METHODOLOGY

Establishment of epithelial and fibroblast cell cultures and cell lines from primary renal cancer nephrectomies

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Abstract

Renal cell carcinoma (RCC) is one of the most lethal urogenital cancers and effective treatment of metastatic RCC remains an elusive target. Cell lines enable the in vitro investigation of molecular and genetic changes leading to renal carcinogenesis and are important for evaluating cellular drug response or toxicity. This study details a fast and easy protocol of establishing epithelial and fibroblast cell cultures or cell lines concurrently from renal cancer nephrectomy tissue. The protocol involves mechanical disaggregation, collagenase digestion and cell sieving for establishing epithelial cells while fibroblast cells were grown from explants. This protocol has been modified from previous published reports with additional antibiotics and washing steps added to eliminate microbial contamination from the surgical source. Cell characterisation was carried out using immunofluorescence and quantitative polymerase chain reaction. Eleven stable epithelial renal tumour cell lines of various subtypes, including rare subtypes, were established with a spontaneous immortalisation rate of 21.6% using this protocol. Eight fibroblast cell cultures grew successfully but did not achieve spontaneous immortalisation. Cells of epithelial origin expressed higher expressions of epithelial markers such as pan-cytokeratin, cytokeratin 8 and E-cadherin whereas fibroblast cells expressed high α-smooth muscle actin. Further mutational analysis is needed to evaluate the genetic or molecular characteristics of the cell lines.

Keywords: cell line; epithelial cells; fibroblast; renal cancer

Introduction

Renal cell carcinoma (RCC) comprises of 2–3% of all human malignancies and the incidence is increasing worldwide (Znaor et al., 2015). The incidence of RCC is higher in western countries (North America, Europe, Australia and New Zealand) compared with Asian countries in general (Znaor et al., 2015). The most common subtypes of RCC are clear cell (70–80%) followed by papillary (10%), chromophobe (5%) and collecting duct RCC (1%) (Moch, 2013). RCC with sarcomatoid or rhabdoid transformation is not a recognised subtype of RCC as sarcomatoid or rhabdoid features can be found in all histologic subtypes of RCC (Delahunt et al., 2013). Both of these histological transformations in RCC are associated with aggressive tumours and poor prognosis. Radiation and chemotherapy has limited efficacy on RCC. The current approved targeted therapies for metastatic RCC suppress angiogenesis by inhibiting vascular endothelial growth factor or platelet-derived growth factor-mediated pathways. However, most patients eventually develop resistance to these drugs and require second or third-line therapies (Buczek et al., 2014). Immune checkpoint inhibitors are the most recent approved for treatment of metastatic RCC. Nevertheless, since these drugs are relatively new, the long-term clinical outcome is unknown at the moment. Hence, the genetic and molecular changes leading to the pathogenesis of RCC and development of therapy resistance are still not well understood.

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Abbreviations: α-SMA, α-smooth muscle actin; AQP-1, aquaporin-1; CK8, cytokeratin 8; CO2, carbon dioxide; ccRCC, clear cell renal cell carcinoma; DMEM, Dulbecco’s modified Eagle’s medium; FAP, fibroblast activation protein; FBS, fetal bovine serum; FFPE, formalin fixed paraffin embedded; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RCC, renal cell carcinoma; THP, Tamm-Horsfall protein
Cell lines provide an avenue for in vitro investigation into the molecular and genetic aspects of renal cancer carcinogenesis and pre-clinical studies for evaluating drug response or toxicity at a cellular level. Primary cell cultures offer the advantage of being more biologically similar to the tumour while continuous cell lines are homogenous and could be subcultured indefinitely. Both have their advantages and are integral for in vitro experimentation. At present, most commercially available RCC cell lines are established from Caucasians, such as ACHN, A-498, Caki-1, Caki-2, 769-P and 786-O. Asians and Caucasians differ in the incidence of RCC and their response to targeted treatment and immunotherapy (Naito et al., 2010; Lee et al., 2014; Znaor et al., 2015). Hence, there might be some differences in the underlying molecular mechanisms of RCC cells in Caucasians and Asians. The advantage of establishing cell lines from any research centre’s population is that patient information at diagnosis, tumour aggressiveness or patient clinical outcome of the corresponding cell lines will be known. In addition, the molecular responses of the established cell lines will likely conform to the characteristics of the study population.

In this study, a fast and simple method of establishing normal kidney epithelial cortex, RCC epithelial and fibroblast cell cultures or cell lines from primary tumours or nephrectomy specimen collected at surgery is described, with emphasis on RCC epithelial cell lines. The outcome of the cell line establishment and methods of initial cell characterisation are also presented here. This protocol has been modified from previous published reports with additional antibiotics (Primocin and Mycozap) and washing steps added to eliminate yeast/mold, bacterial and mycoplasma contamination present from the surgical source (Ebert et al., 1990; Shin et al., 2000; Park et al., 2004; Perego et al., 2005; Grimwood and Masterson, 2009; Vesey et al., 2009; Valente et al., 2011). In addition, this is the first report to present the details and protocols of simultaneous establishment of RCC, normal kidney and RCC-associated fibroblast cell cultures or cell lines from multiple trials/tumours.

### Methods and materials

RCC tissue and normal kidney samples were collected from consented patients who have undergone nephrectomy for the removal of kidney tumour. Ethical approval was obtained from the University of Malaya Medical Centre (UMMC) Ethics Committee (Ref: 848.17) and written informed consent was obtained for each patient.

### Materials for cell line establishment

Table 1 lists out the solutions or media, which were required for the establishment of cell cultures or cell lines. Unless stated otherwise, high glucose Dulbecco’s Modified Eagle’s Medium was purchased from Nacalai Tesque (Japan) and cell culture materials or supplements were obtained from Gibco (USA).

### Tissue collection

Tissue collection was carried out aseptically with the help of the urologist surgeon and pathologist. The urologist surgeon confirmed the location of the RCC lesions during tissue collection and the pathologist confirmed the pathological diagnosis after tissue processing. RCC tissue samples were taken within the tumour region, away from the tumour margin to avoid contamination with normal kidney cells. Tumour samples were collected from the viable fleshy soft part of the tumour, avoiding necrotic, fibrotic or haemorrhagic areas. Normal kidney samples were collected from the outer kidney cortex with macroscopically normal appearance, which was furthest away from the tumour lesion. Tissue collection was carried out within an hour of the tumour or kidney removal from the patient. Figure 1 illustrates an example of a resected kidney with RCC from which tumour tissue was taken for cell line establishment. Each tissue sample was cut in two pieces with one part for formalin fixation [formalin fixed paraffin embedded (FFPE) slides] and one part for cell line

| Table 1 Solutions or media used in the establishment and maintenance of cell cultures and cell lines |
|---|---|
| Solution/media | Contents |
| Tissue collection media | DMEM with high glucose 4.5 g/L and sodium pyruvate supplemented with antibiotic–antimycotic 3 x (penicillin 300units/mL, streptomycin 300 µg/mL and amphotericin B 0.75 µg/mL) |
| Phosphate-buffered saline pH 7.4 | Purchased as a 10x stock solution from First Base Laboratories |
| Collagenase solution | Collagenase type II 1 mg/mL in tissue collection media |
| Culture medium I | DMEM with high glucose 4.5 g/L and sodium pyruvate supplemented with 10% FBS, 1x antibiotic–antimycotic, Primocin 100 µg/mL (Inivivogen, USA) and Mycozap prophylactic 1x (Lonza, USA) |
| Culture medium II/Normal culture medium | DMEM with high glucose 4.5 g/L and sodium pyruvate supplemented with 10% FBS and 1x antibiotic–antimycotic 0.25% Trypsin-EDTA solution |
| Cryomedia | DMEM plus 10% FBS and 10% dimethyl sulfoxide |

DMEM, Dulbecco’s modified Eagle’s medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum.
establishment. The RCC tissues for cell line establishment (sized 0.5–1.5 cm³) were placed in separate 50 mL centrifuge tubes with 5 mL of ice-cold tissue collection media.

Tissue processing

Tissue samples were transported to the laboratory within an hour after collection and tissue processing was performed aseptically in a Class II biosafety cabinet. Kidney tumour and normal tissue were processed separately using a similar protocol. Tissue samples were first placed on a Petri dish where fat tissue and visible blood clots were dissected out. Tissue samples were then washed and agitated in cold PBS pH7.4 in sterile 50 mL tubes 4–5 times to remove any remaining blood. At this point, it is possible to store the tissue in culture medium I at 4°C overnight (=8–12 h) and continue processing the next day. However, it is advisable to proceed to the next steps immediately if time allows.

Tissue samples were placed on 60 mm Petri dishes and minced into 1 mm³ pieces with sterile blades. Next, tissue for establishment of epithelial cells was digested with collagenase (Establishment of epithelial cell lines). However, for fibroblast cell establishment, the minced pieces were washed in PBS and placed directly in culture flasks for propagation, without collagenase digestion (Establishment of fibroblast cell lines).

Establishment of tumour and normal epithelial cell cultures or cell lines

Procedures were performed at room temperature (=27°C) unless stated otherwise. Tumour and normal epithelial cells were processed using a similar protocol. Tissue fragments were transferred to clean 50 mL tubes and washed twice in fresh cold tissue collection media by centrifuging at 300g for 5 min. The supernatant was removed each time, and after the second wash, approximately 5 mL of collagenase solution was added to each tube. The tubes containing the tissue fragments were agitated using a shaking incubator at 37°C for 45 min-1 h. After enzymatic digestion, the digested tissue from each sample was sieved through a 70 µm cell strainer (SPL Life Sciences, South Korea) into a clean 50 mL tube to remove undigested tissue and glomeruli. The 50 mL tubes containing the cells were centrifuged for 5 min at 300g and the supernatant was pipetted off. The cells were re-suspended in pre-warmed culture medium I and transferred to 25 cm² culture flasks (SPL Life Sciences). Typically, cells from each sample were seeded in two 25 cm² flasks. Cell viability was not determined at this stage and seeding density was not tightly controlled. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. After an overnight incubation (12–24 h), the culture medium in the flasks was pipetted off along with any unattached cells and blood cells.

Figure 1 An example of nephrectomy specimen taken before resection of tissues for cell culture or cell line establishment. The tumour tissue for cell line establishment was taken from a tumour area clear of necrosis or haemorrhage. The section with normal kidney tissue was distinguishable from the tumour lesion.

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Attached cells were gently washed once with pre-warmed wash media and replaced with fresh culture medium I. This procedure was repeated after 48 and 72 h until the flasks were free of unattached cells and debris. Subsequently, culture medium was changed every 2–3 days until confluence. On day 7 onwards, culture medium II was used instead of culture medium I.

Yeast and bacterial contamination were tested using the Cell Culture Contamination Detection Kit (Molecular Probes, Thermo Fisher, USA) and presence of mycoplasma was detected using the MycoFluor™ Mycoplasma Detection Kit (Molecular Probes, Thermo Fisher).

Establishment of cancer-associated fibroblast cell cultures

Fibroblast cells were grown using the tissue explant technique. Tumour tissue minced into 1 mm³ pieces were placed in a 25 cm² culture flask and pre-warmed culture medium I was added before incubation at 37°C in a humidified atmosphere of 5% CO₂. After an overnight incubation (12–24 h), the culture medium in the flasks was pipetted off along with any unattached cells and debris. Attached tissue pieces were gently washed once with pre-warmed wash media and replaced with fresh culture medium I. This procedure was repeated after 48 and 72 h until the flasks were free of unattached cells and debris. On day 7 onwards, culture medium II was used instead of culture medium I. Fibroblast cells typically migrate out from the explant after 5–10 days. If growth was slow, 5 ng/mL of fibroblast growth factor (Merck Millipore, USA) was added to the culture medium to encourage fibroblast growth.

Cell culture maintenance and subculture

Cells were grown to 80–90% confluency before passaging. Cell culture medium was removed and 1 mL of 0.25% trypsin-ethylenediaminetetraacetic acid was added to each 25 cm² flask. After 5 min incubation at 37°C, the flasks were gently tapped to detach cells and trypsin reaction was stopped with the addition of 1 mL culture medium II. Cells were pelleted by centrifuging at 200 g for 5 min and re-suspended in culture medium II before seeding into a new 25 cm² flask. Passaging was carried out in a ½ split ratio. For cryopreservation, cells were re-suspended in 1 mL cryomedia, frozen at −80°C overnight and stored at −190°C in a liquid nitrogen tank. Cells were reactivated from cryopreservation by thawing at 37°C in a water bath and centrifuging the cells at 200 g for 5 min. The cells were re-suspended in culture medium II and seeded into a 25 cm² flask.

Cell characterisation by immunofluorescence staining

General cell morphology was viewed under an inverted microscope. Cells were seeded at 1 × 10⁵/well in a 24-well plate. One sterile 9 mm cell culture coverslip (SPL Life Sciences) was placed in each well before seeding. After 24 h, the growth medium was removed and cover slips were washed 2× with phosphate-buffered saline (PBS). Cells grown on cover slips were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature (RT). The cells were then washed with PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (H₂O₂) in PBS for 5 min. After washing with PBS, the cells were incubated with primary antibody diluted to the optimised concentration in PBS overnight at 4°C. The dilution for pan-cytokeratin (PCK-26)(Abcam, USA) was 1:150, α-smooth muscle actin (α-SMA) (1A4) (Dako, USA) was 1:800, aquaporin-1 (AQP-1) (B-11) (Santa Cruz, USA) was 1:150 and Tamm–Horsfall protein (THP) (B-2) (Santa Cruz) was 1:200. The cells were washed with PBS and incubated with secondary antibody (anti-mouse/rabbit Alexa Fluor 488 from Thermofisher, USA) (1:200 dilution) before cover slips were mounted on slides using Vectashield aqueous mounting medium with 4',6-diamidino-2-phenylindole.

Immunohistochemistry staining was also carried out using pan-cytokeratin (Abcam, USA) (1:200 dilution), α-SMA (1:400 dilution), AQP-1 (1:150) and THP (1:150) in FFPE normal kidney and clear cell renal cell carcinoma (ccRCC) tissue collected from the operating theatre (results not shown). This was done to determine the localisation and expression pattern of both antibodies in intact kidney tissue.

Quantitative polymerase chain reaction (qPCR)

The expressions of epithelial and fibroblast markers were determined in established cell lines using qPCR. Briefly, RNA was extracted from cell lines using the Trizol reagent (Invitrogen, USA) according to the manufacturer’s instructions. The extracted RNA was evaluated for RNA concentration, A260/A280 and A260/230 values using Nanophotometer (Implen, USA). Acceptable A260/A280 was 1.8–2.2 and A260/230 was 2.0–2.2. If not immediately used, the RNA was stored at −80°C until analysis. Complementary DNA (cDNA) conversion was achieved using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher) according to the manufacturer’s instructions.

qPCR reaction was performed with the EvaGreen qPCR Mix Plus (Solis Biodyne, Estonia). The run method used was as follows:

Holding Stage—95°C, 15 min
Cycling—denaturation 95°C, 15 s
Annealing—60°C, 1 min
Elongation—72°C, 20 s.
Gene expression level was quantified by the comparative $C_T$ method. The difference between the $C_T$ values ($\Delta C_T$) of the gene of interest and the housekeeping gene was calculated for each experimental sample. The housekeeping gene used was 18s rRNA. The expressions of epithelial markers cytokeratin 8 (CK8) and E-cadherin, as well as fibroblast markers $\alpha$-SMA, fibroblast activation protein (FAP) and vimentin were evaluated. Vimentin was expected to be expressed in ccRCC epithelial cells as well. Primer sequences are shown in Table 2. Primer sequences were obtained from PrimerBank, a validated public online resource for PCR primers provided by collaboration between Massachusetts General Hospital and Harvard Medical School. Gene expressions of established cell lines were compared with the equivalent messenger RNA levels found in a representative ATCC RCC epithelial cell line (ACHN). All experiments were carried out in triplicates.

### Results

#### Establishment of renal tumour and normal kidney cell culture or cell lines

After optimising the establishment method, 11 epithelial tumour cell lines spontaneously immortalised out of 51 trials (from different patients). Therefore, the spontaneous immortalisation rate of tumour epithelial cells was 21.6% with the optimised protocol. Cells were considered immortalised if they could be passaged beyond the 10th passage. Most cells senesced and stopped proliferating after three to five passages. Out of the spontaneously immortalised cell lines, seven were clear cell RCC (ccRCC) variants, two were ccRCC with sarcomatoid transformation, one was mostly undifferentiated RCC with some papillary features and one was non-RCC Ewing’s sarcoma (Table 3). Each cell line had distinctive morphology ranging from polygonal epithelial to spindle-shaped and elongated cells (Figure 2).

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### Table 2

Primer sequences for quantitative polymerase chain reaction of epithelial and fibroblast marker expressions in cell cultures or cell lines

| Genes      | Primers | Sequence                                      |
|------------|---------|------------------------------------------------|
| 18s rRNA   | Forward | 5'-CAGCCACCCGAGATTGAGCA-3'                    |
|            | Reverse | 5'-TGTAGCGAGGCGGGTG-3'                        |
| CK8        | Forward | 5'-CAGAGCTCTACAGTGGCT-3'                      |
|            | Reverse | 5'-GGGTTCGAGGAGTTATAGG-3'                     |
| E-cadherin | Forward | 5'-CTCTGTTGACCCTACTG-3'                       |
|            | Reverse | 5'-GCTGAGACATTGAAAGCT-3'                      |
| $\alpha$-SMA | Forward | 5'-TGACAGCTATGTGGCAGTG-3'                    |
|            | Reverse | 5'-GGTCTTTGGACATCCCGT-3'                      |
| FAP        | Forward | 5'-TGCGTGGAGGATCTA-3'                         |
|            | Reverse | 5'-CCAGAGGAGCTG-3'                            |
| Vimentin   | Forward | 5'-CCAGAGGAGCTG-3'                            |
|            | Reverse | 5'-CCAGAGGAGCTG-3'                            |

### Table 3

Patient characteristics of spontaneously immortalised cell lines

| Cell lines  | Gender | Age | T stage | N stage | M stage | Grade | Subtype                        |
|-------------|--------|-----|---------|---------|---------|-------|---------------------------------|
| UMRCC1      | F      | 54  | 3a      | 0       | 1       | 3     | ccRCC                           |
| UMRCC2      | M      | 38  | 2a      | 0       | 0       | 3     | ccRCC                           |
| UMRCC3      | F      | 55  | 2b      | 0       | 0       | 2     | ccRCC                           |
| UMRCC4      | F      | 67  | 2a      | 0       | 0       | 2     | ccRCC                           |
| UMRCC5      | M      | 42  | 2b      | 1       | 0       | 4     | ccRCC with sarcomatoid transformation |
| UMRCC6      | M      | 67  | 4       | 1       | 1       | 4     | ccRCC with sarcomatoid transformation |
| UMRCC7      | M      | 69  | 3a      | 0       | 1       | 2     | ccRCC                           |
| Ewing1      | M      | 32  | 2b      | 0       | 0       | –     | Ewing’s sarcoma                 |
| UMRCC8      | M      | 64  | 1b      | 0       | 1       | 1     | ccRCC                           |
| UMRCC9      | F      | 62  | 1b      | 0       | 1       | 1     | ccRCC                           |
| UMRCC10     | M      | 62  | 2b      | 0       | 1       | 4     | Mostly undifferentiated RCC with some papillary features |

All immortalised cell lines were maintained for more than 20 passages. ccRCC, clear cell renal cell carcinoma; F, female; M, male.
Nine immortalised cell lines were from patients with stage T2 tumours and above, whereas two were stage T1b with metastasis. In total, 6/11 (54.5%) immortalised cell lines were from patients who had metastasis at presentation. The cell lines were from tumours with grade 2 and above except for two with grade 1 and metastasis (both T1b tumours). Figure 3 displays examples of cell lines of various subtypes, which grew successfully and their different cell morphologies under the inverted microscope.

Normal epithelial kidney cortex cells (proximal tubule cells) were relatively easy to grow and proliferated at a faster rate than the tumour epithelial cells. After the first passage, tumour epithelial cells usually reached confluence in 3–15 days while normal epithelial kidney cortex cells reached confluence in 2–5 days with a 1:2 split ratio for passaging. If there was normal epithelial kidney cell contamination in the tumour cell culture, they would usually be morphologically distinctive and outgrow the tumour cells at initial passages (Figure S1). Therefore, it was important to avoid the tumour margin during tissue collection. None of the normal kidney cortex cells achieved spontaneous immortalisation.

Out of 18 trials of growing RCC cancer-associated fibroblast cells, none has immortalised at the moment. Eight fibroblast cell cultures were successfully passaged once, with one cell culture proliferating up to nine passages before senescence. The rest either grew too slowly and could not reach confluency to be passaged, were overtaken by epithelial cells or the explant did not attach well. Compared with RCC epithelial cells, RCC fibroblasts were harder to grow and contamination of fibroblast cells in RCC epithelial cultures was rarely an issue. Morphologically, RCC epithelial and fibroblast cells were distinguishable (Figure 2).

**Immunofluorescence characterisation of cell cultures or cell lines**

RCC epithelial cells stained strongly positive for the epithelial marker, pan-cytokeratin and negative for the fibroblast marker, α-SMA (Figure 4). Fibroblast cells stained strongly for α-SMA and negative for pan-cytokeratin. Normal kidney cortex cells (proximal tubule cells) stained positive for pan-cytokeratin and AQP-1 (proximal tubular epithelial cell marker) (Figure 4) while staining negative or weakly for α-SMA and the distal tubule marker THP (images not shown).

**qPCR characterisation of cell cultures or cell lines**

qPCR analysis revealed higher epithelial marker expressions in these selected examples of RCC epithelial cell lines compared with fibroblast cells (Figure 5). These
Figure 3  The morphology of cultured cells at confluency (<passage 10) and their tumour counterparts (H&E staining) of various subtypes. RCC, renal cell carcinoma.
Figure 4 Examples of DAPI and Alexa fluor 488 staining of established RCC epithelial, RCC fibroblast and normal kidney cortex cell cultures or cell lines. Commercial ACHN cell line was used as a comparison. (A, C, E and G) Staining for pan-cytokeratin; (B, D and F) staining for α-SMA. (H) Staining for AQP-1 in normal kidney cortex cells. α-SMA, α-smooth muscle actin; AQP-1, aquaporin-1; DAPI, 4',6-diamidino-2-phenylindole; RCC, renal cell carcinoma.
epithelial cell lines were designated UMRCC1 onwards. UMRCC1, UMRCC2, UMRCC6, and UMRCC10 showed higher epithelial marker (CK8 and E-cadherin) and lower fibroblast marker (α-SMA) expressions compared with Fibroblast 1 and 2. The RCC epithelial cell lines had lower FAP expression compared with the fibroblast cells except UMRCC6, which was established from a ccRCC tumour with sarcomatoid transformation. All cell lines have mixed vimentin (fibroblast marker) expression as ccRCC epithelial cells are known to express this protein.

Discussion

Epithelial RCC cell lines were established from the primary tumour tissue of RCC patients with a spontaneous immortalisation rate of 21.6% using this protocol. This was slightly higher than the 12.7% rate obtained by Ebert et al. (1990), which reported the spontaneous immortalisation rate of RCC cells (Ebert et al., 1990). In this study, immortalised cell lines were from tumours with more clinically aggressive characteristics such as larger tumours (stage T2 and above), higher grade (grade 2 and above) or has metastasised. The established cell lines were confirmed to be epithelial cells with higher expressions of epithelial markers such as pan-cytokeratin, CK8 and E-cadherin (Valente et al., 2011; Subramaniam et al., 2013). These cells also exhibit lower expression of fibroblast marker α-SMA (Goodpaster et al., 2008). Interestingly, UMRCC6, a RCC cell line with sarcomatoid differentiation, had higher FAP expression compared with the other epithelial cell lines evaluated. FAP is marker of activated fibroblast and is also expressed by cancer-associated fibroblasts. In primary RCC tumours, FAP is expressed on stromal fibroblasts and is shown to be associated with tumour aggressiveness, including sarcomatoid transformation (Errarte et al., 2016). Morphologically, UMRCC6 cells were slightly spindle-shaped and expressed high epithelial markers.

Using this protocol, kidney tumour cell lines of various subtypes were successfully established, including uncommon types like ccRCC with sarcomatoid transformation, largely undifferentiated RCC and Ewing’s sarcoma, which is a non-RCC kidney tumour. A multilocular cystic RCC cell culture was successfully grown for three passages, but did not achieve spontaneous immortalisation. Most commercially available cell lines are of the common subtype, ccRCC, such as Caki-1, Caki-2, 769-P and 786-0, while the subtypes of ACHN and A-498 cell lines were unclear (Brodaczewska et al., 2016). Hence, these newly established cell lines could provide a valuable resource as in vitro models for rare kidney tumour subtypes. To the authors’ knowledge, there are no commercial RCC with sarcomatoid features or Ewing’s sarcoma of the kidney cell lines, which are easily available.
Normal kidney cell cultures established from the same patients can be used as controls for in vitro experiments, as they represent the non-malignant counterpart of the tumour cell line. In addition to established cell cultures or cell lines (≥ passage 1), cryo-preserved primary cultures (passage 0) are available for studies with the likelihood that they retain the molecular profile of the corresponding tissue (Perego et al., 2005). The above reasons emphasize the advantage of establishing cell cultures or cell lines at research centres if patient samples are accessible.

Several protocols for RCC and kidney cell culture or cell line establishment have been reported, utilising various techniques such as the gradient centrifugation technique, enzymatic digestion or explant method (Ebert et al., 1990; Shin et al., 2000; Park et al., 2004; Perego et al., 2005; Vesey et al., 2009; Valente et al., 2011). Based on the trials during this protocol optimisation, the growth of the epithelial cells before the first passage for the explant method was slower compared with using enzymatic tissue digestion. Therefore, the epithelial protocol described here entails enzymatic digestion followed by cell sieving. Contamination with fibroblast cells was seldom an issue in this study as RCC tumour associated fibroblast cells were more difficult and slower to grow than the epithelial cells. Fibroblast cells were more successfully grown using the explant method, similarly described by previous groups (Grupp and Muller, 1999; Grimwood and Masterson, 2009). Due to the limited publications on cancer-associated fibroblasts in RCC, the optimisation of the fibroblast establishment protocol or technique could be pursued further. This is because cancer-associated fibroblasts are known to interact with cancer cells to promote tumour growth and progression (Shiga et al., 2015).

Compared with previously reported protocols, additional antibiotics and washing steps were added as a precaution to prevent bacterial and mycoplasma growth as primary cultures from human tissues can suffer from contamination issues (Ebert et al., 1990; Shin et al., 2000; Vierck et al., 2000; Park et al., 2004; Perego et al., 2005; Vesey et al., 2009; Valente et al., 2011; Arul et al., 2014). Using this protocol, coating of culture flasks is not required and washed tumour/normal kidney tissue can be left overnight in culture medium at 4°C before further tissue processing with good success rate. This allows for more convenient tissue processing of specimens collected from operation cases, which are carried out in the evenings or at night. Further experimentation and molecular or phenotypic characterisation will be required for evaluating the different subtypes of renal tumours. Molecular and phenotypic characterisation will also determine the genetic or phenotypic stability of the cell lines.

Conclusions

In summary, the protocol described in this paper allows for simultaneous establishment of RCC, normal kidney and RCC-associated fibroblast cell lines or cultures from nephrectomy specimens with a good success rate of spontaneous immortalisation for RCC epithelial cell lines. Tissue location selection from the surgical specimen is important and morphological, immunofluorescence and qPCR characterisation can be carried out to determine the epithelial or fibroblastic origin of the cells. Further characterisation via mutational analysis can be performed next to determine the genetic mutational or molecular features of the cell lines.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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