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Epitope Recognition in the Human–Pig Comparison Model on Fixed and Embedded Material

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Summary

The conditions and the specificity by which an antibody binds to its target protein in routinely fixed and embedded tissues are unknown. Direct methods, such as staining in a knock-out animal or in vitro peptide scanning of the epitope, are costly and impractical. We aimed to elucidate antibody specificity and binding conditions using tissue staining and public genomic and immunological databases by comparing human and pig—the farmed mammal evolutionarily closest to humans besides apes. We used a database of 146 anti-human antibodies and found that antibodies tolerate partially conserved amino acid substitutions but not changes in target accessibility, as defined by epitope prediction algorithms. Some epitopes are sensitive to fixation and embedding in a species-specific fashion. We also find that half of the antibodies stain porcine tissue epitopes that have 60% to 100% similarity to human tissue at the amino acid sequence level. The reason why the remaining antibodies fail to stain the tissues remains elusive. Because of its similarity with the human, pig tissue offers a convenient tissue for quality control in immunohistochemistry, within and across laboratories, and an interesting model to investigate antibody specificity. (J Histochem Cytochem 63:805–822, 2015)

Keywords

antigen, epitope, immunohistochemistry, swine, quality control

Introduction

Diagnostic immunohistochemistry (IHC) is a technique stably embedded in the daily practice of human pathology worldwide. The vast majority of IHC tests are done on tissue which has been fixed in formalin, embedded in paraffin (FFPE), and stored at room temperature. The introduction of a heat-mediated process for the retrieval of the immune reactivity of the tissue (antigen retrieval; AR) (Shi et al. 1991) has further consolidated this practice. Nowadays, IHC is the most versatile of the companion diagnostics needed for individualized therapy (Taylor 2014).

The diagnostic use of IHC requires standardization by adoption of common analytical protocols (both pre-analytical and analytical) and quality control (QC) programs, as suggested by the College of American Pathologists (Fitzgibbons et al. 2014; Goldstein et al. 2007; Hardy et al. 2013; Robb et al. 2014). In Europe, at least two initiatives (NordicQC in Denmark and NEQAS in UK) run voluntary QC tests for IHC. QC tests are run on human tissue remnants from the

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operating theater, which are used as external control tissue samples; although, there are severe procurement and quality limitations for normal noble tissues such as brain, brainstem, heart, and some endocrine organs (pituitary, parathyroid). In addition, ethical considerations and nation-specific rules restrict the exchange of material across Europe (Riegman and van Veen 2011; van Veen et al. 2006).

Despite the widespread use of this technique, the most difficult task is to validate the staining in situ, given that a surprisingly high number of antibodies are not specific for the intended target (Bradbury and Plückthun 2015). At the beginning, immunizing an animal with a biochemically purified abundant antigen and staining with a relatively low-level sensitivity method was enough to produce results with acceptable specificity. However, the advent of the Human Genome project brought the need to produce specific antibodies against molecularly discovered antigen-carrying proteins, often expressed at low level and bearing sequence domains that are shared with other unrelated proteins.

The introduction of methods of synthetic peptide immunization with unique sequences from the desired protein as well as monoclonal antibody production effective in FFPE tissues (Jones et al. 1993) has consolidated the presence of IHC in daily practice. Antibodies raised against synthetic peptides may recognize continuous epitopes in FFPE material (Jones et al. 1993; Sompuram et al. 2006). Indeed, indirect evidence from an analysis of rabbit antisera produced against recombinant sequences of 50–150 amino acids showed that target-specific components of antisera directed against linear epitopes correctly identifies denatured targets on a western blot, whereas those against conformational epitopes do not (Forsström et al. 2015).

Furthermore, the binding of anti-peptide antibodies may be inhibited through competition with the immunizing peptide. However, this does not guarantee specificity (Holmseth et al. 2012), and specificity is therefore implied by a complex combination of indirect evidence (Bordeaux et al. 2010; Smith and Womack 2014). Then again, probing a knock-out experimental animal or employing a gene-silenced cell line, while the most stringent, are often impractical and costly solutions.

Ideally, one would need an identically processed tissue as that of the diagnostic human biopsy, which contains controlled and known variations of the antibody epitope and abundant similar background noise-producing bystander proteins to mimic the staining conditions in the human material. The use of an animal substitute for this task may represent a convenient solution for exploration. The extremely low probability that two unrelated but antigenically identical proteins may be represented in another species preserving the similarity of the unrelated sequences will make a differential immunostaining across the two species the evidence that the antibody is not recognizing an epitope uniquely identifying a protein. On the other hand, conserved proteins performing an identical function in related species may be found identically distributed and therefore similarly stained in the same tissues.

The domestic pig (*Sus scrofa*) is farmed across Europe for human consumption. Overall, the pig genome is 84.1% homologous to the human genome (Fang et al. 2012), one of the closest taxa after the primates. The pig and human genomes diverged 97 million years ago; yet the porcine genome has extensive similarities with the human genome and, thus, it represents an interesting disease model (Groenen et al. 2012). About 250 genes were gained or lost in each species after divergence, most notably the interferon-associated genes and olfactory genes, among others (Fang et al. 2012; Groenen et al. 2012). Genes conserved in the pig related to the cardiovascular function or drug response are the most studied.

The aim of this study was, therefore, to test human diagnostic antibodies on similar, but not identical, swine orthologous targets in order to understand how antibodies bind to FFPE tissue, as well as determine whether these are continuous linear or discontinuous epitopes, which are the epitope variations permissive for binding, and whether porcine tissue could be used for QC in IHC.

Materials & Methods

Tissues and Ethics Statement

Mature, 8-month-old castrated pigs (*Sus scrofa domesticus*) and 10-year-old bulls *(Bos taurus*) were sacrificed in abattoirs certified by the local Health Authority (ASL).

Formalin-fixed, paraffin-embedded (FFPE) fully anonymous human leftover material is exempt from the San Gerardo Institutional Review Board (IRB) approval as per Hospital regulations (ASG-DA-050 Donazione di materiale biologico a scopo di ricerca e/o sperimentazione, May 2012).

The specimens were fixed overnight at room temperature in phosphate-buffered 10% formalin, pH 7.2–7.4 (Bio-Optica, Milano, Italy). The tissue was dehydrated through a graded alcohol series, transferred to D-limonene (Bioclear; Bio-Optica) and embedded in paraffin. Three-µm sections were cut and placed on positively charged glass slides, baked, deparaffinized in xylene, and then rehydrated through a graded alcohol series and brought to water until further use.

Selected specimens were snap-frozen, sectioned in a cryostat (Leica Microsystems, Milan, Italy) and acetone-fixed.

Routine immunostaining was performed along diagnostic runs on a modified pig "Multi-sausage" tissue block (Battifora 1986) containing a human specimen relevant for the staining protocol as a control.

Immunohistochemistry (IHC) Protocols

Inclusion criteria for antibodies were: i) performance on FFPE material, ii) directed at non-polymorphic antigens in the human, iii) relevant for diagnosis or research, and iv) well characterized in terms of specificity. Antibodies against human pathogens and gene mutation-specific were excluded.

Sections were stained as per individual antibody dilutions and protocols established in our laboratory for diagnostic human IHC on a Dako Autostainer Link 48 (Dako, Glostrup, Denmark) with established protocols as suggested by the manufacturer. None required retrieval with proteases.

For individual manual stains, see Supporting Information S1.

Surveys of the reactivity of antibodies raised against human antigens on swine tissue fixed by routine methods have been published previously (Brodersen et al. 1998; Chianini et al. 2001; Debeer et al. 2013; Driessen et al. 2002; Faldyna et al. 2007; Jacobsen et al. 1993; Lauweryns and Van Ranst 1987; Lauweryns et al. 1987; Sierralta and Thole 1996; Tanimoto and Ohtsuki 1996).

Scoring Criteria

Results were scored for subcellular distribution (nuclear, cytoplasmic, membranous) and combinations of subcellular staining and intensity. In addition, the architectural distribution of the stained cells within structured tissues was noted (e.g., proliferating germinal centers versus mantle or interfollicular areas, basal cells in intestinal glands versus apical luminal cells, nerve cells and fibers within smooth muscle layers, among others).

A stain was scored positive if (1) the stain was qualitatively and quantitatively different from the occasional homogeneous faint and diffuse non-specific negative control staining; and (2) both the expected subcellular localization and the architectural distribution were maintained in the porcine tissue, compared with the reference human reactivity. For antigens ubiquitously expressed or known to have a tissue- or subunit-dependent variegation (e.g., S-100 (Takahashi et al. 1984)), putative taxonomic variations were accepted, if the subcellular distribution and at least some of the human pattern was maintained in the pig.

Sequence Retrieval and Alignment

The epitope sequence, provided by the manufacturer or collected from the published literature, was used for the human–pig amino acid (aa) sequence comparison and is reported in Table 1. In the absence of a known sequence, a 100–300-aa sequence was used if the epitope was generically listed as "C-" or "N-terminus". In the absence of either, the whole protein sequence was used. Only the shorter of these three choices was used for the analysis. Details and additional information is contained in Supporting Information Table S1.

Human protein sequences and respective codes were obtained by querying UNIPROT (Universal Protein Resource) (www.uniprot.org) (last accessed on March 24, 2015, tutorial at http://www.uniprot.org/help/). The sequence was queried in UNIPROT for the *Sus* taxa, the animal sequence aligned and inspected for the degree of similarity. In addition, the human sequence in FASTA format was aligned with BLAST (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov) with default settings (last accessed on March 24, 2015, tutorial at ftp://ftp.ncbi.nlm.nih.gov/pub/factsheets/HowTo_BLASTGuide.pdf) for the taxa required (human, swine, rat, others). The sequence was blasted, filtered for the taxonomic species and aligned in order to obtain the percent identity. Sequence alignments of discrepant cases (antibody positive / low identity or vice versa) were reviewed and visually inspected. The term "partially conserved" will be used throughout for substitutions of amino acids between groups of strongly similar properties with scoring >0.5 in the Gonnet PAM 250 matrix (see http://www.uniprot.org/help/sequence-alignments) (Mount 2008).

UniProtKB/Swiss-Prot database was preferred over UniProtKB/TrEMBL entries whenever available. Antibodies directed against multiple proteins (e.g., basic keratins) or carbohydrates were not considered for alignment.

The protein codes and the links are available as a supplementary Excel table (Supporting Information Table S1).

Epitope prediction modeling was used in selected cases with the IEDB Analysis Resource platform (http://tools.immuneepitope.org/bcell/) (last accessed on March 24, 2015, tutorial at http://tools.immuneepitope.org/bcell/help/). Human and porcine sequences were modeled with three algorithms (Emini surface accessibility scale, Kolaskar and Tongaonkar antigenicity scale and BepiPred Linear Epitope Prediction) in order to predict their epitope spatial disposition and accessibility; the latter was usually given a positive score on the y-axis. The data were imported in an Excel spreadsheet and a graph generated for the region of interest.

Virtual Whole-Slide Imaging

Stained slide images were acquired with an Aperio CS whole slide scanner (Leica Microsystems, Italy) at 20× and 40×. Individual single stain images in light microscopy were acquired with the ImageScope software (Aperio), optimized for contrast with Adobe Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA), and mounted with Adobe Illustrator.

Table 1. Targeted Proteins, Antibodies Used, Sequences, Similarity and Results.

Target	Source	Antibody	Clone	Epitope	UNIPROT Human	UNIPROT Swine	Similarity Hu/Sw	Tested	Class
Actin, alpha skeletal muscle	11	IgG1	HHF35	unknown	P68133	P68137	100%	POS	Basic cytoskeletal
Actin, aortic smooth muscle	5	IgG2a	1A4	10 N-term aa	P62736	C7AI81	100%	POS	Basic cytoskeletal
ALK (CD246)	4	Rb mAb	D5F3	C-term (cytoplasmic dom. aa 1060–1620)	Q9UM73	K7GQT6	83%	NEG	Receptor signaling
ALK (anaplastic lymphoma kinase; CD246)	5	IgG3	ALK1	aa 1359–1460 (419–520 chimera)	Q9UM73	K7GQT6	83%	NEG	Receptor signaling
ALK (CD246)	9	IgG1	5A4	aa 419–520 (chimera)	Q9UM73	K7GQT6	99%	NEG	Receptor signaling
ALK (CD246)	11	Rb mAb	SP8	tyrosine kinase catalytic domain & C-terminus	Q9UM73	K7GQT6	91%	NEG	Receptor signaling
AMACR	5	Rb mAb	13H4	unknown	Q9UHK6	F1SP14	78%	NEG	Cytoplasmic; misc
BCL-2	5	IgG1	Bcl-2-100	aa 41–54	P10415	A5A790	100%	POS	Apoptosis-related
BCL6	5	IgG1	PG-B6p	aa 3–484	P41182	F1FSH8	93%	NEG	TF
BCL6	6	IgG2b	LN22	aa 1–350	P41182	F1FSH8	93%	POS	TF
BCL6	9	Rb poly	BCL6 (N3)	aa 3–484	P41182	F1FSH8	93%	POS	TF
BRCA-1	7	IgG1	MS110	aa 1–304	Q3B891	A5A751	82%	NEG	Cell cycle; DNA replication
CA125	5	IgG1	M11	unknown	N/A	N/A	N/A	NEG	Cell–cell interaction
Calcitonin	5	Rb poly		unknown	P01258	A6P7L6	61%	POS	Cytoplasmic; misc
Calponin 2	5	IgG1	CALP	unknown	Q99439	Q08094	92%	POS	Basic cytoskeletal
Calretinin	5	IgG1	DAK-Calret 1	unknown	P22676	F1S3E7	97%	NEG	Cytoplasmic; misc
CCND1 (Cyclin D1)	11	Rb mAb	SP4	C-term (100 aa)	P24385	F1RY77	98%	NEG	Cell cycle; DNA replication
CD10	5	IgG1	56C6	external domain (52–750)	P08473	K7GMJ2	94%	POS	Receptor signaling
CD117 (c-kit)	5	Rb poly		aa 963–976 (C-term)	P10721	Q2HWD6	90%	POS	Receptor signaling
CD138	5	IgG1	MI15	ectodomain (aa 105–112)	P18827	M5DFN4	77%	NEG	Cell–cell interaction
CD14	10	Rb poly	HPA002127	aa 229–370	P08571	A2SW51	72%	NEG	Cell–cell interaction
CD141	1	Rb mAb	EPR4051	C-term (150 aa)	P07204	B3STX8	69%	NEG	Receptor signaling
CD15	5	IgM	C3D-1	Lewis-X	N/A	N/A	N/A	NEG	Cell–cell interaction
CD16	6	IgG2a	2H7	External domain (aa 17–208)	P08637	Q28942	60%	NEG	Cell–cell interaction
CD16	9	IgG1	DJ130c	unknown	P08637	Q28942	63%	NEG	Cell–cell interaction
CD163	11	IgG1	10D6	Domain 1–4 (N-term)	Q86VB7	Q28942	90%	NEG	Cell–cell interaction
CD163L1	9	Rb mAb	EPR6539	Intracellular	Q9NR16	J9T9K7	55%	NEG	Cell–cell interaction
CD1a	5	IgG1	O10	unknown	P06126	A0ZPR3	63%	NEG	Cell–cell interaction; Immun
CD2	11	IgG1	TS2/18	unknown	P06729	F1SAX9	60%	NEG	Cell–cell interaction; Immun
CD20	5	IgG2a	L26	Intracellular (aa 1–56; aa 106–120; aa 210–297)	P11836	I3LDX9	73%	NEG	Cell–cell interaction; Immun
CD23	5	IgG1	DAK-CD23	aa 48–248	P06734	B8YM31	64%	NEG	Cell–cell interaction; Immun

(continued)

Table 1. (continued)

Target	Source	Antibody	Clone	Epitope	UNIPROT Human	UNIPROT Swine	Similarity Hu/Sw	Tested	Class
CD271 (NGF-R p75)	1	Rb mAb	EP1039Y	unknown	P08138	F1RVT6	72%	POS	Receptor signaling
CD3 epsilon	5	Rb poly	poly	aa 156–168	P07766	P79264	92%	POS	Cell–cell interaction; Immun
CD30	5	IgG1	Ber-H2	unknown	P28908	F1RF73	58%	NEG	Receptor signaling
CD31	5	IgG1	JC70A	unknown	P16284	Q95242	58%	NEG	Cell–cell interaction
CD34	1	Rb mAb	EP373Y	C-term (100 aa)	P28906	K7GKN6	93%	NEG	Cell–-cell interaction
CD34	5	IgG1	QBEnd10	Class II CD34	P28906	K7GKN6	63%	NEG	Cell–cell interaction
CD4	6	IgG1	1F6	External domain	P01730	Q6R3N4	59%	NEG	Cell–cell interaction; Immun
CD43	5	IgG1	DF-T1	unknown	P16150	D9MNC9	50%	NEG	Cell–cell interaction
CD44	10	Rb poly		aa 176–313	P16070	F1SGT4	55%	NEG	Cell–cell interaction
CD45	5	IgG1	PD726 + 2B11	multiple	P08575	Q6SZ85	43%	NEG	Receptor signaling
CD45	9	IgG1	Bra-55	Extracellular (aa 24–575)	P08575	Q6SZ85	20%	NEG	Receptor signaling
CD5	5	IgG1	4C7	External domain aa 25–372	P06127	F1RIA2	62%	NEG	Cell–cell interaction; Immun
CD5	5	IgG1	CD5/54/F6	aa 474–495	P06127	F1RIA2	91%	POS	Cell–cell interaction; Immun
CD56	5	IgG1	123C3.D5	unknown	P13591	K7GMV4	97%	POS	Cell–cell interaction
CD57	5	IgM	TB01	unknown	Q96E93	F1SLW9	70%	POS*	Cell–cell interaction
CD68	5	IgG1	KP1	unknown	P34810	F1S4M0	72%	NEG	Cytoplasmic; misc
CD68	5	IgG3	PGM1	unknown	P34810	F1S4M0	69%	NEG	Cytoplasmic; misc
CD68	9	Rb poly	CD68 (H-255)	aa 100–354	P34810	F1S4M0	69%	POS	Cytoplasmic; misc
CD7	11	IgG1	MEM-186	unknown	P09564	M5DNE0	47%	NEG	Cell–cell interaction; Immun
CD79a	5	IgG1	JCB117	Extracellular (aa 33–143^)	P11912	K7GM80	93%	POS (subpop)	Cell–cell interaction; Immun
CD79a	10	IgG1	HM47	aa 208–222	P11912	K7GM80	93%	POS	Cell–cell interaction; Immun
CD8 alpha	5	IgG1	C8/144B	13 C-term aa	P01732	F1SVD3	59%	NEG	Cell–cell interaction; Immun
CD99 (HBA 71)	5	IgG1	12E7	unknown	P14209	F1RQ20	37%	NEG	Cell–cell interaction
CDKN1A (p21)	11	IgG2b	CP74	aa 1–80	P38936	I3LK35	83%	NEG	Cell cycle; DNA replication
CDKN1B (p27)	11	IgG1	DCS-72.F6	aa 83–204	P46527	I3LIR2	81%	POS	Cell cycle; DNA replication
CDKN1B (p27)	11	Rb poly		C-term (100 aa)	P46527	I3LIR2	86%	POS#	Cell cycle; DNA replication
CDKN2A (p16)	9	IgG2a	F-12	unknown	P42771	Q9TSY1	55%	NEG	Cell cycle; DNA replication

(continued)

Table 1. (continued)

Target	Source	Antibody	Clone	Epitope	UNIPROT Human	UNIPROT Swine	Similarity Hu/Sw	Tested	Class
CDKN2A (p16)	9	IgG2a	JC8	unknown	P42771	Q9TSY1	55%	NEG	Cell cycle; DNA replication
CDX2	1	IgG1	CDX2-88	unknown	Q99626	D0V4H7	96%	POS	TF
CEA	5	IgG1	II-7	unknown	P06731	K7GKS4	66%	NEG	Cell–cell interaction
CFTR	9	IgG1	M3A7	aa 1370–1380	P13569	Q6PQZ2	91%	NEG	Receptor signaling
Chromogranin A	5	IgG2b	DAK-A3	aa 210–439	P10645	F1SD66	72%	POS	Cytoplasmic; misc
Cleaved Caspase 3	4	Rb poly	Asp175		P42574	Q95ND5	90%	POS	Apoptosis-related
Cleaved Caspase 3	4	Rb mAb	5A1E		P42574	Q95ND5	90%	POS	Apoptosis-related
Cleaved Notch	4	Rb mAb	D3B8	Val1744	P46531	F1SB08	50%	NEG	TF
Cleaved Notch	4	Rb poly		Val1744	P46531	F1SB08	50%	NEG	TF
Cleaved PARP	4	Rb mAb	D64E10	Asp214	P09874	I3LDH3	94%	NEG	TF
CTNNB1	2	IgG1	#14	aa 571–781 (mouse)	B4DGU4	Q8WNW4	99%	POS	Cell–cell interaction
Cytokeratin-A 34BETA E12	5	IgG1	34bE12	Ker 1, 5, 10, 14	N/A	N/A	N/A	POS	Basic cytoskeletal
Cytokeratins AE1-AE3	5	IgG1 (pooled)	AE1-AE3	multiple	N/A	N/A	N/A	POS	Basic cytoskeletal
Cytokeratins 5/6	5	IgG1	D5/16 B4	unknown	N/A	N/A	N/A	POS	Basic cytoskeletal
Cytokeratins 8/18	11	IgG2a	5D3	unknown	N/A	N/A	N/A	NEG	Basic cytoskeletal
Desmin	11	IgG1	D33	unknown	P17661	P02540	98%	POS	Basic cytoskeletal
E-Cadherin	5	IgG1	NCH-38	unknown	P12830	C6EVT4	84%	POS	Cell–cell interaction
E2-2/TCF4	10	IgG2a	6H5-3	aa 31–331	P15884	F1S1Z1	99%	POS	TF
E2A/E47/TCF3	10	Rb poly	N-649	aa 1–649	P15923	F1SDI1	71%	NEG	TF
EMA	5	IgG2a	E29	APDTRP repeat on Mucin-1	P15941	F1RGR9	40%	POS[¥]	Cell–cell interaction
Emerin	9	Rb poly	(FL-254)	aa 3–254	P50402	I3LHY0	50%	POS	Cell cycle; DNA replication
ER alpha	5	IgG1	1D5	N-term (aa 24–575)	P03372	Q29040	92%	POS	TF
Foxp3	1	IgG1	236A/E7	unknown	Q9BZS1	Q6BBQ1	90%	NEG	TF
FRMD6	10	Rb poly		unknown	Q96NE9-3	F1SFF5	96%	POS	Cell–cell interaction
GFAP	5	Rb poly		unknown	P14136	F1RR02	93%	POS	Basic cytoskeletal
gH2AX	4	Rb mAb		phospho-epitope	N/A	N/A	N/A	POS	Apoptosis-related
gH2AX	7	IgG1	JBW301	phospho-epitope	N/A	N/A	N/A	POS	Apoptosis-related
HER2/ErbB2	5	Rb poly		unknown	P04626	K7GS43	91%	NEG	Receptor signaling
HER2/ErbB2	11	IgG1	e2-4001	intracellular (aa 676–1255)	P04626	K7GS43	94%	NEG	Receptor signaling
HER2/ErbB2	11	Rb mAb	SP3	extracellular (aa 23–652)	P04626	K7GS43	89%	NEG	Receptor signaling
HTR2B	10	Rb poly		aa 240–326	P41595	F1SMV8	91%	POS	Receptor signaling
ID1	3	Rb mAb	BCH-1/195-14	unknown	P41134	B3W6M6	91%	NEG	TF
ID2	3	Rb mAb	BCH-3/9-2-8	unknown	Q02363	Q2VIU1	98%	POS	TF
ID3	3	Rb mAb	BCH-4/17-3	unknown	Q02535	B3W6M8	96%	NEG	TF
Inhibin alpha	5	IgG2a	R1	aa 1–32	P05111	P04087	83%	POS	Cytoplasmic; misc
IRF4	1	Rb mAb	EP5699	unknown	Q15306	A0MZ86	93%	POS	TF
IRF8	10	Rb poly		aa 90–211	Q02556	Q6T5D3	88%	POS	TF
Ki-67	5	IgG1	MIB 1	aa 1101–1112 (FKELF)	P46013	I3LNN3	40%	POS	Cell cycle; DNA replication
Ki-67	8	IgG2a	UMAB107	aa 1160–1493	P46013	I3LNN3	45%	POS	Cell cycle; DNA replication
Ki-67	11	Rb mAb	SP6	C-term (150 aa)	P46013	I3LNN3	92%	NEG[#]	Cell cycle; DNA replication
KRT18 (Cytokeratin 18)	1	Rb mAb	EPR1626	C-term (100 aa)	P05783	F1SGG1	93%	NEG	Basic cytoskeletal

(continued)

Table 1. (continued)

Target	Source	Antibody	Clone	Epitope	UNIPROT Human	UNIPROT Swine	Similarity Hu/Sw	Tested	Class
KRT18 (Cytokeratin 18)	5	IgG1	DC-10	unknown	P05783	F1SGG1	79%	NEG	Basic cytoskeletal
KRT19 (Cytokeratin 19)	9	Goat poly	M-17	C-term (mouse)	P08727	F1S0J8	84%	NEG	Basic cytoskeletal
KRT20 (Cytokeratin 20)	5	IgG2a	Ks 20.8	unknown	P35900	Q29218	75%	NEG	Basic cytoskeletal
KRT7 (Cytokeratin 7)	1	Rb mAb	EPR1619Y	N-term (200 aa)	P08729	F1SGI7	57%	POS	Basic cytoskeletal
KRT7 (Cytokeratin 7)	5	IgG1	OV-TL 12/30	unknown	P08729	F1SGI7	65%	POS	Basic cytoskeletal
MART1 (Melan A)	5	IgG1	A103	unknown	Q16655	F1SMM1	73%	NEG	Cytoplasmic; misc
MCM5	9	IgG2b	E-10	aa 1-30	P33992	I3LR86	77%	POS	Cell cycle; DNA replication
Mel-18/PCGF2	9	Goat poly	MEL-18 (C20)	C-term	P35226	F2Z5D1	99%	NEG	Cell cycle; DNA replication
MMR MLH1	5	IgG1	ES05	unknown (210 aa)	P40692	D3K5L8	91%	NEG	Cell cycle; DNA replication
MMR MSH2	11	IgG1	FE11	C-term	P43246	D3K5K3	95%	POS	Cell cycle; DNA replication
MMR MSH6	1	Rb mAb	EPR3945	C-term	P52701	I3LHZ9	91%	POS	Cell cycle; DNA replication
MMR PMS2	9	Rb poly	(C-20)	C-term (300 aa)	P54278	F1RFM9	78%	POS	Cell cycle; DNA replication
Mucin-2	9	IgG1	Ccp58	5 tandem repeats	N/A	N/A	N/A	NEG	Cell–cell interaction
Mucin-6	9	IgG1	CLH5	tandem repeats	N/A	N/A	N/A	NEG	Cell–cell interaction
MYC	1	Rb mAb	Y69	N-term	P01106	Q29031	94%	POS	TF
MYC	9	Rb poly	(N-262)	aa 1–262	P01106	Q29031	94%	POS	TF
Myeloperoxidase	5	Rb poly		unknown	P05164	K7GRV6	87%	POS	Cytoplasmic; misc
Napsin A	1	Rb mAb	EPR6257	aa 60–90	O96009	F1RH37	84%	NEG	Cytoplasmic;misc
NF Pool	5	IgG1	2F11	unknown	N/A	N/A	N/A	POS	Basic cytoskeletal
NKX2-1 (TTF-1)	5	IgG1	8G7G3/1	unknown	P43699	F1SHK3	97%	POS	TF
Pax5	6	IgG1	1EW	unknown	Q02548	F1ST83	97%	NEG	TF
Pax5	11	Rb mAb	SP34	C-term (aa 251–261)	Q02548	F1ST83	63%	POS	TF
Perforin	11	IgG1	5B10	C-term (100 aa)	P14222	F1SUB6	75%	POS	Cytoplasmic; misc
Pmel 17 (HMB 45)	5	IgG1	HMB45	unknown	P40967	Q4LE84	79%	NEG	Cytoplasmic; misc
PRDM1 / Blimp-1	4	rat IgG2a	6D3	aa 255–395 (mouse)	O75626	F1RYP9	74%	NEG	TF
PRG Progesterone receptor	5	IgG1	PGR 636	unknown	P06401	D0EWS6	85%	NEG	TF
Pro-opiomelanocortin (ACTH)	5	IgG1	02A3	aa 1–39 (N-term)	P01189	P01192	100%	NEG	Cytoplasmic; misc
Prolactin (PRL)	5	Rb poly		unknown	P01236	P01238	79%	POS	Cytoplasmic; misc
PSA	11	Rb poly		unknown	P07288	P00752	59%	NEG	Cytoplasmic; misc
PTEN	4	Rb mAb	138G6	C-term	P60484	B8XSI6	99%	POS	Receptor signaling
S-100 alpha chain	11	IgG2a	4C4.9	unknown	P23297	K7GQ84	73%	POS	Cytoplasmic; misc
S-100 alpha+beta	5	Rb poly		unknown	N/A	N/A	N/A	POS	Cytoplasmic; misc
Somatotropin (Growth hormone)	5	Rb poly		unknown	P01241	P01248	68%	POS	Cytoplasmic; misc
Synaptophysin	5	IgG1	DAK-SYNAP	C-term	P08247	F1RW46	94%	POS	Cytoplasmic; misc
TdT	5	Rb poly		unknown	P04053	F1SBG2	86%	POS	Cell cycle; DNA replication
TFF3 (intestinal trefoil factor)	9	IgG1	H-425	unknown	Q07654	Q29183	80%	POS	Cell–cell interaction
Thyroglobulin	11	Rb poly		unknown	P01266	D1KKB3	74%	POS	Cytoplasmic; misc

(continued)

Table 1. (continued)

Target	Source	Antibody	Clone	Epitope	UNIPROT Human	UNIPROT Swine	Similarity Hu/Sw	Tested	Class
Thyrotropin subunit beta (TSH)	11	IgG1	ZMTS2	unknown	P01222	P01224	89%	NEG	Cytoplasmic; misc
TP53	5	IgG2b	DO-7	aa 1–45	P04637	Q9TUB2	46%	POS	Cell cycle; DNA replication
TP53	12	IgG1	Pab 1801	aa 32–79	P04637	Q9TUB2	72%	NEG	Cell cycle; DNA replication
TP63	5	IgG2a	4A4	aa 1–205 (N-term)	Q9H3D4	I3LP4	99%	POS	Cell cycle; DNA replication
TP63	9	Rb poly	H-137	aa 15–151	Q9H3D4	I3LP4	98%	POS	Cell cycle; DNA replication
TP63 p40 (DN63)	7	Rb poly		aa 5–17	Q9H3D4-2	I3LPD4	99%	POS	Cell cycle; DNA replication
Vimentin	5	IgG1	V9	unknown	P08670	P02543	98%	POS	Basic cytoskeletal
vWF (von Willebrand Factor VIII)	5	Rb poly		unknown	P04275	Q28833	75%	POS	Cytoplasmic; misc
WT1 Protein	5	IgG1	6F-H2	aa 1–181	P19544	O62651	99%	POS*	TF
ZEB1	10	Rb poly		aa 498–627	P37275	F1RWD4	84%	POS	TF

All antibodies listed are from mouse, except when differently noted [rabbit (Rb); rat; goat]. The epitope is listed if known. Note that the % similarity between human and pig is calculated for the shortest sequence known for the pig, for the full-length protein if the epitope is unknown. Ventral and diffuse prostate and testis were obtained from a bull. Under the "Tested" column: *Differences in selected tissues; see text; #Non-specific staining, see text. ¥Different cell type stained. Abbreviations: poly, polyclonal; mAb, monoclonal antibody; N-term, N-terminal; C-term, C-terminal; POS, positive; NEG, negative; TF, transcription factor; Immun, immunity; misc, miscellaneous.

Results

Overall Similarity and Impact on FFPE-Stained Samples

The average similarity score (mean ± SD) for the full list of proteins that were evaluated with a panel of antibodies was 79.2% ± 17.4% (range, 20% CD45 to 100% actins, BCL2), and this percentage was conserved among antibodies routinely used for human diagnostics or reagents used for research projects. Similar percentages were reported in broader, genome-wide comparisons of human and pig sequences (Dawson 2012).

Positive results, scored as specified, were recorded for 74/146 individual antibodies (50.7%). The positive-staining antibodies tended to cluster in the higher similarity group of targets (Table 1).

The proteins against which the antibodies were raised were representative of a broad collection of targets, heterogeneous in function, and cellular and subcellular localization. In order to gain insight into whether there was any preferential reactivity by target, we subdivided the targets into broad categories, and each group was plotted according to the degree of similarity and the positive or negative result in FFPE (Fig. 1). As reported previously (Fang et al. 2012; Groenen et al. 2012), some groups diverged more than others during evolution (e.g., the immune-associated molecules); however, within each group, FFPE-reactive antibodies were related to a higher degree of similarity of the target (Fig. 1). None of the secondary reagents used reacted with the swine tissue (not shown).

Specificity and Differential Reactivity for Human versus Porcine Tissues

The correlation between the degree of similarity and the positive staining of fixed material (Fig. 1) was accompanied by a generally highly conserved distribution of staining within the tissue at the cellular and subcellular levels (with few exceptions, see below) (see examples in Supporting information Fig. S1).

Four out of 74 positively staining antibodies (5.4%) either presented additional reactivity or lacked some stained cell types in the human-pig comparison. These are illustrated in Figures 2 through 4. These results were replicated on frozen sections (not shown).

Human Diagnostic IHC Control Use of Swine Tissue

The pig may be a convenient source of positive external control for important diagnostic antibodies. We focused on targets important for diagnostics or therapy and difficult to standardize in QC schemes. About half of routinely used human diagnostic antibodies, including antibodies against ER, MYC, and Ki-67 (Supporting Information Fig. S1), were found to stain porcine FFPE tissue in an identical fashion to that of human tissue.

Next, we focused on antigens for which no normal tissue is currently used as a control. Anaplastic lymphoma kinase (ALK) protein is aberrantly expressed in hematolymphoid and solid tumor cancers, where genetic lesions

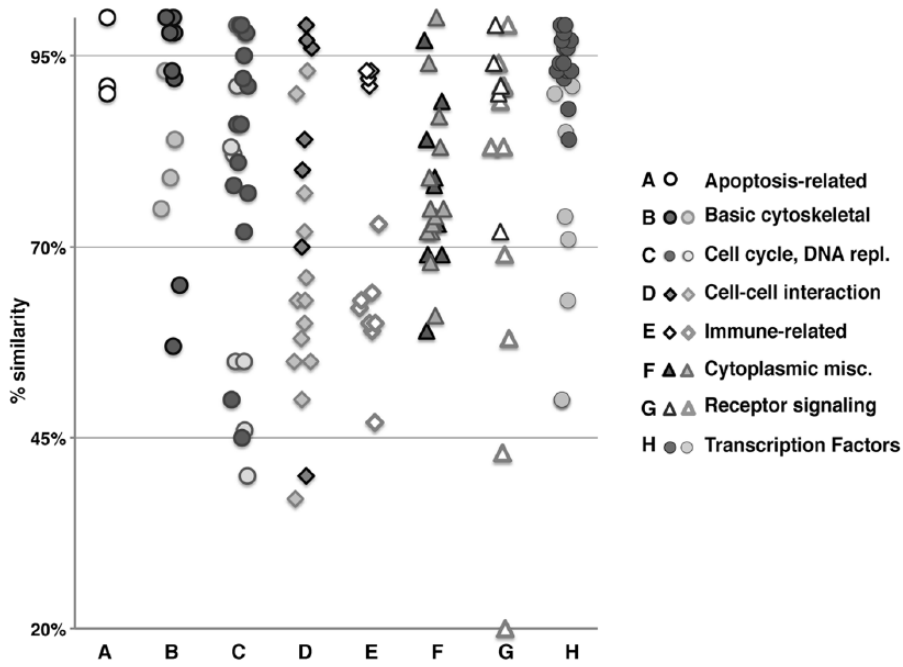


Figure 1. Degree of similarity (human to porcine) versus FFPE staining using anti-human antibodies, categorized by protein class type. The human proteins against which antibodies have been raised are grouped by broad classes and plotted on a similarity scale. Within each class, darker symbols represents FFPE-staining antibodies, lighter ones non-staining antibodies.

cause overexpression (Roskoski 2013). Besides this, ALK has been shown to be expressed as a transcript in the human small intestine (Morris et al. 1994; Tennstedt et al. 2014) and as a protein in the central nervous system and pons (Pulford et al. 1997). Public repositories of cDNA microarray data (BioGPS, http://biogps.org) (last accessed on October 2nd, 2014) report ALK expression in adrenal tissue (GeneAtlas U133A, gcrma; probe 208212_s_at), in astrocytes (Primary Cell Atlas; probe 208212_s_at) and heterogeneously at low levels in normal tissue, including the brain (Barcode on normal tissues).

We tested four different antibodies on three samples of human and one sample of swine small intestine; swine brain cortex, brainstem and cerebellum; and swine and bovine adrenal and failed to convincingly detect a specific signal for ALK. The reason why the ALK gene is transcribed, but not translated in normal adult tissues is unknown.

Similarly, we could not detect Her2 signal with three antibodies on pig breast and heart, which differs from that reported in the human (Fuchs et al. 2003).

Epitope Mapping across Species

For 84 antibodies, the entire epitope has been mapped, the region identified in broader terms (C- or N-term) or the epitope can be inferred from the immunizing peptide: We considered this group as informative. Forty-four antibodies in this group recognized FFPE material, significantly for targets above a 60% similarity limit (40/70 ≥60% similar vs 4/14 <60%; Chi square $p = 1.66 \times 10^{-9}$): This may represent a useful threshold to identify antibodies positive on FFPE material across species.

To gain insight into the amino acid substitutions within the epitope, we selected and examined a small group of antibodies whose epitope was relatively short (less than 24 aa; Fig. 5). All of the non-identical sequences bearing conserved amino acid substitutions allowed binding of the human-specific antibodies. The CD8 epitope, which has numerous non-conserved amino acid substitutions (Fig. 5) did not. Thus, the linear composition of an FFPE-proof epitope would allow changes in the sequence as long as these were completely or partially conserved.

Three antibodies positive on FFPE, for which the degree of similarity of the target was below 60% and thus expected to be negative, were further investigated, revealing possible cross-reactivity with proteins of the same biological function.

Keratin 7 has no direct homologous sequence in the pig; the nearest homology is to Keratin 75 (65% identity, 85% coverage), and the antibody labelled stratified epithelium but also mesothelial spleen-lining cells, consistent with keratin and analogous to that of human keratin 7 staining.

No porcine sequence close to the human Emerin could be found by BLAST search, despite the identical staining pattern. However, the human sequence has a low-coverage, 50% homology with a swine protein of similar nuclear membrane distribution, Inner nuclear membrane protein Man1, which itself is 93% similar to the human counterpart (Supporting Information Fig. S1); cross-reactivity with porcine Man1 may explain an identical immunostaining pattern.

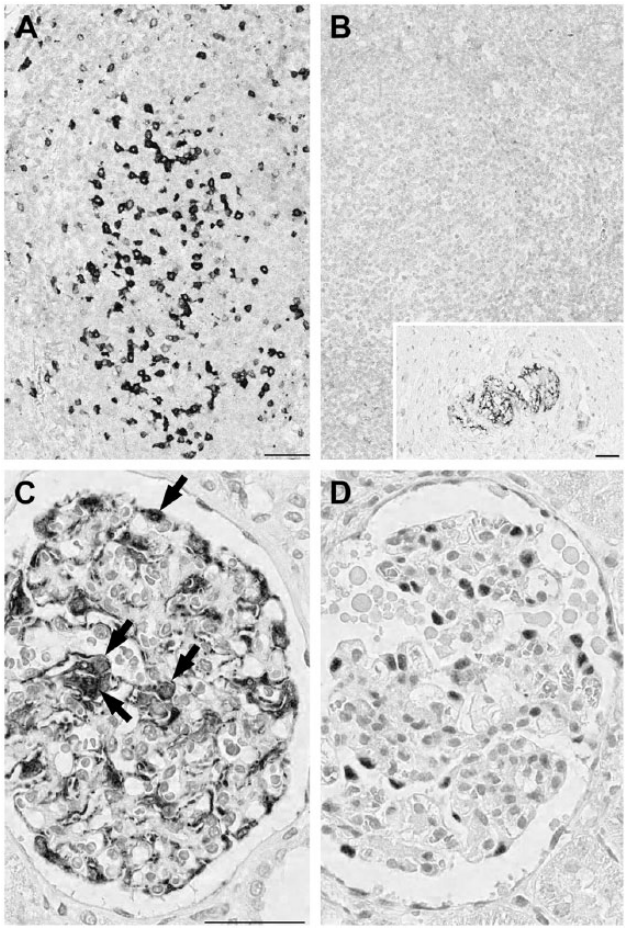


Figure 2. Differential reactivity for anti-human antibodies on swine tissue. (A, B) CD57 decorates follicular helper T cells in human tonsil (A), but not in porcine (B). Swine neural cells in Auerbach plexi are stained (inset). (C, D) Wilms' tumor 1 (WT1) stains the cytoplasm and nuclei (arrows) in the human kidney glomerulus (C) but only the nuclei are stained in the porcine kidney (D). Scale, 100 μm.

The anti EMA (Muc-1) antibody we used (E29) is directed against an APDTRP epitope (Price et al. 1998), sensitive to glycosylation, and broadly distributed: It did not stain epithelial cells but positively identified sparse mononuclear cells. We classified it as positive; however, the type of antigen and the different tissue distribution in the pig suggest cross-reactivity via similar glycosylation of unrelated proteins.

No preferential representation of antibody type (polyclonal, rabbit monoclonal, mouse isotype) was noticed in the low-similarity binders in this group.

Among the antibodies negative on FFPE swine material, the CD20 L26 antibody is directed against a non-defined cytoplasmic portion of the MS4A1 protein; the three cytoplasmic portions of the protein, aa 1–56, 106–120 and 210–297 have, respectively, 75%, 60% and 81% similarity with swine MS4A1. Syndecan-1 (CD138), which shows 77% similarity in human and swine, has lower similarity (67%) in the aa 100–140 ectodomain, the region where the MI15 and B-B4 antibodies compete for binding (Dore et al. 1998).

Antibodies staining human tissue may not recognize FFPE swine tissue if they are directed against a swine-restricted conformational epitope, which does not survive fixation and embedding, or because the epitope is missing altogether. Frozen sections from unfixed material should provide epitopes in a native form and would thereby allow an investigation as to why this group of reagents did not stain the routinely treated porcine material. To this end, 19 antibodies were tested: all were negative on FFPE material, 13 are directed at proteins with ≥75% similarity, and 3 were used as positive controls. These antibodies were tested on frozen sections from porcine small intestine (Supporting Information Table 1). Only 3 antibodies of the 19 reacted positively: anti-Keratin 20, anti-cleaved PARP and anti-BCL6 clone PGB6p.

Epitope Projection Comparison between Human and Pig

Raw similarity percentage of the linear sequence of the porcine counterpart of the human epitope may not fully explain the binding of an antibody raised against another mammal. To further elucidate the conditions for binding of anti-human antibodies in pig tissue, 13 sequences identified by seven negative and five positively staining antibodies (as controls) were plotted and compared via the linear epitope prediction algorithms. The seven human-restricted sequences were chosen among the ones with 70% similarity or greater and shorter than 24 amino acids. Five out of the seven negative antibodies showed a noticeable difference in the conformation of the linear antigenic sequence in the pig with at least one modeling method (Fig. 6). This was not observed for those antibodies that showed positive staining. Eleven known Ki-67 repeats in the human and in the pig were aligned, despite the partially conserved amino acid substitutions in the pig (Fig. 5).

Because the prediction algorithms were unable to completely explain the failure of binding of some highly homologous epitopes, we re-examined the sequence details, starting with the anti-Ki-67 MIB 1 motif, recognized in humans, pig, dog, but not in rat or mouse (Endl and Gerdes 2000). The BepiPred algorithm successfully aligned the anti-Ki-67 motifs of human, pig and rat (Supporting Information Fig. S2). However, inspection of these sequences revealed a non-conserved amino acid sequence in the rat and mouse motifs that was not present in the corresponding porcine or dog motifs. This non-conserved amino acid substitution is close to the central core of the motif, and has been described as deleterious for this

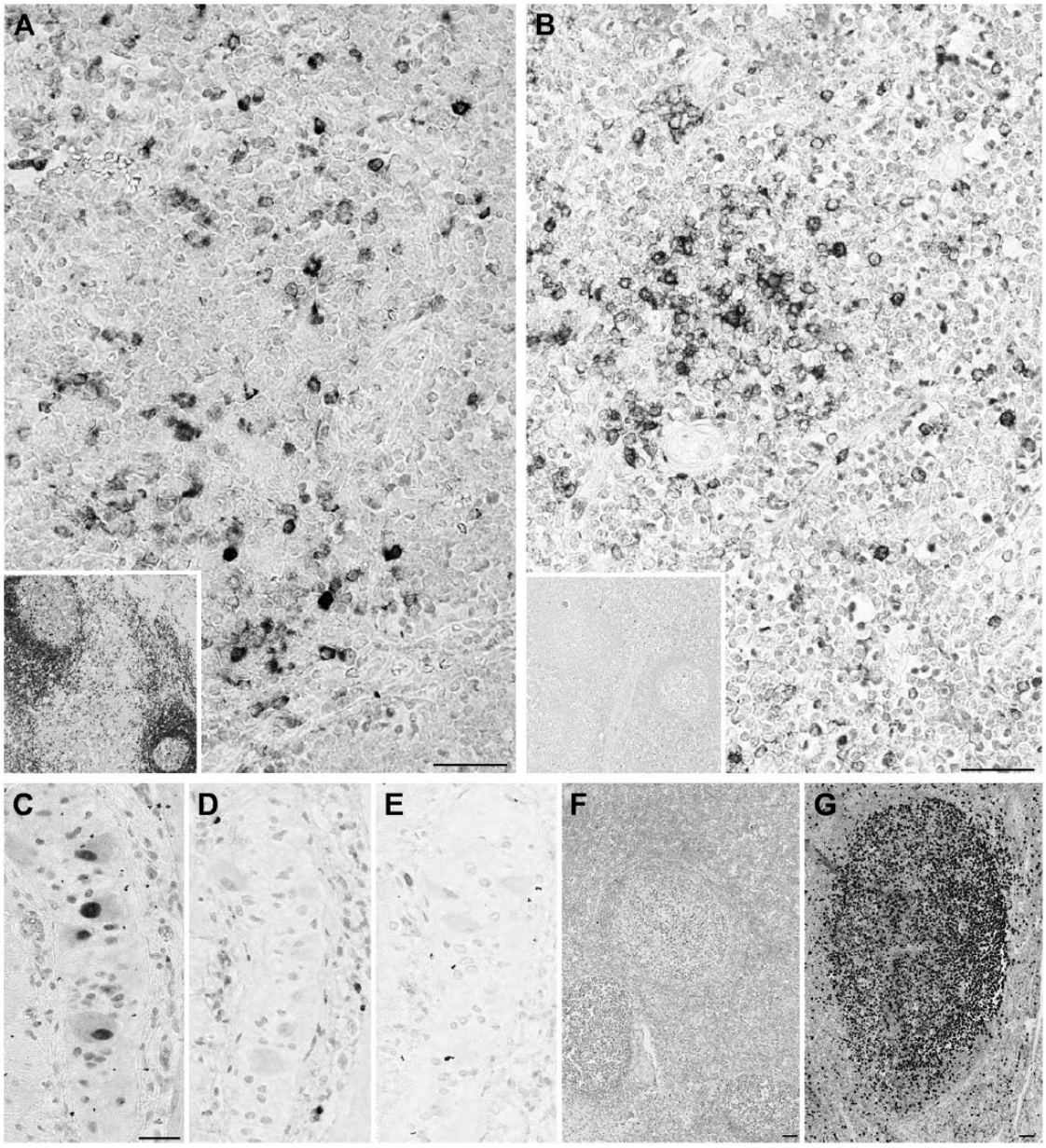


Figure 3. Heterogeneous staining patterns of anti-human antibodies on swine. (A) CD79a HM57 antibody stains scattered lymphocytes in the swine thymic medulla and all lymph nodal B cells (inset). (B) CD79a JCB11 antibody stains scattered lymphocytes in the swine thymic medulla but none in the lymph node (inset). (C) Ki-67 SP6 antibody stains the nuclei in an Auerbach neural plexus. (D, E) The same nuclei are unstained with anti-Ki-67 MIB 1 (D) and anti-MCM5 mouse antibody (E). (F, G) Ki-67 SP6 antibody weakly stains proliferating nuclei in porcine lymph node (F) but strongly in human tonsil (G). The C-terminal 250-aa sequence of human Ki-67 past the 16 repeated motifs (the immunogen for this monoclonal) has less than 40% similarity with the swine counterpart. Scale, 100 μm

anti-Ki-67 antibody binding (Kubbutat et al. 1994). Therefore, amino acid changes known to alter the epitope recognition, as shown by peptide screening, may not be taken into account by the algorithms we have employed.

Re-examination of the amino acid alignments for an additional two highly homologous epitopes showed three non-conserved amino acid substitutions in the porcine Proopiomelanocortin (POMC) within the first 8 amino acids and one at aa 23. However, Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), which also did not show positive staining in the pig, had only one partially conserved substitution in the 10-amino acid epitope and an uninformative profile with the algorithms (Fig. 6 and Supporting Information).

Thus, besides non-conserved amino acid substitutions, there are other unknown factors which prevent the binding

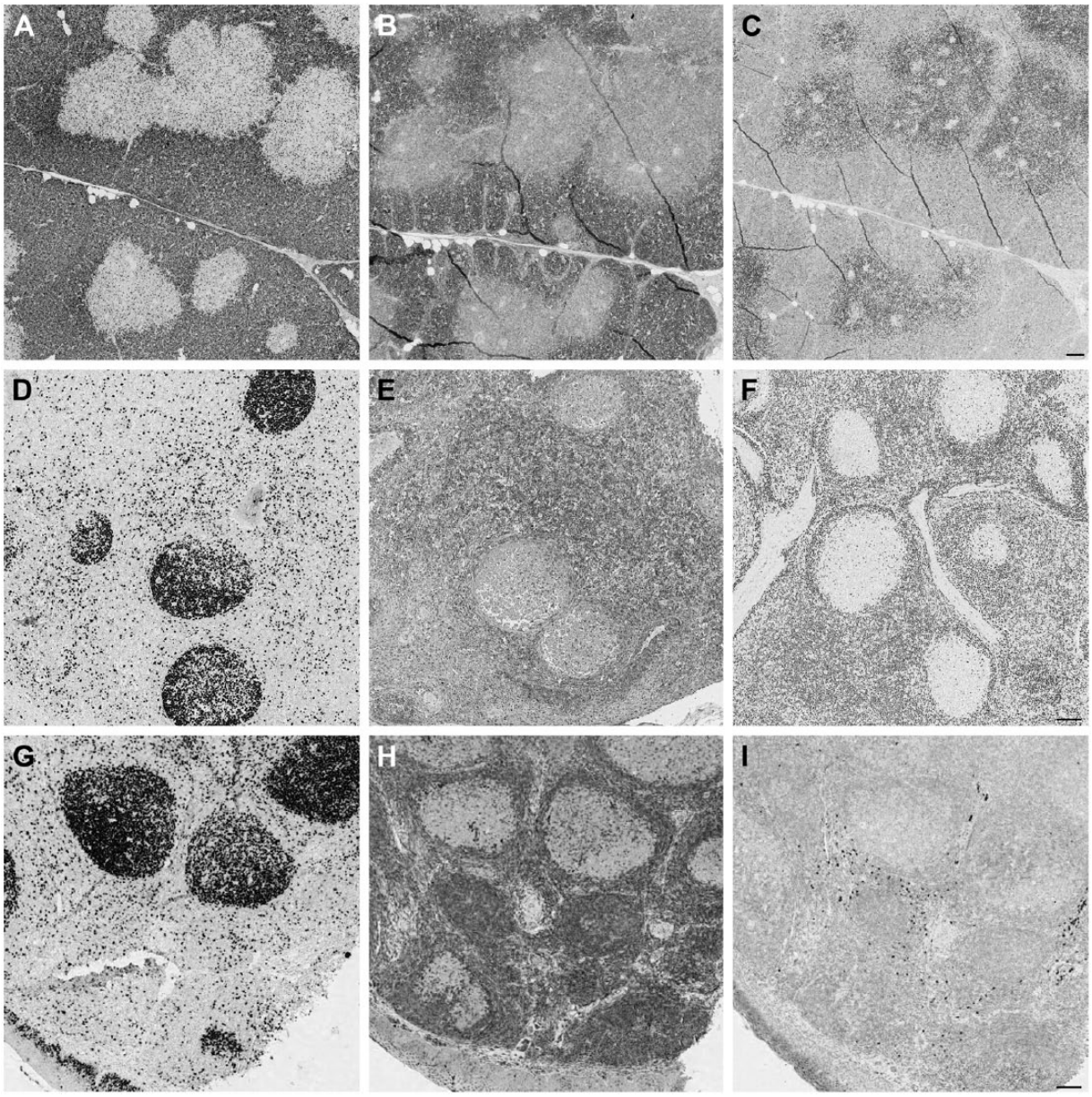
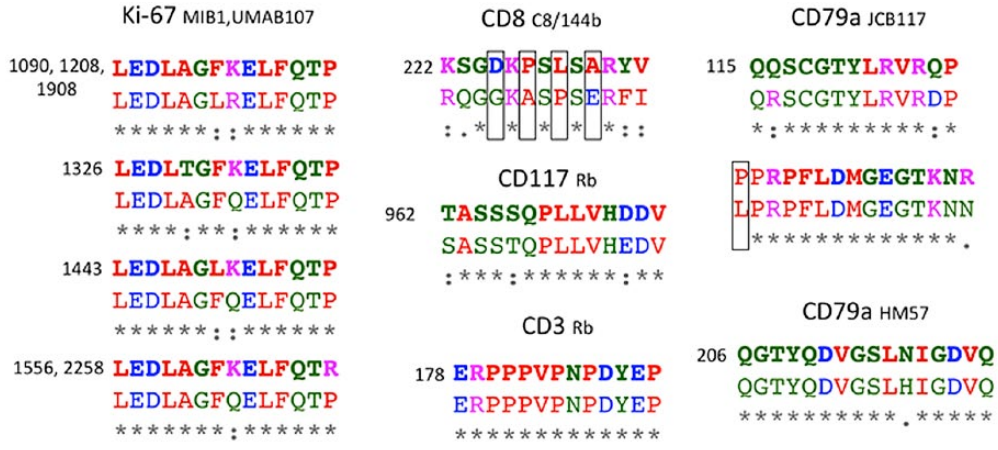


Figure 4. CDKN1B (p27) antibodies stain opposite compartments in swine thymus. Porcine thymus (A, B and C), lymph node (D, E and F) and human tonsil (G, H and I) are stained with Ki-67 MIB 1 antibody (A, D and G), mouse anti-p27 (B, E and H) and rabbit anti-p27 (C, F and I). Note the opposite staining pattern of the mouse (B) and rabbit (C) reagents in the thymus. In the peripheral swine and human tissues, proliferating germinal center cells are unstained. Note the reversed staining intensity of both anti-p27 antibodies with porcine and human tissue. Double staining for Ki-67 and both anti-p27 antibodies showed that proliferating corticothymocytes were mutually exclusively labeled by the rabbit antibody and co-expressed CDKN1B with the mouse reagent; this co-expression is the expected distribution (Nagahama et al. 2001). Scale, 100 μm

of human-specific antibodies to FFPE porcine epitopes and these cannot be entirely predicted by the available algorithms.

For 62 antibodies, no epitope specificity was available, they were directed against determinants shared by two or more proteins (e.g. acidic keratins), or they were simply polyclonal antibodies raised against pooled antigens (e.g. S-100). This group of reagents was therefore considered not informative for the epitope analysis part of the study, except for contributing to a trend for antibodies to stain preferably targets with higher similarity: only 3 out of the 23 antibodies that were positive on swine tissues were directed at targets less than 70% similar (Chi square $p= 0.022$).



Residue	Colour	Properties
AVFPMILW	RED	Small (small+ hydrophobic (incl.aromatic -Y))
DE	BLUE	Acidic
RK	MAGENTA	Basic - H
STYHCNGQ	GREEN	Hydroxyl + sulfhydryl + amine + G
Others	GREY	Unusual amino/imino acids etc
Bold / Plain		**Human** / Pig

Symbol	Properties
*	Single, fully conserved residue
:	conservation btw groups of strongly similar properties - score > 0.5 (Gonnet PAM 250 matrix)
.	conservation btw groups of weakly similar properties - score =< 0.5 (Gonnet PAM 250 matrix.)

Figure 5. Epitope composition of six anti-human antibodies positive on swine formalin-fixed, paraffin-embedded (FFPE) material. Human (bold, top) and swine (light, bottom) sequence alignments for six protein epitopes are shown, with the antibody name at the top of each alignment. The position of the first amino acid in the human sequence is reported at the top left. The CD79a/JCB117 epitope has been split into two parts for graphic reasons. The only negatively staining antibody is CD8. Boxes highlight non-conserved substitutions. The legend is drawn from http://www.uniprot.org/help/sequence-alignments and http://www.ebi.ac.uk/Tools/msa/clustalo/help/faq.html#24. The color-coding sequence display was obtained by sequence alignment with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (last accessed on March 24, 2015).

Discussion

By using naturally occurring amino acid variations introduced during evolution in related species, we have shown that antibodies tolerate partially conserved amino acid substitutions but fail to stain near-identical sequences that harbor subtle changes in epitope accessibility or show non-conserved substitutions. This understanding was possible to discern whenever a reasonably detailed information of the putative epitope was available. These results have been previously obtained only in vitro by synthetic peptide scanning (Geysen et al. 1984; Kubbutat et al. 1994). Starting with the reactivity on human FFPE material—considered the "default" status (i.e. 100% staining, 100% specific)—we generated a catalog of information that applies to each antibody applied to FFPE swine tissues in general and to FFPE-specific antibodies as a whole.

FFPE-Proof Antibodies Are Largely Interpretable by a Linear Epitope Model and Accommodate Epitope Variations

Epitopes can be either linear or continuous. Because we applied epitope modeling, which assumes a linear composition, and we found that these models can interpret the tissue reactivity of the antibodies, our findings reinforce the assumption that these antibodies largely recognize linear epitopes in porcine and, by extension, human fixed and embedded tissue. The suggestion that FFPE antigens are linear has been made previously; they all survive FFPE processing, high heat during antigen retrieval (AR), and even 2-mercaptoethanol and SDS treatments (Gendusa et al. 2014), and are therefore denaturation-resistant. FFPE-proof antibodies are generated against synthetic peptides (Jones et al. 1993; Mason et al. 1989) or denatured antigens (Wang

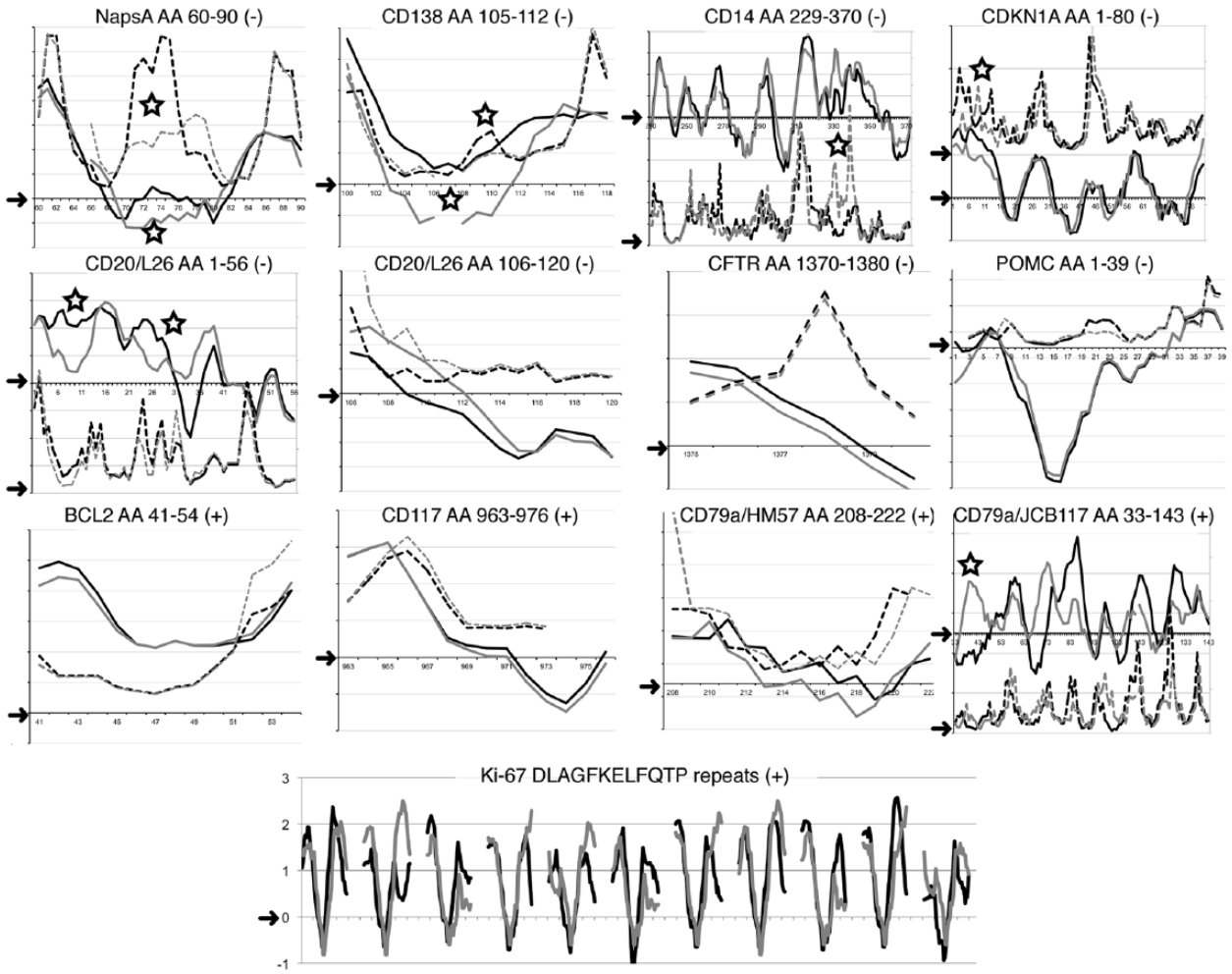


Figure 6. Epitope prediction for 13 sequences from 7 negative and 5 positively staining antibodies. The individual amino acid (aa) score obtained with two algorithms (BepiPred: continuous line; Emini: dashed line) for each antibody target sequence is plotted on the y-axis for the human (black) and the pig (grey). For graphic clarity, two different scales (left and right) are occasionally produced in the same graph for each model. Arrow on the y-axis points to the 0 value above which the algorithm predicts the epitope (see Supporting Information for individual plots). Each amino acid position number is listed on the x-axis. The antibody and clone, the amino acid target human sequence and the staining results (+ or -) is listed at the top of each graph. Eleven Ki-67 repeats BepiPred scores were plotted by aligning the human and corresponding porcine dodecapeptide sequences, flanked by 13 amino acids and an empty space for graphic rendering. The open star marks the areas of noticeable differences between human and pig with at least one prediction model. The top two rows depict antibodies negative on FFPE and frozen swine material: in all but three (CD20 AA 106-120, CFTR, POMC), noticeable conformational changes in the pig may highlight an inaccessible sequence for the swine counterpart. The bottom two rows depict positively staining antibodies. A conformational change at the N-terminus of the CDKN1A and of the CD79a-JB117 epitope may not affect the binding to the remaining sequence. Notice that the known Ki-67 target sequence FKELF, at the center of each repeat, has a negative score with the BepiPred model. Scores obtained with the Kolaskar and Tongaonkar antigenicity scale are not shown because this has been found to be informative in few cases. Sequence gaps and terminal missing sequences with the Emini algorithm are shown. The CD20 aa 210–297 cytoplasmic sequence, which is not informative, is not shown.

et al. 2005), another suggestion that they detect linear continuous epitopes, rather that conformation-dependent, discontinuous ones (Barlow et al. 1986; Fowler et al. 2011; Kringelum et al. 2013).

For some antibodies, the immunogenic sequence is known. Antibodies in this group bound targets with an overall similarity as low as 60%. This suggests that antibodies selected to react on FFPE tissue allow quite some variation in the sequence composition of the target, taking into account that the score derives from a matrix calculation for individual amino acid substitutions, not the simple identical/non-identical ratio.

Some epitopes, perfectly aligned according to the algorithms and highly similar to the human, were not detected in

fixed or frozen swine material: We do not have a good explanation for this observation. Alternative splicing may be one explanation, but the pig genomic database does not have enough information to further investigate this possibility. Post-transcriptional protein modifications or complexes with other proteins may be a cause; the mosaic staining by the CD79a antibody JCB117 may be a good example.

One last additional reason for the differential reactivities of the antibodies on human and swine FFPE material may be the idiosyncrasy of the paratope for a unique binding milieu, going undetected by still imprecise modeling tools (Ponomarenko and Bourne 2007). Part of the required binding sequences may be located on adjacent protein loops, as shown for some therapeutic antibodies (de Weers et al. 2011); swine-specific changes in these sequences may prevent FFPE staining.

For these reasons, it will be impossible to predict whether an anti-human antibody will bind a related mammal, except by making an educated guess by epitope sequence similarity and staining the tissue.

We ruled out a suboptimal AR condition for the negative staining, because some of the highly similar (>80%) protein targets such as CD34, Cytokeratins, Mucin, Napsin A, Progesterone Receptor, were negative despite being abundantly expressed and detectable even without AR.

The epitope sequences did survive fixation, embedding and AR, which reverses many of the formalin-induced chemical bonds that prevent antibody access to the epitope. Not all bonds are resolved; among these may be bonds further stabilized by dehydration (Fowler et al. 2008), as shown by human FFPE-proof targets detected on frozen section only in the pig. Interestingly, only one out of three antibodies raised against the same N-terminal half of the BCL6 protein was fixation-sensitive in the pig, further suggesting that the stable, AR-insensitive bond had a local effect but did not modify the neighboring binding sites or the overall antigenic sequence conformation of porcine N-terminal BCL6, another hint at the linearity of the FFPE epitopes.

Porcine Tissue Can Be a Source of Reference FFPE Material for Human Quality Control

None of the 146 antibodies tested yielded any sort of "background" or non-specific staining; only four of the 74 that did stain resulted in a tissue or cellular staining pattern that could be classified as substantially different from that in human tissue, namely EMA, WT1, CD57 and the Ki-67 antibody SP6. The specificity of WT1 cytoplasmic staining in human endothelial cells is discussed (Carpentieri et al. 2002), and was absent in the swine tissue, suggesting that the cytoplasmic staining is spurious. Non-specific (cytoplasmic) staining of Ki-67 antibodies in non-human cells has been described previously (Falini et al. 1989).

For the vast majority of positively staining antibodies, swine tissue provided a histological and immunohistochemical staining identical to human tissue.

Swine tissue from abattoirs is thus a human-like material that can be used as an external control tissue and is nearly ubiquitous, cheap, freely exchangeable and free of the ethical constraints that limit the use of human tissue. This is most important for noble organs (brain, brainstem, heart) or small tissues (endocrine glands, ganglia) that are not commonly available in sufficient quantity as a healthy human control tissue. In addition, swine tissues may be used to standardize the detection of therapy-modifying targets (estrogen receptors (Sierralta and Thole 1996), MYC (Kluk et al. 2012), for example) in daily practice for antibodies proven to stain the pig.

Antibodies directed against mutated proteins (V600E BRAF (Capper et al. 2011), R132H IDH1 (Capper et al. 2010)), pathogens (EBV, KSHV, TB, among others) or proteins overexpressed because of a tumor-specific genetic modification (e.g., promoter swapping by chromosomal translocation for ALK or gene amplification for Her2) still need human pathological tissue or cell blocks from cultured cell lines. For some antigens that are structurally more divergent during evolution (CD30, immune receptors (Dawson 2012)), either different taxa may be investigated or human tissue still may be needed.

A minority of antibodies recognizing the FFPE porcine tissue showed a substantial differential reactivity, justifying in those few cases a concern about the monospecificity of the antibody, which is worth further investigation. To the contrary, the specificity of the vast majority of the remaining reagents currently used for diagnosis on human samples, accommodating epitope variations at least 50% of the time, is an encouraging finding; these antibodies may be used on occasionally heavily mutated targets, such as melanoma and lung cancer (Schumacher and Schreiber 2015), because of non-synonymous mutations. As we add more antibodies tested on both species, we keep finding reliable antibodies and, rarely, suspicious, unexpected staining (data not shown).

Not every manufacturer details the specificity of the epitope against which the antibody is directed, often because it is considered proprietary information, despite abundant evidence that, for a single immunogen, there are a variety of unique individual epitopes, each one recognized by a monoclonal antibody or an antiserum (Geysen et al. 1984). We have shown, however, that the availability of that information may help in choosing one reagent over another for the use across species or for validation studies (Bordeaux et al. 2010; Smith and Womack 2014). Ideally, the datasheet accompanying an antibody vial should carry the complete immunogen sequence and information on whether the antibody recognizes FFPE mammalian tissue other than human, preferably obtainable from abattoirs.

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Author's Contributions

GC and MMB designed the experiments. MB, AF, DDA, AGR procured and examined essential animal tissue. CRS, RG, LR, LT, AM performed immunohistochemical tests and histopathology preparations. MB, SV, CRS, RG scored the IHC preparations and annotated the results. MMB performed the genomic analysis and protein structure analysis and prediction. GC and MMB wrote the manuscript. All authors have read and approved the final manuscript.

Competing Interests

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