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Production and characterization of domain-specific monoclonal antibodies against human ECM1

Ya Li [a], Yanqing Li [a], Junli Zhao [a], Dongyang Wang [a], Qinwen Mao [b], Haibin Xia [a, *]

[a] Co-Innovation Center for Qinba Regions' Sustainable Development, College of Life Sciences, Shaanxi Normal University, 199 South Chang'an Road, Xi'an 710062, Shaanxi, PR China
[b] Department of Pathology, Northwestern University Feinberg School of Medicine, 303 E. Chicago Avenue, Chicago, IL 60611, USA

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ABSTRACT

Human extracellular matrix protein-1 (hECM1), a secreted glycoprotein, is widely expressed in different tissues and organs. ECM1 has been implicated in multiple biological functions, which are potentially mediated by the interaction of different ECM1 domains with its ligands. However, the exact biological functions of ECM1 have not been elucidated yet, and the functional study of ECM1 has been partially hampered by the lack of sensitive and specific antibodies, especially those targeting different ECM1 domains. In this study, six strains of monoclonal antibody (MAb) against hECM1 were generated using purified, prokaryotically-expressed hECM1 as an immunogen. The MAbs were shown to be highly sensitive and specific, and suitable for western blot, immunoprecipitation assays and immunohistochemistry. Furthermore, the particular ECM1 domains recognized by different MAbs were identified. Lastly, the MAbs were found to have neutralizing activities, inhibiting the proliferation, migration and metastasis of MDA-MB-231 cells. In conclusion, the domain-specific anti-ECM1 MAbs produced in this study should provide a useful tool for investigating ECM1's biological functions, and cellular pathways in which it is involved.

1. Introduction

Extracellular matrix protein 1 (ECM1), which was originally derived from the osteogenic mouse stromal cell line MN7 [1–3], is a secreted glycosylated protein with a molecular weight of 85 kDa. The human ECM1 (hECM1) gene is mapped to chromosome 1q21 outside of the epidermal differentiation complex region [4,5]. hECM1 has four splice variants present – ECM1a (540 aa), ECM1b (415 aa), ECM1c (559 aa) and ECM1d (57 aa) – with ECM1a being the most widely expressed [2,6,7]. The full-length hECM1 contains five different regions, a signal peptide of 19 amino acids, an N-terminal cysteine-free domain, two tandem repeats and a C-terminal domain. The latter three domains have the typical CC-(X_{7-10})-C arrangement, and this organization generates "double-loop" domains involved in ligand-binding functions [7]. ECM1 was reported to have a few binding partners, e.g., perlecan [8], fibulin-1 [9,10], cartilage oligomeric matrix protein [11] and matrix metalloproteinase 9 [12]. ECM1 has also been implicated in multiple physiological functions, such as cell proliferation [13], angiogenesis [14,15], migration [16] and metastasis [15–20]. However, the exact biological functions of ECM1 remain unclear, and the functional study of ECM1 has been hampered partially by the lack of sensitive and specific antibodies, especially those targeting different ECM1 domains.

To facilitate the elucidation of ECM1's functions, domain-specific monoclonal antibodies (MAbs) against hECM1 were generated and characterized. The results showed that these MAbs retained high affinity for hECM1 and were suitable for a wide range of biochemical assays.

2. Material and methods

2.1. Cell culture

HEK 293 (human embryonic kidney cell line), MDA-MB-231 (breast adenocarcinoma cell line), U87MG (human glioblastoma-astrocytoma, epithelial-like cell line) and SP2/0 myeloma cells

* Corresponding author. Department of Biochemistry, College of Life Sciences, Shaanxi Normal University, 199 South Chang'an Road, Xi'an 710062, PR China. Tel./fax: +86 29 85310272.
E-mail address: hbxia2001@163.com (H. Xia).

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were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured by Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 1% penicillin-streptomycin and 1% L-glutamate. Mouse SP2/0 myeloma cells were cultured in RPMI 1640 medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum. The cell lines were cultured in a humidified chamber with 5% CO_2 at 37 °C.

2.2. Establishment of ECM1 knockout MDA-MB-231 cell line

To generate ECM1 knockout MDA-MB-231 cell line, four pairs of the single guide RNAs (sgRNAs) targeted to exon 6 and exon 7 of human ECM1 gene were designed. The sequences of sgRNA 1–4 were listed in Table 1. The sgRNAs 1–4 were cloned into sgRNA expression vector pU6-sgRNA. The positive clones were confirmed by restriction enzyme digestion and sequencing. The resultant plasmids were named pU6-hECM1-sgRNA1, pU6-hECM1-sgRNA2, pU6-hECM1-sgRNA3 and pU6-hECM1-sgRNA4, respectively. The sgRNA2 was shown to have best activity by T7 endonuclease I (T7EI) assay. Then pU6-hECM1-sgRNA2 with highest efficiency was digested by *Kpn* I and *Spe* I and ligated with a Cas9 expression vector driven by a CMV promoter through *Kpn* I and *Spe* I. The resulting plasmid was called phECM1-sgRNA2-CMV-Cas9. The donor vector was constructed according to the previous publication [21], which included a up and down homologous arm and two cassettes for positive and negative selection. The obtained plasmid was called pUC19/ECM1 donor. The primers used for amplifying homologous arms were listed in Table 1. MDA-MB-231 cells were co-transfected with phECM1-sgRNA2-CMV-Cas9 and pUC19/ECM1 donor by electroporation and then screened in the presence of G418 (200 μg/mL), then MDA-MB-231 cell clones were further screened in the culture medium containing GCV (1.0 mg/mL). Single cell clone with hECM1 knockout was obtained through limited dilution after positive and negative selection, then further verified by PCR and sequencing. The obtained clone was named $hECM1^{-/-}$ MDA-MB-231 cell line.

2.3. Plasmid construction

The full length of hECM1, the full length of hECM1 without a signal peptide and each domain of human ECM1 (hECM1) were generated by PCR using the primers shown in Table 1. All PCR products were gel-purified and cloned into pGEM-T easy vector (Promega, Madison, WI). The positive clones were confirmed by restriction enzyme digestion and sequencing. The resultant plasmids were named pGEMT-hECM1, pGEMT-hECM1-no sp., pGEMT-hECM1-N-terminal, pGEMT-hECM1-Repeat I, pGEMT-hECM1-Repeat II and pGEMT-hECM1-C-terminal. The full length of hECM1 digested from pGEMT-hECM1 was subcloned into adenoviral E1 shuttle vector pAd5-E1-CMV-MCS-Flag by restriction sites *Cla* I and *Xba* I. The resultant plasmid was named pAd5-E1-CMV-hECM1-Flag. The full length of hECM1 without a signal peptide digested from pGEMT-hECM1-no sp. was subcloned into prokaryotic expression vector pET-28a (+) (Novagen, San Diego, CA) through restriction sites *Cla* I and *Xba* I. The obtained plasmids were called pET28a-hECM1-6His. N-terminal, Repeat I and Repeat II domains of hECM1 digested from pGEMT-hECM1-N-terminal, pGEMT-hECM1-Repeat I and pGEMT-hECM1-Repeat II were cloned into prokaryotic expression vector pGEX-4T-3 (Amersham Pharmacia Biotech, Piscataway, NJ), and the C-terminal domain of hECM1 digested from pGEMT-hECM1-C-terminal was cloned into pET-28a (+). The resultant plasmids were named pGEX-4T-3-hECM1-N-terminal, pGEX-4T-3-hECM1-Repeat I, pGEX-4T-3-hECM1-Repeat II and pET28a-hECM1-C-terminal.

2.4. Expression of recombinant ECM1 proteins with different lengths

The pET28a-hECM1-6His plasmid was transformed into *Escherichia coli* Rosetta and the recombinant plasmids of pGEX-4T-3-hECM1-N-terminal, pGEX-4T-3-hECM1-Repeat I, pGEX-4T-3-hECM1-Repeat II and pET-28a-hECM1-C-terminal were transformed into *E. coli* BL21 (DE3). The corresponding recombinant protein was induced by isopropyl- β-D-1-thiogalactopyranoside (Sigma, St. Louis, MO), and was purified by the Ni-NTA Purification System (Qiagen, Valencia, CA). All prokaryotic expressed

Table 1
All primers used for amplifying hECM1 and establishing hECM1 knockout cell line.

Primer	Sequence
Full length of ECM1	Forward: 5′-TATCGATATGGGGACCACAGCCAGA-3′ Reverse: 5′-TTCTAGATTCTTCCTTGGGCTCAGA-3′
Full length of ECM1 without signal peptide	Forward: 5′-TATCGATATGGCCTCTGAGGGAGGC-3′ Reverse: 5′-TTCTAGATTCTTCCTTGGGCTCAGA-3′
ECM1 N-terminal	Forward: 5′-TATCGATATGGCCTCTGAGGGAGGC-3′ Reverse: 5′-TTCTAGACTGGGCTGCATTCCAGGAC-3′
ECM1 Repeat I	Forward: 5′-TATCGATATGCACTGCCAACAGGACCG-3′ Reverse: 5′-TTCTAGAGTAGTGTGGCTGGGGAGCT-3′
ECM1 Repeat II	Forward: 5′-TATCGATATGCAGCTCCGGGCCTG-3′ Reverse: 5′-TTCTAGAATAGTTGGGGTAAGGAGCCCG-3′
ECM1 C-terminal	Forward: 5′-TATCGATATGGACCGGGACATCTTG-3′ Reverse: 5′-TTCTAGATTCTTCCTTGGGCTCAG-3′
ECM1 sgRNA 1	Forward: 5′-ACCGCCAGCACTGCCAACAGGAC-3′ Reverse:5′-AAACGTCCTGTTGGCAGTGCTGG-3′
ECM1 sgRNA 2	Forward: 5′-ACCGGGATGGCTTCCCCCCTGGG-3′ Reverse:5′-AAACCCCAGGGGGGAAGCCATCC-3′
ECM1 sgRNA 3	Forward: 5′-ACCGTTCTGTGAGGCCGAGTTCT-3′ Reverse:5′-AAACAGAACTCGGCCTCACAGAA-3′
ECM1 sgRNA 4	Forward: 5′-ACCGAGCTACTGACCCCCTACAA-3′ Reverse:5′-AAACTTGTAGGGGGTCAGTAGCT-3′
ECM1 up homologous arm	Forward: 5′-AGGTACCATCCCTGCTCCTTGGTGC-3′ Reverse:5′-TATCGATTGGAATGGGGAAAACAGTG-3′
ECM1 down homologous arm	Forward: 5′-AGTCGACGTAAGTGGGCGTCCCAGC-3′ Reverse:5′-AAGATCTGGTGTGAAAGGGGCAGGGGT-3′