MONOCLONAL ANTIBODIES IN IMMUNODIAGNOSIS AND IMMUNOTHERAPY
Volume 35, Number 5, 2016

DOI: 10.1089/mab.2016.0029

Short Communication

Generation of Pax1/PAX1-Specific Monoclonal Antibodies

Regina Feederle,[1] Josef-Karl Gerber,[2] Amy Middleton,[3] Emily Northrup,[4] Ralf Kist,[3,5] Elisabeth Kremmer,[6] and Heiko Peters[3]

Pax genes encode an evolutionary conserved group of transcription factors with multiple roles during embryonic development and for cell type specification in normal and malignant tissues of the adult organism. In mice, *Pax1* is required for the formation of specific skeletal structures as well as for the development of a fully functional thymus. In humans, the *PAX1* locus has been linked to otofaciocervical syndrome, idiopathic scoliosis, and to a higher susceptibility for androgenic alopecia. In addition, the methylation status of *PAX1* has recently emerged as a sensitive marker for predictive screening of cervical cancer. To provide a reagent for reproducible detection of Pax1 expression, we have generated rat monoclonal antibodies (MAbs) against the murine Pax1 protein. MAbs of one clone (clone 5A2) specifically detect mouse Pax1 protein in Western blot analyses. Moreover, the anti-Pax1 MAbs cross-react with human PAX1 protein and are applicable in immunohistochemical detection procedures using paraformaldehyde/formalin-fixed tissues embedded in paraffin. The anti-Pax1 MAbs provide a reliable reagent for reproducible Pax1/PAX1 protein expression analyses and, therefore, may help to improve diagnostic protocols in clinical settings involving deregulated expression of Pax1/PAX1.

Keywords: Pax1, rat monoclonal antibodies, mouse, human, Pax9

Introduction

PAX GENES REGULATE patterning and development of a broad spectrum of organs during mammalian embryogenesis.[1] Several lines of evidence implicate important roles of Pax genes for the specification of progenitor cells and for maintenance of progenitor cell fate, including that of cancer cells.[1,2] Sequence similarities, related expression patterns, and overlapping functions during mouse development placed *Pax1* and *Pax9* into a specific group within a family of nine Pax genes identified in mammals.[3] Analyses of a series of spontaneous and targeted *Pax1* mutant alleles in mice demonstrated the important role of *Pax1* for the development of the vertebral column, sternum, and scapula.[4] In addition, expression of *Pax1* in thymic epithelial cells was shown to be essential for normal thymocyte development.[5,6] Although the functions of the human *PAX1* gene have not been analyzed in great detail, genome-wide association studies have implicated the *PAX1* locus on chromosome 20p11 to be involved in male-pattern baldness[7] and in idiopathic scoliosis.[8] Moreover, a mutation in *PAX1* has been linked to otofaciocervical syndrome,[9] and numerous recent studies corroborated the initial report showing that the methylation status of *PAX1* may serve as a predictive marker in cervical cancer.[10]

Materials and Methods

Production of rat monoclonal antibodies recognizing Pax1

A full-length murine *Pax1* cDNA (MGI:97485; NCBI Gene: 18503) linked to *glutathione-S-transferase* (*GST*) was cloned into the plasmid pGEX (GE Healthcare, Germany). After confirmation of sequence integrity, the resulting plasmid was transfected into *E. coli* BL21 (DE3; Agilent Technologies, Santa Clara, CA) and recombinant GST-tagged Pax1 protein was expressed and purified as described elsewhere.[11] Approximately 50 μg of GST-Pax1 fusion protein dissolved in phosphate buffered saline (PBS) was emulsified

[1]Institute for Diabetes and Obesity, Monoclonal Antibody Core Facility and Research Group, Helmholtz Zentrum München GmbH, German Research Center for Environmental Health, Munich, Germany.
[2]Institute of Experimental Genetics, Helmholtz Zentrum München GmbH, German Research Center for Environmental Health, Neuherberg, Germany.
[3]Institute of Genetic Medicine, Newcastle University, International Centre for Life, Newcastle upon Tyne, United Kingdom.
[4]Research Unit Comparative Medicine, Helmholtz Zentrum München GmbH, German Research Center for Environmental Health, Neuherberg, Germany.
[5]Centre for Oral Health Research, School of Dental Sciences, Newcastle University, Newcastle upon Tyne, United Kingdom.
[6]Institute of Molecular Immunology, Helmholtz Zentrum München GmbH, German Research Center for Environmental Health, Munich, Germany.

in an equal volume of incomplete Freund's adjuvant (containing 5 nmol CpG2006; TIB MOLBIOL, Berlin, Germany) and injected both intraperitoneally and subcutaneously into Lou/C rats. After 6 weeks, a boost with 50 μg of fusion protein without Freund's adjuvant was given 3 days before fusion. Fusion of the myeloma cell line P3X63-Ag8.653 with rat immune spleen cells was performed according to standard procedures.[12] P3X63-Ag8.653 cells were cultured at 37°C in a humidified 5% CO_2 incubator in standard medium RPMI 1640 (supplemented with 1% glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin, and 2.5% fetal calf serum (FCS; Sigma, Taufkirchen, Germany). Hybridoma cells were cultured in standard medium supplemented with 20% FCS and 2% HT supplement (Life Technologies, Darmstadt, Germany).

Hybridoma supernatants were tested in a solid-phase enzyme-linked immunoassay (ELISA) using GST-Pax1 fusion protein. Ninety-six-well polystyrene plates were coated with a mouse anti-GST antibody (5 μg/mL) overnight at room temperature. After one wash with PBS, unbound sites were blocked with 2% FCS in PBS for 20 minutes and the plates were washed again. Pax1-GST fusion protein was added (2 μg/mL) for 30 minutes at room temperature, whereas unrelated GST-tagged protein served as negative control. After washing off unbound protein, the hybridoma supernatants (1:10 diluted) were added and incubated for 30 minutes. After another wash with PBS, the plates were incubated with a biotinylated mouse antirat secondary antibody (Dianova, Hamburg, Germany), washed once with PBS, and then incubated with horseradish peroxidase (HRP)-coupled avidin (1 μg/mL) (Vector Laboratories). After five washes with PBS, 3,3′,5,5′-tetramethylbenzidine substrate (ThermoFisher Scientific, Inc.) was added and the absorbance was measured at 650 nm with a microplate reader (Tecan, Switzerland). The hybridoma cells of Pax1-reactive supernatants were cloned at least twice by limiting dilution. The IgG subclass was determined with ELISA, with mouse antirat kappa light chain antibodies as capture and HRP-coupled mouse antirat IgG subclass-specific antibodies for detection.

Tissue preparation and Western blot analyses

To generate test samples for Western blot and immunohistochemical analyses, wild-type mice on ICR (CD-1) genetic background were bred and maintained at specific pathogen-free animal facilities at the Helmholtz Zentrum München or at Newcastle University. Experiments were carried out in accordance with German Animal Welfare Legislation and the Government of Upper Bavaria, Germany, or under licenses issued by the Home Office, United Kingdom. Mice were mated and the pregnant females sacrificed by cervical dislocation. For Western blot analyses, the developing vertebral columns (including the neural tube) were dissected out at embryonic day (E) 11.5, frozen in liquid nitrogen, and stored at −80°C until further use. The tissue was lyzed in RIPA buffer (150 mM NaCl, 0.5% NP40, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 20 mM Tris HCl pH7.5, 1× protease inhibitor mix (Sigma, Taufkirchen, Germany). After sonication (3 × 30 sec), proteins were denatured in Laemmli buffer (50 mM Tris-HCl pH6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM beta mercaptoethanol) for 5 min at 95°C, separated on a 10% SDS-polyacrylamide gel, and electroblotted onto a nitrocellulose membrane (GE Healthcare, Germany). After blocking for 30 min in 5% skimmed milk in PBS/0.1%Tween (PBST), blots were incubated with monoclonal antibody (MAb) hybridoma supernatants (diluted 1:10) or isotype controls (rat IgG1 and rat IgG2a) in 0.1% Tween in PBS for 16 h at 4°C. After washing several times with 0.1% PBST, blots were incubated for 1 hour with secondary mouse antirat IgG1 (ATCC TIB170) or mouse antirat-IgG2a (ATCC TIB173) coupled to HRP. Antibody binding was observed using an enhanced chemiluminescence (ECL) detection reagent (ThermoFisher Pierce, Austria).

Immunohistochemistry

Processing of mouse embryos, embedding, sectioning, and immunohistochemical detection was carried out as described.[13,14] Sagittal sections of human embryos were obtained from the MRC/Wellcome Trust-funded Human Developmental Biology Resource [HDBR,[15]], with appropriate maternal written consent and approval from the National Research Ethics Service (NRES) Committee North East—Newcastle and North Tyneside 1 (REC reference 08/H0906/21 + 5). HDBR is regulated by the United Kingdom Human Tissue Authority (HTA; www.hta.gov.uk) and operates in accordance with the relevant HTA codes of practice. Secondary antibodies, diluent, and staining reagents were purchased from Dako (Dako, Agilent Technologies, Santa Clara, CA). After deparaffinization and antigen retrieval in citric acid buffer, anti-Pax1 (clone 5A2) or anti-Pax9 [clone 7C2[13]] rat MAbs were diluted 1:40 in Dako diluent (Dako S3022) and applied to tissue sections for 40 minutes. After three washes with TBS buffer, HRP-conjugated rabbit antirat IgGs (Dako, P0450) were applied at 1:200 dilution for 40 minutes, followed by application of the rabbit-specific Envision+ detection system (Dako) for another 40 minutes and staining with DAB (3,3′-diaminobenzidine; K4010; Dako).

Results and Discussion

To generate a defined reagent for Pax1 expression analyses, we generated rat MAbs. Hybridoma supernatants from four clones gave a strong signal in the ELISA and were further tested by Western blot analyses and by immunohistochemistry on paraffin-embedded embryonic mouse tissue sections. Some of these clones have been used in two previous studies; however, their specificities and cross-reactivities have not been tested.[14,16] In protein extracts prepared from the mouse embryonic vertebral column, the supernatant of clone 5A2 (rat IgG2a/k) produced a single band at ∼42 kD (Fig. 1A), consistent with previous results obtained using polyclonal antibodies.[17] In contrast, no band was produced with IgG2a isotype control (Fig. 1A). Importantly, antibodies of clone 5A2 did not produce additional bands and did not detect the slightly smaller and closely related Pax9 protein, which is coexpressed with Pax1 in the vertebral column.[18] Pax9-specific MAbs (clone 7C2[13]) detect the Pax9-specific protein in the same protein extract at the expected size of ∼38 kD (Fig. 1A). Although we did not map the exact protein epitope that is recognized by antibodies of clone 5A2, the results show that these MAbs specifically react with Pax1 protein.

We next tested whether anti-Pax1 MAbs are applicable for immunohistochemical detection of Pax1 in tissue samples that have been routinely fixed in 4% paraformaldehyde or formalin. At E13.5 of mouse development, strong Pax1-specific staining was detected in the developing

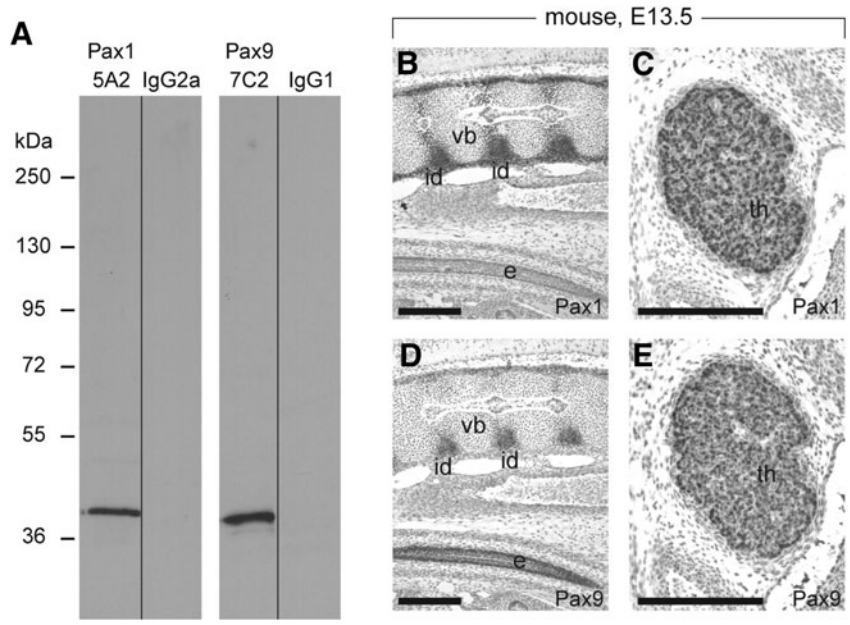

FIG. 1. Detection of mouse Pax1 protein. **(A)** Western blot analysis of vertebral column protein extract at E11.5. Rat MAbs from clone 5A2 generate a single, Pax1-specific signal at 42 kD, whereas Pax9-specific antibodies [clone 7C2[13]] detect a 38 kD protein in these extracts. Individually, corresponding isotype antibodies were included as negative controls. **(B–E)** Immunohistochemical detection of Pax1 **(B, C)** and Pax9 **(D, E)** in midsagittal sections **(B, D)** and parasagittal sections **(C, E)** of mouse embryos at embryonic day 13.5 (E13.5). Pax1 expression is detectable in the nuclei of intervertebral disk primordia of the developing vertebral column **(B)** and in epithelial cells of the embryonic thymus **(C)**. **(D, E)** In the intervertebral disk primordia and thymus, Pax9 is expressed in a pattern similar to that of Pax1. Pax9 is also expressed in the embryonic esophageal epithelium **(D)**, which is negative for Pax1 **(B)**. id, intervertebral disk; nt, neural tube; e, esophagus; th, thymus; vb, vertebral body; MAbs, monoclonal antibodies. Scale bars: 200 μm.

intervertebral disks and in a dense epithelial network of the thymus anlage (Fig. 1B, C). Control staining with Pax9-specific antibodies[13] on adjacent sections revealed a similar expression pattern (Fig. 1D, E); however, additional expression was detectable in the esophagus epithelium (Fig. 1D), which is negative for Pax1 (Fig. 1B). These results agree with previously published expression patterns of Pax1 and Pax9 in the mouse embryo[18] and further support the notion that the anti-Pax1 MAbs specifically react with Pax1 protein. Importantly, these antibodies cross-react with the human PAX1 protein (Fig. 2). Immunohistochemical staining on sections prepared from human embryos at Carnegie

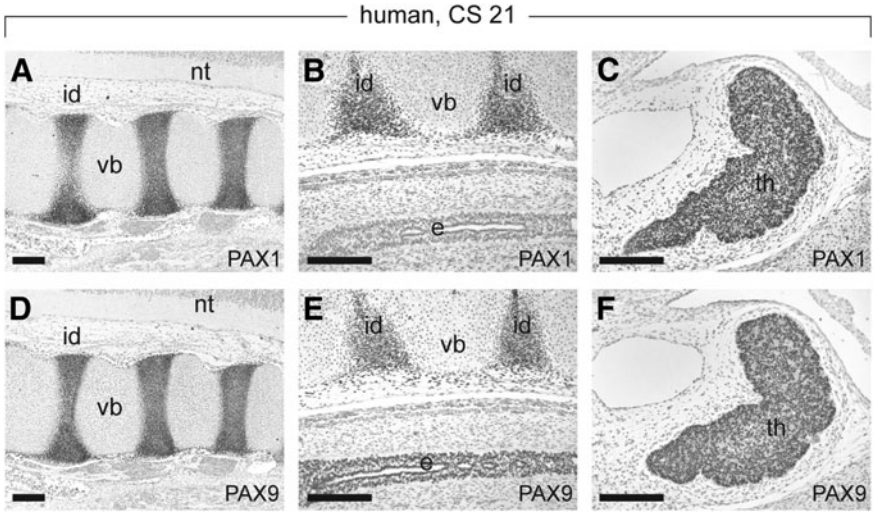

FIG. 2. Immunohistochemical detection of PAX1 and PAX9 in sagittal sections of human embryos at CS 21 (equivalent to approximately E15.5 of mouse development). Expression of PAX1 is detectable in the intervertebral disk **(A, B)** and in the thymus **(C)**. Human PAX9 protein is expressed in a similar pattern **(D–F)** but, as in the mouse, expression is also found in the embryonic esophagus **(E)**, in which PAX1 expression is missing **(B)**. id, intervertebral disk; nt, neural tube; e, esophagus; th, thymus; vb, vertebral body. Scale bars: 200 μm.

stage 21 (CS21) expresses PAX1 in the developing intervertebral disks (Fig. 2A, B) and in thymic epithelial cells (Fig. 2C), but not in the esophagus epithelium (Fig. 2B). Likewise, human PAX9 protein is expressed in the same pattern as observed in the mouse embryo (Fig. 2D–F).

Together, the rat anti-Pax1 MAbs generated in this work specifically detect Pax1. To our knowledge, these are the first Pax1-specific MAbs that have been demonstrated to be applicable for expression studies in formalin-fixed and paraffin-embedded tissue. The robust signals produced, together with minimal batch-to-batch variability, suggest anti-Pax1 MAbs derived from clone 5A2 to represent a reliable reagent for expression analyses during embryonic development as well as for diagnostic detection of Pax1/PAX1 in clinically relevant conditions.

Acknowledgments

The human embryonic material was provided by the Joint MRC/Wellcome Trust (grant number 099175/Z/12/Z) HDBR (www.hdbr.org).

Author Disclosure Statement

No competing financial interests exist.

References

1. Blake JA, and Ziman MR: Pax genes: Regulators of lineage specification and progenitor cell maintenance. Development 2014;141:737–751.
2. Mahajan P, Leavey PJ, and Galindo RL: PAX genes in childhood oncogenesis: Developmental biology gone awry? Oncogene 2015;34:2681–2689.
3. Stapleton P, Weith A, Urbánek P, Kozmik Z, and Busslinger M: Chromosomal localization of seven PAX genes and cloning of a novel family member, PAX-9. Nat Genet 1993;3:292–298.
4. Wilm B, Dahl E, Peters H, Balling R, and Imai K: Targeted disruption of Pax1 defines its null phenotype and proves haploinsufficiency. Proc Natl Acad Sci U S A 1998;95:8692–8697.
5. Wallin J, Eibel H, Neubüser A, Wilting J, Koseki H, and Balling R: Pax1 is expressed during development of the thymus epithelium and is required for normal T-cell maturation. Development 1996;122:23–30.
6. Su DM, and Manley NR: Hoxa3 and pax1 transcription factors regulate the ability of fetal thymic epithelial cells to promote thymocyte development. J Immunol 2000;164: 5753–5760.
7. Brockschmidt FF, Heilmann S, Ellis JA, Eigelshoven S, Hanneken S, Herold C, Moebus S, Alblas MA, Lippke B, Kluck N, Priebe L, Degenhardt FA, Jamra RA, Meesters C, Jöckel KH, Erbel R, Harrap S, Schumacher J, Fröhlich H, Kruse R, Hillmer AM, Becker T, and Nöthen MM: Susceptibility variants on chromosome 7p21.1 suggest HDAC9 as a new candidate gene for male-pattern baldness. Br J Dermatol 2011;165:1293–1302.
8. Sharma S, Londono D, Eckalbar WL, Gao X, Zhang D, Mauldin K, Kou I, Takahashi A, Matsumoto M, Kamiya N, Murphy KK, Cornelia R; TSRHC Scoliosis Clinical Group; Japan Scoliosis Clinical Research Group, Herring JA, Burns D, Ahituv N, Ikegawa S, Gordon D, and Wise CA: A PAX1 enhancer locus is associated with susceptibility to idiopathic scoliosis in females. Nat Commun 2015;6:6452.
9. Pohl E1, Aykut A, Beleggia F, Karaca E, Durmaz B, Keupp K, Arslan E, Palamar M, Yigit G, Özkinay F, and Wollnik B: A hypofunctional PAX1 mutation causes autosomal recessively inherited otofaciocervical syndrome. Hum Genet 2013;132:1311–1320.
10. Lai HC, Lin YW, Huang TH, Yan P, Huang RL, Wang HC, Liu J, Chan MW, Chu TY, Sun CA, Chang CC, and Yu MH: Identification of novel DNA methylation markers in cervical cancer. Int J Cancer 2008;123:161–167.
11. Harper S, and Speicher DW: Purification of proteins fused to glutathione S-transferase. Methods Mol Biol 2011;681: 259–280.
12. Köhler G, and Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975;256:495–497.
13. Gerber JK, Richter T, Kremmer E, Adamski J, Höfler H, Balling R, and Peters H: Progressive loss of PAX9 expression correlates with increasing malignancy of dysplastic and cancerous epithelium of the human oesophagus. J Pathol 2002;197:293–297.
14. Kist R, Watson M, Crosier M, Robinson M, Fuchs J, Reichelt J, and Peters H: The formation of endoderm-derived taste sensory organs requires a Pax9-dependent expansion of embryonic taste bud progenitor cells. PLoS Genet 2014;10:e1004709.
15. Gerrelli D, Lisgo S, Copp AJ, and Lindsay S: Enabling research with human embryonic and fetal tissue resources. Development 2015;142:3073–3076.
16. Sonnesen L1, Nolting D, Kjaer KW, and Kjaer I: Association between the development of the body axis and the craniofacial skeleton studied by immunohistochemical analyses using collagen II, Pax9, Pax1, and Noggin antibodies. Spine (Phila Pa 1976) 2008;33:1622–1626.
17. Peters H, Doll U, and Niessing J: Differential expression of the chicken Pax-1 and Pax-9 gene: In situ hybridization and immunohistochemical analysis. Dev Dyn 1995;203:1–16.
18. Neubüser A, Koseki H, and Balling R: Characterization and developmental expression of Pax9, a paired-box-containing gene related to Pax1. Dev Biol 1995;170:701–716.

Address correspondence to:
Heiko Peters
Institute of Genetic Medicine
Newcastle University
International Centre for Life
Newcastle upon Tyne NE1 3BZ
United Kingdom

E-mail: heiko.peters@ncl.ac.uk

Received: May 31, 2016
Accepted: September 4, 2016