-transfected neighboring cells — had not been established using the bicistronic pCAGIG-V5-NKX6.2-IRES-EGFP construct. Testing this directly in oligodendrocyte-like cells as a first step would have been confounded by the low transfection efficiency expected in such cells. We

therefore began with HEK293 cells as a proof-of-concept and positive control model: they are highly transfectable, well-characterized for HP transfer studies, and were used as the primary system by Lee *et al.* (2019). This approach allowed us to confirm transfer under conditions optimized for sensitivity before moving to the disease-relevant oligodendrocyte system. HEK293 cells were transfected with pCAGIG-V5-NKX6.2-IRES-EGFP, which simultaneously expresses V5-tagged NKX6.2 and an EGFP reporter (Figure 12A), enabling unambiguous identification of transfected donor cells (GFP+/V5+) versus putative transfer recipients (V5+/GFP-).

Immunofluorescence analysis revealed that transfected (GFP+) cells displayed a predominantly nuclear V5 signal, consistent with the expected subcellular localization of NKX6.2 as a transcription factor (Figure 12B). V5+/GFP- cells were identified in all five experiments, consistent with intercellular HP transfer of NKX6.2.

Quantification across five independent experiments ($n = 6,831$ cells total) revealed a mean transfection efficiency of $8.1 \pm 1.2\%$ and a mean transfer frequency (ratio of V5+/GFP- recipient cells to GFP+ donor cells; V5+GFP-/GFP+ ratio) of $1.5 \pm 1.3\%$ (Figure 12C). By comparison, Lee *et al.* reported transfer frequencies of approximately 2–4% for the majority of the 85 secretory homeoproteins detectable in HEK293 cells, with EN2 as an outlier above 10% [18]. NKX6.2 therefore appears to be transfer-competent under these conditions, though the high inter-experiment variability calls for cautious interpretation and replication.

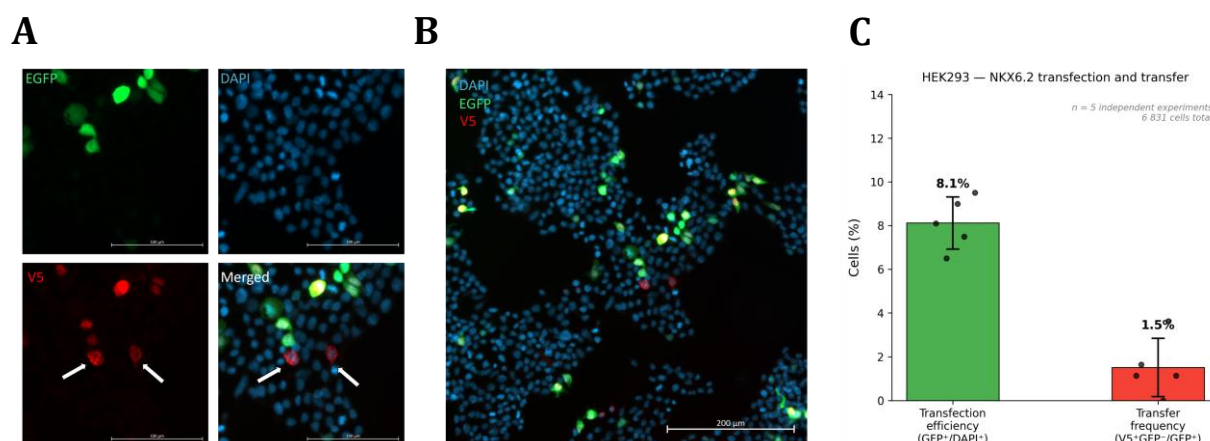


FIGURE 12 TRANSFECTION AND INTERCELLULAR TRANSFER OF NKX6.2 IN HEK293 CELLS. (A) HIGHER-MAGNIFICATION REPRESENTATIVE IMAGES SHOWING INDIVIDUAL CHANNELS (EGFP, DAPI, V5) AND MERGED VIEW. WHITE ARROWS INDICATE V5+/GFP- CELLS EXHIBITING CYTOPLASMIC V5 SIGNAL IN THE ABSENCE OF EGFP, CHARACTERISTIC OF TRANSFER RECIPIENTS. SCALE BAR: 100 μ M. (B) OVERVIEW OF A REPRESENTATIVE FIELD SHOWING DAPI (NUCLEI, BLUE), EGFP (TRANSFECTED DONOR CELLS, GREEN), V5 (NKX6.2, RED), AND MERGED CHANNELS. SCALE BAR: 200 μ M. (C) QUANTIFICATION OF TRANSFECTION EFFICIENCY (GFP+/DAPI+, 8.1%) AND TRANSFER FREQUENCY (V5+GFP-/GFP+, 1.5%). BARS: MEAN $\pm$ SD; POINTS: INDIVIDUAL EXPERIMENTS. $N = 5$ INDEPENDENT EXPERIMENTS, 6,831 CELLS IN TOTAL.

2. Characterization of P19 oligodendrocyte-like differentiation as a model for NKX6.2 transfer

NKX6.2 is a homeodomain transcription factor whose expression in the postnatal central nervous system has been reported primarily in oligodendrocyte-lineage cells [3, 8, 9]. This lineage

association makes the oligodendrocyte compartment the primary relevant recipient cell type for NKX6.2 HP transfer in the context of SPAX8. P19 mouse embryonal carcinoma cells were selected as an oligodendrocyte-like recipient model. Compared to HEK293 cells — which are of renal embryonic origin and lack neural identity — and to HOG cells — a glioma-derived line — P19 cells can acquire OLIG2 immunoreactivity under retinoic acid and growth factor stimulation, representing a potentially more relevant cellular context for HP transfer to oligodendrocyte-lineage cells [32].

P19 cells were treated with 1 μ M RA for four days (Days –4 to 0) to initiate neural commitment. Cells were then dissociated and replated in differentiation medium containing 10 ng/mL hbFGF (to support glial progenitor proliferation [29]), 10 ng/mL PDGF-AA (to activate PDGFR α signalling in oligodendrocyte progenitor cells [29,30]), and B27 supplement (to support oligodendrocyte survival and maturation in serum-free conditions [31]). Differentiation was assessed by OLIG2 immunofluorescence at Days 9, 12, and 14 post-replating.

Immunofluorescence analysis at Days 9, 12, and 14 of differentiation revealed the progressive differentiation of OLIG2-immunoreactive cells: approximately 15% at Day 9, ~25% at Day 12, and ~40% at Day 14 (Figure 13B). It is important to note that OLIG2 is not exclusive to the oligodendrocyte lineage — it is also expressed in motor neuron progenitors of the ventral pMN domain [27] — and OLIG2 positivity alone does not confirm oligodendrocyte-specific commitment. Additional markers (O4, Sox10, MBP) would be required to precisely define the identity of OLIG2+ cells obtained under this protocol.

While the partial OLIG2 induction (~40% positive cells by Day 12) does not yield a homogeneous oligodendrocyte culture, this heterogeneity is not necessarily a drawback for a transfer assay. The OLIG2+ and OLIG2– subpopulations can serve as internal controls for each other within the same well, allowing comparison of HP transfer frequency between oligodendrocyte-like and non-oligodendrocyte-like cells. Further optimization of the differentiation protocol and validation with additional identity markers would be required before using P19 cells in a transfer assay.

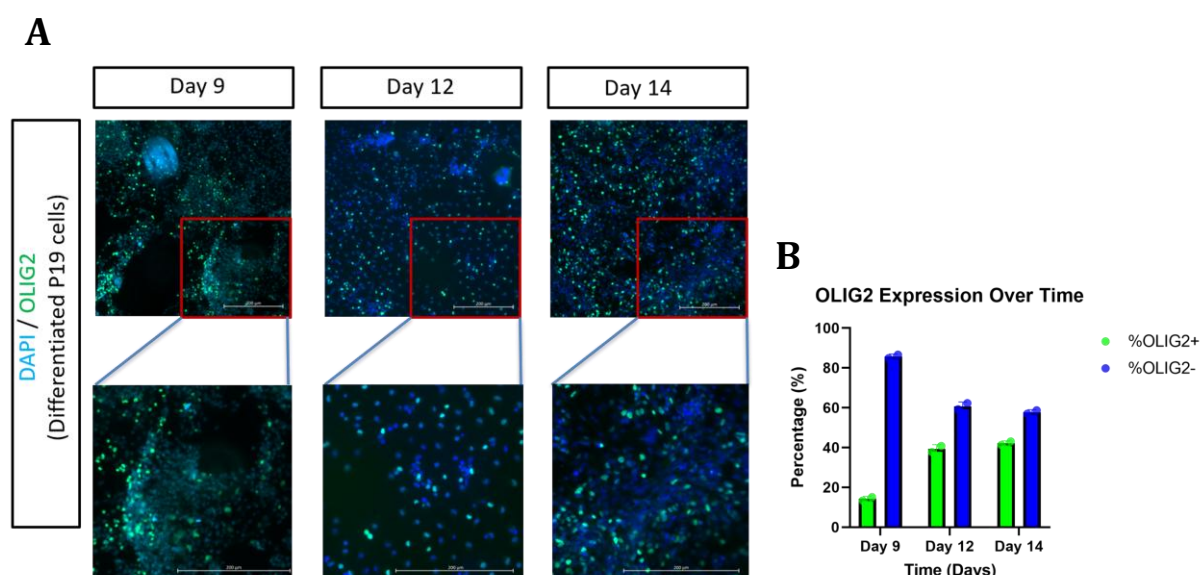


FIGURE 13 OLIGODENDROCYTE-LINEAGE DIFFERENTIATION OF P19 CELLS. (A) REPRESENTATIVE IMMUNOFLUORESCENCE IMAGES AT DAYS 9, 12, AND 14 POST-REPLATING. UPPER PANELS SHOW OVERVIEW FIELDS; LOWER PANELS SHOW HIGHER-MAGNIFICATION VIEWS OF THE BOXED REGIONS. DAPI (CYAN) LABELS NUCLEI; OLIG2 (GREEN) MARKS OLIGODENDROCYTE-LINEAGE CELLS. SCALE BAR: 200 μ M (OVERVIEW); 100 μ M (MAGNIFICATION). (B) QUANTIFICATION OF THE PERCENTAGE OF OLIG2+ AND OLIG2– CELLS AT EACH TIME POINT. OLIG2+ CELLS INCREASE FROM ~15% AT DAY 9 TO ~40–42% AT DAYS 12 AND 14. BARS: MEAN $\pm$ SD; POINTS: INDIVIDUAL EXPERIMENTS.

3. NKX6.2 transfection efficiency in HOG cells is low and no intercellular transfer is detected

Having validated the NKX6.2 transfer paradigm in HEK293 cells (Figure 12), we next asked whether the same phenomenon occurs in an oligodendrocyte-like cellular context — a prerequisite for its potential therapeutic relevance in SPAX8. HOG (Human Oligodendroglioma) cells were selected as the oligodendrocyte-relevant model for several reasons. First, HOG cells are derived from a human oligodendroglioma and constitutively express oligodendrocyte lineage markers, making them an established *in vitro* surrogate for mature oligodendrocytes that does not require a prolonged induction protocol, unlike P19 cells (Figure 8). Second, their human origin is appropriate given that SPAX8 is a human disease. Third, unlike primary oligodendrocyte cultures, HOG cells are commercially available and do not require a prolonged differentiation induction. The same jetPRIME-based transfection protocol used for HEK293 cells was applied to HOG cells with the bicistronic pCAGIG-V5-NKX6.2-IRES-EGFP construct, allowing direct comparison of transfer efficiency between the two cell types under identical experimental conditions.

To improve transfection efficiency, several parameters were systematically optimized across six independent experiments, including the jetPRIME: DNA ratio (standard, x2, x3) and the incubation duration (24 h and 72 h) (Table 1). Despite these optimizations, transfection remained sparse. A total of 432 GFP+ cells were identified across all conditions in selected fields, and no V5+/GFP- cells were detected (Figure 14B). GFP+ cells were also counted in supplementary fields not retained for imaging (n = 221 cells); none displayed a V5+/GFP- phenotype. The absence of V5+/GFP- cells across this donor pool does not allow conclusions about whether HOG cells are biologically resistant to NKX6.2 uptake, given the insufficient number of donor cells to detect transfer at the frequency observed in HEK293 cells (~1.5%).

V5 immunoreactivity was detected in GFP+ transfected HOG cells, confirming expression of the V5-NKX6.2 fusion protein from the pCAGIG-V5-NKX6.2-IRES-EGFP construct (Figure 14A). However, GFP+ transfection efficiency in HOG cells remained very low across all conditions tested (Table 1), indicating that HOG cells are refractory to jetPRIME-mediated lipofection under the conditions used.

Condition	jetPRIME:DNA ratio	Duration	GFP+ cells	V5+/GFP- cells
Exp. 1	Standard	24 h	11	0
Exp. 2	Standard	24 h	32	0
Exp. 3	x2	24 h	40	0
Exp. 4	x3	24 h	52	0
Exp. 5	x2	72 h	122	0
Exp. 6	x2	24 h	175	0
Total	—	—	432	0

Table 1. Summary of HOG transfection conditions and GFP+ cell counts.

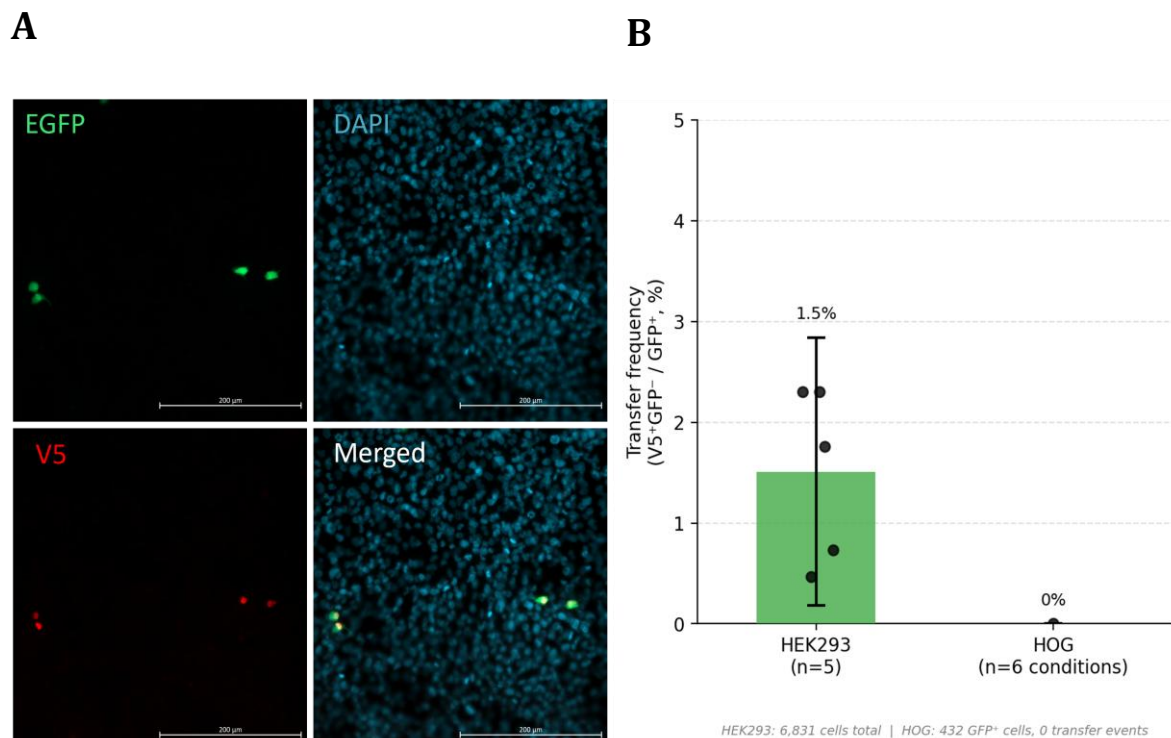


FIGURE 14 ATTEMPTED TRANSFECTION AND INTERCELLULAR TRANSFER OF NKX6.2 IN HOG CELLS. (A) REPRESENTATIVE IMMUNOFLUORESCENCE IMAGES OF A HOG CELL FIELD SHOWING INDIVIDUAL CHANNELS (EGFP, DAPI, V5) AND MERGED VIEW. RARE GFP+ CELLS ARE DETECTED, AND NO V5+/GFP- RECIPIENT CELLS ARE OBSERVED. SCALE BAR: 200 μm. (B) COMPARISON OF TRANSFER FREQUENCY (V5+GFP-/GFP+) BETWEEN HEK293 (1.5% MEAN ± SD, N = 5 EXPERIMENTS, 6,831 CELLS TOTAL) AND HOG CELLS (0%, N = 6 CONDITIONS, 432 GFP+ CELLS, 0 TRANSFER EVENTS).

With 432 GFP+ donor cells identified in imaged fields across six experiments, the donor pool was too small to reliably detect transfer events at the frequency observed in HEK293 cells (~1.5%), which would predict at most ~6 transfer recipients across the entire dataset. It is therefore not possible to conclude whether HOG cells are biologically resistant to NKX6.2 uptake or whether the sample size was simply insufficient to capture such rare events. The low transfection yield also precluded Western blot analysis of transfected HOG cell lysates; this analysis is deferred pending improved transfection efficiency. Increasing the number of donor cells through improved transfection or stable expression is a prerequisite before drawing conclusions on HOG permissiveness.

4. Pilot co-culture experiment reveals no detectable uptake of NKX6.2 by HOG cells from HEK293-conditioned medium

The low transfection efficiency in HOG cells (Figure 14) made the standard bicistronic transfer assay difficult to interpret in this cell type. Rather than excluding HOG cells as a recipient model, we designed a pilot co-culture experiment to test whether HOG cells could internalize NKX6.2 protein secreted by transfected HEK293 donor cells, circumventing the HOG transfection bottleneck.

HEK293 cells were transfected with pCAGIG-V5-NKX6.2-IRES-EGFP for 6 h, after which the transfection medium was replaced with standard culture medium and HOG cells were added in direct co-culture. The transfection efficiency observed in HEK293 donor cells in this co-culture

experiment (~3–5%) was substantially lower than the ~8% mean obtained in the standard transfer assay, and well below the 50–80% typically reported for jetPRIME under standard conditions. Several non-mutually exclusive explanations can be proposed. First, replacement of transfection medium at 6 hours — a step to remove residual jetPRIME before co-culture — may have interrupted complex uptake. Future experiments should ensure optimal transfection conditions and, if variability persists, consider the use of stably transduced HEK293 lines.

Successful transfection of HEK293 cells was confirmed by the presence of GFP+/V5+ donor cells and V5+/GFP– transfer recipient cells on HEK293 coverslips (Figure 15A, left panel). On HOG coverslips, V5+/GFP+ cells were also identified (Figure 15A, right panel). A potential limitation of this design is that HEK293 cells — which grow as a loosely adherent monolayer — could detach and migrate onto HOG coverslips during co-culture, generating V5+/GFP+ signals originating from donor HEK293 cells rather than from HP transfer. V5+/GFP+ signal on HOG coverslips should therefore be interpreted with caution; only V5+/GFP– cells on HOG coverslips would constitute unambiguous evidence of transfer to HOG recipient cells; no such cells were identified in the present experiment.

This result is consistent with observations made for other secretory homeoproteins: Lee et al. showed that HPs in conditioned medium are largely retained at the surface of donor cells via heparan sulfate proteoglycans (HSPGs) [18]. In direct cell-cell contact experiments (such as Figure 12), HSPGs on the donor cell surface can present NKX6.2 directly to adjacent recipient HSPGs without requiring free diffusion. In the conditioned medium setting used here, freely diffusible NKX6.2 would be required; HSPG sequestration at the donor cell surface possibly prevented this. Adding heparin to release HSPG-bound NKX6.2 could in principle help, but would simultaneously compete with recipient cell-surface GAGs for NKX6.2 binding, potentially impairing internalization — a paradox that would need to be resolved empirically.

In retrospect, a more informative positive control would have consisted of non-transfected HEK293 cells on separate coverslips exposed to the same conditioned medium. Detection of V5 signal in these HEK293 recipient cells — which are known to support HP transfer (Figure 12) — would have confirmed that the conditioned medium contained sufficient concentrations of NKX6.2 to enable transfer towards recipient cells. This control is recommended for future experiments.

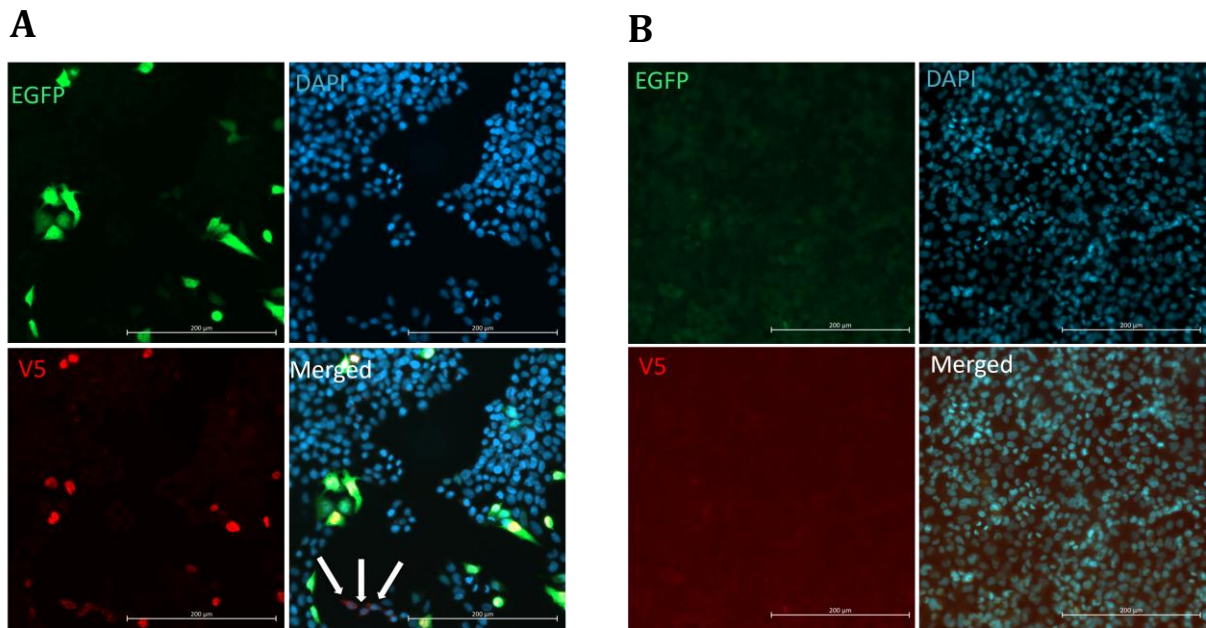


FIGURE 15 PILOT CO-CULTURE EXPERIMENT: EXPOSURE OF HOG CELLS TO NKX6.2-CONDITIONED MEDIUM. (A) POSITIVE CONTROL: REPRESENTATIVE IMMOFLUORESCENCE IMAGES OF HEK293 DONOR COVERSLEIPS SHOWING INDIVIDUAL CHANNELS (EGFP, DAPI, V5) AND MERGED VIEW. GFP+/V5+ TRANSFECTED DONOR CELLS AND V5+/GFP- TRANSFER RECIPIENTS ARE DETECTED. WHITE ARROWS IN THE MERGED PANEL INDICATE RECIPIENT CELLS. SCALE BAR: 200 μ M. (B) HOG COVERSLEIP: REPRESENTATIVE IMAGES SHOWING EGFP, DAPI, V5 (DISPLAYED AT MAXIMUM INTENSITY), AND MERGED CHANNELS. NO EGFP OR V5 SIGNAL IS DETECTED ABOVE BACKGROUND, INDICATING ABSENCE OF NKX6.2 TRANSFER TO HOG RECIPIENT CELLS. SCALE BAR: 200 μ M.

5. *In ovo* electroporation confirms NKX6.2 expression but produces non-interpretable V5 immunostaining in the chick neural tube

The *in vitro* experiments described above yielded no detectable NKX6.2 transfer to HOG cells in any condition tested. The absence of detectable transfer in HOG cells and in the conditioned medium co-culture does not demonstrate that NKX6.2 is incapable of intercellular transfer in oligodendrocyte-lineage contexts; nor does it demonstrate the contrary. These negative results reflect technical constraints — principally the inability to achieve adequate transfection in HOG cells — rather than a resolved biological answer. Consistent with this, NKX6.2 transfer was robustly detected in HEK293 cells under the same construct and protocol, confirming the assay can detect transfer when donor cell numbers are sufficient.

We therefore used *in ovo* electroporation of the developing chick neural tube as a complementary *in vivo* model to test whether NKX6.2 HP transfer is detectable between neural cells in a living tissue. The chick embryo was chosen for several reasons. Electroporation directly introduces plasmid DNA into a subset of neural progenitors by an electric field, entirely bypassing the jetPRIME lipofection efficiency problem encountered in HOG cells. Furthermore, electroporation results in mosaic expression of the genes of interest, well suited to study intercellular transfer. Finally, the chick embryo is experimentally accessible, cost-effective, and compatible with the CAG promoter driving expression in pCAGIG-V5-NKX6.2-IRES-EGFP. Fertilised chick eggs at HH12 (~48 hours of incubation) were windowed under conditions as aseptic as the open-egg configuration permits and the neural tube was injected with a solution containing pCAGIG-V5-NKX6.2-IRES-EGFP supplemented with Fast Green dye as a tracer. Electroporation was performed using six square-wave pulses of 50 ms duration at 24 V in low-voltage mode (400 ms interpulse

interval). Eggs were resealed and incubated for a further 24 hours before embryo collection. Embryos were then fixed in 4% PFA, cryoprotected in PBS-sucrose, embedded in gelatin, and cryosectioned for immunofluorescence analysis with anti-V5 antibody.

EGFP fluorescence was readily detected in cells of the electroporated side of the neural tube, confirming successful plasmid delivery and bicistronic transgene expression (Figure 16A, B). V5 immunostaining revealed signal in the electroporated region partially co-localising with GFP, consistent with NKX6.2-V5 protein expression in transfected cells. However, V5 signal was also detected in areas devoid of GFP expression and, critically, did not co-localise with DAPI-positive nuclei in these regions. This pattern was observed consistently across all analysed sections and is indicative of non-specific background antibody binding rather than genuine NKX6.2 protein detection (Figure 16A, B). Crucially, however, this non-specific signal was consistently extracellular — associated with acellular tissue components rather than with DAPI-positive nuclei — and is therefore qualitatively distinguishable from a genuine intracellular transfer event. Authentic V5+/GFP- transfer recipients would be expected to show discrete, intracellular V5 immunoreactivity co-localising with DAPI-positive nuclei, a pattern qualitatively distinct from the diffuse extracellular background observed.

The unelectroporated contralateral wall of the neural tube serves as a background control for non-specific immunostaining, not as a readout for intercellular HP transfer. The relevant zone for transfer detection is the ipsilateral (electroporated) zone: a V5+/GFP- cell within this zone would indicate transfer from an electroporated GFP+/V5+ cell to a non-transfected neighbour. Systematic examination of the ipsilateral zone revealed no discrete intracellular V5 signal in GFP-negative cells. A diffuse V5 signal was observed at the basal surface of the neural tube epithelium, consistent with non-specific binding to the basal lamina rather than intracellular transfer. No unambiguous V5+/GFP- transfer recipient cells were identified. These results indicate that, under the conditions tested, no intercellular transfer of NKX6.2 was detectable in the chick neural tube.

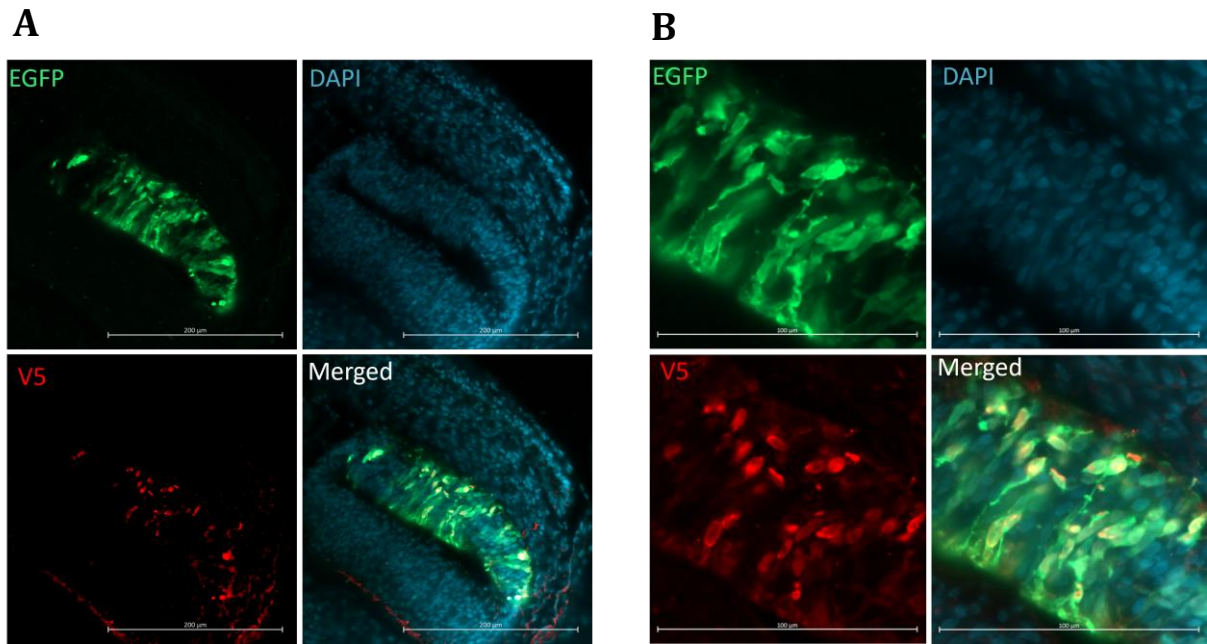


FIGURE 16 *IN OVO* ELECTROPORATION OF *pCAGIG-V5-NKX6.2-IRES-EGFP* IN THE CHICK NEURAL TUBE. (A) REPRESENTATIVE IMMUNOFLUORESCENCE CRYOSECTION SHOWING THE ELECTROPORATED REGION OF THE NEURAL TUBE. CHANNELS SHOWN: EGFP (GREEN), DAPI (CYAN), V5 (RED), AND MERGED. GFP+ CELLS ARE CONFINED TO THE ELECTROPORATED SIDE. NON-SPECIFIC BACKGROUND STAINING IS OBSERVED IN THE V5 CHANNEL IN REGIONS LACKING DAPI-POSITIVE NUCLEI. SCALE BAR: 200 μ M. (B) HIGHER-MAGNIFICATION VIEW OF THE ELECTROPORATED ZONE. GFP+ CELLS (GREEN) CO-EXPRESS V5 (RED), CONFIRMED BY PARTIAL CO-LOCALIZATION IN THE MERGED PANEL, CONSISTENT WITH EXPRESSION OF THE BICISTRONIC *pCAGIG-V5-NKX6.2-IRES-EGFP* CONSTRUCT. SCALE BAR: 100 μ M.

Discussion

The experimental results of this study are discussed in the order presented: beginning with the demonstration of NKX6.2 HP transfer in HEK293 cells [Section 1], followed by the evaluation of P19 cells as an oligodendrocyte-like recipient model [Section 2], then addressing the technical constraints encountered with HOG cells [Section 3], conditioned medium co-culture [Section 4], and *in ovo* electroporation [Section 5]. Final sections consider broader implications for the SPAX8 rescue hypothesis and recommend future experimental directions.

1. NKX6.2 is a secretory homeoprotein capable of intercellular transfer in HEK293 cells

The primary aim of this study was to determine whether NKX6.2 behaves as a secretory homeoprotein and, if so, whether its intercellular transfer could be detected in oligodendrocyte-relevant experimental models. The most unambiguous result obtained was the detection of NKX6.2 transfer in HEK293 cells (Figure 12), establishing that wild-type NKX6.2 can act as a secretory homeoprotein within the *pCAGIG-V5-NKX6.2-IRES-EGFP* system. The transfer frequency of $1.5 \pm 1.3\%$ across five independent experiments is consistent with values reported for confirmed secretory homeoproteins in similar co-expression paradigms, and the stochastic variability (SD nearly equal to the mean) is expected for a process dependent on the number of secreting cells, the local extracellular concentration of protein, and the rate of HSPG-mediated uptake by neighboring cells [18]. This result confirms the validity of the experimental system and

demonstrates that NKX6.2 thus joins the class of secretory homeoproteins (sHPs) identifiable by this assay, in agreement with its detection in heparin-supplemented conditioned medium reported by Lee et al. [18].

A notable feature of the transfer data is the predominantly cytoplasmic localization of V5 signal in GFP-negative recipient cells. This cytoplasmic distribution has been reported for other transferred homeoproteins and may reflect retention in the cytoplasm prior to — or instead of — nuclear import. Whether the transferred NKX6.2 protein retains the capacity to translocate to the nucleus and exert transcriptional repressor activity in recipient cells was not addressed in this study and remains an important open question for assessing the biological relevance of the transfer.

The transfer frequency observed in this study (mean $1.5 \pm 1.3\%$, range approximately 0.5–3.0%) is comparable to, though slightly below, the median transfer frequency reported by Lee et al. for the 85 sHPs in their dataset that exhibited detectable transfer in the HEK293 system (~2–4% for most sHPs, with outliers above 10% for EN2 and below 1% for several C2H2 zinc finger-homeodomain proteins) [18]. NKX6.2 therefore falls within the lower-to-middle range of the transfer efficiency distribution, which is consistent with its classification as a transfer-competent but not exceptionally efficient sHP. Several factors may account for the position of NKX6.2 in this distribution. First, the experiment was performed with a bicistronic construct (pCAGIG-V5-NKX6.2-IRES-EGFP) in which NKX6.2 and EGFP are co-translated from a single mRNA transcript; IRES-dependent translation of the downstream EGFP is generally less efficient than cap-dependent translation of the upstream ORF, and the IRES element may also affect the overall translational load on the cell, potentially reducing the total amount of V5-NKX6.2 available for secretion. However, since Lee et al. employed the same bicistronic construct, this factor is shared across both datasets and cannot explain the relative difference in transfer frequency. Second, the high cytoplasmic retention of V5-NKX6.2 in non-transfected recipient cells — rather than the predominantly nuclear localisation expected for a transcription factor — may indicate that the V5 tag or the protein context interferes with nuclear import of the transferred protein in the HEK293 cellular environment. Alternatively, this cytoplasmic distribution may reflect the timing of fixation: if cells were fixed shortly after transfer, the protein may not yet have had sufficient time to translocate to the nucleus. Third, the cellular conditions required for optimal HEK293 transfection — in particular cell confluency — were not always consistent across experiments. Non-homogeneous coverslips, including areas where cells had partially detached, would reduce both the effective number of donor cells and the density of recipient cells available for quantification, contributing to the stochastic inter-experimental variability observed ($SD \approx \text{mean}$). Fourth, overexpression of NKX6.2 in a non-native cellular context could conceivably affect the viability of a fraction of transfected cells, selectively reducing the GFP+ donor pool and thereby leading to an underestimation of the true transfer frequency. These considerations do not diminish the significance of the positive transfer result but collectively suggest that the efficiency observed here may represent a lower bound on the true transfer frequency of untagged endogenous NKX6.2.

2. Evaluation of P19 cells as an oligodendrocyte-like model: rationale, results, and perspectives

P19 mouse embryonal carcinoma cells originate from a C3H/He mouse embryonic teratocarcinoma. They retain pluripotency under standard culture conditions but can be directed toward neural and glial identities by retinoic acid. The differentiation protocol used here employed a single phase of RA exposure (1 μM , Days –4 to 0) followed by replating in growth

factor-supplemented medium; the OLIG2-immunoreactive cells obtained — approximately 40% of the differentiated population by Day 12 — were used as a surrogate readout of oligodendrocyte-lineage induction. A critical caveat, however, is that OLIG2 expression is not restricted to the oligodendrocyte lineage. OLIG2 is expressed not only in oligodendrocyte-lineage cells but also in motor neuron progenitors of the ventral pMN domain at early developmental stages [27]. The OLIG2+ cells obtained in RA-treated P19 cultures may therefore not represent only oligodendrocyte-lineage cells, and their identity cannot be established without additional markers. Characterization with later-stage oligodendrocyte markers — including Sox10, O4, and MBP — would be a prerequisite before any OLIG2-based readout in P19 cells could be interpreted as evidence of oligodendrocyte differentiation, and before P19 cells could be used as a recipient model in a transfer assay relevant to SPAX8.

The P19 differentiation system was not ultimately used in a transfer assay in the present study, as further protocol optimization and identity characterization are required. Its use in future transfer experiments — particularly as a recipient in a HEK293-donor/P19-recipient co-culture design — remains a recommended next step (Section 8).

3. HOG cells are refractory to transfection: a technical bottleneck, not a biological barrier

The inability to detect NKX6.2 transfer in HOG cells (Figure 14) must be interpreted in light of a critical confound: the very low number of transfected donor cells in HOG cultures. With only 432 GFP+ cells identified across six systematically optimized experiments, the extracellular pool of secreted NKX6.2 available to drive detectable transfer was almost certainly negligible. Extrapolating from the mean transfer frequency of 1.5% observed in HEK293 cells, a pool of 432 total donor cells divided across six experiments would be expected to yield approximately 6–7 transfer events in total — a number at or below the detection threshold of manual immunofluorescence counting, particularly against any background staining. The present data therefore do not permit a conclusion on whether HOG cells are biologically resistant to NKX6.2 uptake, or simply lacked sufficient secreted NKX6.2 in the extracellular milieu to drive detectable transfer. These two possibilities carry very different implications for the therapeutic hypothesis, making their distinction a priority for future work.

The resistance of HOG cells to jetPRIME-mediated lipofection is consistent with the known properties of oligodendrocyte-lineage cells, which are notoriously refractory to cationic lipid-based transfection [33]. This resistance has been attributed to the high lipid content of oligodendrocyte membranes [34], which may interfere with lipoplex formation or endosomal escape. HOG cells also express cell-surface HSPGs [35], which could further impair lipoplex uptake. Viral transduction — particularly AAV serotypes or lentiviral vectors optimised for oligodendrocyte-lineage cells [36] — represents the most promising alternative delivery strategy.

4. Absence of NKX6.2 uptake from conditioned medium: HSPG sequestration as the most parsimonious explanation

The conditioned medium co-culture experiment (Figure 15) was designed to decouple the transfection bottleneck from the transfer assessment by using HEK293 cells as a dedicated NKX6.2-secreting source. While this design successfully bypassed the HOG transfection problem, it introduced its own limitations. The complete absence of V5 signal on HOG coverslips is consistent with a major role for HSPG-mediated sequestration of secreted NKX6.2 on the donor

cell surface, as described by Lee et al. [18]. In the absence of competing free heparin, secreted NKX6.2 is expected to accumulate on donor cell HSPGs and on the substrate, reducing the pool of freely diffusing protein available to reach remote HOG cells. Importantly, this sequestration is not absolute: the successful HEK293-to-HEK293 transfer observed in Figure 12 demonstrates that NKX6.2 can nonetheless be captured by neighboring recipient cells in a direct co-culture setting, where proximity allows cell-surface-presented NKX6.2 to engage directly with adjacent cell HSPGs without requiring sustained free diffusion. The conditioned medium design, by contrast, specifically requires NKX6.2 to diffuse freely across the culture volume to reach a remote coverslip — precisely the pathway that HSPG sequestration impairs. The failure of the indirect design therefore does not exclude the possibility that HOG cells could capture NKX6.2 in a direct co-culture configuration.

This interpretation is further supported by the geometry of the experimental design. In the indirect co-culture configuration used here, HOG cells on coverslips were not in physical contact with HEK293 donor cells, relying exclusively on freely diffusing, non-HSPG-bound NKX6.2 reaching the coverslip surface over a 24-hour period. Even if some NKX6.2 desorbed from HSPG chains, the effective concentration available to HOG cells at a distance would be substantially diluted across the culture volume. This contrasts sharply with the HEK293-to-HEK293 transfer paradigm (Figure 12), where donors and recipients are in direct proximity and intermingled, allowing cell surface-presented NKX6.2 to engage directly with neighbouring cell HSPGs — a key step in the transfer mechanism [18]. The combined effect of distance, HSPG sequestration, and the single replicate ($n = 1$) means that this pilot result, while informative, cannot be used to draw conclusions about the intrinsic permissiveness of HOG cells to NKX6.2 uptake. A properly controlled experiment should include: a direct co-culture condition in which HEK293 and HOG cells are mixed on the same coverslip; and adequate replication ($n \geq 3$). The positive internal control obtained in the donor wells (GFP+/V5+ donors and V5+/GFP- transfer events in HEK293; Figure 15A) demonstrates that the experimental approach is in principle functional and that NKX6.2 was actively being transferred in the donor compartment at the time of the experiment.

An additional, conceptually distinct limitation concerns the intrinsic properties of HOG cells as potential HP transfer recipients. The capacity of a cell to capture secreted homeoproteins depends critically on the composition and sulfation patterns of cell-surface heparan sulfate proteoglycans [18,35]. HOG cells derive from a human oligodendroglioma and, like many tumor-derived lines, may display a substantially altered surface proteoglycan profile relative to primary oligodendrocytes. Two distinct recipient-side mechanisms could account for the absence of V5 signal on HOG coverslips, independently of the distance constraint. First, HOG cells may lack the specific GAG sulfation patterns required for homeoprotein capture and internalization, resulting in insufficient surface binding and failure of uptake. Second, HOG cells may express GAGs with high affinity for NKX6.2 that sequester the protein at the cell surface without triggering internalization, such that all available NKX6.2 remains bound externally and no intracellular V5 signal accumulates. These two mechanisms — receptor absence versus surface sequestration without internalization — produce identical negative readouts by immunofluorescence and are experimentally distinguishable only by surface-versus-intracellular V5 staining under permeabilized and non-permeabilized conditions. Establishing which mechanism operates in HOG cells is a relevant question for future work.

Adding free heparin to the conditioned medium — while potentially releasing HSPG-bound NKX6.2 from donor cell surfaces — might also compete with recipient cell-surface GAGs for NKX6.2 binding, thereby impairing internalization. The practical balance between heparin-mediated release from donor HSPGs and competition with recipient GAGs would need to be determined empirically. An alternative approach that avoids this paradox is to apply purified

recombinant NKX6.2 protein directly to recipient cells in standard medium, bypassing the conditioning step entirely.

5. *In ovo* electroporation: successful gene delivery with non-interpretable V5 immunostaining

The *in ovo* electroporation experiment (Figure 16) achieved its primary technical goal — delivery of the pCAGIG-V5-NKX6.2-IRES-EGFP construct to cells of the HH12 chick neural tube — as evidenced by robust EGFP fluorescence confined to the electroporated side. The interpretation of the V5 immunofluorescence signal, however, was complicated by a high background level that was predominantly extracellular — associated with ECM components and proteoglycans in the neural tube lumen and surrounding tissue. This pattern is consistent with non-specific secondary antibody binding to extracellular matrix components, a recognized challenge in embryonic tissue sections when anti-mouse secondary antibodies are applied in the presence of endogenous immunoglobulins. Crucially, at certain locations in the sections it was difficult to reliably distinguish background from rare discrete signals that could, in principle, reflect genuine transfer events — including possible uptake by differentiating neurons in the electroporated territory. The current antibody conditions therefore do not permit confident identification or exclusion of genuine transfer events, and no conclusion about the presence or absence of NKX6.2 transfer in the neural tube can be drawn from this experiment alone. An optimised protocol — including use of Fab fragment secondaries or substitution of the V5 tag with HA or FLAG — would be required before the *in ovo* model can serve as a reliable transfer readout.

A key limitation of the current *in ovo* design is the choice of developmental stage. HH12 does not represent a stage at which oligodendrocyte-lineage cells are present in the chick neural tube: OPC specification in the chick spinal cord begins around HH26–28 (~E5) [28]. At HH10–12, OLIG2-expressing cells in the ventral domain belong primarily to the pMN progenitor domain, giving rise to motor neurons at this stage [27]. The HH12 model therefore does not offer direct access to the oligodendrocyte-lineage cellular context relevant to SPAX8. Furthermore, since electroporation is technically feasible only up to approximately HH15–16, the chick *in ovo* model cannot be used to target stages at which OPCs are present, representing a fundamental limitation of this approach for studying NKX6.2 transfer in an oligodendrocyte-lineage context.

Independently of these technical obstacles, the *in ovo* experiment contributed positively to the study by demonstrating that the pCAGIG-V5-NKX6.2-IRES-EGFP vector is competent for electroporation-mediated *in vivo* gene delivery in the neural tube. The electroporation itself was technically successful, as evidenced by EGFP fluorescence restricted to the electroporated side, the expected neural tube morphology, and the absence of gross developmental anomalies attributable to the electroporation procedure. These observations validate the *in ovo* approach as a viable future platform for investigating NKX6.2 transfer in an *in vivo* context, provided that the antibody and readout issues identified above are addressed. The chick neural tube model is particularly attractive for this purpose because it permits genetic mosaic experiments in which a defined fraction of cells express a donor construct while neighbouring cells remain non-transfected, replicating the natural minority-donor geometry in which homeoprotein transfer has been most convincingly demonstrated in the CNS [19,21]. The use of an orthogonal non-immunological readout — such as a nuclear fluorescent reporter gene driven by an NKX6.2 target promoter — would allow future *in ovo* experiments to bypass the antibody background problem entirely.

6. Transfer of NKX6.2 is confirmed in one system but may be cell-context-dependent

Taken together, the results of this study are consistent with NKX6.2 acting as a secretory homeoprotein capable of intercellular transfer in HEK293 cells, while revealing the technical and biological challenges that must be overcome before this capacity can be demonstrated in oligodendrocyte-lineage contexts. The HEK293 result provides proof-of-concept for the HP transfer assay but does not constitute evidence that NKX6.2 transfer occurs in oligodendrocytes or in the CNS context relevant to SPAX8.

An important caveat on the HEK293 transfer result is that this cell type, while widely used in HP transfer studies and validated by Lee *et al.* as a transfer-competent system [18], is a transformed human embryonic kidney cell line with properties that differ substantially from oligodendrocytes. HEK293 cells lack the lipid-rich membrane specializations characteristic of myelinating oligodendrocytes, and their surface HSPG composition and abundance may differ from oligodendrocytes in ways that influence the dynamics of NKX6.2 secretion and capture. The HEK293 result therefore provides proof-of-concept that NKX6.2 is transfer-competent in at least one cell system, but it does not constitute evidence that transfer will occur in an oligodendrocyte context, nor that it occurs at the frequency required for a therapeutically meaningful effect.

The molecular basis for cell-context-dependent secretion of homeoproteins has been partially elucidated by Lee *et al.*, who demonstrated that hydrophobic amino acid residues located outside the homeodomain — in positions that are conserved among secretory homeoproteins (sHPs) but divergent in non-secretory homeoproteins (nsHPs) — are required for secretion [18]. Substitution of these hydrophobic positions (typically phenylalanine, leucine, or methionine) with the acidic residue glutamate reproducibly abolished secretion of EN2, OTX2, PAX6, and VAX1 without affecting their nuclear localization or transcription factor activity, while the reciprocal substitution in nsHPs conferred secretory capacity [18]. Whether NKX6.2 possesses such conserved hydrophobic residues outside its homeodomain, and whether these are present in a configuration compatible with secretion machinery recognition, remains to be determined by systematic mutagenesis analogous to that described in the same study.

The demonstration that NKX6.2 transfer is detectable in HEK293 cells but not in HOG cells raises an interpretative question that is distinct from the technical limitations discussed above: even in the absence of the transfection bottleneck, would HEK293 and oligodendrocyte-lineage cells behave identically as transfer recipients? The answer is likely no, and understanding the mechanistic basis for this difference is of direct relevance to the therapeutic scenario. HEK293 cells possess a generalist plasma membrane composition characteristic of epithelial-derived cell lines, with a relatively fluid lipid bilayer and no significant myelin-specific lipid enrichment. Oligodendrocyte membranes, by contrast, are among the most specialized in the mammalian brain, with a markedly elevated galactocerebroside and sulfatide content that confers the extraordinary insulating properties of compact myelin [34]. The net charge and packing density of the oligodendrocyte outer leaflet may differ substantially from that of HEK293 cells, with direct consequences for the electrostatic interaction between the cationic helix 3 of NKX6.2 and the membrane surface — the initiating step of the transfer mechanism [15,16]. Whether the oligodendrocyte membrane is more or less permissive than the HEK293 membrane to helix 3-mediated translocation is an empirically open question that cannot be answered by extrapolation from the HEK293 result alone, and that should be addressed directly by comparing transfer efficiencies between the two cell types under identical conditions once the HOG transfection bottleneck is resolved.

7. Implications for the SPAX8 rescue hypothesis and limitations of the current experimental evidence

The central hypothesis motivating this study requires that: (i) wild-type NKX6.2 is capable of intercellular HP transfer; (ii) the transferred protein reaches oligodendrocyte-lineage cells in sufficient quantity; (iii) the transferred protein retains or regains nuclear localization in recipient cells; (iv) nuclear NKX6.2 exerts transcriptional repressor activity in the recipient context; and (v) this activity is sufficient to partially rescue the differentiation or survival deficit caused by endogenous loss-of-function. The present study provides initial support for condition (i) in HEK293 cells. Conditions (ii)–(v) remain untested. Importantly, wild-type NKX6.2 could also reach NKX6.2-deficient cells if delivered exogenously to the CNS, or by other means bypassing HP transfer.

The significance of this positive result must be contextualized appropriately. Prior to this study, no experimental evidence confirmed that NKX6.2 functions as a secretory homeoprotein. The structural prediction — based on the conservation of the helix 3 transfer domain — provided a rational prior, but the secretory behavior of full-length homeoproteins cannot be inferred from structural homology alone. Transcription factors expressed *in vivo* are subject to competing nuclear retention signals, protein-protein interactions with co-repressors, and post-translational modifications that can sequester even structurally transfer-competent proteins within the nucleus. The HEK293 transfer data demonstrate that none of these potential barriers completely suppresses the secretory competence of NKX6.2 when expressed from a strong constitutive promoter, and that the protein, once secreted, retains sufficient structural integrity to penetrate the plasma membrane of adjacent recipient cells. This places NKX6.2 among the experimentally validated secretory homeoproteins — alongside EN1 [25], EN2 [26], OTX2 [20,21], and the 85 sHPs characterized by Lee et al. [18] — and grounds the therapeutic hypothesis in direct experimental evidence for the first time. The confirmation of this first mechanistic step does not guarantee that the complete rescue chain will operate efficiently in oligodendrocytes, but it establishes that the entry point to this chain is biochemically accessible in the NKX6.2 protein.

From a disease perspective, the therapeutic scenario envisaged for SPAX8 involves transfer from NKX6.2-expressing wild-type oligodendrocytes (or exogenously supplied recombinant protein) to NKX6.2-deficient mutant cells in the same compact white matter environment. The minimum effective dose of transferred NKX6.2 required to rescue myelination-related transcriptional programs, and the fraction of oligodendrocytes that would need to be rescued, remain entirely unknown and cannot be inferred from existing data.

8. Perspectives and recommended future experiments

The experimental foundation established by this study identifies two immediate priorities. The first is to establish a western blot protocol for NKX6.2 in conditioned medium — the single most critical missing piece, as it would directly confirm whether NKX6.2 is secreted from donor cells at detectable levels, and whether the absence of transfer signal in recipient cells reflects an absence of protein in the medium or a failure of uptake. The second priority is to resolve the transfection bottleneck in HOG cells by switching to an electroporation-based approach (e.g., Amaxa Nucleofector with HOG-optimized settings) or lentiviral transduction of the V5-NKX6.2-IRES-GFP cassette, which would enable the transfer assay to be executed in an oligodendrocyte-relevant context with a sufficient donor population. Once these two conditions are satisfied, the conditioned medium experiment should be repeated with free heparin added to the collection medium, to displace HSPG-bound NKX6.2 and test whether HOG cells are capable of NKX6.2 uptake when the protein is made freely available. A direct co-culture design in which HEK293

donors and HOG recipients are seeded on the same coverslip would additionally eliminate the spatial separation that likely contributed to the absence of signal in Figure 15.

P19 mouse embryonal carcinoma cells represent a possible oligodendrocyte-like recipient model for future transfer experiments. Under the RA and growth factor protocol described in Section 2, approximately 40% of cells acquired OLIG2 immunoreactivity by Day 12; however, OLIG2 expression is not exclusive to oligodendrocyte-lineage cells [27]. Whether these cells recapitulate oligodendrocyte-relevant HP uptake biology — including HSPG expression profile and surface GAG composition required for efficient HP internalization — remains unknown. Use of P19 cells in a transfer assay would therefore need to be accompanied by careful characterization of recipient cell identity (Sox10, O4, MBP) and surface proteoglycan biology before conclusions relevant to SPAX8 can be drawn.

Optimization of the *in ovo* model represents a secondary priority once the HOG and western blot limitations are resolved. The antibody protocol should be adapted — either by switching to an alternative epitope tag (HA or FLAG) or by combining species-matched serum blocking with Fab fragment secondary antibodies to reduce tissue background. Since electroporation is not technically feasible beyond HH15–16, reaching stages at which OLIG2-positive progenitors are present in the ventral neural tube would require viral transduction — such as *in ovo* AAV injection — as an alternative delivery method to make the model relevant to oligodendrocyte-lineage transfer. A positive detection in this optimized model would provide strong evidence for NKX6.2 transfer in an *in vivo* context; a negative result, conversely, would consolidate the interpretation that NKX6.2 transfer is restricted to permissive cell systems and does not occur in organized neural tissue, directly informing the feasibility of paracrine rescue in the CNS.

Beyond these immediate priorities, demonstration of functional transfer — defined as the capacity of transferred NKX6.2 to exert transcriptional repressor activity in recipient cells — would substantially strengthen the biological relevance of the transfer observation. Since NKX6.2 acts as a transcriptional repressor [11], a reporter strategy based on its activity must be designed accordingly: a synthetic luciferase cassette containing NKX6.2-responsive repressor elements would show reduced luciferase output upon NKX6.2 binding. The elevation of MBP expression sometimes attributed to NKX6.2 activity is more likely indirect — reflecting de-repression of a negative MBP regulator — rather than direct transcriptional activation. Any reporter design should account for this repressor logic to avoid misinterpretation.

Any transcriptional reporter experiment designed to assess NKX6.2 activity in recipient cells requires a design that decouples transfer efficiency from transcriptional activity, since reduced reporter signal could reflect reduced transfer, reduced repressor activity, or both. The simplest approach is to measure both readouts independently in the same experiment: (1) quantify transfer by V5 immunofluorescence in GFP-negative recipient cells — independent of transcriptional activity; and (2) quantify repressor activity by luciferase assay in the same co-culture. This dual-readout design would allow any deficit observed in the reporter assay to be attributed specifically to transfer failure or to a downstream impairment in transcriptional engagement.

From a translational perspective, these results raise the possibility that wild-type NKX6.2 — if deliverable to NKX6.2-deficient oligodendrocytes in sufficient quantity and in the correct temporal window — might partially rescue their differentiation deficit. From a therapeutic perspective, the intended recipients of exogenous NKX6.2 in SPAX8 would be cells carrying a loss-of-function NKX6.2 allele, not normal oligodendrocytes. Any therapeutic application would require demonstration of transfer in primary oligodendrocytes or OPCs derived from disease-relevant models, which remains an important priority for future work.

A complementary approach that would bypass the transfection bottleneck entirely is the use of purified recombinant NKX6.2 protein. Several secretory homeoproteins — including EN1 [25], EN2 [26], and OTX2 [20,21] — have been applied as purified recombinant proteins to recipient cells, bypassing the need for donor cell transfection. Applying recombinant NKX6.2 directly to HOG cells would enable assessment of membrane uptake and intracellular localization independently of the current transfection bottleneck.

Finally, a critical appraisal of the methodological limitations of this study is necessary to contextualize its conclusions appropriately. The principal limitation is the absence of a positive-control transfer experiment run in parallel with every HOG-recipient experiment: without a concurrent demonstration that HEK293-to-HEK293 transfer is detectable on the same experimental day, any negative result in HOG cells cannot be unambiguously attributed to a biological property of HOG cells rather than to batch-to-batch variation in transfection efficiency or antibody performance. A practical implementation would be to place a coverslip of non-transfected HEK293 recipient cells directly onto a monolayer of transfected HEK293 donor cells in the same dish, providing a concurrent positive control for transfer under identical conditions. Future experiments should incorporate such an internal HEK293 positive control for every session. A second methodological improvement would be to use OLIG2 immunostaining as a cell-identity marker in a direct mixed co-culture of HEK293 donors and HOG recipients seeded on the same coverslip: OLIG2 is not expressed in HEK293 cells but is constitutively expressed in HOG cells, making it a straightforward tool to distinguish the two populations at the imaging stage and to restrict transfer quantification to OLIG2+/V5+/GFP- events — a more rigorous and efficient readout than relying on morphological criteria alone. Finally, FACS-based quantification of V5+/GFP- recipient cells would substantially increase throughput, reduce observer bias, and permit statistically robust sample sizes. Taken together, these methodological improvements would substantially strengthen the conclusions that can be drawn from any future NKX6.2 transfer study.

Conclusion

The present study set out to investigate whether NKX6.2, a homeodomain transcription factor whose loss of function underlies SPAX8 leukodystrophy, can undergo intercellular homeoprotein transfer — a property that would support the feasibility of a protein-based rescue strategy. Using a bicistronic V5-NKX6.2-IRES-EGFP expression system in HEK293 cells, we obtained results consistent with intercellular HP transfer: V5+/GFP- cells were identified in non-transfected recipient cells at a mean frequency of $1.5 \pm 1.3\%$ across five experiments, reproducible under standardized conditions. This finding is consistent with NKX6.2 behaving as a secretory HP and places it within the class of homeoproteins capable of transfer under cell culture conditions.

The remaining experimental results — the absence of transfer in HOG cells, the lack of V5 signal on HOG coverslips exposed to NKX6.2-conditioned medium, and the non-interpretable V5 staining in the *in ovo* model — are each explicable by specific, identifiable technical limitations rather than by a biological inability of NKX6.2 to transfer. The HOG transfection failure prevented the generation of sufficient donor cells; the conditioned medium design required NKX6.2 to diffuse freely across the culture volume, a pathway impaired by HSPG sequestration at the donor cell surface; the *in ovo* antibody background precluded signal interpretation; and the HH12 embryo lacks the oligodendrocyte-lineage cells relevant to SPAX8. None of these results constitute evidence against NKX6.2 transfer in oligodendrocyte-relevant contexts. Rather, they define a clear experimental roadmap whose execution — optimised HOG transfection, direct co-culture of HEK293 donor and HOG recipient cells on the same coverslip, a later-stage *in vivo* model with viral

delivery, and a functional reporter assay — would directly resolve the central question left open by this work.

Beyond its primary experimental aim, this study establishes that the pCAGIG-V5-NKX6.2-IRES-EGFP system functions as expected for transfer detection, confirming the transfer paradigm in a clean positive control, and identifying the specific experimental bottlenecks encountered in each model system. These findings, together with a thorough characterisation of P19 oligodendrocyte-like differentiation as a partial but heterogeneous model, provide a coherent experimental framework on which subsequent studies can build. The study also contributes to the broader hypothesis that intercellular homeoprotein transfer could form the basis of a non-cell-autonomous, genome-independent rescue strategy for SPAX8 — a concept that gains plausibility from the demonstration that NKX6.2 is transfer-competent, even if its efficacy in oligodendrocyte-relevant systems remains to be established.

From a broader perspective, this work underscores both the promise and the challenges of homeoprotein-based therapeutic strategies for transcription factor deficiency disorders. The demonstration that homeoproteins can travel between cells and engage transcriptional targets in recipients — established for EN1 in the motor neuron context [25] and for OTX2 in the visual cortex [21] — represents a paradigm shift in thinking about the boundary between cell-autonomous and non-cell-autonomous gene regulation. Extending this paradigm to the myelination field, where the relevant cell type is the oligodendrocyte rather than the neuron, raises new biological questions about the geometry, efficiency, and cell-type specificity of HP transfer in compact white matter. The present work provides an initial experimental foothold in that direction. The completion of the experiments proposed in the Discussion section will be necessary to determine whether NKX6.2 transfer in oligodendrocytes is a biologically real phenomenon, and if so, whether it occurs at an efficiency that could be harnessed for therapeutic benefit in SPAX8 leukodystrophy.

These results motivate future work in more disease-relevant cellular contexts. Whether the extracellular environment of compact white matter — with its distinct HSPG repertoire and dense lipid-rich membrane architecture — supports or restricts HP diffusion and internalisation remains unknown, and cannot be assumed from cell culture results. This represents an important open question for the translational development of HP-based rescue strategies in leukodystrophy.

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