Acta Biochim Biophys Sin 2014, 46: 163–166 | © The Author 2013. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmt131.
Advance Access Publication 8 December 2013

Lab Note

Preparation of polyclonal antibody highly specific for mouse BRD7 protein and its application

Heran Wang[1†], Ran Zhao[1†], Ming Zhou[1*], Chi Guo[1], Yukun Liu[1], Shihe Jiang[1], Xiayu Li[2], Wei Xiong[1], Jian Ma[1], Shuping Peng[1], Xiaoling Li[1], David C. Schmitt[3], Ming Tan[3], and Guiyuan Li[1*]

[1]Cancer Research Institute, Central South University, Changsha 410078, China
[2]Department of Gastroenterology, The Third Xiang-Ya Hospital, Central South University, Changsha 410013, China
[3]Mitchell Cancer Institute, University of South Alabama, Mobile, AL 36604, USA
[†]These authors contributed equally to this work.
*Correspondence address. Tel: +86-731-84805412; Fax: +86-731- 84805383;
E-mail: zhouming2001@163.com (M.Z.)/E-mail: ligy@xysm.net (G.L.)

BRD7 was first identified as a novel bromodomain gene related to nasopharyngeal carcinoma (NPC) [1,2]. Overexpression of BRD7 in NPC cells inhibits cell growth and cell cycle progression from G1 to S phase by transcriptional regulation of important molecules involved in Ras/MEK/ERK, Rb/E2F, and β-catenin pathways [3–5]. BRD7 is mainly localized in the nucleus and possesses a functional nuclear localization signal sequence [5]. It has been identified as a subunit of the SWI/SNF chromatin-remodeling complexes and is involved in the regulation of transcription as a transcription factor [6,7].

BRD7 is highly conserved in multiple animal species, of which, the mouse may be an excellent animal model to investigate the function of BRD7. In our recent study, we have developed a BRD7 knock-out mouse model using C57BL mice (data not shown), which is being used to investigate the *in vivo* biological function of BRD7. In addition, BRD7 is a member of the bromodomain family, which consists of several members, such as BRD2, BRD3, BRD9, and BRDT, and all of these proteins have one or more bromodomains that are strikingly homologous to each other [8]. Therefore, a highly specific antibody to BRD7 that could not only recognize mouse BRD7 but also differentiate BRD7 protein from other members of the bromodomain family is necessary for the functional investigation of BRD7 in the mouse model.

In this study, we first aligned BRD7 protein sequences from multiple species using the ClustalW program, and the percentage of amino acid sequence identity compared with human is shown in **Fig. 1A**. This analysis supports that BRD7 protein is highly conserved among mammalian species, such as human, monkey, cow, and mouse. Interestingly, the mouse BRD7 is composed of 651 amino acids and has 88% sequence identity with the human homolog. Phylogenetic analysis with the maximum-likelihood method revealed that the human BRD7 grouped with the monkey homolog, while the mouse BRD7 represents a closely related group (**Fig. 1B**). Based on the analysis of homology and phylogenetics, and the evaluation of economic cost and maneuverability, the mouse would be an excellent animal model for investigation of BRD7 function. We further compared the homology of the bromodomain in each member of bromodomain family, such as BRD2, BRD3, BRD4, BRD7, BRD9, and BRDT, and found that the bromodomain in BRD7 has more than 60% amino acid identity shared with the other members of the bromodomain family, especially the amino acid identity is up to 84% between BRD7 and BRD9 (**Fig. 1C**), suggesting that the immunogenic region from BRD7 used for antibody preparation should exclude the bromodomain. We further predicted the immunogenicity region by bioinformatics and found that the region from amino acids 240–360 has more B-cell epitopes and lower homology with other proteins (data not shown), suggesting that this region named as mBRD7M is suitable for antibody preparation. The diagram of mBRD7 protein was shown in **Fig. 1D**, where the positions of mBRD7 bromodomain and mBRD7M fragment used for immunization were indicated, respectively, and the amino acid sequence of mBRD7M fragment is shown in **Fig. 1E**.

To incorporate the peptides coded by amino acids 240–360 of mBRD7, we constructed a prokaryotic expression vector. The mBRD7M fragment from nucleotides 718 to 1080 encoding the 121 amino acids of the middle region of the mBRD7 protein was amplified by polymerase chain reaction. After digestion with *Eco*RI and *Xho*I (Promega, Madison, USA), the fragment was inserted into the pGEX-4T-1 vector (Amersham Pharmacia Biotech, Buckinghamshire, UK), and the recombinant constructs were confirmed by digesting with *Eco*RI and *Xho*I and DNA sequencing (data not shown). The prokaryotic expression plasmid of pGEX-4T-mBRD7M was

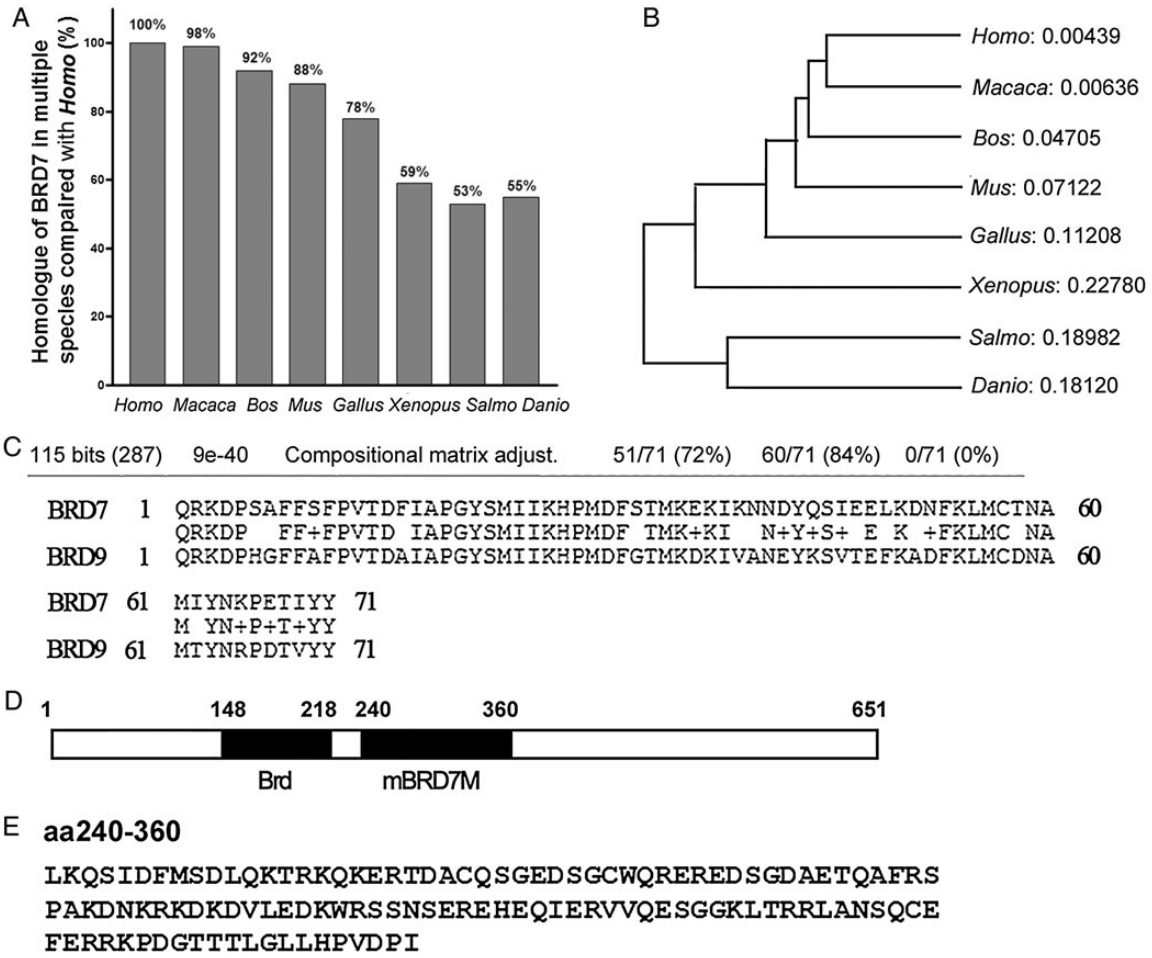

Figure 1. Sequence alignment and phylogenetic analysis of BRD7 homologs using ClustalW software (A) Percentage of amino acid sequence homolog of BRD7 protein from different species compared with humans. The amino acid identities are shown on the top of the column. (B) Phylogenetic analysis of BRD7 homologs using full-length amino acid sequences. The consensus tree was generated by the maximum-likelihood analysis (PHYLIP 3.6a3). The numbers next to each species indicate evolutionary distance, which measures the evolutionary divergence between two homologous sequences. (C) Homolog comparison of bromodomains between BRD7 and BRD9. The amino acid sequences of bromodomain in BRD7 and BRD9 were shown in the first lane and third lane, respectively, and the middle lane represents identical amino acids. Up to 84% identity of amino acid sequence between them was found. (D) The diagram of mouse BRD7 protein. The positions of bromodomain and mBRD7M fragment used for immunization were indicated, respectively. (E) The amino acid sequence of mBRD7M fragment used for immunization.

introduced into *Escherichia coli* BL21 according to standard protocol. Isopropylthio-β-galactoside (IPTG; Promega) was used to induce to the expression of the fusion protein. As expected, the GST-BRD7M fusion protein was highly and rapidly expressed in *E. coli*, and efficient expression of BRD7M could be achieved with 0.5 mM IPTG for 3.5 h at 37°C. Western blot analysis was performed to confirm the expression GST-mBRD7M with glutathione *S*-transferase (GST) antibody. **Figure 2A** shows that a main band with a molecular mass of 39 kDa was detected. After being purified by affinity chromatography, a band of the recombinant GST-BRD7M protein could predominantly be seen in the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel stained with Coomassie bright blue (Sigma, St Louis, USA), indicating that GST-mBRD7M was highly purified (**Fig. 2B**). The total amount of purified protein was >2 mg in 600 μl mixture, and the protein remained intact with no proteolytic degradation during the purification procedure.

Two rabbits were immunized with the purified protein. After five booster immunizations, the anti-sera were harvested 100 days post-primary immunization and subjected to affinity purification. A GST-Sepharose 4B affinity chromatography column was used in the purification system. Enzyme-linked immunosorbent assay (ELISA) results showed that the final titer of the purified rabbit anti-BRD7 serum from these two rabbits was 1 : 100,000, and the titer of flow through was 1 : 2000 or 1 : 500 (**Fig. 2C**). Rabbit serum collected before the day of primary immunization was applied as a negative control. Results from SDS–PAGE combined with bovine serum albumin (Sigma) showed that the estimated concentration of BRD7 antibody was 525 μg/ml for Rabbit 1 and 600 μg/ml for Rabbit 2 (**Fig. 2D**). Western blot results showed that these anti-sera were able to recognize recombinant

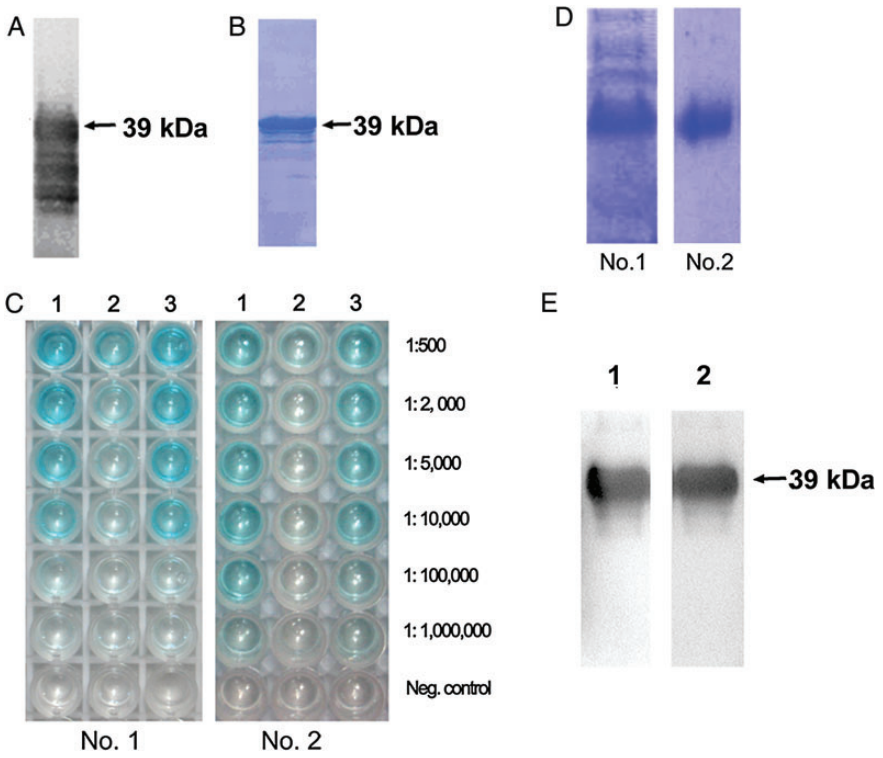

Figure 2. Purification of GST-mBRD7M fusion protein and preparation of anti-mBRD7 serum (A) GST-BRD7M fusion protein with a molecular mass of 39 kDa was detected by western blot with GST antibody. (B) GST-mBRD7M was highly purified by affinity chromatography. A band of recombinant GST-BRD7M protein could predominantly be seen in the SDS–PAGE gel stained with Coomassie bright blue. (C) The final titer of the purified anti-BRD7 serum was detected by ELISA assay. Lane 1: serum; lane 2: flow through; lane 3: purified antibody. No.1 and No. 2 represent number of rabbits used for preparation of anti-BRD7 serum, respectively. (D) The estimated concentration of anti-BRD7 serum was determined by SDS–PAGE stained with Coomassie bright blue. (E) The anti-sera were confirmed to recognize recombinant GST-BRD7M fusion protein by western blot assay. Lane 1. GST antibody; lane 2: mBRD7 antibody.

GST-BRD7M fusion protein with a molecular mass of 39 kDa, and the same was recognized by the anti-GST monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, USA) (**Fig. 2E**), suggesting that mBRD7 antibody had been successfully generated.

After obtaining the purified mBRD7 antibody, we performed western blot and immunofluorescence assays to further confirm the specificity. First, we transfected the constructs of pIRES-Neo3-Flag-mBRD7 into 3T3 cells and the blank vector was used as a control. Two days after transfection, we collected the cells and extracted the protein for western blotting analysis. Three kinds of antibodies, GST, mRRD7, and Flag, were used to simultaneously detect the expression of mBRD7. GST was used as a control. As shown in **Fig. 3A**, we found that the purified mBRD7 antibody could recognize the exogenously expressed Flag-BRD7, with a molecular mass of about 85 kDa, which corresponds to that recognized by monoclonal anti-Flag (Sigma) in the above cells (**Fig. 3A**, right lane 2) and has no cross-reactivity with GST (**Fig. 3A**, left lane 2). Moreover, weak endogenous BRD7 band was shown in 3T3 cells (**Fig. 3A**, middle lane 1) and merged with the exogenous BRD7 in BRD7-transfected cells (**Fig. 3A**, middle lane 2). Moreover immunofluorescence assay was further carried out to investigate the expression and subcellular localization of endogenous mBRD7 protein in 3T3 cells. Results showed that mBRD7 protein recognized by mBRD7 antibody is localized in the nucleus in a dotted distribution pattern (**Fig. 3B**), which is similar to the subcellular localization of the human BRD7 protein reported previously [5]. Furthermore, immunohistochemistry assay was performed to detect the application of BRD7 anti-sera with testis sections from adult mouse, and pre-immune sera were used as a control. Results showed that BRD7 was highly expressed in multiple steps of spermatids and localized in the nucleus (**Supplementary Fig. S1**).

In summary, we explored an approach to generate a highly specific polyclonal antibody against mouse BRD7 and showed that the prepared BRD7 antibody is able to specifically recognize both endogenous and exogenous protein. Moreover, this antibody could be used for the detection of BRD7 protein via western blot analysis, immunofluorescence, and immunohistochemistry assays. In particular, the prepared mBRD7 antibody will be helpful for studying the bio-functions of endogenously or exogenously expressed BRD7 protein in mouse models by *in vitro* or *in vivo* experiments.

Supplementary Data

Supplementary data is available at *ABBS* online.

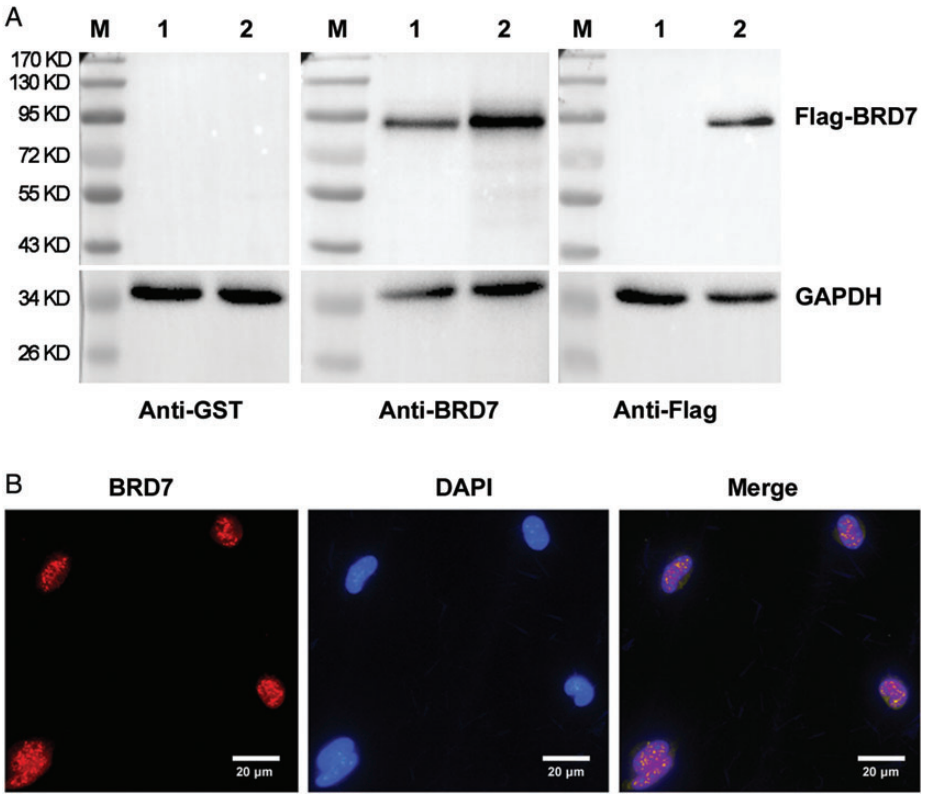

Figure 3. mBRD7 antibody specifically recognizes endogenous and exogenous mBRD7 protein (A) Expression of mBRD7 protein in NIH3T3 cells with transfection of blank vector or mBRD7 construct by western blot assay with different antibodies to GST, mBRD7, and flag, respectively. The right panel shows the western blot results with anti-GST, the middle panel shows the results with anti-BRD7 sera, and the left panel is with anti-flag. Lane 1: 3T3 cells with the transfection of blank vector; lane 2: 3T3 cells with the transfection of mBRD7 construct. (B) Expression and subcellular localization of endogenous mBRD7 protein in 3T3 cells was detected with mBRD7 antibody by immunofluorescence assay. Left panel: BRD7; middle panel: DAPI staining; right panel: merge of the left and middle panels.

Funding

This work was supported by grants from the National Natural Science Foundation of China (Nos. 81071686, 81171934, 81328019), the Programme of Introducing Talents of Discipline to Universities of China (No. 111-2-12), the Project Sponsored by the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry (the 44th), and the Free Exploration Program of Central South University of China (No. 2013zzts073).

References

1. Yu Y, Zhu S, Zhang B, Zhou M, Xiang J, Li X and Li G, *et al*. Growth suppression of nasopharyngeal carcinoma cell line through transfection of *BRD7* gene. Chin J Cancer 2001, 20: 569–574.
2. Peng C, Zhou J, Liu HY, Zhou M, Wang LL, Zhang QH and Yang YX, *et al*. The transcriptional regulation role of BRD7 by binding to acetylated histone through bromodomain. J Cell Biochem 2006, 97: 882–892.
3. Zhou J, Ma J, Zhang BC, Li XL, Shen SR, Zhu SG and Xiong W, *et al*. *BRD7*: a novel bromodomain gene, inhibits G1-S progression by transcriptionally regulating some important molecular involved in Ras/MEK/ERK and E2F pathways. J Cell Physiol 2004, 200: 89–98.
4. Peng C, Liu HY, Zhou M, Zhang LM, Li XL, Shen SR and Li GY, *et al*. BRD7 suppresses the growth of nasopharyngeal carcinoma cells (HNE1) through negatively regulating beta-catenin and ERK pathways. Mol Cell Biochem 2007, 303: 141–149.
5. Zhou M, Liu HY, Xu XJ, Zhou HD, Li XL, Peng C and Shen SR, *et al*. Identification of nuclear localization signal that governs nuclear import of BRD7 and its essential roles in inhibiting cell cycle progression. J Cell Biochem 2006, 98: 920–930.
6. Kaeser MD, Aslanian A, Dong MQ, Yates JR, III and Emerson BM. BRD7, a novel PBAF-specific SWI/SNF subunit, is required for target gene activation and repression in embryonic stem cells. J Biol Chem 2008, 283: 32254–32263.
7. Harte MT, O'Brien GJ, Ryan NM, Gorski JJ, Savage KI, Crawford NT and Mullan PB, *et al*. BRD7, a subunit of SWI/SNF complexes, binds directly to BRCA1 and regulates BRCA1-dependent transcription. Cancer Res 2010, 70: 2538–2547.
8. Filippakopoulos P, Picaud S, Mangos M, Keates T, Lambert JP, Barsyte-Lovejoy D and Felletar I, *et al*. Histone recognition and large-scale structural analysis of the human bromodomain family. Cell 2012, 149: 214–231.