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HOST-VIRUS INTERACTIONS
IN
PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME
VIRUS INFECTION

GEORGE SORENSEN
BSc, MSc

Submitted for the degree of
Doctor of Philosophy

College of Medicine and Veterinary Medicine
Roslin Institute, University of Edinburgh

2014
Preface

I hereby acknowledge that this thesis represents my own work and includes nothing that is the outcome of work done by others, or in collaboration with others, except where specifically indicated in the text. The contents have not previously been submitted in candidature for any other degree or qualification. This dissertation does not exceed the word limit length of 100,000 words.

Name: .................................................. Date: .........................
Acknowledgements

I wish to express my deep gratitude to my future wife Katy, thank you for always believing in me and for your unwavering support over the past four years, a feat that easily exceeds that of researching a doctorate. I do not believe that I would be in this position without your love and backing, thank you.

Also, to my mother and father, I am truly grateful for your support and incessant nagging to get on and submit!

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Thank you to Alison Wilson for your friendship and always being there to help and give advice (09:00 to 15:00, Monday to Wednesday of course)! Thanks also go to Heather Finlayson for help in the lab and trying to get me organised.

This research was funded by a BBSRC Case studentship with Pfizer Animal Health (Zoetis). I wish to thank both organisations for their financial backing which made this work possible.

Thank you to Chris Pendleton (http://www.pendleton.co.uk) for allowing me to reproduce his wonderful watercolour prints and add a bit of colour to this thesis. Chris Pendleton retains the copyright to all of the watercolour prints herein.
Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is a rapidly evolving virus that has significant economic and welfare implications for the pig industry. Vaccination strategies have proved largely ineffective in controlling PRRSV, in some cases even reverting to virulence. An increasing body of evidence suggests a host genetic basis for PRRSV resistance so there is a need to examine the role of host genetics in a biologically relevant in vitro cell culture system. However, PRRSV research is inhibited by the current scarcity of suitable in vitro culture systems.

With the aim of developing a convenient in vitro model, porcine bone marrow-derived macrophages (BMDM) were evaluated as a PRRSV cell culture system. BMDM were found to be highly permissive to Type I PRRSV and amenable to genetic manipulation. BMDM proved to be excellent cells for virus production, producing significantly higher titres of PRRSV than commonly used alternative cell types. Surprisingly, PRRSV entry into BMDM was found to be independent of both the prototypic PRRSV receptors, CD163 and CD169, providing further evidence for the existence of alternate PRRSV entry mechanisms in primary cell types.

To explore the genetics of pig susceptibility to PRRSV, network-based analysis of host transcriptional datasets, following PRRSV challenge, revealed important differences in co-regulated gene pathways between samples from pigs with different PRRSV-permissiveness. These pathways included genes with important, recently characterised, anti-pathogen activities.

The incorporation of network-based transcriptional analysis and published genetic variation data led to the identification of a member of the guanylate binding protein family, GBP-1, as a candidate host gene involved in controlling PRRSV replication. Overexpression of GBP-1 in BMDM revealed a significant anti-PRRSV function for this protein. Further investigation of published genetic variation in GBP-1 suggested a potential role of this gene in PRRSV tolerance.

The results presented in this thesis provide evidence for an alternate PRRSV entry pathway in a biologically relevant cell type. The discovery of a highly PRRSV-infectable cell type with potential for genetic manipulation adds a useful new tool to the area of PRRSV research. The identification of GBP-1 as a novel anti-viral protein with a significant inhibitory effect on PRRSV infection, together with genetic variation in this gene, prompts further research into the genetic basis for PRRSV resistance.
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### Abbreviations

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<tbody>
<tr>
<td>AM</td>
<td>alveolar macrophage</td>
</tr>
<tr>
<td>APA</td>
<td>alternative polyadenylation</td>
</tr>
<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BMDC</td>
<td>bone marrow-derived macrophage</td>
</tr>
<tr>
<td>BMDM</td>
<td>bone marrow-derived macrophage</td>
</tr>
<tr>
<td>BM-imDC</td>
<td>bone marrow immature dendritic cell</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT-enhancer-binding protein</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CSF-1</td>
<td>colony stimulating factor 1</td>
</tr>
<tr>
<td>Cy3</td>
<td>cyanine 3 dye</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>dpi</td>
<td>days post-infection</td>
</tr>
<tr>
<td>DUR</td>
<td>Duroc (pig breed)</td>
</tr>
<tr>
<td>EAV</td>
<td>equine arteritis virus</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GBP</td>
<td>guanylate binding protein</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GEBV</td>
<td>genomic estimated breeding value</td>
</tr>
<tr>
<td>GED</td>
<td>GTPase effector domain</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GO</td>
<td>gene ontology</td>
</tr>
<tr>
<td>GOF</td>
<td>gain-of-function</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>HAM</td>
<td>Hampshire (pig breed)</td>
</tr>
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<td>HCV</td>
<td>Hepatitis C virus</td>
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<tr>
<td>HIV-1</td>
<td>human immunodeficiency virus 1</td>
</tr>
<tr>
<td>hpi</td>
<td>hours post-infection</td>
</tr>
<tr>
<td>hpt</td>
<td>hours post-transfection</td>
</tr>
<tr>
<td>IAV</td>
<td>influenza A virus</td>
</tr>
<tr>
<td>IER</td>
<td>immediate-early response</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity Pathway Analysis</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
</tr>
<tr>
<td>ISG</td>
<td>interferon-stimulated gene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>ISRE</td>
<td>interferon-sensitive response element</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
</tr>
<tr>
<td>LD</td>
<td>linkage disequilibrium</td>
</tr>
<tr>
<td>LG</td>
<td>large-G domain</td>
</tr>
<tr>
<td>LOF</td>
<td>loss-of-function</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LR</td>
<td>Landrace (pig breed)</td>
</tr>
<tr>
<td>LW</td>
<td>Large White (pig breed)</td>
</tr>
<tr>
<td>M1</td>
<td>classically activated macrophage</td>
</tr>
<tr>
<td>M2</td>
<td>alternatively activated macrophage</td>
</tr>
<tr>
<td>MAVS</td>
<td>mitochondrial antiviral signalling</td>
</tr>
<tr>
<td>Mb</td>
<td>megabases (millions of base pairs)</td>
</tr>
<tr>
<td>MCL</td>
<td>Markov cluster algorithm</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MX</td>
<td>myxovirus resistance</td>
</tr>
<tr>
<td>nsSNP</td>
<td>non-synonymous SNP</td>
</tr>
<tr>
<td>PAS</td>
<td>polyadenylation site</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PIE</td>
<td>Pietrain (pig breed)</td>
</tr>
<tr>
<td>QC</td>
<td>quality control</td>
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<tr>
<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
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<tr>
<td>QTL</td>
<td>quantitative trait locus</td>
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<tr>
<td>RLH</td>
<td>RIG-like helicase</td>
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<td>RIVitro</td>
<td>Roslin Institute <em>in vitro</em></td>
</tr>
<tr>
<td>RIVivo</td>
<td>Roslin Institute <em>in vivo</em></td>
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<tr>
<td>RMA</td>
<td>robust multi-array average</td>
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<td>SCF</td>
<td>stem cell factor</td>
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<td>sgmRNA</td>
<td>sub-genomic RNA</td>
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<td>SLA-DR</td>
<td>swine leukocyte antigen-DR</td>
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<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<td>SSC(#)</td>
<td><em>Sus scrofa</em> chromosome location</td>
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<tr>
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<td>swine workshop cluster 3</td>
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<tr>
<td>STAT1</td>
<td>signal transducer and activator of transcription 1</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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<tr>
<td>TRIM</td>
<td>tripartite motif family</td>
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<tr>
<td>USE</td>
<td>upstream sequence element</td>
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<tr>
<td>VL</td>
<td>viral load</td>
</tr>
<tr>
<td>VLIG</td>
<td>very large inducible GTPases</td>
</tr>
<tr>
<td>WG42</td>
<td>body weight gain from 0 to 42 dpi</td>
</tr>
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CHAPTER ONE

AN INTRODUCTION TO PRRSV BIOLOGY AND CONTROL
1.1 **Porcine reproductive and respiratory syndrome**

1.1.1 *PRRSV discovery and spread*

During the late 1980s, outbreaks of a previously unrecognised disease emerged in pig production units across the USA (Keffaber, 1989; Loula, 1991). The clinical presentation included: severe reproductive losses, post-weaning pneumonia, reduced growth performance and increased mortality (Hill, 1990). Concurrent attempts to identify the aetiology of these outbreaks were unsuccessful, the origin of the moniker “Mystery Swine Disease” (MSD), by which it became known at the time (Hill, 1990; Reotutar, 1989).

Shortly after the first disease outbreaks were reported in the USA, cases exhibiting comparable clinical signs occurred across the Atlantic, firstly in Germany in November 1990 (OIE, 1992). No definitive evidence linked the German outbreak with the American MSD. Over 3,000 outbreaks of the German disease were reported in May 1991 and the outbreak continued unabated, spanning Europe over the following 4 years (Baron *et al*., 1992; Bøtner *et al*., 1994; Edwards *et al*., 1992; OIE, 1992; Pejsak & Markowska-Daniel, 1996; Plana Duran *et al*., 1992; Valícek *et al*., 1997). Disease outbreaks with apparently analogous clinical signs also occurred on the Asian continent in parallel with the American and European epidemics, both in Japan in 1988 (Hirose *et al*., 1995) and in Taiwan in 1991 (Chang *et al*., 1993).

A causal relationship between a pathogen and MSD was first established in 1991, when the clinical signs of MSD were experimentally reproduced upon infection with a previously uncharacterised RNA virus named “Lelystad Virus” (Terpstra *et al*., 1991b; Wensvoort *et al*., 1991). Shortly afterwards, the causative virus was also isolated in the USA and designated BIAH-001 and in Canada, with the IAF-EXP 91 isolate. At this point, the name “Swine Infertility and Respiratory Syndrome” was coined to describe the reproductive and respiratory disease symptoms (Collins, 1991; Collins *et al*., 1992; Dea *et al*., 1992a). Some early researchers also named the virus “swine arterivirus” (Albina *et al*., 1998b; Legeay *et al*., 1997). It should be noted that “swine arterivirus” is more in keeping with the guidelines put forward for virus nomenclature by the International Committee on Taxonomy of Viruses (http://ictvonline.org). However, the name “porcine
reproductive and respiratory syndrome (PRRS) virus” (PRRSV) was introduced by European researchers around 1991 and has since been adopted as the accepted term for the virus (Terpstra et al., 1991a).

PRRS has been estimated to account for annual losses of between $560 and $664 million in the USA alone (Holtkamp et al., 2012; Neumann et al., 2005). Additional veterinary, biosecurity and other outbreak related costs have also been predicted to total $477.79 million annually in the USA (Holtkamp et al., 2012). Accurate estimates of the cost of PRRS to the UK pig industry are not readily available. However, around 79% of breeder to finisher units in the UK are estimated to be either affected by PRRSV and/or using vaccination to try and control it (Evans et al., 2008). 11.1 million pigs are held in the UK (www.bpex.org.uk; 2013 figures), in an industry worth £984 million (www.ukagriculture.com; 2010 figures). Nevertheless, the UK pig industry is in severe economic decline, with a reduction in deadweight sow prices in recent years. Therefore, the financial pressure exerted by PRRS is significant.

1.1.2 PRRSV types and phylogeny

Based on genetic and antigenic characteristics, PRRSV is divided into European (type I) and North American (type II) genotypes (Mardassi et al., 1994). These two genotypes, represented by the prototype viruses Lelystad and VR-2332 respectively, can be discriminated based on significant differences in nucleotide sequence (Han et al., 2007; Meulenberg et al., 1993b). Type I and type II virus genotypes share 50 to 60% nucleotide sequence identity, with as much as 20% nucleotide sequence variation between individual strains of the same genotype (Han et al., 2006). These genotypes have been both geographically and genotypically distinct, although both now have a worldwide distribution and their diversity is rapidly increasing (Dea et al., 1992a; Murtaugh et al., 2010).

As the virus continues to evolve, we are presented with new clinical variations. PRRSV has an estimated mutation rate of between $1.1$ and $3.3 \times 10^{-2}$ substitutions per site per year (Prieto et al., 2009) and was found to have the highest synonymous substitution rate of 49 RNA viruses tested in one study (Hanada et al., 2004). Aided by this mutation rate and consequent heterogeneity, evidence has
recently emerged of highly virulent PRRSV strains circulating in the USA, China and Eastern Europe (Karniychuk et al., 2010; Murtaugh, 2009; Tian et al., 2007). The emergence of porcine high fever disease in China has been linked to a novel PRRSV strain belonging to the type II genotype (Tong et al., 2007). Affected farms experienced high morbidity and up to 100 % mortality. These viral isolates possessed a 30 amino acid deletion in non-structural protein (nsp) 2, which appeared to represent a novel determining factor for virulence that may explain the high pathogenicity of these strains (Tian et al., 2007).

Phylogenetic analysis of nucleocapsid sequences of Eastern European PRRSV isolates have assigned them to the type I virus genotype, but grouping separately from all other type I genotype sequences (Stadejek et al., 2006; Stadejek et al., 2008). Thus, a division of the type I genotype into three subtypes has been proposed: a pan European subtype 1 and Eastern European subtypes 2 and 3 (Stadejek et al., 2008). A Belarusian PRRSV isolate, designated “Lena”, is a highly pathogenic Eastern European subtype 3 virus which has been shown to differ from European and North American strains at both genetic and antigenic levels (Karniychuk et al., 2010).

Estimates in the literature for the most recent common ancestor of the PRRSV go back as far as 1880 ± 15 years (Forsberg, 2005). Following the evolution of ancestral PRRSV, the divergence of type I PRRSV is thought to have occurred between 1947 and 1968 (Stadejek et al., 2013). The most recent common ancestor of type II PRRSV is predicted to have emerged later, between 1977 and 1981 (Shi et al., 2010). Despite the apparently simultaneous outbreak of the PRRSV epidemic on both sides of the Atlantic in the late 1980s, the virus must have been circulating in some form or host prior to its emergence in the scientific literature. The 3’-terminal non-coding region of the Quebec PRRSV type II reference strain appears to be more closely related to equine arteritis virus (EAV) and lactate dehydrogenase-elevating virus (LDV) than to the type I PRRSV strains (Mardassi et al., 1994), suggesting the possibility of recombination between arteriviruses, possibly in other mammalian hosts, in the evolution of PRRSV.
1.1.3 PRRSV structural biology

PRRSV is an enveloped virus with a diameter of 50 to 65 nm and a loosely organised, non-isometric, linear nucleocapsid core of 25 to 35 nm (Benfield *et al.*, 1992; Dokland, 2010). The virus has a compact, positive-sense RNA genome of 15.1 to 15.5 kb, transcribed to produce seven sub-genomic mRNA (sgmRNA) transcripts (Conzelmann *et al.*, 1993). PRRSV belongs to the family *Arteriviridae* which, together with the *Coronaviridae*, form the order *Nidovirales* (Figure 1.1; Cavanagh, 1997). The *Arteriviridae* are composed of EAV, LDV, simian hemorrhagic fever virus and PRRSV (Figure 1.1). This group exhibits several important properties relevant to the study of viral pathogenesis, such as cytopathic replication in macrophages and the ability to establish both asymptomatic infection and severe and fatal disease (Plagemann, 1996).

The PRRSV genome contains at least 10 open reading frames (ORFs) that encode biologically significant polypeptides, including a large viral replicase (ORF1a and ORF1b) that is expressed from the genomic RNA, and several major proteins expressed from nested 3´ coterminal sgmRNAs (Figure 1.2A). With the exception of the smallest, all of these sgmRNAs are polycistronic (Yuan *et al.*, 2000). The major sgmRNAs encode four glycoproteins (GP2, GP3, GP4 and GP5; ORFs 2, 3, 4 and 5, respectively), an unglycosylated membrane protein (M; ORF 6) and a nucleocapsid protein (N; ORF 7) (Conzelmann *et al.*, 1993; Meng *et al.*, 1994; Meulenberg *et al.*, 1993b; Murtaugh *et al.*, 1995; Nelsen *et al.*, 1999). A short ORF (ORF5a) has also been identified which overlaps with ORF5 (Firth *et al.*, 2011). The viral transcriptome also contains minor sgmRNA transcripts with orfs for which no protein product has yet been identified (Meng *et al.*, 1996; Nelsen *et al.*, 1999). All strain-specific viral RNAs possess identical 5´ and 3´ regions containing small ORFs, for which no function has yet been assigned (Allende *et al.*, 1999; Meulenberg *et al.*, 1993a; Nelsen *et al.*, 1999).
Figure 1.1 | Phylogeny of the Nidovirales. Unrooted phylogenetic tree of RNA-dependent RNA polymerase (RdRp) domains of the Nidovirales and other (super) groups of RNA viruses. Taken from Snijder et al. (2005).
Each sgRNA is formed by the mechanism of discontinuous transcription (Figure 1.2D), which involves sense and antisense base pairing of transcription-regulating sequences (TRS) (Van Marle et al., 1999). The PRRSV leader TRS is a hexa-nucleotide, UUAACC, which is conserved across PRRSV genotypes (Meng et al., 1996; Meulenberg et al., 1993a; Nelsen et al., 1999). The Nidovirus discontinuous synthesis model has been reviewed in detail by Sawicki et al. (2007). Briefly, negative-strand RNA synthesis starts from the 3′ end of the genomic RNA and attenuates at the TRS body site, where the transcriptase complex and nascent RNA jump to the leader TRS, probably via a mechanism of similarity-assisted RNA recombination (Pasternak et al., 2006; Sawicki & Sawicki, 1995; Sawicki et al., 2007; Spaan et al., 1983). A significant body of experimental data has emerged in support of this negative-strand extension model (den Boon et al., 1996; Hussain et al., 2005; Pasternak et al., 2006; Pasternak et al., 2001; Sethna et al., 1989; Van Marle et al., 1999).

The full-length PRRSV RNA is used for the translation of two open reading frames: ORF1a and ORF1b, which occurs immediately upon entry. Translation of ORF1a yields the pp1a polyprotein. ORF1b is expressed through a ribosomal frame shift, leading to the formation of the large pp1ab polyprotein (Figure 1.2A; den Boon et al., 1995; Meulenberg et al., 1993b). pp1a and pp1ab are processed by viral proteases to release 14 non-structural proteins (nsps; Figure 1.2A), which include: four proteases (nsp1α, nsp1β, nsp2 and nsp4), the RdRp (nsp9), a helicase (nsp10) and an endonuclease (nsp11) (den Boon et al., 1995; Snijder & Meulenberg, 1998; van Aken et al., 2006; Ziebuhr et al., 2000). The function of several of the PRRSV nsps is still unknown. Nsp2 is a multi-domain protein, consisting of N-terminal cysteine protease, middle hypervariable and transmembrane (TM) domains and a C-terminal tail (Han et al., 2009). The cysteine protease is capable of both cis and trans cleavage activity and is essential for viable virus recovery (Han et al., 2009). Nsp2 is the most variable nsp, with the majority of the polymorphisms concentrated in the middle, hypervariable region. Arterivirus nsp2 localises to the perinuclear region in some cell types, where it associates with intracellular membranes to form double membrane vesicles where virus replication and transcription take place (Pedersen et al., 1999; Snijder & Meulenberg, 1998; Van Der Meer et al., 1998).
PRRSV possesses a repertoire of seven structural proteins, the three major structural proteins being: GP5, M and N (Figure 1.2B and C). The small, 15 kDa N protein is encoded by ORF7 and is expressed in abundance during productive infection, constituting up to 40% of the total protein content of the PRRSV virion (Bautista et al., 1996). N protein is highly basic and interacts with the viral RNA during replication, in the assembly of the viral particle (Rowland & Yoo, 2003b). N protein exhibits considerable size polymorphism in type 1 PRRSV strains (Stadejek et al., 2008). Intriguingly, given its pivotal role in virus replication, a deletion of at least 40 nucleotides following the ORF7 stop codon, within the three prime untranslated region (3’UTR), has been shown to be tolerated without a distinguishable effect on virus viability in vitro (Sun et al., 2010).

The 25 kDa GP5 primary envelope protein (Figure 1.2C), encoded by ORF5, is the most variable of the PRRSV proteins (Murtaugh et al., 1995). GP5 is involved in receptor recognition and contains a major neutralisation epitope (NE) within the N-terminal ectodomain (Ansari et al., 2006; Ostrowski et al., 2002; Plagemann et al., 2002; Plagemann, 2004; Wissink et al., 2003).

The envelope protein (M; Figure 1.2C) is the most genetically conserved PRRSV structural protein and is involved in viral assembly and budding. M protein and GP5 form heterodimers in the endoplasmic reticulum (Mardassi et al., 1996). The resulting heterodimers are incorporated into viral particles, where they are involved in virus infectivity. The M/GP5 complex has also been identified as a viral ligand interacting with the N-terminal, immunoglobulin (Ig)-like domain of the PRRSV cellular attachment receptor, sialoadhesin (CD169) (An et al., 2010; Delputte & Nauwynck, 2004; Delputte et al., 2007; Van Breedam et al., 2010a). The M protein is also capable of inducing PRRSV-neutralising antibodies (NAbs) (Bastos et al., 2002).

The minor envelope proteins GP2 (29 to 30 kDa) and GP4 (31 to 35 kDa) are type I membrane proteins encoded by ORFs 2a and 4, respectively (Dokland, 2010; Meulenberg, 2000). The GP4 protein is critical for mediating inter-glycoprotein interactions and, along with GP2a, serves as the viral attachment protein responsible for mediating interactions with the CD163 virus receptor and subsequent PRRSV uncoating (Das et al., 2010).
The 2b (or E) protein, expressed from a second ORF (ORF2b) contained entirely within ORF2 (Figure 1.2A), is similar to the E protein in EAV and is a minor structural component of the PRRSV envelope (Wu et al., 2001; Wu et al., 2005). E is a non-glycosylated, 70 to 73 amino acid protein that is predicted to be involved in virus fusion and internalisation through comparison with similar proteins in other viruses (Dokland, 2010).

GP3 (ORF3) has a size of 42 to 50 kDa and shares approximately 54% to 60% amino acid identity between North American and European isolates (Meng et al., 1995). GP3 is the most glycosylated PRRSV protein, with seven putative N-linked glycosylation sites that are well conserved among strains of both PRRSV genotypes (Gonin et al., 1998). GP3 is known to be a structural protein of a type I PRRSV strain (Meulenberg et al., 1995) and a non-structural protein of a type II strain (Gonin et al., 1998). However, a structural role for GP3 in PRRSV type II has also been suggested, given its ability to induce NAbs (Cancel-Tirado et al., 2004; Jiang et al., 2008). In addition, partial protective immunity to PRRSV has been reported in vivo following the immunisation of animals with a recombinant baculovirus expressing ORF3 (Urniza et al., 1997) or with recombinant fowlpox expressing ORF3 and ORF5 (Shen et al., 2007). de Lima et al. (2009) have also presented evidence that GP3 is incorporated into virus particles in a type II PRRSV strain. Overall, it is likely that GP3 represents just a small fraction of the PRRSV structural proteins, consistent with the notion that GP3 is one of the minor structural glycoproteins of the Arteriviridae (Meulenberg, 2000). A subset of viral GP3 has also been shown to be secreted into cell culture supernatant in a non virion-associated, membrane-free form (Mardassi et al., 1998).
Figure 1.2 -- figure legend overleaf--
Figure 1.2 | PRRSV genome organisation, expression strategy and virion structure. A. The PRRSV ORFs are shown, with the corresponding names of the structural proteins annotated. The lower panel depicts the fourteen non-structural proteins produced post-processing of the pp1a and pp1b polyproteins translated from ORF1a and ORF1b, respectively. The functions of the non-structural proteins are annotated, where known. B. The structural relationships of the genome and sgmRNAs are shown. The leader and TRS, as well as the ribosomal frameshifting element found in the genome-length mRNA, are annotated. The translated region of each mRNA is demarked by a black line and translationally silent regions by a green line. C. PRRSV virion structure and the relative positions of the structural proteins, modified from its original form at ExPASy (ExPASy, 2013). D. Model of coronavirus discontinuous transcription taken from van Marle et al. (1999). Discontinuous negative-strand synthesis (i), yields the subgenomic (sg) (-) strand, involving the translocation of the nascent (-) strand to the genomic leader TRS (ii), where base pairing occurs and transcription is resumed to add the anti-leader sequence to the sg (-) strand. The sg (-) strand is then used as the template for sg mRNA synthesis (iii).
1.1.4 PRRS: clinical signs and gross pathology

PRRSV is the causative agent of two disparate syndromes: reproductive and respiratory. Infected farms may experience one or both presentations. A variety of factors may influence the clinical signs of disease, including: host genetics, circulating PRRSV strains, herd health status (including the presence of other pathogens), stock age and pregnancy status.

Sows

Gilts and sows infected with PRRSV may present with symptoms of inappetence and fever in up to 30% of infected animals, or may show no clinical signs. During the acute stage of infection, fever often occurs and abortions may ensue in pregnant animals, most commonly in individuals infected during the last trimester of pregnancy. Virus-induced reproductive failure may present clinically as an increase in the number of open sows, a reduction in farrowing rate and delayed return to oestrus as well as abortions, mummified foetuses and weak or still-born piglets (Benfield et al., 1999; Christianson et al., 1993; Collins et al., 1992; Mengeling et al., 1994; Rossow et al., 1999; Rowland et al., 2003). Highly virulent PRRSV strains may increase the frequency of abortion and death in sows (Halbur et al., 1998a).

A second acute phase of infection can occur at around a week following the onset of clinical signs. Late-term reproductive failure occurs as a result of PRRSV infection of the foetus in gestating sows. This phase can persist for one to four months within a farm and result in 5 to 80% reproductive failure in pregnant sows at 100 to 118 days of gestation (Benfield et al., 1999). Premature farrowing routinely results in variable numbers of normal and weak-born pigs, as well as dead pigs in various stages of mummification.

Natural infection of the foetus with PRRSV is initiated at around 90 days of gestation, with sows possessing an apparent resistance to placental or trans-placental PRRSV infection before this time (Karniychuk & Nauwynck, 2013). Following productive maternal viremia and PRRSV replication in the endometrium, the virus is able to cross the placenta and foetal infection occurs (Karniychuk & Nauwynck, 2013). The exact mechanism of trans-placental infection is currently unknown.
However, the mechanism may be akin to the LDV-infected “Trojan Horse” macrophage which, upon intra-vaginal injection, migrates directly to the placenta, as well as establishing a systemic infection (Cafruny & Bradley, 1996). Sows experimentally inoculated with PRRSV have been shown to have a greater proportion of CD169-positive macrophages in the endometrium and placenta than uninfected animals, due to de novo CD169 expression on local CD163-positive macrophages (Karniychuk et al., 2013). Given that the pig foetus becomes immuno-competent at around 70 days of gestation, PRRSV infection occurs in an environment containing functional B- and T-cells (Salmon, 1984; Šinkora & Butler, 2009).

Foetuses which are infected in utero, but which survive to term, can exhibit the most severe form of respiratory disease with mortality sometimes reaching 100% within three weeks of birth (Feng et al., 2001; Rossow, 1998; Rossow et al., 1994). The complex pathology following exposure to PRRSV in utero represents a unique form of the disease, referred to as “congenital PRRS” (Rowland et al., 2003).

Boars

Adult boars infected with PRRSV can exhibit symptoms including: fever, lethargy, inappetence, decreased libido and respiratory distress. Some boar deaths have been attributed to highly virulent PRRSV variants (Ni et al., 2012). Nevertheless, few clinical signs are generally observed in adult boars infected with PRRSV. A decline in semen quality may following infection. However, given evidence that semen quality remains within acceptable limits following PRRSV infection, it is unclear as to whether this adversely impacts conception rates (Lager & Halbur, 1996; Prieto et al., 1996a; Prieto et al., 1996b; Swenson et al., 1994; Yaeger et al., 1993). Most significantly, PRRSV present in semen from infected boars can be transmitted to sows by natural mating or through artificial insemination (AI). The likelihood of transmission of PRRSV present in semen appears to be dependent on the quantity of virus, with a greater dose required in semen compared to intra-nasal inoculation (Hermann et al., 2005; Prieto & Castro, 2005). However, infected boars also shed virus in saliva and other excretory routes.
Suckling pigs

Symptoms of acute PRRSV infection in pigs infected either in utero or as neonates include: listlessness, inappetence and starving, fever, watery diarrhoea, laboured breathing, lethargy, oedema or swelling of the eyelids and a discoloration of the ears or hindquarters (Gordon, 1992; Hopper et al., 1992; White, 1992). Incidences of pre-weaning mortality can reach between 40 and 80 % (Linhares et al., 2012; Piras et al., 2005). Less common clinical signs can include tremors, doming of the forehead and haemorrhage from navels (Gordon, 1992; Hopper et al., 1992; Keffäber, 1989; Loula, 1991; White, 1992). PRRSV can also be directly transmitted to suckling piglets via the mammary gland secretions of infected sows (Wagstrom et al., 2001a).

Weaned and grower/finisher pigs

The most commonly observed clinical signs in weaned and grower/finisher pigs include: lethargy, inappetence, dyspnoea and discoloration of the skin on the ears or hindquarters. Reductions in average daily gain and feed efficiency often vary between individuals, resulting in uneven groups which constitutes the primary source of economic loss in this system (Moore, 1990; Pejsak et al., 1997; White, 1992). Often an increased incidence of endemic secondary bacterial and viral infections is observed, influencing the severity of the clinical disease.

1.1.5 Role of PRRSV in other porcine diseases

Diseases most commonly reported alongside PRRS include: streptococcal meningitis, Glasser’s disease, septicaemic salmonellosis, exudative dermatitis, bacterial bronchopneumonia and sarcoptic mange (Benfield et al., 1999). A recent Chinese study has also reported a case of hepatitis E virus and PRRSV co-infection (Mao et al., 2013). Enzootic pneumonia is characterised by infection with Mycoplasma hyopneumoniae and other pathogenic bacteria. This, in concert with PRRSV, porcine circovirus type 2 (PCV2) and/or swine influenza virus (SIV) leads to a multifactorial syndrome known as the porcine respiratory disease complex (PRDC). Current understanding of PRDC is limited, but it has emerged as a serious and complex health problem for the swine industry.
Postweaning multisystemic wasting syndrome (PMWS) is another multifactorial syndrome, with PRRSV and PCV2 co-infection being the most common and clinically severe form of this syndrome (Allan et al., 1999; Harms et al., 2001; Pallarés et al., 2002).

The term porcine circovirus-associated disease (PCVAD) is in use to represent the range of disease symptoms attributed to PCV2, in which PRRSV also plays a role. However, the precise mechanism of interaction between PRRSV and PCV2 in the pathogenesis of PCVAD remains to be determined.

Proliferative and necrotising pneumonia (PNP), characterised by the proliferation of type II pneumocytes and coagulates of necrotic cells in the alveoli, has been associated with particular strains of SIV (Dea et al., 1992b; Girard et al., 1992; Morin et al., 1990). However, the discovery of PNP occurred simultaneously with the emergence of PRRS and studies have since indicated that PRRSV may indeed be the key aetiological agent of this condition (Drolet et al., 2003).

An additive, or potentiating, effect has also been observed between PRRSV and some bacterial and viral agents (Benfield et al., 1999). In these cases, PRRSV co-infection results in enhanced disease severity in comparison to either individual infection. Reported polymicrobial, or potentiating disease combinations include: *M. hyopneumoniae*, porcine respiratory coronavirus, PCV2, SIV and Aujeszky’s disease virus (Allan et al., 2000; Rovira et al., 2002; Shibata et al., 2003; Thacker et al., 1999; Van Reeth et al., 1996, 2001). High fever PRRSV has also been linked to the co-infection of pigs with the Reston ebolavirus (Barrette et al., 2009). Some authors have speculated that the inhibition of macrophage phagocytosis upon PRRSV binding to its entry receptor, CD169, may be important in the apparent increased susceptibility of PRRSV-infected animals to other pathogen co-infections (De Baere et al., 2012).

1.1.6 PRRSV transmission and epidemiology

It is likely that PRRSV originally entered domestic pig (*Sus scrofa domesticus*) populations through contact with a wildlife species. One hypothesis for the origin of PRRSV is that a mutant of the closely related murine Arterivirus, LDV, infected wild boar in central Europe. These animals functioned as intermediate hosts
and records show that wild boar were exported to the USA in 1912. The virus then evolved separately on each continent in the wild boar populations for around 70 years, before entering the domestic pig population (Plagemann, 2003).

The pig and wild boar, or feral pigs, remain the only animals known to succumb to natural PRRSV infection. Mallard ducks have been reported as being susceptible to the virus (Zimmerman et al., 1997) but subsequent attempts to reproduce this finding have failed (Trincado et al., 2004). The susceptibility of other members of the Suoidea (Sus spp., babirusa, peccary and warthog) to PRRSV infection remains to be established. The infection of wild boar or feral pigs with PRRSV is thought to vary between geographical regions and does not correlate with domestic pig population density (Reiner et al., 2009). Additionally, there are no reports of wild boar exhibiting clinical signs of PRRSV infection and limited substantiation of their role in the transmission of PRRSV to domestic pigs.

Given the extensive replication of PRRSV in the respiratory tract, aerosol transmission is likely to play a role in spreading the virus. There are reports of airborne virus spread over distances of up to 20 km (i.e. De Jong et al., 1991). However, aerial transmission is more likely to be relevant over distances of less than 3 km and during cold, winter weather conditions (Alonso et al., 2013; Komijn et al., 1991; Le Potier et al., 1997). The primary transmission route for PRRSV is via close contact between carrier and susceptible pigs (Albina, 1997). Nose-to-nose contact or contact with urine or faeces presents an important source of infection (Rossow et al., 1994). Transmission via insemination with contaminated semen is also well documented and there are epidemiological reports which incriminate AI centres as the origin of many disease outbreaks (Edwards et al., 1992; Le Potier et al., 1997). These data have led to a widespread call for the PRRSV-free certification of AI centres, which has been undertaken in some countries with great success (Vangroenweghe et al., 2008). Dams exposed to PRRSV during late gestation can also shed virus in mammary secretions (Wagstrom et al., 2001b).

PRRSV often spreads rapidly within a herd, leading to a high seroprevalence (85 to 95 %) within two to three months (Terpstra et al., 1992). Subsequently, the virus can persist for many weeks and even up to 16 months in some infected farms (Auvigne et al., 1994). This virus persistence can be due to the infection of naïve
animals, the presence of persistently infected animals or the infection of piglets following the passing of passive immunity (Albina et al., 1994; Terpstra et al., 1992). However, spontaneous elimination of PRRSV has been observed in some cases (Freese et al., 1994) and reports suggest that some farms have remained free of infection despite having purchased replacement pigs from infected farms (Potter, 1994).

1.1.7 PRRSV control strategies

Vaccines are readily available, and routinely used in pig production, against pathogens such as porcine parvovirus and *M. hyopneumoniae*. An inexpensive and effective PRRSV vaccine that could be administered alongside other immunisations would be invaluable in limiting both the economic and welfare implications of PRRS.

The two primary PRRSV vaccine strategies employ modified-live virus (MLV) or inactivated virus and there are more than 20 vaccines currently on the market worldwide (Rowland et al., 2012). Inactivated PRRSV vaccines are generally ineffective, whereas MLV vaccines are effective in protecting immunised pigs against homologous circulating virus, with minimal protection afforded against heterologous isolates (Huang & Meng, 2010; Murtaugh & Genzow, 2011). The relatively poor protective immunity achieved following vaccination against PRRSV is a function of the virus’s capacity to generate broad genetic diversity, combined with several strategies to evade innate and adaptive immune responses (Rowland et al., 2012). This means that, at the very least, vaccines must be used in conjunction with biosafety and other disease control measures.

There have also been cases of commercial live vaccine strains reverting to virulence under farm conditions. Most famously, in Denmark more than 1,100 herds were simultaneously vaccinated with the Ingelvac PRRSV Vet vaccine (Boehringer Ingelheim) in 1996. Within several months the majority of herds experienced increased abortion rates and weak or stillborn piglets. PRRSV was isolated and found to have originated from the vaccine virus strain (Nielsen et al., 2001). The timing of vaccination is also important, with one study observing a decrease in pigs born alive and weaned and an increase in the number of stillborn and mummified pigs.
following vaccination with a PRRSV MLV during the last four weeks of gestation (Dewey et al., 1999).

In a recent study, Calzada-Nova et al. (2012) compared the efficacy of the same MLV vaccine strain, propagated on different host cell lines, in conferring protection against subsequent PRRSV challenge. Virus propagated on an alveolar macrophage (AM)-like cell line resulted in quicker PRRSV clearance from immunised pigs than from animals immunised with MLV propagated on the MA104-derived simian kidney epithelial cell line, MARC-145 (Calzada-Nova et al., 2012). The representation of virus quasispecies possessing a glycosylation site at an important position in the GP2 protein also differed between vaccine virus propagated on each cell type (Calzada-Nova et al., 2012). These data suggest that the host cell used for virus propagation is an important additional consideration in the production of efficacious PRRSV vaccines. The genetic background of individual pigs may also influence their response to vaccination, as seen with PCV2 (Potter et al., 2008).

Several other methods for the control of PRRSV involve management practices. The depopulate/repopulate method involves the culling of all pigs in a production facility, regardless of age or weight. Following disinfection, the unit is restocked with PRRSV-free animals and monitored closely. Herd closure and rollover is another, less costly, PRRSV control measure. In this case, the introduction of naïve animals is prevented for at least six months, allowing circulating virus to be removed from the herd following the establishment of immunity. Some intensive farming systems in pig-dense regions employ the use of HEPA filtration in sealed production units, which can almost completely prevent aerosol transmission of PRRSV (Alonso et al., 2013; Dee et al., 2006). This system has the additional benefits of removing other airborne pathogens and not being influenced by regional health status, but is extremely costly to implement and out of reach of smaller producers.

The genetic improvement of commercial pig breeds for a more robust immune response to PRRSV may also prove to be an important future control measure.
1.2 Host genetic improvement for PRRSV control

Research has shown that there is a genetic component involved in the effectiveness of pigs in responding to, controlling and abolishing PRRSV infection (Ait-Ali et al., 2007; Doeschl-Wilson et al., 2009; Greiner et al., 2000; Halbur et al., 1998b; Lewis et al., 2009a; Lewis et al., 2009b; Lewis et al., 2007; Lowe et al., 2005; Lunney & Chen, 2010; Lunney et al., 2011; Petry et al., 2005; Petry et al., 2007; Reiner et al., 2010; Vincent et al., 2005, 2006). This indicates that naturally-existing genetic variation in PRRSV resistance may occur.

Firstly, it is important to consider the distinction between the terms “tolerance” and “resistance”. PRRSV-tolerant pigs would host a viral infection but show a smaller reduction in production traits and/or reduced clinical signs of disease. Whereas, PRRSV-resistant pigs would be less likely to be infected by PRRSV. The distinction between these states, in practical terms, is that PRRSV-resistant pigs could aid in the eradication of the virus, given that they would be less likely to be hosts for virus replication. PRRSV-tolerant pigs would still host a productive virus infection, so PRRSV would still be circulating and could be spread to naïve, non-tolerant pigs. An additional concern of PRRSV-tolerance is that a virus with a high variability, such as PRRSV, could potentially circumvent the tolerance mechanism. Mechanisms causing the introduction of random point mutations, such as the relatively low fidelity of the RdRp of RNA viruses, result in virus genetic variation and the potential emergence of virus “escape variants”. Alternatively, the co-existence of tolerant pigs and PRRSV could result in reduced selection pressure on the virus to evolve. Theoretical models suggest that epidemiological feedback for tolerance may be positive, i.e. as the frequency of tolerant hosts increases in the population, pathogen transmission and prevalence increases, resulting in further selection for tolerant hosts and the fixation of tolerance gene variants (Best et al., 2008; Lefèvre et al., 2011; Roy & Kirchner, 2000). Breeding for resistance has similar issues to some of those afflicting PRRSV vaccine development, such as the application of intense selection pressure to the virus, promoting the survival of only those virus genotypes able to circumvent the resistance mechanism. However, this is likely to be more of an issue in the case of vaccines, as breeding for resistance
applies selection pressure in a slower manner. An additional beneficial use of host genetics in the control of PRRSV could be through the dissection of any genetic basis to the efficacy of PRRSV vaccines. However, this strategy is still likely to be hampered by the primary reason that PRRSV vaccination strategies are currently ineffective: virus heterogeneity (Huang & Meng, 2010).

In order to robustly identify cellular genes and pathways involved in mounting an effective host response to PRRSV infection, precise and comprehensive analyses of phenotypic characteristics are required. Experimentally, this necessitates the use of large numbers of animals infected in carefully controlled conditions and monitored for robust and relevant suites of parameters, although field studies involving large numbers of animals may also be effective. In the USA, this is the aim of the PRRS Host Genetics Consortium (PHGC) (Lunney et al., 2011). The PHGC employ a nursery-pig model, continuing to 42 days post-infection, to assess pig resistance/susceptibility to primary PRRSV infection and viral persistence. Thus far, over 2000 pigs have been assessed in this way. Through the statistical analysis of PHGC data, pigs may be segregated into four extreme categories. These categories are: PRRS resistant - low virus/high weight gain, PRRS susceptible - high virus/low weight gain, PRRS tolerant - high virus/high weight gain and the less thrifty phenotype - low virus/low weight gain animals (Lunney et al., 2011). Given that PRRSV virus load and weight gain traits are both heritable (Boddicker et al., 2012), this further demonstrates the potential of selective breeding for PRRSV resistance or tolerance traits. However, selection would ideally be possible on a trait in uninfected pigs that is correlated with their response to infection. Heritability estimates for the effect of PRRSV infection on the percentage of live pigs born to infected sows are relatively low, ranging from 0.12 to 0.15 (Lewis et al., 2009a). Heritability estimates for viremia and survival in weaners have been found to be 0.096 and 0.26, respectively (Biffani et al., 2010; Vukasinovic & Clutter, 2010).

In the past decade, aided by the publication of the pig genome (Groenen et al., 2012), genetic research has shifted towards the identification of genes involved in pig health and disease traits, rather than production traits (Archibald et al., 2010a; Edfors-Lilja et al., 2000; Green, 2009; Lunney, 2007; Mellencamp et al., 2010). Advanced genome-wide studies using single-nucleotide polymorphism (SNP) chips...
are able to genotype thousands of variable loci simultaneously. This has facilitated genome-wide association studies and has resulted in the identification of SNPs associated with PRRSV-resistance traits (Boddicker et al., 2012). Microarray or RNA-seq technologies for profiling the transcriptomes of animals or cells infected with pathogens are also capable of identifying genes and pathways associated with, or regulating, the host response to pathogen infection and can complement genotyping data.

Preliminary analysis of PHGC data has revealed a region on Sus scrofa chromosome 4 (SSC4) that is correlated with both lower virus load and higher weight gain in PRRSV-challenged pigs (Boddicker et al., 2012). This result suggests that selecting animals for a PRRSV resistance trait may not necessarily compromise growth efficiency. Thus, breeding programs could be established to develop pigs with robust growth characteristics and increased resistance to PRRSV infection. Additionally, the results of multiple studies show that pig lines selected for improved reproductive traits (dam line robust) are more resistant to the detrimental effects of PRRSV than those lines selected for lean growth and efficiency (Lunney & Chen, 2010). However, more research is required in order to identify the mechanistic basis driving the effect of genetic variation in the SSC4 genomic region. Identifying the causal genetic variation would aid the understanding of the potential host resistance mechanism and permit marker-assisted selection based on the causal genetic variation, which should be applicable across populations.

Given the reliance of viruses, as obligate intracellular parasites, on an infectable host cell for genome replication, the infection process is contingent on cellular receptors and proteins. Thus, one approach to render a host cell resistant to infection is to remove or mask an essential receptor. However, due to the co-evolution of virus and host, viruses often exploit cellular receptors with additional, important biological functions. Thus, the removal of these proteins may severely compromise the viability of the host. However, studies indicate that this strategy may be effective in particular cases, such as for Escherichia coli in pigs (Hu et al., 1993). One complication of this approach, in the context of PRRSV, is the evidence of multiple receptors for PRRSV: CD169, CD163 and others. An important recent study was the first to provide evidence of a PRRSV receptor-null pig (Prather et al., 2013).
Prather and colleagues generated a knockout pig with no CD169 expression on the surface of its AM. Interestingly, the authors reported that the absence of CD169, despite being an important PRRSV entry mediator \textit{in vitro}, had no measurable effect on viremia, clinical disease course or histopathology (Prather \textit{et al.}, 2013). These factors highlight the challenges of a targeted genetic modification approach to the generation of PRRSV-resistant pigs.

Host genetic manipulation, either targeted or through screening and selective breeding programs, is likely to be most effective when employed in concert with additional PRRSV control measures, such as vaccination and biosecurity. However, when used as a part of a multi-pronged approach, host genetics is likely to be an integral part of the solution to PRRSV control.

1.3 Host response to PRRSV infection

1.3.1 PRRSV host cell tropism

PRRSV has a highly restricted cellular tropism, primarily replicating in tissue-specific macrophages, including the AM and other cells of the monocyte/macrophage lineage (Lamontagne \textit{et al.}, 2001; Pol \textit{et al.}, 1991). PRRSV can also replicate in testicular germ cells of infected boars, including: spermatids, spermatocytes and multi-nucleated cells (Sur \textit{et al.}, 1997), as well as the macrophages of the intestinal lamina propria (Haynes \textit{et al.}, 1997; Yaeger \textit{et al.}, 2002). A change in tissue tropism can occur later in the course of PRRSV infection, shifting from multiple sites of PRRSV replication, although primarily in the AM, to preferential replication in tonsil and lymph node tissue (Rowland \textit{et al.}, 2003). Continuous replication in regional lymph nodes facilitates efficient virus transmission via oral and nasal secretions, or in semen, during persistent infection (Christopher-Hennings \textit{et al.}, 1998). Despite the continuous viral replication in some tissues, PRRSV does not establish a true steady-state persistent infection, as the virus is eventually cleared by around 150 days post-infection (dpi) in most animals (Allende \textit{et al.}, 2000).
1.3.2 *Macrophages and the mononuclear phagocyte system*

In a series of classical studies performed more than a century ago, Ilya Mechnikov began to fully comprehend the capabilities of phagocytes (Gordon, 2008; Karnovsky, 1981; Mechnikov, 1908; Tauber, 2003). Then, in the late 1960s, van Furth and Cohn defined the mononuclear phagocyte system (MPS) as non-granulocytic, myeloid cells that play a role in homeostasis, tissue remodelling and the regulation of both innate and adaptive immunity (Van Furth & Cohn, 1968). The MPS has traditionally been divided into three cell types: monocytes, terminally differentiated macrophages and dendritic cells (DCs). All of which are comprised of diverse sub-populations, defined by their phenotypes and anatomic microenvironment.

In mammals, the actively hematopoietic red bone marrow (BM) shifts from all bone cavities, to being restricted to the axial skeleton and the proximal ends of the femur and humerus, with age and maturity. The remaining bone cavities are gradually repopulated by non-hematopoietic mesenchymal cells which accumulate lipid drops, known as yellow BM (Moore & Dawson, 1990). There is an additional intermediate stage where BM cells (BMC) can lose their fat and then resume hematopoiesis under certain conditions (Gurevitch et al., 2007). The majority of available myeloid cell differentiation data are derived from human or murine studies. Murine hematopoietic stem cells comprise a small fraction of BMC. These cells give rise to two oligopotent cell types: the common lymphoid progenitor cells and the common myeloid progenitors that differentiate into the megakaryocyte-erythrocyte progenitors and granulocyte-monocyte progenitors (Akashi et al., 2000; Kondo et al., 1997; Kondo et al., 2003). The differentiation of these progenitor cells is determined by the combined effects of intrinsic growth factors and extrinsic signalling pathways, as a result of regulatory cytokine stimulation (Zhu & Emerson, 2002).

In humans and mice, several cytokines have been shown to act in concert as differentiation factors in monocytes and granulocytes. Early stages of differentiation are regulated by granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-6, stem cell factor and Flt3 ligand. These factors enhance the development of multiple lineages, whereas the final stages of differentiation into monocytes and granulocytes are stimulated by colony-stimulating factor 1 (CSF-1) and GM-CSF,
respectively (Barreda et al., 2004; Emerson, 2002; Lewis & Harriman, 1996). Under steady-state, un-stimulated conditions BM stromal cells constitutively secrete, and present on their surfaces, low levels of hematopoietic growth factors. This supports basal hematopoiesis and the maintenance of blood cell counts. However, under stress or stimulated conditions, stromal cells secrete increased quantities of these factors in response to inflammatory mediators produced at sites of inflammation by activated macrophages. The consequence is an increase in the number of circulating neutrophils and monocytes (Barreda et al., 2004; Ezquerra et al., 2009; Zhu & Emerson, 2002).

Historically, it has been assumed that the majority of macrophages are derived from the circulating pool of blood monocytes. However, it is becoming increasingly clear that there are alternative mechanisms for the maintenance and replenishment of local macrophage populations. Tissue-resident macrophage populations, including: AM, splenic macrophages and liver Kupffer cells are now known to be maintained by additional local proliferation, particularly under steady-state conditions (Crofton et al., 1978; Landsman et al., 2007; Sawyer et al., 1982; Tarling et al., 1987; Varol et al., 2008; Wijffels et al., 1994).

1.3.3 Host immune response to PRRSV

The ability of PRRSV to infect pigs in the presence of a fully functional immune system is achieved through an archetypal and multifaceted adaptation to its host. The delayed and defective patterns of both innate and adaptive immune response underlies the long-term persistence of PRRSV in infected pigs (Batista et al., 2004).

Innate immune response

The atypical immune response to PRRSV suggests that the virus robustly modulates it. PRRSV is highly susceptible to inhibition by type I IFNs, with exogenous IFN-α or IFN-β reducing viral replication in AM in vitro (Albina et al., 1998a; Overend et al., 2007). However, PRRSV is able to impede IFN-α production and attenuate the IFN response to other pathogens (Albina et al., 1998a; Buddaert et al., 1998). PRRSV is also capable of inhibiting double-stranded (ds) RNA-mediated
up-regulation of IFN-β gene transcription (Miller et al., 2004). These characteristics allow the virus to replicate efficiently, invade neighbouring cells and establish a prolonged infection.

The mechanism behind the viral inhibition of IFN-α production remains to be fully elucidated. However, it is not mediated through widespread transcriptional repression (see Chapter 3 of this thesis). Functional screening of the PRRSV nsps has identified four proteins with a suppressive effect on type I IFN, namely: nsp1, nsp2, nsp4 and nsp11 (Kim et al., 2010). Nsp1 has the potential to inhibit IFN-β promoter activity and nsp1β and nsp11 have the ability to inhibit IRF3-mediated, type I IFN production (Beura et al., 2010). Nsp11 blocks the phosphorylation of IRF3 and IκB, preventing their nuclear translocation and subsequently inhibiting IFN production (Sun et al., 2012). Conversely, PRRSV N has been shown to stimulate NF-κB in a dose-dependent manner (Lee & Kleiboeker, 2005) and nsp2 also activates NF-κB in vitro (Fang et al., 2012). However, PRRSV nsp1α has been reported to inhibit the activation of NF-κB in another study (Song et al., 2010) and nsp1α interacts with PIAS1, a negative regulator of STAT1, in yeast-two-hybrid assays (Sun et al., 2012). Nsp11 is also able to degrade IFN-β promoter stimulator 1 (IPS-1) mRNA in PRRSV-infected cells, resulting in the suppression of type I IFN induction through the consequent inhibition of the RIG-I signalling pathway (Luo et al., 2008; Sun et al., 2012). IPS-1 also functions as a conduit between type I IFN induction and apoptosis, providing a plausible hypothesis for the regulation of apoptosis by PRRSV.

The adaptive immune response to a pathogen depends, in part, on a robust innate immune response. Therefore, the suboptimal host innate immune response upon PRRSV infection is likely to result in the adaptive immune response being ineffective in providing full immunity. The adaptive immune response to PRRSV infection is characterised by the delayed emergence of NAbs (Lopez & Osorio, 2004), a brief cell-mediated immune response (Xiao et al., 2004) and slow development of a virus-specific IFN-γ response (Meier et al., 2003).

Humoral immune response
Circulating antibodies against PRRSV are detectable from as early as 5 to 7 dpi (Yoon et al., 1995). PRRSV-specific IgM peaks at 14 dpi and then declines, becoming undetectable by 42 dpi, and IgG concentrations peak at 21 to 49 dpi (Loemba et al., 1996). However, the observed IgM and IgG response does not correspond to NAb production (Yoon et al., 1994). NAbs are generally detected later than 28 dpi against both type I and type II PRRSV strains and even then, often only at low antibody titres (Diaz et al., 2005; Meier et al., 2003; Yoon et al., 1994). A protective level of immunity is only achieved following at least 3 months of PRRSV infection in the majority of animals (Kimman et al., 2009).

PRRSV NEs have not been fully characterised. However, GP2, GP3, GP4, GP5 and M have all been implicated as inducers or targets of NAbs (Ansari et al., 2006; Cancel-Tirado et al., 2004; Kim & Yoon, 2008; Plagemann, 2004; Vanhee et al., 2011; Yang et al., 2000). As a consequence of its high genomic variability, PRRSV shows great antigenic heterogeneity (Bautista et al., 1993; Drew et al., 1995; Yang et al., 1999). Random changes in, or around, the amino acid sequence of NEs, stimulated by the virus’s high mutation rate and then selected by immune pressure, can generate neutralisation-resistant mutants (Costers et al., 2010; Martínez-Lobo et al., 2011; Prieto et al., 2009). PRRSV variants with heavily glycosylated GP5 are less sensitive to neutralisation (Ansari et al., 2006; Faaberg et al., 2006). The existence of virus “immunotypes” with differing capacities to suppress or escape host immunity have also been demonstrated (Darwich et al., 2011).

As a result of the humoral immune response, pigs are able to develop strong homologous immunity, preventing re-infection by the same strain of virus for at least 600 days after the initial infection (Lager et al., 1997). Additionally, the passive transfer of PRRSV NAbs to gestating sows can confer sterilising immunity to challenge with a homologous virulent strain (Osorio et al., 2002). However, heterologous protection is inconsistent and less complete (Lager et al., 1997; Lager et al., 1999). NAbs may also be involved in the resolution of viremia through cytolysis or apoptosis of PRRSV-susceptible macrophages (Lopez & Osorio, 2004). The exhaustion of permissive macrophages through this process may result in the confinement of PRRSV to the macrophage-rich lymph nodes, a process that has been described for the closely related LDV in mice (Diaz et al., 2006).
Non-NAbs are an important asset for PRRSV, given that antibody-dependent enhancement (ADE) takes place in AM (Yoon et al., 1996). ADE occurs when non-NAbs facilitate virus entry into host cells, leading to increased infectivity. In the case of PRRSV, the method of ADE may be due to the coating of the virus, subsequently enhancing the uptake of viral particles by permissive macrophages.

**Cell-mediated immunity**

PRRSV-infected pigs develop a relatively rapid humoral response. However, these initial antibodies do not confer protection and can even mediate the ADE of disease. In contrast to the rapid humoral response, the development of NAbs is delayed and the generation of cell-mediated immune responses, including PRRSV-specific IFN-γ-secreting cells, is erratic.

The detection of serum-NAbs and IFN-γ-secreting cells shows a defective and erratic pattern in experimental trials (Mateu & Diaz, 2008). One study failed to detect IFN-γ-secreting cells until 8 to 10 weeks post-infection, with their frequency gradually increasing until 48 weeks post-infection and remaining relatively stable until 98 weeks post-infection (Meier et al., 2003). The delayed development of PRRSV-specific IFN-γ secreting cells has also been noted in other studies, following either infection or vaccination with PRRSV (Diaz et al., 2005, 2006). However, despite being slow to develop, the specific cell-mediated response to PRRSV is important and may provide a degree of clinical protection in experimental settings (Bautista & Molitor, 1997; Meier et al., 2003). Clinical protection following immunisation with PRRSV vaccines has also been shown to be associated with the activation of the cell-mediated immune response (Martelli et al., 2009).

Researchers have attempted to identify the PRRSV epitopes responsible for the induction of the cell-mediated immune response. Immunodominant T-cell epitopes have been identified in PRRSV GP5 and M proteins, both being able to induce interferon IFN-γ production in peripheral blood mononuclear cells obtained from PRRSV-infected pigs (Vashisht et al., 2008; Wang et al., 2011).

Thus, PRRSV proteins exert a range of stimulatory and inhibitory effects on key members of the host innate immune response. The overall result is that the virus
is able to control the innate immune response without shutting off host transcriptional activity, enabling it to establish a productive and prolonged infection. Additional important factors contributing to the success of PRRSV in infecting immunocompetent animals are: the exploitation of non-NAbs for ADE and the delayed emergence of NAbs against the virus. Additionally, the delayed development of PRRSV-specific IFN-γ secreting cells leads to a relatively ineffective cell-mediated immune response in most cases.

1.4 PRRSV-host interacting proteins and their roles in infection

1.4.1 Virus attachment and internalisation

An important early interaction in the PRRSV infection process occurs through the cell surface receptor heparan sulphate. Heparan sulphate glycosaminoglycans (GAGs) on the macrophage surface function as PRRSV attachment factors, promoting the concentration of virions on the cell surface and increasing infection efficiency (Figure 1.3a). The source of the interaction between the viral envelope and heparan sulphate molecules on the host cell surface appears to be the PRRSV M/GP5 complex, which binds heparin (Delputte et al., 2002). Despite the role of heparan sulphate on the host cell surface in PRRSV infection, this molecule is not essential. A complete block of virus infection cannot be obtained in AM through pre-incubation with either heparin or heparan sulphate (Delputte et al., 2002; Vanderheijden et al., 2001).

An additional early interaction occurs between PRRSV and the CD169 cell surface receptor. Early virus attachment occurs predominantly through the interaction with heparan sulphate, followed by a gradual increase in binding to CD169 (Figure 1.3b; Delputte et al., 2005). Sialic acids on the viral M/GP5 heterodimer interact with the N-terminal domain of CD169, present on the surface of AM, triggering virus internalisation (An et al., 2010; Van Breedam et al., 2010a). This engagement of CD169 by PRRSV can inhibit the phagocytic capacity of AM in vitro (De Baere et al., 2012). Both the sialic acid-binding activity of CD169 and the sialic acids on the surface of the PRRSV virion are important in the infection of AM (Delputte & Nauwynck, 2004). Pre-incubation of AM with heparin, together with an
anti-CD169 antibody, effectively blocks virus attachment. Therefore, heparan sulphate and CD169 are accepted to be the main factors involved in PRRSV attachment to AM (Delputte et al., 2005).

Following binding of PRRSV, first to heparan sulphate and then to CD169 (Figure 1.3b), the virus is internalised via the process of clathrin-mediated endocytosis (Figure 1.3c), allowing the virus to enter the endocytic pathway (Nauwynck et al., 1999; Vanderheijden et al., 2003).

The expression of CD169 in several non-permissive cell lines renders these cells able to bind and internalise PRRSV virions (e.g. Delrue et al., 2010). However, a productive infection does not occur in these cells due to a lack of nucleocapsid disassembly. This indicates a requirement for additional macrophage-specific factors for productive PRRSV infection flowing virus binding and internalisation.

1.4.2 Virus uncoating and genome release

Productive PRRSV infection requires the release of the virus genome into the cytoplasm of the host cell. For this step to occur, a pH drop within the virus-containing endosome is required (Figure 1.3c; Kreutz & Ackermann, 1996). In AM, PRRSV virions appear to travel no further than the early endosomes (pH 6.0 to 6.5), suggesting that the virus releases its genome in the early endosome (Van Gorp et al., 2009).

CD163 is a scavenging receptor that is expressed on the plasma membrane and in the early endosome; colocalising with PRRSV in the latter compartment (Figure 1.3d; Van Gorp et al., 2009). CD163 appears to play a crucial role in virus genome release in AM (Figure 1.3e; Van Gorp et al., 2008). Expression of exogenous porcine CD163 in several otherwise non-permissive cell types leads to productive PRRSV infection, indicating that this molecule functions during the PRRSV replication process (Calvert et al., 2007; Delrue et al., 2010; Wang et al., 2013a). The physical interaction between PRRSV and CD163 is mediated through the viral GP2 and GP4 glycoproteins (Das et al., 2010), with CD163 scavenger receptor cysteine-rich (SRCR) domain 5 being essential for this interaction and subsequent PRRSV infection (Van Gorp et al., 2010b).
Figure 1.3 | A model of PRRSV entry into AM. PRRSV first attaches to heparan sulphate GAGs on the cell surface (a), before establishing a more stable attachment through binding to the N-terminal domain of CD169 via the viral M/GP5 complex (b). Following attachment to CD169, the virus is internalised via clathrin-mediated endocytosis (c). PRRSV is now present in the early endosome, where binding to CD163 and acidification of the endosome (d) is essential for uncoating and subsequent genome release into the cytoplasm (e). Figure adapted from Van Breedam et al. (2010b).
The transfection of a CD163 deletion mutant missing the cytoplasmic domain has been shown to be sufficient to render cells infectable by PRRSV. Furthermore, the absence of the cytoplasmic tail notably enhanced virus replication in this system (Lee & Lee, 2010). The mechanism behind these findings is unclear. However, it could potentially be a result of improper folding of tailless CD163, altering its function as a receptor for PRRSV, or a result of changes in downstream host signalling that are conducive to virus replication (Lee & Lee, 2010). It has been demonstrated that CD163 can cycle between the plasma membrane and early endosomes (Schaer et al., 2006). Therefore, CD163 present at the cell surface may have the potential to facilitate PRRSV entry as well as uncoating and genome release. CD163 can also be released in a soluble form (sCD163) and taken back into cells (Van Gorp et al., 2010a). Therefore, it may be possible for sCD163 to act as an internalisation receptor if bound to the virus, negating the requirement for CD169 (Prather et al., 2013).

In summary, although the classical PRRSV entry mechanism into AM, involving CD163, CD169 and heparan sulphate, has been widely studied, much of this process still remains to be elucidated. The apparent dispensability of key proteins in various cell types, poses the question of if there are additional molecules and processes involved in PRRSV entry that remain to be uncovered.

1.4.3 PRRSV genome replication, assembly and egress

Once the PRRSV genome has been released into the cytoplasm then replication can take place, with direct translation of the positive-sense RNA genome by host cellular machinery (Dea et al., 2000). The translation of ORF1a and 1b results in polyproteins 1a and 1b which are subsequently cleaved into 14 non-structural proteins, including the replication-critical virus RdRP (Ziebuhr et al., 2000). The RdRP initiates replication by generating a full length negative-sense RNA genome, which is used as the template for positive-sense RNA synthesis (Pasternak et al., 2006). The resultant positive-sense RNA is used for further synthesis of nsps and the PRRSV sgmRNAs serve to express the structural proteins from ORFs located in the 3’-proximal third of the genome, which are not accessible for ribosomes engaged in genome translation. Newly synthesized genomes are
encapsidated by the N protein. Progeny virions acquire their envelope by budding into the lumens of membranes of the endoplasmic reticulum-to-Golgi pathway (Dea et al., 2000; Pasternak et al., 2006). Intraluminal virions are then released from the host cell via exocytosis, through the fusing of the vesicle wall and the cytoplasmic membrane (Dea et al., 1995; Thanawongnuwech et al., 1997).

In summary, of all of the stages of the PRRSV replication cycle, the entry and uncoating processes have been the primary focus of research. This research has concentrated on studying the cellular receptors involved in direct interactions with the virus and several of these receptors are now relatively well characterised. However, other important stages of the PRRSV replication cycle still remain to be fully elucidated, such as the mechanisms of discontinuous transcription and egress.

1.4.4 The prototypic PRRSV receptors: CD163 and CD169

i) CD169

The Ig-type lectin, CD169 (sialoadhesin, Siglec-1 or Sn), was initially identified as a sialic acid-dependent, sheep erythrocyte receptor expressed by murine macrophages (Crocker & Gordon, 1986). It is now also characterised in humans, rats and pigs (Crocker & Gordon, 1989; Dijkstra et al., 1985; Hartnell et al., 2001; Vanderheijden et al., 2003). The Ig-type lectins are expressed, with the exception of Siglec-4 and Siglec-6, on distinct subsets of haematopoietic cells (Crocker et al., 2007). CD169 is constitutively expressed on specific subsets of tissue-resident macrophages in the spleen, BM, lymph nodes, liver, colon and lungs (Crocker et al., 1991; Crocker et al., 1994; Crocker & Gordon, 1989; Hartnell et al., 2001).

CD169 is one of the largest members of the Ig superfamily, with an extracellular region consisting of 16 constant Ig domains, following an N-terminal, variable Ig-like domain (Crocker et al., 1994; Delputte et al., 2007). The biological and structural characteristics of porcine CD169 suggest that this molecule is also a sialic acid-binding lectin (Delputte & Nauwynck, 2004; Vanderheijden et al., 2003).

CD169 is highly expressed on inflammatory macrophages and has been implicated in breast carcinoma, peripheral and central nervous system demyelination, autoimmune uveoretinitis, rheumatoid arthritis, proliferative glomerulonephritis,
multiple sclerosis and human immunodeficiency virus (HIV) infection, suggesting a role for CD169-positive macrophages in these conditions (Hartnell et al., 2001; Ikezumi et al., 2005; Ikezumi et al., 2006; Ip et al., 2007; Jiang et al., 2006; Kobsar et al., 2006; Nath et al., 1999; Pulliam et al., 2004; Wu et al., 2009). Overall, these data are consistent with a potentially important role of CD169 in the modulation of T-cell function and activation, as well as in the capacity of a phagocytic receptor in the clearance of sialylated pathogens (Crocker et al., 2007). A xenogeneic innate immune receptor function has recently been suggested for CD169, helping to explain the recognition of foreign epitopes by macrophages upon xenotransplantation (Brock et al., 2012).

Unlike most siglecs, CD169 lacks the tyrosine-based signalling motifs that are implicated in signal transduction and endocytosis and has a cytoplasmic tail that is poorly conserved among mammalian species (Hartnell et al., 2001; O’Reilly & Paulson, 2009). These factors may suggest a primary role of CD169 as a binding partner in cellular interactions, as opposed to cell signalling. Conversely, a role in cell signalling could be inferred from published data demonstrating the ability of CD169 to internalise a specific antibody (Vanderheijden et al., 2003) as well as to bind and phagocytose sialylated Neisseria meningitides (Jones et al., 2003). As a result of the apparent involvement of CD169 in immunity, its endocytic capability and constitutive expression on macrophages, research is being conducted to assess its potential for use in the targeted delivery of toxins or antigens (Delputte et al., 2011).

CD169 has been shown to play a role in PRRSV infection in multiple studies as a receptor mediating virus attachment and internalisation (Delputte & Nauwynck, 2006; Delputte et al., 2011). Additionally, PRRSV binding to a soluble form of the four N-terminal Ig-like domains of CD169 has recently been demonstrated in vitro (Chen et al., 2013). However, conflicting data have also emerged, showing that CD169 is not required for PRRSV infection in vivo (Prather et al., 2013). Nevertheless, CD169 is probably an important PRRSV receptor in AM and is implicated in a multitude of host inflammatory and signalling roles.

ii) The macrophage scavenger receptor CD163
The scavenger receptors (SRs) are a large group of trans-membrane, cell surface glycoproteins involved in the recognition and endocytosis of polyanionic structures of either endogenous or exogenous origin (Sarrias et al., 2004). The range of ligands bound by SRs is diverse, including microbial structures and a plethora of endogenous molecules (Plüddemann et al., 2007). SR expression is restricted to macrophages, DCs and certain other cell types (Murphy et al., 2005). SRCR-containing proteins have been described in a variety of divergent organisms, such as: invertebrates (sponges, echinoderms and insects) and vertebrates (human, mouse, rat, pig, cow, rabbit, sheep, avian, amphibian and fish) and even in algae (Dangott et al., 1989; Goldberger et al., 1987; Mayer & Tichy, 1995; Pancer et al., 1997; Wheeler et al., 2008).

The SRCR superfamily has over 30 assigned members, divided into groups A and B. Group A SRCR domains are encoded by at least two exons and contain six cysteine residues, with group B SRCR domains containing eight cysteine residues and each being encoded by a single exon (Martínez et al., 2011). However, CD163 is a notable exception, as SRCR domain 8 contains only six cysteine residues. Thus, CD163 is considered to be in a subgroup of SRCR group B. The precise biological function of the SRCR domain remains to be determined, but is likely to involve the mediation of protein-protein interactions.

Structurally, the CD163 protein has an ectodomain consisting of nine SRCR domains in tandem, with SRCR domains 6 and 7 being separated by a proline-serine-threonine (PST)-rich interdomain segment (Figure 1.4). A short linker section connects SRCR domain 9 with a TM domain and cytoplasmic tail (Figure 1.4). The cytoplasmic tail of human CD163 is subject to alternative splicing, generating at least three splice variants of differing lengths (Law et al., 1993; Ritter et al., 1999b). Splice variants affecting the extracellular domains of human CD163 have also been reported (Law et al., 1993).

CD163 is a TM type I glycoprotein expressed by monocytes in the circulation and up-regulated during differentiation to macrophages, being widely used as a marker of the monomyeloid lineage (Backe et al., 1991; Buechler et al., 2000; Morganelli & Guyre, 1988; Philippidis et al., 2004; Pulford et al., 1992; Sanchez et al., 1999; Sulahian et al., 2000). CD163 is highly expressed on tissue-resident
(Fabriek et al., 2005; Lau et al., 2004; Pulford et al., 1992; Van den Heuvel et al., 1999; Zwadlo et al., 1987), alternatively activated (M2) (Lee et al., 2008; Nagorsen et al., 2007; Ohri et al., 2009; Shabo et al., 2008) and pro-angiogenic macrophages (De Palma et al., 2005; Venneri et al., 2007). The use of a variety of antibodies against CD163, prior to its cloning and full characterisation, has resulted in the existence of multiple aliases for this molecule in the scientific literature, including: CD163 (Kishimoto et al., 1997), AM-3K (Komohara et al., 2006; Zeng et al., 1996), Ber-MAC3 (Backe et al., 1991), ED2 antigen (Dijkstra et al., 1985), GHI/61 (Pulford et al., 1992), HbSR (Kristiansen et al., 2001), M130 (Ritter et al., 1999a), p155 (Morganelli & Guyre, 1988), RM3/1 (Zwadlo et al., 1987), SM4 (Pulford et al., 1992) and Ki-M8 (Radzun et al., 1987).

Perhaps the most established function of CD163 is its ability to bind and endocytose haemoglobin-haptoglobin (HbHp) complexes. This interaction has been mapped to the third SRCR domain (Figure 1.4; Madsen et al., 2004). Upon binding to CD163, the heme group is converted into iron and bilirubin by heme oxygenase-1 (HO-1) (Kristiansen et al., 2001; Nielsen et al., 2010). HO-1 expression can be induced by glucocorticoids and IL-10, which also induce CD163 expression (Philippidis et al., 2004; Wagener et al., 2003). Furthermore, the binding of HbHp complexes to CD163 triggers the secretion of IL-10 (Philippidis et al., 2004). These data point to the existence of a positive-feedback loop that controls the coordinated up-regulation of a system that is activated upon the release of hemoglobin into the circulation, with the role of protecting tissues from heme-mediated damage (Madsen et al., 2004; Ritter et al., 2001; Van den Heuvel et al., 1999). Aside from this protective function, heme clearance from the circulation could also reduce the availability of iron to pathogens, consequently helping to fight infection (Madsen et al., 2004; Weaver et al., 2006).

CD163 expression is regulated via various, complex mechanisms; for example, CSF-1 strongly induces CD163 expression on monocytes. However, upon the differentiation of monocytes to DCs using GM-CSF and IL-4, CD163 expression is suppressed (Buechler et al., 2000). The binding of LPS to toll-like receptor (TLR) 4 induces the expression of IL-6 and IL-10 and, in turn, CD163 expression (Weaver
et al., 2007; Weaver et al., 2006). However, LPS also directly induces CD163 ectodomain shedding, resulting in sCD163, which circulates in the plasma and whose physiological role is unknown (Droste et al., 1999; Hintz et al., 2002; Møller et al., 2002).

Most of the CD163 SRCR domains have been implicated in a variety of binding roles (Figure 1.4). As with other SR superfamily members, CD163 is capable of binding both Gram-positive and Gram-negative bacteria in its role as a pattern recognition receptor (PRR) (Fabriek et al., 2009). This binding motif has been mapped to CD163 SRCR 2 (Figure 1.4; Fabriek et al., 2007). Binding of bacteria by CD163 results in the production of pro-inflammatory cytokines, including TNFa, suggesting that it is a bacteria-sensing PRR (Fabriek et al., 2009).

As discussed earlier in this Chapter, CD163 is a receptor for PRRSV in AM (Calvert et al., 2007). Its direct interaction with PRRSV has been mapped to the fifth SRCR domain (Figure 1.4; Van Gorp et al., 2010b). However, a recent study reported the generation of soluble CD163 domains, with SRCR 5 alone not resulting in PRRSV binding, but SRCR domains 5 to 9 together did bind PRRSV (Figure 1.4; Chen et al., 2013). CD163 also mediates and is essential for successful infection of porcine macrophages by African swine fever virus (ASFV) (Sanchez-Torres et al., 2003). The responsible interaction appears to occur through one, or all, of the three CD163 N-terminal SRCR domains: 1, 2 or 3 (Figure 1.4; Sanchez-Torres et al., 2003). CD163 can also bind and sequester TNF-like weak inducer of apoptosis (TWEAK), hampering its ability to induce apoptosis of tumour cells (Chicheportiche et al., 1997). Bover et al. (2007) predicted a total of 44 putative interacting sites between TWEAK and CD163, which were located within eight of the nine extracellular SRCR domains (Figure 1.4).

The exact role of CD163 in the immune response remains unclear, as it elicits differing responses dependent on stimuli and context. Overall, CD163 appears to be a multifaceted immunomodulator that is capable of inducing or suppressing the immune response. The plethora of roles of CD163 SRCR domains in protein binding and pathogen-receptor interactions suggests that further research is likely to uncover additional functions of this important macrophage receptor.
Figure 1.4 | Structure of the porcine CD163 protein and known functions of the SRCR domains.
1.5 The current state of PRRSV research

The exploitation of a heparin-like molecule by PRRSV was identified as far back as 1997 (Delputte et al., 2002; Jusa et al., 1997), CD169 was identified as a PRRSV entry receptor in 1998 (Duan et al., 1998a; Duan et al., 1998b) and in 2007 CD163 was shown to play an integral part in the infection of AM (Calvert et al., 2007). These seminal publications have led to an acceptance within the field of PRRSV research of the aforementioned three molecules being the cornerstones of the PRRSV infection process in AM, the most biologically relevant cell culture system currently available.

However, despite the identification and characterisation of convincing receptors for PRRSV in a variety of cell types over the past 15 years, much is still unclear about the PRRSV infection process. Additional molecules are being implicated in PRRSV entry and infection. Simian vimentin interacts with the PRRSV N-protein on the cell surface (Kim et al., 2006) and CD151 may be involved in the fusion of the viral envelope and the endosome and its overexpression can render cells permissive to PRRSV infection (Huang et al., 2013; Shanmukhappa et al., 2007). Porcine DC-SIGN may also be involved in the transmission of PRRSV from infected cells (Huang et al., 2009). In addition, both the aspartic protease cathepsin E and an unidentified trypsin-like serine protease, have been implicated in the uncoating of internalised PRRSV and subsequent infection (Misinzo et al., 2008). These findings are further complicated by the apparently contradicting results obtained in different systems and studies. CD169 appears to be dispensable in some cell types (Calvert et al., 2007; Van Gorp et al., 2008) and indeed, in vivo (Prather et al., 2013). A recent study also identified PRRSV-infected CD163-positive/CD169-negative and CD163-negative/CD169-negative cells in the nasal mucosa, indicating that CD163 (and CD169) independent PRRSV infection may be possible (Frydas et al., 2013).

In conclusion, PRRSV has been a priority livestock infectious disease since its emergence in the early 1980s. However, despite the extent of research into the virus, the research community appears to be a long way from providing the solutions required to effectively tackle the disease. Existing vaccines are largely ineffective, especially given the heterogeneity of PRRSV field isolates. There is a current lack of
suitable cell culture systems available to undertake biologically relevant *in vitro* PRRSV research. The difficulties in identifying essential PRRSV receptors which operate across multiple cell types has led to a fragmented research field, with different groups focussing on each putative receptor. Therefore, it is important to foster more inter-disciplinary and collaborative research efforts in order to effectively tackle this costly and damaging disease.

1.6 Thesis scope and outline

There is a scarcity of infectable, biologically relevant and tractable *in vitro* host cell types that are available for PRRSV research. To tackle this, Chapter 2 explored existing options for *in vitro* PRRSV culture, resulting in the discovery of an infectable, bone marrow-derived macrophage (BMDM) primary cell system. Further characterisation of BMDM as a PRRSV host was carried out and revealed a potential alternative PRRSV entry mechanism, independent of several known cellular receptors. Chapter 3 sought to examine the host transcriptional response to PRRSV infection using network-based analysis techniques. These analyses resulted in the identification of biological pathways that were conserved between two contrasting expression datasets. The representation of members of the guanylate binding protein (GBP) family in key, PRRSV-induced transcript clusters, coupled with the fact that loci encoding the GBP proteins map to the chromosomal region associated with variation in viral load and weight gain in response to PRRSV infection (Boddicker *et al.*, 2012) prompted further investigation into the importance of its membership. Chapter 4 introduced porcine GBP-1 and focused on its cloning and overexpression. This led to the discovery that GBP-1 overexpression had an inhibitory effect on PRRSV replication and colocalised with PRRSV nsp2. The targeted introduction of a single nucleotide substitution into the helical domain of GBP-1 resulted in an apparent alteration in the subcellular localisation of this protein. Thus, the GBP family is proposed to contain strong potential candidate genes for the study of a genetic basis for PRRSV resistance.
Chapter Two

Establishing a tractable in vitro culture system for PRRSV

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2.1 Introduction

2.1.1 General introduction

PRRSV research is impeded by a lack of a suitable in vitro cell culture system. Reports of a porcine AM-derived cell line (Lee et al., 2010; Weingartl et al., 2002) and the recently published technique for the generation of porcine BMDM (Kapetanovic et al., 2012) potentially provide new, biologically relevant porcine cell culture systems. This chapter reports attempts to characterise the phenotype of these cells and assess their potential for the growth and study of PRRSV. The ultimate aim of this research was to develop an in vitro culture system for the study of host-virus interactions in PRRSV infection.

2.1.2 PRRSV infectious clones

Viruses engineered to express a reporter gene offer a convenient and reproducible method of rapidly and accurately quantifying virus infection. They are amenable to use in high-throughput assays and do not require a further antibody staining step for detection, saving time, money and also limiting unwanted consequences of repeated wash steps. Several reverse genetics systems for PRRSV that allow the creation of infectious (and potentially modified) virus from cDNA have been published in the scientific and patent literature (Calvert et al., 2002, 2006; Fang et al., 2006; Meulenberg et al., 1998a; Meulenberg et al., 1998b; Nielsen et al., 2003; Yoo et al., 2004).

Fang et al. (2006) reported the generation of an infectious clone of a type I PRRSV isolate known as pSD01-08. In contrast to the prototypic European type I strain PRRSV LV, pSD01-08 was derived from a type I (European) PRRSV parental strain (SDPPIRS 01-08) which was isolated in North America in 2001 (Zeman et al., 1993). SDPPIRS 01-08 was isolated from a group of 8 week-old pigs showing no clinical signs of infection (Zeman et al., 1993). Initial diagnostic sequencing of ORF5 showed a close similarity to PRRSV LV, which identified 01-08 as a type I PRRSV strain. SDPPIRS 01-08 was initially isolated on porcine AM (Zeman et al., 1993) and subsequently adapted to growth in culture by passaging 33 times on MARC-145 cells, at which time the new isolate was renamed 01-08-P33 (Fang et al.,
Despite being isolated on separate continents, 01-08-P33 and LV share significant sequence homology. Nsp9 is the most conserved protein between LV and 01-08-P33, which exhibits 98.9% amino acid identity, with the least conserved being nsp2, at 90.6% amino acid identity (Fang et al., 2004). 01-08-P33 possesses a unique 51 bp deletion in the immunodominant region of nsp2, which was confirmed to be present in the original isolate and, as such, was not a direct result of extended passaging in vitro (Fang et al., 2004). Indeed, as many as 403 codons can be removed from nsp2 without affecting PRRSV viability in vivo (Faaberg et al., 2010).

To derive the infectious clone pSD01-08_CMV, cDNA was generated from the parental virus and cloned into the pACYCI77 plasmid vector (Figure 2.1; Fang et al., 2006). Expression of the clone is driven by a cytomegalovirus major immediate early (CMV) promoter, with RNA polymerase II (Pol II) transcription being terminated downstream of the viral sequences (including a synthetic polyA tail) through the addition of a hepatitis delta virus ribozyme (HDV rbz; Figure 2.1). An enhanced green fluorescent protein (GFP) gene was also inserted, in frame, into the nsp2 deletion site, resulting in the translation of an nsp2-GFP fusion protein (Figure 2.1). In order to confirm the expression of the nsp2-GFP fusion protein, Fang and colleagues stained cells infected with the SD01-08 virus with an nsp2-specific antibody. Two-colour confocal microscopy confirmed the perinuclear colocalisation of both GFP and nsp2, which was also similar to that of nsp2 in the parental virus (Fang et al., 2006). Due to the location of the GFP gene within the nsp2 ORF, the nsp2-GFP fusion protein is immediately translated upon virus entry.
Figure 2.1 | pSD01-08_CMV plasmid structure and transcription mechanism. The PRRSV cDNA, along with 41 nucleotide 3` poly(A) tail, flanked by Ascl and XbaI restriction enzyme sites, has been inserted into the pACYCI77 plasmid vector. Expression is under the control of a CMV promoter, in the place of the T7 promoter described by Fang et al. (2006). The 3` end of the RNA Pol II transcript is generated by the action of a HDV ribozyme. The SD01-08 construct was generated and supplied by Dr. Ying Fang.
2.1.3 Current status of PRRSV vaccine production systems

Although vaccine virus production in AM would be ideal in order to reduce culture-adaptation, these cells cannot realistically be used due to batch variation, the risk of contamination with other pathogens present in primary cultures and the high production costs involved. In commercial PRRSV vaccine production systems the simian MARC-145 cell line is the most commonly used host. However, PRRSV entry into MARC-145 cells has been demonstrated to be different to entry into AM (Delputte et al., 2004). Furthermore, adaptation is required for the efficient growth of PRRSV isolates on MARC-145 cells, which results in genetic changes to the virus (Tan et al., 2001). Based on findings with other viruses, the host cell line can also influence antigen quality and the glycosylation pattern of the viral envelope glycoproteins (Schwarzer et al., 2009).

The efficacy of vaccine strains may also be affected by the host cell line used for their propagation. Calzada-Nova et al. (2012) investigated the ability of a modified live PRRSV vaccine strain to confer protection to young pigs against a PRRSV challenge in vivo. The same vaccine strain was propagated on either the MARC-145 or ZMAC (immortalised AM) cell lines (Calzada-Nova et al., 2012). In this study, MARC-145-grown vaccine-immunised animals exhibited a delay in the clearance of challenge virus from their lungs, as well as a greater virus load compared to ZMAC-grown virus-immunised animals. The dominant quasi-species in the ZMAC-grown virus stock contained a highly conserved N-glycosylation site at position 184 in GP2, which has been implicated in virus-receptor interactions (Das et al., 2010; Das et al., 2011), while this entity was underrepresented in the stock grown on MARC-145 cells (Calzada-Nova et al., 2012). These data suggest that the host cell may not only affect vaccine efficacy, but also result in changes to the PRRSV sequence. Thus, there is a need to develop a readily available, biologically relevant, cost-effective and easily cultured in vitro host cell for PRRSV research and vaccine production.

2.1.4 Bone marrow-derived macrophages

Cells of the MPS are the first line of defence against pathogens. The macrophage represents the tissue compartment of the MPS, along with monocytes
and their BM progenitor cells. Macrophages are critical to both innate and adaptive immune responses, responding to any changes in their environment in order to block pathogen infiltration. The result of tissue damage or pathogen attack is inflammation in the first instance, resulting from the accelerated production of pro-inflammatory cytokines and inflammatory mediators. However, macrophages are also essential in the control of the inflammatory response, avoiding autoimmune damage by the production of anti-inflammatory cytokines and increasing phagocytic activity. It has now been widely accepted that there are at least two phenotypic extremes of polarised macrophages: pro-inflammatory M1 (classically activated) and anti-inflammatory M2 (alternatively activated).

The respiratory host cell for PRRSV is the AM. These cells are commonly extracted as the principal component of bronchoalveolar lavage fluid (BALF) and used to propagate PRRSV for clinical, diagnostic and research purposes. However, there are complications with the in vitro growth of PRRSV on AM, including: individual variation in the permissiveness of cell isolates, reproducibility of results from different cell isolates and difficulties infecting AM with virus strains previously grown on continuous cell lines (mainly MARC-145). The BM is the primary reservoir for hematopoietic cells that give rise to oligopotent myeloid precursors. This reservoir of progenitor cells is responsible for maintaining blood counts under steady-state conditions and accelerated myeloid cell production under inflammatory conditions. Thus, the BM is a potential source of a significant supply of monocyte and macrophage cells if these cells could be extracted and cultured in vitro, under the appropriate conditions to give rise to “terminally differentiated” macrophage cells, akin to AM.

Previously, attempts have been made to culture cells derived from pig BM. Work by Mayer (1983) showed that pig BMC cultured in L929 cell-conditioned medium (a source of murine CSF-1) and horse serum resulted in the generation of cells with the morphological and functional characteristics of macrophages. Carrasco et al. (2001) used BMC isolated from the sternum of 3 to 6 month old pigs, cultured in growth medium supplemented with recombinant porcine (rp) GM-CSF either alone, or in combination with rpTNF-α, for 8 days to differentiate dendritic cells (DCs). Kekarainen et al. (2008) generated BM-derived DCs (BMDCs) for use in
PCV2 infection by culturing BMCs for 8 to 9 days in the presence of GM-CSF, following the protocol established by Carrasco and colleagues (2001). They found the BMDCs to have low levels of CD163 (CD163LO) and to be swine workshop cluster 3 (SWC3) and swine leukocyte antigen (SLA)-DR positive (Kekarainen et al., 2008). Mussá et al. (2011) derived BMDCs for infection with SIV using an 8 day protocol, combining those of Carrasco et al. (2001) and Kekarainen et al. (2008). The resulting BMDCs were characterised and also found to be CD163LO and SWC3 and SLA-DR positive (Mussá et al., 2011). It has recently been reported that porcine BMC can be reliably isolated and cryo-preserved and that their culture in the presence of the hematopoietic growth factor recombinant human (rh) CSF-1 and FBS (the same reagents used in murine and human systems) results in a pure population of BMDM (Kapetanovic et al., 2012).

Emery et al. (1996) published a protocol for the long-term culture of porcine hematopoietic progenitor cells. They found that BMC could be kept in culture for up to seven weeks, with colony-forming units (CFU)-granulocyte/macrophage (CFU-GM) being produced. These CFU-GM could be transplanted to fresh cultures and were confirmed as being active germinal centres for myelopoiesis. This BM culture protocol has significant attraction, with the ability to produce large numbers of cells from a single CFU, enabling research to be carried out using a single stock of primary cells. This could also reduce the number of animals sacrificed for research projects, as well as increasing experimental robustness and reproducibility to closer to that of a continuous cell line.

Studies have been published where BMCs have been differentiated for PRRSV infection. Hou et al. (2012) used L929-conditioned medium to differentiate BMC into BMDM in order to examine the effects of highly pathogenic PRRSV on TNFα release. Chang et al. (2008) generated so-called “BM immature-DCs” (BM-imDCs) from BMC isolated from the humerus and femur of young pigs and cultured in the presence ofrpGM-CSF and rpIL-4. The authors showed that the resultant BM-imDCs were permissive to PRRSV. However, viral replication dynamics were found to be slower than in AM, with a lower percentage of infected cells at all timepoints assayed, and lower viral loads in the supernatant (Chang et al., 2008). Peng et al. (2009) followed up the aforementioned data, using the same culture conditions, to
dissect changes in surface marker expression on BM-imDCs during PRRSV infection. Fraile et al. (2012) also derived BMDCs for use in the context of PRRSV research. The majority of these reports have focussed on “BMDCs” differentiated in the presence of the hematopoietic growth factor GM-CSF or cells differentiated using conditioned medium that is likely to contain a cocktail of growth factors.

2.1.5 Existing continuous cell culture systems for PRRSV

A number of cell types are known to support the in vitro replication of PRRSV (Table 1). The MARC-145 cell line is a derivative of MA-104 African green monkey (Chlorocebus sabaeus) kidney epithelial cells, selected for PRRSV-permissiveness by limiting dilution cell cloning (Kim et al., 1993). As a result of its robust, continuous growth characteristics and ability to support productive PRRSV infection, the MARC-145 cell line is widely used in research and vaccine production (Table 2.1). Additionally, multiple, non-permissive cell lines have been rendered susceptible to PRRSV infection through the plasmid-based over-expression of CD163 alone (Calvert et al., 2007), or CD163 and CD169 (sialoadhesin) together, including the baby hamster kidney (BHK-21) fibroblast cell line and the porcine kidney (PK-15) epithelial cell line (Table 2.1; Delrue et al., 2010). CD163-expressing PK-15 cells have also been generated using the PiggyBac transposon system and are susceptible to PRRSV infection (Wang et al., 2013b). Additionally, the stable transfection of CD151 into PK-15 cells has recently been shown to render them susceptible to PRRSV infection (Huang et al., 2013). However, the forced over-expression of exogenous genes may not preserve biologically relevant host signalling pathways or virus entry mechanisms.

Previously, the only readily available porcine cell line of a biologically relevant origin for studying respiratory PRRSV infection was the 3D4/21 line, which was established following SV40 large T antigen immortalisation of primary porcine AM cultures (Weingartl et al., 2002). However, this cell line also required the over-expression of CD163 in order to become permissive to PRRSV infection (Table 2.1; Lee et al., 2010). Recently, a number of reports have been published that contain work carried out using in-house, immortalised primary cells. Calzada-Nova et al. (2011) revealed that they have developed a AM-like cell line from foetal lung lavage
samples, named ZMAC-4 (Table 2.1). Further details are not forthcoming, although the cells are described in a US patent (Zuckermann, 2008) and a derivative, “ZMAC”, appears to have been used in another recent study (Calzada-Nova et al., 2012). It seems that this cell line is susceptible to PRRSV infection, at least by North American strains. In another study, AM have been transduced with human telomerase reverse transcriptase, allowing them to proliferate indefinitely, without affecting CD163 expression (Sagong et al., 2011). These “PAM-KNU” cells (Table 2.1) are said to be fully permissive to infection by both type I and II PRRSV strains (Sagong et al., 2011). However, the senior author of the Sagong et al. (2011) paper has rejected requests for aliquots of the cell lines (Prof. Alan Archibald, personal communication). Table 2.1 shows a summary of published PRRSV-permissive cell types and their characteristics.

The scarcity of biologically relevant cell culture systems for PRRSV and associated issues with virus production on cells such as MARC-145 prompted research into viable alternative culture systems. Thus, an aim of the work described in this Chapter was to characterise and assess the potential of an existing macrophage-like continuous cell line, with the purpose of utilising the SD01-08 infectious clone as a complete cell culture system for the investigation of host-virus interactions. An additional aim was to attempt to induce macrophage differentiation and, at the same time, render the cells permissive to PRRSV infection. The characterisation of BMDM and their permissiveness to infection by PRRSV was also investigated. Additionally, attempts were made to elucidate the PRRSV entry mechanism into BMDM.
Table 2.1 | A subset of cell types reported to be susceptible to PRRSV infection and their characteristics.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Source</th>
<th>Modifications</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM</td>
<td>Porcine BALF</td>
<td>N/A</td>
<td>• Biologically relevant host cell</td>
<td>• Poor growth of MARC-145-adapted PRRSV</td>
<td>Wenovaart et al., 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Good growth of PRRSV field strains</td>
<td>• Cannot be expanded</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Variability in permissiveness</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>• Contamination issues</td>
<td></td>
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<tr>
<td>BHK-21</td>
<td>Newborn Syrian hamster (Mesocricetus auratus) kidney</td>
<td>CD163 ectopic expression</td>
<td>• Fibroblast cell</td>
<td>• Fibroblast cell</td>
<td>Calvert et al., 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Hamster origin</td>
<td>• Hamster origin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Ectopic expression of CD163 required for permissiveness</td>
<td>• Ectopic expression of CD163 required for permissiveness</td>
<td></td>
</tr>
<tr>
<td>CHO-K1</td>
<td>Chinese hamster (Cricetulus griseus) ovary</td>
<td>CD169 and CD163 ectopic expression</td>
<td>• Easily transfected</td>
<td>• Ectopic expression of PRRSV receptors required for permissiveness</td>
<td>Van Gorp et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Rapid growth in culture</td>
<td>• Hamster origin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Epithelial cell</td>
<td></td>
</tr>
<tr>
<td>CL2621</td>
<td>Proprietary cell line: Boehringer Ingelheim Animal Health</td>
<td></td>
<td></td>
<td>• Poor PRRSV-permissiveness (hence subclone MARC-145 widely used)</td>
<td>Collins et al., 1992</td>
</tr>
<tr>
<td>MA104</td>
<td>African Green Monkey (Chlorocebus spp.) kidney</td>
<td>N/A</td>
<td>• Rapid growth in culture</td>
<td>• Poor PRRSV-permissiveness (hence subclone MARC-145 widely used)</td>
<td>Kim et al., 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Epithelial cell</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>• Monkey origin</td>
<td></td>
</tr>
<tr>
<td>MARC-145</td>
<td>African Green Monkey (Chlorocebus spp.) kidney, subclone of MA104</td>
<td>N/A</td>
<td>• Easily infected with many PRRSV strains</td>
<td>• PRRSV entry mechanism differs to natural host</td>
<td>Kim et al., 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Rapid growth in culture</td>
<td>• Epithelial cell</td>
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<td></td>
<td></td>
<td></td>
<td>• Monkey origin</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>• Extended virus passage leads to culture adaptation and inability to infect AM</td>
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<tr>
<td>NLFK</td>
<td>Feline kidney (Norden Laboratories)</td>
<td>CD163 ectopic expression</td>
<td>• Productive infection of multiple PRRSV strains and genotypes</td>
<td>• Feline origin</td>
<td>Calvert et al., 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Ectopic expression of CD163 required for permissiveness</td>
<td></td>
</tr>
<tr>
<td>PAM-KNU</td>
<td>Porcine BALF</td>
<td>human telomerase-immortalised</td>
<td>• Biologically relevant host cell</td>
<td>• Unclear</td>
<td>Sagong et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Macrophage like?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PK15</td>
<td>Porcine kidney</td>
<td>CD169 and CD163 ectopic expression, CD169 alone, CD151 alone</td>
<td>• Rapid growth in culture</td>
<td>• Ectopic expression of PRRSV receptors required for permissiveness</td>
<td>Van Gorp et al., 2008, Huang et al., 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Porcine origin</td>
<td>• Epithelial cell</td>
<td></td>
</tr>
<tr>
<td>ZMAC</td>
<td>Foetal pig BALF</td>
<td>Unclear</td>
<td>• Macrophage like?</td>
<td>• Unclear</td>
<td>Catzade-Nova et al., 2012</td>
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<td>3D4/21</td>
<td>BALF - SV40 transformed</td>
<td>CD163 ectopic expression</td>
<td>• Porcine origin</td>
<td>• Cell type unclear</td>
<td>Lee et al., 2010</td>
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<td></td>
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<td></td>
<td>• Biologically relevant isolation location</td>
<td>• Ectopic expression of CD163 required for permissiveness</td>
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2.2 Results

2.2.1 Characterisation of the SD01-08 cloned virus

To assess the utility of the SD01-08 GFP infectious clone for routine *in vitro* infection experiments across multiple cell types, virus rescue was undertaken using the pSD01-08_CMV plasmid (Figure 2.1), which has eGFP inserted, in-frame, within an nsp2 deletion site (Fang *et al.*, 2006). Virus-containing supernatant was produced using BHK-21 cells through the lipid-based transfection of the infectious clone plasmid, with expression being driven by a CMV promoter (see Figure 2.1). Following the optimisation of transfection conditions, a large volume of viral supernatant was produced from a single, bulk transfection. This negated the need to passage on, and risk adaptation to, a continuous cell line such as MARC-145.

The infectivity of the resultant virus-containing cell-culture supernatant was initially tested using MARC-145 cells. Cells were infected with an unknown titre of virus-containing cell-culture supernatant in a monolayer and GFP fluorescence was visualised by fluorescence microscopy at 48 hours post-infection (hpi). Infected cells exhibited strong, discrete GFP fluorescence, with no detectable fluorescence in mock infected samples (Figure 2.2). In order to confirm the expression of virus structural proteins (as well as nsp2) immuno-histochemical (IHC) staining was carried out using monoclonal antibody (mAb) SDOW-17, recognising the PRRSV N protein. The use of a horseradish peroxidase (HRP)-conjugated secondary antibody, detected using 3-amino-9-ethylcarbazole (AEC) substrate, confirmed that the observed GFP-positive units corresponded directly to cells containing the PRRSV N protein (Figure 2.2). The correlation between PRRSV N protein staining and GFP-positive cells was tested up until passage 11 on the MARC-145 cell line. No detectable change in the correlation between GFP-positive cells and PRRSV N protein staining was observed over all passages tested, suggesting that the GFP gene was not under strong negative selection pressure within the PRRSV backbone (*data not shown*).

Thus, the SD01-08 cloned PRRSV was successfully rescued using the BHK-21 cell line. Preliminary data suggested that the virus could prove to be a useful tool in further *in vitro* experiments.
Figure 2.2 | SD01-08 infection of the MARC-145 cell line. Microscopic images showing MARC-145 cells at 48 h after infection (or mock infection) with PRRSV SD01-08, imaged either by white light following IHC staining of the PRRSV N protein, using mAb SDOW-17 together with an HRP-conjugated secondary antibody and AEC substrate (left hand panels), or by fluorescence microscopy for GFP (right hand panels).
2.2.2 Characterising a porcine alveolar macrophage continuous cell line

One of the few readily available, continuous, porcine macrophage cell lines is the 3D4/21 line, having been established following SV40 large T antigen immortalisation of BALF cultures (Weingartl et al., 2002). The cell line is able to support the efficient replication of multiple viruses, including: vesicular stomatitis virus (VSV), ASFV and porcine adenovirus 3 (Weingartl et al., 2002). However, despite being derived from porcine BALF, the cells are not natively infectable by PRRSV. The forced overexpression of the CD163 receptor has been shown to render the 3D4/21 cell line permissive to both type I and type II PRRSV (Lee et al., 2010). These authors proposed that 3D4/21\(^{+}\)CD163 cells have the potential to closely mimic in vivo virus-host interactions in PRRSV research and they readily refer to the resultant cell line by the moniker: “porcine alveolar macrophage” (Chen et al., 2013; Lee et al., 2010; Lee & Lee, 2012). However, these cells were not readily available and requests directed to the authors for aliquots were not met with a response. Additionally, the lack of native CD163 expression and PRRSV-permissiveness raises the possibility that this cell line may not be macrophage-like. Accordingly, to evaluate the potential of the 3D4/21 cell line for use as a continuous cell culture system for PRRSV, work was undertaken to characterise the native cell line and elucidate its monomyeloid differentiation state.

Porcine monocytes are a heterogeneous cell population. Chamorro et al. (2005) devised a classification system for porcine monocytes, based primarily on the expression of SWC3, CD14, CD163 and SLA-DR markers (Figure 2.3). The four porcine monocyte subsets proposed by these authors can be interpreted as representing different stages of a maturation pathway (Figure 2.3).
Porcine monocytes can be divided into four subsets using flow cytometry, depending on the presence of absence of staining for four key markers: SWC3, CD14, CD163 and SLA-DR. In addition to these markers, variable levels of expression or secretion (from low to high) of CD11a, wCD11R1, CD29, CD49d, CD61, CD1a, CD80/CD86 and TNFα aid the classification of porcine monocytes. Figure based on phenotypic characterisation data of porcine monocyte subsets published in Chamorro et al. (2005).
Experiments were undertaken to characterise the 3D4/21 cell line and classify its monomyeloid differentiation state, compared to AM. Marker expression in AM and 3D4/21 cells was assessed using antibodies recognising key macrophage surface markers. AM and 3D4/21 cells were stained for the porcine monomyeloid marker SWC3, and analysed by flow cytometry (FC). In FC, ‘side scatter’ is proportional to the granularity of the cells being analysed, with granulocytes having higher side scatter than agranular cells such as lymphocytes. FC data for AM and 3D4/21 were plotted as the side-scatter (cell granularity) against the staining intensity of the SWC3 antibody. FC analysis confirmed that AM were granular cells with high levels of SWC3 staining (Figure 2.4A i). The 3D4/21 cell line exhibited similar granularity to AM, but expressed lower levels of SWC3. An aliquot of the 3D4/21 cells was seeded in growth medium supplemented with rhCSF-1 to investigate the effect of this growth factor on the cell population. After 3 weeks of sub-culture, the rhCSF-1-supplemented cell population was stained again for SWC3 and was found to have higher SWC3 staining levels and increased granularity (Figure 2.4A iii). This increase in SWC3 and granularity appeared to be directly attributed to the presence of rhCSF-1 in the culture medium, as its expression remained unchanged in 3D4/21 cells cultured in otherwise identical, non-supplemented growth medium (Figure 2.4A ii). This alteration of cellular phenotype could be due to an induced differentiation of the original cell line into a more monocyte/macrophage-like cell type, indicated by the apparent increase in levels of SWC3 expression (Figure 2.3). CSF-1 has long been known to induce differentiation into macrophages (Becker et al., 1987). Exposure to rhCSF-1 may also have led to the synchronisation of the cell cycle, explaining the relative homogeneity observed post-CSF-1 addition to the culture medium of a sub-population of 3D4/21 cells (Figure 2.4A iii).

Quantification of the percentage of cells expressing detectable levels of SWC3 protein revealed that the 3D4/21 cell line were 51 % SWC3-positive (Figure 2.4B; blue line), compared to 95 % of AM stained in parallel (Figure 2.4B). The culture of 3D4/21 cells in the presence of rhCSF-1 led to an increase in SWC3 expression to 82 % of cells staining positive (Figure 2.4B; green line).

Macrophages are professional phagocytes, so to assess whether 3D4/21 cells shared this phenotype and to corroborate data published by Weingartl et al. (2002),
phagocytosis assays were performed. These assays were carried out using zymosan particles, which consist of fluorophore-conjugated protein-carbohydrate complexes prepared from yeast cell walls. The 3D4/21 cell line exhibited no detectable phagocytic activity when exposed to zymosan particles under multiple conditions, including when cultured in the presence of porcine serum (data not shown).

Given published data that the unadulterated 3D4/21 cell line was not permissive to PRRSV infection (Lee et al., 2010; Weingartl et al., 2002), the levels of CD163 and CD169 PRRSV receptor expression were measured by antibody staining, followed by FC. No CD163 expression could be detected in the 3D4/21 cell line using mAb 2A10/11. As expected, analysis of AM stained in the same way showed that 91% of these cells expressed the CD163 protein (Figure 2.4B). The lack of detection of CD163 in the 3D4/21 cell line was consistent with the findings of Lee et al. (2010). Staining using mAb 41D3, recognising the CD169 receptor, revealed that 2% of 3D4/21 cells appeared to express minimal levels of the CD169 PRRSV co-receptor (Figure 2.4B). However, 73% of AM expressed readily detectable levels of this protein (Figure 2.4B). The low or null expression of the two prototypic PRRSV receptors confirmed that the 3D4/21 cell line was unlikely to permit PRRSV entry and uncoating.

To test the permissiveness of the 3D4/21 cell line to PRRSV infection, cells were infected at a multiplicity of infection (MOI) of 1.5, alongside AM as a control, with the AM-grown PRRSV H2 virus strain. At 24 hpi, AM and 3D4/21 cells were stained for the PRRSV N protein, along with a fluorescein (FITC)-conjugated secondary antibody for quantification by FC. Despite 38% of the AM staining positive for PRRSV infection, no infection was detected in the 3D4/21 cell line (Figure 2.4B). Thus, the available data corroborate previous findings that the 3D4/21 cell line is not natively permissive to PRRSV infection (Weingartl et al., 2002).

Due to the atypical marker expression when compared to AM, the 3D4/21 cell line appeared to be at an intermediate stage of monomyeloid differentiation (Figure 2.3). This may have resulted from the use of SV40 for the immortalisation of the original clones. SV40 can down-regulate the expression of lineage-specific transcription factors (LTFs) and could potentially be responsible for the non-macrophage phenotype of the 3D4/21 cell line (Pettersson et al., 1995).
Figure 2.4 | Marker expression and PRRSV infection in the 3D4/21 cell line. A. FC plots showing SWC3 expression against side-scatter (log) in i) AM, ii) untreated 3D4/21 cells and iii) 3D4/21 cells cultured in the continuous presence of rhCSF-1 for 3 weeks. B. FC plots showing the difference in staining for SWC3, CD163 and CD169 markers and PRRSV infection in AM and the 3D4/21 cell line. The 3D4/21 plot for SWC3 also shows staining pre- (blue) and post- (green) rhCSF-1 culture. Each plot consists of an isotype-matched control sample (red, vertical bars) and the stained sample (green, diagonal bars). Percentages shown correspond to the percentage of positive cells above a threshold set using each respective isotype-matched control. Plots are representative of multiple replicates.
2.2.3 LTF characterisation

Most myeloid promoters require both the CCAAT/enhancer-binding proteins (C/EBPs) and PU.1 LTFs for efficient activation and subsequent cell differentiation (Friedman, 2002; Tenen et al., 1997). The 3D4/21 cell line was established through the immortalisation of BALF-derived cells, yet the cell line appeared to be at an intermediate or atypical differentiation state, when compared to AM. Measuring the expression of LTFs in the 3D4/21 cell line might therefore provide an insight into the myeloid commitment of these cells. To investigate the expression of key LTFs in the 3D4/21 cell line, quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using primers targeting porcine C/EBP-α, C/EBP-β and PU.1.

Total RNA was extracted from 3D4/21 cells and AM, as a differentiated cell type positive control, and used as a template for cDNA synthesis. qRT-PCR was performed, with no-template and no-reverse transcription controls both returning no Ct values (data not shown). Both beta-actin and GAPDH were used as loading controls in order to ensure that accurate comparative quantification of LTF expression levels could be achieved. Both aforementioned control primers yielded comparable Ct levels between AM and 3D4/21 samples (Figure 2.5). However, Beta-actin levels expression levels were found to be significantly different between AM and 3D4/21 cells using Student’s t-test (Figure 2.5). GAPDH levels were not significantly different between these cell types using the same test, indicating that GAPDH may be more suitable for use as a control in this case. The relative mRNA levels of C/EBP-α, C/EBP-β and PU.1 were measured in AM and 3D4/21 RNA preparations. As expected, given its differentiation state, AM expressed high levels of the PU.1 transcription factor (Figure 2.5). However, the 3D4/21 cell line was found not to express PU.1 within the detection limit of the assay (Figure 2.5). AM expressed relatively low levels of C/EBP-α, when compared to PU.1, but again, the 3D4/21 cell line did not express this mRNA within the detection limit of the assay (Figure 2.5). Conversely, the 3D4/21 cell line apparently expressed a greater abundance of C/EBP-β mRNA than AM (Figure 2.5).
Figure 2.5 | Expression of LTFs in AM and 3D4/21 cells. qRT-PCR analyses of PU.1, C/EBP-α and C/EBP-β in AM and 3D4/21 cells. Data are presented as mean Ct values. Error bars represent the standard deviation of three biological replicates for AM and three technical replicates for 3D4/21 cells. Beta-actin and GAPDH are shown as controls for RNA loading. No-template and no-reverse transcription controls both returned no Ct values. Student’s t-test showed that although Beta-actin expression levels were significantly different (*) between AM and 3D4/21 cells, GAPDH levels did not significantly differ between these cell types (n/s, p <0.05). PU.1, C/EBP-α and C/EBP-β expression was significantly different when comparing levels in AM and 3D4/21 cells.
The apparent early stage of monocyte differentiation of the 3D4/21 cell line, as evidenced by the marker expression (Figure 2.4) and the lack of phagocytic ability, could be interpreted in the context of the atypical LTF expression in these cells. If the phenotype of these cells resulted from their SV40-transformation, then re-instating the function of key LTFs could potentially force a more mature differentiation state. Forced-overexpression of LTFs has been shown to lead to reprogramming of fibroblasts, hematopoietic progenitors and B cells into macrophages (DeKoter & Singh, 2000; Feng et al., 2008; Xie et al., 2004). Additional data indicate that the overexpression of PU.1 up-regulates CSF-1 receptor (CSF1R) expression (Aikawa et al., 2010; DeKoter et al., 1998) and that, upon binding of CSF-1 to its receptor, CSF1R, CD163 is up-regulated (Buechler et al., 2000; Tippett et al., 2011). Given that CD163 is the prototypic PRRSV receptor in AM, this may render the 3D4/21 cell line permissive to PRRSV. To investigate the potential of the overexpression of LTFs in forcing: 1) the differentiation of the 3D4/21 cell line, 2) the expression of CD163 and 3) subsequent PRRSV-permissiveness, lentiviral vectors were generated following the cloning of porcine LTFs into the pSL10 vector (provided by Simon Lillico; Appendix 1A). pSL10 encodes eGFP in the vector backbone, acting as a marker of transduction and permitting rapid titering and enrichment of transduced cells by cell sorting. Stocks of lentivirus encoding porcine PU.1, C/EBP-α and C/EBP-β were produced using HEK-293 as the packaging cell line. The resultant virus stocks were used to transduce the 3D4/21 cell line for stable expression of each porcine LTF. Enrichment of LTF-transduced cells was performed using fluorescence-activated cell sorting, based on the simultaneous expression of the GFP marker. GFP-positive cells were obtained. However, as has been noted previously (DeKoter et al., 1998), the overexpression of LTFs appeared to regulate cell proliferation and the cells grew poorly. This, combined with a poor starting lentivirus titre, led to the overgrowth of LTF-positive clones by contaminating, non-transduced 3D4/21 cells (Appendix 1B). Thus, it was not possible to establish a clonal LTF-expressing 3D4/21 cell population.

The non-macrophage phenotype, taken together with the lack of native permissiveness to PRRSV infection, suggested that the 3D4/21 cell line was unlikely
to prove to be a biologically relevant system for the study of host-virus interactions. Therefore, an alternative cell type for PRRSV culture was explored.

2.2.4 BMDM markers and phenotypic traits of macrophage differentiation

A recent development in pig research is the differentiation of porcine BMC into BMDM using only CSF-1, akin to human and murine systems (Kapetanovic et al., 2012). First, the protocol developed by Kapetanovic et al. (2012) for the collection of porcine BMCs and their differentiation into mature macrophages in vitro was optimised. The original protocol was established for the collection of several large ribs. However, this protocol was extended to the collection of all ribs. A protocol for the collection of BMC from the femur of weaner pigs was also developed. Additional modifications were made to seeding densities, timings and the age of pigs used for BMC collection. In the interest of brevity, these set-up experiments are not reported here, but the final method is detailed in Chapter 6, sections 6.3.3 and 6.3.4.

In order to evaluate the potential of these porcine BMDM as a biologically relevant culture host for PRRSV, the expression levels of key monomyeloid markers were quantified, following their differentiation from BMCs using rhCSF-1 only. BMC from two pigs were cultured for 7 days in the presence of rhCSF-1, prior to antibody staining of the porcine myeloid marker SWC3 or the MHC Class II antigen SLA-DR, followed by FC analysis. AM isolated from the same pigs were stained similarly for comparison. All samples exhibited negligible levels of background fluorescence upon staining with isotype control antibodies (data not shown). Similarly to AM, the majority of BMDM expressed the SWC3 protein (Figure 2.6A). However, substantially fewer BMDM expressed the MHC class II protein SLA-DR, when compared to AM (Figure 2.6A). To compare the phagocytic ability of AM and BMDM, FITC-conjugated E. coli were used. Cells were incubated in the presence of the fluorescent E. coli for 1 h prior to analysis by FC or confocal microscopy. When quantified by FC, the phagocytic capacity of BMDM was found to be greater than AM isolated from the same animal, according to the percentage of cells having internalised E. coli particles (Figure 2.6A). Confocal microscopic images of AM and
BMDM confirmed the high phagocytic capacity of BMDM and the successful internalisation of fluorescent particles (Figure 2.6B).

BMDM were strongly adherent to un-coated laboratory plastic culture dishes and their morphology was similar to AM in their granularity and large, rounded appearance. However, the AM population obtained from BALF was relatively homogeneous, unlike that of BMDM, which exhibited a degree of heterogeneity in size and appearance. Thus, rhCSF-1 BMDM cells displayed certain key macrophage characteristics but were dissimilar to AM in other aspects.

The smaller proportion of BMDM expressing SLA-DR than AM suggested that the BMDM may not be classically terminally-differentiated (Figure 2.3; Chamorro et al., 2005). However, taken with the consistently high levels of SWC3 expression, this confirmed that BMDM were monomyeloid cells with aggressive phagocytic capacity. It is to be expected that the phenotype of in vitro-derived BMDM would differ significantly from their ex vivo macrophage counterparts, the AM.
Figure 2.6 | Characterisation of porcine BMDM. A. FC quantification of the percentage of cells expressing key surface markers on AM and BMDM isolated from the same two animals, identified as Pig1 and Pig2. B. The phagocytic capacity of AM and BMDM, imaged using confocal and phase contrast microscopy at 1 h post-addition of E. coli:FITC particles. Images are of a single representative field. All control samples were negative for background staining and fluorescence. Student’s t-test showed all markers to significantly differ between AM and BMDM from the same pig ($p <0.05$), with the exception of SWC3, whose expression was not significantly different between BMDM and AM isolated from Pig 1.
2.2.5 *BMDM are permissive to type I PRRSV infection*

In order to establish whether BMDM were permissive to PRRSV infection and consequently, if they presented a tractable *in vitro* model for PRRSV research, cells were exposed to two type I (European) PRRSV strains. BMC were cultured for 6 days in the presence of rhCSF-1. At this point the non-adherent cells were discarded and the adherent cells were washed and re-seeded at a suitable cell density, 24 h prior to infection. As a positive control, AM were similarly seeded on tissue culture plates and also incubated for 24 hours prior to infection. AM and BMDM were infected under the same conditions, with either PRRSV H2 or SD01-08 viruses. Cells were incubated for 24 hpi and the infection was assessed. Mock-infected cells exhibited minimal background fluorescence or staining (Figure 2.7). Microscopy of AM at 24 hpi with the recombinant GFP-expressing SD01-08 clone showed numerous fluorescent green cells (Figure 2.7A). Imaging of BMDM revealed similarly strong GFP expression, indicating that BMDM were infectable with this PRRSV strain (Figure 2.7A). Similarly, when cells were infected with the non-recombinant type I PRRSV H2 strain, histochemically stained for the PRRSV N protein and visualised using light microscopy, many AM and BMDM cells stained positive for the virus antigen (Figure 2.7B). These results indicated that BMDM were infectable by two type I PRRSV strains to at least the same level as AM.
Figure 2.7 | Microscopic images of AM and BMDM following infection or mock-infection with PRRSV. A. AM and BMDM were infected with SD01-08 and visualised at 24 hpi by low magnification confocal microscopy. Merged images show both bright-field and GFP channels on the same image, GFP images show only GFP channel fluorescence. Mock-infected cells are shown in the left panel, with infected cells in the right panel. Inset are higher magnification images of single infected cells. B. AM and BMDM were infected with PRRSV H2 and stained, at 48 hpi, for the PRRSV N protein using mAb SDOW-17 along with a secondary antibody labelled with HRP. AEC substrate was utilised for the detection of HRP. Images of mock-infected cells are shown alongside images of infected cells.
Microscopic analysis of cells at low magnification allows one to capture information on the proportion of cells staining positive or expressing virus-associated GFP. However, microscopy can also provide data on the intracellular distribution of viral proteins when higher magnifications are available. Thus, to visualise and compare the intracellular localisation of GFP expression in AM and BMDM infected with the SD01-08 virus, high magnification confocal microscopy was carried out. AM and BMDM were seeded on coverslips, infected with PRRSV SD01-08 and incubated for 24 hpi, when they were fixed and mounted on glass slides in the presence of the 4’,6-diamidino-2-phenylindole (DAPI) nuclear counterstain. Confocal analysis consisted of the capture of z-stacks; or 21 images taken as thin optical-slices through complete cells. These z-stacks were then rendered as a single, pseudo-three-dimensional image, encompassing both DAPI and GFP fluorescence throughout the cell. The resulting images revealed a stark difference in the size of nuclei between AM and some BMDM (Figure 2.8A), although the BMDM population exhibited significant size heterogeneity. The intracellular distribution of the nsp2-GFP fusion protein appeared to be comparable between AM and BMDM (Figure 2.8A). In both cell types, nsp2-GFP was localised in discrete cytoplasmic foci, with some perinuclear fluorescence (Figure 2.8A). These observations are consistent with data that nsp2 localises to the perinuclear region in some cell types, where it associates with double-membrane virus replication compartments (Pedersen et al., 1999; Snijder & Meulenberg, 1998; Van Der Meer et al., 1998).

For the quantitative analysis of a large number of cells, FC provides a useful and robust solution. Therefore, FC was utilised in order to compare and quantify the permissiveness of different BMDM and AM isolates to PRRSV infection. AM and BMDM were infected with PRRSV SD01-08 at MOI 1, as described above, and the number of infected cells was measured according to the percentage of cells expressing GFP at 24 hpi. The infectability of BMDM was considerably greater than AM in both experiments, with a maximum of 93 % and 17 % of cells being infected at 24 hpi in BMDM and AM, respectively (Figure 2.8B). Figure 2.8C illustrates the clear and distinct fluorescence emission peak from the nsp2-GFP fusion protein in two infected cell isolates, representing both AM and BMDM. The percentage of cells represented by the GFP peak is also shown (Figure 2.8C).
Figure 2.8 | Comparison of PRRSV SD01-08 infection of BMDM and AM. A. Pseudo-three-dimensional images rendered from representative high magnification z-stacks of AM or BMDM cells using a Zeiss 710 confocal microscope with the Zen Black software application. Scale bars illustrate the magnitude of the size difference between AM and some BMDM cells. Nuclei are stained in blue with the DAPI counter-stain and green fluorescence originates from PRRSV nsp2-GFP fusion protein expression. B. Percentage of cells expressing GFP at 24 hpi with the SD01-08 virus at MOI 1. Data represent mean FC counts for AM or BMDM derived from different animals and error bars show the standard deviation of three technical replicates. Student’s t-test showed that both BMDM isolates were significantly different to both AM isolates in the percentage of GFP-positive cells (*, p <0.05). C. FC plots illustrating the difference in the GFP signal as measured by FC at 24 hpi in AM and BMDM infected with PRRSV SD01-08 at MOI 1. Two cell isolates are included on each plot, with coloured lines representing cells derived from different animals; an uninfected control sample is included in the BMDM plot (coloured green) to illustrate background fluorescence levels.
2.2.6 BMDM viral load and production of infectious virus

To test if porcine BMDM were truly permissive to PRRSV and if the infections were productive, releasing viral particles into the supernatant, the kinetics of virus replication in AM and BMDM were measured. AM and BMDM were infected with PRRSV SD01-08 at an MOI of 0.01 and the supernatant was harvested over a 48 hour time course. Viral RNA was extracted from the supernatants and the virus titre was estimated by qRT-PCR of PRRSV RNA, relative to a standard curve generated from a virus sample of known tissue culture infectious dose 50 (TCID$_{50}$) titre. By this measure, both cell types supported productive virus replication, but BMDM reproducibly released a greater titre of virus than AM (Figure 2.9A).

Despite not being a physiologically relevant cell type, the monkey kidney epithelial cell line, MARC-145, is one of the most commonly used in vitro culture hosts for PRRSV. Accordingly, the capacity of this cell line to replicate PRRSV was compared to BMDM and AM cells. Following low multiplicity infection with PRRSV SD01-08, MARC-145 cells produced lower amounts of virus than AM at both 24 and 48 hpi but again, BMDM released substantially higher amounts of virus into the cell culture supernatant at both timepoints (Figure 2.9B). At 24 hpi, BMDM released over 60-fold more virus than AM and more than 250-fold more virus than MARC-145 cells (Figure 2.9B). At 36 hpi, BMDM released over 90-fold more virus than AM and more than 400-fold more virus than MARC-145 cells (Figure 2.9B). SD01-08 has been shown to produce virus yields of between 2.1x10$^4$ to 2.8x10$^4$ focus forming units (FFU) per mL on AM (Fang et al., 2006). This is consistent with the estimated titre from AM seen here, as determined by qRT-PCR (Figure 2.9B).
Figure 2.9 | Quantification of released virus in BMDM, AM and MARC-145 cells. A. Cells isolated from four pigs were infected at MOI 0.01 with the un-passaged SD01-08 PRRSV infectious clone and virus in the supernatant was quantified by qRT-PCR at timepoints up to 48 hpi. Error bars represent the standard deviation of four biological replicates. B. Virus production in the supernatant of BMDM, AM and MARC-145 cells infected with the PRRSV SD01-08 GFP infectious clone at MOI 0.01, as measured by qRT-PCR. Error bars represent the standard deviation of four independent biological replicates for BMDM and AM and three technical replicates for MARC-145 cells. Student’s t-test showed that the quantity of PRRSV genome released differed significantly between each cell type at both 24 hpi and 48 hpi (p <0.05).

All data in A and B are expressed as mean, equivalent TCID$_{50}$ values extrapolated using a standard curve (see Materials and Methods) generated by performing qRT-PCR on dilutions of a virus stock of a known (BMDM) TCID$_{50}$/mL.
2.2.7 Assessment of variation in BMDM infectability

To test whether the trend of greater BMDM permissiveness to PRRSV infection was consistent across cells isolated from a variety of individual animals, a library of BMDM cell isolates from 24 animals was tested for infectability. BMDM and AM were seeded in tissue culture plates 24 h prior to infection with either PRRSV H2 or SD01-08 strains. Due to the differing permissiveness of the cells available for PRRSV titration, the MOI used is specified according to the cell type used to titre each virus stock. Infections with SD01-08 were performed at MOI 1, determined by titration on BMDM. The PRRSV H2 isolate was titrated on AM, so infections were performed using (AM) MOI 1.5.

At 24 hpi, cells were fixed and analysed by FC for either direct GFP fluorescence, in the case of PRRSV SD01-08 infection, or staining with an antibody recognising PRRSV N protein for PRRSV H2 infections. In order to test if the observed increased permissiveness of BMDM compared to AM was consistent across multiple cell isolates, 24 BMDM isolates and 18 AM isolates were tested. The percentage of BMDM infected with PRRSV SD01-08 at 24 hpi was found to vary between 59 and 93 % (Figure 2.10A). AM from 18 individuals were found to be between 5 and 34 % infected at 24 hpi with SD01-08 (Figure 2.10A). Additionally, BMDM and AM from 12 and 6 animals, respectively, were infected with PRRSV H2 and the percentage of infected cells at 24 hpi varied from 32 to 40 % and 15 to 21 % respectively (Figure 2.10B).

To test if the increased permissiveness of BMDM to PRRSV infection extended to AM and BMDM isolated from the same animal, both cell types isolated from two animals were infected, as above. At 24 hpi, the percentage of infected cells with either PRRSV SD01-08 or PRRSV H2 was quantified by FC. Again, a clear increase in infectability of BMDM over AM was observed, which extended across both virus strains and animals tested (Figure 2.10 C and D).

Given that pigs are an outbred population and particular breeds vary in their resistance to PRRSV (Ait-Ali et al., 2007; Reiner et al., 2010), it was of interest to assess any breed variation in the permissiveness of BMDM isolates to PRRSV. When the 24 BMDM isolates infected with SD01-08 were arranged according to their breed, it was evident that the ubiquitous commercial crossbred Landrace/Large
White (LRxLW) line encompassed all of the variation of the breeds tested (Figure 2.10E). The Hampshire (HAM) isolates tested exhibited consistently high levels of PRRSV infection (Figure 2.10E). Only two pig lines were represented by the 12 BMDM isolates infected with PRRSV H2, both of which showed similar levels of PRRSV infection at 24 hpi (Figure 2.10F).

Thus, BMDM were consistently permissive to PRRSV infection, with no isolates tested exhibiting resistance. When infected in the same way, BMDM were considerably more infectable by PRRSV than AM. When the isolates available were infected and arranged by breed it was clear that the permissiveness of cell isolates differed, but variation was minimal, all of which was encompassed by the LRxLW line.
Figure 2.10 | Assessment of variation in BMDM infectability and comparison with AM. Box and whisker plots representing data from AM and BMDM isolated from multiple individuals and breeds. A. and B. The percentage of virus-positive BMDM and AM at 24 hpi with SD01-08 and PRRSV H2, respectively, under the same conditions. C. and D. The percentage of PRRSV-infected BMDM and AM isolated from the same animals (either Pig1 or Pig6) at 24 hpi with SD01-08 and PRRSV H2, respectively, under the same conditions. E. and F. The percentage of PRRSV-infected BMDM isolated from a number of different breeds and crossbred lines at 24 hpi with SD01-08 and PRRSV H2, respectively. All data shown are from multiple, independent experiments. N is shown for the number of individual animals represented, with the exception of C and D, where it represents the number of technical replicates. LR is the Landrace breed, LW is Large White and PIE is the Pietrain breed.
2.2.8 Permissiveness of BMDM to transfection

A significant advantage of the use of continuous cell lines in the study of host-virus interactions is the ability to perform gain-of-function (GOF) or loss-of-function (LOF) assays. Primary cells are largely refractive to the transfection of foreign nucleic acid. To further assess the use of porcine BMDM as a cell culture system for PRRSV research, their amenability to transfection was examined.

Traditionally, transfection via electroporation was often the only available method for the efficient introduction of foreign nucleic acid into primary cell types. A development in the area of electroporation is a technique known as Nucleofection. This is a process for the efficient non-viral transfection of primary and hard-to-transfect cells, based on the use of optimised solutions and electrical parameters. For this reason, the Nucleofection technique was selected in order to test the efficiency of transfection of plasmid DNA into porcine BMDM. Nucleofection was used to introduce pMAX_GFP (a GFP-expressing plasmid vector) into BMDM in suspension, these cells were then re-seeded into tissue culture plates and the percentage of cells expressing GFP was measured at 24 hours post-transfection (hpt), either by FC or cell counts of microscopic images. Upon the visualisation of transfected cells by fluorescence microscopy, it was clear that many cells expressed high levels of GFP (Figure 2.11A). No-substrate electroporated control cells exhibited no readily detectable background fluorescence (Figure 2.11A). Following the optimisation of Nucleofection conditions over more than 80 electrical parameters and two solutions, a transfection efficiency of 51 % was achieved with the pMAX_GFP vector, as quantified by FC (Figure 2.11B). Cell viability was an important consideration in the development of the transfection protocol, given the desire to perform infections post-transfection. Therefore, the optimisation procedure additionally utilised a cell proliferation assay. The optimised Nucleofection protocol achieved cell viability of greater than 95 % using the pMAX_GFP plasmid (data not shown). These data indicate that, if sufficiently optimised, this procedure could be used to introduce plasmids encoding exogenous genes into BMDM, in order to assess their impact on PRRSV replication.

RNA interference is a widely-used method of gene knockdown in studies of host-virus interactions, often utilising small interfering RNAs (siRNAs) for this
purpose. To test whether siRNAs could be introduced into BMDM, similarly to plasmid DNA, a standard electroporation reaction was performed. A non-targeting (scrambled) siRNA, labelled with the Cy3 fluorescent dye, was used to quantify transfection efficiency. BMDM were electroporated with siRNA in suspension, re-seeded to tissue culture plates and analysed for Cy3 fluorescence by FC at 24 hpt. No substrate, electroporated control samples were used to set the gates for FC analysis (Figure 2.11B i). In addition, to control for phagocytosis of fluorescently labelled siRNAs or non-specific binding of the dye-conjugated RNA to the cell surface, siRNA was added to some samples and no electroporation was performed. FC analysis of non-electroporated cells, incubated in the presence of siRNA, revealed a substantial increase (around 30 %) in the number of cells expressing Cy3 compared to no substrate control samples (Figure 2.11B ii). This was the case in spite of vigorous washing prior to FC analysis, suggesting a non-specific uptake mechanism or, more likely, adhesion of the dye-conjugated siRNA to the macrophage cell surface. However, it was apparent that the electroporation of siRNA-containing samples resulted in a tight and intense Cy3 signal that was distinct from non-electroporated samples (Figure 2.11B iii). These data suggested that an efficiency of between a conservative figure of 47 % and a maximum figure of 77 % may be obtained for siRNA transfection of BMDM using standard electroporation techniques.

The efficient transfection of BMDM achievable with plasmid DNA through the Nucleofection technique suggests that these cells could be used to study host-virus interactions in vitro. Furthermore, the apparently permissive nature of BMDM to siRNA electroporation also indicates the potential of BMDM for use in routine laboratory experimentation and LOF assays.

Having established that CSF-1-derived BMDM were highly permissive to PRRSV, expressed monomyeloid markers and were also permissive to DNA and RNA transfection, the cell type was characterised further. The ribs present an ideal source of BMC from experimental pigs. However, there are additional significant sources of BMC that could also be utilised. To investigate the differences in cells obtained from additional sources, their marker expression and differentiation potential were assessed.
Figure 2.11 | DNA and RNA transfection of BMDM. A. Fluorescence microscopic images of mock and pMAX_GFP plasmid DNA-nucleofected BMDM (DAPI counterstained) at 24 hpt. B. The mean percentage of cells expressing GFP, following transfection with the pMAX_GFP plasmid, as quantified by FC analysis at 24 hpt. Error bars represent the standard deviation of three biological replicates. C. FC analysis of cells 24 hpt with siRNA. i) BMDM electroporated with no nucleic acid substrate. ii) BMDM incubated in the presence of non-targeting Cy3-labelled siRNA, with no electroporation reaction. iii) BMDM electroporated with non-targeting Cy3-labelled siRNA.
2.2.9 Distinct BMC preparations and their characteristics

Having furthered the observations by Kapetanovic et al. (2012) that BMDM can be successfully derived from sexually/immunologically immature pigs, it was of interest to assess the hematopoietic differentiation potential of adult BMC. Marrow cavities in all the bones of new-born mammals contain functionally active hematopoietic tissue. From the early postnatal period onwards, this hematopoietic tissue is gradually replaced by fatty, yellow bone marrow in all but certain areas, such as the axial skeleton (Moore & Dawson, 1990; Taccone et al., 1995; Waitches et al., 1994). Given this information, the axial skeleton, specifically the rib cage, of mature pigs may prove to be an excellent source of BMC. To test this hypothesis, ribs were collected from a 14 month old gilt following euthanasia by anaesthetic overdose for another project. Cells were isolated as per the standard protocol, with the exception of sectioning the ribs at 100 mm intervals prior to flushing. Cell count data from this experiment demonstrated that a single rib from a 14 month old commercial breed sow could yield in excess of $6 \times 10^8$ BMC. When these counts were extrapolated to the collection of all ribs, a total of up to $1.7 \times 10^{10}$ to $2 \times 10^{10}$ BMC should be achievable (depending on the number of ribs in the pig breed), a figure 140-times greater than the complete ribcage of a weaner pig. The number of BMDM produced from a given number of these BMC was found to be comparable to other ages of pigs and individuals tested, suggesting that the BMC in the ribs of adult gilts are likely to be at least as enriched for actively hematopoietic cells as those of weaners (data not shown). The BMDM derived from the ribs of adult, sexually mature gilts were also highly permissive to PRRSV. Given that BMC could be obtained post-mortem with large yields from a single rib, cells could potentially be collected from commercial slaughter operations for cost-effective and large-scale experimentation.

As well as collecting BMC from the ribs, cells were also isolated from the femurs of juvenile animals. The femurs contained more yellow bone marrow and fatty deposits than the ribs of a weaner pig. However, this disadvantageous characteristic was largely negated following centrifugation, washing and media changes. The total number of BMC isolated from both femurs in a 6 to 8 week old
commercial pig (LWxLR) was typically in excess of $1.5 \times 10^8$ cells and collection could be carried out with little time penalty.

In order to assess the differences in permissiveness to PRRSV infection in BMDM derived from the rib or femur, cells isolated from each location within the same animal were cultured under identical conditions. Virus infection was performed at MOI 1 with either PRRSV H2 or SD01-08 type I strains. FC quantification of the percentage of cells expressing GFP, in the case of SD01-08 infection, or positive for the PRRSV N protein following antibody staining, in the case of PRRSV H2, was performed at 24 hpi. Levels of PRRSV infection with both SD01-08 and PRRSV H2 strains were found to be comparable in BMDM derived from the ribs or femurs of the same animal (Figure 2.12A). BMDM derived from the femur appeared to be marginally more permissive to infection by PRRSV H2 than those derived from the ribs of this animal (Figure 2.12A).

In order to quantify the phagocytic capacity of BMDM derived from the ribs or femur, cells were incubated in the presence of zymosan particles for 1 h. BMDM were then washed and the number of flurophore-conjugated zymosan-positive cells was measured by FC. The phagocytic capacities of BMDM derived from either the ribs or femur were found to be similar (Figure 2.12B). Having established that the permissiveness to type I PRRSV and phagocytic capacity were comparable between BMDM isolated from the ribs or femur, it was of interest to determine if the BMC isolated from these locations possessed the same potential for hematopoietic differentiation. To approximate the “yield” of BMDM achieved from a given number of BMC, the percentage of cells that had adhered to the un-coated plastic culture vessel (taken to be macrophages), relative to the number of non-adherent cells, on day 6 of culture in the presence of rhCSF-1 was calculated using cell count data. Both femur and rib BMDM preparations appeared to possess a similar propensity for myeloid differentiation (Figure 2.12B). Together, these data suggest that the femur of weaner pigs constitutes a further, valuable source of BMDM for PRRSV research.

Since its emergence in PRRSV research (Calvert et al., 2007), the CD163 protein has been established to perform an important function in PRRSV infection (Van Gorp et al., 2009). In the PRRSV respiratory host cell, the AM, CD163 appears
to be essential for a productive infection, permitting the uncoating of PRRSV in the early endosome (Van Gorp et al., 2009; Van Gorp et al., 2010b). Given that in this Chapter porcine BMDM have been shown to be permissive to PRRSV infection to a greater level than AM, the expression of CD163 was measured in both rib and femur BMDM preparations. Following 6 days of culture in the presence of rhCSF-1, the resultant BMDM were permeabilised and stained with the anti-CD163 mAb 2A10/11 before being analysed by FC for the number of cells staining positive for CD163. Contrary to expectations, no significant staining was detected for CD163, indicating extremely low, or null expression of this PRRSV receptor in BMDM (Figure 2.12B).
Figure 2.12 | Characterisation of BMDM derived from the rib or femur. A. The percentage of virus-positive cells in rib or femur-derived BMDM preparations at 24 hpi, as determined by FC. B. The percentage of BMDM having phagocytosed zymosan particles following 1 h incubation and quantified by FC. BMDM “yield” shows the proportion of adherent to non-adherent BMDM after 6 days incubation of either rib or femur BMC in the presence of rhCSF-1 (data derived from cell counts using trypan-blue exclusion). Also shown is the percentage of antigen-positive BMDM following staining for CD163 with mAb 2A10/11 and FC quantification. Error bars represent the standard deviation of three biological replicates. Student’s t-test showed both rib and femur BMDM isolates to significantly differ in the percentage of virus-positive cells (*, p <0.05). However, no significant difference (n/s) was detected between rib and femur BMDM isolates for Zymosan, BMDM yield or CD163 expression.
2.2.10 CD163 and CD169 PRRSV cell membrane receptors

Given the evidence that CD163 and CD169 are receptors for PRRSV in multiple cell types, not least in its respiratory host cell, the AM (Calvert et al., 2007; Van Gorp et al., 2008), and that CD163 was not readily detected in BMDM derived from either rib or femur, the infectability of these cells was puzzling. Therefore, the expression of these receptors on BMDM was further assessed.

In order to measure the expression of the CD169 receptor, cells were stained with the anti-CD169 mAb, 41D3, and analysed by FC alongside an isotype-matched control antibody. Minimal non-specific staining was observed with the isotype-matched control antibody in BMDM or AM (Figure 2.13A). CD169 was readily detected in 73 % of AM, but no detectable levels of CD169 protein were found in BMDM (Figure 2.13A). To analyse intra- and extra-cellular CD163 protein levels in AM and BMDM, FC was performed following permeabilisation and staining using mAb 2A10/11, which recognises an epitope in CD163 SRCR domains 1 to 3. This antibody readily detected CD163 in AM, but failed to detect significant CD163 expression in BMDM (data not shown). The availability of a polyclonal antibody (pAb 1607) that recognises multiple epitopes on the CD163 protein, including SRCR 5, as well as in other uncharacterised domains, prompted the additional use of this antibody to assess CD163 expression in BMDM. No significant signal was evident following staining with an isotype-matched control antibody (Figure 2.13A). pAb 1607 detected CD163 expression in 63 % of AM (Figure 2.13A). However, no staining was observed upon FC analysis of BMDM (Figure 2.13A). Overall, these findings are consistent with those of Kapetanovic et al. (2012), who also detected low to null levels of CD163 in porcine BMDM, suggesting that CD163 is not present at significant levels in BMDM.

To further investigate the apparent absence of CD163 and CD169 PRRSV receptors on BMDM, the transcript abundance of the genes encoding these proteins were assessed. According to data made available by the Pig Tissue Atlas project (Freeman et al., 2012), both CD163 and CD169 (SIGLEC1) mRNAs are present in CSF-1-derived BMDM at appreciably lower levels than in AM (Figure 2.13B). When comparing these normalised expression data, BMDM expressed 17-fold lower levels of CD169 and 8-fold lower levels of CD163 mRNA than AM (Figure 2.13B).
Figure 2.13 | CD163 and CD169 protein and mRNA expression in BMDM and AM. A. FC data for both AM and BMDM, stained using pAb 1607 or mAb 41D3, recognising CD163 and CD169, respectively. Isotype-matched control antibody staining is shown in red with vertical bars, with CD163 or CD169 antibody staining in green with diagonal bars. FC plots are representative of the staining of cells derived from >10 animals. B. Gene expression data taken from the publicly available Pig Tissue Atlas Project (Freeman et al., 2012; http://biogps.org) for SIGLEC1 (CD169) and CD163 mRNAs across multiple tissues, AM and BMDM are highlighted with an asterisk.
The protein and transcript data presented thus far suggested that minimal transcription and translation of CD163 and CD169 occurred in CSF-1-differentiated porcine BMDM. Nevertheless, in order to examine the possibility that the failure to detect appreciable CD163 staining on BMDM resulted from a surface structure blocking the CD163 antibodies from accessing their epitopes, western blots were carried out using pAb 1607 to probe BMDM and AM protein lysates.

Total protein lysates were made from equal numbers of AM and BMDM and neat, 1:3 and 1:10 dilutions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before transferring to nitrocellulose. Western blots were performed using anti-tubulin alpha as a loading control and probing for CD163 with pAb 1607. The intensity of tubulin staining revealed that comparable levels of total protein were loaded for AM and BMDM samples (Figure 2.14A). CD163 was readily detected in AM across a range of total protein concentrations (Figure 2.14A). However, only very low levels of CD163 were detected in the undiluted BMDM lysate (Figure 2.14A). Quantification of protein band intensity and normalisation to the tubulin alpha loading control was performed. When these data were plotted relative to CD163 detection in AM, CD163 levels in BMDM were around 30-times lower than in AM (Figure 2.14B). These data further support the negligible expression of CD163 in CSF-1-differentiated porcine BMDM.
Figure 2.14 | Western blot for CD163 in AM and BMDM. A. CD163 protein in AM and BMDM cells. Three-fold dilutions of AM or BMDM cell lysates were analysed by SDS-PAGE and western blotting for CD163 (using pAb 1607) or tubulin-alpha, as labelled. B. Quantification of CD163 protein band intensity, normalised to the tubulin alpha loading control and plotted relative to the neat protein dilution of AM. Quantification was performed using the Licor Odyssey Image Studio software. Data are representative of two biological replicates.
CD163 ectodomain shedding is known to be induced by inflammatory stimuli (Droste et al., 1999; Weaver et al., 2006), with high levels of sCD163 in the plasma of patients suffering from a variety of diseases and disorders (Møller et al., 2002). Therefore, to test if the protocol employed for FC staining and analysis, involving repeated centrifugation and wash steps, could have resulted in the shedding of CD163, in situ immunofluorescent staining was carried. This procedure reduces the potential disturbance caused to the cells during other staining techniques.

AM and BMDM were seeded on coverslips at an appropriate cell density and incubated for 24 h prior to staining. AM and BMDM were then fixed and stained in parallel with pAb 1607, recognising CD163, or an isotype-matched FITC-conjugated control antibody. All cells were counterstained with the DAPI nuclear stain. Following mounting of the coverslips, cells were visualised by confocal microscopy. Analysis of BMDM and AM stained with the isotype control antibody revealed minimal background staining (Figure 2.15). As expected, the vast majority of AM stained positive for CD163 (CD163\textsuperscript{HI}; Figure 2.15). Microscopic analysis of BMDM stained for CD163 showed that, although a small minority of BMDM did express readily detectable levels of CD163, most cells appeared not to express the protein (Figure 2.15). The CD163 staining pattern in AM was largely cell membrane-associated, with some discrete cytoplasmic foci (Figure 2.15). The few CD163\textsuperscript{HI} BMDM appeared to express the protein in more of a cytoplasmic localisation (Figure 2.15). However, it appeared that some BMDM expressed CD163 in an apparently PRRSV-accessible localisation for virus uncoating (Figure 2.15). Thus, the overall BMDM cell population was CD163\textsuperscript{LO} under routine culture conditions, measured using a variety of different techniques, with a very small CD163\textsuperscript{HI} cell population.
Figure 2.15 | CD163 expression in AM and BMDM stained *in situ*. AM and BMDM were stained with the CD163 antibody pAb 1607 and a FITC-conjugated secondary antibody and visualised by confocal microscopy at 40x magnification. Isotype control samples were stained with a FITC-conjugated isotype-matched control antibody. All samples were co-stained with DAPI. Images are of single representative fields for AM. However, due to the low number of CD163<sup>HI</sup> BMDM, *an atypical CD163 field has been selected* in order to show an example of CD163<sup>HI</sup> BMDM.
The numbers of CD163<sup>HI</sup> BMDM detected by microscopy and FC were seemingly vastly outweighed by the percentage of PRRSV-permissive cells when separately quantified. To investigate this correlation further, FC analysis of BMDM and AM infected with SD01-08 and co-stained for CD163 was carried out. AM and BMDM were infected with PRRSV SD01-08 at MOI 1 for 24 h. At 24 hpi, cells were permeabilised and stained for CD163, using pAb 1607 and a PE-conjugated secondary antibody. Manual compensation was performed on FC data, using the appropriate control samples (unstained, isotype control, infected-only and CD163-only) to set the gates for further analyses. Uninfected, isotype control-stained samples from both cell types showed minimal background fluorescence in either GFP or phycoerythrin (PE) channels (Figure 2.16). A total of 7 % of AM were infected with PRRSV SD01-08 and, within this 7 %, only 1 % appeared to be PRRSV+/CD163-, leaving 6 % both PRRSV+/CD163+ (Figure 2.16). A total of 80 % of AM stained positive for CD163, representing 74 % PRRSV-/CD163+ cells (Figure 2.16). In the case of the BMDM, a total of 3 % of cells stained positive for CD163 and 1 % of these were PRRSV-/CD163+. 51 % of BMDM were found to be infected with PRRSV SD01-08 by FC (Figure 2.16). Within this 51 %, 49 % of cells were found to be PRRSV+/CD163-, with only 2 % of BMDM being both PRRSV+/CD163+ (Figure 2.16). Overall, these data for double-stained BMDM confirmed previous findings in this Chapter, in that the proportion of BMDM permissive to PRRSV infection greatly outweighed those staining positive for CD163. Thus, the high permissiveness of BMDM to PRRSV infection seems very unlikely to be explained by selective infection of a minority population of CD163-positive cells.
Figure 2.16 | AM and BMDM infected with PRRSV SD01-08 and co-stained for CD163. FC plots are shown for uninfected AM or BMDM, stained with a PE-conjugated isotype-matched control antibody. Additional plots show AM or BMDM infected with PRRSV SD01-08 and expressing GFP, co-stained with pAb 1607, recognising CD163 and a PE-conjugated secondary antibody. Plots are divided into four quadrants, representing PRRSV-/CD163-, PRRSV-/CD163+, PRRSV+/CD163- and PRRSV+/CD163+. The x-axis represents PE-log (CD163 or isotype-matched control) intensity and the y-axis represents GFP-log (PRRSV nsp2) intensity.
2.2.11 Blocking PRRSV infection in BMDM

The very low, or absent, CD163 and CD169 protein expression in BMDM, taken with the increased virus susceptibility of these cells when compared to the highly CD163 and CD169-positive AM, suggested that there may be an alternative PRRSV entry pathway in CSF-1-differentiated porcine BMDM. Blocking of PRRSV infection through incubation of AM with CD163 and CD169 antibodies has previously been demonstrated (Van Gorp et al., 2008).

To explore the effect of antibody blocking on PRRSV infection, AM and BMDM were incubated with pAb 1607 (anti-CD163) or the anti-CD169 mAb 41D3 for 1 h. A concentration of 2 μg/100 μL CD163 antibody was used to block PRRSV infection, in line with data presented in previous studies (Calvert et al., 2007; Van Gorp et al., 2008). mAb 41D3 was supplied as a tissue culture supernatant of an unknown concentration (gift from Prof. Hans Nauwynck). Therefore, an estimation of the concentration was made, based on the quantity required for FC staining, and 3.3 μL/100 μL was used for blocking experiments. Untreated samples were incubated in PBS and isotype control-blocked samples were incubated with an isotype control antibody only at a concentration of 2 μg/100 μL. Blocked cells were then infected with either SD01-08 or PRRSV H2 at MOI 1 for 1 h, before being washed and incubated for 10 hpi. A 10 h infection was selected in order to assess infection prior to the completion of the first round of PRRSV replication. At 10 hpi, AM and BMDM infected with both virus strains were stained for the PRRSV N-protein and analysed by FC. The proportion of virus-positive cells in each sample was quantified and normalised to the isotype-blocked sample. Neither untreated nor isotype-blocked samples exhibited a significant (p < 0.05) reduction in infection with either PRRSV strain in AM or BMDM cells (Figure 2.17A). As expected, incubation with either anti-CD163 or anti-CD169 antibodies significantly decreased the proportion of AM infected with both SD01-08 and PRRSV H2 strains (Figure 2.17A). However, neither antibody resulted in a substantial decrease in BMDM infection, although this small decrease was found to be significant using Student’s t-test for anti-CD169 blocking data (Figure 2.17A). Although a decrease in the proportion of infected AM was observed upon incubation with an antibody targeting CD163, this block was not complete (Figure 2.17A). This could indicate that the antibody is not efficient in
blocking virus-interacting domains on the CD163 protein, or that alternative entry mediators exist in AM.

Cytochalasin D is an actin-depolymerisation agent which inhibits clathrin-mediated endocytosis, phagocytosis and macropinocytosis and consequently, PRRSV infection in AM (Nauwynck et al., 1999). To test whether cytochalasin D resulted in a similar inhibition of PRRSV infection in BMDM, AM and BMDM were exposed to varying concentrations of the compound. Cells were incubated in the presence of a range of concentrations of cytochalasin D for 1 h prior to infection. Following infection with PRRSV SD01-08 at MOI 1 for 1 h, the virus was removed and cells were washed prior to the addition of growth medium, also containing cytochalasin D. As a control against the effect of cytochalasin D on other stages of the PRRSV replication cycle, cell culture and infections were performed as normal and cytochalasin D was only introduced following virus internalisation at 90 minutes post-infection. Cells were analysed by FC for the number of GFP-positive cells at 10 hpi and data were normalised to the untreated, infected samples. There was a minor reduction in the relative proportion of infected cells in AM treated with cytochalasin D only at 90 minutes post-infection and no significant difference was observed for BMDM (Figure 2.17B). This small decrease in infectivity in AM may have been due to the presence of DMSO in the stock solution. However, a significant (p <0.05) and dose-dependent decrease in the relative proportion of infected cells was observed in both AM and BMDM following pre-incubation with all concentrations of cytochalasin D (Figure 2.17B).

The absence of a significant reduction in the infection of BMDM following blocking with CD163 or CD169 antibodies further substantiated the hypothesis that type I PRRSV entry into CSF-1-differentiated porcine BMDM occurs independently of this pathway. The comparable level of reduction in the proportion of infected AM and BMDM upon cytochalasin D treatment, indicated that the entry mechanism for PRRSV into BMDM may also involve clathrin-mediated endocytosis.
Figure 2.17 | Blocking of PRRSV infection in AM and BMDM. A. AM and BMDM blocked with media only (untreated), 2 μg/100 μL isotype-control antibody, 2 μg/100 μL anti-CD163 pAb 1607 or 3.3 μL/100 μL anti-CD169 mAb 41D3. Cells were incubated with the treatment in 300 μL minimal media for 1 h prior to infection with SD01-08 or PRRSV H2 at MOI 1. Following antibody staining for the PRRSV N protein at 10 hpi, the number of PRRSV-positive cells was measured by FC and normalised to each isotype control antibody-blocked sample. B. AM and BMDM treated with varying concentrations of the actin-depolymerising agent cytochalasin D and infected with PRRSV SD01-08 at MOI 1. Untreated samples were blocked with media only, treatment control samples had the drug added at 90 minutes post-infection only. The number of GFP-positive cells was measured by FC at 10 hpi and normalised to each untreated control antibody-blocked sample. All error bars represent the standard deviation of three independent technical replicates. Student’s t-test data indicates the significant differences in the relative proportion of virus-positive cells (*, p <0.05) or where differences were not significant (n/s).
2.3 Discussion

The studies in this chapter set out to characterise existing, and develop new, cell culture systems for in vitro PRRSV research. The first objective was to characterise the 3D4/21 cell line and assess its suitability as a porcine-derived model system for research into PRRSV host-virus interactions. Given the lack of suitability of this cell line as an alternative cell culture system, a further objective became to test the infectability of porcine BMDM. A secondary objective became to explore the PRRSV entry mechanism in BMDM.

The recombinant SD01-08 PRRSV infectious clone has been shown to be a valuable research tool and assays carried out using this virus performed comparably to the PRRSV H2 wild-type virus. As expected, the nsp2-GFP fusion protein was strongly expressed in different cell types from early in the infection and correlated with antibody-staining for the PRRSV N protein.

The utility of the 3D4/21 cell line for PRRSV research was assessed. Overall, the available data suggested that the 3D4/21 cell line was likely to fall into subsets I or II of the porcine monocyte classification system proposed by Chamorro et al. (2005). This was based on the presence and absence, respectively, of SWC3 and CD163 in the cell line, indicating an immature differentiation state. Immortalisation was performed by SV40 large T antigen transfection of BALF-derived cultures (Weingartl et al., 2002). BALF, although highly enriched for AM, does not represent a 100% pure macrophage population, with contamination from other cell types being unavoidable. Given the phenotype of the cells and the method of immortalisation, it is possible that the clone selected to derive the 3D4/21 cell line was not an AM.

Hematopoietic lineages are governed by lineage-restricted transcription factors which determine the decision between lymphoid and myeloid cell fates (Xie et al., 2004). However, most of the underlying mechanisms still remain largely unknown. PU.1 is an ets-family transcription factor that is known to be a master regulator of myeloid differentiation that regulates the expression of IL-7Ra, CD45, CD11b, F4/80 and MHC class II proteins, as well as controlling cell proliferation by regulating the expression of the c-fms and IL-7Ra genes which encode receptors for
CSF-1 and IL-7, respectively (Celada et al., 1996; DeKoter & Singh, 2000; Nishiyama et al., 2004). Although not essential for myeloid lineage commitment (Olson et al., 1995), PU.1 promotes the differentiation of myeloid progenitors (Celada et al., 1996; DeKoter et al., 1998). PU.1 regulates GM-CSF-dependent effects on the terminal differentiation of AM, being markedly down-regulated in the AM of GM-CSF<sup>−/−</sup> mice (Shibata et al., 2001). High concentrations of PU.1 specify the macrophage fate in vivo, with alternative cell fates being induced by lower, graded concentrations (DeKoter & Singh, 2000).

Along with PU.1, the C/EBPs regulate the majority of myeloid genes (Friedman, 2002). The forced expression of C/EBP-α and C/EBP-β in B cells has been shown to result in their reprogramming into macrophages (Xie et al., 2004). The mechanism appears to involve C/EBP-mediated inhibition of the B cell commitment factor, Pax5, resulting in the down-regulation of its B cell-restricted target, CD19, and the release of the suppression of CSF1R by Pax5. C/EBPs also synergise with endogenous PU.1, leading to the up-regulation of Mac-1 and other myeloid markers (Xie et al., 2004). Mac-1 activation does not occur in the absence of PU.1 (Xie et al., 2004). Work by Feng et al. (2008) also examined the role of LTFs in the differentiation of fibroblast cells, finding that the myeloid conversion is primarily induced by PU.1, with C/EBP-α acting as a modulator of macrophage-specific gene expression. C/EBP-α or PU.1 are necessary for the formation of myeloid cells in a mouse model because the loss of either gene results in mice lacking macrophages and granulocytes (Koschmieder et al., 2005).

The null expression of PU.1 in 3D4/21 cells may result from their SV40 transformation. The PU.1 binding site is known as the “pu box”, a tissue-specific regulatory DNA element present in many genes expressed in myeloid lineages (Shin & Koshland, 1993). The pu box in the SV40 promoter binds PU.1 with high affinity (Pettersson et al., 1995). This interaction results in the inhibition of PU.1, which may account for the phenotype of the 3D4/21 cell line (Pettersson et al., 1995). The 3D4/21 cell line had a greater relative abundance of C/EBP-β mRNA than AM. C/EBP-β has been shown to induce cell proliferation (Tang et al., 2003) so this may be a result of the continuous nature of the 3D4/21 cell line. The lack of native PRRSV-permissiveness and the aberrant marker and LTF expression, coupled with
the lack of PRRSV receptor expression, makes the 3D4/21 cell line a poor system for the biologically-relevant dissection of host-virus interactions. These data highlight the questionable adoption of the term “porcine alveolar macrophage” to refer to the 3D4/21^CD163 cell line by some researchers (Chen et al., 2013; Lee et al., 2010; Lee & Lee, 2012).

In order to attempt to indirectly induce the expression of CD163, without the need to transfect exogenous CD163 and risk disrupting downstream pathway members, as well as to force myeloid differentiation, LTF overexpression was tested. Issues arose upon the transformation of 3D4/21 cells with lentivirus encoding LTFs, in that it proved challenging to establish a clonal cell population that exhibited robust continuous growth characteristics. For these reasons, further research using the 3D4/21 cell line was abandoned.

The primary respiratory host cell of PRRSV is the AM, a terminally-differentiated, tissue-resident macrophage that is currently the “gold standard” for PRRSV research. In an attempt to develop a novel PRRSV culture system, the utility of CSF-1-derived porcine BMDM for the growth of the PRRSV was evaluated. BMDM could consistently be derived from BMC collected post-mortem using rhCSF-1 and FCS alone. This culture method is most comparable to existing human and murine studies (Kapetanovic et al., 2012). In order to compare in vitro-derived porcine BMDM to AM, a number of markers and phenotypic traits were investigated.

BMDM phagocytosed more aggressively than AM isolated from the same animal and were strongly adherent to un-coated laboratory plastic, but expressed a similar level of SWC3, an antigen specific to myelomonocytic cells (Alvarez et al., 2000). In the pig, MHC genes are termed SLA (Hosokawa-Kanai et al., 2002). BMDM expressed a considerably lower level of SLA-DR, an MHC class II molecule consistently expressed by vascular endothelium (Hosokawa-Kanai et al., 2002) than AM. This could suggest an attenuated antigen-presenting capacity in BMDM.

Previous attempts to produce PRRSV-permissive primary cells from BMC have been reported. These cells were isolated from the femur and humerus of 6 to 8 week old, caesarean-derived, colostrum-deprived pigs. Once isolated, BMC were
cultured in the presence of either GM-CSF alone, or in combination with IL4, while in some cases the cells were also stimulated using LPS. There is significant evidence in the literature that GM-CSF and/or LPS act as stimuli driving the polarisation of macrophages into the M1 inflammatory state. CSF-1 is present in the circulation and in all tissues \textit{in vivo} (Chitu & Stanley, 2006), whereas GM-CSF is inducible by stimuli such as LPS or T cell mitogens, so is likely always to act in the presence of CSF-1 \textit{in vivo}. CSF-1 is thought to be sufficient in order to induce the differentiation into tissue macrophages of an M2 phenotype. M2 macrophages are associated with anti-inflammatory functions linked to wound healing and tissue repair, whereas M1 macrophages have an “activated” phenotype that is associated with greater microbicidal and antigen-presenting activity (Lawrence & Natoli, 2011). A recent study found that M2-polarised monocyte-derived macrophages were highly permissive to infection by type I and type II PRRSV strains of varying virulence (García-Nicolás \textit{et al.}, 2013). Conversely, M1-polarised macrophages were resistant to low pathogenic type I PRRSV strains, but less resistant to type II strains (García-Nicolás \textit{et al.}, 2013). These data could explain the high susceptibility to PRRSV infection observed in CSF-1-derived BMDM in this chapter. In light of the data presented, the BMDM generated using rhCSF-1 only in this study were likely to be M2 polarised. In contrast, previously reported porcine BMDM are likely to have been M1 polarised.

Porcine monocytes can be divided into subsets based on the expression of SWC3, SLA-DR and CD163 markers (Chamorro \textit{et al.}, 2005). Previously reported data have shown that CD163+ and CD163- monocytes are heterogeneous in their expression of SLA-DR (Chamorro \textit{et al.}, 2005). The consistently low levels of SLA-DR expression in BMDM when compared to AM from the same pig in this study suggested that BMDM are phenotypically similar to the SWC3+, CD163-, CD14+ and SLA DR- monocyte subset I, as defined by Chamorro \textit{et al.} (2005). The use of rhCSF-1 to differentiate BMC has potentially led to an M2 macrophage phenotype, thereby possessing attenuated antigen-presenting capabilities, as signified by the low SLA-DR expression in resultant BMDM. However, despite being in agreement with the findings of Kapetanovic \textit{et al.} (Kapetanovic \textit{et al.}, 2012) the null or low CD163 expression in CSF-1-derived porcine BMDM is contrary to data from other species,
which suggest that M2 macrophages should express CD163 in abundance (Gordon, 2003; Mantovani et al., 2002; Ohri et al., 2009). On the other hand, in vitro-derived cells cannot reasonably be expected to be identical to their ex vivo counterparts.

BMDM were readily infectable by both MARC-145-grown PRRSV H2 and un-passaged SD01-08 type I PRRSV strains. The permissiveness of BMDM, as measured by the proportion of infected cells at 24 hpi, was far greater than AM for both aforementioned virus strains. BMDM were routinely >90% infected at 24 hpi, compared to <15% of AM under the same conditions. Quantification of virus genome in the cell culture supernatant of BMDM revealed that they supported virus replication to high relative titres. These titres were significantly higher than both the natural respiratory host cell, the AM, and the commonly used vaccine production and culture host, the MARC-145 cell line. More than 90 and 400-fold greater titres of SD01-08 virus were supported by BMDM compared to both AM and MARC-145 at 36 hpi, respectively.

AM vary in their susceptibility to a productive PRRSV infection (e.g. Ait-Ali et al., 2007; Vincent et al., 2005). For this reason, testing of a battery of BMDM and AM preparations from different animals of varying ages and belonging to various commercial pig lines was undertaken to evaluate the reproducibility of BMDM infectivity. BMDM were shown to be more permissive to infection than AM in all samples, across both SD01-08 and PRRSV H2 virus strains. This heightened permissiveness extended to BMDM and AM isolated from the same animal. Representative BMDM isolates were available from four commercial lines. Segregating the data into each breed revealed minimal breed differences in permissiveness of BMDM across the limited number of samples analysed. However, the HAM breed was consistently highly permissive to infection, although cells isolated from only three individuals were tested.

The permissiveness of BMDM to infection by PRRSV suggested that the PRRSV co-receptors CD163 and CD169 should typically be present. However, this was not found to be the case, with no CD163 or CD169 protein readily detectable by FC in intracellular or extracellular localisations. Western blot for CD163 also revealed minimal protein levels in BMDM lysates. Transcriptional data confirmed a
relative lack of mRNA transcription in un-stimulated BMDM for both CD163 and CD169, compared to AM. The low or null detection of CD163 in BMDM was further confirmed by co-staining PRRSV-infected AM and BMDM with a CD163 antibody. This indicated a discrepancy between the percentage of infected BMDM (51 %) and the percentage of CD163-positive BMDM (3 %). Incubation of BMDM with antibodies recognising CD163 and CD169 led to a significant attenuation of AM infectivity, with only a negligible effect on BMDM infectivity. However, blocking of endocytosis using cytochalasin D led to a similar reduction in infection in AM and BMDM, indicating an endocytic entry mechanism in these cells.

The PRRSV entry and replication cycle was fully functional in BMDM as infectious progeny virus was produced. However, previous studies across multiple cell types have shown that CD163 and CD169 act as receptors or enhancers of PRRSV infection, respectively (Calvert et al., 2007; Van Gorp et al., 2008; and others). Porcine BMDM matured in the presence of rhCSF-1 were CD163 and CD169 low at transcript and protein levels. Whereas, AM were CD163 and CD169 high at both transcript and protein levels. This indicated that PRRSV binding, internalisation and uncoating in BMDM was likely to be CD169 and CD163 independent.

Multiple virus entry pathways for a single virus have been reported before, with alternative receptors and virus entry mechanisms existing for HIV-1 (Pollakis & Paxton, 2012) and influenza A virus (IAV) (de Vries et al., 2011). Alternative PRRSV entry pathways have been alluded to by previous studies. This is due to the apparently dispensable role of CD169 in some cell types (Calvert et al., 2007; Van Gorp et al., 2008) and, recently also in vivo (Prather et al., 2013). CD169 binds and internalises PRRSV virions (Delputte et al., 2005; Delrue et al., 2010; Van Gorp et al., 2008; Vanderheijden et al., 2003). However, this does not result in the initiation of virus replication in the absence of the uncoating receptor CD163 (Vanderheijden et al., 2003). Although higher infectability has been reported in cells transiently coexpressing CD163 and CD169 than expressing CD163 alone (Van Gorp et al., 2008), other authors have failed to demonstrate this CD169-mediated enhancement when using virus strains adapted to growth on cells expressing CD163 alone (Welch & Calvert, 2010). CD163 has been shown to cycle between the plasma membrane
and early endosomes (Schaer et al., 2006), suggesting that CD163 at the cell surface could also facilitate an CD169-independent entry mechanism. These data are supported by the readily detectable CD163 protein staining at the plasma membrane of AM. According to previously published data, the lack of detectable CD169 protein in BMDM may still allow a productive PRRSV infection, but only in the presence of CD163 (Calvert et al., 2007; Van Gorp et al., 2008).

Using a polarised nasal mucosa explant system, Frydas et al. (2013) recently showed that the PRRSV subtype III strain, Lena, infected both CD163+/CD169- and CD163-/CD169- cells in the nasal mucosa. The authors postulated that PRRSV infection began in CD163+/CD169+ cells and then spread to bystander cells, possibly through the phagocytosis of apoptotic cellular remains. However, this phenomenon was not demonstrated for strain LV, indicating a virus or subtype-specific alternative entry mechanism (Frydas et al., 2013). Such an alternative mechanism may occur in BMDM, either through an alternative receptor or entry mediator, or through a non-specific process such as macropinocytosis that negates the need for either CD163 or CD169. This would be supported by the observed blocking of BMDM infection following cytochalasin D treatment, a known inhibitor of macropinocytosis. Macropinosomes rapidly develop early endosome characteristics (Jones, 2007), potentially allowing virus entry. However, an uncoating receptor, such as CD163, would likely still be required for completion of the replication cycle. Additional receptors, including CD151 have also been implicated in PRRSV entry. CD151 may be involved in the fusion of the viral envelope and the endosome through a yet-to-be elucidated mechanism (Shanmukhappa et al., 2007). Recently, ectopic expression of CD151 alone has also been shown to be sufficient to render the non-permissive PK-15 cell line permissive to productive PRRSV infection (Huang et al., 2013). Further work must be carried out to assess the potential role of this protein in PRRSV infection of BMDM.

No significant difference in a number of different markers or PRRSV-permissiveness was evident in BMDM derived from BMC preparations isolated from the ribs of sexually mature adult pigs or the femur or ribs of juvenile animals. This is an important finding as the reduction of animal use in research is highly desirable. Isolation of a greater number of cells from each juvenile animal through the removal
of ribs and femurs could reduce the number of animals euthanised for tissue collection. The ribs of lethally anaesthetised sexually mature gilts yielded large numbers of readily infectable BMDM. This finding suggests that an alternative source of BMC from bones made available by commercial slaughter operations may be feasible, reducing the need to cull experimental animals.

An important tool in the study of host-virus interactions is the ability to perform host genetic manipulation, including GOF or LOF assays. Given the refractive nature of AM to transfection (data not shown), the amenability of BMDM to transfection of plasmid DNA and siRNA with efficiencies of >50 % and between 47 and 77 %, respectively, is an additional benefit of the use of BMDM. The data presented in this chapter indicate that BMDM are amenable to transfection, with minimal impact on cell viability, whilst still being an actively phagocytic primary cell system.

The result of the current shortage of suitable cell culture systems for PRRSV research that are similar to its in vivo cellular target, is the reliance on the capricious AM, xenographic cell culture systems or genetically modified cell-lines. The work in this Chapter has succeeded in producing mature macrophages from BM progenitor cells in vitro. BMDM are an abundant and biologically relevant cell type that are highly permissive to PRRSV infection and transfection and thus, could be used for a plethora of vaccine production, diagnostic or basic research activities in the future. Of particular value is that CSF-1-differentiated porcine BMDM may present an opportunity to explore an efficient alternative PRRSV entry mechanism in a porcine primary cell system. It is also important to establish if the entry mechanism of PRRSV into BMDM is also relevant in vivo.
ANALYSIS OF THE TRANSCRIPTIONAL RESPONSE TO PRRSV INFECTION
Statement of Authorship

It must be stated that the datasets on which the analyses presented in this Chapter rely, were not generated by me. Experiments for the *in vitro* dataset (RIVitro) were performed at the Roslin Institute and have been published previously (Ait-Ali *et al.*, 2007; Ait-Ali *et al.*, 2011). These data are readily available in the public data repository “Array Express” (http://www.ebi.ac.uk/arrayexpress/) under the accession number: E-MTAB-505. I was personally responsible for submitting these data to Array Express on behalf of the authors.

The *in vivo* dataset (RIVivo) is an extension of the work performed by Dr. Craig Lewis (Lewis, 2009) and the microarray data are as yet unpublished, performed under the leadership of Alan Archibald. The *in vivo* animal experiment was performed at the Veterinary Laboratories Agency, while the sample preparation and microarray hybridisation were largely performed by Dr. Alison Wilson at the Roslin Institute. Dr. Alison Wilson was additionally responsible for performing the qRT-PCR experiments to quantify PRRSV RNA and IFN-β induction.

My contribution, presented in the forthcoming Chapter, extends to the complete re-analysis of the publically available RIVitro dataset and the first analysis of the RIVivo dataset. Data that were not collected by me are clearly annotated with their authorship attribution.
3.1 Chapter Introduction

Transcriptional profiling technologies for the pig are becoming more accurate and accessible and will only continue to improve following the publication and annotation of the pig genome sequence (Archibald et al., 2010b; Groenen et al., 2012). Transcriptomics has previously been used to dissect host-pathogen interactions in the pig, suggesting that it has the potential to be an excellent predictive tool (Afonso et al., 2004; Badaoui et al., 2013; de Greeff et al., 2010; Flori et al., 2008; Ledger et al., 2004; Miller & Fox, 2004; Moser et al., 2004; Niewold et al., 2005; Uthe et al., 2007; Wang et al., 2007; Wang et al., 2008; Zhao et al., 2006).

The technological progression of microarray platforms has been mirrored by the development of sophisticated computational analysis techniques. The size of high-throughput datasets has necessitated that standards for recording and storing information about microarray experiments must exist (Brazma et al., 2001). Microarrays are now a mature technology, having existed for well over a decade. However, in recent years the emergence of RNA-Seq as an alternative method for gene expression analysis has threatened to impact the uptake of microarray technology in contemporary studies (Shendure, 2008). Several porcine microarray platforms are commercially available, including: Affymetrix GeneChip Porcine Genome Array (Porcine GeneChip), U.S. Pig Genome Coordination Program array (Rothschild, 2004; Tsai et al., 2006b), Pigoligoarray (http://www.pigoligoarray.org) and the Agilent Porcine Gene Expression Microarray. Affymetrix have also released a newer, more comprehensive “PorGene-1_0-st-v1” array. However, many of these platforms are somewhat lacking in their coverage of the pig transcriptome and the annotation of probe targets.

In order to quantify gene expression, microarrays typically employ oligonucleotide probes of 25 nucleotides in length, which are complementary to their mRNA target sequence. On Affymetrix microarrays, 11 to 20 of these 25 mers are used to target different sequences on the same transcript; each group of probes is referred to as a probe set. As well as perfectly complementary probes (perfect match, or “PM” probes) these microarrays also contain mismatch probes that are identical to PM probes, with the exception of a substitution at nucleotide position 13. These
mismatch probes can aid in identifying nonspecific hybridisation and background signals. The hybridisation of a fluorescently labelled target sample to the microarray chip permits the measurement of the relative fluorescent signal at each probe location, which equates to target abundance.

The Porcine GeneChip array boasts 24,123 probe sets, representing 11,265 unique genes. However, the Porcine GeneChip array files provided by the manufacturer delivered annotation coverage of only 14% of the probe sets on the array, having been developed using the expressed sequence tag (EST) data available in 2004 (Tsai et al., 2006a; Tuggle et al., 2007). Thankfully, attempts have been made to improve the annotation of this array platform, based both on the availability of improved pig genome sequence and homology with the well annotated mouse and human genome sequences (Naraballobh et al., 2010; Tsai et al., 2006a). 99% of the probe sets on the array are now annotated following the 2010 update of a bioinformatics-led Porcine GeneChip annotation dataset (Tsai et al., 2006a). A recent collaborative research effort has developed the “Snowball” microarray, which boasts much greater coverage of the pig genome than previously available (Freeman et al., 2012). The trend towards increased coverage and reduced cost of microarrays has increased their uptake by researchers, particularly those focussed on the host response to pathogens.

Virus infection induces widespread changes in host gene expression programs, involving a wide range of biological processes. Extracellular or endocytosed/phagocytosed foreign organisms can be recognised by PRRs. Additionally, RIG-like helicases (RLHs), such as RIG-I, sense foreign macromolecules and initiate signalling cascades upon interaction with these molecules. The resulting signalling pathways lead to the synthesis and secretion of interferons (IFNs) and interleukins, as well as the activation of caspases, often leading to apoptosis. The binding of viral RNA to RIG-I results in the downstream activation of the mitochondrial antiviral signalling protein (MAVS) and consequently, IFN production. However, some viruses are able to circumvent or disrupt this pathway (Baril et al., 2009; Chen et al., 2007). Defining transcriptional remodelling is a vital component in the study of the host response to viral infection, informing attempts to understand and increase the robustness of the host antiviral
response. Several genome-wide experiments exist in the PRRSV literature, employing a variety of experimental platforms and PRRSV strains (Table 3.1). More genome-wide data are available for type II PRRSV strains than for type I European strains (Table 3.1). The online availability of published microarray datasets facilitates their analysis by other research groups, where appropriate using different methodologies. The re- or meta-analysis of datasets can lead to different interpretations or findings and the highlighting of potentially overlooked trends.

Biolayout Express\textsuperscript{3D} (Biolayout) (Freeman \textit{et al.}, 2007a; Theocharidis \textit{et al.}, 2009) is an application designed to generate and display large, complex network graphs of biologically-derived data. For microarray data, transcripts are represented as nodes, which are connected by edges that infer a degree of co-expression (Theocharidis \textit{et al.}, 2009). Biolayout employs pair-wise transcript comparison of expression profiles calculated using a Pearson correlation matrix. Setting a correlation cut-off value ensures that only those genes that are related in expression to others above this selected threshold are included in analyses. The built-in correlation and Markov cluster algorithm (MCL) (Van Dongen, 2000), together with the ability to visualise and explore very large network graphs, facilitates the analysis of large complex datasets. Genes coding for proteins within biological pathways, both ubiquitous and cell-specific, often group together to form co-expression clusters (Mabbott \textit{et al.}, 2010). Studying these clusters can allow one to infer the function of another gene member of unknown function or lacking annotation, based on its co-expressed kin. As a result, multiple large datasets have been successfully analysed using Biolayout (Freeman \textit{et al.}, 2012; Hume \textit{et al.}, 2010; Kapetanovic \textit{et al.}, 2012; Lacaze \textit{et al.}, 2009; Mabbott \textit{et al.}, 2010; Mabbott \textit{et al.}, 2011; Natividad \textit{et al.}, 2010; Summers \textit{et al.}, 2010).
Table 3.1 | PRRSV transcriptional experiments in the literature. Genome-wide transcriptional experiments investigating the host response to PRRSV are listed, employing a variety of experimental platforms and PRRSV strains. \(^1\) demarcates data deposited in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/). \(*\) demarcates data that are deposited in NCBI’s Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). SAGE stands for “serial analysis of gene expression”.

<table>
<thead>
<tr>
<th>Assay type</th>
<th>Pig breed</th>
<th>PRRSV Strain</th>
<th>PRRSV type</th>
<th>Infectious dose</th>
<th>Accession number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine GeneChip</td>
<td>Landrace; Pietrain</td>
<td>H2</td>
<td>I</td>
<td>MOI=1</td>
<td>E-MTAB-505(^1)</td>
<td>Ait-Ali et al. (2011)</td>
</tr>
<tr>
<td>Porcine GeneChip</td>
<td>Rattlerow-Seghers</td>
<td>LV</td>
<td>I</td>
<td>MOI=10</td>
<td>E-MEXP-1350(^1)</td>
<td>Genini et al. (2008)</td>
</tr>
<tr>
<td>SAGE</td>
<td>Crossbred</td>
<td>VR-2332</td>
<td>II</td>
<td>MOI=10</td>
<td>E-GEOD-10346(^1)</td>
<td>Miller et al. (2008); Miller et al. (2010)</td>
</tr>
<tr>
<td>Pigoligoarray</td>
<td>Not noted</td>
<td>MNW2B, NC Powell, Ingelvac</td>
<td>II</td>
<td>(10^{4.5} \text{TCID}_{50})</td>
<td>E-GEOD-26642(^1)</td>
<td>Joan Lunney, personal communication</td>
</tr>
<tr>
<td>Pigoligoarray</td>
<td>Hampshire x (Duroc x NE Index line)</td>
<td>NVSL 97-7985</td>
<td>II</td>
<td>(50% \text{TCID}_{50})</td>
<td>E-GEOD-25120(^1)</td>
<td>Petry et al. (2007); Bates et al. (2008)</td>
</tr>
<tr>
<td>Pigoligoarray</td>
<td>Crossbred</td>
<td>NVSL 97-7985</td>
<td>II</td>
<td>(10^{3} \text{TCID}_{50})</td>
<td>GSE41144(^*)</td>
<td>Arceo et al. (2013)</td>
</tr>
</tbody>
</table>
In this Chapter, Biolayout was employed in order to adopt a network-based approach to the re-analysis of two PRRSV microarray datasets. This approach was used for both datasets analysed and revealed striking similarities in certain clusters of host transcripts. These key transcript clusters were well conserved, despite the different tissue and study types. The resulting analyses have also provided a unique insight into multiple members of these clusters with unknown functions, or that have not previously been implicated in PRRSV infection. These datasets were enhanced through the implementation of additional bioinformatic and pathway analyses. Genes in the region of recently published, high-confidence SNPs identified as being associated with the host response to PRRSV (Boddicker et al., 2012) were found to be present within key clusters in both datasets. The representation of an antiviral gene family in this SNP region prompted further investigation into its potential role in PRRSV infection and resistance.
3.2 Results

A standardised analysis pipeline was applied to both microarray datasets presented herein. The particulars of each dataset are described, in detail, later in this Chapter. Following normalisation, quality control (QC) and statistical filtering, the transcriptional data were clustered within Biolayout. Each cluster within the network represented a group of transcripts that were highly correlated in their expression over the time course experiment (Figure 3.1). After clustering the network graph and the removal of nodes having no cluster membership, clusters displaying differential temporal expression were identified. Clusters exhibiting up-regulation or down-regulation relative to the control samples were selected for further examination. These clusters were assigned a description based on criteria proposed by Raza (2011). Up-regulation or down-regulation relative to the control samples was denoted by U or D, respectively. Transient (T) implies that any change in expression was reversible, whereas sustained (S) implies that it was maintained over the time course (Table 3.2). The timepoint/s where maximal or minimal transcript expression levels were reached and the duration of the change in expression were also recorded and denoted by numbers (Table 3.2).

Clusters were annotated using Ingenuity Pathway Analysis (IPA) to determine the enrichment of genes belonging to known pathways or biological processes. IPA is not presently available for the pig, so all annotation was based on human RefSeq IDs assigned to the target of each porcine probe set (where available). The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was employed in order to add additional, manually curated data for the understanding of high-level biological processes in each dataset. Manual annotation, through the review of the scientific literature, was also employed in order to assign biological functions to clusters (Figure 3.1).

This approach was carried out for two PRRSV-infection microarray datasets; one in vitro respiratory experiment and one in vivo reproductive experiment (described in detail below).
Figure 3.1 | Microarray clustering and analysis workflow. Workflow diagram summarising the approach taken to assemble a biological network from transcriptional data and outlining the data-driven investigation of the biological functions of resulting clusters of co-ordinately regulated transcripts. “RMA” stands for robust multi-array average.
3.3 RIVitro – in vitro respiratory transcriptional dataset

3.3.1 Experiment introduction

Ait-Ali et al. (2007) performed in vitro PRRSV infection experiments using BALF-derived AM isolated from pigs belonging to five commercial lines, with the aim of identifying any genetic or breed component to PRRSV-susceptibility. PRRSV H2, an AM-grown type I PRRSV strain that results in a pronounced cytopathic effect in vitro (Drew et al., 1997), was employed as the infectious agent in the study. AM were isolated from three-week old piglets obtained from the same high health status pig production unit. Infections were performed at MOI 1, without removing the growth medium from the cells, thus including any non-adherent and contaminating non-macrophage cells in subsequent total RNA preparations.

A difference in PRRSV replication was clearly observed between cell preparations from the genetic lines tested, despite statistical similarity in the levels of multiple cell-surface markers, including the PRRSV internalisation-receptor CD169 (Ait-Ali et al., 2007). However, no data are available for the expression of the prototypic PRRSV receptor, CD163 in these cell preparations. PRRSV-positive cells were quantified using FC and showed a broadly similar beginning of a productive infection at 8 to 12 hpi, continuing to the end of the time course at 30 hpi, by which time 70 to 90 % of cells were infected (Figure 3.2A). The notable exception to this trend was the Landrace (LR) breed, which appeared not to succumb to a significant infection (Figure 3.2A). The normalised fold induction of PRRSV RNA, as measured by qRT-PCR of PRRSV ORF7, revealed more than two orders of magnitude lower accumulation in LR samples, when compared to the Pietrain (PIE) samples (Figure 3.2B). The quantification of infectious virus in the supernatant by TCID<sub>50</sub> assay at 72 hpi revealed that again, the LR cells produced a virus titre more than two orders of magnitude lower than cells from representatives of the other genetic lines (Figure 3.2C).

Following this experiment, total RNA was isolated at a high temporal resolution, up to and including 30 hpi, and analysed using Porcine GeneChip microarrays in order to assess the host transcriptional response to PRRSV infection in respiratory macrophages (Ait-Ali et al., 2011). AM were assayed from two of the
commercial pig lines included in the previous analysis by Ait-Ali et al. (2007). PIE and LR breeds were selected for microarray profiling, allowing the comparative analysis of the host response to PRRSV in cells from individual representatives of each breed (single litter only). The PIE samples exhibited one of the highest PRRSV transcript accumulations and the LR, the lowest, in the original study (Ait-Ali et al., 2007). A first analysis of the resulting transcriptional data has been published (Ait-Ali et al., 2011). For the purpose of this thesis, this transcriptional experiment has been designated Roslin Institute in vitro (RIVitro).

I have undertaken a re-analysis of the RIVitro dataset using a combination of techniques, including biological network and pathway analysis, in order to attempt to extract further valuable data on the host response and any genetic basis for susceptibility to type I PRRSV infection in AM.
Figure 3.2 | PRRSV infection in AM derived from various breeds. A. The percentage of PRRSV-positive AM, as determined by FC of three animals representing each of four pig breeds across an infection time course. The mean standard error is shown. B. Log_{10} intracellular PRRSV RNA fold-induction, as determined by qRT-PCR and normalised to beta-actin mRNA level, over a time course of infection in AM. Data are shown from four individuals representing each of five pig breeds. Error bars represent the standard deviation of the samples representing each breed. C. Endpoint virus titre at 72 hpi, determined by TCID_{50} assay on pooled AM from each breed. AM from four animals representing each of the five pig breeds were pooled prior to infection. Individual figures taken directly from Ait-Ali et al. (2007).
3.3.2 Results

In order to take individual differences in the host response to PRRSV infection into account when examining the RIVitro transcriptional dataset, it was necessary to also consider the dynamics of PRRSV replication in these cell isolates. When examining the induction of PRRSV RNA in the AM of LR and PIE breeds collectively, it was evident that although the LR cells hosted an infection, the relative quantity of virus RNA detected in these cell isolates was lower than in PIE macrophages infected in the same manner (Figure 3.3A). Although the mean PRRSV RNA accumulation data distinguished between the LR and PIE breeds, it was also important to examine individual variation in virus RNA accumulation within each breed. PIE sample 643 hosted the highest PRRSV RNA accumulation of all samples and LR 1286 hosted the lowest (Figure 3.3B). However, the difference between the PIE sample with the lowest RNA induction of its cohort (sample 637) and the LR sample with the highest RNA induction of its cohort (sample 1287) was somewhat less substantial (Figure 3.3B). This was a result of individual, intra-breed variation in the level of PRRSV RNA accumulation. A sharp increase in the quantity of PRRSV RNA was observed in all samples following the 8 hpi timepoint, consistent with the timing of the first round of PRRSV genome replication (Figure 3.3B). However, a LR sample-specific decrease in PRRSV RNA accumulation was noted at 24 hpi, which did not appear to occur to the same extent in the PIE samples (Figure 3.3A and B). Using Biolayout to cluster the PRRSV RNA induction profiles measured in the AM of each of the three representatives of the LR and PIE breeds revealed two distinct clusters (Figure 3.3C). Four samples, composed of two representatives of each breed, were clustered together. However, LR sample 1286 and PIE sample 641 were clustered separately (Figure 3.3C). This indicated both that these samples shared similarities in their PRRSV RNA induction profiles and that the profiles of these two cell isolates were distinct from the other representatives of their breeds (Figure 3.3C).

In summary, although all AM derived from PIE animals hosted higher levels of intracellular PRRSV RNA than LR isolates; considerable intra-breed variation was evident. Therefore, when exploring the host transcriptional response to PRRSV in these cell preparations, this intra-breed variation was taken into consideration.
Figure 3.3 | PRRSV induction in the RIVitro samples: breed and individual differences.
A. Mean intracellular log_{10} PRRSV induction (normalised to beta-actin) in AM representing the LR and PIE breeds, as measured by qRT-PCR. Error bars represent the standard deviation of the mean PRRSV induction for all three representatives of each breed. Student’s t-test showed that LR and PIE isolates were not significantly different in their levels of intracellular PRRSV RNA at all timepoints tested (p <0.05).
B. Log_{10} intracellular PRRSV induction (normalised to beta-actin) as measured by qRT-PCR in the AM of individual animals representing the LR and PIE breeds. Samples beginning with ‘12’ were derived from LR animals and those beginning with ‘6’ from PIE animals.
C. Biolayout network graph of PRRSV RNA induction profiles for all samples presented in B. Nodes are coloured according to their MCL cluster. All PRRSV induction qRT-PCR data were generated by Dr. Alison Wilson.
QC was performed on all microarray samples available in the RIVitro dataset in order to identify any outlying data that could indicate an issue with a particular sample. Using the RMAExpress program (v1.0.5), boxplots of the unadjusted PM intensities were generated, one for each array sample, with log₂ transformation of the PM intensities for ease of interpretation (Figure 3.4A). Upon visualisation of the log₂ intensities of PM probes as boxplots, two microarray samples appeared to be brighter than other arrays in the dataset, producing outlying data points (Figure 3.4A). Plotting smoothed density curves of the same log₂ intensities for each microarray sample provided a clearer representation of the data (Figure 3.4B). In density plots, potential low quality data can be indicated by density curves that are shifted away from the other samples, or show a different shape. The density plots confirmed that two array samples clearly stood out as being different to the rest of the chips (Figure 3.4B). These samples were confirmed to be the same two samples identified as outliers in the boxplot QC analysis (Figure 3.4A). Both samples required removal at this stage, prior to further analysis and normalisation. 46 Porcine GeneChip microarray samples from the RIVitro experiment passed QC and were normalised by robust multi-array average (RMA).
**Figure 3.4 | Pre-normalisation QC of RIVitro microarray samples.**

**A.** Boxplot of log₂ PM probes, by array, for raw data. Each boxplot represents a single microarray sample, allowing the visual inspection of the consistency between samples and the identification of outlying samples.

**B.** Density plot of log₂ PM, by array, for raw data. Each line plotted represents a single microarray sample. Asterisks in **A** and **B** denote the two outlying array samples that were removed from further analyses following QC.
Following RMA normalisation, the RIVitro dataset was transposed within the Biolayout software, facilitating the analysis of the correlation between each array sample, as opposed to the correlation of each transcript across all array samples. MCL clustering of the 46 array sample graph, constructed using a Pearson correlation coefficient cut-off of $r=0.97$, revealed a number of patterns within the data. The network separated into five clusters, based on the expression profile of the entire array sample. Clusters 1 and 2 formed two distinct clusters, interconnected by several edges (Figure 3.5A). The remaining three clusters were smaller and were not connected to the larger Clusters 1 and 2 by any edges at the correlation cut-off used (Figure 3.5A).

As a result of the transposition of the dataset for this analysis, each array sample represented an RNA sample, taken at a particular point in time, following the processing of cells derived from one animal. Therefore, it was possible to annotate the small, 5 cluster, transposed biological network with the breed that each sample was derived from, in order to attempt to identify any trends in the dataset. Colouring the array samples within the network shown in Figure 3.5A, according to their breed of origin, revealed that Clusters 1 to 5 appeared to effectively partition or define the breeds tested in this study (Figure 3.5B). It was clear that Clusters 1 and 2 represented a subset of array samples derived from PIE and LR cells, respectively, only (Figure 3.5). The same was true for Clusters 4 and 5, consisting solely of array samples from the LR and PIE breed cells, respectively. However, Cluster 3 contained array samples derived from both LR and PIE cells, indicating a statistical similarity in some array samples, independent of breed (Figure 3.5).

This initial analysis of the transposed dataset indicated that the transcriptional activity across whole array samples was largely distinguishable between cells derived from the LR and PIE breeds. However, some array samples were clustered together in this analysis, indicating that the overall transcriptional “snapshot” represented by each sample could be comparable between cells from each represented breed in some cases.
Figure 3.5 | Network graphs showing the correlation between each array sample. Annotated network graphs where each node represents a complete array sample. The degree of correlation across the whole sample was considered and influenced the clustering of samples. Nodes are coloured according to their A. MCL cluster or B. breed.
In order to further investigate the host transcriptional activity over the PRRSV infection time course, a network-graph of the complete, RMA-normalised microarray dataset was generated using Biolayout by filtering edges at a Pearson correlation threshold of 0.85. The resultant network graph of 5,712 nodes, connected by 45,884 edges, was clustered using the graph-based clustering algorithm MCL (Van Dongen, 2000) set at an inflation value of 3, resulting in 140 clusters with a membership of 6 or more nodes (transcripts). These 140 clusters formed two, large distinct sub-networks within the biological network (Figure 3.6). Clusters 4 and 5, which were up-regulated towards the middle of the time course, were clustered together, set apart from the two large sub-networks. The remaining clusters that were noted as showing a similar up-regulation mid-infection, were located in close proximity to each other, together with other large clusters such as Clusters 1, 4 and 6 (Figure 3.6). The other sub-network contained clusters that appeared to be primarily bi-phasic in their expression profiles, or were down-regulated early in the infection time course (Figure 3.6).

The 140 clusters generated using the MCL algorithm were manually interrogated and assigned categories based on the timing and directionality of their expression profiles. Following this manual curation, it was evident that there were a number of trends in the dataset, with large numbers of transcripts being induced or repressed at various timepoints. Table 3.2 provides an overview of the major clusters of interest in the RIVitro dataset, along with the number of transcripts, examples of gene members and the biological processes represented by each cluster. Clusters in the table are ordered according to the temporal phase encompassing the change of expression of member transcripts: early up (2 hpi), early up (4 hpi), early down (2 hpi), early down (4 hpi), mid up (8 hpi) and bi-phasic.
Figure 3.6 | Transcriptional network formed from the RIVitro expression dataset. The network was filtered to display only relationships at or above a Pearson correlation threshold of 0.85, resulting in a graph of 5,712 nodes connected by 45,884 edges. The resultant network was then clustered using the graph-based clustering algorithm MCL, set at an inflation value of 3. Nodes (transcripts) belonging to the same cluster share the same colour. Un-clustered (no class) nodes have been removed for visual clarity. Key areas of the network that correspond to the clusters identified in Table 3.2 are annotated. The network graph layout was generated using the FMMM layout algorithm in Biolayout v3.1.
### Table 3.2 | Description of clusters of co-ordinately expressed transcripts in the RIVitro transcriptional dataset.

Annotation of clusters induced or repressed at different timepoints during the RIVitro infection time course, relative to the 0 hpi timepoint. Clusters are grouped according to the time of their induction or repression for ease of interpretation. Representative pathways associated with the transcript membership of each cluster is shown, alongside example gene members in each cluster.

<table>
<thead>
<tr>
<th>Original cluster ID</th>
<th>No. Cluster members</th>
<th>Profile description</th>
<th>Transient/sustained</th>
<th>Max or min expression timepoint</th>
<th>Duration of expression change</th>
<th>Both/Piettrain/Landrace</th>
<th>Example gene member(s)</th>
<th>Processes/functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Column 1]</td>
<td>[Column 2]</td>
<td>[Column 3]</td>
<td>[Column 4]</td>
<td>[Column 5]</td>
<td>[Column 6]</td>
<td>[Column 7]</td>
<td>[Column 8]</td>
<td>[Column 9]</td>
</tr>
</tbody>
</table>
In order to begin the analysis of the network generated using Biolayout and representing co-ordinately regulated transcript clusters, the expression profiles of key, large clusters were examined in the context of both PRRSV induction and pathway enrichment. The enrichment of Gene Ontology (GO) terms in each cluster’s membership was calculated using the GOrilla tool (Eden et al., 2009).

The largest cluster in the RIVitro dataset, Cluster 1, was found to consist of transcripts primarily associated with GO terms involving the regulation of the cell cycle and organelle organisation (Figure 3.7A). Interestingly, this cluster was differentially up-regulated in the PIE samples, before returning to basal expression levels, with only one LR individual (1286) exhibiting a significant up-regulation of transcripts in this cluster (Figure 3.7A). The PIE samples hosted the greatest intracellular PRRSV RNA induction in the study, so this could indicate an increase in cellular metabolism as a result of a productive virus infection. A rapid induction of Cluster 1 transcripts occurred at 2 to 8 hpi, coinciding with the exponential increase in PRRSV genome levels detected at 8 hpi (Figure 3.7A). LR AM isolate 1286 had the lowest PRRSV RNA accumulation of all samples. However, conversely, this sample also exhibited a strong “PIE-like” induction of Cluster 1 transcripts (Figure 3.7A).

Cluster 4, which was primarily composed of host immediate-early immune response transcripts, was differentially induced in LR samples only at 2 hpi, mostly returning to basal levels by 12 hpi (Figure 3.7B). LR sample 1287 exhibited only minimal up-regulation of Cluster 4 transcripts, somewhat akin to the PIE samples (Figure 3.7B). Additionally, AM from this animal exhibited the highest PRRSV genome accumulation of the LR samples. The apparent suppression of transcripts in this cluster in a more productive PRRSV infection (all PIE and one LR sample) highlighted a potential role of this host immediate-early immune transcript cluster in in vitro PRRSV permissiveness. GO terms represented within the membership of this cluster included: response to mechanical stimulus and cell differentiation.

Cluster 5 was a classical virus-responsive host transcript cluster, as confirmed by the enrichment of GO terms involving the defence response to viruses. This cluster was strongly induced at 8 and 30 hpi in PIE samples (Figure 3.7C). However, LR animals exhibited negligible up-regulation of Cluster 5 transcripts, with the
exception of LR AM sample 1287, the LR sample with the most productive PRRSV infection, which exhibited a marginally greater up-regulation than the rest of its breed cohort (Figure 3.7C). Furthermore, the LR samples did not appear to show a bi-phasic expression profile for transcripts in this cluster, where the PIE samples did (Figure 3.7C). The up-regulation of this cluster, containing RNA and pathogen sensing transcripts (Table 3.2), was likely to have been in response to PRRSV genome replication, coinciding with virus replication cycles 1 and 2 or 3.

When examining the transcriptional profiles of clusters in the RIVitro dataset, it was also clear that many large transcript clusters were expressed consistently across breeds. For example, Cluster 6, a cluster of metabolic process transcripts, was consistently induced in all samples, independent of breed (Figure 3.7D).

In conclusion, the examination of clusters with some of the largest transcript memberships revealed that biologically relevant host signalling pathways were represented. In addition, the host transcript expression profiles of some clusters appeared to be associated with the degree and timing of PRRSV RNA synthesis in the same samples.
Figure 3.7 | Expression profiles of representative clusters in the RIVitro network. Graphs show the mean expression level of all transcripts represented by each cluster (unbroken black line, primary y-axis), across the time course of PRRSV infection in Landrace cell isolates: 1286, 1287 and 1288 and Pietrain cell isolates: 637, 641 and 643. Data for log$_{10}$ PRRSV RNA induction (over beta-actin), as measured by qRT-PCR, are overlaid for each animal (broken grey line, secondary y-axis). Cluster description and membership information is also shown to the right of each graph. GO enrichment was calculated using GOrilla (Eden et al., 2009).
Biological function/pathway annotation

Following the identification of clusters of co-ordinately regulated transcripts showing a clear response to infection over the experimental time course, biological functions were assigned, where known, using a combination of manual (literature), software and online database curation. Taking the temporal pattern of expression profiles into account, it was possible to group clusters into categories based on the timing and directionality of their expression profiles (Figure 3.8). RIVitro co-ordinately regulated transcript clusters could be broadly separated into three temporal groups based on their expression profiles: early, mid and bi-phasic (Figure 3.8).

**Early (Figure 3.8A)**

Twelve transcript clusters exhibited an early up-regulation, resulting in a peak of expression at 2 hpi. Four additional clusters exhibited the same trend, but with their peak expression levels at 4 hpi (Table 3.2). Several clusters were also down-regulated early in the experimental infection. One cluster exhibited an early down-regulation, resulting in the lowest expression level at 2 hpi, two clusters exhibited the same trend, but with their lowest expression levels at 4 hpi (Table 3.2). The biological functions of the transcript clusters regulated at these early timepoints were varied, including: cell cycle, immune, transcription and membrane trafficking. Cluster 4, with a membership of 47 probe sets, was a host immediate-early immune response cluster with several notable members, including: JUN (part of the AP-1 complex) and several immediate early response (IER) family members (Table 3.2). This cluster was up-regulated at 2 hpi, preferentially in the LR samples.

**Mid (Figure 3.8B)**

Six clusters exhibited an up-regulation mid-way through the PRRSV infection time course, resulting in a peak of expression at 8 hpi (Figure 3.8B). A large number of transcripts were co-ordinately regulated at this timepoint. Clusters regulated at 8 hpi were enriched for biological functions including: antiviral, virus infection, immune response and cellular processes. This timepoint was likely to correspond with the start of aggressive PRRSV genome replication, with the full PRRSV replication cycle being completed by 12 hpi in AM (Costers et al., 2008).
Figure 3.8 | Temporally grouped average expression profiles of transcript clusters. Time-grouped mean expression profiles of all clusters assigned to the A. early, B. mid and C. bi-phasic temporal groups, separated into LR and PIE breeds.
Cluster 5, with a membership of 38 probe sets, was a major transcript cluster induced mid-PRRSV infection. The mean expression profile of all transcripts in this cluster showed a brief and transient up-regulation at 8 hpi in LR samples, before returning to basal levels by around 12 to 16 hpi and remaining at this level until the final timepoint (Figure 3.9). Notably, Cluster 5 transcripts were also induced at 8 hpi in all samples from the PIE breed, but to a far greater degree than LR cell preparations (Figure 3.9). The expression profile also hinted at a second up-regulation in PIE AM only, towards the end of the infection time course at 30 hpi (Figure 3.9). This cluster had a well-annotated membership, including: GBP-4, DDX58, IRF7, STAT2, MX1, TRIM22 and ISG15. Pathway analysis of Cluster 5 member transcripts confirmed that their primary function appeared to involve the antimicrobial response, inflammatory response and inflammatory disease, with all of these functions being over-represented in this cluster (Figure 3.9).

When the induction of PRRSV RNA (as measured by qRT-PCR) was overlaid on the transcriptional profile of Cluster 5, it was evident that the low PRRSV RNA induction in the LR samples coincided with minimal up-regulation of Cluster 5 transcripts (Figure 3.9). However, in the PIE samples there was a strong induction of Cluster 5 transcripts at 8 hpi, before a return to near basal transcription levels, coinciding with the beginning of the rapid accumulation of PRRSV RNA. Synthesis of PRRSV RNA began in earnest at 8 hpi in all cell isolates, but rapidly tailed off in the LR samples, coinciding with the minimal induction of Cluster 5 transcripts in this breed (Figure 3.3A). Thus, this cluster of co-regulated transcripts was apparently induced upon PRRSV infection and was differentially up-regulated in the PIE breed. The known antiviral and pathogen sensing functions of multiple member transcripts suggest that further investigation into this cluster is warranted.
Figure 3.9 | Summary of RIVitro Cluster 5. The mean expression profile of RIVitro Cluster 5 is shown as an unbroken black line. Box and whisker plots show the variation (median, upper and lower quartiles) and the maximum and minimum values (whiskers) in the mean expression level of this cluster between the three cell isolates available for each timepoint. Mean PRRSV log_{10} RNA induction, as determined by qRT-PCR (performed by Dr. Alison Wilson), is overlaid as a dashed black line. A summary of the cluster timing and membership, as well as pathway analysis data is displayed below the graph.
**Bi-phasic (Figure 3.8C)**

Eleven clusters exhibited a bi-phasic expression pattern (Table 3.2). Many of these clusters were down-regulated early in the infection time course, resulting in their lowest expression levels before 8 hpi, before being up-regulated later in the infection to expression levels that were largely maintained until the end of the time course at 30 hpi (Figure 3.8C). These clusters appeared to represent biological processes such as: RNA degradation/metabolism, apoptosis and virus transcription. The timing of the down regulation of these transcript clusters coincided with virus entry and genome replication. The up-regulation of many of these co-ordinately regulated transcript clusters corresponded to the end of the first cycle of PRRSV replication, with PRRSV-positive cells beginning to be detected at 10 to 12 hpi in this experiment (Figure 3.2A).

**Pathway analysis**

In order to further enhance the annotation of the clusters identified within the biological network thus far and investigate biological pathways that may be represented within the dataset, several pathway depositories were utilised. KEGG is a curated online database of known members of biological pathways. The identification of enriched GO terms in each clusters’ membership was performed using the GOrilla tool (Eden et al., 2009), making comparisons based on the background of the entire microarray. IPA is a commercial software application for analysing ‘omics data and interpreting biological meaning. IPA was used to identify canonical pathways and biological functions in the transcriptional data. Canonical pathways are relatively well characterised metabolic and signalling pathways with some degree of directionality. They are determined based on human manual review of the literature and databases and do not change based on the input dataset.

Differentially expressed clusters from within the biological network (Table 3.2) were used to query the KEGG Pathway database. Probe set redundancy was removed, resulting in a list of KEGG pathways that were over-represented in the dataset. All KEGG pathways containing more than five unique RIVitro transcripts were considered in the analysis and arranged into three groups according to their biological function: virus/pathogen, immune and disease (Figure 3.10). Cellular
process pathways were also abundant in the data, as expected (data not shown). Pathogen/immune/disease-related pathways were over-represented in the RIVitro data. Notably, pathways defined as involved in human T-lymphotropic virus type 1 (HTLV-1), tuberculosis, IAV, herpes simplex virus (HSV) and Epstein-Barr virus (EBV) infections were present in the data (Figure 3.10). A manually curated KEGG pathway was available for IAV. Highlighting the members of the differentially expressed transcript clusters on the KEGG IAV pathway revealed that this pathway clearly included multiple important members of the PRRSV-induced co-expression clusters (data not shown). These data indicated the presence of a strong pathogen response transcriptional signature in the RIVitro dataset.

Of additional interest, given their apparent differences in permissiveness to PRRSV infection, was establishing the biological pathways induced or suppressed in response to infection in LR and PIE breeds. IPA analysis of the clusters that were differentially regulated upon PRRSV infection in either PIE or LR samples revealed that contrasting pathways were represented within these clusters. The clusters that were differentially up-regulated in the less susceptible LR breed were enriched for cell cycle pathways, but also IL-12 signalling and other innate immunity pathways (Table 3.3). Clusters that were differentially up-regulated in the PIE breed upon PRRSV infection were enriched for pattern recognition, innate immunity and interferon signalling, consistent with the greater amounts of PRRSV RNA in these samples (Table 3.3).

Overall, using the KEGG database to interrogate all differentially-regulated transcript clusters confirmed that a strong virus-induced expression signature was detected in the microarray analyses. Furthermore, IPA analysis was performed on coordinately regulated transcript clusters, separated according to breed. These analyses revealed that the transcriptomes of PIE samples, which hosted the highest PRRSV RNA accumulation in this study, were significantly more enriched than the LR cells for canonical pathways involving pathogen sensing and anti-viral immunity.
Figure 3.10 | KEGG pathway analysis of RIVitro clusters of interest. KEGG pathways represented in the clusters of interest in the RIVitro dataset. The number of unique transcripts belonging to each KEGG pathway (>5 transcripts per pathway) is shown and these pathways are arranged into functional categories. 41 pathways were also represented in the “cellular processes” category but were omitted from this figure for clarity.
### Table 3.3 | Enriched canonical pathways in LR and PIE samples

Table showing enriched IPA canonical pathways in differentially expressed (relative to uninfected samples) transcript clusters of interest in LR and PIE samples. Pathway enrichment analysis was performed using IPA. P-values were derived from Fisher’s exact test. Significance was determined according to the $-\log$ of the p-value, with the threshold set at IPA’s default of 0.05 (or 1.3 when expressed as $-\log(p\text{-value})$). The 15 most significantly enriched canonical pathways are shown for each breed.

The associated gene number represents the number of differentially expressed genes that are reported to be involved in the respective canonical pathway.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Canonical Pathway</th>
<th>$-\log(p\text{-value})$</th>
<th>No. genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Landrace</td>
<td>p53 Signaling</td>
<td>5.37</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Glucocorticoid Receptor Signaling</td>
<td>4.4</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>IL-12 Signaling and Production in Macrophages</td>
<td>3.91</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Ceramide Signaling</td>
<td>3.87</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>ATM Signaling</td>
<td>3.83</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>RANK Signaling in Osteoclasts</td>
<td>3.59</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>ILK Signaling</td>
<td>3.37</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>ERK5 Signaling</td>
<td>3.35</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>HGF Signaling</td>
<td>3.34</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>April Mediated Signaling</td>
<td>3.31</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>B Cell Receptor Signaling</td>
<td>3.27</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>MIF Regulation of Innate Immunity</td>
<td>3.22</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>B Cell Activating Factor Signaling</td>
<td>3.22</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>PDGF Signaling</td>
<td>3.2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>IL-17 Signaling</td>
<td>3.09</td>
<td>5</td>
</tr>
<tr>
<td>Pietrain</td>
<td>Activation of IRF by Cytosolic PRRs</td>
<td>6.36</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Role of PRRs in Recognition of Bacteria and Viruses</td>
<td>3.54</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Interferon Signaling</td>
<td>2.72</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Role of RIG1-like Receptors in Antiviral Innate Immunity</td>
<td>2.35</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Extrinsic Prothrombin Activation Pathway</td>
<td>2.23</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Fatty Acid Elongation in Mitochondria</td>
<td>2.18</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>NF-κB Signaling</td>
<td>2.12</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>TREM1 Signaling</td>
<td>2.09</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Retinoic acid Mediated Apoptosis Signaling</td>
<td>1.93</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>IL-10 Signaling</td>
<td>1.86</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>CCR5 Signaling in Macrophages</td>
<td>1.84</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Melatonin Signaling</td>
<td>1.84</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Chemokine Signaling</td>
<td>1.83</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>PDGF Signaling</td>
<td>1.83</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>NF-κB Activation by Viruses</td>
<td>1.75</td>
<td>3</td>
</tr>
</tbody>
</table>
In order to establish infections *in vivo*, viruses must replicate in spite of powerful host immune defence mechanisms including the IFNs. The type I IFNs, IFN-α and IFN-β, are produced in direct response to virus infection. The type II IFN, IFN-γ, is synthesised in response to the recognition of infected cells by activated T lymphocytes and natural killer (NK) cells, rather than being induced directly by virus infection. Probe sets were available on the Porcine GeneChip array targeting IFN-α, IFN-β and IFN-γ, so it was desirable to examine their transcript profiles over the course of this *in vitro* microarray experiment. No IFN transcripts were clustered in the present analyses of the RIVitro dataset. This is commonly observed in such datasets, as IFN transcripts often exhibit “orphan” expression profiles, in that they are not coordinately regulated alongside other genes. Thus, they are not assigned to clusters when using a robust Pearson correlation coefficient cutoff.

Transcripts representing IFN-α and IFN-γ were found not to be differentially regulated in any samples analysed in the RIVitro experiment (Figure 3.11). In contrast, IFN-β transcripts remained at basal levels until 24 hpi, where a rapid induction occurred in some samples (Figure 3.11). The timing of this transcript induction followed at least one round of virus replication (Figure 3.2A). However, the up-regulation only occurred in AM isolated from animals belonging to the PIE breed (Figure 3.11), which also exhibited a more productive PRRSV infection (Figure 3.2C). The induction of the IFN-β transcript was significantly greater in PIE 643 than in the other two representatives of this breed (Figure 3.11). This PIE AM sample also hosted the greatest induction of PRRSV RNA of all samples in the RIVitro experiment (Figure 3.3B).

In conclusion, although IFN-α and IFN-γ transcripts were not induced in the RIVitro experiment, IFN-β was strongly induced in the PIE breed only. The greater PRRSV replication in the PIE samples appeared to correlate with greater IFN-β transcript induction in this breed.
Figure 3.11 | IFN transcriptional profiles across the RIVitro experimental time course. Absolute, normalised expression data are shown for probe sets targeting IFN-α (probe set ID: Ssc.14545.1.S1), IFN-β (probe set ID: Ssc.29006.1.S1) and IFN-γ (probe set ID: Ssc.4093.1.A1). Transcript profiles for all RIVitro AM samples are shown. Samples beginning with “12” and coloured in black relate to LR cell isolates and those beginning with “6” and coloured in grey relate to PIE cell isolates.
3.3.3 Discussion

Consistent with the notable difference in perceived permissiveness of LR and PIE samples to PRRSV infection identified by Ait-Ali et al. (2007), a clear difference was found in the host transcriptional response between these breeds in the RIVitro transcriptional dataset. Samples from the LR breed showed an enhanced transcriptional response, relative to those of the PIE breed, in clusters pertaining to the host immediate-early response, macrophage activation, inflammatory response and apoptosis. The preferential, early induction of Cluster 4 host immediate-early transcripts at 2 hpi in the less PRRSV-susceptible LR samples prompts speculation as to whether the samples from this breed were genetically predisposed to mounting an effective antiviral response to PRRSV infection at an early stage. It is possible that the apparently delayed early transcriptional response to PRRSV infection in the PIE samples allowed the virus to replicate more effectively in these cells, only inducing antiviral transcripts towards the end of the first cycle of virus replication.

Samples from the PIE breed exhibited a transcriptional response of a greater magnitude than the LR breed in clusters representing the IFN response and ISGs, viral replication and infection, amongst others. Cluster 5, consisting of 38 immune transcripts, was notably up-regulated in the PIE samples. This cluster exhibited a rapid induction at 8 hpi and 30 hpi in the PIE-derived AM, coinciding with increased PRRSV RNA detected by qRT-PCR in the same samples at the 8 hpi timepoint. The timing of this transcript induction was likely to have been in response to multistep PRRSV genome replication, given the length of the timecourse employed in this study, likely coinciding with virus replication cycles 1 and 2 or 3. One Cluster 5 member transcript of note was the GTPase, myxovirus resistance (MX1), which is known to be responsible for anti-viral activity against hepatitis B virus, Thogoto virus, La Crosse virus and IAV (Gordien et al., 2001; Kochs & Haller, 1999; Reichelt et al., 2004; Turan et al., 2004) as well as being induced by IFN-α and IFN–β, dsRNA or virus infection (Arnheiter et al., 1990). The up-regulation of this cluster did not appear to be a result of stimulation by IFN and, additionally, did not appear to directly result in the induction of IFN. This suggests that the pathway following the recognition of the virus by the cellular PRRs, such as RIG-I (also a member of Cluster 5) and leading to IFN induction, may be efficiently disrupted by PRRSV.
Transcripts representing an additional large GTPase (GBP-4) were also present in Cluster 5. A genomic region on SSC4 encoding this gene has been implicated in a PRRSV resistance quantitative trait locus (QTL) in a recent large scale in vivo genotyping and infection study (Boddicker et al., 2012). The data presented thus far suggest that the differential expression of Clusters 4 and 5 in less and more PRRSV-susceptible cell isolates, respectively, may be of importance in identifying expression signatures associated with PRRSV resistance.

3.3.4 Conclusions

The identification of important clusters of immune transcripts that were differentially regulated in samples from each of the two breeds represented in this study prompts further investigation into the specific roles of the cluster membership. There may be a genetic basis for the observed consistent lack of efficient PRRSV replication in the LR breed macrophages, which were derived from sibling animals. Along with the implication of a gene cluster on SSC4 in PRRSV infection, it is of interest to attempt to identify any published genetic variation in these genes. Analysis of additional datasets may facilitate the corroboration of the gene clusters identified in this in vitro study in other PRRSV infection transcriptional experiments.
3.4 RIVivo – *in vivo* reproductive transcriptional dataset

3.4.1 Experiment introduction

An *in vivo* transcriptional response experiment has previously been performed at the Roslin Institute (*Alan Archibald, unpublished data*). This experiment focussed on reproductive PRRS. As such, groups of pregnant gilts belonging to either the synthetic B/C commercial sire line (Genus PIC) or LR breed were intra-nasally inoculated with PRRSV and animals were sacrificed at set timepoints over the course of 48 days. Additional gilts from both groups were housed separately, where they were mock-infected and sacrificed in the same way. The inguinal (reproductive) lymph nodes were collected for total RNA extraction and subsequent transcriptional analysis using the Porcine GeneChip microarray platform. A number of phenotypic characteristics were recorded throughout the course of the experiment. PRRSV serology and RNA load data were also available. For the purpose of this thesis, this *in vivo* transcriptional experiment has been designated Roslin Institute *in vivo* (RIVivo).

3.4.2 Results

One measure of infection available from the RIVitro experiment was the accumulation of PRRSV RNA in the inguinal lymph node tissue. This was the same tissue used for the preparation of total RNA for the microarray experiments. All mock-infected gilts were found to be negative for PRRSV RNA by qRT-PCR (Dr. Alison Wilson, *data not shown*). The data for infected animals showed considerable variation between individuals. However, it was clear that both breeds experienced a large increase in the levels of PRRSV RNA, followed by a slow decay. The mean data showed that B/C gilts experienced a more than 800-fold increase in the amount of PRRSV RNA, over beta-actin, on day 6 of the infection time course (Figure 3.12A), compared to a corresponding uninfected animal at the same timepoint. On the same day, LR animals hosted a 400-fold PRRSV RNA induction, half the level of the B/C gilts (Figure 3.12A). However, by the next sampling timepoint at 13 dpi, the mean amount of PRRSV RNA had reduced to 9-fold in B/C animals, but remained higher in the LR gilts (Figure 3.12A), at 160-fold over that of a corresponding
uninfected animal at the same timepoint (*data not shown*). By 27 and 48 dpi, the LR gilts hosted a lower mean PRRSV RNA induction than the B/C animals sacrificed at these timepoints (Figure 3.12A). At the final timepoint in the RIVivo experiment, the B/C gilts had a mean PRRSV RNA induction of 15-fold and the LR animals 13-fold induction, over mock-infected animals (Figure 3.12A). However, Student’s t-test indicated that there was no significant difference between B/C and LR PRRSV induction at any timepoint (p <0.05).

Serology data were also available for the RIVivo experiment, due to serum samples being collected from gilts over the infection time course. Seroconversion was measured using the commercial PRRS IDEXX enzyme-linked immunosorbent assay (ELISA) kit (IDEXX Laboratories). PRRSV infection results in the production of serum antibody, which can then be detected by ELISA. The status of each sample was evaluated by the sample to positive ratio (S/P ratio; see 6.8.1B for explanation). Intra-nasally administered PRRSV was successful in infecting the challenged gilts, as demonstrated by the seroconversion that occurred in all animals exposed to the virus (Figure 3.12B). Control gilts were housed in a separate location and as expected, did not seroconvert (Figure 3.12B).

All experimentally infected animals had seroconverted by 6 dpi (Figure 3.12B). The gilts belonging to the B/C line all remained serum-positive for PRRSV antibody at 15 dpi, whereas one individual from the LR breed became seronegative by 13 dpi, along with an additional animal by 15 dpi (Figure 3.12B). Although the number of replicates was more limited for the B/C line, it appeared that serum levels of PRRSV-reactive antibody may have declined more rapidly in the LR animals (Figure 3.12B).

Changes in live body weight were also recorded during the RIVivo experiment. Despite heterosis in the crossbred B/C line, the LR breed exhibited superior growth in the RIVitro experiment. PRRSV infection resulted in a significant reduction in daily weight gain in both pig lines tested, relative to control animals (p <0.01). However the effect differed between lines (p <0.01) (Lewis, 2009). In the B/C animals, an 84.5 % reduction in daily gain was recorded (reducing from 0.45 to 0.07 kg/day). Contrastingly, the LR animals exhibited a less severe reduction in daily gain of 56.1 % (falling from 0.66 to 0.29 kg/day) during the infection (Lewis, 2009).
Figure 3.12 | PRRSV infection in the RIVivo experiment. A. Mean PRRSV \( \log_{10} \) -fold RNA induction in the inguinal lymph node tissue of experimental animals belonging to the B/C and LR lines, as determined by qRT-PCR and normalised to beta-actin (Dr. Alison Wilson, personal communication). Fold change was calculated relative to mock-infected animals. Error bars represent the standard deviation of samples from all animals available at each timepoint. B. Detection of antibody as a result of PRRSV infection, in serum samples taken from RIVitro animals across the experimental time course. Serology data derived from ELISA S/P ratios obtained using the PRRS IDEXX kit. Animals heterozygous for the SSC4 PRRSV resistance index SNP (WUR10000125) are denoted by an asterisk. Figure based on data originally described by Lewis (2009).
Thus, the LR pigs showed greater apparent PRRSV-tolerance than the B/C line gilts using the crude measure of daily weight gain. LR animals also seroreverted sooner than B/C animals in this experiment and hosted peak levels of PRRSV RNA that were (on average) half the magnitude of those in the B/C animals. However, the limited sample size did not permit the truly robust assessment of differences between these pig lines.

In addition to the collection of phenotypic data over the infection time course, total RNA was isolated from the inguinal lymph nodes of animals culled during the RIVitro experiment, resulting in a total of 23 Porcine GeneChip array samples making up the transcriptional dataset (Dr. Alison Wilson, personal communication). I performed QC on all microarray samples available for the RIVivo dataset in order to identify any outlying data that could indicate an issue with a particular sample. Upon visualisation of the unadjusted, log2-transformed PM intensities as either box (Figure 3.13A) or smoothed density plots (Figure 3.13B), it was clear that no outlying samples could be readily detected. Thus, all 23 available samples passed QC and were collectively normalised by RMA and further processed in these analyses.
Figure 3.13 | Pre-normalisation QC of RIVivo microarray samples. A. Boxplot of log$_2$ PM probes, by array, for non-normalised raw data. Each boxplot represents a single microarray sample, allowing the visual inspection of the consistency between samples and the identification of any outlying samples. B. Density plot of log$_2$ PM by array, for non-normalised raw data. Each line plotted represents a single microarray sample. No samples were discarded following QC.
In order to investigate the host transcriptional activity over the infection time course in the macrophages and associated reproductive lymph tissue of animals from the two represented genetic lines, a network-graph of the complete, RMA-normalised dataset was generated using the Biolayout software by filtering edges at a Pearson correlation coefficient cut-off of $r = 0.85$. The resultant network graph of 9,521 nodes, connected by 189,077 edges, was clustered using the graph-based clustering algorithm MCL (Van Dongen, 2000) set at an inflation value of 3. This resulted in 191 clusters with a membership of 5 or more nodes (transcripts). These 191 clusters formed two, distinct sub-networks within the large biological network, with a small number of additional outlying clusters at one end of the network (Figure 3.14). Clusters that were noted as showing an up-regulation early in the infection time course were located in one of the two primary sub-networks (Figure 3.14). Other clusters, which showed a bi-phasic or late up-regulation, were also clustered in close proximity within this sub-network (Figure 3.14). Clusters of co-ordinately expressed transcripts exhibiting early or mid bi-phasic expression profiles were located together in the other sub-network (Figure 3.14). Cluster 44 was a cluster of note, located on the periphery of the aforementioned sub-network (Figure 3.14).

Each of the 191 clusters in the network were manually interrogated and assigned categories based on the timing and directionality of their expression profiles. Following this manual curation, it was evident that there were a number of trends in the dataset, with large numbers of transcripts being induced or repressed at various timepoints across multiple clusters of interest. Table 3.4 provides an overview of the major clusters of interest in the RIVivo dataset, along with the number of transcripts within each cluster, examples of gene members and the biological processes represented. The clusters in the table are ordered according to the temporal phase encompassing the change of expression of member transcripts: early up (6 dpi), early down (6 dpi), mid up (13 dpi), early/mid bi-phasic and bi-phasic/late up.
Figure 3.14 | Transcriptional network formed from the RIVivo expression dataset. The network was filtered to display only relationships at or above a Pearson correlation threshold of 0.85, resulting in a graph of 9,521 nodes, connected by 189,077 edges. The resultant network was then clustered using the graph-based clustering algorithm MCL, set at an inflation value of 3. Nodes (transcripts) belonging to the same cluster share the same colour. Un-clustered (no class) nodes have been removed for visual clarity. Key areas of the network that correspond to the clusters identified in Table 3.4 are annotated. The network graph layout was generated using the FMMM layout algorithm in Biolayout v3.1.
Table 3.4 | Description of clusters of co-ordinately expressed transcripts in the RIVivo transcriptional dataset. Annotation of clusters induced or repressed at different timepoints during the RIVivo infection time course, relative to the mock-infected control animals. Clusters are grouped according to the time of their induction or repression for ease of interpretation. Representative pathways associated with the transcript membership of each cluster is shown, alongside example gene members in each cluster.

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Biological function/pathway annotation

Following the identification of clusters of transcripts showing a clear response to infection over the experimental time course, biological functions were assigned, where known, using a combination of manual (literature), software and online database curation. Taking into account the temporal pattern of expression, it was possible to group the clusters into categories based on their expression profiles and relate this to the likely biological function of each cluster (Table 3.4). RIVivo co-ordinately regulated transcript clusters could broadly be separated into three temporal groups based on their expression profiles: early (Figure 3.15A), mid (Figure 3.15B) and late/bi-phasic (Figure 3.15C).

Early (Figure 3.15A)

Fourteen clusters exhibited an early up-regulation, resulting in a peak of expression in individuals culled at 6 dpi (Figure 3.15A; Table 3.4). Two clusters were also down-regulated in individuals sacrificed early in the infection, resulting in their lowest expression at 6 dpi (Table 3.4). Table 3.4 demonstrates that the majority of differentially expressed clusters were induced upon PRRSV-infection, with minimal transcript repression.
Figure 3.15 | Temporally grouped average expression profiles of RIVivo transcript clusters. Time-grouped mean expression profiles of all clusters assigned to the A. early, B. mid and C. late/bi-phasic temporal groups, separated into B/C and LR breeds. The C prefix indicates a control timepoint and the I prefix indicates an infected timepoint. Control samples were not available for 6 dpi and 27 dpi timepoints for the B/C line.
One notable early differentially-regulated transcript cluster, Cluster 4, had a membership of 141 well annotated, co-expressed probe sets, including: GBP-1, GBP-2, GBP-4, DDX58, DDX60,IFI44L, IFIHI1, IFIT1, IFIT3, IRF7, ISG15, ISG20, MX1, MX2, STAT1, STAT2, TRIM22 and USP18. Pathway analysis of Cluster 4 member transcripts confirmed that their primary function appeared to involve virus infection, the antimicrobial response and IFN signalling, with all of these functions being over-represented in this cluster (Figure 3.16). This cluster remained at a constant, baseline level in mock-infected control samples (Figure 3.16). However, the expression of Cluster 4 member transcripts was significantly up-regulated by 6 dpi (the first timepoint in this study) in infected samples (Figure 3.16). This was followed by a gradual decrease in transcript abundance to around the level of the mock-infected control animals by 27 to 48 dpi (Figure 3.16). There were notable differences in the induction of this cluster between the two pig lines tested. The B/C line exhibited a lower induction of transcripts in this cluster, slowly returning to around basal levels by 48 dpi. The LR breed had a greater induction of Cluster 4 transcripts at 6 dpi, before returning to basal expression levels by 27 dpi (Figure 3.16).

When compared to the mean PRRSV RNA induction in the same experimental animals, the decrease in the mean expression of Cluster 4 member transcripts appeared to coincide with a decrease in PRRSV RNA, as measured by qRT-PCR of inguinal lymph node tissue (Figure 3.16). The B/C breed had higher mean levels of PRRSV RNA accumulation in the inguinal lymph node tissue than the LR animals, together with lower Cluster 4 transcript induction (Figure 3.16).

In summary, the expression levels of Cluster 4 transcripts at the beginning of the RIVivo time course were higher in the B/C line, consistent with the higher PRRSV RNA induction at 6 dpi, compared to the LR breed gilts. These higher expression levels could be a result of the involvement of Cluster 4 member-transcripts in the response to virus infection.
**Figure 3.16 | Summary of RIVivo Cluster 4.** The mean expression profile of RIVivo Cluster 4 is shown as an unbroken black line. Box and whisker plots show the variation (median, upper and lower quartiles) and the maximum and minimum values (whiskers) in the mean expression level of this cluster between the samples representing each timepoint. Mean PRRSV $\log_{10}$ RNA induction, as determined by qRT-PCR of inguinal lymph node tissue (performed by Dr. Alison Wilson), is overlaid as a dashed black line. Control samples were not available for 6 dpi and 27 dpi timepoints for the B/C line. A summary of the cluster timing and membership, as well as pathway analysis data is displayed below the graph.

**RIVivo Cluster 4 – 141 transcripts**
Early-up in both lines, greater induction in Landrace breed
Maximal expression at 6dpi
Example gene members: GBP1, GBP2, GBP4, DDX58, IFIT, IFIT7, STAT1, Mx1, Mx2

**KEGG:**
Influenza A; Hepatitis C; Herpes simplex infection; Measles; Metabolic pathways

**IPA:**
Top Canonical Pathways: Interferon Signalling; Activation of IRF by cytosolic PRRs
Diseases and Disorders: Dermatological diseases and conditions; Antimicrobial response; Inflammatory response
Mid (Figure 3.15B)

Three clusters exhibited an up-regulation midway through the infection time course, resulting in a peak of expression in animals culled at 13 dpi (Table 3.4). Functional analysis of these clusters suggested involvement in transcriptional and translational control (Table 3.4).

Late/bi-phasic (Figure 3.15C)

Nine clusters had early or mid bi-phasic expression profiles, being both up-regulated and down-regulated at different timepoints between 6 and 27 dpi (Table 3.4). Two clusters had late up/bi-phasic expression profiles, being both up-regulated and down-regulated at distinct timepoints up to 48 dpi (Table 3.4). Transcript clusters regulated at these timepoints were implicated in functions involving cell signalling and proliferation and virus budding (Table 3.4).

Pathway analysis

Member transcripts of RIVivo clusters of interest, identified from within the biological network, were used to query the KEGG Pathway database. Probe set redundancy was removed from the input gene list, resulting in a list of KEGG pathways that were over-represented within the dataset. All KEGG pathways containing more than five unique transcripts were considered in this analysis and grouped according to their biological function: virus/pathogen, immune and disease (Figure 3.17). Cellular process pathways were abundant in the data, as expected (data not shown). Pathogen/immune/disease-related pathways were also over-represented. Members of pathways defined for measles, HTLV-1, IAV, HSV, Hepatitis C virus (HCV) and EBV infections were all present in the data, indicating a strong viral/pathogen transcriptional signature.
Figure 3.17 | KEGG pathway analysis of RIVivo clusters of interest. KEGG pathways represented in the clusters of interest in the RIVivo dataset. The number of unique transcripts belonging to each KEGG pathway is shown and these pathways are arranged into functional categories. 11 pathways were also represented in the “cellular processes” category; these were omitted from this figure.
Probe sets were available on the Porcine GeneChip array that represented IFN-α, IFN-β and IFN-γ, none of which were clustered in the present analyses. No IFN transcripts were differentially regulated in the infected samples analysed from the RIVivo experiment, when compared to mock-infected control samples (Figure 3.18A). IFN-γ transcripts exhibited a minimal early up-regulation, but at negligible levels of expression which were indistinguishable from background noise (Figure 3.18A). The relative lack of IFN-α induction was confirmed independently by qRT-PCR of inguinal lymph node tissue (Figure 3.18B; Dr. Alison Wilson, personal communication). No clear difference was apparent between IFN transcript induction in B/C and LR individuals (Figure 3.18). However, IFN genes can be induced and repressed extremely rapidly, so it is possible that the transcriptional data at this relatively low temporal resolution may have missed any induction that occurred during the experiment.
Figure 3.18 | IFN transcriptional profiles across the RIVivo experimental time course. A. Absolute, normalised expression data are shown for probe sets targeting IFN-α (probe set ID: Ssc.14545.1.S1), IFN-β (probe set ID: Ssc.29006.1.S1) and IFN-γ (probe set ID: Ssc.4093.1.A1). Mean transcript profiles are shown for samples from each pig line. B. Mean fold-induction of IFN-α, over mock-infected animals, in inguinal lymph node tissue. Quantified by qRT-PCR (performed by Dr. Alison Wilson) and normalised to beta-actin.
3.4.3 Discussion

Both serology and the accumulation of PRRSV RNA confirmed that the experimental infections via intra-nasal inoculation were successful. However, differences were apparent in these same data. The initial PRRSV induction in the LR animals was around half that of the B/C animals, but the subsequent decline of viral RNA levels appeared to be slower in these gilts, compared to the B/C animals. When these data were compared to the detection of PRRSV antibody in the serum of infected animals then a different conclusion could be drawn; with LR animals apparently seroreverting sooner than the B/C animals. This suggests that the apparently lower levels of PRRSV replication in the reproductive tissue of the LR gilts, as indicated by the quantification of RNA induction, may also have been indicative of the overall resistance of these animals. Thus, a lower PRRSV burden is likely to have been the cause of the apparently more rapid seroreversion in the LR animals.

No significant levels of IFN mRNA were detected in the inguinal lymph tissue of either LR or B/C gilts in the RIVivo study. However, a large number of transcript clusters were up-regulated early in the RIVivo infection time course. The largest of these, Cluster 4, represented a number of transcripts with known pathogen-sensing, immune-related and IFN-stimulated functions. Other than the members of the “classical” host transcriptional response to virus infection, RIVivo Cluster 4 contained three of the six members of the GBP family of GTPases, predicted to be present within a 1 megabase (Mb) region on SSC4. This genomic region has been implicated in a QTL associated with the host response to PRRSV (Boddicker et al., 2012). The differential up-regulation of Cluster 4 in the LR line, relative to the B/C gilts, corresponded to the lower levels of PRRSV RNA measured in the same tissue. Therefore, the rapid induction of this cluster of immune transcripts and GBP family members may be a factor in the observed lower levels of PRRSV RNA detected in the LR gilts. The mean expression profile of this cluster also returned to basal expression levels in both breeds following the same trend as the observed reductions in PRRSV RNA levels in the same tissue.
3.4.4 Conclusions

The experimental design of the RIVivo study was not conducive to the identification of robust differences in the biological data collected for each of the two pig lines represented in the study. However, the LR animals appeared to be perhaps more tolerant to virus infection when quantified by the less severe loss in daily weight gain compared to the B/C animals. The LR animals also became PRRSV seronegative sooner than the B/C animals, albeit with a low sample number and with data somewhat lacking in temporal resolution. The identification of large clusters of co-ordinately regulated immune transcripts, which were differentially up-regulated in animals with lower PRRSV RNA induction, prompts further investigation into to the involvement of these member transcripts in PRRSV infection and host resistance.
3.5 Comparative discussion

The analysis of two PRRSV-infected, host microarray datasets in this Chapter set out to further investigate the host transcriptional response to PRRSV. The primary objective of the work described in this Chapter was to identify virus-responsive gene clusters and gain insight into the pathways represented by these co-expression clusters. Data were available over a high temporal resolution 30 hour, in vitro, respiratory cell time course and a low temporal resolution, 48 day in vivo, reproductive experiment. Given the evidence supporting a genetic basis for PRRSV resistance, a further objective was to compare and contrast the transcriptional response to PRRSV infection both in samples collected from pig lines that exhibited “resistance” and “susceptibility” to experimental PRRSV infection and also between in vitro and in vivo datasets. Of additional interest was establishing the application of network-based clustering analysis in the investigation of host transcriptional data during PRRSV infection.

In this Chapter, a network-based explorative approach was performed to visualise and analyse two transcriptional datasets. Each study was analysed independently due to the contrasting time-scales, tissue types and experimental designs. Biolayout was used to generate network graphs of transcriptional data from each study and the resulting networks were allocated cluster IDs along with co-ordinately expressed transcripts (probe sets) by the MCL algorithm. The use of mathematical networks, coupled with the pattern analysis provided by the MCL algorithm, enabled an automated and unbiased approach to hypothesis generation. However, a degree of manual curation of the data was required in order to take into account biological meaning. Upon manual analysis of cluster expression profiles, clusters exhibiting differential expression upon PRRSV infection (relative to either the control time course or 0 hpi timepoint) were retained for further analysis. For the RIVitro dataset, these “clusters of interest” had a membership ranging from 103 to 10 nodes, while the RIVivo dataset cluster membership ranged from 141 to 5 nodes.

A strong transcriptional signature for viral response was evident in both RIVitro and RIVivo datasets. Significant transcriptional remodelling occurred in the
RIVitro dataset, with the majority of clusters of interest being up-regulated. Particular timepoints where large-scale expression changes occurred in this experiment were 2 hpi and 8 hpi. The clusters induced at these timepoints represented a number of members of IFN-stimulated and antiviral pathways and biologically, their induction coincided with PRRSV entry and uncoating at around 2 hpi, and genome replication and assembly at around 8 hpi. RIVitro Cluster 5 was enriched for KEGG pathways involving virus infection, antiviral and RIG-I/TLR signalling. The induction of this transcript cluster at 8 hpi was consistent with the PRR detection of the PRRSV dsRNA intermediate, produced during genome replication. However, the potential detection of PRRSV dsRNA at this timepoint did not appear to result in the induction of IFN. This could be due to a lack of mRNA translation or the viral inhibition of key members of the dsRNA-signaling pathway, for instance, PRRSV is known to inactivate IPS-1 (Luo et al., 2008).

Consistent with the observation that PRRSV is able to suppress IFN production (Luo et al., 2008; Murtaugh et al., 2002), no induction of IFN-α and IFN-γ transcripts was observed in the RIVitro dataset. IFN-β transcripts were only significantly up-regulated after 16 hpi in PIE macrophages, following at least one complete round of virus replication.

In the RIVivo dataset, a large number of transcripts were up-regulated by the first sampled timepoint, at 6 dpi. All infected animals had also seroconverted by this day. No probe sets representing IFN-α, IFN-β and IFN-γ were significantly elevated above the level of mock-infected samples and qRT-PCR for IFN-α mRNA also revealed nothing more than fluctuating, <5-fold changes in either direction. The largest of the clusters of interest in the analysis of this dataset, RIVivo Cluster 4, was significantly up-regulated by 6 dpi, after which, there was a gradual decrease in transcript abundance to around the level of the mock-infected control animals by between 27 and 48 dpi. This decrease in overall expression of Cluster 4 member transcripts coincided directly with a marked decrease in PRRSV RNA in the same tissue. KEGG analysis of RIVivo Cluster 4 revealed the enrichment of pathways involving virus infection, antiviral and RIG-I/TLR signalling. These were the same KEGG pathways represented in RIVitro Cluster 5.
Further investigation of RIVitro Cluster 5 and RIVivo Cluster 4 revealed a high level of homology, with significant overlap of transcript members (Figure 3.19A and B). Overall, transcripts belonging to the large RIVivo Cluster 4 were collectively represented in RIVitro Cluster 5 (23 of 33 unique transcripts in cluster), Cluster 43 (9 of 10 unique transcripts in cluster) and Cluster 200 (all 5 transcripts in cluster). Transcripts in these clusters included; Cluster 43: *RAB3C*, *PARP9* and *PARP12* and in Cluster 200: *IFI44L*, *NANS* and *IFIT3*. Both RIVitro Clusters 43 and 200 were differentially up-regulated between LR and PIE cells, with the samples from PIE animals, exhibiting a bi-phasic up-regulation at 8 and 30 hpi, in contrast to generally minimal changes in LR samples (Figure 3.19C). This differential up-regulation in the PIE animals was also consistent with the expression profile of RIVitro Cluster 5. However, the strong induction of RIVitro IFN-related Clusters 5, 43 and 200 in the PIE breed (Figure 3.9 and Figure 3.19C) appeared to have little detrimental effect on PRRSV replication.

Between the primary, conserved, PRRSV-responsive transcript clusters: RIVivo Cluster 4 and RIVitro Cluster 5, three of the five currently annotated members of the porcine GBP family (GBP-1, GBP-2 and GBP-4) were represented and differentially expressed upon PRRSV infection. Improved annotation of the genomic region encoding these proteins and greater microarray genome coverage, with reduced probe set redundancy, may have also revealed additional GBP transcripts clustered in each dataset. The PSICQUIC tool (Aranda et al., 2011) allows concurrent searching of multiple molecular interaction information depositories using a single query and was used to identify published GBP-1 interactors. 241 interactors were found and, when mapped to RIVitro Cluster 5 and RIVivo Cluster 4, showed a 24 and 21 % representation, respectively (*data not shown*). This adds further weight to the putative involvement of the GBP family in these PRRSV-responsive transcript clusters and the capability of Biolayout in identifying biological processes through the principle of co-expression.
Figure 3.19 | Comparison between key transcript clusters in RIVitro and RIVivo datasets. A. Venn diagram illustrating the degree of overlap between RIVivo Cluster 4 and RIVitro Cluster 5. RIVivo Cluster 4 had a total of 110 unique transcripts, 23 of which were shared with RIVitro Cluster 5. RIVitro Cluster 5 had a total of 33 unique transcripts, 23 of these transcripts were shared with RIVivo Cluster 4. B. A list of shared unique transcripts between RIVitro Cluster 5 and RIVivo Cluster 4. C. Mean expression levels of all transcripts represented by RIVitro Clusters 43 and 200 (which contained additional transcript representatives of RIVivo Cluster 4) across the time course of PRRSV infection in LR cell isolates: 1286, 1287 and 1288 and PIE cell isolates: 637, 641 and 643.
Genetic differences in the response to pathogen infection are often related to genetic variation, such as SNPs, in relevant genes. The apparent increased PRRSV-susceptibility of the PIE macrophages, relative to the LR cell isolates, in the RIVitro study may potentially be related to such mutations. In a series of in vivo experiments, Boddicker et al. (2012) identified a region of SSC4 that was strongly associated with both PRRSV viral load (VL) and weight gain from 42 dpi with PRRSV (WG42). This QTL consisted of 33 SNPs, spanning 1 Mb of DNA (based on pig genome build Sscrofa 10.2). 6 SNPs in the QTL region were in perfect linkage disequilibrium (LD) ($r^2 = 1$), meaning that these alleles are always inherited together. These 6 SNPs each explained 99.3% of the variance for the 33 SNP region, with the index SNP being referred to as WUR10000125 (Boddicker et al., 2012).

In order to investigate the role of this region in the transcriptional datasets analysed in this study, the 1 Mb genomic region identified by Boddicker et al. (2012) was located on the pig genome assembly and mapped to probe sets on the Porcine GeneChip array, where available (Table 3.5). The resulting Porcine GeneChip probe sets were further identified within transcript clusters (Table 3.5). Five of the six successfully mapped and clustered probe sets fell into RIVivo Cluster 4 and one probe set was represented in RIVitro Cluster 5. This further corroborates the potential importance of these two co-expressed gene clusters in the host response to PRRSV infection. These five probe sets all targeted members of the GBP family: GBP-1, GBP-2 and GBP-4 (Table 3.5). Further investigation of the 1 Mb region identified by Boddicker et al. (2012) revealed that it encompassed the entire annotated porcine GBP family.

To further explore the possibility of an involvement of the SSC4 PRRSV host response QTL in the present analyses, all animals in the RIVivo study were genotyped for six index SNPs in the region identified by Boddicker et al. (2012). No homozygous G (beneficial) allelic individuals were genotyped among the animals represented in the RIVivo experiment (data provided by Alan Archibald). However, given that a dominance effect has been proposed by Boddicker et al. (2012), it is relevant that 40% of B/C and 25% of LR animals in the study were heterozygous for the beneficial allele (Appendix 2). Unfortunately, no samples remained from the original RIVitro study to allow the genotyping of these donor animals.
Table 3.5 | Genes encoded by the genomic region implicated in the SSC4 PRRSV host response QTL. Annotated genes within the SSC4 1 Mb genomic region identified as being associated with the host response to PRRSV infection by Boddicker et al. (2012), are mapped to probe sets on the Porcine GeneChip array. The clustering of these probe sets in the RIVitro and RIVivo networks, where available, is also shown.
The co-regulation of GBP family members as well as the presence of GBP-1 interactors in key PRRSV-responsive transcript clusters alongside MX1, RIG-I and other known antiviral genes in RIVitro Cluster 5 and RIVivo Cluster 4 justifies further investigation into the role of the GBP protein family in PRRSV infection. Along with the probability that the causative SNP for the VL and WG42 traits identified by Boddicker et al. (2012) falls in the vicinity of a GBP family member, this suggests a plausible role for the GBP gene family in PRRSV infection. Given the differential regulation of the GBP-containing Cluster 5 in the ‘PRRSV-susceptible’ PIE breed in the RIVitro study, there is further potential for an involvement in PRRSV resistance. However, there are some discrepancies between the present interpretation of the nature of the involvement of RIVitro Cluster 5 and RIVivo Cluster 4. RIVitro Cluster 5 was differentially up-regulated in the apparently more permissive PIE breed. Conversely, RIVivo Cluster 4 was differentially up-regulated in the LR breed, which could tentatively be interpreted as being more resistant in this study. Thus, a greater understanding of the putative involvement of these complex, co-ordinately regulated transcript clusters in the host response to PRRSV is required.

Given the growing body of evidence that individual and potentially breed-specific differences exist in the susceptibility of pigs to PRRSV infection (Ait-Ali et al., 2007; Bates et al., 2008; Petry et al., 2005; Petry et al., 2007; Reiner et al., 2010; Vincent et al., 2005, 2006), one hypothesis during the analysis of the RIVitro dataset was that there may be a significant difference in gene expression in the PRRSV ‘resistant’ LR and ‘susceptible’ PIE macrophage preparations. Inter-breed differential regulation was evident in multiple clusters of interest in the RIVitro dataset. RIVitro Cluster 4, consisting of 47 host immediate-early immune response transcripts, was differentially up-regulated at 2 hpi in the LR samples, which experienced consistently attenuated PRRSV replication. RIVitro Cluster 5 was differentially up-regulated in the PIE samples, which exhibited enhanced PRRSV replication compared to the LR samples. The contrasting transcriptional profiles of the primary RIVitro clusters of interest, Clusters 4 and 5 (Figure 3.7), can be interpreted in the context of resistance to infection by also considering the induction of PRRSV RNA in each sample. The intra-breed variation in these two cluster
profiles is also of significant interest. RIVitro Cluster 4 was differentially up-regulated in LR breed macrophages. However, there was limited transcript induction in LR sample 1287 and this same sample hosted the greatest PRRSV induction of its breed cohort. When examining RIVitro Cluster 5, whose transcripts were differentially up-regulated in the PIE breed macrophages, it was evident that LR 1287 again exhibited a contrasting transcription profile to its cohort, in also being up-regulated. Therefore, one tentative interpretation of the limited data available is that the early induction of RIVitro Cluster 4 transcripts may have some protective function against PRRSV infection in AM. Additionally, an upstream master regulator of this putative biological pathway could be implicated in PRRSV resistance.

The members of RIVitro Cluster 5 and RIVivo Cluster 4, while being relatively conserved between the two studies in these analyses, were by no means PRRSV-specific. Zaas et al. (2009) published an “acute respiratory viral” expression signature, consisting of a number of genes that were consistently expressed in response to three respiratory viruses. Several of the genes identified by the authors were also present in the aforementioned primary PRRSV-responsive clusters in the present analyses.

Both RIVitro and RIVivo datasets contained clusters that were enriched for members of the MAVS pathway (Moore & Ting, 2008). MAVS functions as the essential signalling adaptor required for RLH signal transduction following viral infection, leading to the transcriptional activation of IFN-β (Moore & Ting, 2008). Transcripts representing: MX1, RIG-I (aka. DDX58) and DDX60 (which has recently been identified as an antiviral protein (Miyashita et al., 2010)) were clustered in RIVitro Cluster 5 and RIVivo Cluster 4. Additional members of the MAVS pathway uncovered in the current analyses included: IRF7 and ISG15, both of which were present in RIVitro Cluster 5 and RIVivo Cluster 4, and TANK which was clustered in RIVitro Cluster 5. USP18 was also a member of RIVitro Cluster 5 and RIVivo Cluster 4 and is known to have a direct homeostatic role in innate immunity through the specific removal of ISG15 from conjugated proteins (Malakhov et al., 2002; Ritchie et al., 2004). Multiple members of the tripartite motif-containing (TRIM) proteins were also clustered in both datasets. In the MAVS
pathway, TRIM25 is known to function as an E3 ubiquitin ligase of RIG-I, enhancing downstream signalling to IFN-β through interaction with its PRYSPRY domain (Moore & Ting, 2008; Ozato et al., 2008). TRIM25 was not clustered in the present analyses. However, the PRYSPRY domain is shared by all four TRIM proteins whose transcripts were clustered in RIVitro Cluster 5 and RIVivo Cluster 4. At least two of these proteins (TRIM22 and TRIM26) are known to be IFN-responsive and inhibit HIV infection (Barr et al., 2008; Ozato et al., 2008). Thus, the enrichment of key members of the MAVS pathway in key clusters in both datasets suggests that this pathway may play an important role in the host innate immune response to PRRSV, as has recently been reviewed by Sun et al. (2012). If this were the case, an inheritable, genetic basis for reduced or enhanced efficacy of this important pathway could influence the infectability of a livestock line.

The macrophage early response to infection is important in establishing an antiviral state (Akira et al., 2001), especially given that AM are the preferred site of PRRSV infection in vivo (Therrien et al., 2000). In the RIVitro dataset, the general function of the differentially up-regulated clusters in the LR breed (e.g. Cluster 4) pertained to the early response to infection, inflammatory response and macrophage activation, the “first line of defence” against an intracellular pathogen such as PRRSV. These clusters did not appear to be functionally redundant across the LR and PIE breeds. The differentially up-regulated clusters in the PIE breed (e.g. Cluster 5) appeared to be primarily associated with the IFN-response and innate immune response to infection, which are likely to occur as a result of the detection of the PRRSV dsRNA replication intermediate by intracellular PRRs. This suggests that one of the host factors that could have conferred the reduced susceptibility phenotype to the LR breed AM may involve the rapid detection of the virus, followed by an early, macrophage-specific, response (Cluster 4) that does not culminate in the violent induction of the IFN-response. Conversely, in the “more susceptible” PIE samples there appeared to be a weaker classical early transcriptional response to the virus (Cluster 4).

RIG-I is an important PRR for dsRNA (Yoneyama et al., 2004) and was clustered in RIVitro Cluster 5. This cluster was induced at 8 hpi in samples with the
greatest PRRSV genome accumulation. Thus, the attenuated induction of these transcripts in the LR samples may have been a result of the lower PRRSV RNA levels, rather than a contributing factor to the observed LR virus ‘resistance’ phenotype. This hypothesis is strengthened by the observation that the LR macrophages from which the RNA for the transcriptional experiment was derived, have been shown to be similar to other breeds in their response to the dsRNA mimetic polyI:C (Ait-Ali et al., 2011). Although, disappointingly, no direct comparison was made with the PIE breed macrophages (Ait-Ali et al., 2011).

In addition to the RIVitro experiment, the RIVivo dataset also benefitted from having representatives from two pig lines. Given that an in vitro PRRSV-resistance phenotype has been observed in LR samples previously (Ait-Ali et al., 2007), it was of interest to see whether this was a breed-specific trait that could be replicated in vivo. Due to the temporal resolution of the RIVivo study and the consequent small sample size, the impact of PRRSV infection on reproductive performance and other biological traits could not be robustly assessed. An additional complication in attempting to infer the existence of any comparative resistance traits in the RIVivo study was the use of the B/C genetic line in place of the PIE breed that was used in the RIVitro study. For these reasons, the primary data utilised from the RIVitro study were the results of the transcriptional network analyses, which could be compared and contrasted with the RIVitro study data. However, when tentatively considering the weight gain data in the RIVivo study, LR pigs showed signs of tolerance compared to the B/C animals, experiencing a less severe reduction in daily weight gain upon infection. Additionally, the RIVivo LR animals became PRRSV serum-negative sooner and had lower PRRSV RNA induction in the inguinal lymph node tissue than B/C animals. Despite hosting lower PRRSV RNA levels than the B/C line, the LR animals had higher expression levels of the 141 IFN-related Cluster 4 transcripts. Thus, this data could be in agreement with the in vitro findings by Ait-Ali et al. (2007) that the LR breed may be more resistant to PRRSV infection. This could also provide an indication as to the merits of an in vitro screening as a strategy for investigating the host response to PRRSV in vivo.
Conclusions drawn from fold-change analysis of unfiltered transcriptional datasets must be carefully considered. Ait-Ali *et al.* (2011) placed significant weight on the early induction (2 hpi) of IFN-β transcripts in the LR samples. However, when the underlying data are dissected, this observation is misleading. The RMA-normalised absolute expression values for IFN-β transcripts remained below 24 for the duration of the time course. For comparison, the highest absolute expression value for the same transcript was 1,418 in PIE sample 643. In this case, the use of fold change appears to have confused the fact that the ‘induction’ of this transcript at 2 hpi in LR samples was well below the level where it could confidently be distinguished from experimental noise. Ait-Ali *et al.* (2011) observed that the IFN-β transcript accumulated concomitantly with a large spectrum of transcripts in PIE samples. However, the sensitivity of the Biolayout approach in correlating statistically similar expression patterns was able to identify that the expression profile of this transcript was dissimilar to other transcripts on the array. Thus, at the correlation threshold employed in the current study, IFN-β remained un-clustered.

A misinterpretation has also been applied to the expression of MX1 and STAT2 co-regulated transcripts, represented within RIVitro Cluster 5 in the current analyses. Ait-Ali *et al.* (2011) overstate the degree of LR-specific early up-regulation of these transcripts, relating this to the ‘induction’ of IFN-β. The more notable difference in the expression of these transcripts was the rapid and transient up-regulation in PIE samples at 8 hpi, followed by a sustained transcript accumulation up to 30 hpi. This cluster was induced in the LR samples, but primarily later in the time course, at 8 hpi.

The aforementioned points illustrate the merits of employing a network-based approach in the analysis of transcriptional data. The current analyses were able to highlight large groups of co-regulated transcripts and identify clear differences in their expression between samples from the breeds represented in the study. Ait-Ali *et al.* (2011) were able to identify individual transcripts which were up-regulated at similar timepoints, concentrating largely on transcripts with a known function, such as IL-10. However, the sophisticated clustering algorithm employed by Biolayout successfully identified co-expressed clusters across the entire dataset, without the introduction of bias. Resulting clusters could then be assessed for inter-breed
differences. The principle of co-expression means that clustered transcripts are likely to have shared regulation or function, thus identifying biological pathways, not simply individual transcripts. The use of Biolayout has also corroborated previously published findings. The observed up-regulation of RIG-I in both datasets compliments data generated by other studies involving PRRSV (Luo et al., 2008; Zhang et al., 1999). In PRRSV-infected pigs, in vivo, the up-regulation of MX1 mRNA has also previously been detected in several relevant tissues, suggesting that it may play an important role in clinical PRRS (Zhang et al., 1999).

The present study benefitted from the first published use of Biolayout analysis techniques in the interpretation of the host transcriptional response to PRRSV infection. Network clustering analysis resulted in the detection of multiple transcripts and important pathways that were not resolved in previous studies. The current analyses benefit from the use of novel techniques, as well as improved microarray annotation and a genome-wide comparative analysis approach. As a result, major differences in expression profiles have been uncovered between the PIE and LR samples analysed. Similarities have also been identified in key, large transcript clusters between two separate studies, which are likely to represent comparable biological pathways. These analyses have also resulted in the generation of a ‘short-list’ of candidates for validation in secondary assays, specifically the members of the GBP protein family.

Previously the LR breed has been shown to be less susceptible to PRRSV infection than the PIE breed (Ait-Ali et al., 2007). These apparently breed-specific differences in susceptibility to PRRSV may be related to their strongly contrasting host responses to PRRSV, which have been corroborated in the current analysis of the RIVitro dataset. These data also provide an indication as to the merits of in vitro screening as a strategy for investigating the host response to PRRSV in the pig. Therefore, by dissecting their differing host responses to PRRSV infection in several major transcript clusters identified through the use of Biolayout, it may be possible to uncover genetic factors that confer PRRSV resistance.
CHAPTER FOUR

THE ROLE OF THE CELLULAR GUANYLATE BINDING PROTEINS IN PRRSV INFECTION
4.1 Introduction

4.1.1 General Introduction

For some time, genes associated with the host immune response have been investigated as potential targets for the genetic improvement of animals for disease resistance (Van der Zijpp, 1983; Warner et al., 1987). Given the difficulties in developing effective vaccines against PRRSV, host genetics is likely to play an integral role in combating the economic and welfare effects of PRRS. The analysis of transcriptional datasets, in combination with statistical association data linking key GTPases with PRRSV infection and resistance, warranted further investigation into these genes. The ultimate aim of this research was to inform future attempts at genetic improvement of commercial pig breeds for PRRSV resistance traits.

4.1.2 IFN-induced gene products

One notable feature of PRRSV infection is that type I IFN, interleukin-1 or TNF-α inflammatory cytokine expression is not significantly induced in the lungs of infected pigs (Thanawongnuwech et al., 2001; Van Reeth et al., 1999). PRRSV infection does not induce significant IFN-α expression at either transcript or protein levels in AM, either in vivo or in vitro (Kimman et al., 2009). IFN-β is induced by PRRSV at the transcript level in AM in vitro (Genini et al., 2008), but transcript copies do not correlate with protein levels, suggesting that there are likely to be post-transcriptional mechanisms of suppression involved (Lee et al., 2004). IFN-γ is induced shortly after atypical PRRSV infection and has been attributed to NK cell activation (Wesley et al., 2006). These data are in contrast with other observations that exogenous IFN-γ is able to effectively inhibit PRRSV replication (Bautista & Molitor, 1999; Rowland et al., 2001).

The modulation of the expression of IFN-induced gene products in infected cells is a common approach to the characterisation of antiviral proteins. Studies of this type have been able to demonstrate that murine Mx1 confers resistance to IAV in vitro (Staeheli et al., 1986) and Thogoto virus (Haller et al., 1995) and Dhori virus (Thimme et al., 1995) in vivo. Additionally, murine Mx2 confers resistance to VSV (Zürcher et al., 1992) in vitro. Human MxA confers resistance to IAV, VSV (Pavlovic et al., 1990) and measles (Schnorr et al., 1993) in vitro and to Thogoto

4.1.3 GTPases: mediators of the innate immune response

GTPases have increasingly emerged as being intimately involved with the innate immune response. The GTPases are divided into four major families: the very large inducible GTPases (VLIGs), the large GTPases (p65 or GBPs), the small (p47) GTPases and the Mx proteins (Figure 4.1). Many of these proteins are expressed in macrophages and induced upon LPS stimulation (Figure 4.1A). At least 47 members of this IFN-γ-inducible GTPase superfamily have been mapped to the mouse and human genomes (Kim *et al*., 2011).

The VLIGs are unusual in that they have a molecular mass of around 280 kDa, the largest known GTPases in any species, and that the VLIG ORF is encoded on a single large exon (Klamp *et al*., 2003; MacMicking, 2004). ESTs have been assembled and mapped to VLIG-1 in the pig (Klamp *et al*., 2003). The exact function of this GTPase family remains to be dissected. However, it is clear that VLIG-1 is induced by both type I and II IFNs (Klamp *et al*., 2003). The p47 GTPase, or immunity related guanosine triphosphatases (IRG), family is a group of 47–48 kDa IFN-responsive proteins (Figure 4.1B). 6 murine IRGs have been identified, with only one, IRGM, being present in the human genome (Kim *et al*., 2012a; Taylor *et al*., 2004). The Mx proteins (Figure 4.1B) are powerful antiviral proteins which inhibit a broad range of viruses, including: influenza viruses, VSV, Thogoto virus, HIV-1 and La Crosse virus (Frese *et al*., 1995; Haller & Kochs, 2011; Liu *et al*., 2013; Pavlovic *et al*., 1990). MxA has even been implicated in fulfilling a barrier function in preventing the introduction of IAV zoonoses into the human population (Zimmermann *et al*., 2011). The established importance of GTPases in human and murine pathogen defence raises questions as to their potential roles in the pig.
Figure 4.1 | The IFN-induced GTPases and their tissue expression. A. Tissue mRNA expression patterns of porcine GBP-1 and murine IIGP1 and MxA. Data derived from publicly available datasets available from http://www.biogps.org. B. Crystal structure of a key member of each group of GTPases. GBP-1 and MxA taken from Gao et al. (2011) and IIGP1 taken from Ghosh et al. (2004).
4.1.4 The guanylate-binding proteins: structure, sequence and activity

The human GBP family consists of seven members located in a gene cluster on chromosome 1 (Olszewski et al., 2006). The porcine GBP family is also located together as a gene cluster in a relatively poorly annotated area of SSC4. Of the GBP family, human (h) GBP-1 and hGBP-3 exhibit the highest degree of homology (87% amino acid identity). The best characterised member of the GBP family is GBP-1. The gene encoding GBP-1 is highly conserved between mammalian species, with human, mouse and pig sequences having an overall nucleotide homology of 76% and an amino acid identity of 69% between these species.

GBP-1 belongs to the dynamin superfamily of large GTPases. The class of dynamin-like GTPases mediates basic cellular processes involving membrane remodelling, such as the scission of membrane vesicles (Haller & Kochs, 2011; Low & Löwe, 2010; Praefcke & McMahon, 2004). Most dynamin-like GTPases are composed of an amino-terminal (N-terminal) GTPase (G) domain, a central middle domain and a carboxy-terminal (C-terminal) GTPase effector domain (GED) (Gao et al., 2011).

G-domains act as molecular switches, having distinct conformations in their active (GTP-bound) and inactive (GDP-bound) states (Vetter & Wittinghofer, 2001). Proteins possessing a functional G-domain can often be inactivated by specific GTPase proteins that catalyse the conversion of GTP to GDP, thereby rendering the G-domain-containing protein inactive. Conformational changes in the G-domain are subsequently transmitted to proximal protein domains or interacting proteins, leading to GTPase-dependent regulation of protein function. Certain GBPs, including hGBP-1 and hGBP-2, possess a unique ability to bind GTP and convert it further, to GMP, using the same reaction mechanism (Ghosh et al., 2006; Schwemmle & Staeheli, 1994). GMP is the major product at 37°C, with GDP becoming favoured at lower temperatures (Rani et al., 2012).

The p47 IRGs are primarily host immediate-early genes (IEGs). Induction of IEGs does not require de novo protein synthesis and is mediated by pre-existing transcription factors. Thus, many IEGs are maximally induced within 30 minutes of stimulation (Tullai et al., 2007). In contrast, the GBPs require de novo protein synthesis and secondary transcription factors, such as IRF-1, to recognise ISREs in
their promoters (Briken et al., 1995; Lew et al., 1991). Thus, human GBP family members can also be induced by type I IFNs through the ISGF-3 complex, which also binds ISREs (Colombo & Pang, 1982). However, current evidence suggests that this activation is weaker in mice (Boehm et al., 1998) and no data are available for the pig. The augmentation of IFN-γ-mediated GBP-1 induction by TNF-α and IL-1 in mice is mediated through the NF-κB binding site in its promoter (Naschberger et al., 2004). Given the self-activating nature of the large GTPases, including the Mx proteins and GBP family members, the mechanism for their negative regulation is intriguing and remains to be elucidated (Hunn et al., 2011).

GBP-1 has been previously shown to be prenylated in vivo (Nantais et al., 1996). hGBP-1, hGBP-2 and hGBP-5 possess a ‘CaaX’ prenylation motif at their C-terminal end (where “C” represents cysteine, “a” is an aliphatic amino acid and “X” is any amino acid), which suggests an affinity for cellular membranes. However, murine (m) GBP-1 also possesses an identical ‘CaaX’ motif and is poorly prenylated, if at all (Stickney & Buss, 2000). hGBP-1 possesses an additional region rich in basic amino acids directly upstream of the ‘CaaX’ motif, which may increase its affinity for cellular membranes (Britzen-Laurent et al., 2010; Resh, 2006; Rocks et al., 2005).

Dimerisation and tetramerisation of members of the GBP family has been demonstrated (Britzen-Laurent et al., 2010). The GTPase activity of hGBP-1 has been shown to be strongly increased by this dimer and tetramer formation, indicating the importance of self-association in this protein (Ghosh et al., 2006; Schwemmle & Staeheli, 1994). Britzen-Laurent et al. (2010) showed that the association of hGBPs with cellular membranes requires prenylation and also dimerisation. hGBP-1 localises diffusely as a monomer and is associated with membranous structures as a dimer, partially accumulating at the plasma membrane (Britzen-Laurent et al., 2010). hGBP-1 requires GTP binding, additional IFN-γ-stimulated cofactors and isoprenylation for Golgi residence (Modiano et al., 2005). The plasma membrane-association of hGBP-1 supports a possible role in signal transduction, consistent with its ability to inhibit endothelial cell spreading and migration (Weinländer et al., 2008).
Dimerisation functionally connects the regulation of membrane association with the GTPase cycle of GBPs in living cells. GBP-1 hydrolyses GTP into GDP and GMP in a two-step process. GBP-1 first dimerises upon binding to GTP, when it is transiently present as a tetramer during the GDP-bound transition phase. Following this, GDP is cleaved into GMP, resulting in dimer dissociation (Kunzelmann et al., 2006; Prakash et al., 2000a; Wehner & Herrmann, 2010). Therefore, the mixed pattern of localisation of GBP-1 may reflect dynamic and transient association with membranes depending on the GTP hydrolysis cycle and consequently, its dimerisation state.

Despite the absence of a leader peptide, the hGBP-1 protein has been shown to be secreted in some cell types (Bleiziffer et al., 2012; Naschberger et al., 2006). Secretion occurs via a non-classical pathway, independently of both isoprenylation and GTPase activity, and does not require additional IFN-induced factors (Naschberger et al., 2006). ATP-binding cassette transporter 1, a glyburide-sensitive transporter, has been proposed as a likely candidate for the mediation of hGBP-1 secretion (Naschberger et al., 2006). Given structural and sequence similarity between hGBP-1 and porcine (p) GBP-1, pGBP-1 is also likely to be secreted via a similar, non-classical secretion pathway. The secretion of GBP-1 indicates the potential for extracellular functions of this protein. Such functions have been proposed to be either indirect: altering the extracellular GTP pool and subsequently affecting cell growth and differentiation, or direct: activating cells or blocking pathogen entry by binding to cellular receptors (Naschberger et al., 2006).

4.1.5 GBP-1: functional and disease effects

Despite being among the first IFN-inducible proteins identified, the function of GBP-1 is still largely unknown. Given its IFN-responsive expression pattern it is perhaps unsurprising that a plethora of anti-pathogen activities have been attributed to this protein. However, elucidating the role of GBP-1 is complicated by the fact that it acts in apparently opposite fashions in different tissues and through different protein domains. For instance, in glioblastoma cell lines GBP-1 acts as a positive regulator of matrix metalloproteinase 1 (MMP-1) expression, while in endothelial cells, GBP-1 inhibits its expression (Guenzi et al., 2003; Li et al., 2011).
GBPs are being found to have increasingly diverse functional effects, including the inhibition of endothelial cell proliferation, MMP expression and intestinal epithelial development (Schnoor et al., 2008; Vestal & Jeyaratnam, 2011; Weinländer et al., 2008). At least four mouse GBPs (mGBPs 1, 6, 7 and 10) are involved in macrophage immunity to listerial or mycobacterial infection (Kim et al., 2011). GBP-1 recognises ‘non-self’ lipid droplets through an absence of IRGM proteins (Haldar et al., 2013). GBP-1 acts as a direct tumour suppressor in colorectal carcinoma and the loss of its expression in affected cells can indicate tumour evasion from the IFN-γ-dominated Th1 immune response (Britzen-Laurent et al., 2013).

The large-G (LG) domain appears to mediate many of the anti-pathogen activities of GBP-1, with these affects being largely attenuated through mutations causing a loss of GTPase function (eg. Itsui et al., 2009). However, the C-terminal helical domain of hGBP-1 also plays an important biological role. In some cell culture systems the C-terminal helices and not the LG domain, induce the expression of MMP-1 (Li et al., 2011). The helical domain, alone, is also able to induce cell invasion of glioblastoma multiforme and inhibit endothelial cell proliferation (Guenzi et al., 2001; Li et al., 2011).

Antiviral activity of hGBP-1 and mGBP-2 proteins against the VSV, HCV and EMCV ssRNA viruses has been demonstrated (Anderson et al., 1999; Carter et al., 2005; Itsui et al., 2009). GBP-1 knockdown has been shown to result in an increase in HCV RNA replication (Itsui et al., 2009). Furthermore, the high degree of sequence homology between many GBP family members indicates that further research may reveal an overlap in anti-pathogen function.

A summary of known anti-pathogen and other activities attributed to members of the GBP family is shown in Table 4.1.
### Table 4.1 Published functions of GBP family members. Citations in the literature involving anti-pathogen and other activities of GBP family members.

<table>
<thead>
<tr>
<th>GBP</th>
<th>Experimental host</th>
<th>Pathogen</th>
<th>Pathogen type</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>h1</td>
<td>Human</td>
<td>Influenza A Virus</td>
<td>orthomyxovirus</td>
<td>overexpression of hGBP1, inhibited IAV replication in vitro, interacts directly with viral NS1</td>
<td>Zhu et al., 2013</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td>Vesicular Stomatitis Virus</td>
<td>arbovirus</td>
<td>overexpression of hGBP1 mediated resistance to VSV cytopathic effect in vitro</td>
<td>Anderson et al., 1999</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td>Encephalomyocarditis Virus</td>
<td>cardiovirus</td>
<td>overexpression of hGBP1 mediated resistance to EMCV cytopathic effect in vitro</td>
<td>Anderson et al., 1999</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td>Hepatitis C Virus</td>
<td>hepacivirus</td>
<td>GBP-1 (guanine binding domain) directly binds the finger domain of the HCV protein NS5B, inhibiting its GTPase activity and antiviral effect.</td>
<td>Itirli et al., 2009</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td>Hepatitis C virus replicon</td>
<td>hepacivirus (replicon)</td>
<td>GBP-1 exhibited antiviral activity against an HCV replicon in HuH-7 cells in vitro</td>
<td>Itirli et al., 2006</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td>Bacterial meningitis</td>
<td>various</td>
<td>secreted hGBP-1 in cerebral spinal fluid of patients with bacterial meningitis in vivo</td>
<td>Nanchanger et al., 2005</td>
</tr>
<tr>
<td>h1, h2</td>
<td>Human</td>
<td>Chlamydia trachomatis</td>
<td>gram-negative bacterium</td>
<td>Potentiates anti-chlamydial effects of IFN-γ, may be target of chlamydial cytotoxin and localises to chlamydial inclusion membranes</td>
<td>Tietzel et al., 2009</td>
</tr>
<tr>
<td>h5</td>
<td>Human</td>
<td>Salmonella enterica, Listeria monocytogenes</td>
<td>gram-negative and gram-positive bacterium, respectively</td>
<td>GBPS required for CASK-1 activation in response to these pathogens</td>
<td>Shenoy et al., 2012</td>
</tr>
<tr>
<td>m1</td>
<td>Mouse</td>
<td>Dengue virus</td>
<td>mosquito borne flavivirus</td>
<td>In RAW264.7 cells, intracellular Dengue Virus (DENV) loads were significantly higher in GBP1 silenced cells, indicating an antiviral response to DENV infection</td>
<td>Pan et al., 2012</td>
</tr>
<tr>
<td>m1, 2, 3, 5, 7, 9</td>
<td>Mouse</td>
<td>Toxoplasma gondii</td>
<td>Protozoa</td>
<td>associate with intracellular Toxoplasma, gondii upon IFN-γ stimulation</td>
<td>Degradini et al., 2007</td>
</tr>
<tr>
<td>m1, 6, 7, 10</td>
<td>Mouse</td>
<td>Listeria monocytogenes, Mycobacterium bovis BCG</td>
<td>gram-positive bacterium</td>
<td>reversed IFN-γ-mediated inhibition of replication upon siRNA knockdown</td>
<td>Kim et al., 2011</td>
</tr>
<tr>
<td>m2</td>
<td>Mouse</td>
<td>Vesicular Stomatitis virus</td>
<td>arbovirus</td>
<td>Murine GBP-2 inhibited the replication of VSV in NIH-3T3 murine fibroblast cells</td>
<td>Carter et al., 2005</td>
</tr>
<tr>
<td>m2</td>
<td>Mouse</td>
<td>Encephalomyocarditis Virus</td>
<td>cardiovirus</td>
<td>Murine GBP-2 inhibited the replication of EMCV in NIH-3T3 murine fibroblast cells</td>
<td>Carter et al., 2005</td>
</tr>
<tr>
<td>m2</td>
<td>Mouse</td>
<td>Toxoplasma gondii</td>
<td>Protozoa</td>
<td>mGBP2-deficient mice possess a marked immune susceptibility to T. gondii</td>
<td>Degradini et al., 2013</td>
</tr>
<tr>
<td>m5</td>
<td>Mouse</td>
<td>Listeria monocytogenes, Mycobacterium bovis BCG</td>
<td>gram-positive bacterium</td>
<td>as above, but to a lesser extent</td>
<td>Kim et al., 2011</td>
</tr>
<tr>
<td>m6</td>
<td>Mouse</td>
<td>Salmonella enterica</td>
<td>gram-negative bacterium</td>
<td>Promotes Salmonella enterica serovar Typhimurium-induced pyroptosis in RAW264.7 cells</td>
<td>Rupprecht and Cardelli, 2008</td>
</tr>
</tbody>
</table>

**Other activities**

<table>
<thead>
<tr>
<th>GBP</th>
<th>Experimental host</th>
<th>Pathogen</th>
<th>Pathogen type</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>h1</td>
<td>Human</td>
<td></td>
<td></td>
<td>hGBP-1 is the specific mediator of the anti-proliferative effect of inflammatory cytokines on endothelial cells.</td>
<td>Ganzetti et al., 2001</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td></td>
<td></td>
<td>Human GBP1 inhibits endothelial cell invasion by down-regulating matrix metalloproteinase-1</td>
<td>Ganzetti et al., 2003</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td></td>
<td></td>
<td>Overexpression in HUVECs inhibits apoptosis</td>
<td>Pammner et al., 2006</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td></td>
<td></td>
<td>Reduced ability of HUVECs to form capillaries in culture</td>
<td>Ganzetti et al., 2003</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td></td>
<td></td>
<td>Inhibits HUVEC spreading on fibronectin</td>
<td>Weinländer et al., 2008</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td></td>
<td></td>
<td>Enhances gloma cell invasion through MMP1 induction</td>
<td>Li et al., 2011</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td></td>
<td></td>
<td>Localizes with CAR in the tight junctions of intestinal epithelial cells and regulates barrier function</td>
<td>Schnaar et al., 2008</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td></td>
<td></td>
<td>Modulates the migration and invasion of Oral cavity squamous cell carcinoma cells in vitro</td>
<td>Yu et al., 2011</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td></td>
<td></td>
<td>Overexpression in mice significantly reduces mammary carcinoma tumor growth.</td>
<td>Lipkin et al., 2010</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td></td>
<td></td>
<td>Able to inhibit colorectal carcinoma tumor growth in vivo</td>
<td>Britten-Laurier et al., 2013</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td></td>
<td></td>
<td>Stable expression of a GBP1 transgene in an ovarian cancer cell line (OVCAR8) was sufficient to confer moderate paclitaxel resistance</td>
<td>Duan et al., 2006</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td></td>
<td></td>
<td>Estrogen overexpression of GBP5 totally increases docetaxel sensitivity</td>
<td>Desarnaud et al., 2011</td>
</tr>
<tr>
<td>h1</td>
<td>Human</td>
<td></td>
<td></td>
<td>Cytokine-induced GBP-1 retards epithelial cell proliferation</td>
<td>Capaldo et al., 2012</td>
</tr>
<tr>
<td>h2</td>
<td>Human</td>
<td></td>
<td></td>
<td>Inhibits cell spreading, requirement for GTP binding and possibly dimerisation</td>
<td>Balasubramanian et al., 2011</td>
</tr>
<tr>
<td>m2</td>
<td>Human</td>
<td></td>
<td></td>
<td>Stimulates proliferation of NIH-3T3 murine fibroblast cells</td>
<td>Gorbatcheva et al., 2002</td>
</tr>
<tr>
<td>m2</td>
<td>Human</td>
<td></td>
<td></td>
<td>Provides resistance to paclitaxel-induced death in vitro</td>
<td>Balasubramanian et al., 2006</td>
</tr>
<tr>
<td>m2</td>
<td>Human</td>
<td></td>
<td></td>
<td>Inhibits spreading of NIH 3T3 and B16 melanoma cells on fibronectin</td>
<td>Messmer-Blust et al., 2010</td>
</tr>
<tr>
<td>m2</td>
<td>Human</td>
<td></td>
<td></td>
<td>Inhibits Rac activation by integrin engagement</td>
<td>Messmer-Blust et al., 2010</td>
</tr>
<tr>
<td>m2</td>
<td>Human</td>
<td></td>
<td></td>
<td>Inhibits PI3-K activation by integrin engagement</td>
<td>Messmer-Blust et al., 2010</td>
</tr>
</tbody>
</table>
4.1.6 The GBPs and PRRSV

Despite the varied anti-viral effects that have been demonstrated for GBP-1, this protein has not been directly implicated in PRRSV infection to date. GBP-1 transcripts were differentially-regulated upon infection in both PRRSV transcriptional datasets analysed in Chapter three of this thesis. The three GBP family members represented in the microarray analyses were co-expressed alongside known antiviral signalling and sensing genes, providing suggestive evidence for the involvement of this gene family in the host immune response to PRRSV.

Three microarray probe sets targeting different sequences on the GBP-1 mRNA were available on the Porcine Genome microarray, all three of which exhibited the same profile, but detected varying levels of transcript expression (e.g. Figure 4.2B). Probe set SSc.29054.1.A1 consistently detected the highest levels of expression of the GBP-1 transcript. In the RIVivo dataset, GBP-1 mRNA, as detected by probe set SSc.29054.1.A1, was induced, relative to control samples, during PRRSV infection in animals representing both genetic lines (Figure 4.2A). However, animals from the LR breed exhibited the greatest increase in transcript abundance (>5-fold) when compared to the B/C line animals, at around 3-fold induction (Figure 4.2A). Overall, GBP-1 mRNA levels tended to peak at the 6dpi timepoint in animals from both breeds sampled (Figure 4.2A). When individuals that were heterozygous for the WUR10000125 PRRSV resistance allele were highlighted, no clear trend in the expression of GBP-1 was evident in this study (Figure 4.2A).

In order to assess the expression of GBP-1 in the RIVitro PRRSV-infection dataset, GBP-1 transcriptional profiles in AM isolated from individual animals representing the LR and PIE breeds were plotted. A degree of variation was observed between samples, particularly at the 0 hpi timepoint in the PIE cell isolates (Figure 4.2B). In two of the PIE samples, GBP-1 expression appeared to be down-regulated early upon infection with PRRSV and the other sample, 637, was steadily up-regulated to the end of the time course (Figure 4.2B). A limitation of the RIVitro study is not having a mock infected control time course, making it challenging to discern if the observed variability between animals was genuine or an artefact of the experimental protocol. However, differences were evident between samples from the
LR and PIE breeds (Figure 4.2B). GBP-1 was induced at 12 hpi in the three LR samples, whereas in the PIE samples, 8 hpi appeared to be a pivotal timepoint (Figure 4.2B). On the whole, a greater differential regulation of GBP-1 transcripts was observed in the AM from the PIE breed (Figure 4.2B). In the RIVitro dataset, cells from PIE animals also succumbed to a more productive PRRSV infection (Figure 3.3B).

In conclusion, PRRSV infection has been shown to stimulate the expression of GBP-1 mRNA in both *in vitro* and *in vivo* experiments. The apparent differential regulation of this gene in animals with varying levels of permissiveness to PRRSV infection poses the question of whether this gene is involved in the host response to PRRSV infection. Additionally, if this is the case, then what are the mechanisms behind its varying expression levels between different samples?

### 4.1.7 Genetic variation in GBP genes

Several SNPs have been identified in porcine GBP genes. Ma *et al.* (2008) published the discovery of four SNPs in the pGBP-1 gene; two synonymous mutations in exon 8 and 11 and two non-synonymous (ns) mutations resulting in amino acid substitutions in exon 2 (corresponding to the LG domain of the protein). The authors also discovered three SNPs in the pGBP-2 gene. Association analysis revealed that one of these SNPs in the pGBP-1 gene and one SNP in pGBP-2 were significantly associated with red blood cell count, haemoglobin concentration and haematocrit traits (Ma *et al.*, 2008). In a follow-up study, Chen *et al.* (2012) sampled several diverse pig populations for SNPs in a region spanning exon 2 of the pGBP-1 gene and found three novel coding SNPs. The authors identified six haplotypes, none of which were present in the commercial breeds sampled, indicating a possible loss of genetic variation in these breeds at this locus (Chen *et al.*, 2012).
Figure 4.2 | Transcriptional regulation of GBP-1 during PRRSV infection. A. pGBP-1 (probe set ID: SSc.29054.1.A1) transcriptional profiles for animals belonging to B/C and LR lines in the RIVivo PRRSV-infection dataset. RIVivo SNP-genotyped individuals that were heterozygous for the WUR10000125 PRRSV resistance allele are highlighted in orange; all other animals were homozygous wild-type for this SNP. B. pGBP-1 transcriptional profiles across the time course of PRRSV infection in AM isolated from individual animals representing the LR and PIE breeds in the RIVitro dataset. Data from the three probe sets targeting pGBP-1 and available on the Porcine Genome microarray are shown.
In the pig, all currently annotated GBP family members are located in a gene cluster between 139 and 140 Mb on SSC4 (Figure 4.3A). This region coincides with the genomic region consisting of 33 SNPs, identified by Boddicker et al. (2012) as being associated with the host response to PRRSV challenge. As discussed in the previous chapter, a single SNP, designated WUR10000125, captured the majority of effects of this region, with 99.4 % of variation in the 33 SNP window genomic estimated breeding value (GEBV) for VL and 99.3 % of the variation for WG42 (Boddicker et al., 2012). GEBVs provide a measure of the breeding potential of an animal for a specific trait and are expressed in the same units as the recorded trait. Referring to the nucleotide at the WUR10000125 locus, the homozygous G genotype was desirable for WG42 and VL traits and the homozygous A genotype was undesirable. When Boddicker and colleagues plotted the GEBVs for the 33 SNP window for WG42 and VL according to their genotype at index SNP WUR10000125, a clear separation of the A:A and G:G genotypes was evident (Figure 4.3B). In addition, the separation of the A:G genotype indicated a possible dominance effect of the G allele in heterozygotes (Figure 4.3B; Boddicker et al., 2012). The homozygous G animals clustered together with both the highest weight gain and lowest viral load in the study (Figure 4.3B). The A:G genotype animals also exhibited positive weight gain and lower virus load. Finally, the homozygous A genotype animals hosted both higher viral load and weight loss upon infection (Figure 4.3B). The frequencies of the desirable G:G genotype and A:G were low in the 565 LRxLW barrows (boars that have been castrated prior to reaching sexual maturity) genotyped by Boddicker and colleagues, at 2.7 and 16.1 %, respectively (Figure 4.3B). The reduction in PRRSV-associated costs for the US pig industry by having A:G versus A:A genotype growing pigs, has been predicted to lie between $79.7 and $564.4 million (Boddicker, 2013).

The availability of crystal structure and sequence data, together with the well-characterised functions in innate immunity, makes the GBP-1 gene a potential candidate for investigation of the genetic basis underlying disease resistance. This, coupled with the PRRSV-specific findings in the transcriptional analyses carried out in Chapter 3 of this thesis and the putative involvement of pGBP-1 in PRRSV resistance traits, made pGBP-1 a strong candidate for further investigation.
Figure 4.3 | QTL associated with the host response to PRRSV challenge. A. Schematic representation of the currently annotated genes encoded by the 1 Mb region spanning 139 to 140 Mb on SSC4. B. Scatter plot showing GEBVs for the SSC4 host response QTL for both BW gain and VL. Animals are grouped by their genotype at index SNP WUR10000125. N represents the number of animals sampled by Boddicker and colleagues, belonging to the genotype in question. B. has been modified from its originally published form in Boddicker et al. (2012).
4.2 Results

Chapter 3 of this thesis described the transcriptional response to PRRSV and identified several clusters of host transcripts that were differentially regulated in pigs more or less susceptible to PRRSV infection. The identification of members of the GBP family within key transcript clusters and the existence of published SNP association data implicating these genes in the host response to PRRSV prompted further investigation into the role of these proteins, specifically GBP-1, in PRRSV infection.

4.2.1 in silico analysis of the WUR10000125 SNP

The WUR10000125 (WG42 and VL) SNP was mapped to the pig genome and identified as being a non-coding mutation in the 3′UTR of the pGBP-1 gene (Figure 4.4). In order to predict the potential effect of the WUR10000125 SNP on pGBP-1 expression, in silico bioinformatics analyses were carried out on the published 3′UTR sequence for pGBP-1, to predict and annotate any features present. It was clear that the GBP-1 3′UTR contained predicted binding sites for multiple microRNAs (miRNAs), as well as several AU-rich elements (AREs). AREs are known to target mRNAs for degradation and miRNAs also often direct mRNA degradation or prevent translation (Shaw & Kamen, 1986). In addition to these sequence elements, two identical ‘AAUAAA’ polyadenylation signals (PAS) were present in the pGBP-1 3′UTR (Figure 4.4). This appears to be the first indication of the existence of multiple PAS in the pGBP-1 sequence. The presence of multiple PAS implied that alternative polyadenylation (APA) might occur in the processing of the pGBP-1 3′UTR. However, published sequence data for the pGBP-1 3′UTR implied the exclusive use of the distal PAS by the polyadenylation machinery, which raised questions as to the function of the newly predicted PAS, proximal to the stop codon.

Notably, the WUR10000125 SNP was identified as being directly adjacent to the proximal PAS (Figure 4.4). APA alleles that provide shorter transcripts can be important hereditary causes of disease (Thomas & Sætrom, 2012). Due to the presence of two putative PAS, it was reasoned that APA could result in at least two
pGBP-1 mRNA variants: a full-length (~1030 bp) 3’UTR, or a truncated 3’UTR of ~580 bp in length.

Pre-mRNA made in the nucleus by RNA Pol II undergoes a series of post-transcriptional processing steps to produce mature mRNA, which is then exported to the cytoplasm. Pre-mRNA processing is inefficient, resulting in the rapid degradation of pre-mRNA in the nucleus, most of which never reaches the cytoplasm (Edwalds-Gilbert et al., 1997). Shorter transcripts are often processed more efficiently than longer mRNAs (Tian et al., 2005). Thus, small changes in RNA processing efficiency, such as transcript truncation via APA, can influence gene expression. Following the capping and polyadenylation of pre-mRNA and export to the cytoplasm, transcripts can also be targeted by miRNAs via binding sites in the 3’UTR. Thus, another potential factor increasing the stability of an mRNA can be the loss of miRNA binding sites as a result of transcript truncation caused by APA (Sandberg et al., 2008; Thomas & Sætrom, 2012).

The use of alternative PAS can be tissue or developmentally specific (Edwalds-Gilbert et al., 1997). PAS strength can also directly influence the amount of cytoplasmic RNA produced from a given transcript (Denome & Cole, 1988). The sequence directly upstream of the PAS, the upstream sequence element (USE) (containing WUR10000125), has also been shown to enhance 3’-end processing efficiency (Brackenridge & Proudfoot, 2000; Carswell & Alwine, 1989; Danckwardt et al., 2007; DeZazzo et al., 1991; Moreira et al., 1995; Valsamakis et al., 1991; Venkataraman et al., 2005). Thus, the additional predicted PAS in the 3’UTR of pGBP-1 prompted speculation as to the occurrence of APA in this mRNA. The location of the WUR10000125 SNP in the USE, directly upstream of the proximal PAS suggested that this SNP may influence the use of this predicted alternative PAS by the polyadenylation machinery and result in pGBP-1 3’UTR variants (Figure 4.4).
Figure 4.4 | Prediction of features within the pGBP-1 3’UTR sequence. Diagram representing the entire published 3’UTR sequence of pGBP-1. Predicted features are annotated, including: miRNA binding sites, AREs, U-rich element (URE) and PAS. The WUR10000125 SNP is annotated and sits directly adjacent to the predicted proximal PAS. The nucleotide sequence of the proximal predicted PAS is also enlarged at the top of the figure, along with the WUR10000125 SNP allele. Nucleotide base numbering begins following the pGBP-1 coding sequence stop codon.
4.2.2 Alternative polyadenylation of pGBP-1

In order to investigate the potential APA of GBP-1, PCR was performed. Porcine cDNA generated from total RNA isolated from RIVivo spleen tissue (see Chapter 3 of this thesis) using oligo d(T)\textsubscript{16} was used, as the WUR10000125 genotypes of these animals were known (Appendix 2). Samples were available from animals either wild-type (A:A) or heterozygous (A:G) for the WUR10000125 SNP. No known homozygous G:G samples were available. However, the suggestion of a dominance effect in the data presented by Boddicker \textit{et al.} (2012) indicated that the use of heterozygous samples was still likely to be relevant in assessing the function of the WUR10000125 SNP. Two primer sets were designed to amplify regions of the pGBP-1 3'UTR. One primer set was designed to amplify the region of the pGBP-1 3'UTR downstream of the stop codon, but upstream of the WUR10000125 SNP and its adjacent putative PAS (Figure 4.5A). The second primer set was designed to span the WUR10000125 SNP and putative proximal PAS (Figure 4.5A). Thus, the comparison of the relative abundance of these PCR products could potentially indicate both the use of the proximal putative PAS and any notable differences in the use of this predicted PAS in animals heterozygous for the WUR10000125 allele.

PCR was carried out using both primer sets on cDNA samples from each animal and products were visualised by agarose gel electrophoresis. This revealed the successful amplification of PCR products across all samples using both primer sets, indicating that the proximal predicted PAS was not utilised exclusively in any samples tested (Figure 4.5B). A clear difference was observed between the fluorescence intensity of each of the two PCR products for some animals, e.g. pig ID: 1666 (Figure 4.5B). The intensity of each band on the agarose gel was quantified and the ratios between the intensity of primer sets 1 and 2 were calculated for each sample. These ratios were plotted and segregated into animals that were either homozygous A:A (n= 15) or heterozygous A:G (n= 7), according to their genotype at the WUR10000125 allele. The ratio calculations suggested a subtle effect, with the heterozygous animals apparently exhibiting less of a difference between ‘long’ and ‘short’ 3'UTR PCR product intensities (Figure 4.5C). These data could support the hypothesis of a disruption to the USE of the predicted proximal PAS as a result of the WUR10000125 SNP, leading to GBP-1 transcripts with largely full length
3`UTRs. The homozygous A:A animals exhibited a greater variability in the ratio between product intensities for ‘long’ and ‘short’ 3`UTR PCR products, suggesting that the favoured 3`UTR length in certain of these individuals might be shorter, with fewer transcripts possessing a 3`UTR continuing further than the newly predicted, proximal PAS. However, the 95% confidence intervals of the populations plotted on the graph overlapped, indicating that the population means were not statistically significant.

Overall, these data indicated that there was no clear-cut difference in the polyadenylation of pGBP-1 as a result of the WUR10000125 SNP, although they did not rule out a subtle effect on pGBP-1 transcript 3`UTR length resulting from the WUR10000125 polymorphism. However, due to the high LD in the 1 Mb genomic region encoding the GBP family, it was of interest to explore coding SNPs in the region whose effects could be more accurately quantified.
Figure 4.5 | Effect of the WUR10000125 SNP on GBP-1 polyadenylation. A. Schematic representation of the GBP-1 3’UTR, showing the relative positions of PCR primer sets 1 (FW1 and REV1) and 2 (FW2 and REV2). B. Agarose gel image of PCR products resulting from the amplification of cDNA samples using primer sets 1 and 2 (as in A). The pig IDs correspond to the animals represented in the RIVitro experiment. Animals heterozygous for the WUR10000125 allele are denoted by an asterisk, full genotypes can be found in Appendix 2. C. The ratio of agarose gel band intensities of PCR product 1 to PCR product 2 for animals heterozygous for WUR10000125 allele or homozygous wild type was determined by densitometry. Bars represent the median and 95% confidence intervals.
4.2.3 rs80955804: a non-synonymous SNP in pGBP-1

The challenges involved in investigating the, at best, subtle effects of APA of the pGBP-1 mRNA, given the potential existence of tissue-specific differences and the limited availability of homozygous G:G WUR10000125 samples, prompted an alternative approach. The high LD in the region encoding GBP-1 suggested that additional SNPs in this region are also likely to be associated with WUR10000125. Investigation of published variation in the pGBP-1 gene highlighted a missense mutation at nucleotide position 1486 in the pGBP-1 coding sequence, with the dbSNP accession number: rs80955804. This mutation (g1486a) results in an amino acid substitution at position 496 from alanine to threonine (A496T). To assess the extent of any potential protein structural changes resulting from this amino acid substitution, the structure of the pGBP-1 monomer was predicted based on the model available for hGBP-1 (PDB ID: 1DG3; Prakash et al., 2000a) and nucleotide sequence data generated for pGBP-1 in this Chapter. hGBP-1 and pGBP-1 were found to share a high degree of structural homology (91.1 % sequence similarity, Z-score: 0.554). The rs80955804 SNP was then mapped to the pGBP-1 protein model in order to assess its impact on overall monomer structure (Figure 4.6A and B). The rs80955804 amino acid polymorphism was found to be located within the α12 helix of GBP-1, but had no clear visual effect on monomer structure (Figure 4.6C).
Figure 4.6 | pGBP-1 monomer structure and rs80955804 amino acid polymorphism. A. Cartoon (ribbon with stick side-chains) model of the pGBP-1 protein. B. Closeup of the tip of the helical domain, showing the rs80955804 A496T amino acid substitution highlighted in red. C. Cartoon representation of wild type pGBP-1 (red) overlaid on the g1486a mutant pGBP-1 (blue), with structural domains and α-helices annotated. The rs80955804 A496T amino acid substitution is highlighted in yellow. pGBP-1 was modelled based on the hGBP-1 protein crystal structure data, publically available as PDB ID: 1DG3 (Prakash et al., 2000a) using a translation of the pGBP-1 nucleotide sequence derived from the sequencing of RIVivo pig ID: 1662 in this Chapter (GenBank accession number: KF977840).
The alpha-helical nature of the C-terminus of GBP-1 posed the question of whether an amino acid substitution may affect coiled-coil formation and subsequently alter the protein structure. Several algorithms are available for the prediction of coiled-coils, many of which exist as online tools. The COILS program predicts the presence of α-helical coiled-coil domains in proteins (Lupas et al., 1991). Prediction was performed for both wild type pGBP-1 and g1486a mutant sequences and confirmed that both protein sequences had a high probability of coiled-coil formation in the α12 helix, under the highest stringency scanning window (28 residues). However, when COILS data for wild type and SNP sequences were overlaid, only a minor difference in the probability of coiled-coil formation in the α12 helix (resulting from the single amino acid substitution) was evident (Figure 4.7A).

Heptad repeats are characteristic of α-helical coiled-coil motifs. The heptad repeat pattern for pGBP-1 was identified based on published data for hGBP-1 (Syguda et al., 2012). Figure 4.7B shows the residues in the α12 helical region of pGBP-1, according to the heptad repeat pattern. The indicated positions a to g refer to the respective locations on the helical wheel projection in Figure 4.7C. The residues at positions a and d are predominantly hydrophobic, positions e and g are charged or polar and positions b, c and f vary in their charge. This pattern is interrupted three times, which may prevent over-winding of the supercoil (Syguda et al., 2012). The rs80955804 (A496T) SNP is indicated in Figure 4.7B by a red box. The alanine amino acid at this position is conserved across the majority of porcine and human GBP family members, with the exception of pGBP-2 (Appendix 3). Threonine, which is substituted in this position as a result of the rs80955804 missense SNP (Figure 4.7B), is polar and is not present at either position a or d in human or porcine wild type GBP-1 sequences. Additionally, polar side chains at the a and d positions can destabilise coiled-coil structures (Choi et al., 2012).

An amino acid substitution can impact on the biological function of a protein. To investigate this, multiple online tools were used to analyse the effect of the rs80955804 A496T substitution on pGBP-1. PROVEAN (Choi et al., 2012) found the substitution at A496T in pGBP-1 to be significantly in excess of the “deleterious” threshold of -2.5, with a score of -3.154. PolyPhen-2 is a tool for the prediction of the
impact of nsSNPs on protein function (Adzhubei et al., 2010). Within this tool, the HumDiv-trained model found the A496T substitution to be “possibly damaging”, with a score of 0.539 (sensitivity: 0.88, specificity: 0.91). The SIFT tool predicts the effect of variation on protein function based on sequence homology and physicochemical similarity between alternate amino acids (Ng & Henikoff, 2001). SIFT also predicted the A496T substitution to be deleterious, with a probability of 0.02 (deleterious threshold <0.05), based on an alignment of 91 sequences. These analyses suggested that the SNP might have a deleterious functional effect on pGBP-1.

An important further consideration for any functional impact of the pGBP-1 rs80955804 SNP is on self-association. hGBP-1 is known to form homodimers in vivo (Abdullah et al., 2010; Britzen-Laurent et al., 2010; Prakash et al., 2000b; Wehner et al., 2012) and tetramers in vitro (Syguda et al., 2012). hGBP-1, hGBP-2 and hGBP-5 are also thought to form heterodimers (Britzen-Laurent et al., 2010). hGBP-1 is predicted to form a head-to-tail homodimer, which buries 2890 Å² of surface area, and a head-to-head homodimer, which buries 2140 Å² (Prakash et al., 2000b). The head-to-tail dimer (Prakash et al., 2000b) contacts along the α12 helix, an interaction that could potentially be disrupted by a change in polarity caused by the rs80955804 A496T substitution within the α12 helix (Figure 4.7D). However, modelling the head-to-tail dimer suggested that amino acid position 496 was unlikely to be involved with dimer formation in this particular model (Figure 4.7D). Nevertheless, a theoretical tetramer formation model has also recently been published for hGBP-1, based on biochemical data, which proposes the interaction of tail-to-tail GBP-1 dimers along the α12 helix (Figure 4.7E; Syguda et al., 2012). This tetramer formation model suggested another potentially damaging functional effect of the rs80955804 missense SNP, given its position towards the tip of the pGBP-1 α12 helix. However, no robust model currently exists for GBP-1 tetramerisation, so it was not possible to fully examine the effect of the substitution.

The results of the in silico analyses suggested that the rs80955804 SNP may result in a minor deleterious conformational change to the pGBP-1 monomer and also affect protein function and potentially tetramer formation. To assess whether the wild-type pGBP-1 or the mutant exerted an anti-viral effect on PRRSV, in vitro studies were carried out.
Figure 4.7 | Assessment of the impact of the rs80955804 SNP on pGBP-1 structure and self-association. A. COILS analysis of wild-type pGBP-1 and the pGBP-1 A496T mutant. Plot shows the highest probability of coiled-coil formation to be at the C-terminal helices α12/13, as predicted by COILS (Lupas et al., 1991), using a scanning window of 28 residues. B. GBP-1 heptad repeat table. “A” at position d in heptad is substituted by “T” as a result of the rs80955804 SNP, indicated by a red box. Figure based on Syguda et al. (2012) and adapted for pGBP-1. C. Helical wheel projection of a heptamer showing inter-helical packing interactions. The view is from the N terminus, heptad positions are labelled a to g and correspond to these position designations in B. Reproduced from Liu et al. (2006). D. pGBP-1 head-to-tail dimer model, modelled in YASARA using a model provided by Prof. Dr. Alfred Wittinghofer (personal communication). rs80955804 A496T substitution highlighted in yellow. E. Theoretical hGBP-1 tetramer formation model adapted from Syguda et al. (2012). The predicted areas of contact between the α12 helices are circled. All pGBP-1 sequences were derived from the sequencing of RIVivo pig ID: 1662 (GenBank accession number: KF977840).
4.2.4 Generation and overexpression of pGBP-1 constructs

No commercially available anti-porcine GBP-1 antibodies were available at the time of this study. Two commercial anti-hGBP-1 antibodies were tested but failed to detect pGBP-1, despite the protein sequence homology between these species (data not shown). Therefore, cloning of the pGBP-1 gene into a V5-tagged mammalian expression vector was undertaken in order to facilitate subsequent protein detection. The pGBP-1 gene was cloned from cDNA generated from the spleen tissue of pregnant gilts represented in the RIVivo study (Chapter 3 of this thesis). The pGBP-1 gene was successfully cloned from six animals and, following nucleotide sequencing, all six were found to be identical and to be wild type for both WUR10000125 and rs90955804 SNPs (data not shown). The cloned sequence was found to share 99.5% amino acid identity with a published pGBP-1 sequence (NCBI ID: NM001128473). As a result of the minor, but reproducible, discrepancies between published and sequenced pGBP-1, this nucleotide sequence was submitted to GenBank as accession number: KF977840 (Appendix 4). Appendix 5 presents the full amino acid sequence translation, with differences from the published pGBP-1 sequence highlighted.

The pGBP-1 clone used in further experiments was derived from pig ID: 1662, this pGBP-1 sequence was cloned into the mammalian expression vector pEF6/V5-His-TOPO. pGBP-1 was additionally cloned in-frame with both V5 and His tags, with a deleted stop codon, for the generation of a pGBP-1_V5 fusion protein. Site-directed mutagenesis was also performed on pGBP-1_V5 in order to replicate the rs80955804 SNP, by substituting nucleotide g1486a, resulting in the missense mutation A496T. All constructs were verified by direct sequencing.

In order to test the expression of the V5-tagged genes, lipid-based transfection was performed in the commonly used Chinese hamster ovary (CHO-K1) mammalian epithelial cell line (Figure 4.8). CHO-K1 cells were seeded onto glass coverslips 24 hours prior to transfection. Control transfections were performed with either no vector (mock) or the manufacturer-supplied pEF6_lacZ_V5 plasmid, as an irrelevant gene expression control vector. For pGBP-1 samples, cells were transfected with either wild type or g1486a mutant V5-tagged constructs. Cells were fixed and stained at 24 hpt with an anti-V5 antibody and the DAPI nuclear counter-
stain. Low magnification confocal microscopy revealed high levels of staining of all tagged proteins at 24 hpt (Figure 4.8A). In addition to the microscopic analysis of transfected CHO-K1 cells, samples transfected under the same conditions were incubated until 48 hpt and stained with an anti-V5 antibody for FC analysis. FC analysis at 48 hpt revealed apparently lower levels of tagged protein staining at this later timepoint (Figure 4.8B).

In order to confirm that the intended proteins were being expressed along with the V5 tag, western blots were performed using an anti-V5 antibody to probe protein lysates of transfected CHO-K1 cells. Total protein lysates were made from equal numbers of CHO-K1 cells, transfected in duplicate, and separated by SDS-PAGE before transferring to nitrocellulose. Western blots were performed using anti-beta-actin as a loading control and probing for the V5 tag. The intensity of beta-actin staining revealed that comparable levels of total protein were loaded for all samples (Figure 4.8C). Probing membranes with an anti-V5 antibody revealed no detection of the V5 tag in mock-transfected samples (Figure 4.8C). However, V5 tagged protein was readily detected in both replicates of all transfected CHO-K1 samples (Figure 4.8C). Additionally, all tagged proteins appeared to conform to their expected atomic mass, including the lacZ control (Figure 4.8C). A full-length protein also appeared to be translated from the GBP-1 g1486a mutant vector (Figure 4.8C).
Figure 4.8 | Lipid-based transfection of GBP and control plasmids in CHO-K1 cells. A. CHO-K1 cells stained with an anti-V5 mAb and an Alexa Fluor 594-conjugated secondary mAb on coverslips. Cells were visualised by low magnification confocal microscopy at 24 hpt, following co-staining with DAPI. B. CHO-K1 cells fixed and permeabilised, prior to staining with an anti-V5 antibody and Alexa Fluor 594-conjugated secondary mAb and analysis by FC at 48 hpt. FC plots correspond to the sample descriptions in A. C. V5-tagged protein expression in CHO-K1 cells, transfected in duplicate. Plasmid-transfected CHO-K1 total cell lysates were analysed by SDS-PAGE and western blotting for V5 or beta-actin, as labelled. Membranes were stained with an IR dye-conjugated secondary Ab for detection on the LI-COR Odyssey system.
4.2.5 Co-expression of GBP-1 and PRRSV in CHO-K1

As a first assessment of the impact of GBP1_wt or GBP1_g1486a on PRRSV, these plasmids were transfected into the uninfactable CHO-K1 cell line, alongside the SD01-08 PRRSV reverse genetics plasmid. CHO-K1 cells are capable of producing infectious virus when transfected with the PRRSV reverse genetics plasmid. However, they are not natively permissive to PRRSV infection.

CHO-K1 cells were seeded on coverslips and co-transfection was performed using plasmids expressing either: V5-tagged GBP1_wt, GBP1_g1486a or lacZ, together with the SD01-08 GFP-expressing PRRSV infectious clone plasmid. At 24 hpt cells were fixed and stained with an anti-V5 antibody and then mounted on glass slides in the presence of the DAPI nuclear stain. High-magnification confocal imaging of the fluorescence in transfected cells detected no significant background staining or auto-fluorescence in mock-transfected samples (Figure 4.9A i). Analysis of lacZ-transfected samples revealed that this V5-tagged protein was distributed diffusely throughout the cytoplasm (Figure 4.9A ii). Fluorescence from the PRRSV nsp2-GFP fusion protein in these cells was also diffusely distributed throughout the cytoplasm. However, the GFP signal also extended to the nucleus (Figure 4.9A ii). Nuclear staining could be explained by published observations that several PRRSV proteins localise to the nucleus of infected cells (Fang & Snijder, 2010; Rowland & Yoo, 2003a). In CHO-K1 cells co-transfected with GBP1_wt and SD01-08 plasmids, the staining pattern of the V5-tagged GBP1_wt protein appeared to be predominantly discrete and granular (Figure 4.9A iii). The fluorescence from the PRRSV nsp2-GFP fusion protein in these cells was distributed across the cytoplasm and nucleus, but also existed in a granular localisation, apparently similar to that of GBP1_wt (Figure 4.9A iii). In the case of GBP1_g1486a-transfected cells, the staining pattern of the V5-tagged GBP1_g1486a protein also appeared to accumulate in a predominantly discrete and granular localisation (Figure 4.9A iv). In these cells, the PRRSV nsp2-GFP fusion protein was also distributed in a granular localisation, appearing to overlap with GBP1_g1486a staining (Figure 4.9A iv).

These data confirmed that the co-transfection of the SD01-08 infectious clone plasmid alongside other host gene plasmids was possible. In order to quantify the overlap between both GBP1_wt and GBP1_g1486a protein staining and PRRSV-
nsp2 fluorescence observed by high magnification confocal imaging, colocalisation analyses were performed. High magnification confocal images were captured using consistent microscope settings and the overlap coefficient (OC) (Manders et al., 1993) was calculated using the Zen 2011 software package (v7.0, Carl Zeiss MicroImaging; also see 6.7.4). The purpose of the OC is to characterise the degree of overlap between two channels in a microscope image, with a value of 1 representing perfect overlap. The OC is most useful when analysing colocalisation in cells with a similar ratio of pixels in each channel. For the calculation of the OC, data were utilised for the colocalisation of green (nsp2-GFP) and red (V5-tagged protein) pixels across the whole cell, in all available images. This analysis confirmed the observed lack of colocalisation between lacZ and PRRSV nsp2-GFP (Figure 4.9B). However, the OC revealed significant colocalisation of both GBP1_wt and GBP1_g1486a with PRRSV nsp2 (Figure 4.9B). The OC scores indicated that this colocalisation was marginally stronger in the case of GBP1_wt than for GBP1_g1486a (Figure 4.9B).

Given the evidence for the colocalisation of both GBP1_wt and GBP1_g1486a with PRRSV nsp2 upon co-transfection into CHO-K1 cells, as well as the known antiviral function of multiple GBP family members, it was of interest to assess any effect of GBP1_wt and GBP1_g1486a on PRRSV. The uninfectable CHO-K1 cell line was again utilised for the transfection of the SD01-08 GFP-expressing infectious clone plasmid, alongside V5-tagged GBP-1 and control plasmids. Cells were fixed at 24 hpt and the numbers of cells expressing the PRRSV nsp2-GFP fusion protein were quantified by FC. As expected, negligible background fluorescence was detected in mock-transfected samples (Figure 4.9C). The relative proportion of GFP-positive cells was calculated by normalising data to the percentage of GFP-expressing cells upon transfection with SD01-08 only. In this way, it was evident that the co-transfection of lacZ with the PRRSV SD01-08 infectious clone plasmid resulted in a minimal decrease in the proportion of GFP-positive cells compared to SD01-08 transfection alone (Figure 4.9C). However, co-transfection of SD01-08 and GBP1_wt resulted in a substantial decrease in the proportion of GFP-expressing cells (Figure 4.9C). Co-transfection of SD01-08 and the mutant GBP1_g1486a also resulted in a similar decrease to the wild-type GBP-1.
in the proportion of nsp2-GFP-expressing cells, relative to SD01-08 alone (Figure 4.9B).

Overall, data derived from the co-transfection of GBP-1 and SD01-08 in the uninfected CHO-K1 cell line show that both GBP1_wt and GBP1_g1486a colocalised with PRRSV nsp2 in vitro. Furthermore, given that GFP expression is linked to the translation of the PRRSV nsp2 protein, occurring at the beginning of the infection process, the measured reduction in the number of GFP-positive cells suggested that the over-expression of GBP-1 was inhibitory to PRRSV. However, little difference was witnessed in this apparent inhibitory effect, between GBP1_wt and the GBP1_g1486a mutant.
Figure 4.9 -- figure legend overleaf--
Figure 4.9 | Co-expression of GBP-1 and SD01-08 nsp2 in the CHO-K1 cell line. A. High magnification confocal microscopic images of CHO-K1 cells either: i) mock transfected or expressing the PRRSV nsp2-GFP fusion protein along with either ii) lacZ, iii) GBP1_wt or iv) GBP1_g1486a. Cells were stained and imaged at 24 hpt. Red fluorescence represents staining with an anti-V5 antibody and an Alexa Fluor 594-conjugated secondary antibody. Green fluorescence represents the direct expression of the PRRSV nsp2-GFP fusion protein. Three colour merge images of the cells shown in ii, iii and iv are shown in B, where the intensity of staining is shown in the third dimension. C. Quantification of the colocalisation of V5-tagged lacZ, GBP1_wt or GBP1_g1486a proteins with the PRRSV nsp2-GFP fusion protein, as measured using the OC. Data are derived from the analysis of confocal images of transfected CHO-K1 cells. D. Quantification of the proportion of GFP-expressing CHO-K1 cells when transfected alone of alongside plasmids expressing lacZ, GBP1_wt or GBP1_g1486a. Data were derived from FC analysis of GFP-expressing cells at 24 hpt and are normalised to the percentage of cells expressing nsp2-GFP upon SD01-08 transfection alone. Error bars represent the standard deviation.
The colocalisation of GBP-1 and SD01-08 nsp2 and the observed reduction in the number of PRRSV-positive CHO-K1 cells in the presence of transfected GBP-1, supported the need for further analysis of the subcellular localisation patterns of wild type and mutant GBP-1 proteins, both in the presence and absence of SD01-08. In order to test this, further high magnification confocal microscopy was carried out on CHO-K1 cells. Cells were fixed at 24 hpt with V5-tagged GBP-1 or lacZ control plasmids, either alone or alongside the SD01-08 PRRSV infectious clone plasmid. All samples were stained with an anti-V5 antibody. Serial optical slices were taken through multiple cells representing each treatment type. These Z-stack images were then rendered to produce pseudo-three-dimensional images of the three-colour staining patterns: the DAPI-stained nucleus, the SD01-08 nsp2-GFP fusion protein and the V5-tagged proteins (Figure 4.10).

No detectable green or red fluorescence was observed in samples that were not transfected with SD01-08 or V5-tagged plasmids, respectively (Figure 4.10 i). The transfection of the SD01-08 plasmid alone resulted in nsp2-GFP fusion protein expression throughout the cytoplasm, in discrete granules (Figure 4.10 ii). The pattern of V5-staining in lacZ-transfected cells, both alone and alongside SD01-08, confirmed the initial findings in Figure 4.9, exhibiting a diffuse cytoplasmic, non-nuclear distribution in both cases (Figure 4.10 iii and iv). When GBP1_wt-transfected cells were analysed, it was clear that two distinguishable subcellular staining patterns were represented (Figure 4.10 v). GBP1_wt staining often appeared in a granular pattern throughout the cytoplasm (Figure 4.10 v a), possibly associated with the membranes of vesicular structures, as previously observed with hGBP-1 (Britzen-Laurent et al., 2010). In other cells, this cytoplasmic staining showed an obvious weighting towards the perinuclear region (Figure 4.10 v b). However, markedly different staining patterns were observed in some cells co-transfected with GBP1_wt and SD01-08. The majority of SD01-08-negative cells showed a granular GBP1_wt staining pattern throughout the cytoplasm (Figure 4.10 vi; left cell). Conversely, in cells co-transfected with both GBP1_wt and SD01-08, a condensed distribution of SD01-08 nsp2, with GBP1_wt staining surrounding the exterior of the apparent virus replication site was observed (Figure 4.10 vi; right cell).
GBP1_g1486a single-transfected cells exhibited a V5 staining pattern that was granular and distributed throughout the cytoplasm, but with an apparently greater weighting towards the perinuclear region than GBP1_wt (Figure 4.10 vii a). However, two distinguishable subcellular GBP1_g1486a staining patterns were represented, with some cells showing an almost completely perinuclear distribution and little dispersed cytoplasmic staining (Figure 4.10 vii b). Cells co-transfected with both GBP1_g1486a and SD01-08 PRRSV infectious clone plasmids demonstrated a similar staining pattern to GBP1_wt, with a condensed distribution of SD01-08 nsp2 and GBP1_g1486a staining around the exterior of the potential PRRSV replication site (Figure 4.10 viii).

In conclusion, the colocalisation of GBP-1 and PRRSV nsp2 identified in Figure 4.9 was confirmed and a potential shift in the subcellular localisation of both GBP-1 and PRRSV nsp2 upon co-transfection was observed. A possible subtle difference in the localisation of GBP1_wt and the mutant GBP1_g1486a was also perceived in the absence of SD01-08 co-transfection.
Figure 4.10 | Subcellular localisation of GBP-1 in the presence or absence of co-transfected SD01-08 plasmid. CHO-K1 cells were transfected with the V5-tagged lacZ, GBP1_wt and GBP1_g1486a plasmids either alone, or in combination with the PRRSV SD01-08 GFP-expressing infectious clone plasmid. High magnification confocal microscopic images were taken following staining of cells at 24 hpt. Multiple, stacked images were rendered to create pseudo-three dimensional images of complete cells. Red fluorescence represents staining with an anti-V5 antibody and an Alexa Fluor 594-conjugated secondary antibody. Green fluorescence represents direct imaging of the PRRSV nsp2-GFP fusion protein. Blue fluorescence represents DAPI nuclear staining. a and b denote examples of the dispersed and perinuclear, respectively, subcellular staining patterns of the V5-tagged GBP1_wt and the GBP1_g1486a proteins.
In order to assess the reproducibility of the observed change in GBP-1 protein localisation upon SD01-08 co-transfection, multiple biological and technical repeats of single and co-transfections were performed in CHO-K1 cells. Confocal images were collected from multiple fields of view and the image IDs were automatically randomised in order to perform single-blind scoring of the subcellular localisation patterns. The subcellular staining patterns of fluorophores representing both V5-tagged constructs and SD01-08 nsp2 were assigned to either ‘dispersed’ or ‘perinuclear’ categories.

Firstly, the V5-staining pattern was assessed, either with or without co-transfected SD01-08. Quantification of a large number of cells in this way confirmed that lacZ was present in a dispersed distribution in the presence or absence of SD01-08 co-transfection (Figure 4.11A). Most (80%) GBP1_wt-transfected cells contained largely dispersed staining when transfected alone. However, the co-transfection of SD01-08 led to a marked shift in the subcellular GBP1_wt staining pattern, to around 50% of cells showing a perinuclear staining distribution (Figure 4.11A). The scoring of large numbers of cells revealed that the distribution of GBP1_g1486a was perinuclear in 58% of cells when transfected alone, compared to just 20% of GBP1_wt-transfected cells (Figure 4.11A). A minimal (5%) increase in the proportion of GBP1_g1486a-transfected cells exhibiting a perinuclear staining pattern was observed upon SD01-08 co-transfection (Figure 4.11A).

The intracellular distribution of PRRSV nsp2 was also scored when transfected alone, or in combination with GBP-1 or lacZ plasmids. When transfected alone, the nsp2-GFP fusion protein appeared to be present exclusively in a dispersed localisation pattern (Figure 4.11B). No significant change was noted in the subcellular distribution of nsp2-GFP fluorescence upon co-transfection with the lacZ control plasmid (Figure 4.11B). However, the localisation of SD01-08 nsp2 changed markedly in the presence of co-transfected GBP1_wt or GBP1_g1486a. The co-transfection of GBP1_wt resulted in a shift in PRRSV nsp2 from being exclusively dispersed to 49% of cells exhibiting a perinuclear distribution (Figure 4.11B). The co-transfection of the mutant GBP1_g1486a together with the SD01-08 infectious clone also resulted in a shift in PRRSV nsp2 to 44% of cells exhibiting a perinuclear distribution of this viral non-structural protein (Figure 4.11B).
Figure 4.11 | Subcellular localisation of GBP-1 and PRRSV nsp2. A. Subcellular distribution of V5-tagged constructs transiently transfected into CHO-K1 cells, either alone or alongside the SD01-08 PRRSV infectious clone plasmid. B. Subcellular distribution of the SD01-08 nsp2-GFP fusion protein following transient transfection of the SD01-08 PRRSV infectious clone plasmid into CHO-K1 cells, either alone or along with V5-tagged constructs. All cells were visualised at 24 hpt by confocal microscopy at 63x magnification and scored (single-blind) for their pattern of subcellular fluorophore distribution into either “dispersed” or “perinuclear” categories. Examples of dispersed” or “perinuclear” staining distributions can be seen in Figure 4.10 vii a and b, respectively. Error bars represent the range of counts from two independent biological replicates. N is shown for the total number of cells scored per condition.
Overall, the scoring of a large number of images revealed that, in the absence of co-transfection or external stimuli, the intracellular localisation pattern of GBP1_g1486a was biased towards a perinuclear distribution. Whereas, a greater proportion of cells expressing the wild-type GBP-1 protein appeared to show a diffuse staining pattern under the same conditions. However, both GBP1_wt and GBP1_g1486a were similarly localised upon co-transfection of the SD01-08 infectious clone. The change in localisation upon co-transfection was mirrored by the PRRSV nsp2-GFP fusion protein, which shifted from a dispersed localisation in un-transfected and lacZ-transfected cells to around half of all cells scored exhibiting a perinuclear bias when co-transfected with either GBP1_wt or GBP1_g1486a. Taken together with the reduction in nsp2-GFP-positive cells after overexpression of either pGBP-1 protein, these data are suggestive of a biologically relevant, probably inhibitory, interaction between pGBP-1 and PRRSV.

4.2.6 Validation of GBP-1 antiviral activity in BMDM

As detailed in this Chapter thus far, the transfection of either GBP1_wt or GBP1_g1486a, along with the PRRSV reverse genetics plasmid, resulted in a reduction in the proportion of SD01-08 nsp2-positive CHO-K1 cells. Additionally, a colocalisation of both GBP1_wt and GBP1_g1486 was observed, as well as a shift in the localisation of both proteins to a perinuclear distribution upon co-transfection with PRRSV SD01-08. To extend these studies and assess the effect of the overexpression of GBP-1 constructs on virus infection in a biologically relevant system, PRRSV permissive host cells were utilised.

Previous findings in Chapter 2 of this thesis demonstrated that porcine BMDM were highly susceptible to a productive PRRSV infection. Another important consideration was that porcine BMDM were also found to be permissive to foreign DNA transfection in preliminary experiments. Having established a Nucleofection protocol that was amenable to plasmid transfection in a 96 well plate format in Chapter 2, an experimental protocol was developed to assess the impact of the over-expression of GBP-1 on PRRSV infection.

LacZ, empty vector control, GBP1_wt or GBP1_g1486a plasmids were transfected into BMDM in suspension using the Nucleofection method. BMDM were
then re-seeded at an appropriate density and incubated for 24 hours prior to infection at MOI 1 with PRRSV SD01-08 virus. At 24 hpi with the PRRSV SD01-08 virus, BMDM were fixed and stained using the DAPI nuclear counterstain and analysed by low magnification confocal microscopy for expression of the PRRSV nsp2-GFP fusion protein. Preliminary examination of microscopic images revealed no detectable GFP fluorescence in uninfected samples and a large number of GFP-positive cells in both no-substrate electroporated and lacZ expression control samples (Figure 4.12A). However, a clear reduction in the number of GFP-positive cells was evident in the cells overexpressing GBP1_wt or GBP1_g1486a constructs (Figure 4.12A). In order to more accurately quantify the observed difference in the proportion of infected cells as a result of GBP-1 transfection, cell counts were performed using microscopic images. All DAPI-stained nuclei in multiple fields of view, across several biological and technical replicates were counted, as were the number of GFP-positive cells. When the proportion of GFP-positive cells were normalised to the infected, no-electroporation samples, these data revealed that Nucleofection-only (no substrate) control samples showed a reduction in infection of around 25%, compared to infected-only samples (Figure 4.12B). A similarly reduced level of infection was also observed following electroporation with lacZ or empty vector control plasmids (Figure 4.12B). This indicated a lack of non-specific effects caused by the DNA transfection itself, suggesting that the reduction in infection rate was likely due to an effect of the electroporation procedure on the infectability of the cells. However, GBP1_wt and GBP1_g1486a transfection resulted in a substantial decrease in the number of GFP-positive cells when compared to empty vector, lacZ or no-substrate samples (Figure 4.12B). No clear difference was detected between the numbers of GFP-positive cells in GBP1_wt or GBP1_g1486a-transfected samples (Figure 4.12B). It was also necessary to further control for any functional effect of the 14 amino acid V5 tag and 6 amino acid His tag added to the GBP-1 constructs to facilitate staining of the protein products. In order to do this, an un-tagged GBP1_wt plasmid was generated and confirmed to have an otherwise identical nucleotide sequence to the tagged GBP1_wt. When this un-tagged GBP1_wt plasmid was transfected into BMDM and these cells were infected with PRRSV SD01-08 virus, the numbers of PRRSV-positive cells were
found to be directly comparable to V5-tagged GBP1_wt (Figure 4.12B). Thus overexpression of pGBP-1 is inhibitory to PRRSV infection in a biologically plausible cell type.

Given the nature of the SD01-08 infectious clone used in these studies, GFP-positive cells were taken to be infected with PRRSV. Thus, an inhibitory effect of GBP-1 on GFP itself could give the impression of a genuine inhibitory effect on virus replication. In order to control for an effect of GBP1_wt or GBP1_g1486a on the expression of GFP, a CMV promoter-driven GFP control plasmid (pMAX_GFP) was co-transfected into BMDM alongside lacZ or GBP-1 plasmids. At 24 hpt, BMDM were analysed by fluorescence microscopy and cell counts were performed using the resultant images. These cell counts revealed that 50 % of BMDM expressed detectable levels of GFP, as a result of the transfection of the pMAX_GFP plasmid, when co-transfected with the lacZ expression control plasmid (Figure 4.12C). Additionally, no significant effect was observed on the proportion of cells expressing GFP when the pMAX_GFP plasmid was co-transfected alongside either GBP1_wt or GBP1_g1486a constructs (Figure 4.12C).

In conclusion, GBP1_wt and GBP1_g1486a overexpression resulted in a significant reduction in the number of PRRSV-infected cells in the fully permissive BMDM. This may be due to an inhibitory effect of GBP-1 on PRRSV replication, as the overexpression of GBP1_wt or GBP1_g1486a in the same cell type failed to reduce the proportion of GFP-expressing cells when co-transfected with a GFP-expression control plasmid.
Figure 4.12 | SD01-08 infectious clone replication in GBP-1-transfected BMDM. A. Microscopic images of BMDM transfected with control or GBP-1 plasmids and then infected with PRRSV SD01-08 virus at MOI 1. All images were captured at 24 hpi using a Zeiss LSM710 confocal microscope at 10x magnification. B. Cell count data from microscopic images of BMDM transfected with GBP-1 and control constructs and infected with PRRSV SD01-08 virus at MOI 1 for 24 h. The mean proportion of GFP-positive cells is shown, along with the standard error of the mean. Counts are normalised to infected mock-treated samples. C. The percentage of GFP-positive BMDM at 24 hpt with the pMAX_GFP plasmid and co-transfected with either lacZ control or GBP-1 plasmids. Data were derived from cell counts performed on fluorescence microscopic images.
4.2.7 Quantification of the effect of GBP-1 on released PRRSV

Upon establishing that the overexpression of both porcine GBP1_wt and GBP1_g1486a significantly reduced the proportion of CHO-K1 and BMDM expressing the PRRSV nsp2-GFP fusion protein, the effect of these host proteins on the production of extracellular virus was assessed. BMDM were transfected with GBP1_wt (both with and without V5/His tags), GBP1_g1486a or control plasmids and incubated for 24 hpt. Following this incubation, BMDM isolated from two animals were infected at low multiplicity (MOI 0.01) with PRRSV SD01-08 virus for a further 24 h. At this point, virus RNA was purified from the cell culture supernatant in all samples. The resultant viral RNA preparations were analysed by qRT-PCR using primers targeting PRRSV Orf5 (encoding the major envelope protein GP5), which is widely exploited for the analysis of PRRSV genetic variation and molecular epidemiology. The use of a consistent volume of cell culture supernatant in all RNA preparations permitted the comparison of the relative abundance of PRRSV genome in the supernatant of each transfected BMDM sample. However, dilutions of virus-containing supernatant of a known TCID_{50}/mL were also analysed by qRT-PCR in the same way, allowing the generation of a standard curve and consequent calculation of relative virus titre in test supernatants.

Analysis of the qRT-PCR data revealed that the transfection of the lacZ irrelevant gene expression control plasmid resulted in a minimal, but statistically significant (p <0.05) reduction in the relative titre of released virus from BMDM, compared to infected-only, mock-treated samples (Figure 4.13). However, a >100-fold reduction in the relative titre of released virus was observed upon transfection of GBP1_wt or GBP1_g1486a plasmids (Figure 4.13). Once again, no significant difference was observed between the effects of GBP1_wt in its un-tagged or V5/His-tagged forms (Figure 4.13; p <0.05). Transfection of the GBP1_g1486a mutant plasmid appeared to result in a marginally greater reduction in free PRRSV genome copies, when compared to GBP1_wt transfection (Figure 4.13). This effect was corroborated by the finding that the relative virus titre produced by GBP1wt_V5-transfected BMDM was significantly different from that produced by GBP1mut_V5-transfected BMDM (p <0.05) using Student’s t-test.
Overall, these data show that the observed decline in the infectivity of BMDM upon overexpression of GBP1_wt and GBP1_g1486a also appeared to correspond to the quantity of released virus, indicating an inhibitory effect on PRRSV replication.
Figure 4.13 | Relative virus titre in the supernatant of GBP-1-transfected BMDM. BMDM derived from two pigs were transfected, by Nucleofection, with lacZ control, GBP-1 wild type (tagged and un-tagged) or GBP1_g1486a plasmids. At 24 hpt, cells were infected (+V) at MOI 0.01 with the un-passaged SD01-08 PRRSV infectious clone. Virus in the supernatant was quantified by qRT-PCR at 24 hpi. Error bars represent the standard deviation of two biological replicates. Data are expressed as mean, equivalent TCID\textsubscript{50} values extrapolated using a standard curve (see 6.5.3) generated by performing qRT-PCR on dilutions of a virus stock of a known (BMDM) TCID\textsubscript{50}/mL. Student’s t-test showed that the relative virus titre produced by mock-transfected BMDM was significantly different from that produced by lacZ-transfected BMDM (*, p <0.05). The relative virus titre produced by GBP1wt_V5-transfected BMDM was significantly different from that produced by GBP1mut_V5-transfected BMDM (*, p <0.05).
qRT-PCR is a sensitive tool for the detection of virus genome. However, this technique does not take progeny virus viability or infectivity into consideration. For this reason, the transfected and PRRSV SD01-08-infected BMDM supernatants used to produce virus RNA for qRT-PCR-quantification were concurrently assayed by TCID$_{50}$ on naïve BMDM. Five 10-fold dilutions were performed with supernatants from two independent biological replicates, performed in triplicate, and used to inoculate naïve BMDM in triplicate. At 36 hpi, BMDM were fixed and stained with the DAPI nuclear counterstain. Each well was analysed by fluorescence microscopy for expression of the nsp2-GFP fusion protein. Initial analysis of the resultant microscopic images revealed that no detectable background fluorescence was present in the BMDM inoculated with any dilutions of supernatant derived from uninfected mock samples (Figure 4.14). In contrast, BMDM infected with supernatant derived from lacZ or mock-transfected BMDM were highly GFP-positive at all dilutions of virus-containing inoculum (Figure 4.14). However, BMDM infected with supernatant from GBP1_wt and GBP1_g1486a-transfected samples exhibited a severe reduction in the proportion of GFP-expressing cells by only the third serial dilution (10$^{-3}$) of the inoculum (Figure 4.14). There appeared to be little difference between the proportion of cells infected with supernatant from BMDM transfected with either tagged or un-tagged GBP1_wt (Figure 4.14).

The five-fold dilution series (10$^{-5}$) was insufficient to calculate the endpoint for control samples, negating the calculation of TCID$_{50}$ (Figure 4.14). The use of an alternative approach, such as enumeration as an infectious unit assay using cell counts from microscopic images and the Poisson distribution, was prevented due to the 36 h time course used, which allowed several rounds of PRRSV replication to take place. Therefore, the approach used to approximate the difference in virus titre between control and GBP-1-transfected samples was semi-quantitative. Nevertheless, a clear difference of over 2 log$_{10}$ of infectivity was apparent between control and GBP1_wt or GBP1_g1486a samples (Figure 4.14).

In summary, the data resulting from the dilution of transfected and infected BMDM supernatants as inoculum on fresh BMDM further corroborate the inhibitory effect of transfected GBP-1 on PRRSV replication.
Figure 4.14 | Titre of GBP-1-transfected, PRRSV SD01-08-infected BMDM supernatant on BMDM. Fluorescence microscopic images of BMDM 36 hpi with supernatants derived from BMDM transfected with GBP-1 or control plasmids, followed by infection with PRRSV SD01-08 virus. Images are representative of the $10^3$ (3-fold) serial dilution of virus-containing supernatant with the exception of mock +V and lacZ, which represent the $10^5$ (5-fold) dilution.
4.3 Discussion

The aim of the work discussed in this Chapter was to evaluate the potential of GBP-1 as an antiviral host gene involved in PRRSV infection. An analysis of the impact of GBP-1 on PRRSV infection in several different systems was performed, as well as investigating the effect of genetic variation on GBP-1 function. Having established porcine BMDM as novel primary cell PRRSV culture system, this was exploited to explore the role of GBP-1 on PRRSV in a biologically relevant cell type. The data reported in this Chapter suggest that wild-type GBP-1 exerted a strong antiviral effect on PRRSV, but the mutation introduced did not significantly alter this effect. Nevertheless, there is a need to consider the effect of variation in the GBP-1 gene, given that it has been shown to be an effective anti-PRRSV host protein. Furthermore, the investigation into the genetic variation that exists in diverse pig breeds has the potential to inform selective breeding programs for PRRSV tolerance or resistance.

Mapping of the PRRSV host response-associated WUR10000125 SNP to the 3′UTR of GBP-1 prompted investigation into the potential impact of this mutation. *In silico* analysis of the sequence surrounding the WUR10000125 SNP revealed that the SNP fell directly adjacent to a putative additional PAS in the 3′UTR of GBP-1. The sequence, AATAAA, was identical for both PAS, with the distal PAS apparently being utilised by the polyadenylation factors in published GBP-1 sequences. APA of the pGBP-1 mRNA at the proximal PAS would result in the loss of predicted binding sites for miR339-5p and miR2320 as well as two predicted AREs. As a result, the shorter 3′UTR could potentially increase mRNA stability, influencing GBP-1 gene expression. Preliminary PCR analysis of 3′UTR transcript abundance in samples from heterozygous WUR10000125 and wild-type animals revealed that the proximal PAS was not utilised exclusively in any animals. Nonetheless, the data suggested a potential difference in the relative abundance of “short” and “long” 3′UTRs in WUR10000125 SNP individuals, consistent with some use of the upstream PAS. However, these data were not fully conclusive and an alternative approach for identifying the effect of genetic variation in pGBP-1 was explored.

The high LD in the SSC4 region identified by Boddicker *et al.* (2012) as being associated with the host response to PRRSV, complicates the identification of
a single causative mutation resulting in the observed PRRSV resistance phenotype. Additionally, the sensitivity of a SNP genotyping study is directly related to the coverage of the SNP-chip over the area in question. For these reasons, and because it supplied a simple, readily testable hypothesis, it was decided to investigate a published missense SNP in the CDS of GBP-1, known as rs80955804. The amino acid at this SNP position was largely conserved and bioinformatic approaches revealed a possible detrimental effect of the rs80955804 polymorphism on GBP-1 protein function and the coiled coil domain. Additionally, an amino acid substitution at this position could potentially influence GBP-1 tetramerisation. Given the uncertainties presented thus far, the functional effect of the rs80966804 SNP was investigated through the cloning of porcine GBP-1 and the generation of the GBP1_g1486a mutant, replicating the rs80966804 SNP.

Following the successful transfection of CHO-K1 cells with GBP-1 constructs, it was evident that a difference existed in the subcellular localisation of the wild-type and mutant proteins. GBP1_wt staining was mainly in a diffuse pattern, with GBP1_g1486a exhibiting a higher proportion of perinuclear staining. The membrane association of hGBP-1 has been shown to be related, in part, to both its GTPase activity and its ‘CaaX’ prenylation motif (Britzen-Laurent et al., 2010). Given the location of the rs80955804 SNP, it is highly unlikely that this mutation would impact GTPase activity (not in close proximity of the LG domain) or prenylation (full length protein still translated, with intact C-terminus) of pGBP-1. However, the mutation could potentially affect its dimerisation and tetramerisation state, which have both been shown to influence the association of GBPs with cellular membranes (Britzen-Laurent et al., 2010; Vöpel et al., 2010). Heterodimer formation regulates the sub-cellular localisation of GBPs, with hGBP-1 having been shown to recruit hGBP-2 and hGBP-5 into its own cellular compartment (Britzen-Laurent et al., 2010). The difference in subcellular localisation between GBP1_wt and GBP1_g1486a observed in CHO-K1 cells appeared to be related to the single missense mutation introduced in the α12 helix to mimic the rs80955804 SNP. hGBP-1 employs two different binding sites for self-assembly. One binding site is located within the LG domain and is triggered by the binding of GTP. Enzymatic activity leads to a structural shift making buried sites on α12/13 available for interaction with
another α12/13 domain, leading to coiled-coil formation of two α12/13 subdomains (Syguda et al., 2012). Additionally, hGBP-1 α12/13 helices have been shown to interact with the LG domain to facilitate tetramer formation (Vöpel et al., 2010). Thus, the pGBP-1 rs80955804 SNP may affect GBP-1 homo- or hetero-dimerisation through disruption of the helical domain, presenting in these experiments as an alteration in subcellular localisation pattern.

A direct interaction of GBP-1 on PRRSV replication was supported by the inhibition of virus replication upon co-transfection of the SD01-08 infectious clone and GBP-1 plasmids into the CHO-K1 cell line. The GBP1_wt and GBP1_g1486a staining patterns suggested the formation of antiviral protein ‘clusters’ of high expression around the sites of PRRSV nsp2 accumulation. An important caveat is that this could have been, in part, a result of the anti-V5 antibody being unable to efficiently access its epitope in these regions. Nevertheless, the observed colocalisation of GBP-1 with SD01-08 nsp2 upon co-transfection, along with a change in the localisation of GBP-1 and the PRRSV nsp2-GFP fusion protein to a perinuclear localisation indicated that GBP-1 may interfere with the localisation of PRRSV proteins (including the nsp2-GFP fusion protein). This has been previously described for hGBP-1 in the case of IAV nsp 1 (Zhu et al., 2013). Additionally, this is a strategy employed by the antiviral GTPase Mx1, which sequesters Thogoto virus (a member of the Orthomyxoviridae) nucleocapsids from nuclear import, preventing viral genome transcription (Kochs & Haller, 1999). Arterivirus nsp2 has been shown to act as a membrane anchor for the assembly of multi-protein replication complexes (Snijder et al., 2001). Therefore, the redirection of PRRSV nsp2 to the perinuclear region and possible formation of GBP-1 protein ‘clusters’ around the viral protein could potentially inhibit the formation of PRRSV replication complexes.

To test the effect of pGBP-1 on a true PRRSV infection, experiments were performed in BMDM. Both pGBP-1 constructs tested significantly reduced the permissiveness of these cells to infection by PRRSV. This effect appeared to be a direct inhibition of virus replication, confirmed by the lack of effect of GBP-1 transfection on control GFP expression. Additionally, quantification of released virus by qRT-PCR and serial dilution infectivity assays both revealed a dramatic reduction in released virus in GBP1_wt and GBP1_g1486a-transfected BMDM. The
significant inhibition of PRRSV replication seen upon overexpression of GBP1\(_{\text{wt}}\) and GBP1\(_{\text{g1486a}}\) in BMDM confirmed the earlier observations in a fully infectable, permissive cell type. Little difference was detected between the anti-PRRSV effects of wild type and mutant GBP-1. Thus, not supporting the simple hypothesis that the nonsynonymous SNP explains the phenotypic effects correlated with the WUR10000125 SNP. However, the apparent impact of the g1486a mutation on pGBP-1 localisation, possibly mediated through tetramerisation or heterodimerisation with other GBP\(_s\), provides further insight into the important functional domains of the pGBP-1 protein.

In order to fully characterise the causative SNP for the PRRSV host-response QTL, the LD must be broken in the 1 Mb region of SSC4. This is likely to require full sequencing of this genomic region in multiple animals with the WUR10000125 SNP and exhibiting the PRRSV resistance phenotype, followed by bioinformatic and functional experimentation. LD varies significantly between pig breeds, especially between the centres of pig domestication. Chinese breeds exhibit much lower LD than European breeds and modern breeding programs have further increased the LD in the latter (Amaral \textit{et al.}, 2008). LD in European breeds extends over large haploblocks of up to 400 kb, with haploblocks of less than 10 kb in Chinese breeds (Amaral \textit{et al.}, 2008). For this reason, it may be of value to attempt to dissect, and break, the LD in the SSC4 QTL region by looking at the sequence variation in this region in a variety of genetically distinct pig breeds.

Genotyping data have been gathered by the pig HapMap project for the WUR10000125 SNP in pig samples spanning 71 breeds and 29 geographical regions (Groenen \textit{et al.}, 2010; Ramos \textit{et al.}, 2009). Plotting the frequency of the beneficial G allele at this SNP location, according to the geographical origin of the sample, indicated an East-West divide (Figure 4.15). The Eastern pig breeds sampled in this study had a higher mean beneficial (G) allele frequency than inbred Western breeds (Figure 4.15). This may prove to be an additional benefit of conducting research into a host-genetic basis for PRRSV resistance using animals from these divergent pig breeds.
Figure 4.15 | Global distribution of WUR10000125 G-allele frequency. SNP genotyping data for pigs sampled by the Porcine HapMap project (Groenen et al., 2010) are coloured by the mean ‘G’ (beneficial) allele frequency at the WUR10000125 PRRSV-resistance locus and shown according to their geographical sampling location. Pig-dense regions are further annotated with the allele frequency of all breeds sampled at that locality.
The complex issues surrounding the control of PRRS have placed an impetus on host genetics as a potential measure for limiting the economic and welfare impacts of this costly disease. The data presented in this Chapter have revealed a novel antiviral target for pGBP-1, adding to the, already large repertoire of known anti-pathogen activities of the GBPs. The investigation of a published missense SNP in pGBP-1 revealed a minimal impact on the antiviral activity of the protein. However, the alteration of subcellular localisation as a result of this SNP enhances our limited knowledge of the function of the GBP-1 protein domains.

In addition to the in vitro antiviral effect of pGBP-1, the presence of an index SNP for a convincing PRRSV resistance trait in the vicinity of pGBP-1, as well as 32 additional SNPs spanning all known members of the porcine GBP family, prompts further investigation as to the role of GBP-1 in PRRSV infection. The differential regulation of pGBP-1 in more and less PRRSV-resistant individuals also poses the question of whether this gene could be a target for genetic manipulation or selective breeding efforts with the aim of reducing the economic burden of PRRS on the pig industry.
CHAPTER FIVE

OVERVIEW OF MAIN FINDINGS, CONCLUDING REMARKS AND FUTURE PERSPECTIVES
The work described in this thesis began by attempting to improve existing cell culture systems for \textit{in vitro} PRRSV research. In the course of these investigations, an additional aim became to elucidate alternative PRRSV entry pathways into primary cell types. The analysis of genome-wide transcriptional data looking at the dynamics of the host response to virus infection prompted the further examination of candidate genes as targets for the genetic improvement of pigs for PRRSV resistance. The investigation of one such candidate resulted in the discovery of a novel anti-PRRSV gene. Overall, the results presented in this thesis provide new insights into host-virus interactions in PRRSV infection, both in the case of virus receptors and immune-responsive proteins. The majority of studies were carried out or verified on biologically relevant porcine primary cells, facilitated by the use of the BMDM model system developed during the research.

Chapter two of this thesis reported the characterisation of the 3D4/21 cell line, a cell line that is referred to in the literature as being a “porcine alveolar macrophage” line (Chen \textit{et al.}, 2013; Lee \textit{et al.}, 2010; Lee & Lee, 2012). The aim of this work was to establish the use of a biologically relevant continuous cell line in order to advance the study of host-virus interactions in PRRSV research. However, the use of the 3D4/21 cell line was found to be untenable, due to the non-macrophage-like phenotype and aberrant expression of LTFs and PRRSV receptors. Porcine BMDM were investigated as an alternative to the use of continuous cell lines and were found to be fully permissive to type I PRRSV infection, to a greater degree than other infectable cell types tested. Moreover, the transfectability of BMDM and the large numbers in which they could be derived, reinforced the utility of these primary porcine macrophage cells in routine \textit{in vitro} experimentation. Finally, the data presented in Chapter two have uncovered the existence of an alternative entry mechanism for PRRSV into a porcine primary cell type, independent of the prototypic PRRSV receptors CD169 and CD163.

Important questions were raised as a result of the findings in Chapter two. Firstly, if the PRRSV entry mechanism into BMDM does not require the CD163 and CD169 receptors, how is the virus gaining entry and uncoating? Given that the infection of BMDM is more productive than the other permissive cells tested, is this
alternative entry mechanism more efficient and is this pathway also utilised in vivo? In this regard, it would be of interest to attempt to elucidate the proteins exploited by PRRSV for entry into BMDM. One possible future experiment would employ the virus overlay protein binding assay (VOPBA), where BMDM proteins transferred to nitrocellulose membranes are probed with virus in order to detect binding partners. The VOBPA procedure has previously been used to identify receptors for viruses such as: mouse hepatitis virus (Boyle et al., 1987), lymphocytic choriomeningitis virus (Borrow & Oldstone, 1992) and visna virus (Dalziel et al., 1991). This technique has also demonstrated the binding of PRRSV to simian vimentin (Kim et al., 2006), which may perform a receptor function in the infection of the MARC-145 monkey epithelial cell line (Kim et al., 2006), so it may be a feasible route forward.

The work presented in Chapter three examined two genome-wide, porcine transcriptional datasets in an attempt to further our understanding of the host response to PRRSV infection through network-based analysis techniques. The comparative analysis of in vitro respiratory and in vivo reproductive PRRSV infection datasets revealed clusters of co-expressed transcripts that were conserved between studies. The representation of apparently PRRSV resistant animals in one dataset gave an additional dimension to the interpretation of the observed differences in the transcriptional response to infection between the individuals sampled. Furthermore, the data-driven analyses reported in Chapter three highlighted genes in key PRRSV-induced clusters that have recently been implicated in PRRSV resistance in genetic association studies. Members of the GBP family were represented in the network analysis of both datasets. Loci encoding the porcine GBP proteins map to a chromosomal region associated with variation in viral load and weight gain in response to PRRSV infection.

The results described in Chapter three also raised further questions. The identification of conserved transcript clusters in separate microarray datasets and differences in key clusters which appeared to be associated with the susceptibility of cell preparations to PRRSV infection, prompts further investigation into their membership. The integration of genotype data with microarray profiling of infected animals could provide new insights into the genetic basis for the observed individual
variation in the susceptibility and host response to PRRSV infection. This type of data may be made available by the PHGC in the future, facilitating such an approach.

Chapter four focussed on the cloning and overexpression of porcine GBP-1. Informatic analyses of the GBP-1 nucleotide sequence revealed the potential for alternative polyadenylation to occur, resulting in 3`UTR variants. Overexpression of GBP-1 along with a PRRSV infectious clone demonstrated colocalisation, as well as an apparent change in subcellular localisation of both PRRSV nsp2 and GBP-1 upon co-transfection with a PRRSV plasmid clone. A powerful antiviral effect was also observed upon the overexpression of GBP-1 in BMDM, followed by PRRSV infection. Testing the functional effect of the introduction of a published SNP to the porcine GBP-1 gene showed that it did not significantly influence the anti-PRRSV activity of the protein but did appear to alter its subcellular localisation pattern.

Several questions arose as a result of the work presented in Chapter four. Firstly, what is the mechanism behind the observed antiviral effect of pGBP-1 on PRRSV replication? The dramatic reduction in the proportion of infected BMDM upon pGBP-1 transfection suggested that GBP-1 may be resulting in the priming of surrounding un-transfected cells through a yet to be elucidated mechanism. The primary aim of Chapter four was to establish the potential of the GBP family as targets for the genetic improvement of pigs for PRRSV resistance. The data generated in this Chapter suggest that, along with the implication of the chromosomal region encoding the GBP family in PRRSV resistance traits, at least one family member can exert an antiviral effect on PRRSV in vitro. Thus, further investigation into naturally existing genetic variation in the genomic region encoding the GBP family in animals exhibiting resistance to PRRSV may prove to be a valuable approach in informing pig breeding programs.

Given the existence of multiple published PRRSV entry mediators, as well as the discovery of an apparently alternative entry pathway in BMDM, receptor research is unlikely to provide an answer to the problem of PRRSV. This assertion has also been confirmed in a CD169 PRRSV receptor knockout pig where the ablation of CD169 expression had no measurable effect on measured aspects of
PRRSV infection, including viremia (Prather et al., 2013). However, BMDM provide an opportunity to better understand possible alternative PRRSV entry mechanisms into primary cells. Given the overall lack of efficacy of PRRSV vaccines in providing heterologous protection and the ineffective host immune response mounted against the virus, it may be more productive to focus on enhancing the pig’s immune response to PRRSV. The differential regulation of biological pathways in animals from different breeds, which also possess varying degrees of PRRSV resistance, provides a better understanding of the immune response to PRRSV. The availability of robust QTLs in genomic regions encoding an entire family of immune-related genes led to the identification of the GBP protein family. The discovery of the GBP-1 protein as having an antiviral effect on PRRSV both validates the use of BMDM as an experimental system for the investigation of host-virus interactions, and provides a candidate gene with an anti-PRRSV effect located within a QTL associated with PRRSV resistance traits.
CHAPTER SIX

MATERIALS AND METHODS
### 6.1 Materials

#### 6.1.1 General reagents

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<tr>
<th>Reagent</th>
<th>Supplier</th>
<th>Product code</th>
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<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Sigma-Aldrich</td>
<td>A2153</td>
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<tr>
<td>Cell Fix (10X)</td>
<td>Becton-Dickinson</td>
<td>340181</td>
</tr>
<tr>
<td>CellWash</td>
<td>BD Biosciences</td>
<td>349524</td>
</tr>
<tr>
<td>Distel High Level Laboratory Disinfectant</td>
<td>Tristel</td>
<td>N/A</td>
</tr>
<tr>
<td>Distilled water, DNase/RNase Free</td>
<td>GIBCO</td>
<td>10977</td>
</tr>
<tr>
<td>DMEM</td>
<td>GIBCO</td>
<td>61965-026</td>
</tr>
<tr>
<td>Ethanol, Absolute, Analytical reagent grade</td>
<td>Fisher Scientific</td>
<td>E/0650DF/17</td>
</tr>
<tr>
<td>Fix and Perm, (Solution B)</td>
<td>Life Technologies</td>
<td>GAS-004</td>
</tr>
<tr>
<td>GMEM</td>
<td>GIBCO</td>
<td>21710-025</td>
</tr>
<tr>
<td>Heat-inactivated Foetal Bovine Serum (HI-FBS)</td>
<td>PAA</td>
<td>N/A</td>
</tr>
<tr>
<td>L-Glutamine 200 mM (100x)</td>
<td>GIBCO</td>
<td>25030-024</td>
</tr>
<tr>
<td>Opti-MEM medium</td>
<td>GIBCO</td>
<td>11058-021</td>
</tr>
<tr>
<td>Paraformaldehyde 16 % w/v</td>
<td>Alfa Aesar</td>
<td>43368</td>
</tr>
<tr>
<td>Penicillin/Streptomycin (pen/strep) 10,000 U/mL</td>
<td>GIBCO</td>
<td>15140-122</td>
</tr>
<tr>
<td>Phosphate buffered saline (PBS), 10x, pH 7.2</td>
<td>GIBCO</td>
<td>70013-032</td>
</tr>
<tr>
<td>Propan-2-ol, Analytical reagent grade</td>
<td>Fisher Scientific</td>
<td>P/7500/17</td>
</tr>
<tr>
<td>rhCSF-1</td>
<td>Prof. Hume lab, gift</td>
<td>N/A</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>GIBCO</td>
<td>61870-010</td>
</tr>
<tr>
<td>Saponin</td>
<td>Fluka BioChemika</td>
<td>47036</td>
</tr>
<tr>
<td>SOC</td>
<td>Invitrogen</td>
<td>15544-034</td>
</tr>
<tr>
<td>Sodium Azide</td>
<td>Sigma-Aldrich</td>
<td>S2002</td>
</tr>
<tr>
<td>Sterile water for irrigation, hypotonic</td>
<td>Baxter</td>
<td>UKF7114</td>
</tr>
<tr>
<td>SYBR Safe DNA gel stain (10,000X in DMSO)</td>
<td>Invitrogen</td>
<td>S33102</td>
</tr>
<tr>
<td>TEMED</td>
<td>Sigma Aldrich</td>
<td>T9281</td>
</tr>
<tr>
<td>Triton X100</td>
<td>Sigma Aldrich</td>
<td>T8787-250ml</td>
</tr>
<tr>
<td>TRIzol Reagent</td>
<td>Life Technologies</td>
<td>15596-026</td>
</tr>
<tr>
<td>Trypan Blue Solution</td>
<td>Sigma Aldrich</td>
<td>T8154</td>
</tr>
<tr>
<td>TrypLE Express Enzyme 1X, no phenol red</td>
<td>GIBCO</td>
<td>12604-013</td>
</tr>
<tr>
<td>Trypsin EDTA, 1x solution</td>
<td>Sigma Aldrich</td>
<td>T3924-100ml</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Sigma Aldrich</td>
<td>P9416</td>
</tr>
<tr>
<td>0.5M EDTA</td>
<td>GIBCO</td>
<td>15575-038</td>
</tr>
</tbody>
</table>
6.1.2 Enzymes

DNA restriction enzymes were supplied by Agilent Technologies, Roche, and New England Biolabs.

6.1.3 Oligonucleotides

A) Lineage-specific transcription factor – qRT-PCR quantification

<table>
<thead>
<tr>
<th>Oligo ID</th>
<th>Sequence (5’ to 3’)</th>
<th>Source</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PU.1_rtPCR_F [104-122]</td>
<td>CAG GGG ACC TGA CAG GCT C</td>
<td>Life Technologies</td>
<td>DST</td>
</tr>
<tr>
<td>PU.1_rtPCR_R [187-209]</td>
<td>GTC ATA GGG AAC CAG GTC TTC TG</td>
<td>Life Technologies</td>
<td>DST</td>
</tr>
<tr>
<td>C/EBP-A_rtPCR_F [528-545]</td>
<td>GCG CTG GCC GGC CTC TTT</td>
<td>Life Technologies</td>
<td>DST</td>
</tr>
<tr>
<td>C/EBP-A_rtPCR_R [621-642]</td>
<td>CAG TGC GCG ATC TGG AAC TGC A</td>
<td>Life Technologies</td>
<td>DST</td>
</tr>
<tr>
<td>C/EBP-B_rtPCR_F [908-926]</td>
<td>TGC GCA ACC TGG AGA CGC A</td>
<td>Life Technologies</td>
<td>DST</td>
</tr>
<tr>
<td>C/EBP-B_rtPCR_R [1001-1023]</td>
<td>ACA AGT TCC GCA GGG TGC TGA G</td>
<td>Life Technologies</td>
<td>DST</td>
</tr>
<tr>
<td>Beta-Actin_F [319-338]</td>
<td>GAG AAG CTG TGC TAC GTC GC</td>
<td>Alison Wilson, gift</td>
<td>DST</td>
</tr>
<tr>
<td>Beta-Actin_R [397-417]</td>
<td>CCA GAC AGC ACT GTG TTG GC</td>
<td>Alison Wilson, gift</td>
<td>DST</td>
</tr>
<tr>
<td>GAPDH_F [164-180]</td>
<td>TTC CAC GGC ACA GTC AA</td>
<td>Alison Wilson, gift</td>
<td>DST</td>
</tr>
<tr>
<td>GAPDH_R [246-263]</td>
<td>GCA GGT CAG GTC CAC AA</td>
<td>Alison Wilson, gift</td>
<td>DST</td>
</tr>
</tbody>
</table>

B) Lineage-specific transcription factor - coding sequence cloning

<table>
<thead>
<tr>
<th>Oligo ID</th>
<th>Sequence (5’ to 3’)</th>
<th>Source</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pig PU.1 FW [1-27]</td>
<td>ATG TTA CAG GCG TGC AAA ATG GAA GGG</td>
<td>Life Technologies</td>
<td>DST</td>
</tr>
<tr>
<td>pig PU.1 REV [1016-1039]</td>
<td>CCG GCG AGG GGT TAA TGC TAT GGC</td>
<td>Life Technologies</td>
<td>DST</td>
</tr>
<tr>
<td>pig C/EBP-A FW [1-25]</td>
<td>ATG GAG TCG GCC GAC TTC TAC GAG G</td>
<td>Life Technologies</td>
<td>DST</td>
</tr>
<tr>
<td>pig C/EBP-A REV [1073-1095]</td>
<td>CCA CTC CCT GGG TCC CAG GCA G</td>
<td>Life Technologies</td>
<td>DST</td>
</tr>
<tr>
<td>pig C/EBP-B FW [1-21]</td>
<td>ATG CAA CGC CTG GTG GCC TGG</td>
<td>Life Technologies</td>
<td>DST</td>
</tr>
<tr>
<td>pig C/EBP-B REV [1049-1069]</td>
<td>CCC GGA GTC ACT GTG GCC GC</td>
<td>Life Technologies</td>
<td>DST</td>
</tr>
</tbody>
</table>
C) **PRRSV genome quantification qRT-PCR (ORF5)**

<table>
<thead>
<tr>
<th>Oligo ID</th>
<th>Sequence (5' to 3')</th>
<th>Source</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRRSV_Orf5_F</td>
<td>GCT TTA CCC GGT TGC CAC TCA TA</td>
<td>Alex Brown, gift</td>
<td>DST</td>
</tr>
<tr>
<td>[214-237]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRRSV_Orf5_R</td>
<td>GTA CAT ACC GCC CGC CAA CAA ATC</td>
<td>Alex Brown, gift</td>
<td>DST</td>
</tr>
<tr>
<td>[333-357]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D) **GBP-1 coding sequence cloning (pEF6 vector)**

<table>
<thead>
<tr>
<th>Oligo ID</th>
<th>Sequence (5' to 3')</th>
<th>Source</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBP1_cds 1 F</td>
<td>ATG GCC TCA AAG GTG CAC</td>
<td>Invitrogen</td>
<td>DST</td>
</tr>
<tr>
<td>[1-18]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBP1_cds 1 R</td>
<td>TTA GCT CAG GAA ACA TTC TTT CT T</td>
<td>Invitrogen</td>
<td>DST</td>
</tr>
<tr>
<td>[1800-1823]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBP1_cds_V5 R</td>
<td>GCT CAG GAA ACA TTC TTT CT T TG</td>
<td>Invitrogen</td>
<td>DST</td>
</tr>
<tr>
<td>[1803-1823]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E) **GBP-1 site-directed mutagenesis**

<table>
<thead>
<tr>
<th>Oligo ID</th>
<th>Sequence (5' to 3')</th>
<th>Source</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGBP1_g1486a_s</td>
<td>TGA GAG CTG AAG CTA CAC AGG CTG CAG CC</td>
<td>Sigma Aldrich</td>
<td>PAGE</td>
</tr>
<tr>
<td>pGBP1_g1486a_as</td>
<td>GGC TGC AGC CTG TGT AGC TTC AGC TCT CA</td>
<td>Sigma Aldrich</td>
<td>PAGE</td>
</tr>
</tbody>
</table>

F) **GBP-1 3'UTR-length PCR**

<table>
<thead>
<tr>
<th>Oligo ID</th>
<th>Sequence (5' to 3')</th>
<th>Source</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBP1_cds 1 F</td>
<td>ATG GCC TCA AAG GTG CAC</td>
<td>Invitrogen</td>
<td>DST</td>
</tr>
<tr>
<td>[1-18]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBP1_cds 1 R</td>
<td>TTA GCT CAG GAA ACA TTC TTT CT T</td>
<td>Invitrogen</td>
<td>DST</td>
</tr>
<tr>
<td>[1800-1823]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBP1_cds_V5 R</td>
<td>GCT CAG GAA ACA TTC TTT CT T TG</td>
<td>Invitrogen</td>
<td>DST</td>
</tr>
<tr>
<td>[1803-1823]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

G) **Non-targeting control siRNA**

<table>
<thead>
<tr>
<th>Oligo ID</th>
<th>Sequence (5' to 3')</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silencer Cy3 Labeled Negative Control No. 1 siRNA</td>
<td>non targeting (scrambled)</td>
<td>AM4621, Ambion</td>
</tr>
</tbody>
</table>
6.1.4 Immunological reagents

A) Primary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Label</th>
<th>Host species</th>
<th>Application</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-pig CD169</td>
<td>FITC</td>
<td>mouse</td>
<td>FC</td>
<td>41D3, gift from Prof. Hans Nauwynck</td>
</tr>
<tr>
<td>anti-pig Monocyte/Granulocyte (SWC3a)</td>
<td>PE</td>
<td>mouse</td>
<td>FC</td>
<td>Southern Biotech, 4525-09</td>
</tr>
<tr>
<td>anti-pig SLA class II DR</td>
<td>FITC</td>
<td>mouse</td>
<td>FC</td>
<td>Rural Technologies</td>
</tr>
<tr>
<td>anti-pig mAb CD163 2A10/11</td>
<td>FITC</td>
<td>mouse</td>
<td>FC/IF</td>
<td>AbD Serotec, MCA2311F</td>
</tr>
<tr>
<td>anti-pig mAb CD163 2A10/11</td>
<td>PE</td>
<td>mouse</td>
<td>FC</td>
<td>AbD Serotec, MCA2311PE</td>
</tr>
<tr>
<td>anti-human pAb CD163</td>
<td>none</td>
<td>mouse</td>
<td>FC/IF/IHC/WB</td>
<td>RnD Systems, 1607-CD-050</td>
</tr>
<tr>
<td>SDOW-17</td>
<td>FITC</td>
<td>mouse</td>
<td>FC/IF/IHC</td>
<td>RTI, SDOW17-F</td>
</tr>
<tr>
<td>Beta-Actin</td>
<td>none</td>
<td>mouse</td>
<td>WB</td>
<td>Thermo Scientific, RB-9421-P0</td>
</tr>
<tr>
<td>anti-V5</td>
<td>none</td>
<td>mouse</td>
<td>FC/IF/WB</td>
<td>Invitrogen, 46-0705</td>
</tr>
<tr>
<td>anti-Beta-actin (reacts with Chinese Hamster)</td>
<td>none</td>
<td>mouse</td>
<td>WB</td>
<td>Abcam, 8226</td>
</tr>
<tr>
<td>anti-tubulin alpha (reacts with pig)</td>
<td>none</td>
<td>rat</td>
<td>WB</td>
<td>AbD Serotec, MCA77G</td>
</tr>
</tbody>
</table>

B) Secondary and isotype control antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Label</th>
<th>Host species</th>
<th>Isotype</th>
<th>Application</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse isotype control</td>
<td>none</td>
<td>mouse</td>
<td>IgG2a, κ</td>
<td>FC</td>
<td>BD Pharmingen, 554126</td>
</tr>
<tr>
<td>anti-mouse</td>
<td>FITC</td>
<td>rabbit</td>
<td>IgG</td>
<td>FC/IF</td>
<td>AbD Serotec</td>
</tr>
<tr>
<td>anti-mouse</td>
<td>AlexaFluor 594</td>
<td>rabbit</td>
<td>IgG (H + L)</td>
<td>FC/IF</td>
<td>Invitrogen, A11062</td>
</tr>
<tr>
<td>anti-rabbit</td>
<td>HRP</td>
<td>rabbit</td>
<td>IgG (H + L)</td>
<td>IHC</td>
<td>RnD Systems, FIN1012081</td>
</tr>
<tr>
<td>anti-mouse</td>
<td>HRP</td>
<td>rabbit</td>
<td>IgG (H + L)</td>
<td>IHC</td>
<td>Dako Denmark, P0260</td>
</tr>
<tr>
<td>anti-rat</td>
<td>none</td>
<td>goat</td>
<td>IgG (H + L)</td>
<td>Blocking</td>
<td>Invitrogen, A10536</td>
</tr>
<tr>
<td>anti-Mouse</td>
<td>IRDye 800CW</td>
<td>donkey</td>
<td>IgG (H + L)</td>
<td>WB</td>
<td>LI-COR, 926-32212</td>
</tr>
<tr>
<td>anti-Mouse</td>
<td>IRDye 680CW</td>
<td>donkey</td>
<td>IgG (H + L)</td>
<td>WB</td>
<td>LI-COR, 926-68072</td>
</tr>
<tr>
<td>anti-rat</td>
<td>IRDye 680CW</td>
<td>goat</td>
<td>IgG (H + L)</td>
<td>WB</td>
<td>LI-COR, 926-68076</td>
</tr>
</tbody>
</table>
6.1.5 Solutions and media

A) Eukaryotic tissue culture media

Complete culture media composition:

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Media composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMC/BMDM</td>
<td>RPMI 1640, Glutamax, 10 % v/v HI-FBS, 1:100 pen/strep, 10,000 U/mL rhCSF-1</td>
</tr>
<tr>
<td>AM</td>
<td>RPMI 1640, Glutamax, 10 % v/v HI-FBS, 1:100 pen/strep</td>
</tr>
<tr>
<td>3D4/21</td>
<td>RPMI 1640, 4.5 g/L D-glucose, 2.383 g/L HEPES, 1:100 L-glutamine, 1.5 g/L Sodium Bicarbonate, 110 mg/L Sodium Pyruvate, NEAA, 10 % v/v HI-FBS, pen/strep (optional: 10,000 U/mL rhCSF-1)</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>RPMI, 10 % v/v HI-FBS, 1:100 Pen/Strep</td>
</tr>
<tr>
<td>BHK-21</td>
<td>GMEM (BHK-21, Gibco), 1:100 L-glutamine, 1:200 Tryptose Phosphate Broth, 5 % v/v HI-FBS, 1:100 pen/strep</td>
</tr>
<tr>
<td>MARC-145</td>
<td>DMEM, 10 % v/v HI-FBS, 1:100 L-glutamine, 1:100 pen/strep</td>
</tr>
<tr>
<td>HEK-293</td>
<td>DMEM, 10 % v/v HI-FBS, 1:100 pen/strep</td>
</tr>
</tbody>
</table>

Freezing media (continuous cell lines):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete culture medium</td>
<td>6.7 mL</td>
</tr>
<tr>
<td>HI-FBS</td>
<td>1.3 mL</td>
</tr>
<tr>
<td>DMSO</td>
<td>2 mL</td>
</tr>
</tbody>
</table>

Cells were diluted 1:1 in freezing mix and their usual complete growth medium.

Freezing media (primary cells):

90 % v/v HI-FBS + 10 % v/v DMSO. Cell pellets resuspended in freezing mix only.

B) Prokaryotic culture media

All prokaryotic culture media, such as LB broth and LB-agar was prepared and supplied by the Central Services Unit within the Roslin Institute.
C) Other solution and buffers

**DNA loading buffer (10x):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>50 % (v/v)</td>
</tr>
<tr>
<td>EDTA</td>
<td>100 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>1 % (w/v)</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.1 % (w/v)</td>
</tr>
<tr>
<td>Xylene cyanol</td>
<td>0.1 % (w/v)</td>
</tr>
</tbody>
</table>

**Agarose gel Tris-acetate-EDTA (TAE) running buffer (10x):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-acetate</td>
<td>0.4 M</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

**8 % polyacrylamide gel:**

<table>
<thead>
<tr>
<th>Component</th>
<th>8 % Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 % polyacrylamide (4°C)</td>
<td>2.64 mL</td>
<td>1.3 mL</td>
</tr>
<tr>
<td>4x Resolving/Stacking buffer</td>
<td>2.5 mL</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>MiliQ water</td>
<td>4.72 mL</td>
<td>6.1 mL</td>
</tr>
<tr>
<td>10 % APS</td>
<td>100 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>TEMED (4 °C)</td>
<td>10 µL</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

**Western blot 10X running buffer:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>30.3 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>144 g</td>
</tr>
<tr>
<td>SDS</td>
<td>10 g</td>
</tr>
</tbody>
</table>

Solution at pH 8, diluted with water for 1X running buffer.
Western blot transfer buffer:
90 % v/v 1x running buffer and 10 % v/v methanol.

Western blot 2X loading buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 % Glycerol</td>
<td>20 mL</td>
</tr>
<tr>
<td>SDS</td>
<td>4 g</td>
</tr>
<tr>
<td>1M Tris pH8</td>
<td>10 mL</td>
</tr>
<tr>
<td>DTT</td>
<td>3.08 g</td>
</tr>
<tr>
<td>Water</td>
<td>to 100 mL</td>
</tr>
</tbody>
</table>

PBS (Tween20, 0.1 %):

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>1 L</td>
</tr>
<tr>
<td>Tween20</td>
<td>0.1 % (v/v)</td>
</tr>
</tbody>
</table>

Western blot blocking solution:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skimmed milk powder</td>
<td>2.5 g</td>
</tr>
<tr>
<td>PBS (Tween20, 0.1 %)</td>
<td>to 50 mL</td>
</tr>
</tbody>
</table>

Red blood cell lysis buffer (for preparation of AM and BMDM)

<table>
<thead>
<tr>
<th>Component</th>
<th>1000ml</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KHCO₃</td>
<td>1.0 g</td>
<td>10 mM</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>8.29 g</td>
<td>155 mM</td>
</tr>
<tr>
<td>0.5M EDTA</td>
<td>200 μL</td>
<td>0.1 mM</td>
</tr>
</tbody>
</table>

This was then dissolved in 500 mL dH₂O, adjusted to pH 8.0 and made up to 1 L. 50 mL volumes were filter sterilised using 0.2 μm syringe filters and stored at 4 °C.

70 % ethanol:
70 % (v/v) absolute ethanol, 30 % (v/v) dH₂O
Tissue culture PBS (1X):
100 mL 10x PBS (pH 7.2) + 900 mL sterile water for irrigation

50 mM Acetate buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M acetic acid</td>
<td>74 mL</td>
</tr>
<tr>
<td>0.2 M sodium acetate</td>
<td>176 mL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>750 mL</td>
</tr>
</tbody>
</table>

AEC (3-Amino-9-ethylcarbazole) substrate:
One AEC tablet was dissolved in 2.5 mL N,N-dimethylformamide. 0.5 mL of this solution was then added to 9.5 mL 50 mM acetate buffer, with a further 5 µL of 30 % (w/v) hydrogen peroxide added immediately prior to use.

FC PBS:
(2.5 % (v/v) FBS, 0.01 % (v/v) sodium azide)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>482.5 mL</td>
</tr>
<tr>
<td>FBS</td>
<td>12.5 mL</td>
</tr>
<tr>
<td>Sodium azide (1 %)</td>
<td>5 mL</td>
</tr>
</tbody>
</table>

FC PBS w. Saponin:
(0.5 % (v/v) Saponin, 0.1 % BSA (v/v), 0.01 % (v/v) azide)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>440 mL</td>
</tr>
<tr>
<td>BSA (10 %)</td>
<td>5 mL</td>
</tr>
<tr>
<td>Sodium azide (1 %)</td>
<td>5 mL</td>
</tr>
<tr>
<td>Saponin (5 %)</td>
<td>50 mL</td>
</tr>
</tbody>
</table>

50 % glycerol:
50 % (v/v) glycerol, 50 % (v/v) nuclease-free dH2O
6.1.6  *Eukaryotic cells used*

A)  Continuous cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK-21</td>
<td>Gift from Prof. David Hume lab</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>Gift from Prof. David Hume lab</td>
</tr>
<tr>
<td>HEK-293</td>
<td>Gift from Dr. Simon Lillico</td>
</tr>
<tr>
<td>MARC-145</td>
<td>ATCC, CRL-12231</td>
</tr>
<tr>
<td>3D4/21</td>
<td>Gift from Prof. Hans Nauwynck</td>
</tr>
</tbody>
</table>

B)  Primary cells

AM were isolated in our laboratory, either by myself, Dr. Alison Wilson or Dr. Tahar Ait-Ali. BMC were either collected by myself from animals euthanised in our laboratory, or provided as a gift by Dr. Ronan Kapetanovic. Sexually mature gilt provided by Dr. Simon Lillico.

6.1.7  *Virus stocks*

AM-grown PRRSV H2 virus stocks were generated by Dr. Alison Wilson and provided as a gift. SD01-08 CMV plasmid (see 6.1.9) provided as a gift by Dr. Ying Fang, virus rescue performed myself.

6.1.8  *Drugs and compounds*

- Cytochalasin D from *Zygosphorium mansonii* (C8273, Sigma Aldrich). Dissolved in DMSO to a stock concentration of 2mM.
- *E. coli* (K-12 strain) BioParticles®, Fluorescein Conjugate (E-2861, Molecular Probes)
- Zymosan A *S. cerevisiae* BioParticles, Fluorescein Conjugate (Z2841, Molecular Probes)
6.1.9 Plasmids

- **pSD01-08_CMV_GFP** (Fang *et al.*, 2006). Gift from Dr. Ying Fang.
- **pGEM®-T Easy Vector System I** (A1360, Promega)
- **pEF6/V5-His TOPO TA expression kit** (K9610-20, Life Technologies, Carlsbad, California)
- **pEF6_GBP1wt** (untagged)
- **pEF6_GBP1wt_V5**
- **pEF6_GBP1_g1486a_V5**
- **pEF6_lacZ_V5**
- **pMAX_GFP** (Lonza)

6.2 Molecular cloning and nucleic acid preparation

6.2.1 Bacterial culture and glycerol stock preparation

Following transformation, bacteria were streaked on LB agar plates containing the antibiotic appropriate to the plasmid insert. Following an overnight incubation at 37 °C, single colonies were picked using sterile 10 μL pipette tips. These pipette tips were then used to inoculate a 5 mL LB broth culture (following colony PCR if appropriate) containing appropriate antibiotics in a 15 mL polypropylene tube. These tubes were placed on their side in a shaking incubator at 37 °C for 8 hours to overnight, after which, an aliquot of this starter culture could be used to inoculate a large overnight culture at 1:1000 dilution (i.e. 250 μL starter culture + 250 mL LB broth). At this point, a glycerol stock was also prepared by adding an equal volume of bacterial starter culture to 50 % glycerol (v/v in dH₂O), on wet ice. Glycerol stocks were stored at -80 °C.

6.2.2 Ligation of DNA

A) pGEM-T Easy vector system

Freshly amplified PCR products were used in ligation reactions using 2x reaction buffer, following the manufacturer’s recommendations.
B) pEF6/V5-His TOPO TA expression kit

Primers were designed according to the manufacturer’s guidelines and PCR cycling was performed as normal, followed by agarose gel purification (see 6.2.13). Fresh, gel purified PCR products were ligated into pEF6/V5-His TOPO TA as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product</td>
<td>4 μL</td>
</tr>
<tr>
<td>Salt solution</td>
<td>1 μL</td>
</tr>
<tr>
<td>pEF6/V5-His TOPO TA</td>
<td>1 μL</td>
</tr>
</tbody>
</table>

The reaction was incubated for 5 minutes at room temperature prior to freezing at -20 °C overnight. The following day, 2 μL of each ligation was transformed into XL1-Blue chemically competent cells as below.

6.2.3 Transformation of XL1-Blue competent cells

1.5 mL tubes were incubated on ice and SOC medium was pre-heated to 42 °C. XL1-Blue competent cells (200249, Stratagene) were thawed gently on ice, gently mixed and transferred in 100 μL aliquots into each pre-chilled tube. 1.7 μL β-mercaptoethanol was added to each aliquot of competent cells, swirled gently and incubated on ice for 10 minutes, swirling every 2 minutes. 2 μL DNA was added to each cell aliquot, swirled gently and incubated on ice for a further 30 minutes. Tubes were then subjected to a heat-pulse in a 42 °C water bath for exactly 45 seconds before incubating the tubes on ice for 2 minutes. 500 μL preheated 42 °C SOC medium was added to each tube and incubated at 37 °C for 1 hour in a shaking incubator at 225 rpm.

For high copy-number plasmids, 100 μL and 25 μL (+ 75 μL SOC medium for the same total volume) were plated onto two separate antibiotic-containing LB agar plates per respective reaction. For low copy-number plasmids the 500 μL transformation reaction was centrifuged at 380 x g for 7 minutes and the cell pellet was resuspended in 200 μL SOC medium. Then, 100 μL and 50 μL (+ 50 μL SOC medium for the same total volume) of the concentrated transformation reaction were spread on two separate antibiotic-containing LB agar plates per reaction. LB agar
plates were incubated at 37 °C overnight until adequate growth of bacterial colonies was observed.

6.2.4 Preparation of plasmid DNA
Small-scale preparation of plasmid DNA was performed on 4 mL overnight cultures of *E. coli* grown in the presence of the appropriate antibiotic, using the PureYield Plasmid Miniprep System (A1223, Promega) or QIAprep Spin Miniprep Kit (27104, Qiagen) according to the manufacturer’s instructions. Large-scale DNA preparation of transfection-grade plasmid DNA was prepared using the EndoFree Plasmid Maxi kit (12362, Qiagen) according to the manufacturer’s instructions. An overnight 100 to 250 mL culture was used as starting material, depending on the plasmid copy-number. The concentration of DNA prepared was determined by measuring the absorbance of DNA in water at 260 nm with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). The purity of the preparation was estimated by comparison of OD 260 nm to OD 280 nm ratios.

6.2.5 PCR
PCR reactions were carried out either in semi-skirted PCR plates (AB-0990, Thermo Scientific) or 0.2 mL PCR tubes (AB-0266, Thermo Scientific). Reactions were performed in a total volume of 10 μL, or scaled up to 50 μL as required.

A master mix was prepared according to the number of reactions, including a negative control with water instead of DNA, and accounting for pipetting loss:

<table>
<thead>
<tr>
<th>Master mix</th>
<th>10 μL reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>2.92 μL</td>
</tr>
<tr>
<td>10xbuff + 20 mM Mg²⁺</td>
<td>1 μL</td>
</tr>
<tr>
<td>2 mM dNTPs</td>
<td>1 μL</td>
</tr>
<tr>
<td>10 pmol/μl primerF</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>10 pmol/μl primerR</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>5U/μl FastStart Taq</td>
<td>0.08 μL</td>
</tr>
<tr>
<td>DNA + dH₂O</td>
<td>4 μL</td>
</tr>
</tbody>
</table>
The master mix was mixed thoroughly and briefly centrifuged, before adding 6 μL of mix to each tube containing DNA in dH2O. Tubes or plates were sealed and placed in an MJ Research PC-225 Thermal Cycler. Cycling programs were run with the heated lid option, as follows for a standard 30 cycle program:

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>5 min</td>
<td>95 °C</td>
<td>Denaturation / Taq activation</td>
</tr>
<tr>
<td>Step 2</td>
<td>30 s</td>
<td>95 °C</td>
<td>Denaturation</td>
</tr>
<tr>
<td>Step 3</td>
<td>30 s</td>
<td>Usually 58 °C</td>
<td>Primer annealing</td>
</tr>
<tr>
<td>Step 4</td>
<td>60 s/kb</td>
<td>72 °C</td>
<td>Elongation</td>
</tr>
<tr>
<td>Step 5</td>
<td></td>
<td></td>
<td>Go to Step 2 an additional 29 times</td>
</tr>
<tr>
<td>Step 6</td>
<td>10 min</td>
<td>72 °C</td>
<td>Final extension</td>
</tr>
</tbody>
</table>

PCR products were used immediately for cloning into TA expression vectors. Unused PCR products were stored at -20 °C.

6.2.6 GBP-1 3’UTR PCR

In order to assess the PAS of the GBP-1 mRNA 3’UTR, cDNA generated from all animals in the RIVivo PRRSV infection experiment was used (Dr. Alison Wilson, gift). SNP genotypes were available for all animals (Appendix 2). Samples from both reproductive lymph node and spleen of each animal were used. The same quantity of total RNA was used in the RT-PCR for cDNA synthesis, allowing direct comparison of cDNA samples. Standard PCR was performed using the two primer sets, either spanning or downstream of the putative PAS site within the GBP-1 3’UTR (see 6.1.3 F for details of primers). PCRs were performed as in 6.2.5, but in a total volume of 25 μL, with 2 μL cDNA template per reaction. PCR thermal cycling conditions were as in 6.2.5, but with an annealing temperature of 58 °C and 25 cycles. Resultant PCR products were immediately loaded onto a 1 % agarose gel for electrophoresis and quantification.

6.2.7 Bacterial colony PCR

Direct PCR of a bacterial colony was performed in order to perform a diagnostic digest or to directly sequence an insert. PCR reactions were performed as normal (see 6.2.5), with the exception of the addition of dH2O in the place of DNA. 10 μL
master mix was added to PCR tubes or plates and a sterile 10 μL pipette tip was used to pick a colony from a plate of colonies post-transformation. The pipette was briefly dipped into the PCR reaction and then streaked on a correspondingly labelled quadrant of an LB agar plate. PCR cycling was performed as usual and the LB agar plate was incubated so that it could be used to inoculate a liquid culture following the results of the colony PCR.

6.2.8 *Reverse-transcription (RT-PCR):*

Total RNA (isolated using the Trizol reagent) was used for cDNA synthesis using TaqMan Reverse Transcription Reagents (Life Technologies, N8080234) kit in a 50 μL reaction volume. 1 μg RNA was made up to a volume of 19.25 μL in dH₂O and added to 30.75 μL RT reaction mix as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x buffer</td>
<td>5 μL</td>
</tr>
<tr>
<td>MgLl2</td>
<td>11 μL</td>
</tr>
<tr>
<td>dNTPs</td>
<td>10 μL</td>
</tr>
<tr>
<td>Random hexamers or oligo d(T)₁₆</td>
<td>2.5 μL</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>1 μL</td>
</tr>
<tr>
<td>Reverse transcriptase</td>
<td>1.25 μL</td>
</tr>
</tbody>
</table>

Thermal cycling parameters were as follows:

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>25 °C</td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>48 °C</td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>95 °C</td>
<td></td>
</tr>
</tbody>
</table>

Resultant cDNA was stored at -20 °C.

6.2.9 *Host gene expression quantification by qRT-PCR:*

qRT-PCR detection was performed using the SYBR Green I dye. Platinum SYBR Green qPCR SuperMix (Life Technologies, 11733-046) was used according to the manufacturer’s recommendations, with the exception of performing reactions in a
scaled down total volume of 25 μL. 2.5 μL freshly prepared cDNA was added to 22.5 μL qRT-PCR reaction mixture, composed of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW primer (10 μM)</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>REV primer (10 μM)</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>9 μL</td>
</tr>
<tr>
<td>Rox reference dye</td>
<td>0.1 μL</td>
</tr>
<tr>
<td>Platinum SYBR Green qPCR SuperMix-UDG</td>
<td>12.5 μL</td>
</tr>
</tbody>
</table>

Standard curves for each primer with serial dilutions of cDNA were included on each plate. Thermal cycling was performed on a MX3000 machine (Agilent Technologies), collecting data for SYBR green and using ROX as the reference dye. The following thermal cycling parameters were employed:

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time/sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50°C</td>
<td>120</td>
</tr>
<tr>
<td>1</td>
<td>95°C</td>
<td>120</td>
</tr>
<tr>
<td>40</td>
<td>95°C</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>30</td>
</tr>
</tbody>
</table>

Data were analysed using the MxPro software (Agilent Technologies)

6.2.10 **Restriction enzyme digest**
DNA was digested for 1 hour with 3 to 5 units (per μg of DNA) of the appropriate enzyme. Enzymes were used in the manufacturer’s recommended buffer and digestion was carried out at the recommended temperature.

6.2.11 **Agarose gel electrophoresis**
1 % agarose gels were used for electrophoresis and were prepared by dissolving agarose in TAE. SYBR Safe DNA Gel Stain was added to the molten agarose at a 1:10,000 dilution for the visualisation of DNA. Gels were submerged in TAE buffer and samples loaded in DNA loading buffer (6.1.5C). Gels were run at 10 V/cm for approximately 90 minutes and visualised using the GelDoc system (Bio-Rad).
6.2.12 Quantification of agarose gel band intensity
Gel band quantification was performed using the “Measure” functionality in the ImageJ software (1.47v, NIH, USA). Images of agarose gels captured using the same exposure settings using the GelDoc system (Bio-Rad), were loaded into the ImageJ software and identical sized boxes were placed around the fluorescent bands on the image, representing each PCR product. The measure function was used and background intensity was subtracted from all measurements.

6.2.13 Gel purification of DNA
DNA fragments were separated by agarose gel electrophoresis. The fragments were visualized by UV trans-illumination and the required fragments were excised using a sterile scalpel. Agarose slices were transferred to a 1.5 mL microtube and DNA was extracted using either QIAquick Gel Extraction (Qiagen, 28704) or MinElute Gel Extraction (Qiagen, 28604) kits, according to manufacturer’s instructions.

6.2.14 Cloning of porcine GBP-1
cDNA generated from total RNA extraction of spleen tissue was used for the PCR cloning of GBP-1 (cDNA provided by Dr. Alison Wilson). cDNA used was from the spleen of a Landrace pregnant gilt from the RIVivo experiment, ID:1662, with a homozygous A:A genotype at the WUR1000125 locus (Appendix 2). PCR was performed using primers amplifying the entire coding sequence (for primer details see 6.1.3 D). The cloning PCR was performed in a 50 μL reaction volume with thermal cycling conditions, as in 6.2.5, but with an extension time of 90 seconds and an annealing temperature of 58 °C. The resulting PCR-products were analysed by agarose gel electrophoresis and a single band was excised and purified. The low abundance of GBP-1 in all tissues tested resulted in the need to further amplify the GBP-1 coding sequence. This was achieved by the ligation of the purified PCR-product into the pGEM-T-Easy plasmid vector and subsequent growth and plasmid purification. The resultant, high copy-number pGEM-T-Easy_GBP1(1662)cds plasmid was then used as the template for further manipulation. The GBP-1(1662) coding sequence was either directly ligated into pEF6/V5-His TOPO for un-tagged,
or had the stop codon removed by PCR (for primer details see 6.1.3 D) for cloning in-frame with V5/His tags in the pEF6/V5-His TOPO vector, for tagged versions. Sequencing of the GBP-1(1662) clone revealed 99.5 % amino acid identity to published porcine GBP-1 sequences (Appendix 5) and the nucleotide sequence was deposited as GenBank accession number: KF977840.

6.2.15 Site-directed mutagenesis (SDM)
SDM was performed using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, 200523). Mutagenic primers were designed using the QuikChange Primer Design Software (http://www.agilent.com/genomics/qcpd) according to the specific mutation parameters (see 6.1.3E). Primers were diluted to a working concentration of 100 ng/μL. DNA template used was pEF6_GBP1(1662)_V5/His (clone 1) at a concentration of 10 ng/μL (serially diluted).

The sample reaction was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x reaction buffer</td>
<td>5 μL</td>
</tr>
<tr>
<td>dsDNA template (at 10 ng/μL)</td>
<td>1 μL</td>
</tr>
<tr>
<td>Sense primer (at 100 ng/μL)</td>
<td>1.25 μL</td>
</tr>
<tr>
<td>Antisense primer (at 100 ng/μL)</td>
<td>1.25 μL</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>1 μL</td>
</tr>
<tr>
<td>QuikSolution</td>
<td>3 μL</td>
</tr>
<tr>
<td>Nuclease-free dH₂O</td>
<td>37.5 μL</td>
</tr>
</tbody>
</table>

1 μL PfuUltra HF DNA polymerase (2.5U) was then added to the reaction mix and thermal cycling was performed using the following parameters:

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95 °C</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95 °C</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>55 °C</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68 °C</td>
<td>60 seconds/kb plasmid length</td>
</tr>
</tbody>
</table>
The sample was then cooled to 37 °C, when 1 μL DpnI enzyme was added and the sample was incubated for 1 hour at 37 °C in order to digest parental supercoiled dsDNA. 2 μL sample was transformed using XL1- Blue chemically competent cells as normal and grown overnight at 37 °C on LB-Ampicillin plates. Transformants were picked and grown in liquid culture overnight when DNA was extracted as normal and purified DNA was screened for the correct mutation by sanger sequencing (see 6.2.16). A 100 % success rate was achieved for the g1486a mutation.

6.2.16 DNA sequencing
DNA sequencing was performed by DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

6.3 Eukaryotic cell culture

6.3.1 Cell passage
All cell types were incubated at 37 °C and 5 % CO₂ for routine growth. When continuous cell lines reached confluency, the medium was removed and the monolayer was washed with PBS. Warmed trypsin EDTA or room-temperature TrypLE Express was added to the flask and incubated at 37 °C until cells had fully detached. Cells were washed in complete growth medium, followed by centrifugation at 380 x g for 5 minutes. The cell pellet was then gently resuspended in complete medium and cells were counted at a 1:10 dilution in 0.1 % v/v trypan blue solution using a haemocytometer, when necessary, prior to use or re-seeding for continued culture at an appropriate density.

6.3.2 Cell thawing
Frozen cells were retrieved from the -155 °C freezer and transported to the laboratory on wet ice. Cells were thawed rapidly in a 37 °C water bath until a small amount of ice still remained within the tubes. Cells were then transferred to a 50 mL polypropylene tube and warmed PBS or RPMI-1640 (for BMC only) was added
dropwise to a volume of 50 mL. Cells were then centrifuged at 380 x g for 5 minutes, resuspended in an appropriate volume of growth medium, counted and seeded at an appropriate cell density into tissue culture plates.

6.3.3 Bone marrow cell isolation

BMC were obtained from pigs from a number of different breeds and ages. BMC in pigs younger than 12 weeks of age were collected post-mortem following sedation, stunning by captive bolt and pithing. After evisceration, the body cavity was flushed with sterile PBS and all ribs were removed after severing the costochondral junction and the dorsal section of the rib at the costovertebral joint. The intact ribs were removed fully and their intercostal muscles were dissected away with Mayo curved scissors to avoid epithelial or fibroblastic contamination of subsequent cultures. The ribs from each animal were placed in a zip-loc bag on wet ice prior to processing. Femurs were dissected out in a similar fashion. For rib collection from mature animals euthanized following embryo donation, two central ribs were removed through an incision on the flank of the animal, shortly following euthanasia by anaesthetic overdose.

Bones were transferred to a biological safety cabinet and sterilised in a bath of 70 % ethanol. They were then transferred to a bath of dH2O to remove traces of ethanol, before being allowed to dry briefly. For the ribs of weaner pigs, the intact bones had both ends removed using Rongeur’s or large, bypass loppers. For the ribs of adult pigs or femurs of juvenile pigs, the bones were sectioned, in addition to having the ends removed, to permit flow-through of flushing medium. For all bones, a gas sterilised, 20G Jamshidi Bone Marrow Biopsy Needle was inserted into the exposed BM cavity and the cavity was flushed with flushing medium (RPMI 1640 + GlutaMax, no FBS, no antibiotics). The flush was collected in sterile 50 mL Falcon tubes and the flush was repeated with fresh flushing medium from the opposite end of the same rib section. The flush supernatant was centrifuged at 380 x g for 10 mins in order to pellet the cells and the supernatant was discarded. Red-cell lysis buffer (see 6.1.5C) was then added in order to remove any red cell contamination. All tubes were then topped up with sterile PBS and centrifuged again at 380 x g for 10 mins. The pellet was then counted and re-suspended in primary cell freezing mix (see
6.1.5A) and frozen at -20 °C for 24 h, then -80 °C for 24 h before finally moving to -155 °C for long term storage.

6.3.4 BMDM differentiation
BMDM were removed from the -155 °C freezer and thawed in a 37 °C water bath before adding 50 mL room temperature PBS dropwise, centrifuging at 380 x g for 10 mins and re-suspending the cell pellet in culture and differentiation media (consisting of RPMI 1640 + Glutamax + 10 % v/v HI-FBS + pen/strep + 10,000 U/mL rhCSF-1). Cells were grown at an approximate density of 1.5 x 10⁶ cells per mL in square plastic dishes (un-treated plastic surface) for 6 days in the presence of rhCSF-1. After this period, the supernatant, containing all non-adherent cells, was removed and discarded. The remaining, adherent BMDM were dislodged from the plate by jetting with sterile PBS in a 20 mL syringe through an 18G blunt mixing needle. Any remaining adherent cells were dislodged using a cell scraper or TrypLE Express (following a PBS wash). The BMDM were then pelleted and resuspended at an appropriate density for subsequent experiments.

6.3.5 Alveolar macrophage isolation
Juvenile pigs were sedated using ketamine and euthanised by captive bolt, followed by pithing, to ensure the humane dispatch of the animals. The chest was opened post-mortem, and the trachea, lungs and heart were removed intact, with the mouth of the trachea clamped with locking forceps. The lungs and heart were placed in a covered beaker for transport to sterile conditions in a Biosafety Category 2 laboratory.

Alveolar lavage was carried out by introducing around 750 mL of 37 °C PBS into the lungs through a sterile funnel securely inserted into the mouth of the trachea. The lungs were gently massaged for around 30 seconds, before the PBS was poured directly from the trachea, into a sterile bottle. This process was repeated 2-3 times. Lavage fluid was then transferred into 50 mL polypropylene tubes and centrifuged for 10 minutes at 380 x g. The supernatant was removed into a waste beaker containing a Haz-Tab chlorine disinfection tablet (H8811, Guest Medical, Kent, UK), taking care not to disturb the cell pellet. Red blood cell contamination was
removed by adding 5 mL red-cell lysis buffer (6.1.5C) for up to 5 minutes. Tubes were then topped up with PBS and centrifuged once more. The cell pellet was carefully resuspended, typically into twenty 1 mL cell aliquots per pig, in primary cell freezing mix (6.1.5A) and frozen at -20 °C for 24 h, then -80 °C for 24 h before finally moving to -155 °C for long term storage.

6.3.6 Plasmid lipofection

A) Lipofectamine 2000 (SD01-08 virus production)

For the production of virus-containing cell culture supernatant, a 90 % confluent 75 cm² flask of BHK-21 cells was prepared in the absence of pen/strep in the media. Transfections were performed with the SD01-08 infectious clone plasmid (CMV promoter) using Lipofectamine 2000 transfection reagent (11668-019, Invitrogen).

For transfection of one 75 cm² flask of BHK-21 cells, 30 μg plasmid DNA was mixed with 2 mL Opti-MEM medium. 75 μL Lipofectamine was added to 2 mL Opti-MEM medium and incubated for 5 minutes at room temperature. Following the 5 minute incubation, the DNA and Lipofectamine-containing solutions were combined to make a 4 mL volume of transfection complex solution. This solution was incubated for a further 20 minutes at room temperature to allow DNA-lipid complexes to form. During this incubation period, the growth medium was removed from the culture flask and the cells were washed once in Opti-MEM medium. Following the 20 minute incubation period, the transfection complexes were added to the cell culture flask and incubated for 6 hours at 37 °C and 5 % CO₂. After 6 hours the flasks were topped up with 10 mL growth medium (no pen/strep). Cell culture supernatant was harvested at 48 hpt and centrifuged briefly to pellet cell debris, before being aliquoted and stored at -80 °C.

B) FuGENE HD

For the transfection of the CHO-K1 cell line seeded on coverslips in a 24-well plate, cells were seeded at a density of 5 x 10⁴ cells per well 24 hours prior to transfection, in growth medium without pen/strep.
For transfection of one well of a 24-well plate, FuGENE HD transfection reagent (E2311, Promega) was allowed to equilibrate to room temperature prior to use. Directly prior to transfection, 26 μL of 0.02 μg/μL plasmid solution (0.55 μg DNA in 26 μL total volume) was prepared in OptiMEM media in a 1.5 mL microtube. 1.7 μL FuGENE HD reagent was added directly into the plasmid solution, without touching the sides of the tube. The solution was mixed by pipetting up and down 15 times and incubated at room temperature for 10 minutes before the direct addition of 25 μL to the growth medium and mixing. Cells were then incubated for 24 hours at 37 °C in a 5 % CO₂ incubator.

For qRT-PCR quantification of virus genome in the cell culture supernatant, the growth medium was removed at 4 hpt and cells were washed thoroughly with PBS before the addition of fresh complete growth medium.

6.3.7 Plasmid nucleofection

For the transfection of BMDM with plasmid DNA, nucleofection was performed using the 96-well Shuttle System (AAM-1001S, Lonza) as a plate-handler, coupled to, and powered by, a Nucleofector II device (AAB-1001, Lonza). Prior to the Nucleofection reaction, supplement was added to Solution P4, as follows for a single 20 μL reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution P4</td>
<td>16.4 μL</td>
</tr>
<tr>
<td>Supplement</td>
<td>3.6 μL</td>
</tr>
</tbody>
</table>

The 96-well shuttle software (Lonza) was used to create a plate map, determining the nucleofection program to be performed on each well, including no-electroporation controls and empty wells. The optimum program for both cell-viability and transfection efficiency was determined to be DP-163, using the Primary Cell P4 nucleofection solution. 96-well plates were prepared containing 110 μL growth medium (without pen/strep) and incubated at 37 °C in a 5 % CO₂ incubator, ready to receive nucleofected cells.

TrypLE Express, or jetting and scraping were employed to dislodge BMDM from untreated plastic culture dishes following 6 days of culture in the presence of
rhCSF-1. Cells were washed in PBS, centrifuged at 380 x g for 5 minutes and the cell pellet resuspended in an appropriate volume of PBS for counting at 1:10 dilution in Trypan Blue solution. Nucleofections were typically performed at 2 x 10^5 cells per reaction, in a volume of 20 μL supplement-containing nucleofection solution, using the 96-well shuttle device nucleo-cuvette plates. Thus, a cell number appropriate to the number of reactions being performed was aliquoted into separate 1.5 mL microtubes per condition. The microtubes were centrifuged at 380 x g for 5 minutes. The supernatant was carefully removed and the cell pellet was resuspended in the 20 μL of Solution P4 (containing supplement) for each reaction to be performed. DNA was added to the cell-containing nucleofection solution at this point, at a concentration of 500 ng/reaction. 20 μL for each sample was transferred to the bottom of each well of a sterile nucleo-cuvette plate. The plate was tapped gently on the bench to ensure that nucleofection reactions were in contact with the electrodes in the base of the wells. The lid on the nucleo-cuvette plate ensures sterility, so the plate was removed from the biological safety cabinet at this point and loaded into the 96-well shuttle device and pulsed using the DP-163 program. A nucleofection report was generated, allowing the assessment of the success of the nucleofection. The plates were rested at room temperature for 10 minutes following nucleofection. Following this, the plates were moved into a biological safety cabinet and 80 μL pre-warmed growth media (without pen/strep) was added to each well and pipetted up and down. 90 μL was then transferred from the nucleo-cuvette plates to a pre-incubated 96-well tissue culture plate, containing 110 μL growth media (without pen/strep). These 96-well plates were incubated at 37 °C in a 5 % CO₂ incubator.

6.3.8 siRNA electroporation

BMDM were harvested as for routine sub-culture and a cell count was performed using a haemocytometer. Cells were pelleted at 380 x g for 5 mins and resuspended in RPMI only, to achieve a concentration of 1 x 10^6 cells/mL. 1 μL of 10 μM siRNA stock was resuspended in a final volume of 10 μL of sterile, endotoxin-free PBS and added to the bottom of cuvettes prior to the addition of cells. The cell suspension was agitated by flicking, prior to the addition of 200 μL (2 x 10^5 cells) to the siRNA-containing cuvette, giving a final siRNA concentration of 50 nM. Cells were
incubated in the presence of siRNA in their cuvettes for 5 mins at room temperature. Cuvettes were tapped to resuspend cells and placed into the electroporation port of a Gene Pulser II (BioRad) and pulsed at 250 V, with high capacitance set to 500 μF and a resistance of 1000 Ω. Following the electroporation reaction, 500 μL media was added to cuvettes and BMDM were resuspended and added to 14 mL complete medium. The cells were washed by pelleting at 380 x g for 5 mins and resuspending the cell pellet in pre-warmed complete medium. Cells were then plated at the desired cell density for onward culture.

6.4 Virus work

6.4.1 Generation of SD01-08 viral stock
SD01-08 infectious cell-culture supernatant was generated by transfection of the infectious clone plasmid (CMV promoter) into BHK-21 cells by lipofection, as outlined in 6.3.6A.

6.4.2 Virus infection
For virus infection of adherent cells, cells were seeded at an appropriate density, according to the size of the culture vessel and cell type, 24 h prior to PRRSV infection. Infections were performed with SD01-08-GFP virus, a Lelystad-like cloned virus (Fang et al., 2006) or PRRSV H2 (Drew et al., 1997). Complete medium was removed and cells were washed with PBS. Infections were carried out in minimal volumes, adjusting virus stocks with growth medium to the desired concentration for the appropriate MOI, as determined by TCID$_{50}$. Cells were typically incubated with virus at 37 °C for 60 minutes prior to the removal of the inoculum and overlaying of complete growth medium.

6.4.3 Quantification of virus stocks by TCID$_{50}$

A) Virus infections
AM or BMDM were seeded in 96 well culture plates at a density of $1 \times 10^5$ cells/well in complete growth media (100 µL volume per well) at 24 h prior to infection. The following virus dilutions, in complete growth medium, were made:

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/10</td>
</tr>
<tr>
<td>2</td>
<td>1/100</td>
</tr>
<tr>
<td>3</td>
<td>1/1000</td>
</tr>
<tr>
<td>4</td>
<td>1/10000</td>
</tr>
<tr>
<td>5</td>
<td>1/100000</td>
</tr>
<tr>
<td>6</td>
<td>1/1000000</td>
</tr>
</tbody>
</table>

50 µL of each dilution was used to inoculate multiple replicate wells of the plate seeded with cells (making a total of 150 µL per well). Plates were transferred to a 37 °C incubator (5 % CO$_2$) for 36 hpi.

**B) PRRSV antibody staining**

After 36 hours incubation at 37 °C, wells were either examined for GFP expression under a fluorescence microscope (for PRRSV SD01-08 virus infected cells) or stained using the SDOW17-A monoclonal antibody for light microscopic analysis of PRRSV H2-infected cells.

For *in situ* PRRSV antibody staining, supernatants were removed after 48 hpi and wells were washed with 0.9 % v/v NaCl (100 µL/well) and then air dried under a heat lamp for 2 h. Cells were washed with PBS (100 µL/well) and fixed using 1 % (w/v) paraformaldehyde in PBS for 10 minutes at room temperature (100 µL/well). The PBS wash was repeated and the PRRSV monoclonal antibody SDOW17-A was added (50 µL/well) at a 1:250 dilution in 0.5 M NaCl, 1 % Tween 80 and incubated at 37 °C for 1 h. The antibody was removed and wells were washed a further three times with PBS, 0.5 % v/v Tween 80 (100 µL/well). HRP-conjugated rabbit anti mouse secondary antibody was added (50 µL/well) at a 1:150 dilution in 0.5 M NaCl, 1 % v/v Tween 80 and incubated in the dark at 37 °C for 1 h. Wells were washed a further three times with PBS, 0.5 % v/v Tween 80 and 50 µL/well of AEC substrate ($6.1.5C$) was added. Following a 15 minute incubation in the dark at 37 °C, the substrate was removed and the wells washed three times with PBS, 0.5 % v/v...
Tween 80 (100 µL/well) before viewing under a light microscope. Cells positive for the SDOW17-A epitope on the PRRSV N-protein stained pink/purple in colour.

C) TCID\textsubscript{50} calculation

The Spearman-Karber method was used to calculate TCID\textsubscript{50}/mL titre, based on the proportion of wells of each virus dilution containing PRRSV-positive cells. The equation used was as follows:

\[
\log\left( \frac{\text{TCID}_{50}}{\text{Volume unit}} \right) = \text{Area} + d_1 - \log(V_{\text{test}}) = \frac{d_f}{2} p_1 + d_f \sum_{i=2}^{N} p_i + \frac{d_f}{2} p_{N+1} + d_1 - \log(V_{\text{test}}) \\
= \frac{d_f}{2} + d_f \sum_{i=2}^{N} p_i + d_1 - \log(V_{\text{test}})
\]

Where:

- \(d_1\) = the log of the highest dilution where all the wells are positive
- \(N\) = the number of dilutions after \(d_1\) where some wells are still positive
- \(D_f\) = the dilution factor
- \(d_f\) = the log of the dilution factor, \(D_f\)
- \(p_i\) = the fraction of positive wells for dilution \(i\) \((1 \leq i \leq N+1)\).
- \(V_{\text{test}}\) = the volume of the test substrate

The TCID\textsubscript{50} figure was converted to PFU/mL, in order to permit the estimation of MOI, by multiplying the titre by 0.7.

6.4.4 Blocking of virus infection

For antibody blocking of AM or BMDM in a 12-well plate format, anti-CD163 pAb 1607 (0.2 mg/mL), mAb 41D3 or goat anti-rat IgG isotype control antibodies were made up at appropriate concentrations in 300 µL minimal media (RPMI 1640 + Glutamax only). pAb CD163 was used at a concentration of 2 µg/100 µL, 41D3 was used at 3.3 µL/100 µL and the isotype control antibody was used at 2 µg/100 µL. Complete growth media was removed from the cells and they were washed with PBS, the antibody-containing media was then added in a volume of 300 µL to each treated well of a 12-well plate and incubated at 37 °C for 1 hour. The antibody-containing media was then removed and the cells washed in PBS prior to
the addition of SD01-08 or PRRSV H2 at MOI 1. The cells were incubated for a further hour at 37 °C in the presence of the virus inoculum, then the inoculum was removed and the cells were washed in PBS to remove un-bound virus particles. Then complete growth media (RPMI 1640 + Glutamax + pen/strep + 10 % v/v FBS) was added and the cells were incubated until 10 hours post-infection at 37 °C when they were subjected to flow cytometric analysis.

For cytochalasin D treatment, blocking was performed as above. AM and BMDM were treated with varying concentrations (2 μM, 4 μM and 10 μM) of cytochalasin D. However, the complete growth medium added post-infection also contained cytochalasin D. The “treatment control” sample was only exposed to cytochalasin D at 90 minutes post-infection, following endocytosis of PRRSV particles.

6.5 RNA analysis

6.5.1 Isolation of total RNA from adherent cells

3 mL TRIzol Reagent was added to a 90 % confluent 75 cm² flask. This cell-lysate-containing liquid was then transferred to a new nuclease-free microtube and centrifuged for 10 min at 12,000 x g at 5 °C. The supernatant (avoiding aspirating the pelleted cell debris) was carefully transferred to a new nuclease-free microtube and incubated at room temperature for 5 minutes. In a fume hood, 200 μL chloroform was added and the sample was shaken vigorously for 15 seconds. The sample was then incubated at room temperature for a further 2 to 3 minutes. Following this incubation, the sample was centrifuged for 15 min at 12,000 x g at 5 °C. The upper aqueous phase was carefully transferred to a clean microtube and RNA was precipitated by the addition of 500 μL isopropanol and incubation at room temperature for 10 minutes. The sample was then centrifuged for 10 min at 12,000 x g at 5°C and the supernatant aspirated from the RNA pellet. The pellet was then washed with 1 mL 75 % ethanol, vortexed and centrifuged for 5 min at 7,500 x g at 5 °C. The pellet was then allowed to air-dry and resuspended in a suitable volume of nuclease-free dH₂O. RNA samples were quality checked by measuring OD₂₆₀ and OD₂₈₀ ratio of a 1:100 dilution on a Nanodrop ND-1000 spectrophotometer. Samples
were then subjected to a cleanup using the RNeasy MinElute Cleanup Kit (74204, QIAGEN), as per the manufacturer’s recommendations. Resultant RNA was immediately stored at -80 °C.

6.5.2 Isolation of virus RNA from cell culture supernatant

RNA was isolated from 140 μL cell culture supernatant using the QIAamp Viral RNA Mini Kit (52904, Qiagen, Hilden, UK) according to the manufacturer’s instructions (using carrier RNA and eluting in 60 μL volume of supplied elution buffer).

6.5.3 Quantification of PRRSV genome by one-step qRT-PCR

RNA purified using the QIAamp Viral RNA Mini Kit (52904, Qiagen, Hilden, UK) was used as the template in qRT-PCR reactions. qRT-PCR was performed using the SuperScript III Platinum SYBR Green qRT-PCR kit (11736-051, Life Technologies) as per the manufacturer’s instructions (scaled down to a reduced volume of 25 μL). Briefly, the reaction master mix (for one 25 μL reaction), was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SuperScript III RT/Platinum Taq Mix (including RNase OUT)</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>2X SYBR Reaction Mix</td>
<td>12.5 μL</td>
</tr>
<tr>
<td>PRRSV_Orf5_F</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>PRRSV_Orf5_R</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>ROX Reference Dye</td>
<td>0.1 μL</td>
</tr>
<tr>
<td>nuclease-free dH2O</td>
<td>1 μL</td>
</tr>
<tr>
<td>RNA template</td>
<td>10 μL</td>
</tr>
</tbody>
</table>

Standard curves were also included on each plate and consisted of virus infection medium at a known TCID₅₀ and viral RNA at a known concentration, both were serially diluted in nuclease-free water 2.5 μL of each dilution was loaded into each well, along with 7.5 μL nuclease-free water and the reaction master mix. Thermal cycling was performed on a MX3000 machine (Agilent Technologies) using the following protocol:
<table>
<thead>
<tr>
<th>Temperature/°C</th>
<th>Time(min:sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>48</td>
</tr>
<tr>
<td>Step 2</td>
<td>95</td>
</tr>
<tr>
<td>Step 3</td>
<td>95</td>
</tr>
<tr>
<td>Step 4</td>
<td>60</td>
</tr>
<tr>
<td>Step 5</td>
<td>go to step 3, 39 times</td>
</tr>
<tr>
<td>Step 6</td>
<td>40</td>
</tr>
<tr>
<td>Step 7</td>
<td>95</td>
</tr>
<tr>
<td>Step 8</td>
<td>60</td>
</tr>
<tr>
<td>Step 9</td>
<td>95</td>
</tr>
<tr>
<td>Step 10</td>
<td>25</td>
</tr>
<tr>
<td>Step 11</td>
<td>25</td>
</tr>
<tr>
<td>Step 12</td>
<td>25</td>
</tr>
</tbody>
</table>

Data were collected for both SYBR-Green and ROX, with ROX being used as the reference dye and were analysed using the MxPro software (Agilent Technologies). The standard curve used to extrapolate the relative TCID50/mL from Ct values was as follows:
6.6 Protein analysis

6.6.1 Western blot

A) Sample preparation
Protein lysates were produced by adding a suitable volume of 2X loading buffer, following the removal of growth medium from adherent cell cultures. Lysates were stored at -80 °C prior to use. Directly prior to loading, protein lysates were thawed and subjected to 95 °C in a heat block for 5 minutes, vortexed extensively and heated for a further 5 minutes at 95 °C. Samples were then centrifuged at 14,000 rpm in a micro-centrifuge for 1 minute in order to pellet remaining debris.

B) Running and transfer
Resolving gels with 8% stacking gels were cast in a ‘mini-PROTEAN 3’ protein gel apparatus (Bio-Rad) and stored overnight at 4 °C prior to use. Prepared samples were loaded at appropriate dilutions and the gels were electrophoresed vertically at 120 V until the bromophenol blue marker dye ran out of the stacking gel and into the resolving gel. At this point, the voltage was increased to 150 V and run until the ladder markers reached the desired position on the gel. Proteins were transferred to a nitrocellulose membrane alongside an ice pack to avoid overheating. Transfer was performed at 100 V for 1 h.

C) Probing
Post-transfer, the nitrocellulose membrane was trimmed to a minimal area and incubated in blocking solution for 30 minutes at room temperature on a rocking stage. Blocking solution was removed and the membrane was washed four times for 10 minutes each in PBS (Tween20, 0.1 % v/v) in a container on a rocking platform. Up to two nitrocellulose membranes were then placed, facing outwards, in a heat-sealed plastic bag containing primary target and loading control antibodies together at appropriate dilutions in 5 mL PBS (Tween20, 0.1 % v/v). Air was removed from the bag and it was fully sealed and placed on a rocking platform for 1 h at room temperature. Following the antibody incubation, the membranes were removed from the bag and washed four times for 10 minutes each in PBS (Tween20, 0.1 % v/v) in a
container on a rocking platform. Appropriate infrared dye-conjugated secondary antibodies (for details see 6.1.4 B) were then added in a volume of 50 mL in PBS (Tween20, 0.1 % v/v) and incubated for 30 minutes on a rocking platform in the dark. The membranes were then washed vigorously 6 times in PBS (Tween20, 0.1 % v/v) prior to imaging.

D) Imaging
For imaging, membranes were placed face-down in the scanner of a LI-COR Odyssey Quantitative Fluorescent Imaging System (LI-COR Biosciences, Lincoln, Nebraska) in reverse orientation and scanned at the wavelength appropriate to the infrared dye-conjugated secondary antibodies used.

6.6.2 Immunohistochemical staining:
Cells were infected prior to permeabilisation and staining with the SDOW-17 monoclonal antibody and a horseradish peroxidase secondary antibody. The AEC substrate was utilised for detection of HRP staining and cells were visualised using a light microscope. For detailed staining procedure see 6.4.3 B.

6.6.3 Phagocytosis assay
Cells were seeded 24 hours prior to treatment at an appropriate cell density. 24 hours post-seeding, FITC-labeled zymosan A BioParticles or E. coli:FITC particles were added to the growth medium at a ratio of 10 particles/cell. Cells were incubated in the presence of the treatment for 1 hour at 37 °C to allow particle uptake. Cells were then washed four times in cold PBS to stop phagocytosis and remove un-incorporated particles. Cells were fixed in freshly prepared 4 % v/v paraformaldehyde in PBS for 10 minutes at room temperature and washed with PBS prior to microscopic visualisation or FC quantification.

6.6.4 Fluorescence-activated cell sorting (FACS)
Cells were stained with the antibody of interest, as normal, and suspended at a density of 2 x 10^7 cells in OptiMEM serum-free medium in 12 x 75 mm polypropylene FACS tubes. Unstained and isotype-matched samples were also
prepared to aid the gating of positive cells. Samples were filtered through 35 μm filter-top sterile FACS tubes prior to running on the FACS machine in order to remove clumps and fragments. 200 μL complete medium was placed into sort collection tubes for cells to “splash down” into post-sort. FACS was performed by Bob Fleming using the BD FACS Aria (BD Biosciences, San Jose, USA).

6.6.5 Flow cytometry

A) Preparing and staining PRRSV-infected cells for FC

Growth medium was removed into a waste container containing 10 % v/v Distel in water. Cells were washed cells once in warmed (37 °C) PBS and a suitable volume of trypsin/EDTA was added. Cells were then incubated for 5 minutes at 37 °C, or until all cells were detached from the culture vessel. The cell-containing solution was transferred to a 15 mL tube and a suitable volume of FC PBS was added, in order to dilute the trypsin/EDTA. Tubes were wiped with 1 % (v/v) Distel in water and centrifuged for 5 mins at 380 x g. The supernatant was then removed into a waste container containing 10 % v/v Distel in water. Cells were counted and aliquoted into 12 x 75 mm polypropylene FACS tubes at 1 x 10^6 cells per tube.

For SD01-08 analysis, cells were resuspended in 500 μL 0.4 % v/v PFA in PBS at this point and stored at 4 °C in the dark prior to FC analysis. For analysis of PRRSV H2, 200 μL of cell fix (diluted 1/10 in dH2O in a glass container) was added to all tubes. Samples were incubated at room temp for 15 minutes in the dark. All tubes were washed in FC PBS w. saponin and centrifuged for 5 mins at 380 x g. The supernatant was removed and cells were resuspended in 100 μL Fix and Perm Solution B, containing 2 μL mouse serum, to block non-specific binding. Tubes were incubated for 10 minutes in the dark at room temperature. 1 μL SDOW17-FITC antibody was then added to each tube and incubated in the dark for 1 hour at room temperature. Cells were then washed in FC PBS w. saponin and centrifuged for 5 mins at 380 x g. Cells were resuspended in 500 μL 0.4 % (v/v) paraformaldehyde in PBS and stored at 4 °C in the dark prior to FC analysis.
B) Antibody staining of cells for FC analysis
Staining of cells was performed using standard procedures as outlined in 6.7.2, with the exception of performing all staining and wash steps in 12 x 75 mm polypropylene FACS tubes, as in 6.6.5A.

C) Flow-cytometric analysis
FC of stained, fixed cell populations was performed with either CyAn ADP Analyzer (Beckman Coulter) or Fortessa (Becton Dickinson) machines. Initial calibration of forward and side scatter was performed using the unstained cell populations to determine the ‘live’ cell gate. Background fluorescence intensity was determined using the samples stained only with secondary or isotype-matched control antibodies, and these values were used to set the lower collection limit. FC was performed on each sample until a suitable number of cells in the ‘live’ gate were counted. Data were processed using the FC data analysis and display program Summit (v4.3, Dako Colorado Inc).

6.7 Imaging techniques

6.7.1 Fluorescence microscopy
Analysis of fluorescence in cells at 10x magnification was performed using a Zeiss Axiovert25 microscope.

6.7.2 Confocal microscopy
Cells seeded at least 24 hours previously on glass coverslips in 24-well plates were used for staining. Cells were washed twice in situ with PBS and fixed using an appropriate volume of fresh 4 % v/v PFA in PBS for 10 minutes at room temperature. Cells were washed a further two times in PBS and then permeabilised using PBS (0.1 % v/v TRITON X-100) for 4 minutes at room temperature. Cells were washed twice in FC PBS with Saponin and the primary antibody was added at an appropriate dilution in FC PBS with Saponin. Following a 25 minute incubation at 37 °C, the primary antibody solution was removed and cells were washed twice in FC PBS w. Saponin. The secondary antibody, if required, was then added at an
appropriate dilution in FC PBS w. Saponin. Following a further 25 minute incubation at 37 °C, the secondary antibody solution was removed and cells were washed twice in FC PBS w. Saponin. Chambered cover-slides were imaged using a confocal microscope with a minimal covering of PBS to prevent drying of the cells. Glass coverslips were mounted to glass slides prior to microscopic visualisation. Images were analysed using the Zen 2011 (Black) software (v7.0, Carl Zeiss MicroImaging).

6.7.3 Coverslip mounting
For coverslip mounting, coverslips were washed in FC PBS, lifted from the well of a 24-well plate using a bent needle and a pair of forceps. Coverslips were dipped into PBS, dabbed dry and placed face-down on a drop of VectaShield, containing the DAPI nuclear counterstain (Vector Laboratories, H-1200), on a glass microscope slide (Shandon superfrost Plus, positively charged microscope slides, 6776214, Thermo Scientific). Coverslips were left at room temperature for 5 minutes, before nail varnish was used to secure and seal the edges of the coverslip to the glass microscope slide. Slides were allowed to cure prior to visualisation and were stored in the dark at 4 °C.

6.7.4 Colocalisation analysis
Colocalisation analysis was performed using the Zen 2011 (Black) software (v7.0, Carl Zeiss MicroImaging). The overlap coefficient (Manders et al., 1993) was calculated based on the colocalisation of red and green pixels across the entire cell, based on the following equation:

\[
\frac{\Sigma (Ch1_i)(Ch2_i)}{\sqrt{\Sigma (Ch1_i)^2(Ch2_i)^2}}
\]

All images used in these analyses were captured and analysed using the same settings, such as laser power, gain and contrast.
6.7.5 Single blind scoring of GBP-1 localisation patterns

Images were captured of fluorescent cells using identical microscope settings and saved with a known image ID related to their subject matter. All filenames were then renamed randomly using a freely available batch script, “RandomNames.bat”, written by Jason Faulkner (available at: http://www.howtogeek.com/57661/stupid-geek-tricks-randomly-rename-every-file-in-a-directory/). The original file names were automatically recorded in a single text file, which was relied upon for translating the file names following single blind scoring of staining patterns in the images. The localisation of SD01-08 or GBP-1 was scored as being either “dispersed” or “perinuclear”, according to the pattern of antibody staining.

6.8 Informatics

6.8.1 Microarray methodology
A) RIVitro-specific methods

The method for preparation of the RIVitro microarray dataset analysed in this thesis is published by Ait-Ali and colleagues (Ait-Ali et al., 2007; Ait-Ali et al., 2011) and briefly described below. BALF was recovered, post-mortem, from each of three LR and three PIE pigs as described previously (Ait-Ali et al., 2007). BALF was analysed, with 90% of cells staining positive for macrophage markers (Ait-Ali et al., 2007). Briefly, alveolar macrophages from each breed were infected with the H2 virus isolate (Drew et al., 1997) at an MOI of 1. RNA was isolated from AM at eight timepoints (0, 2, 4, 8, 12, 16, 24 and 30 hpi) for each sample and their transcriptional profiles were assessed using Affymetrix Porcine Genome Arrays.

I removed three microarray samples from the RIVitro dataset following QC:

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<tr>
<td>197_0021_Tahar_195_1287T2.CEL</td>
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</table>
B) RIVivo-specific methods

The following section describes the method of the unpublished RIVivo reproductive PRRSV experiment (Alan Archibald, personal communication).

The animals used in the RIVivo study were sourced from the breeding company PIC (now Genus PIC) from a high health status breeding herd. PRRSV-challenge experiments were carried out within the animal facilities at the VLA laboratory. Upon arrival at the VLA laboratory all pigs were free from PRRSV. The study was undertaken following strict ethical codes of practice, and within the terms of a project licence granted under the Animal (Scientific Procedures) Act 1986.

The gilts used for the RIVivo study were 9 to 10 months old. The gilts had Regumate Porcine (Janssen Animal Health) orally administered for 18 days prior to insemination. NB: Regumate is used for the synchronisation of oestrus and improvement in litter size in sexually mature gilts.

PRRSV-negative gilts in early pregnancy from two different lines (LR and synthetic B/C) were randomly allocated to two treatments: control and inoculated. The animals were housed and monitored for 11 days prior to intranasal inoculation with the H2 PRRSV strain (Drew et al., 1997) on day 52 of gestation, just into the second trimester. Infected gilts received $1 \times 10^{5.85}$ TCID$_{50}$ per nostril of PRRSV H2 virus in a volume of 1.5 mL and mock-infected control gilts received 1.5 mL per nostril of RPMI media containing 10 % v/v FBS (Lewis et al., 2010; Lewis, 2009). Phenotypic information was recorded prior to infection and for up to 48 dpi. Gilts were euthanised throughout the experiment for tissue and RNA collection on days -7, 0, 2, 13, 27, and 48. Phenotypic measures recorded included: body weight, rectal temperature, numbers of viable foetuses, mummified and re-absorbed foetuses, serology (by ELISA using the PRRS IDEXX kit) and cell counts.

Below is a timeline of the RIVitro experiment, including sampling dates and gestation timings:
Below is a summary of the number of animals remaining, by day, during the RIVivo sampling time course:

![Graph showing number of animals remaining by day]

Two control gilts and one infected gilt from the B/C breed were found to not be pregnant upon post-mortem examination. This is consistent with an expected 20% return rate in gilts i.e. the experiment began with 20 gilts of the B/C breed, so a 20% return rate means that 4 gilts were expected not to fall pregnant following insemination. However, all Landrace animals were found to be pregnant on post-mortem examination. Samples from non-pregnant animals were excluded from the microarray experiment. In order to determine the status of a sample using the PRRS IDEXX kit, S/P ratios were utilised. S/P ratios of greater than 0.5 were considered to be positive. S/P ratios were calculated using the following formula:

\[
S/P \text{ ratio} = \frac{\text{Sample mean (mean optical absorbance) – negative control mean}}{\text{Positive control mean – negative control mean}}
\]
6.8.2 Analysis of microarray data

I performed all of the following analyses on the previously generated RIVitro and RIVivo microarray datasets.

Data from 46 arrays for RIVitro and 23 arrays for RIVivo were normalised using the RMA expression measure (Irizarry et al., 2003). This consisted of background adjustment and quantile normalisation followed by median-polish, using the standalone GUI program RMAExpress (http://rmaexpress.bmbolstad.com/). Density and box-plots were generated in order to confirm the efficacy of the normalisation process and visually detect and remove any outliers. For the network analysis described here in, the data remained unfiltered, as low intensity signals are fundamentally highly variable and generally these data do not feature in co-expression networks generated from relatively large datasets. Following normalisation, samples were ordered according to pig breed and timepoint, respectively. The dataset was annotated using the 2010 version of the annotation developed by Tsai et al. (2006a) (freely available at: http://www4.ncsu.edu/~stsai2/annotation/). The dataset was saved as an ‘.expression’ file containing a unique identifier for each row of data (gene symbol concatenated to probe set ID), followed by columns of gene annotations used as class-sets for the overlay and analysis of information with respect to the graph, and finally normal scale normalised data values for each sample, each column of data being derived from a different sample. These files were then loaded into the network analysis tool BioLayout Express3D (Freeman et al., 2007b).

A pairwise Pearson correlation matrix was calculated for each probe set on the array as a measure of similarity between the signal derived from different probe sets. All Pearson correlations with \( r \geq 0.7 \) were saved to a ‘.pearson’ file and a correlation cut off of \( r = 0.85 \) was used to construct a graph containing 5,712 nodes (probe sets) connected by 45,884 edges (correlations above the threshold) for RIVitro, and 9,521 nodes connected by 189,077 edges for RIVivo. Graph layout was performed using a modified Fruchterman-Rheingold algorithm (Fruchterman & Reingold, 1991) in three-dimensional space in which nodes representing genes/transcripts are connected by weighted, undirected edges representing correlations above the selected threshold. The resultant graph was clustered using
MCL (Van Dongen, 2000), which has been demonstrated to be one of the most effective graph-based clustering algorithms available (Brohee & van Helden, 2006). An MCL inflation value of 3 was used as the basis of determining the granularity of clustering, as it has been shown to be optimal when working with highly structured expression graphs (Freeman et al., 2007b). Clusters were named according to their relative size, the largest cluster being designated “Cluster1”.

Graphs of each dataset were explored in order to understand the significance of the gene clusters and their relevance to the host response to PRRSV infection. Clusters were annotated if the genes within it suggested a known function shared by multiple members of the cluster. These analyses were supplemented by KEGG and IPA database analysis and manual review of the literature.

6.8.3 Pathway Analysis

A) Ingenuity Pathway Analysis (IPA)
The human and mouse-based IPA platform (Qiagen) was used to analyse the biological pathways represented by members of cluster groups in the microarray datasets. Pathway analysis of pig transcripts was achieved by mapping them to their corresponding human orthologue and annotating with the human RefSeq ID.

B) KEGG
The KEGG “Search & Color Pathway” database (http://www.genome.jp/kegg/tool/map_pathway2.html) was queried using the NCBI gene IDs of the members of transcript clusters derived from the microarray analyses.

C) Gene ontology
GO enrichment analysis was performed using the online tool GOriilla (http://cbl-gorilla.cs.technion.ac.il/). An unranked membership list of each cluster was input as the target dataset, with all the genes on the Porcine Genome Array input as the background dataset. Gene names were then used to determine Homo sapiens GO-term enrichment in the target dataset. A p-value threshold of $10^{-3}$ was used.
6.8.4 Other bioinformatic tools

A) 3' UTR feature prediction

Various online tools were used to annotate predicted features in the porcine GBP-1 3' UTR, including:

http://www.targetscan.org/vert_60/
http://regrna.mbc.nctu.edu.tw/html/about.html
http://www.imtech.res.in/raghava/polyapred/help.html

B) Coiled-coil prediction

The probability of coiled-coil formation was predicted using COILS (Lupas et al., 1991), available from: http://embnet.vital-it.ch/software/COILS_form.html. A scanning window of 28 residues was utilised.

6.8.5 Sequence analysis and annotation

Sequence analysis and annotation, including the generation of vector maps, was performed using the SeqMan (DNASTAR, v10.1.0), SnapGene Viewer (v2.1.2) or Geneious (Biomatters, v7.0.5) software packages.

6.8.6 EST contig assembly

ESTs mapped to porcine GBP-1 were downloaded from UniGene (http://www.ncbi.nlm.nih.gov/uniGene/), consisting of 147 sequences at the time of analysis. These sequences were assembled using ‘pro assembler’ in the SeqMan software (DNASTAR, v10.1.0) using the settings described in Al-Ahmadi et al. (2009). This resulted in the assembly of a single large contig containing 131 sequences. Four EST sequences were not assembled.

6.8.7 Protein modelling

YASARA (Yet Another Scientific Artificial Reality Application; http://www.yasara.org/index.html) was used to perform homology modelling of GBP-1 and assess the impact of mutations on dimer formation. Modelling was based on available crystal structures of the hGBP-1 monomer and head-to-tail and head-to-head dimers (Prof. Dr. Alfred Wittinghofer, personal communication and Prakash et
al., 2000a). Homology modelling of porcine and human GBP-1 utilised a porcine GBP-1 consensus sequence obtained from nucleotide sequencing data generated myself (Genbank accession number: KF977840).

6.8.8 Geographical mapping of genotype data
Genotype data were derived from the pig HapMap project (Groenen et al., 2010) and supplied by Prof. Alan Archibald (personal communication). The geographical location of each sample was assigned a code and the mean allele frequency for each geographical location was plotted on a world map. Mapping was performed using InstantAtlas Desktop (GeoWise, Edinburgh).
APPENDICES
Appendix 1 | A. pSL10 plasmid map (Dr. Simon Lillico, personal communication). B. 3D4/21 cells transduced with PU.1 lenti virus, expressing the GFP transduction marker.
Appendix 2 | SSC4 PRRSV host response QTL genotypes of RIVitro animals. The animals used in the RIVivo study were genotyped for 6 index SNPs (WUR10000125, MARCO056249, ALGA0029524, ASGA0023344, ASGA0023349 and ALGA0029538) in the 1 Mb region on SSC4 associated with the host response to PRRSV (Boddicker et al., 2012). “?” indicates that the genotyping assay failed to establish the genotype at this allele. Given the perfect LD in the region in question, heterozygosity at any locus can reasonably be taken to indicate heterozygosity at all six index SNPs in the case of missing data points. Data were provided by Prof. Alan Archibald (personal communication). Heterozygotes are highlighted.

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Appendix 3 | Protein sequence alignment of porcine GBP family members, with comparison to hGBP-1. The rs80955804 SNP location (conserved alanine in all but GBP-2) is highlighted in a yellow box and appears at position 500 in this alignment.

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<td>hGBP1</td>
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[Image of protein sequence alignment]

Identity:
- The rs80955804 SNP location (conserved alanine in all but GBP-2) is highlighted in a yellow box and appears at position 500 in this alignment.
Appendix 4 | Nucleotide sequence of the GBP1(1662) coding sequence (deposited as GenBank
accession no: KF977840) with the g1486a SNP (rs80955804) SNP highlighted in red.

ATGGCCTCAAAGGTGCACATGCCCGAACCACAGTGCCTCATTGAGAACATCAATGGGCGA
CTGGCGGTGAACCCGAAAGCGCTGAAGCTCCTGTCTGCCATCAAGCAGCCCCTGGTGGTG
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AAGAACAAGGGCTTCTCTCTGGGCTCCACGGTGCAGTCTCACACAAAGGGCATCTGGATG
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CTGGGGGATGTTGAGAAGGGTGACAACCAGAACGACTCCTGGATCTTCGCCCTGGCAATT
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AAGGATTCCAGGAAGAGAACAGACGACTTCATAATGAAATACAAAATCTCCAGAAGAAGA
TGAAAAAATCAAAGAAAGAATGTTTCCTGAGCTAA

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Appendix 5 | Amino acid sequence translation and alignment of the coding sequences of published pGBP-1 sequence (GenBank accession number: NM001128473) and the GBP1(1662) sequence (deposited as GenBank accession number: KF977840). Sequences show 99.5% amino acid identity. Amino acid differences between the two sequences are highlighted in yellow. The A496T SNP (rs80955804) is denoted by a blue box at position 496.
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determines the noninflammatory and protective transcriptional response of macrophages to hemoglobin. *Circulation research* 99, 943-950.


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