A Type I Monoclonal Antibody Identifies a Common Epitope on Multiple Isoforms of the Porcine CD34 Antigen

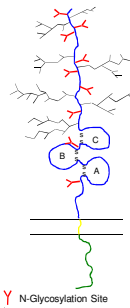
T.W. Jensen[1], D.A. Swanson[1], L.A. Rund[2], K. Kim, S.G. Clark-Deener[3], L.B. Schook[2]

[1]Institute for Genomic Biology, [2]Department of Animal Sciences, [3]Veterinary Medicine, University of Illinois at Urbana-Champaign 61801

Abstract

CD34 is an important marker for the identification and enrichment of stem and progenitor cell subsets used in a variety of cell-based therapies but there are no commercial monoclonal antibodies available that cross-react with pig CD34 (pCD34). We have produced a monoclonal antibody which recognizes multiple isoforms of pig CD34. Balb/c mice were immunized with a fusion protein encompassing the extracellular region of porcine CD34. After screening a panel of 480 monoclonal antibodies we identified 1 antibody, 3G7, capable of recognizing porcine CD34 expressed in CHO cells, hematopoietic cells, adipose derived stromal vascular cells, and endothelial progenitor cells. The epitope recognized by 3G7 is sensitive to both glycoprotease and neuraminidase treatment, classifying 3G7 as a Type I CD34 antibody. We have previously identified multiple isoforms of porcine CD34 resulting from multiple single nucleotide polymorphisms in the CD34 gene. The 3G7 antibody was able to recognize two isoforms of pCD34 differing by 6 amino acids. Both isoforms had similar sensitivity to both glycoprotease and neurominidase treatment. Western blot analysis of protein extracts from CD34-expressing CHO cells and enriched CD34+ primary cells demonstrates that pig CD34 expressed in these mammalian cells has an apparent electrophoretic mobility of approximately 100kD. The Type I 3G7 monoclonal antibody recognizes multiple isoforms of pCD34 providing a robust reagent to identify CD34+ cells in porcine tissues. This antibody will expand the potential of the swine model for use in preclinical trials of stem cell therapies.

Background



Human (NP001764.1) to Pig (NP999251.1) CD34 protein alignment

Extracellular Region:

Transmembrane Region:

Intracellular Region:

Due to low sequence identity in the extracellular region of the CD34 protein, attempts at identifying antibodies that exhibit species cross reactivity have failed. In the pig model there has been one previous report of an anti-porcine CD34 monoclonal antibody (Layton et al. Exp. Hem. 2007). Sakurai et al. (Exp. Hem. 2006) identified an anti-bovine CD34 antibody sensitive to a single amino acid substitution resulting from a SNP. Because we have identified multiple SNP variations in pCD34 we have generated a robust monoclonal antibody that cross reacts with pCD34 varying by 6 amino acids.

Methods

Animals and tissue collection: Porcine tissues were collected from Yorkshire crossbred pigs approximately 3 months old. Adipose derived stromal vascular cells (ADSCs) were obtained from the back fat of test animals. Four-week-old BALB/c mice (University of Illinois Immunolgical Resource Center) were used for monoclonal antibody production. All work was performed with approval from University of Illinois Institutional Animal Care and Use Committee (IACUC).

Cloning and sequencing of the porcine CD34 gene and CD34 protein expression: Single stranded cDNA synthesis was performed using AMV reverse transcriptase (Promega, Madison, WI) and a primer specific to porcine CD34: 5'-CCG GTA CCT CAC AGT TCA GTA TCT GCC ACC-3' or Oligo dT primers. The region encoding porcine CD34 was amplified by PCR using the forward primer 5'-CCG CTA GCA GGA TGC TCC GCA GG-3' and reverse primer 5'-CCA AGC TTC AGT CAC AGT TCA GTA TCT GCC A-3'. PCR products were subcloned into pGEM-T Easy vector (Promega) for sequencing. Individual clones were sequenced at the U. of Ill. Core DNA Sequencing Facility (UICDSF).

• Two sequences of pCD34 were identified varying by 6 amino acids (5 extracellular amino acids).

SNP distribution between Sequence 1 (and) 2	Resulting amino acid change
A (108) G	--
A (112) G	Ile (38) Val
G (130) A	Ala (44) Thr
G (159) A	--
C (165) T	--
T (247) C	Ser (83) Pro
C (413) T	Ala (138) Val
T (420) C	--
A (868) C	Asn (290) His
T (1082) C	Val (361) Ala
G (1170) A	--

The extracellular domain of porcine CD34 was amplified by PCR using the primers 5'-CGA CTA GGG TAC CGC TGT GAA CAG CTC GAC TGT-3' and reverse primer 5'-TAG TAA GCT TGC TTT CGG GAG TAG CTC TGG TG-3'. The product was subcloned into the pQE2 vector (Qiagen). M15 production bacteria was used to generate His-tagged extracellular pCD34. Products were purified on Ni-NTA columns.

Full protein coding regions were then subcloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA) and used to transfect Chinese hamster ovary (CHO) cells.

Antibody generation: Balb/c mice were inoculated with the extracellular domain of Sequence 2 at the University of Illinois Immunological Resource Center. Spleen cells were harvested and fused with Sp1 cells following standard protocols. Antibody containing media was screened against the original immunogen using an enzyme-linked immunosorbent assay (ELISA) with Sequence 2.

Flow cytometry: For antibody typing enzyme treatment, cells were treated with either 20mg O-sialoglycoprotein endopeptidase from *Pasteurella haemolytica* (Cedarlane Laboratories, Ontario, Canada) or 0.1 units neuraminidase from *Vibrio cholerae* (Sigma Aldrich) for 30 minutes at 37ºC followed by two rinses with DMEM media containing 10% FBS. Cells were analyzed on a BD Biosciences LSR II flow cytometer.

Results

Antibody Screening: Antibodies were initially screened by ELISA. Positive clones were tested by flow cytometry using a a goat anti-mouse IgG1 secondary conjugated to R-phycoerythrin (Invitrogen) for reactivity to bone marrow and freshly isolated adipose cells.

• One clone (3G7) was reactive to an appropriate percentage of bone marrow and adipose cells (Figure 1).

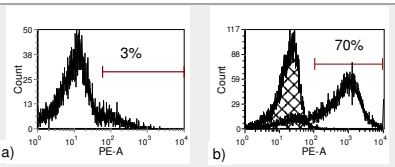


Figure 1. Antibody 3G7 identifies subpopulations of cells in both a) bone marrow and b) freshly isolated adipose cells in expected percentages.

To confirm reactivity to the original antigen used to generate 3G7, a Western blot was run using protein from the bacteria used to generate the original antigen.

• In a Western blot, 3G7 labeled the extracellular region of both Sequence 1 and Sequence 2 pCD34 produced in bacteria but not a control His-tagged protein (Figure 2).

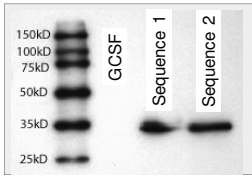


Figure 2. Antibody 3G7 labels the extracellular domain of both Sequence 1 and 2 pCD34 with the expected molecular weight of 31kD but not a similarly His-tagged control protein GCSF, thus confirming the antibody is not reactive to the Histidine tag sequence.

• 3G7 identifies a protein from adipose cell extracts with an apparent electrophoretic mobility of 100kD, consistent with the highly gycosylated forms of CD34 identified in both mouse and human. (Fig 3).

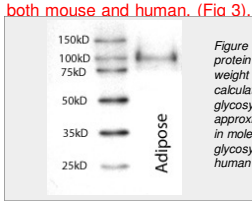


Figure 3. Antibody 3G7 labels a protein with an apparent molecular weight of approximately 100kD. The calculated molecular weight of non-glycosylated porcine CD34 is approximately 38kD. The increase in molecular weight due to glycosylation is consistent with both human and mouse CD34.

• 3G7 binds to both sequences of pCD34 expressed on the surface of CHO cells by flow cytometry. This binding is eliminated after glycoprotease treatment (Fig 4).

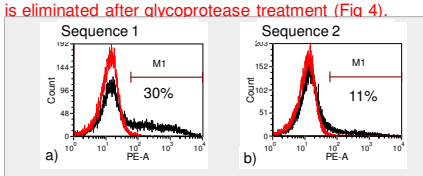


Figure 4. Antibody 3G7 binding is reduced to baseline after treatment with glycoprotease for both a) Sequence 1 and b) Sequence 2. Red lines indicate glycoprotease treated cells. Glycoprotease treated cells statistically similar to baseline controls

CHO cell constructs were enriched for pCD34 expression via FACS using 3G7 antibody. Binding of 3G7 to culture expanded, enriched cells is inhibited by neuraminidase.

• 3G7 is classified as a Type I CD34 antibody due to its sensitivity to both glycoprotease and neuraminidase treatment (Figs 4 and 5).

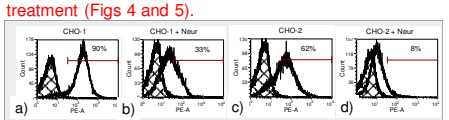


Figure 5. CHO cells expressing both a) Sequence 1 and c) Sequence 2 are enriched and culture expanded. 3G7 binding to both b) Sequence 1 and d) Sequence 2 is inhibited by treatment with neuraminidase.

Endothelial progenitor cells (EPCs) cultured from peripheral blood are stained with EPCs markers CD31, LDL receptor, and CD34.

• 3G7 can also be used in immunohistochemical assays to identify pCD34 (Fig 6).

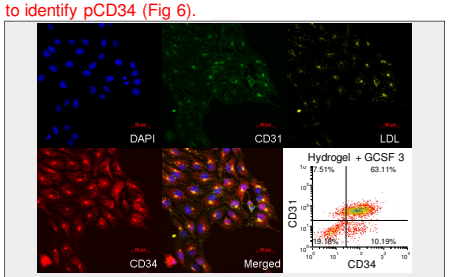


Figure 6. Porcine EPCs are CD34+ as identified by the 3G7 antibody. CD34 is expressed in common with other EPC markers including CD31 and LDL receptor.

Conclusions

The monoclonal antibody 3G7 is a robust reagent for identifying porcine CD34 in applications including Western Blot, Flow Cytometry, and Immunohistochemical staining. It recognizes a common epitope on pCD34 proteins varying, due to SNP variations, by 5 amino acids in the extracellular region. This antibody will be useful for procedures in the pig model designed to replicate clinical trials utilizing CD34+ stem cells as well as other applications requiring identification of CD34+ cells.

Acknowledgments

• This work was funded by grants from the USDA (AG 2008-34480-19328 and 538 AG 2009-34480-19875) and by a Fellowship from Carle Foundation Hospital.

ILLINOIS