Compartmentalization and Interaction of Pax5 and Pax6 in Brain of Mice

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Research Article

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Abstract

Many transcription factors play important roles to maintain the microenvironment, integrity of the blood-brain barrier, the neurons-glia interaction, activities of microglia, composition of cerebrospinal fluid, metabolic activities, concentration of neurotransmitters, presence of inflammatory and anti-inflammatory cytokines, ischemia, stress, aging, neurological disorders, and diseases. The Paired box transcription factors and multifunctional proteins, Pax6 and Pax5 are expressed in brain. They regulate several regulators from cell cycle to cell death. The Pax5, a B-cell lineage-specific activator protein (BSAP), is expressed in the cerebellum, cerebral cortex, hippocampus, olfactory bulb, third ventricles, and choroid plexus. The Pax5 has been observed down-regulated in autism, mental retardation, and Glioblastoma multiforme. The Pax6 affects genes of neurodegeneration, immunological surveillance, and energy homeostasis in brain of mice. The Pax5 and Pax6 recognize several similar DNA sequences and regulate the expression of genes in a tissue-specific manner. Therefore, it is presumed that Pax5 and Pax6, are compartmentalized in brain of mice. Results indicate interactions, cell and tissue-specific compartmentalization, and co-localization of Pax5 and Pax6 in the cerebral cortex, cerebellum, and hippocampus in brain of mice.

Introduction

The Paired box family transcription factors prove critical for development, growth, differentiation, organogenesis, and maintaining functional anatomy of vital organs like eye, nose, limb, muscles, kidney, vertebral column, and brain [1–3]. During development, paired box genes regulate cell fate specification, proliferation, and/or migration of neuroectodermal precursor cells. The overexpression of Pax genes appears to elicit the transformation of rat fibroblasts *in vitro* [4–6]. Among them, Pax5 and Pax6 have mainly been described as critical for the development and maintaining functional anatomy of the brain.

The Pax5 has been implicated in B-cell malignancies, dedifferentiation to uncommitted progenitors in the bone marrow, and BCR signaling. The targeting of Pax5 in mice causes defects in midbrain, abolition of lymphopoiesis, and incomplete V-H gene recombination in the adult bone marrow. The impact of Pax5 has been observed on neuronal development, non-hematological astrocytomas, medulloblastoma, and neuroblastomas malignancies. The Pax5 also regulates NF- κ B, genes involved in actin-remodeling [7], and the immune pathway in the development of Glioblastoma multiforme [8]. Recent reports suggest multiple binding sites for Pax5 in the promoter of Ionized calcium-binding adapter molecule 1 (*Iba1*), which regulates actin-bundling, membrane ruffling, cell migration, and phagocytosis in activated microglia. The Pax5 interacts with proteins essential for neuronal and glial differentiation. The interaction of Pax5 with Iba1 indicates Pax5-mediated transcriptional regulation of Iba1 in brain and impact on microglia-mediated immunity in brain of mice [9].

The Pax6 regulates astroglial de-differentiation, neural and glial proliferation in the central nervous system [10–11]. The expression of astrocytes markers GFAP and S100 β have been proposed to be regulated by Pax6 as their expression was observed downregulated in Pax6 knockdown condition [12].

The Pax6 binds to promoter sequence elements of *Gfap*, *S100 β* , *Tmem119* [13] and interacts with Iba1 both at the genetic and protein level [14].

The consensus recognition sequence of the Pax6 paired domain deviates primarily only at one position from that of Pax5, and the two proteins exhibit largely different binding specificities for genes [15] in a tissue-dependent manner. Since, both Pax5 and Pax6 are expressed in the brain, it is presumed that they may have similar DNA binding affinity on genes being expressed in that regions. The information about association of Pax5 and Pax6 in brain is lacking. They may co-localize and physically interact apart from their distinct expression pattern. Observations favor compartmentalization, co-localization, and interactions of Pax5 and Pax6 in brain of mice.

Material And Methods

Animal model

The male adult albino mice of AKR strain were used for the experiments. Mice were maintained at 25 $\pm$ 2°C as per the guideline of the Institutional Animal Ethical Committee in the animal house of the department. The mice were sacrificed by euthanasia and cervical dislocation to dissect out brain for experimental purposes. The experiments were carried out three times using brain of adult male mice (n = 5) per experiment.

In silico analysis of interacting proteins and promoter sequence elements of Pax5 and Pax6

The Pax5 and Pax6 interacting proteins in mice were analyzed using <http://string-db.org/> and evaluated by interaction scores shown in the string database. Annotation of Pax5 and Pax6 biological functions and signaling pathways were based on the interacting proteins, separately. The promoter sequences of *Mus musculus* Pax5 and Pax6 were retrieved from Eukaryotic Promoter Database (<https://epd.vital-it.ch/index.php>). The transcription factor search motifs for binding of Pax5 on *Pax6* promoter sequence and Pax6 on *Pax5* promoter sequences were analyzed.

Chromatin-Immunoprecipitation (ChIP) with anti-Pax5 and anti-Pax6 in brain of mice

The Chromatin Immunoprecipitation was performed as described earlier [13]. Briefly, the lysate of the adult brain was prepared. Cross-linking and chromatin preparation from lysate was done by 1% formaldehyde. The cross-linking reaction was stopped by adding 125mM glycine. Nuclear extract was collected by centrifugation at 10,000xg for 10 min. Nuclear lysis was performed in ChIP-lysis buffer followed by sonication. Typically four rounds, 30-sec pulse with 1 min rest in between rounds at output 5.0 (LABSONIC L, B. Braun Biotech International GmbH, Germany). The desired DNA fragment was between 0.5kb to 1kb in length. The supernatant after sonication containing chromatin was incubated for 4 hours at 4°C for Immunoprecipitation with anti-Pax5 antibody (anti-mouse, sc-13146, Santa-Cruz Biotech, USA) and anti-Pax6 antibody (anti-mouse, sc-32766). In control, anti-human IgG (HPO-1, Merk, India) was used. After centrifugation at 10,000xg, reverse linking was performed by adding 120mM NaCl

and incubation at 65°C for 1 hour. DNA obtained through Immunoprecipitation was purified through the phenol: chloroform purification method. The pulled DNA was checked on 1% agarose gel. Chromatin prepared without antibody was reverse linked to obtaining input DNA. The qPCR was performed to calculate fold enrichment of promoter *Pax5* and *Pax6* gene in input DNA, anti-Pax5 and anti-Pax6 pulled DNA and negative control. The primers sequence used were Pax5F, 5' CGGACCATCAGGACAGGA 3', Pax5R, 5' GGGCTCGTCAAGTTGG 3'; Pax6F, 5'GAGGTCAGGCTTCGCTAATG 3', Pax6R, 5' TCCAACAGCCTGTGTTGTTC 3'.

Analysis of the interaction of Pax5 and Pax6 by Co-Immunoprecipitation (Co-IP) in brain of mice

For Co-Immunoprecipitation, 50µl of Protein-A bead was taken into a spin column and washed twice with 1ml 1X IP buffer by centrifugation at 3000rpm for 20 seconds. 2µg of anti-Pax5 (anti-mouse, sc-13146, Santa-Cruz Biotech, USA) and anti-Pax6 (anti-mouse, sc-32766, Santa-Cruz Biotech, USA) diluted to 200µl in buffer were added to each column containing resin, respectively. The columns were incubated at 4°C for 30 minutes. After 30 minutes, the resin was washed with 1ml cold 1X IP buffer thrice and 150µl of adult mice brain tissue lysate was added to each column, respectively. Then columns were incubated for 1hr at 4°C and washed with cold 1X IP buffer thrice. In the negative control, the beads were incubated with antibodies and lack brain tissue lysate. After washing, 100µl of sample loading buffer was added to the resin, mixed well and the suspension was transferred into the 1.5ml microfuge tube [14, 16]. Samples were heat-denatured for 5 minutes, centrifuged for 1 min at 3000rpm and 50µl of the samples were resolved through 12% SDS-PAGE and analysed by Western blotting for Pax5 and Pax6-reactive peptide band, respectively.

Analysis of expression and co-localization of Pax5 with Pax6 in brain of mice

The antigen retrieval of cryo-sections was done in 0.1% trypsin+0.1% CaCl₂ for 10 min. Sections after antigen retrieval were blocked with 1% BSA for 1 hour. For double labeling, anti-Pax5 (anti-mouse, sc-13146, Santa-Cruz Biotech, USA, 1:500 dilution) and anti-Pax6 (anti-mouse, sc-32766, Santa-Cruz Biotech, USA, 1:200 dilution) antibodies were used at 4°C for overnight. The sections were washed with PBS and probed with TRITC (red) goat anti-mouse IgG secondary antibody, FITC (green) conjugated goat anti-mouse IgG secondary antibody (1:2000 dilution) (Merk, India), separately for 2h each for detecting Pax5 and Pax6 immunoreactivity. In the negative control, slides were stained with TRITC (red) goat anti-mouse IgG secondary antibody, FITC (green) conjugated goat anti-mouse IgG secondary antibody (1:2000 dilution) without incubating with primary antibody. The slides were washed with PBS with Tween 20 (0.02%) and counterstained with DAPI (Molecular Probe) for nuclear staining as previously described [14, 16]. Imaging was performed using a fluorescence microscope (Evos FLc) and confocal microscope (Zeiss LSM 780). Image analysis was performed by Zen software.

Results

Pax5 and Pax6 show interacts and is involved in various signaling pathways

Data curation for interacting proteins (Figure 1) and functional enrichment analysis (Supplementary Table 1-2) show some common associations of Pax5 and Pax6 with the regulation of gene expression, related to metabolic processes, cell differentiation, neurogenesis, gliogenesis, cell proliferation, and chromatin organization. The Pax5 has been involved with programmed cell death, response to growth factor, somatic diversification of immune receptors, whereas Pax6, with genes of eye morphogenesis, regionalization, stem cell differentiation, cell fate commitment, generation of neurons along with other functions indicating a potential role of Pax5 and Pax6 in the regulation of neuronal and glial homeostasis. The pathway enrichment analysis showed that apart from individual roles in several different pathways, Pax5 (Figure 2) and Pax6 (Figure 3) are commonly involved in transcriptional misregulation of cancer, pathways of cancer, viral carcinogenesis, Epstein-Barr virus infection, signaling pathway regulating pluripotency of stem cells, gastric cancer, Cushing's syndrome melanogenesis, basal cell carcinoma, and TGF-beta signaling pathway.

Pax5 and Pax6 interact with each other at gene and protein levels in the brain of mice

The multiple binding sites of Pax5 to the promoter sequence elements of *Pax6* and two binding sites of Pax6 to the promoter sequence elements of *Pax5* were predicted. The Pax5 binds to the -1751, -1715, -1692, -1536, -201, -75, +117 and +945 sequence position of *Pax6*. Similarly, Pax6 binding sites have been observed at the -1736 and +488 sequence position of *Pax5*. Chromatin Immunoprecipitation showed 2.5 fold enrichment of *Pax6* and 2.3 fold enrichment of *Pax5* promoter sequence element in anti-Pax5 and anti-Pax6 pulled down DNA, respectively as compared to anti-IgG pulled down DNA (Figure 4A-B).

Western blot analysis using anti-Pax5 and anti-Pax6 showed the presence of Pax6 in anti-Pax5 pulled down protein (Figure 5A; lane 2) and Pax5a/b in anti-Pax6 pulled down protein (Figure 5B; lane 2) where crude tissue lysate was taken as a positive control (Figure 5A-B; lane 3). The antibody-column complex without incubating with tissue lysate was taken as negative control (Figure 5A-B; lane 1). This indicates the physical interaction of Pax5a/b and Pax6 in the brain of mice at the protein level.

Pax5 and Pax6 compartmentalize and co-localize in the brain of mice

The Pax5 and Pax6 in the brain of mice show localization in Purkinje cells (PuC), granular cells (GC), stellate cells (SC), parallel fibres (PF) in cerebellum (Figure 6A). They co-localize in pyramidal cells (PyC), radial glial cells (RGC), stellate cells (SC) in cerebral cortex (Cc) (Figure 6B), in basket cells (BC), adult granular cells (AGC), radial glial cells (RGC) in hippocampus (Figure 6C). In the cerebellum, localization of Pax5 and Pax6 was observed throughout the cell body of Purkinje cell. Apart from Purkinje cells, localization of Pax5 and Pax6 were observed in basket cells, stellate cells of the molecular layer, and granule cells respectively show differential co-localization (Figure 6A). In the cerebral cortex, localization of Pax5 and Pax6 compartmentalized and shows co-localization at some regions on the periphery of

pyramidal cells and glial cells whereas stellate cells were observed as complete positive for Pax5 and Pax6 (Figure 6B). In the hippocampus, localization of Pax5 and Pax6 were observed in basket cells (BC), granule cells (AGC), and radial glia cells (Figure 6C).

Discussion

Analysis of signaling pathways of Pax5 and Pax6 based on interacting proteins neural network indicates their involvement in transcriptional misregulation and opposite role of Pax5 and Pax6 in cancer malignancies. The misappropriate expression of Pax5 leads to malignancies of gliomas and medulloblastomas which correlates positively with cell proliferation and inversely with neuronal differentiation [4, 17] whereas Pax6 act as a glioma tumor suppressor [18–19]. The involvement of Pax5 and Pax6 in TGF- β signalling pathway indicates the association of Pax5 and Pax6 in neuroprotective and immune modulator functions [20]. It also correlates with Huntington's disease pathway and modulations in DNA methylation pattern of Pax6 [21] and the association of Pax5 expression with clinical covariates of disease [22]. Pax6 acts as a downstream target of the Wnt/ β -catenin pathway, and β -catenin/Pax6 signalling which is critical for self-renewal and neurogenesis of radial glia/neural stem cells during neocortical development [23]. However, Pax5 functions as a transcription factor in canonical Wnt signalling which determines cellular fate in the cerebral cortex and hippocampus [24–25]. Conditional knockout of Pax5 in GABAergic neurons [25] in mice indicates the necessity of Pax5 in normal ventricular development. It has also been observed down-regulated in bipolar disorders and autism spectrum disorder [26].

Results of Chromatin Immunoprecipitation (ChIP) support binding of Pax5 on the promoter sequences of *Pax6* and the Pax6 on the control region of Pax5. It also suggests transcriptional regulation of Pax5 and Pax6 by each other, respectively. The observation on the physical interaction of Pax5a/b and Pax6 may be a cooperative binding or complex for other regulations of pathways. The physical association of Pax5 and Pax6 may also influence their own expression. The pattern of cell-specific expression and co-localization of Pax5 and Pax6 in cerebellum, cerebral cortex, and hippocampus indicates their compartmentalization in brain of mice. The differential co-localization of Pax5 and Pax6 were observed in parallel fibre, stellate cells, glial cells of the molecular layer and granule cells of the granular layer in cerebellum. The expression of Pax6 and Pax5 in granule cells may be associated with ataxia, learning and memory [27]. In the cerebral cortex, the glial cells showed differential localization of Pax5 and Pax6. The Pax6 compartmentalize in some regions at the periphery where it co-localized with Pax5, which has higher expression at some regions in the periphery and basal expression throughout the cell in pyramidal cells whereas stellate cells showed co-localization of Pax5 and Pax6. Pyramidal cells are the building blocks for high-level functions like memory and consciousness and are thought of as the mover and shaker of the brain [28] correlate with significant electrophysiologic changes and cognitive decline with age [29–30]. In the hippocampus, the co-localization of Pax5 and Pax6 were observed in the granule cells and basket cells of dentate gyrus except for a cell that showed Pax6 expression throughout the cell and a very low signal of Pax5 in the middle of the cell. Granule cells significantly contribute to learning and memory, which decrease with aging [27]. Adult-born granule cells (aGCs) shown positive for Pax5 and

Pax6 have been implicated in cognition and mood actively participate in the encoding of novel information [31]. The heterogeneous population of cells in brain showed cells positive for Pax5 and Pax6, respectively. They may have an independent function and cells positive for both Pax5 and Pax6 may have co-operative functions too in the brain.

Conclusion

Results indicate compartmentalization and co-localization of Pax5 and Pax6 in the cerebral cortex, cerebellum, and hippocampus in brain of mice. Pax5 and Pax6 also interact physically in brain of mice both at genetic and protein levels. Thus, apart from independent roles, Pax5 and Pax6 co-operate to maintain the functional anatomy of the brain (Figure 7).

Declarations

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Compliance with Ethical Standards

Disclosure of potential conflicts of interest: Author's declare no conflict of interest.

Research involving Human Participants and/or Animals: Not applicable

Informed Consent: Not applicable

Consent to Participate: Not applicable

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Figures

Figure 1

In silico analysis showed a neural network of top 100 Pax5 (A) and Pax6 (B) interacting proteins

Figure 2

Figure 2

Based on in silico analysis for Pax5 interacting proteins, involvement of Pax5 predicted in diverse biological pathways are listed.

Figure 3

Figure 3

Based on in silico analysis for Pax6 interacting proteins, involvement of Pax6 predicted in diverse biological pathways are listed.

Figure 4

Figure 4

In silico analysis of binding of Pax5 and Pax6 to the genetic sequence element of promoter sequence element of Pax6 and Pax5. Evaluation of enrichment of *Pax6* and *Pax5* promoter sequence in ChIP DNA with anti-Pax5 (A) and anti-Pax6 (B) and IgG pulled DNA (-ve control) in brain of mice. Data are represented as mean $\pm$ SEM and different superscripts denote significant difference ($p \leq 0.05$) in respect to control (independent-samples t-test).

Figure 5

Figure 5

Analysis of the interaction of Pax5 with Pax6 in brain of mice by Co-Immunoprecipitation. Western blot analysis with anti-Pax5 and anti-Pax6 from samples Co-Immunoprecipitated with anti-Pax5 (A; lane 2) and anti-Pax6 (B; lane 2) from brain of mice. Crude protein lysate was taken as input (positive control) (A-B; lane 3) and antibody-column complex without protein lysate was taken as -ve control (A-B; lane 1).

Figure 6

Figure 6

Photomicrographs of Pax5 and Pax6 positive cells in C, Cerebellum (A), Cc, Cerebral Cortex (B) and H, Hippocampus (C) in brain of mice. In the cerebellum, Pax5 (red) and Pax6 (green) positive cells are Purkinje cells (PuC), basket cells (BC), stellate cells (SC), parallel fibre (PF) and granule cells (GC) in brain of mice (A). In the cerebral cortex, Pax5 (red) and Pax6 (green) positive cells are pyramidal cells (Pyc), glial cells in brain of mice (B). In the hippocampus, Pax5 (red) and Pax6 (green) positive cells are basket cells (BC), granule cells (GC), glia, microglia, adult granule cell (AGC) in brain of mice (C).

Figure 7

Figure 7

Diagrammatic representation showing interaction and co-localization of Pax5 and Pax6 with each other in brain of mice.

Supplementary Files

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