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Preparation and Application of Monoclonal Antibody Against hNDRG2

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Abstract The full-length hNdrg2 cDNA-coded 357 amino acids was cloned and expressed in *Escherichia coli* strain DH5α as a 6× His-tagged protein. The purified 6× His-fusion protein was used to immunize mice for preparing monoclonal antibodies (mAb) against N-myc downstream-regulated gene 2 (Ndrg2). A hybridoma secreting a monoclonal antibody against Ndrg2 was obtained and named FMU-Ndrg2.3. Western blot analysis confirmed that this mAb is specific only to Ndrg2 but not to Ndrg1, Ndrg3, and Ndrg4-B. Some tissue distribution features of Ndrg2 proteins, such as thyroid, kidney, testis, prostate, and pancreas islets, were present by immunohistochemistry.

Keywords N-myc downstream-regulated gene · Immunohistochemistry · Fusion protein · Monoclonal antibody against hNdrg2

Introduction

The existence of a gene family often indicates the important function of its members. Human Ndrg2 belongs to the N-myc downstream-regulated gene (Ndrg) family, a new family of differentiation-related genes. This family comprises four recently identified members: Ndrg1, Ndrg2, Ndrg3, and Ndrg4 [1]. The biological function of human Ndrg gene family need to be explored further [2].

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Ndrg1 is widely expressed in many tissues. The expression of Ndrg1 in some tumor cells can be downregulated by overexpressed N-myc. The overexpression of human Ndrg1 can decrease cell proliferation rate, enhance cell differentiation, and suppress metastatic potency of the cancer cells [3–5]. Recently, a group [6] reported a nonsense mutation in the Ndrg1 gene as the cause of hereditary motor and sensory neuropathy-Lom, a severe peripheral neuropathy characterized by Schwann cell dysfunction and progressive axonal loss in the peripheral nervous system. It is presumed that the function of Ndrg1 in the peripheral nervous system may be important for axonal survival.

Human Ndrg2 was cloned from human brain glioma cDNA library as a downregulated gene by subtractive cloning [7]. Ndrg2 is highly expressed in adult brain, salivary gland, muscle, and heart but is undetectable in proliferative tissues such as the colon and bone marrow [7]. We previously reported that Ndrg2 expression levels were significantly reduced in 56% and 100% of human glioblastoma tissue samples and cell lines, respectively, when compared to that of the normal brain or the low-grade glioma samples [8].

The tumor suppressor activity of Ndrg2 gene was confirmed in Ndrg2 gene transiently transfected cell line HGC-27, which is an undifferentiated gastric mucosa gland carcinoma and did not express NDRG2 gene. It was found that the expression of Ndrg2 gene could suppress colony formation in soft agar and induce apoptosis in gastric carcinoma cells. The down-regulated expressions of cyclin D1 and cyclin E also were observed in transfected cells [9].

There are some other reports recently implying the important function of Ndrg2 in cell proliferation and cell differentiation. It was showed that Ndrg2 might be involved in the differentiation process of dendritic cells, monocytes, $CD34^+$ precursor, and leukemia cells [10]. The phosphorylation of Ndrg2 at 332 Ser by PKCθ led to the inhibition of Akt-mediated phosphorylation of Ndrg2 at the 348 Thr. The crosstalk might indicate that Ndrg2 protein was an important molecular in lipid-activated PKCθ interference with insulin action [11]. But the mechanism of Ndrg2 action in tumor cells remains unclear.

Human Ndrg members are highly homologous except for their C- and N-terminal regions. Amino acid sequences of Ndrg1, Ndrg3, and Ndrg4 proteins were similar to that of Ndrg2, showing 54%, 55%, and 56% identity to Ndrg2, respectively [12]. Therefore, it would be difficult to generate a monoclonal antibody against Ndrg2 but not to other family members.

The present study was designed to generate a monoclonal antibody against Ndrg2 so as to investigate its function and cellular location. For this purpose, we expressed and purified the 6×His-tagger hNdrg2 fusion protein in *Escherichia coli* strain DH5α [13]. A hybridoma was produced by using 6xHis-tag hNDRG2 fusion protein as immunogen. The specificity of monoclonal antibodies (mAb) to Ndrg2 was confirmed by Western blot and immunohistochemical staining. The expression of Ndrg2 in human testis, epididymis, prostate, pancreas, thyroid gland, and kidney were displayed here.

Material and Methods

Construction of Expression Vector and Protein Expression in *E. coli*

The full-length (1,200 bp) wild-type hNdrg2 cDNA from pGEM-T vector was subcloned into the downstream of 6×His tag of bacterial expression vector pRSET (Invitrogen, USA) with *EcoR*I and *BamH*I digestion. The correct reading frame was confirmed by sequencing. The expression of 6× His–hNDRG2 fusion protein was induced in *E. coli* strain DH5α with 0.5 mmol/l of isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma, USA) at 37 °C for 1 to 4 h in Luria–Bertani medium containing 50 μg/ml ampicillin (Sigma, USA).

The full-length coding region of hNDRG1, hNDRG3, and hNDRG4-B cDNAs were amplified by reverse-transcription polymerase chain reaction, respectively, from human brain cDNA library (Clontech). The cDNAs with correct sequence were cloned into pRSET same as above, respectively. The expression of 6× His–hNdrg1, 6× His–hNdrg3, and 6× His–hNdrg4-B fusion proteins were done with same procedure as 6× His–hNdrg2 fusion protein [14].

Purification of 6× His–hNDRG2 Fusion Protein

Bacteria cell pellets were harvested and resuspended in ice-cold sodium chloride–Tris–ethylene diamine tetraacetic acid (EDTA) buffer (10 mmol/l of Tris–HCl, 150 mmol/l of NaCl, 1.0 mmol/l of EDTA, 1.0 mmol/l of phenyl methyl sulfonyl fluoride and 0.1 mg/ml of lysozyme, pH8.0) on ice for 20 min. Dithiothreitol and Sarkosyl were added to 5.0 mmol/L and 1.5%, respectively on ice until the solution became viscous. The lysate were then sonicated (30~40 output, 1 min ×2 at 4 °C) and centrifuged at 10,000×*g* for 20 min at 4 °C. Triton X-100 was added to the supernatant to 2% (*v*/*v*). The solution containing fusion protein was filtrated with 0.45-nm membrane and then loaded on Ni-NTA agarose column according to the manufacture's instructions (Qiagen). 6× His–hNdrg2 fusion protein was collected and identified by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Generation of Hybridoma

Female BALB/c mice (8 weeks old) were immunized with 20 μg of 6× His–hNdrg2 fusion protein in complete Freund's adjuvant by subcutaneous (s.c.) injection. Subsequently, immunizations were carried out twice with 20 μg of the proteins in incomplete Freund's adjuvant by s.c. and intraperitoneal (i.p.) injection, respectively, at 3-week intervals. Ten days after the third immunization, the antiserum titers were determined by indirect enzyme-linked immunosorbent assay (ELISA). The positively immunized mice were boosted with 20 μg of 6× His–hNdrg2 fusion protein by i.p. injection. Three days later, splenocyte from immunized mice and SP2/0 myeloma intraperitoneal hernia (cultured in Roswell Park Memorial Institute 1640 containing 10% fetal calf serum) were fused in the presence of PEG (MW4000, Merck, Darmstadt, Germany). The positive hybrids were selected by ELISA and subcloned four times using limiting dilution method. Monoclonal antibody was collected as supernatant of the hybridoma culture. The antibody was named as FMU-Ndrg2.3.

Western Blot

Immunoblotting was performed using the recombinant hNdrg1, hNdrg2, hNdrg3, and hNdrg4-B fusion proteins expressed in *E. coli*, respectively. Briefly, proteins were separated on 12% SDS-PAGE and transferred onto a 0.2-μm nitrocellulose membrane. Nonspecific binding was blocked with 5% nonfat milk in Tris-buffered saline Tween-20 for 1 h at 37 °C. FMU-Ndrg2.3 was used at a 1:500 to 1:4,000 dilution and horseradish peroxidase (HRP)-conjugated rabbit antimouse secondary antibody was used in a 1:2,000 dilution. The incubation of both primary and secondary antibodies was done at 4 °C overnight and at room temperature for 2 h, respectively. Chemiluminescent substrate kit (SuperSignal West Pico, PIERCE) was used to detect immunoblot signal on nitrocellulose membrane.

Immunohistochemistry

The standard avidin–biotin–peroxidase complex staining method was adopted. Briefly, the sections were dewaxed in xylene and dehydrated through descending concentrations of ethanol, immersed in 0.3% H_2O_2–methanol for 30 min, and washed with phosphate-buffered saline (PBS).

As primary antibody, dilutions were optimized for each tissue to obtain maximum signal: background for hNdrg2 protein. The slides were incubated with primary antibody at 4 °C overnight. Nonspecific staining was defined by substituting a nonimmune mouse immunoglobulin G (IgG) antibody for the hNDRG2-specific antibodies as the primary antibody. After washing, sections were incubated in HRP-labeled rabbit antimouse IgG secondary antibody (1:100 dilution; Santa Cruz Biotechnology, Inc.), rinsed, and washed in PBS. Sections were developed in a 0.005-M Tris solution containing 0.03% diaminobenzidine and 0.006% H_2O_2 for 15 min. Finally, the sections were counterstained with Gill's hematoxylin and mounted in dinitro-*p*-xylene mountant (BDH, Poole, UK).

Results

Identification of Recombinant Fusion Proteins

The recombinant 6× His-tagged Ndrg2 fusion protein was expressed in *E. coli* with correct molecular weight (42 kDa) after being induced with 0.5 mmol/l of IPTG for 1 to 4 h at 37 °C (Fig. 1a). The fusion protein was purified by Ni-NTA agarose column, which showed a good quality (Fig. 1b) for working as immunogen both for mouse immunization and ELISA. The

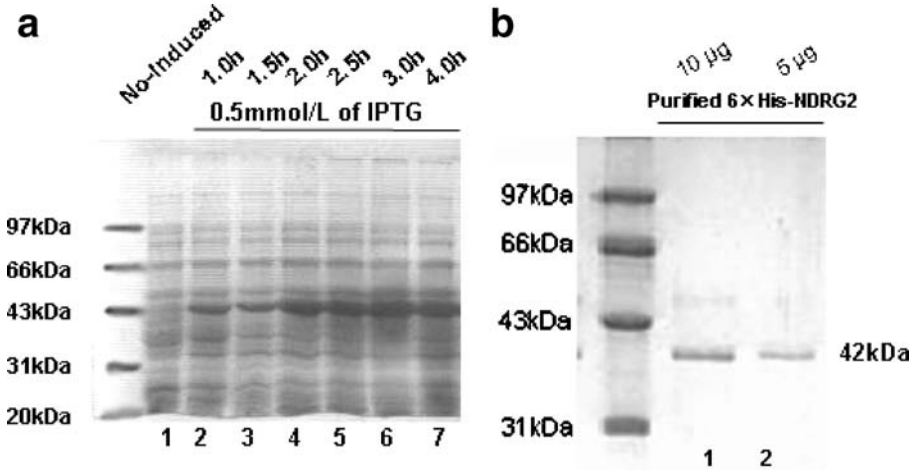

Fig. 1 *Protein expression analysis of recombinant fusion proteins by SDS-PAGE*. **a** 6× His-tagged Ndrg2 expression in *E. coli* was induced by adding 0.5 mmol/l of IPTG for 1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 h (lane 2 to lane 7), respectively. The protein concentration of bacterial cell pellets was measured by bicinchoninic acid protein assay (Pierce, Rockford, IL, USA); 50-μg aliquots of lysates were separated on 12% SDS-PAGE and then stained with Coomassie Blue. MK stands for molecular weight marker. **b** Purification of 6×His-NDRG2 fusion protein by Ni-NTA affinity chromatography. The protein concentration of purification products was measured by BCA protein assay; 10-μg aliquots (lane1) and 5-μg aliquots (lane2) of purification products were separated on 12% SDS-PAGE and then stained with Coomassie Blue

expression of other Ndrg2 family members (6× His-tagged fusion proteins) were shown in Fig. 2a. The molecular weight of these fusion proteins are as expected, 41 kDa for Ndrg1, 42 kDa for Ndrg2, 43 kDa for Ndrg3, and 40 kDa for Ndrg4-B, respectively. All four Ndrg protein expressions were determined by Western blot analysis using an anti-His-tag antibody.

Specificity Analysis of FMU-Ndrg2.3 by Western Blot

In order to analyze whether hNdrg2 mAb can recognize hNdrg2 specifically, all of recombinant hNdrg1, hNdrg2, hNdrg3, and hNdrg4-B fusion proteins expressed in *E. coli*

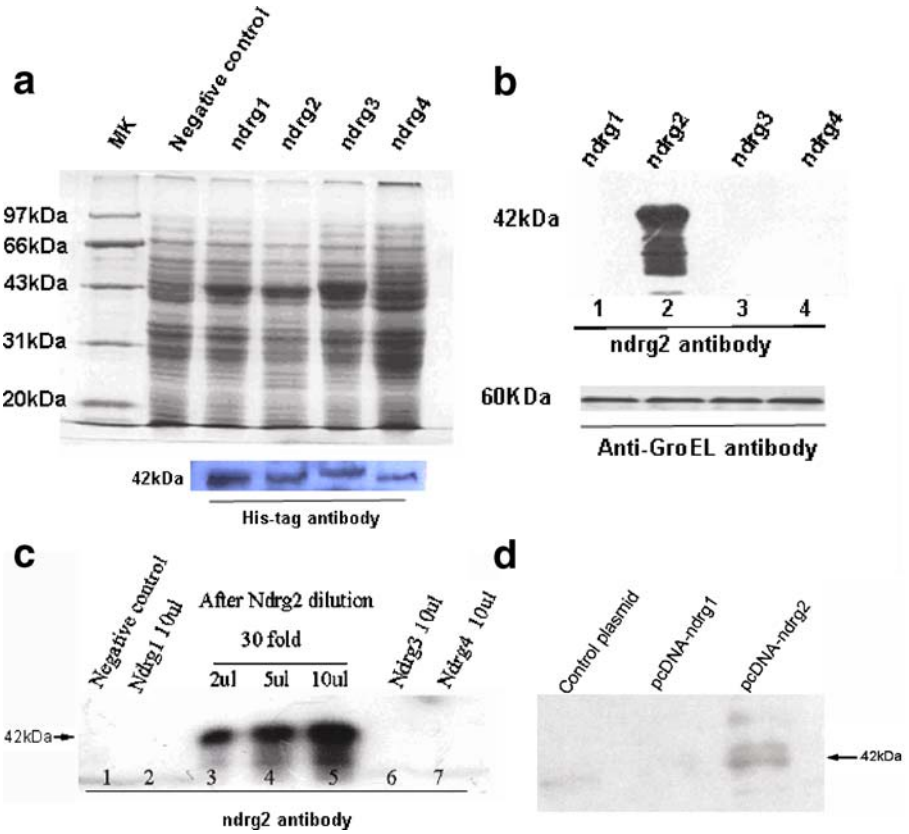

Fig. 2 *Analysis of FMU-Ndrg2.3 mAb by Western blot*. **a** 10 μl (50-μg aliquots) of each 6× His-tagged Ndrg1, Ndrg2, Ndrg3, and Ndrg4 was separated by 12% SDS-PAGE and stained with Coomassie Blue. MK stands for molecular weight marker. Negative control is the lysate from a vector without insert transformed *E. coli*. All four Ndrg protein expressions were determined by Western blot analysis using an anti-His-tag antibody. **b** Ten microliter (50-μg aliquots)of the bacterial lysate producing Ndrg1, Ndrg2, Ndrg3, and Ndrg4 fusion proteins were separated and were run into well of gel. After 12% SDS-PAGE separation, the proteins were transferred into NC membrane. *FMU-Ndrg2.3 mAb* (1:500) was used as the primary antibody for blotting. Equal loading of protein in each gel well was confirmed by blotting the gel with an antibody recognizing GroEL (a housekeeping gene product of *E. coli*). **c** Loading volume run onto SDS-PAGE well was different. Loading volume of bacterial lysate expressing ndrg1, ndrg3, and ndrg4B was 10 μl, but loading volume of bacterial lysate expressing ndrg2 was 2, 5, and 10 μl after the lysate was diluted 30 fold. **d** Western blot analysis of recombinant pcDNA-ndrg1 and pcDNA-ndrg2 separately transformed 293 cell line. The primary antibody was *FMU-Ndrg2.3 mAb* (1:500) and secondary antibody was HRP-labeled rabbit antimouse IgG (1:2,000)

were separated by 12% SDS-PAGE and subjected to Western blot analysis by the *FMU-Ndrg2.3* mAb we obtained. The immunoblot analysis clearly showed that the monoclonal antibody is specific against hNdrg2. There was no cross-reaction with hNdrg1, hNdrg3, and hNdrg4-B (Fig. 2b,c).

For determining whether *FMU-Ndrg2.3* mAb can also distinguish different member in mammalian cells, it was used to analyze Ndrg1 and Ndrg2 proteins in pcDNA-ndrg1- and pcDNA-ndrg2-transfected 293 cells. Only Ndrg2 protein could be detected by *FMU-Ndrg2.3* mAb but not Ndrg1 protein (Fig. 2d).

FMU-Ndrg2.3 mAb obtained from final cloned hybridoma cells was used at a 1:1,000 to 1:4,000 dilutions for Western blot. The recombinant hNdrg2 fusion proteins in bacteria lysates can be detected well in different dilutions. 6× His-tagged Ndrg2 protein from 5 μl of lysate can be detected even with 1:4,000 dilution of *FMU-Ndrg2.3* mAb (Fig. 3).

Tissue Distribution Features of Human Ndrg2 Demonstrated by Immunohistochemical Staining

We next investigated NDRG2 expression in different human tissues by immunohistochemistry using the *FMU-Ndrg2.3* mAb. The immunohistochemical specificity of *FMU-Ndrg2.3* mAb on human tissues was confirmed by staining the continuous sections of normal human liver with this mAb and nonimmune mouse serum was used as negative control. Strong staining of Ndrg2 protein can be seen clearly in the cytoplasm of hepatocytes and no immunoreaction appeared in the negative control (Fig. 4a,b).

The distributions of Ndrg2 protein in some human tissues were displayed by using *FMU-Ndrg2.3* mAb staining here. Ndrg2 protein expression was detected in both cortex and medulla of normal kidney (Fig. 5a). Ndrg2 signal was clearly seen in the proximal convoluted tubule and distal convoluted tubule. Immunoreaction was 1ocated at the cytoplasm of tubules and not in the nucleus. No expression was detected in the glomerular and periglomerular region, descending straight part of the proximal tubule, nephric loop, and ascending straight part of the distal tubule.

Immunoreactivity of Ndrg2 presented in Leydig cells of human testis and a positive immunoreaction was located at the cytoplasm (Fig. 5b) and not in the nucleus. No expression was detected in the dermatogenic cells.

All epididymal ducts were immunoreactive positive for Ndrg2, where the staining was predominantly observed in the cytoplasm of the pseudostratified epithelium (Fig. 5c). No expression was detected in tall columnal or cuboidal cells in the efferent ducts.

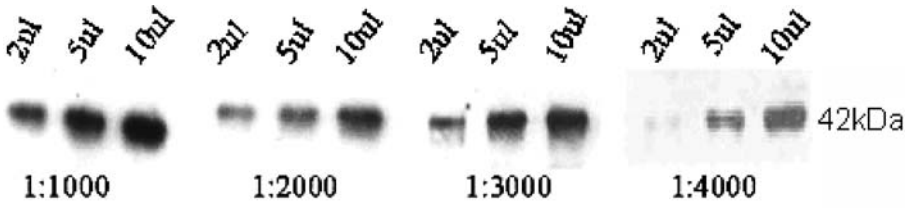

Fig. 3 *Western blot analysis of titer of monoclonal antibody against ndrg2*. The total protein of the bacteria expressing recombinant 6× His-tagged ndrg2 plasmid was run in 12% SDS-PAGE. The bacterial lysate was run into well of gel with 2, 5, and 10 μl (50-μg aliquots), respectively, after the lysate was diluted 30-fold. *FMU-Ndrg2.3 mAb* was diluted to 1:1,000–1:4,000, respectively. HRP-labeled rabbit antimouse IgG was diluted to 1:2,000

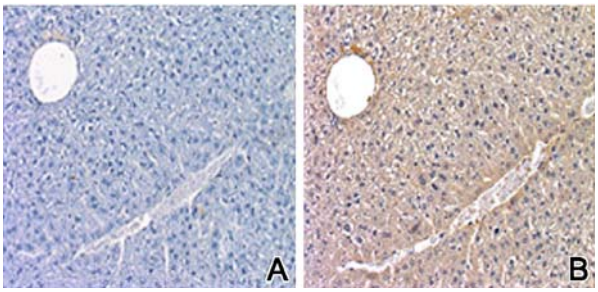

Fig. 4 *Immunohistochemical staining of NDRG2 protein in normal human liver.* Paraffin section of normal human liver stained with *FMU-Ndrg2.3 mAb* (diluted 1:50; **b**) and nonimmune mouse serum for negative control (**a**)

Strong immunoreactivity was located in the epithelium of the glandular lining, including columnar, cuboidal, squamose, and pseudostratified epithelium (Fig. 5d), but the scattered stromal histiocytes and other nonepithelial elements in the normal prostatic gland were negative for Ndrg2.

Cytoplasmic Ndrg2 staining was observed in the follicular epithelial cells of the normal thyroid gland (Fig. 5e), but the scattered stromal histiocytes and other nonepithelial elements were negative for Ndrg2.

Ndrg2 proteins are presented in the islets of Langerhans of human pancreas but not in the acinus of exocrine pancreas. All endocrine cells of the islets were immunoreactive positive (Fig. 5f). Positive staining was 1ocated at the cytoplasm of endocrine cells and not in the nucleus.

Tissue Distribution Features of Mouse Ndrg2 Demonstrated by Immunohistochemical Staining

To determine whether this monoclonal antibody can also recognize the ndrg2 protein in mouse tissues, an immunohistochemistry analysis was performed to detect the expression of ndrg2 protein in mouse distinct tissues. We found that the NDRG2 protein expression pattern in normal mouse kidney was similar to normal human kidney; positive signal appeared in the proximal convoluted tubule and distal convoluted tubule. (Fig. 6a).

The dark-brown reaction signals appeared in the base part the epithelia cells of the uterine gland mostly (Fig. 6b) and nuclei were not stained. There were no positive staining in epithelia cells of the mucosa and the endometrium stroma in the mouse uterus.

In skeletal muscle, Ndrg2-positive immunoreaction was located at the cytoplasm of sarcolemma and cross-striae of skeletal muscle cells (Fig. 6c). Ndrg2 was observed in the sarcolemma, intercalated discs, and cross-striae of cardiocytes (Fig. 6d).

Same as human, Ndrg2 were mainly located in the central region of pancreatic islets in mouse (Fig. 6e). There were no Ndrg2 immunoreactivity in the acinar cells and the duct cells of the exocrine pancreas.

In the small intestine, Ndrg2 were expressed predominantly at villus epithelium (Fig. 6f), and epithelia cells at the crypts were only weakly stained with Ndrg2 antibody. There was no positive staining in the lamina propria of the intestinal villi.

Discussion

Ndrg family is highly conserved in plants, invertebrates, and mammals, indicating the important functions of this gene family. More and more evidences show that Ndrg2 have a

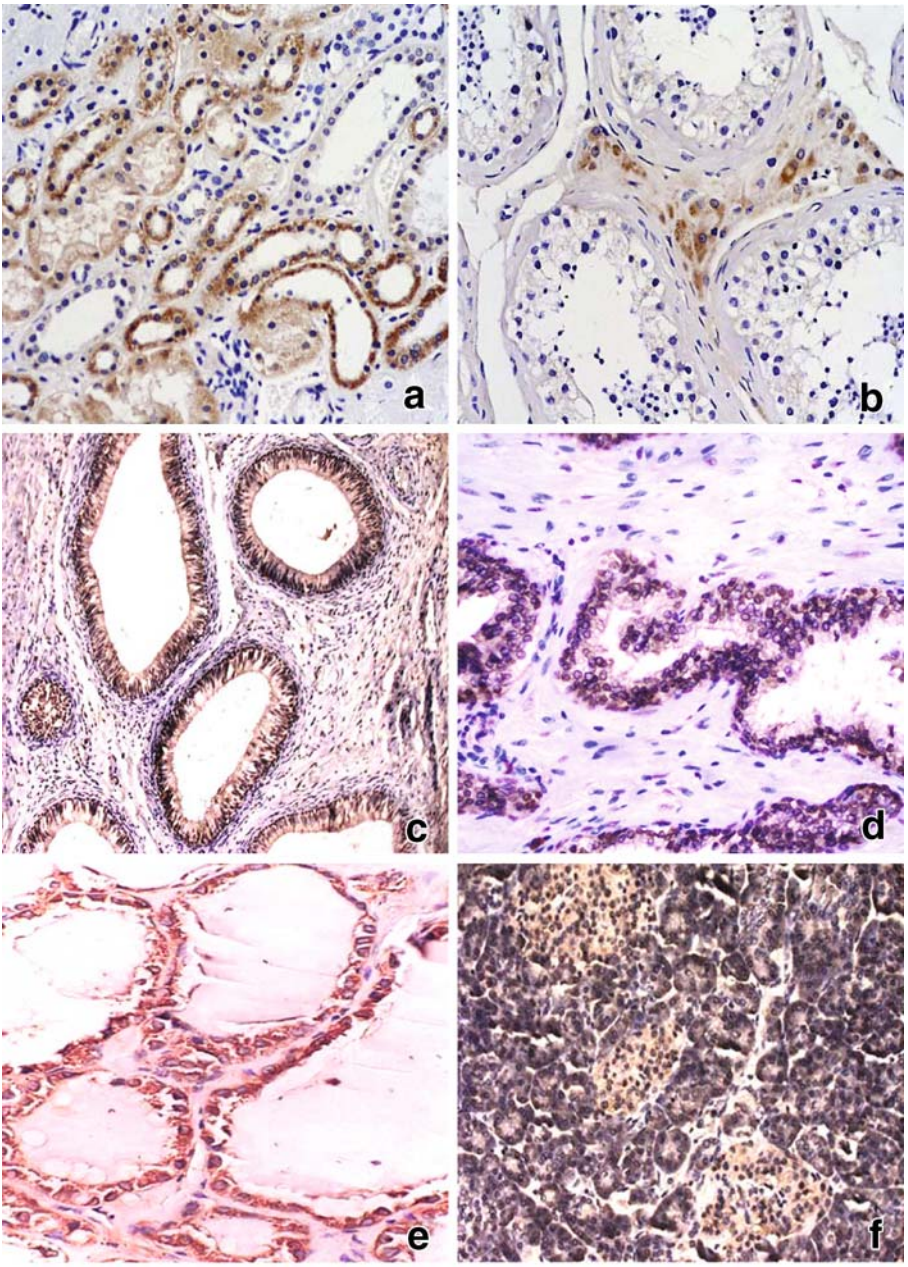

Fig. 5 *Immunohistochemical distribution of NDRG2 protein in normal human tissues*. **a** kidney (proximal and distal convoluted tubule); **b** testis (Leydig cell); **c** epididymis (epididymal ducts); **d** prostatic gland (epithelium of the glandular lining); **e** thyroid gland (follicular epithelial cell); **f** pancreas (islets)

wide spectrum of functions in regulating cell growth and differentiations. It is important to have highly specific antibody for further exploring the function of Ndrg2. It is also helpful if the tissue distribution of Ndrg2 protein can be characterized well.

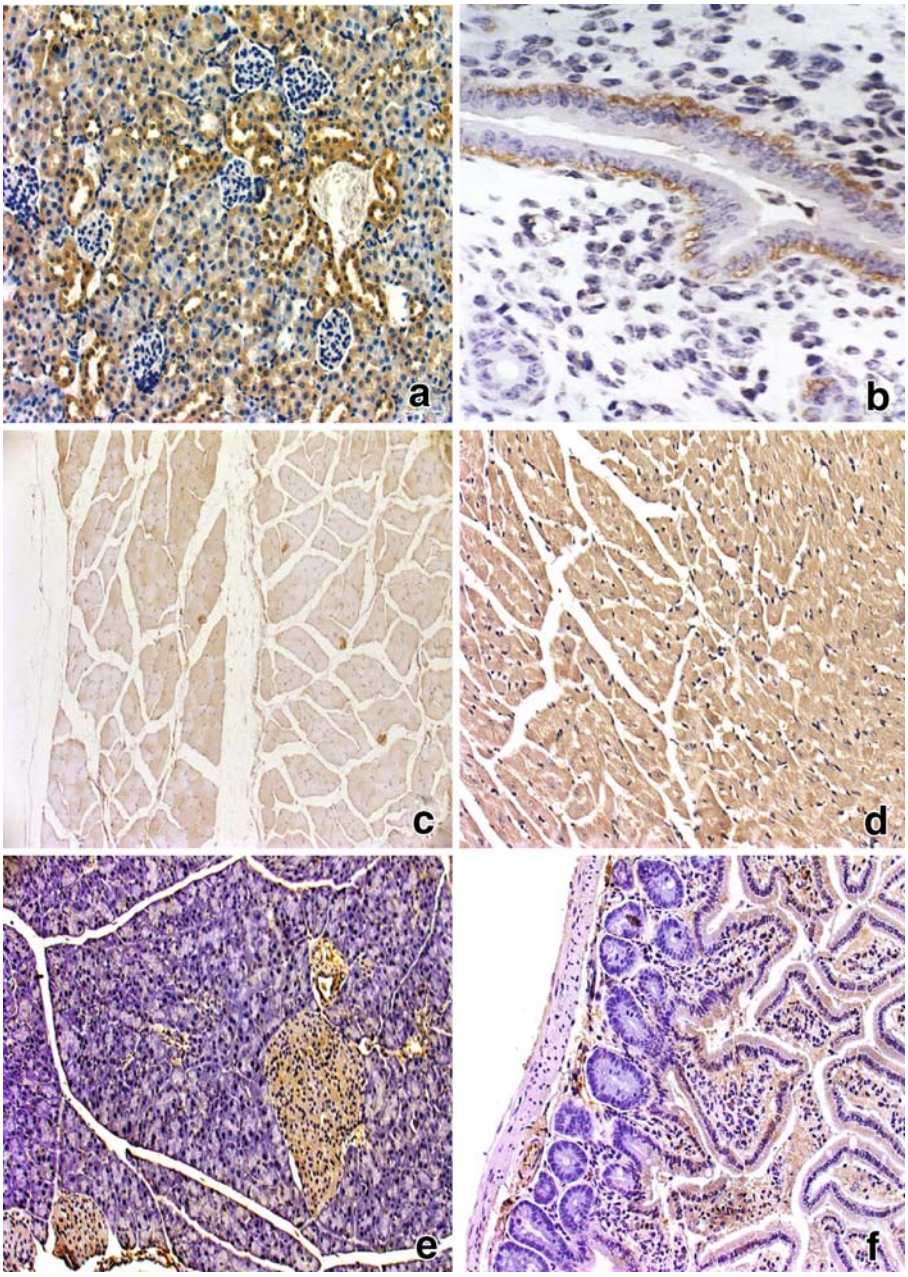

Fig. 6 *Immunohistochemical distribution of NDRG2 protein in normal mouse tissues*. **a** kidney (proximal and distal convoluted tubule); **b** uterus (the uterine gland); **c** skeletal muscle tissue (the sarcolemma and cross-striae of skeletal muscle cells); **d** heart (the sarcolemma, intercalated discs, and cross-striae of cardiocytes); **e** pancreas (the pancreatic islets); **f** small intestine (the villus epithelium)

In the present study, we generated a mAb, FMU-ndrg2.3. The data showed that this mAb is a specific anti-Ndrg2 antibody. There is almost no cross-reaction to other family members of Ndrg in Western blot assay and immunohistochemical staining. The antibody also can be used for detecting mouse and human embryo Ndrg2 in immunohistochemical staining [15, 16]. Therefore, we provided a very useful tool for Ndrg2 study.

We demonstrated some of tissue distribution features of Ndrg2 protein in human or in mouse. In the case of kidney, Ndrg2 mainly located in the proximal convoluted tubule and distal convoluted tubule. The result further confirmed the previous data reported by Sheerazed Boulkroun. In their paper, Ndrg2 mRNA was shown to be expressed specifically in the collecting duct, the site of aldosterone-regulated sodium absorption [17].

The expression analysis of Ndrg2 protein in some normal human or mouse tissues revealed some new and noteworthy information, such as Ndrg2 protein is located in Leydig cells of human testis, in epididymal ducts, in the islets of Langerhans of human, and in mouse pancreatic islets. The functional meaning of Ndrg tissue distribution will be studied further.

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