

Research progress of the transcription factor Brn4 (Review)

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Abstract. Brain 4 (Brn4) is a transcription factor belonging to the POU3 family, and it is important for the embryonic development of the neural tube, inner ear and pancreas. In addition, it serves a crucial role in neural stem cell differentiation and reprogramming. The present review aimed to summarize the chromosomal location, species homology, protein molecular structure and tissue distribution of Brn4, in addition to its biological processes, with the aim of providing a reference of its structure and function for further studies, and its potential use as a gene therapy target.

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1. Introduction

The transcription factor brain (Brn)4 is a member of the POU protein family. In 1984, Parslow *et al* (1) first identified a highly conserved sequence ~70 base pairs upstream of the transcription initiation site of the immunoglobulin heavy and light chain genes, which was considered to be the area involved in the transcriptional regulation of gene expression. Subsequently, DNA elements with similar functions and highly conserved sequences were found at the origin of adenoviral DNA replication and at the promoters of nucleolar small RNA and Histone 2B genes. Given the similarity of the 150-160 amino acids in the sequences in the mammalian proteins, pituitary-specific positive transcription

factor 1, Oct1, 2 and nematode Uncoordinated-86, the homologous sequence was named the POU domain (an acronym of the transcription factors) (2).

The POU domain is a binding site for DNA binding proteins. It comprises two subdomains linked by a 15-56 amino acid sequence junction region: i) The N-terminus, which contains the POU-specific domain (POU_S) comprising 76-78 amino acids, and ii) the C-terminus, which comprises the POU homology domain (POU_H) composed of 60 amino acids (3). Proteins with a POU domain are named POU proteins, and serve as transcription factors that have important roles in embryonic development and cell fate-directed processes by binding to specific nucleotide sequences and regulating the transcription of downstream target genes (4). According to the degree of homology of the POU domain, the POU gene can be divided into six classes, which encode the POU1-6 protein families, respectively, each of which contains different members (5).

The POU3 family contains four members, Tst-1 [also known as POU domain, class 3, transcription factor 1 (POU3F1), Oct6, Test1, and SCIP], Brn1 (also known as POU3F3 and Oct8), Brn2 (also known as POU3F2 and Oct7) and Brn4 (also known as POU3F4, Oct9, deafness 3 and deafness X-linked 2). Tst-1 was originally identified in rat testes and glial cells, as well as in the developing brain and skin (5), and it is an important regulator of neurogenesis and epidermal differentiation (6). It has a dual regulatory function, not only does it activate neural lineage genes (such as Sox2), but it also represses neural-inhibitory signals (such as Wnt and bone morphogenetic protein), thus inhibiting cell proliferation (7,8). The degradation of POU3F1, caused by numerous factors such as DNA damage, starvation, and oxidative stress, was reported to induce neural tube defects (9). Brn1 is involved in the development of the central nervous system, and its loss-of-function mutations were identified to cause developmental delays, intellectual disability and impairments in language skills (10). It was also found to be expressed in the developing kidney (11). In previous studies, Brn1 knockout mice were discovered to have neurological defects, including locomotor and auditory impairments, but also a thicker ascending loop of the limb of Henle and decreased numbers of nephrons, thus resulting in renal failure and perinatal death (12,13). Brn2 has been reported to serve an important role in the development of the central nervous system; it has been associated with bipolar disorder (14), and it promotes neurogenesis by regulating the expression levels of neurotrophin 3 (15). Brn2 knockout mice

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were also revealed to suffer from cognitive impairment and decreased numbers of newborn neurons were found in the dentate gyrus of the hippocampus (16). Another previous study suggested that Brn2 may promote human gastric carcinoma cell proliferation, migration and invasion by binding to the promoter of tumor-associated NADH oxidase (17). It was also shown to serve a role in the development of hepatocellular carcinoma through the long non-coding RNA brain cytoplasmic RNA 1/microRNA-490-3p/Brn2 axis (18). Brn4, also known as POU3F4, Oct9, deafness 3 and deafness X-linked 2, was named after it was initially discovered in brain tissue (19). The following sections of the present review aimed to concisely summarize the research progress on Brn4 regarding its chromosomal location and species homology, protein molecular structure and tissue distribution, and related biological processes.

2. Chromosomal location and species homology

Brn4 is a single copy gene located on the human chromosome X q21.1, spanning 3,867 nucleotides. Two polydeoxyadenine nucleotides in the POU3 family gene sequence are considered to be formed by the reverse transcription of mRNA and re-entry into chromosomal sequences via genetic recombination (20). In the absence of introns, Brn4 mRNA leaves the nucleus and enters the cytoplasm without undergoing post-transcriptional splicing, thus resulting in rapid gene expression (20). Brn4 was formed before the human and rodent gene duplication that occurred during evolution (5); therefore, it has high homology between humans and mice, and investigations using rodent models can provide valuable references for studies of clinical diseases. In addition, a previous study of a lactose operon reporter gene identified the presence of ≥ 2 positive *cis*-regulatory elements of 6 kb in length upstream of the promoter sequence in Brn4 (21), indicating that Brn4 may be highly expressed at certain stages of development and may be important in histogenesis.

3. Protein molecular structure and tissue distribution

The transcription factor Brn4 is a DNA-binding protein that recognizes a specific octamer motif (ATGCAAAT) (4). Brn4 comprises 361 amino acids and has a molecular weight of ~ 39.43 kDa. Both the POU_S and POU_H domains of Brn4 contain a helix-turn-helix motif, which can form a hairpin structure that binds DNA sequences and exerts biological effects such as transcriptional activation (Fig. 1) (4). During development in mammals, the POU3 gene family has been reported to be widely expressed in ectoderm-derived tissue, such as the nervous system and auditory vesicles (22). In addition, Brn4 has been detected in the embryonic pancreas and adult glucagon cells (23,24). A previous study investigating the role of the POU3 gene during development in *Xenopus laevis* found that Brn4 was expressed in the embryonic neural tube, brain, developmental ear vesicles, distal renal tubules and connective tubules (11). RNA sequencing results of the genome-wide analysis of 272 mouse cell and tissue types revealed the widespread expression of Brn4 during nervous system development, including within the neural crest, neuroectoderm, hypothalamus, pituitary, hippocampus and

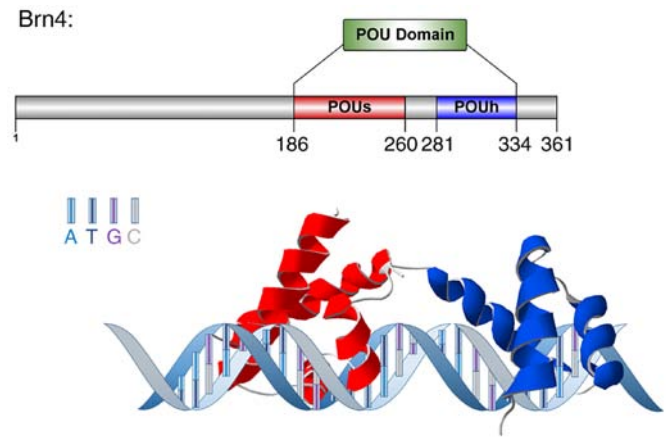


Figure 1. Structure of the Brn4-POU domain bound to DNA. POU_S and POU_H form a helix-turn-helix motif, which can form a hairpin structure combined with the DNA sequence to exert biological effects. The red represents POU_S and the blue represents POU_H. Brn4, brain 4; POU_H, POU homology domain; POU_S, POU-specific domain.

inner ear (5). However, in contrast to the extensive distribution in embryonic development, in adult animals, Brn4 expression was reported to be limited to a small part of the forebrain, including the supraoptic nucleus and paraventricular nucleus of the thalamus.

4. Related biological processes of Brn4

Inner ear development. The inner ear is part of the anatomical structure of the ear and is located in the cavity of the sacral rock (25). Through a series of complicated canals, it forms a structure called the labyrinth, which consists of the bony and membranous labyrinths (25). The bony labyrinth contains the vestibular system, which is associated with balance, and the cochlea, which is associated with hearing (26). The cochlea converts sound into nerve impulses that transmit external signals to the central nervous system through the auditory nerve, thus resulting in hearing (26). Inner ear development is a complex process involving a series of morphological changes caused by the interaction between ectodermal epithelial cells and mesenchymal cells (27).

Brn4 is abundantly expressed in the mesenchymal cells of the embryonic ear (Fig. 2), which not only benefits the survival of spiral ganglion neurons (SGNs), but also stimulates SGNs to extend axon bundles and eventually form synapses with surrounding hair cells (28). The gap junctions between cochlear cells facilitate K⁺ transmission, thus resulting in a high resting potential for sensory hair cell excitation (29). Brn4 was reported to promote the assembly and localization of connexins at the cochlear support cell borders, thereby maintaining proper intra-cochlear lymphatic potential for the efficient transmission of sound (22). Mice with targeted mutations in Brn4 demonstrated abnormal development in the inner ear interstitial compartment and showed signs of numerous behavioral changes associated with auditory and vestibular system function defects, including vertical nodding movement, gait abnormality and hearing loss (30). As both the temporal bone and cochlea originate from mesenchymal cells of the embryonic ear, anatomical and histological analyses

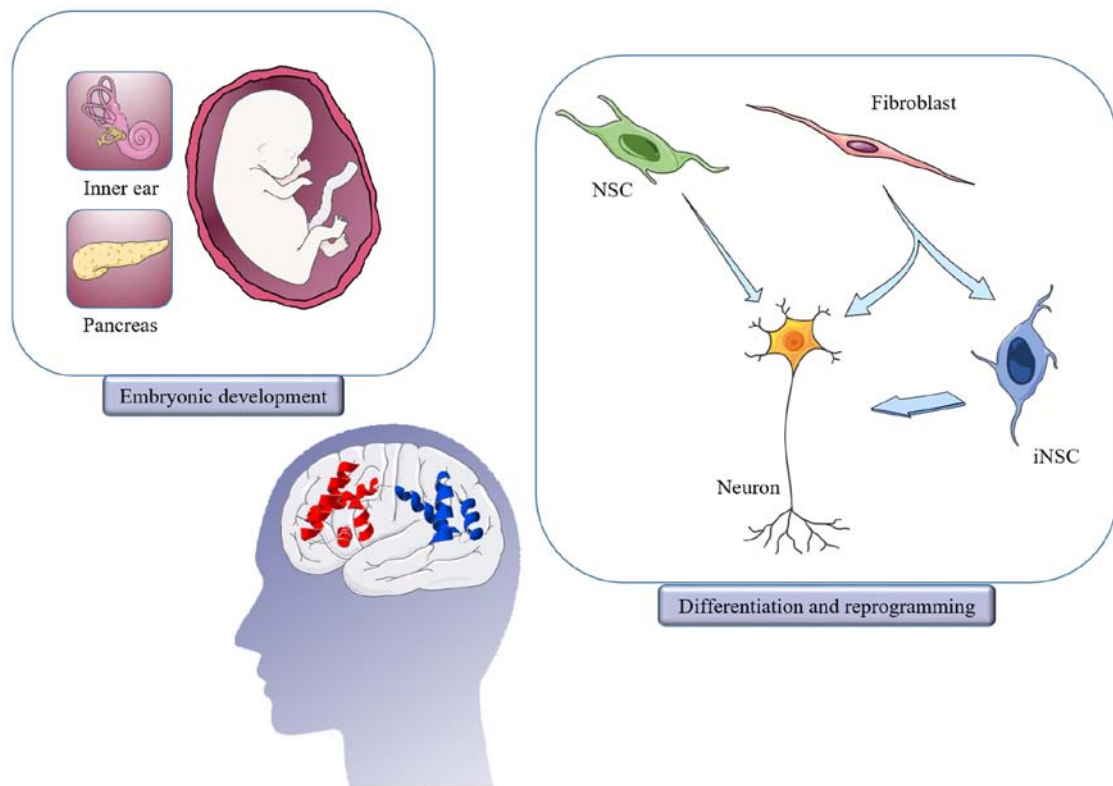


Figure 2. Reported biological functions of Brn4. Brn4 reportedly promotes the development of the embryonic inner ear and pancreas. It can also induce NSCs to differentiate into neurons and help reprogram fibroblasts into iNSCs. Brn4, brain 4; iNSC, inducible NSC; NSC, neural stem cell.

have indicated that Brn4 mutant mice have temporal bone dysplasia, enlargement of the inner ear canal, constriction in the bony labyrinth, thinning of the chamber wall, cochlear dysplasia, reduction in cochlear coiling and changes in the size and shape of the stapes footplate (30).

DFN3 is the most common type of X-chromosome-linked hereditary hearing loss (31). More than two decades ago, Brn4 was found to be a specific pathogenic factor in this disease (32-34). The pathogenic molecular mechanism of DFN3 simultaneously involves the sound conduction and sensory system and is based on structural changes in the Brn4 protein. Imaging examinations in patients have also revealed that Brn4 mutations can result in cochlear morphological abnormalities, internal auditory canal expansion and thickening of the stapes footplate (35). Cerebrospinal fluid was observed to flow into the vestibule through the dilated inner auditory canal, and consequently promoted perilymphatic gusher following the removal of the stapedial footplate during inner ear surgery (36). The disease is characterized by severe mixed hearing loss in males, whereas female carriers usually do not experience deafness. However, female carriers may have varying degrees of hearing threshold changes, mild semi-circular canal dilatation and postural abnormalities (37). In previous years, genetic sequencing of families with DFN3 has revealed that almost all affected individuals have Brn4 gene segments with nonsense, missense or frameshift mutations, and even *de novo* genome deletion, thus resulting in abnormal protein structure and function (37-44). Chromosome translocations and deletions have also been detected upstream of Brn4 (up to 900 kb upstream), suggesting that some *cis*-regulatory elements may be present in these regions to regulate the gene

expression (45). Imaging analyses of patients with DFN3 with Brn4 mutations revealed that the patients also had a high incidence of hypothalamic malformation, in addition to inner ear structural abnormalities such as stapes fixation and multiple vestibular diverticulum (46,47).

Although Brn4 gene mutations have been widely confirmed to cause severe inner ear dysplasia, its specific molecular mechanism remains unclear. Moreover, how to effectively prevent the occurrence and development of this genetic disease and how to correct the abnormal cochlear structure caused by the mutation of Brn4 require further research.

Pancreas development. The pancreas originates from the dorsal and ventral buds of the endoderm, which are later referred to as the dorsal and ventral lobes; these develop into the 'tail' and 'head' of the adult pancreas, respectively (48). The pancreas functions as both an exocrine and endocrine gland. The endocrine function involves the pancreatic islets, which lack a fixed form and are found scattered throughout the pancreas (49). The most representative cells in the islets are glucagon-secreting α cells (15-20% of islet cells) and insulin-secreting β cells (65-80% of islet cells) (50). The balance of insulin and glucagon is crucial for the maintenance of blood glucose homeostasis (50).

Islet α cells are the first endocrine cells formed in the developing pancreas on embryonic day 9.5 in mice, and Brn4 was revealed to promote their differentiation (Fig. 2) (51-53). Islet α cells first express the proglucagon gene, and the produced proglucagon is further processed into glucagon, which increases blood glucose levels (53). Previous studies identified that the proximal G1 promoter element of the

proglucagon gene, an AT-rich DNA sequence, was the binding site for Brn4 (24). Upregulated expression levels of Brn4 have been detected in islet cell lines producing glucagon and rat pancreatic islet cells, thus indicating its importance in pancreatic development. Heller *et al.* (23) reported that Brn4, expressed in the E10 pancreas (Fig. 2), was the only confirmed islet α cell-specific transcription factor. However, Brn4 deletion did not generate abnormalities in pancreatic bud formation, pancreatic islet α cell numbers or physiological function in mice. Therefore, the synthesis and secretion of glucagon appears to depend on the participation of numerous factors, which may overlap in function.

Clinically, diabetes is typically treated with oral medication or insulin injection (54). The complications of diabetes have long been a prominent cause of human disabilities such as musculoskeletal abnormalities and microangiopathy (54,55). One previous study discovered that the differentiated islet cells from human marrow mesenchymal cells highly expressed pancreatic transcription factors, such as Brn4, in induced islet-like cell aggregates, suggesting that Brn4 may be targeted to induce the regeneration of islet cells to treat clinical diseases including diabetes, pancreatic cancer and abnormal glucose metabolism (56).

Differentiation of neural stem cells. Self-proliferating neural stem cells (NSCs) are present throughout life in mammalian brains. These cells are mainly distributed in the subventricular zone and the hippocampal subgranular zone, and they can differentiate into neurons and glial cells (57). Since Brn4 was found to mediate striatal neuron precursor differentiation in 1999 (58), its role in neural regeneration has attracted significant attention (Fig. 2). Nuclear receptor-related 1 (Nurr1), a midbrain dopaminergic (DA) neuron-specific transcription factor, was reported to promote the differentiation of NSCs into DA neurons, increase the levels of dopamine in the striatum and ameliorate behavior defects in rats with Parkinson's disease. The expression of Brn4 also promoted the Nurr1-induced significant increase in the viability and maturity of DA neurons (59). Brn4 was also discovered to induce the expression of glial cell line-derived neurotrophic factor, which contributed to the survival and maturation of DA neurons, together with its receptors, GDNF family receptor α -1 and Ret (60). In addition, following denervation surgery, the expression levels of Brn4 in the hippocampus of rats were found to be upregulated, and reached a peak at 14 days post-injury (61). Functional experiments have indicated that Brn4 not only promotes the differentiation of NSCs into neurons but also enhances the maturation of neurons (61,62). This change in Brn4 may be due in part to the activation of the PI3K/AKT signaling pathway caused by upregulated expression levels of insulin-like growth factor (63). In addition, the paired box protein Pax 6/barrier-to-autointegration factor complex was demonstrated to drive neurogenesis by directly activating the expression of transcription factors, such as Brn4, during neurodevelopment (64). Investigations to determine target genes of Brn4 using RNA sequencing have revealed that, alongside the expression of Brn4, the expression levels of genes involved in neuronal development and maturation were upregulated, whereas those of genes associated with maintaining the pluripotency of NSCs were downregulated.

Furthermore, C-terminal-binding protein 2 and Notch2 were suggested to be direct targets of Brn4 (65,66).

Fetal brain tissue can be used for cell replacement therapy in neurodegenerative diseases, such as Parkinson's disease, but it is limited by donor deficiency and cannot be standardized (67). Fortunately, the induced differentiation into specific cell groups after the expansion of NSCs *in vitro* can solve this problem. For example, in both the midbrain and the hippocampus, Brn4 promoted the differentiation of NSCs into neurons (Fig. 2) and the maturation of new neurons (58-62,68). Therefore, Brn4 may provide a target for novel drugs in the treatment of neurodegenerative diseases.

Reprogramming. In 1998, Thomson *et al.* (69) proposed that blastocyst-derived embryonic stem cells may be used as a clinical treatment for diseases such as type 1 diabetes and Parkinson's disease. Since this discovery, the cell transplantation theory has gradually become a research focus. However, there are ethical problems in obtaining stem cells from embryos, and transplant rejection is common during allogeneic transplantation (69). Therefore, how to obtain cell sources conveniently and quickly and eliminate rejection reactions has become an urgent problem that must be solved. In 2006, Takahashi and Yamanaka (70) identified four factors, Oct3/4 (also known as POU5F1), Krüppel-like factor 4 (KLF4), Sox2 and c-Myc together referred to as OKSM, that induced embryonic or adult mouse fibroblasts to differentiate into induced pluripotent stem cells (iPSCs) *in vitro*; the results were subsequently confirmed in human cells (71). However, the activation of c-Myc, a proto-oncogene, was revealed to result in the formation of tumors in mice transplanted with iPSCs (70). Subsequently, some researchers found that induced NSC (iNSC) colonies were produced after culturing mouse iPSCs induced by OKSM in NSC-specific medium for ≥ 8 days (72). As with Oct4, Brn2 and 4 are also members of POU protein family and have important effects on nervous system development (5). Therefore, some researchers are focusing on the roles of Brn2 and 4 in cell reprogramming.

The co-overexpression of Brn2, Sox2 and forkhead box G2 was identified to be able to reprogram human or mouse fibroblasts and astrocytes into neural precursor cells, which are also called induced DA precursors, owing to their DA differentiation ability (73-75). Under spontaneous neuronal differentiation condition, these induced neural precursor cells were observed to differentiate into glutamatergic and GABAergic neuronal subtypes (73). In previous studies, Brn2 combined with guanine nucleotide-binding protein subunit β -like protein and myelin transcription factor 1-like protein was used to reprogram somatic cells into functional neurons (76-79). A previous study suggested that Brn2 itself may be sufficient to achieve the aforementioned somatic cell reprogramming effect, and that changes in cell culture conditions determine the direction of induction (80).

Brn4, in combination with Sox2, KLF4 and c-Myc is called BSKM; BSKM was found to successfully induce fibroblast reprogramming into iNSCs (Fig. 2). These cells, after being transplanted into the stem cell pool in the adult mouse brain, exhibited true pluripotency in terms of proliferation and differentiation into neurons and glial cells (81,82). The reprogramming efficiency was further improved by

the addition of E47 or transcription factor 3 (83). Oct4 and Brn4 were discovered to bind to Sox2 and form a dimer in a similar manner by cooperativity by sequencing, a method to determine the cooperativity parameters of transcription factors (84). Although both OKSM and BSKM can reprogram somatic cells into iNSCs (85), they exhibit several differences; for example, owing to the subtle differences between Oct4 and Brn4 in the POU_s domain (86), BSKM was observed to directly transform somatic cells into iNSCs without requiring the state of transient iPSCs induced by OKSM (Fig. 2) (81,86). Thus, BSKM may have lower tumorigenicity and an enhanced clinical practical value.

Moreover, reprogramming human or mouse fibroblasts or astrocytes into functional neurons or neuronal precursor cells by Brn4 is not uncommon (87-89), and these studies suggested that neurons induced by Brn4 *in vitro* could be used as cell resources for substitutive therapy to treat nerve injury or neurodegenerative diseases. However, numerous issues require further investigations and research, such as whether induced neurons can completely replace the damaged cells; whether these cells have the long-term characteristics of nerve cells in producing and conducting excitation; and how to accurately intervene to modulate the direction, cycle and speed of cell differentiation.

5. Conclusion

With further medical advancements, the survival rate of patients with various genetic, chronic and nervous system diseases has increased significantly (90). However, improving patient comfort and wellbeing is currently the ultimate goal of medical care (90,91). As aforementioned, Brn4 was discovered to promote the survival and synaptic growth of SGNs in the inner ear (28) and maintain proper intra-cochlear lymphatic potential by regulating connexins (22). However, to the best of our knowledge, its downstream target genes and the specific molecular mechanisms leading to structural abnormalities of the inner ear remain unclear. It was hypothesized that Brn4 may not only promote the growth of neurons, but it may also directly affect the signal transduction between cells and serve an important role in the osteogenic differentiation of the inner ear. Brn4^{-/-} mice were reported to exhibit vertical nodding and an abnormal gait (30), thereby suggesting that Brn4 might affect the sense of balance and voluntary movement function.

Although Brn4 was once considered a specific transcription factor of islet α cells that upregulates the expression of the proglucagon gene, the pancreatic development of Brn4^{-/-} mice is not significantly altered, and Brn4 expression levels were also upregulated in insulin-like cells with the function of decreasing blood glucose in diabetic model mice (23,24,51,52,56). However, to the best of our knowledge, the mechanism via which Brn4 maintains blood glucose stability and the quantitative balance among different islet cell types has not been reported. It can be suggested that Brn4 may provide an adequate energy supply for insulin synthesis in islet β cells by promoting glucagon secretion in islet α cells. Accordingly, the feedback regulation of both hormones and blood glucose levels would ultimately maintain Brn4 homeostasis.

Brn4 was identified to not only promote the differentiation of NSCs and the maturation of newborn neurons (59-61,63-66),

but also induced somatic cells to transform into neural lineage cells (87-89). However, the majority of the previous studies have focused on the number rather than the function of neurons following Brn4-induced differentiation. Further in-depth studies are required to determine whether the neurons induced by Brn4 have the long-term function of generating and conducting excitation; whether they produce effective synaptic connections with surrounding cells after being transplanted into the injured site; and how to improve the conversion ratio of neurons.

The microenvironment affects cell fate transitions; therefore, Brn4-mediated downstream gene transcription may provide a suitable microenvironment for neurogenesis, and the expression changes observed in numerous neural lineage-related genes following Brn4 treatment support this hypothesis (65). Previously, Brn4 was revealed to be a driving factor in neuroendocrine differentiation, thus promoting the occurrence and development of castration-resistant prostate cancer, together with Brn2. It was reported to be used as both a predictive marker of advanced castration-resistant prostate cancer and as a novel target to address the issue of resistance to enzalutamide treatment (92).

Due to its specificity of distribution and its high degree of homology across species, Brn4 is clearly important in development and biological functions including promoting the differentiation of NSCs into neurons and inducing reprogramming of somatic cells (Fig. 2). However, to the best of our knowledge, multiple aspects of the regulatory mechanism of its downstream biological processes remain unknown. Elucidation of the links between Brn4 and clinical diseases will require further investigations and experimental data. The present review aimed to concisely summarize the reported structure and biological properties of Brn4, with the aim of providing novel ideas for research in related fields.

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Authors' contributions

YW collected the data and drafted the manuscript. XuZ and JW assisted with manuscript preparation. GJ and XiZ revised the final manuscript. XiZ conceived and designed the idea for this paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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