



## Characterisation of Crandell-Rees Feline Kidney (CRFK) cells as mesenchymal in phenotype

J.S. Lawson<sup>a,\*</sup>, H.M. Syme<sup>b</sup>, C.P.D. Wheeler-Jones<sup>a</sup>, J. Elliott<sup>a</sup>

<sup>a</sup> Comparative Biomedical Sciences, The Royal Veterinary College, Royal College Street, London NW1 0TU, UK

<sup>b</sup> Clinical Sciences and Services, The Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Herts, AL9 7TA, UK

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### ABSTRACT

The Crandell-Rees Feline Kidney Cell (CRFK) is an immortalised cell line derived from the feline kidney that is utilised for the growth of certain vaccinal viruses. Confusion exists as to whether CRFK are epithelial or mesenchymal in phenotype. The aim of this study was to characterise CRFK cells via immunofluorescence, enzyme cytochemistry, western blotting, RT-qPCR for S100A4 and comparison to primary feline proximal tubular epithelial cells (FPTEC) and feline cortical fibroblasts (FCF). CRFK cells were of fusiform morphology and appeared similar to FCF. CRFK expressed the mesenchymal intermediate filament (IF) protein vimentin together with two cell adhesion molecules associated with feline fibroblasts (CD29 and CD44), and lacked expression of the epithelial IF cytokeratin, myogenic IF desmin and endothelial marker von Willebrand factor (vWF). In addition, CRFK did not demonstrate brush border enzyme activity typical of FPTEC. S100A4 gene expression, implicated in both neoplastic transformation and epithelial to mesenchymal transition, was highly upregulated in CRFK in comparison to the primary feline renal cells. CRFK appear phenotypically similar to fibroblasts, rather than tubular epithelial cells, and may have undergone neoplastic transformation or epithelial-to-mesenchymal transition after extensive passaging. This finding may have potential implications for future research utilising this cell line.

The Crandell Rees Feline Kidney Cell (CRFK) is an immortalised cell line isolated in 1964 from the cortical kidney tissue of a 12 week old kitten (Crandell et al., 1973). CRFK are utilised for the growth of certain feline vaccinal viruses (Lappin et al., 2005). Proteins derived from CRFK cells have been suggested to persist in vaccines, and there is some evidence of an association between vaccination and the development of chronic kidney disease (CKD) in cats, possibly due to the development of antibodies against CRFK cell antigens (Finch et al., 2016; Lappin et al., 2005; Whittemore et al., 2010). CRFK have also been utilised as an in vitro model for the study of profibrotic factors on the feline tubular epithelium (van Beusekom and Zimmering, 2018).

Despite the importance of this cell line, there is uncertainty in the literature regarding CRFK phenotype, with publications variably describing CRFK as fibroblasts (Dietrich et al., 2011; Munk et al., 2007) or epithelial cells (Pratelli, 2011; van Beusekom and Zimmering, 2018), without clear justification. CRFK were originally described as epithelial based upon morphology, presumably indicating a tubular epithelial derivation (Crandell et al., 1973). It is unknown whether they remain representative of their original phenotype. This study characterised

CRFK cells via immunofluorescence, western blotting, enzyme cytochemistry, RT-qPCR for S100A4 and comparison to primary feline proximal tubular epithelial cells (FPTEC) and feline cortical fibroblasts (FCF).

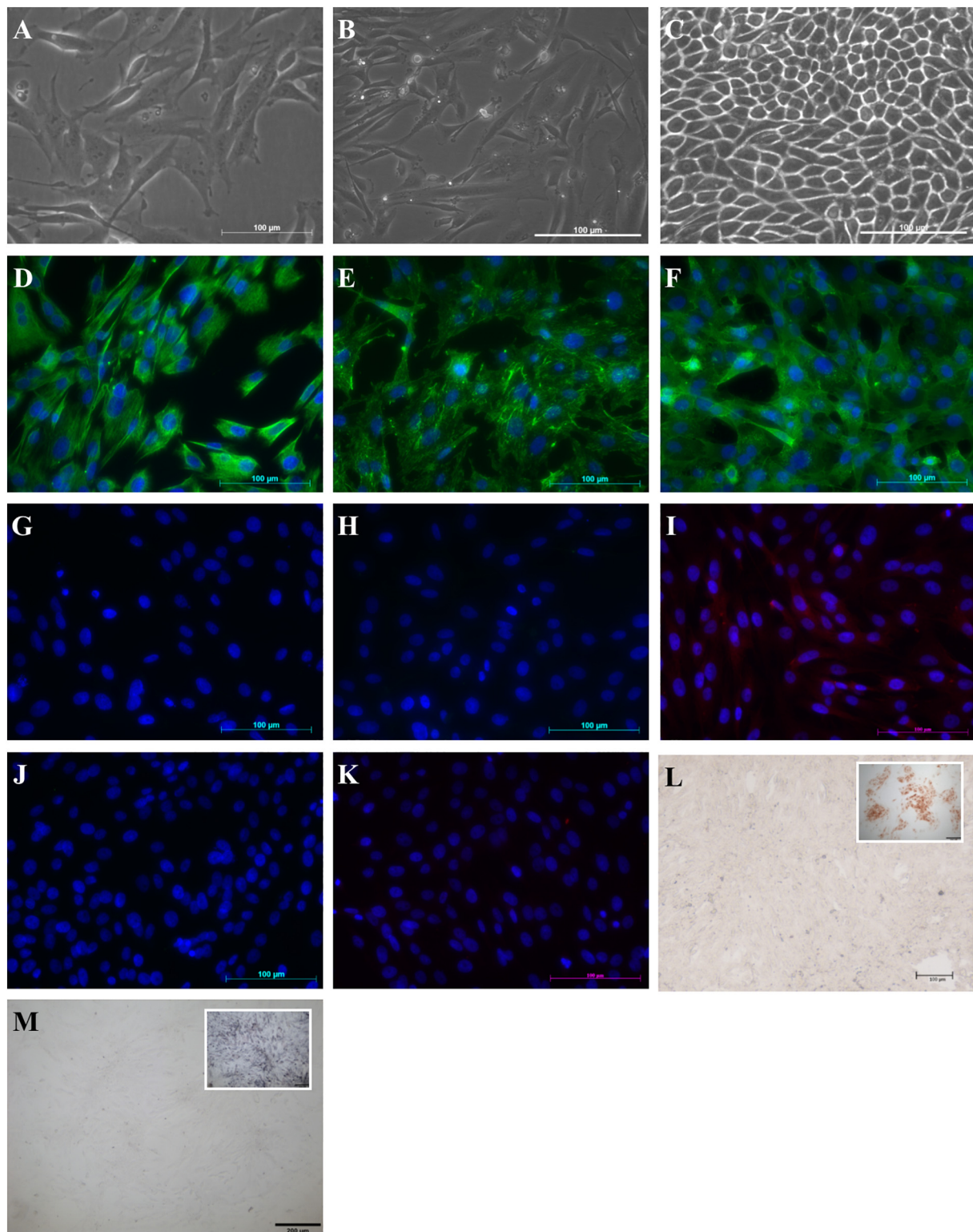
Cryopreserved CRFK cells were purchased at passage 185 (CCL-94, ATCC) and cultured according to the manufacturer's protocol.<sup>1</sup> FPTEC, FCF and human umbilical vein endothelial cells (HUVEC) were cultured for comparison as previously described (Lawson et al., 2018a; Lawson et al., 2018b). Cells were assessed for the expression of the marker proteins cytokeratin AE1/AE3 (Dako), vimentin (Dako), desmin (Dako), von Willebrand factor (vWF)(Dako), CD44 (ABD Serotec) and CD29 (Bio-rad) by immunofluorescence, and cytokeratin AE1/AE3, vimentin and vWF by western blotting as previously described (Lawson et al., 2018a). Cells were stained for GGT activity using a modified version of a previously published protocol (Rutenburg et al., 1969), and ALP activity using a commercially available substrate solution (SIGMAFAST™ BCIP®/NBT, Sigma Aldrich).

RNA was extracted from CRFK, FPTEC and FCF using a column based kit (Genelute™ Mammalian Total RNA Miniprep Kit, Sigma-

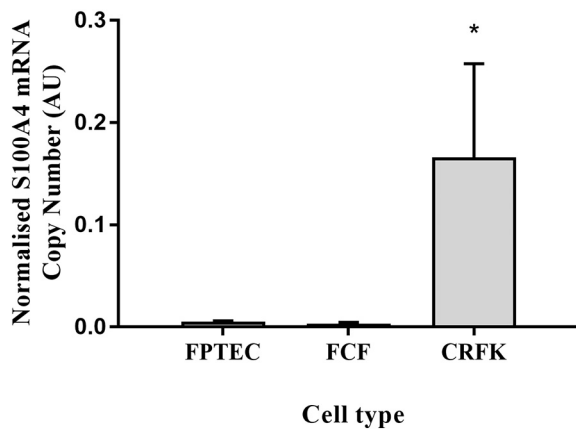
\* Corresponding author.

E-mail address: [Jlawson@rvc.ac.uk](mailto:Jlawson@rvc.ac.uk) (J.S. Lawson).

<sup>1</sup> [https://www.lgcstandards-atcc.org/Products/All/CCL-94.aspx?geo\\_country=gb#culturemethod](https://www.lgcstandards-atcc.org/Products/All/CCL-94.aspx?geo_country=gb#culturemethod).



**Fig. 1.** CRFK cells exhibit fusiform morphology (A) and appear more similar to primary feline fibroblasts (B) than feline proximal tubular cells (C). CRFK were positive for the mesenchymal markers vimentin (D), CD29 (E), and CD44 (F), and negative for the epithelial marker cytokeratin AE1/AE3 (G), myogenic marker desmin (H) and endothelial marker vWF (I). Both mouse IgG (G) and rabbit IgG (H) isotype controls were negative. CRFK cells did not demonstrate either ALP (L) or GGT (M) activity (FPTEC positive control inset). Greyscale photomicrographs were collected using a DMIRB inverted microscope with samples illuminated using an EBQ100 light source and an AxioCam ICm1 monochrome camera controlled through Axiovision software version 4.8.2. Immunofluorescence images were collected using a DM4000B upright microscope with samples illuminated using an EBQ100 light source and filter cubes A4 and L5 (all from Leica Microsystems) and an AxioCam MRm monochrome camera controlled through Axiovision software version 4.8.2 (Carl Zeiss Ltd). Cell nuclei were stained with DAPI (blue). Colour photomicrographs for the enzyme activity experiments were collected using an Axiovert 135 inverted microscope (Carl Zeiss Ltd., Cambridge, UK) and an Infinity 3-3UR colour camera (Lumenera, Ottawa, ON, Canada) controlled through Image Pro Insight software version 9.1.4 (Media Cybernetics, Rockville, MD, USA). Images are representative of three experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** Graph illustrating S100A4 mRNA expression in FPTEC, FCF and CRFK as measured by RT-qPCR. FPTEC and FCF demonstrated a similar, low level of S100A4 mRNA expression. The CRFK cell line demonstrated significantly greater S100A4 mRNA expression as measured by one-way ANOVA ( $P = .013$ ). The bars represent the mean normalised mRNA copy number and whiskers represent the standard deviation,  $n = 3$  (cells isolated from 3 different cats in the case of the primary cells or experiments undertaken on 3 batches of CRFK cells).

Aldrich). Expression of S100A4 was quantified by RT-qPCR and normalised to GAPDH/RPS7 using previously published primers (Lawson et al., 2018b). Data are expressed as mean fold change relative to untreated control and statistical significance was evaluated by one-way analysis of variance (ANOVA) with post-hoc Dunnett's test. All experiments were carried out in triplicate.

CRFK cells were of fusiform morphology, with cells demonstrating a multipolar or bipolar cell shape at lower densities (Fig. 1A). Cells initially formed monolayers, but did not exhibit contact inhibition at confluence. CRFK cultures appeared similar to that of FCF (Fig. 1B), and were distinctly different to the “cobblestone” monolayers formed by FPTEC (Fig. 1C). CRFK were positive for vimentin, CD44 and CD29 by immunocytochemistry (Fig. 1D, E, F), and negative for cytokeratin AE1/AE3, desmin and vWF (Fig. 1G, H, I). CRFK were also positive for vimentin expression, and negative for cytokeratin AE1/AE3 and vWF, assessed by western blotting (data available on request). CRFK did not demonstrate activity of either GGT or ALP (Fig. 1L,M). CRFK cells exhibited significantly higher S100A4 expression (30–50 $\times$ ) than both FCF and FPTEC ( $P = .013$ ) (Fig. 2).

The results of this study suggest that CRFK are of mesenchymal, rather than an epithelial, phenotype. CRFK expressed the mesenchymal intermediate filament (IF) vimentin, which labels fibroblasts in the feline kidney (Yabuki et al., 2010). CRFK also expressed two cell adhesion molecules, CD29 and CD44, associated with feline fibroblasts (Lawson et al., 2018b; Mumaw et al., 2015). There was no expression of the epithelial IF cytokeratin, which labels feline distal tubular/collecting duct cells in situ, and FPTEC in vitro (Bland et al., 2017; Lawson et al., 2018a). Furthermore, CRFK did not demonstrate expression of the brush border enzymes ALP or GGT, which are present in differentiated feline proximal tubular epithelial cells. CRFK were also negative for the myogenic IF desmin and the endothelial marker vWF, which labels interstitial capillaries in feline kidneys (Lawson et al., 2018a). Altogether, the expression profile of CRFK is identical to that of FCF (Lawson et al., 2018b), and is not suggestive of tubular epithelial, glomerular or endothelial origin.

S100A4 is a gene implicated in neoplastic transformation, with upregulation of expression contributing to tumour growth and metastasis (Yang et al., 2012; Zhou et al., 2005). S100A4 expression has also been associated with epithelial-to-mesenchymal transition, a process by which epithelial cells dedifferentiate and gain mesenchymal characteristics (Okada et al., 1997). S100A4 expression was highly

upregulated in CRFK cells, suggesting that CRFK may have originally been epithelial, but have undergone extensive phenotypic and genotypic drift after long-term culture, resulting in a form of neoplastic transformation enabling indefinite proliferation. There are, however, other possible explanations for the discrepancy in the reportedly original epithelial phenotype and the current mesenchymal phenotype. Original cultures were established with no attempt at cell selection, and it is possible that long-term culture selected for faster growing, fibroblastic cells, which overgrew the original epithelial cell population. It is also possible that contamination of the CRFK cell line has occurred at some point, as numerous cell lines have been found to be cross-contaminated with faster growing cell types (Buehring et al., 2004). Further information on the status of CRFK could be gained from DNA profiling or examining expression of additional marker proteins, but such work was beyond the scope of the current study.

In summary, CRFK appear phenotypically similar to fibroblasts and characterisation was not supportive of the originally reported epithelial phenotype. The findings of this study raise concerns over the use of CRFK as an in vitro model for the study of the feline renal tubular epithelium. This study also provides supporting information for future investigation of the link between CRFK lysates in feline vaccines and the development of CKD.

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#### Declaration of competing interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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