# Report on completed EPIZONE short-term mission

Visitor: Dr. Sylvia Kohler

Home institution: Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler Institute, Federal Research Institute for Animal Health, Greifswald – Island of Riems, Germany

Home institution supervisor(s): Dr. Sandra Blome, Institute of Diagnostic Virology

Host institution: Division of Functional Genetics, The Roslin Institute, University of Edinburgh, Edinburgh, Scotland, United Kingdom

Host institution supervisor(s): Dr Tom Burdon (Head of Division), Dr Stephen Meek (Research Fellow), Dr Tom Watson (Research Associate)

Duration: 01 May 2023 – 30 June 2023

# Generation, cultivation and differentiation of induced pluripotent stem cells from domestic pig and warthog

## Background

African swine fever (ASF) is caused by a big-sized, double-stranded DNA virus (ASFV) distinct from classical swine fever virus though showing similar severe clinical symptoms and high mortality in swine and wild boar. Hence, ASFV represents a global threat to pig livestock and wild suids, and options for controlling and eradicating this disease remain highly limited. ASFV preferentially infects porcine cells of the monocyte/macrophage lineage associated with lymphocyte depletion and massive cell death (apoptosis and necrosis) in lymphoid tissues. In contrast, warthogs display remarkable resistance to virulent ASFV infection and are considered as the natural mammalian host of ASFV. However, the virulence determinants and hostpathogen interactions of ASFV are far from being fully understood. To date, primary swine macrophages are the gold standard for ASFV research involving significant drawbacks, such as laboratory and ethical constraints. Therefore, the generation and use of macrophages from induced pluripotent stem cells (PSCdMs) from different suid species can help to identify potential factors or mechanisms contributing to ASFV susceptibility and resistance to fill important knowledge gaps. Consequently, identification of relevant biological mechanisms may have promise for disease control in Eurasian swine populations.

Dr Tom Burdon and his group at The Roslin Institute has established a pluripotent stem cell (PSC)-based platform for the generation and experimental manipulation of macrophages from susceptible and naturally resilient pig species (Meek et al., 2022; Watson, 2022). These porcine PSCdMs exhibit molecular and functional characteristics of ex vivo primary macrophages and can be productively infected by pig pathogens, including African swine fever virus (ASFV) (Meek et al., 2022). In order to expand their research on other resilient pig species, Burdon`s group received several warthog cell lines from the Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler Institute, in January/February this year, including:

PHA-B-1-R (CCLV-RIE 1557, brain, male, adult)
PHA-T-1-R (CCLV-RIE 1471, trachea, male, adult)
PHA-K-3-R (CCLV-RIE 1558, kidney, female, adult)
WAZI-1-R (CCLV-RIE 1459, skin, male, adult)

## Objectives

The aim of the EPIZONE short-term mission (STM) in the research group of Dr Tom Burdon was to:

(1) learn laboratory techniques to generate, cultivate and differentiate induced pluripotent stem cells (iPSCs) from domestic pig and warthog into macrophages.
(2) transfer the techniques to the Friedrich-Loeffler Institute to establish a platform for researchers interested in utilizing iPSC technology and for providing researchers with specifically differentiated iPSCs.

The work was conducted during a 2-month STM and followed the detailed plan of the professional activities submitted in the application form to EPIZONE. Since the generation and differentiation of iPSCs requires a labor-intensive and time-consuming protocol, the research mission started at different steps of the overall workflow (Fig. 1).

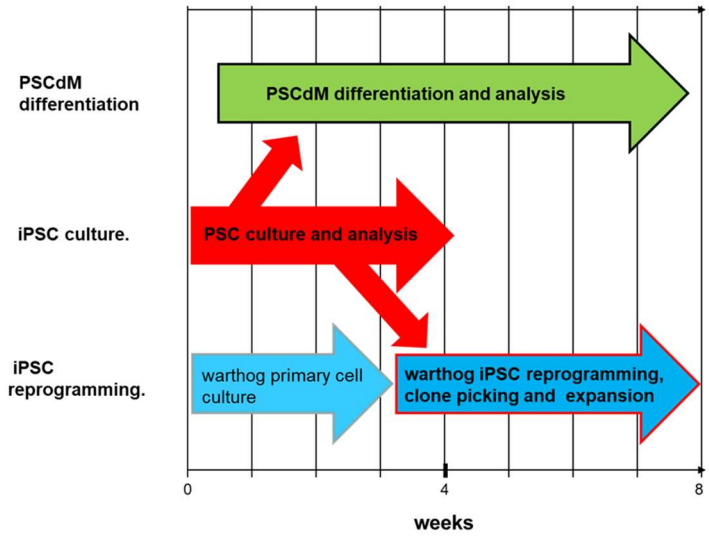


Fig. 1: Project schedule.

## Methods

1. iPSC cultivation
    1. culturing iPSCs (incl. medium and fibroblast feeder cell preparation)
    2. enzymatic dissociation of iPSC cultures and subculturing (assessment of undifferentiated status by morphology and optimal growth)
    3. cryopreservation and thawing of iPSCs

2. Reprogramming of iPSCs
    1. electroporation of warthog primary cell lines with reprogramming factor expressing vectors and GFP vector control, respectively
    2. assessment of reprogramming efficiency by morphology and stem cell marker expression using RT-qPCR and flow cytometry (GFP vector control)
    3. seeding and cultivation of electroporated cells on gamma-irradiated fibroblast feeder cells
    4. picking and expansion of colonies with characteristic stem cell morphology, cryopreservation of stably propagatable iPSC-like clones

3. Differentiation of iPSCs into macrophages – three stage protocol
    1. induction of the formation of embryoid body (EB)-like aggregates containing mesoderm and haematopoietic progenitors by incubating warthog iPSCs with the cytokines BMP4, VEGF, bFGF, and SCF in low-attachment tissue culture (TC) plates
    2. induction of myeloid differentiation by plating EB-like aggregates onto gelatin-coated TC plates in presence of CSF-1 and IL-3, microscopic monitoring of production of myeloid cells
    3. production of mature macrophages by cultivation of myeloid monocyte-like cells in the presence of pig M-CSF and confirmation of macrophage identity by RT-qPCR

    Due to delays in the generation of warthog iPSC-like clones, the third stage – production of mature macrophages – was solely performed with the well-established pig iPS cell line Pa9.

**Results**

1. warthog cell line PHA-B-1-R

Warthog brain cells were transfected with reprogramming factor expressing vectors or GFP control vector, respectively. However, the transfection efficiency was very low (14%) as assessed by flow cytometry. Hence, reprogrammed brain cells were thawed from cell culture stocks frozen 7 days after transfection and cultivated on γ-irradiated fibroblast feeder cells. After 19 to 26 days, 25 individual colonies were picked by pipette. Of these, 19 clones showed a typical PSC morphology (Fig. 2A), were expanded, cryopreserved, and tested for their potential to differentiate into macrophages. After incubation with the cytokine cocktail, twelve clones formed reasonable embryoid bodies (Fig. 2B) and were transferred into macrophage induction medium. The iPSC clones showed variable cellular outgrowth and monolayer formation but no myeloid cell production after 12 days, which usually marks the time point of early appearance of macrophages, till the end of my visit (Fig. 2C-F).
Outcome: Unfortunately, none of the PHA-B-1-R iSPC clones showed production of macrophage-like cells.

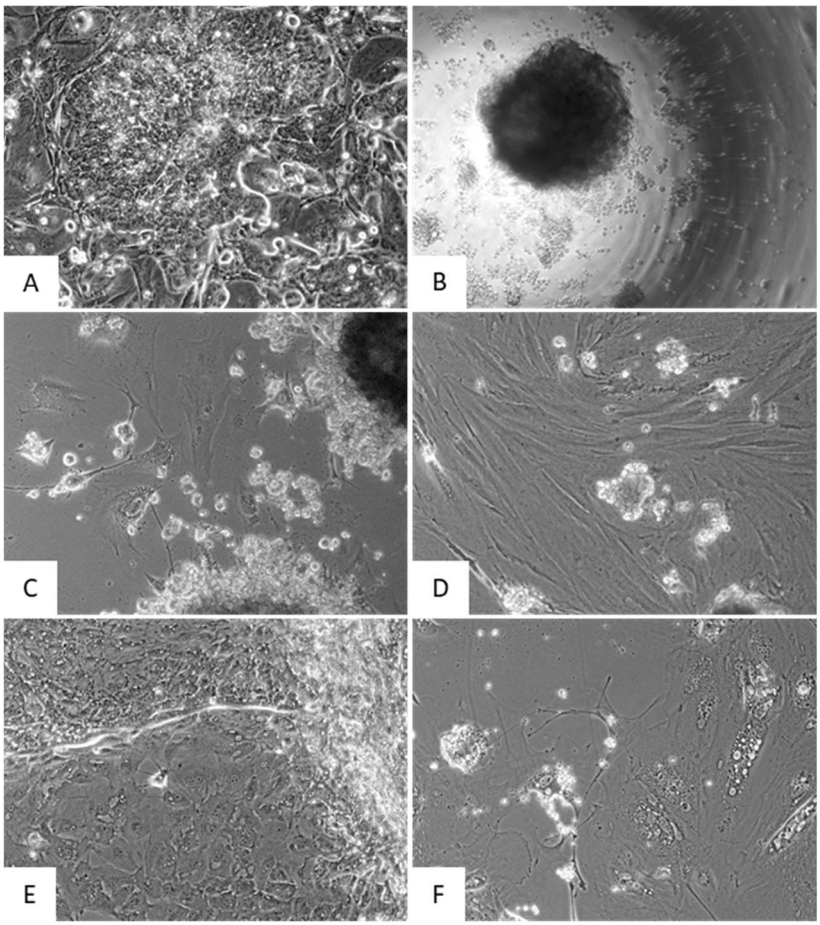


Fig. 2: Colony morphology (A), EB formation (B) and cellular outgrowth after macrophage induction (C-F) of PHA-B-1-R iPSC clones. A) C4, p3, 5 dpp; B) C1, 1 dpi; C) C19, 17 dpi, no outgrowth; D) C4, 17 dpi, fibroblasts; E-F) C14, 12 dpi (E) and C17, 12 dpi (F), different cell types but no myeloid cells. A, C-F) 100x magnification; B) 50x magnification.

2. warthog cell line PHA-T-1-R

Warthog trachea cells were transfected with reprogramming factor expressing vectors or GFP control vector, respectively, and reprogrammed trachea cells were thawed from cell culture stocks frozen 7 days after transfection and cultivated on γ–irradiated fibroblast feeder cells. The transfection efficiency was 30-40% in both experiments as measured by flow cytometry. After 17 to 20 days, 45 individual colonies were picked by pipette but displayed slow outgrowth and different morphologies during cultivation (Fig. 3A-C). Therefore, only seven clones could be assessed for macrophage differentiation. One clone showed no cellular outgrowth after plating of EBs (Fig. 3D) and six other clones were induced in the last week of my visit.
Outcome: Four of the six PHA-T-1-R iPSC clones induced gave rise to macrophage-like cells (Fig. 3E).

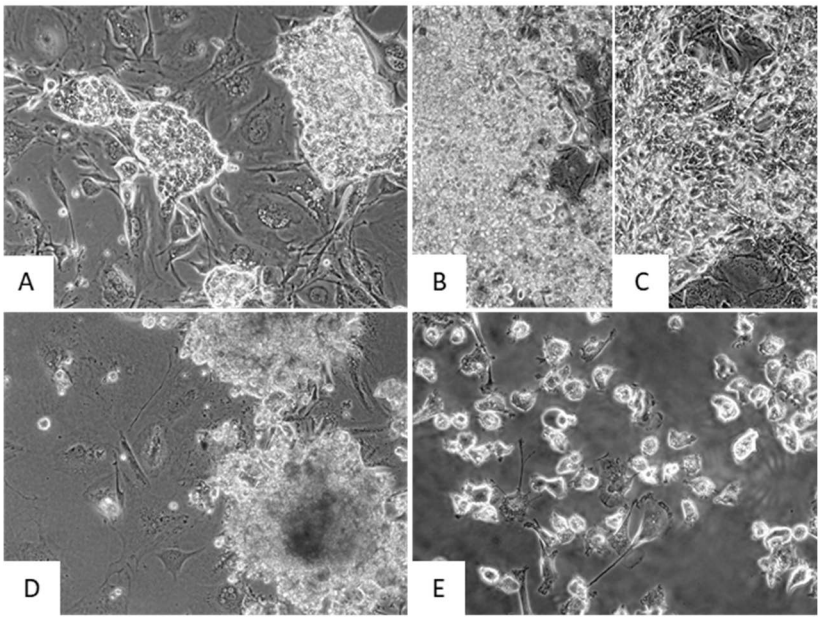


Fig. 3: Colony morphology (A-C) and cellular outgrowth after macrophage induction (D) of PHA-T-1-R iPSC clones. A) C2, p4, 7 dpp; B-C) C8, p6, before (B) and after 2x wash with PBS (C); D) C1, 16 dpi, no outgrowth; E) C7, dpi unknown. A-D) 100x magnification; E) 200x magnification.

3. wart hog cell lines PHA-K-3-R and WAZI-1-R

Warthog kidney and skin cells were transfected with reprogramming factor expressing vectors or GFP control vector, respectively. The transfection efficiency was up to 48% (PHA-K-3-R) and 33% (WAZI-1-R, in two separate experiments). Despite the acceptable transfection efficiency, only eight colonies from reprogrammed PHA-K-3-R cells and three very small colonies from reprogrammed WAZI-1-R cells could be picked after 23-29 days and did not expand further under prolonged culture conditions.

4. pig iPS cell line Pa9

In order to produce macrophages from Pa9 iPSCs, immature macrophages were transferred into macrophage maturation medium 20 days post induction and cultivated for further five days. RT-qPCR was performed and showed the expression of cell surface proteins characteristic for macrophage-like cells (Fig. 4).

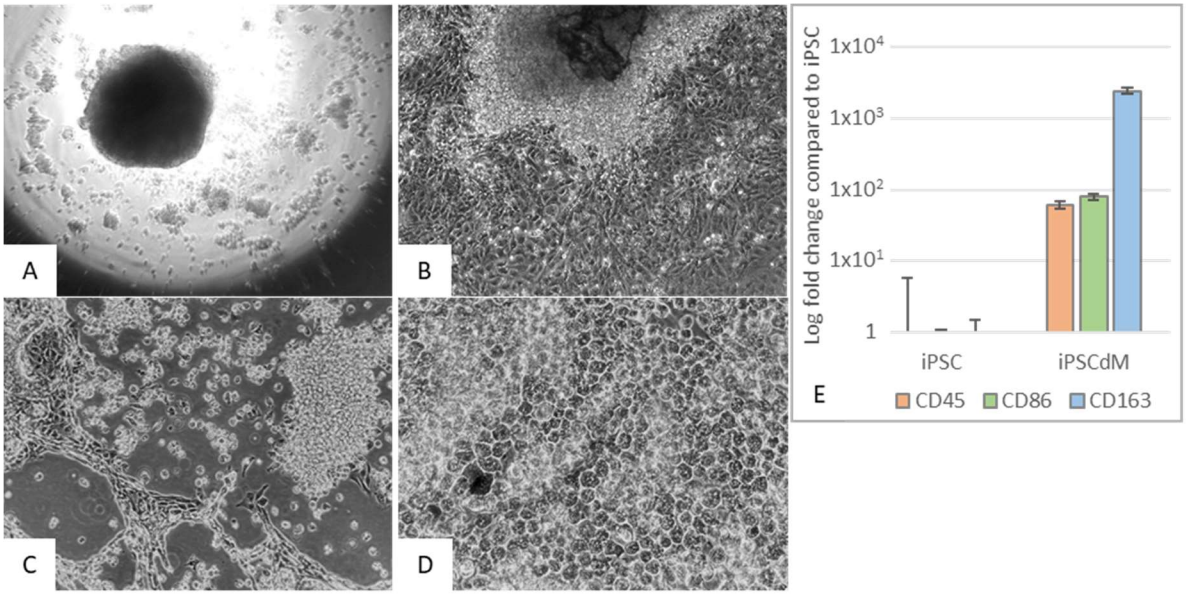


Fig. 4: EB formation (A), cellular outgrowth after macrophage induction (B-D) and expression of macrophage markers (E) of Pa9 iPSC. A) 3 dpi; B) 8 dpi; C) 12 dpi, early macrophage production; D) 25 dpi, peak macrophage production. A, C-D) 100x magnification; B) 50x magnification. E) RT-qPCR data showing expression of macrophage related genes (CD45, CD86, CD163) cultured for five days in macrophage maturation medium. Fold change was determined relative to non-induced Pa9 iPSCs.

**Disucssion**

During the last few weeks of my lab visit, Dr Meek figured out that the macrophage base medium currently used in the lab and purchased during the pandemic due to production shortage was less effective than the medium originally mentioned in the protocol. Hence, all warthog iPSC clones will be assessed for macrophage production in the originally-formulated medium.

**Work Achievements and Personal Experiences**

In summary, I achieved all the proposed objectives of my short-term mission which included the generation, cultivation and differentiation of pig and warthog iPSCs into macrophages and neuronal cells (not shown). Importantly, after further intensive assessment of the promising candidates generated during my lab visit, the warthog iPSC(s) might serve as an unlimited source of experimentally tractable macrophages for studying host-ASFV interactions. Furthermore, I received (and still do) a lot of advices and suggestions which I already started to apply in my research work. In addition, I gained insights into a range of interesting, high-quality research activities at the Roslin Institute.
Personally, the short-term mission in Edinburgh strengthened my ability to manage complex challenges regarding family and work issues, to demonstrate flexibility, reliability and professionalism in a foreign scientific environment as well as to easily connect with people of varying backgrounds, job levels and titles. The STM was science mixed with some funny chats about German/English words and breath-taking leisure activities with Tom Burdon and his lab members, e.g. dancing traditional Ceilidh or going hiking (Fig. 5). I was warmly welcomed and equally warmly waved/hugged goodbye. Many thanks to both Toms, Stephen, and all the other lab members!

Fig. 5: Hiking tour in the Pentland Hills with some of the group members (I am 3rd from the right). © C. Abeykoon

## Appendix

### References:

Meek S, et al. 2022. Stem cell-derived porcine macrophages as a new platform for studying host-pathogen interactions. BMC Biol. 20(1): 14. doi: 10.1186/s12915-021-01217-8.
Watson TM. 2022. Development of pluripotent stem cells from domestic and wild suidae for the study of host pathogen interactions in vitro. PhD thesis. dx.doi.org/10.7488/era/3248.

### Abbreviations:

| | |
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| bFGF | basic fibroblast growth factor |
| BMP4 | bone morphogenetic protein 4 |
| C | clone |
| dpi | days post induction |
| dpp | days post passaging |
| EB | embryoid body |
| CSF-1 | colony-stimulating factor 1 |
| GFP | green fluorescent protein |
| IL-3 | interleukin 3 |
| iPSC | induced PSC |
| M-CSF | macrophage colony-stimulating factor |
| p | passage number |
| PBS | phosphate-buffered saline |
| PSC | pluripotent stem cell |
| PSCdM | PSC derived macrophage |
| SCF | stem cell factor |
| TC | tissue culture |
| VEGF | vascular endothelial growth factor |