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FROM THE BENCH

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Protocol for the long-term culture of human primary keratinocytes from the normal colorectal mucosa

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Abstract

Procedures for in vitro culturing of human primary keratinocytes from normal colon mucosa specimens have not been fully feasible, thus far. The protocol described herein allows primary keratinocytes from small tissue fragments of colorectal mucosa biopsies to grow in vitro. The procedure develops in three steps: (a) the enzymatic digestion of the tissue biopsy; (b) the use of cloning rings to purify primary keratinocyte colonies, (c) a defined keratinocyte medium to grow these cells in long-term culture. Our cultural method enables normal primary keratinocytes to be obtained by simple and rapid techniques. In our culture condition, primary keratinocytes express specific epithelial markers. Colorectal mucosa keratinocyte colonies require approximately 2 weeks to grow. Compared with previous approaches, our protocol provides a valuable model of study for human primary keratinocytes from normal colorectal (NCR) mucosa both at the cellular and molecular levels. It is well known, that different mutations occurring during the multistep process of carcinogenesis in the NCR mucosa, are strictly associated to the onset/progression of the colorectal carcinoma. On this ground, normal keratinocytes grown with our protocol, may represent an innovative tool to investigate the mechanisms that lead to colorectal carcinoma and other diseases. Our innovative procedure may allow to perform comparative investigations between normal and pathological colorectal cells.

KEYWORDS
colorectal mucosa, defined medium, keratinocytes, long-term culture, primary epithelial cells

1 | INTRODUCTION

The colon, which is the first part of the large intestine, represents the final tract of the digestive canal. Its main function is to maintain physiological homeostasis, by regulating the absorptive and secretory processes (Bachmann & Seidler, 2011). These functions are guaranteed by epithelial cells lining the colonic mucosa (Bloushtain-Qimron, Yao, Shipitsin, Maruyama, & Polyak, 2009) including keratinocytes, the most abundant epithelial cell lineage in both the small intestine and colon (Gallo & Hooper, 2012). Alterations in the normal growth and function of these cells are strictly associated with the onset/progression of severe pathological conditions, such as inflammatory diseases and colon cancer (Gradel et al., 2009; Kim & Chang, 2014; Okamoto & Watanabe, 2016; Pastorelli, De Salvo, Mercado, Vecchi, & Pizarro, 2013). Thus far, most of what is known about normal colon epithelial cell characteristics has been derived from

studies carried out on cell cultures obtained from tissue biopsies taken from experimental animals (Evans, Flint, & Potten, 1994), and human cancer specimens (Rousset, 1986). Experimental data obtained with human cell lines from colorectal carcinomas, such as Caco-2, HT29, and T84, which may spontaneously differentiate into intestinal-like cells (Moberg, Bell, Wahrer, Haber, & Hariharan, 2001; Peck et al., 2016), are limited by the cancerous nature of these cellular models (Whitehead, Vaneeden, Noble, Ataliotis, & Jat, 1993). In recent years, many attempts have been made to set up, isolate and grow epithelial cells from normal human colorectal mucosa specimens. However, these methods have shown several limitations, such as low cell yield in primary culture and the presence of contaminating fibroblasts in the epithelial culture (Deveney et al., 1996; Lichti, Anders, & Yuspa, 2008; Rasmussen, Thomas-Virnig, & Allen-Hoffmann, 2013; Vidrich, Ravindranath, Farsi, & Targan, 1988).

The setting up and development of homogeneous cultures of human primary mucosa-derived colon keratinocytes as a model of study for investigating the cellular and molecular biology of normal keratinocytes are therefore of interest.

In this report, we describe an effective, simple and rapid protocol to set up long-term cultures of viable and isolated human primary keratinocytes from normal colorectal (NCR) mucosa. This method may enable comparative investigations between using normal and pathological colorectal cells, including carcinoma-derived keratinocytes, both at the cellular and molecular levels.

2 | MATERIALS AND METHODS

2.1 | Colorectal tissue specimens

Small fragments (10–20 mm^3) of colorectal mucosa biopsies (NCR) were obtained from the resection margins (about 2–5 cm from the edge of the tumor) of colectomy specimens from 15 patients who underwent colorectal cancer surgery. NCR samples were collected for research purposes. Informed written consent was obtained from patients. Human specimens were used in agreement with the institutional biosafety protocol requirements. Anonymously collected NCR mucosa tissue samples were coded with indications of the anatomical area of the colorectum, age, and gender. Specifically, we harvested 15 biopsies from the following areas of the colorectum: cecum (2/15), ascending colon or right colon (8/15), sigmoid colon or sigma (2/15), and rectum (3/15). The County Ethics Committee, Ferrara, approved the study, assigning the number 151078, including the methods used. Every biopsy was immediately transferred into a 50 ml conical tube containing 10 ml of cool (4°C) Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 medium (DMEM F12, Cat. No. BE12-719F; Lonza, Milan, Italy) serum-free medium supplemented with antibiotics, 800 U/ml penicillin–streptomycin (Cat. No. DE17–602E; Lonza) and 0.25 μg/ml Amphothericin B (Cat. No. 17–836E; Lonza; see the "Reagent and equipment details" Supporting Information). Specimens were transferred to the laboratory within 2 hr for setting up the NCR tissue culture.

2.2 | Primary colorectal mucosa keratinocyte culture set up

A detailed protocol of NCR tissue culture set up is reported in the "Detailed Protocol" Supporting Information. Briefly, NCR tissue fragments were washed profusely in Dulbecco's phosphate-buffered saline (DPBS) 1× (Cat. No. BE17-513F; Lonza) supplemented with antibiotics, minced, and digested overnight (ON) with 0.7% type II collagenase (Col II; Cat. No. CSL-2; Worthington Biochemical Corporation, Lakewood, NJ) at 37°C, 5% CO_2. Suspended cells were washed in DPBS 1×, counted and seeded in T25 flasks (T0) with DMEM F12 containing 10% fetal bovine serum (FBS; Cat. No. ECS0180L; EuroClone, Milan, Italy), then left to attach ON. The following day, unattached cells were recovered and reseeded into a six-well plate (T1) and left to attach for a further 2 days. Primary cultures were grown for an additional 5 days, changing the medium twice weekly. This culture period allows the formation and growth of early keratinocyte colonies.

2.3 | Primary colorectal mucosa keratinocyte isolation and expansion

At Day 7, the DMEM F12 medium containing 10% FBS for NCR keratinocyte cultures was substituted with a mixture of DMEM F12 medium and defined keratinocyte serum-free basal medium (dKSFM; Cat. No. 10785-012; Thermo Fisher Scientific, Waltham, MA) in a 1:1 ratio, 5% FBS for further 7 days, changing the medium twice a week. To efficiently identify and isolate primary keratinocyte colonies visualized under an inverted phase-contrast microscope (Olympus CK2-TR; Olympus, Tokyo, Japan), small circles were drawn with a marker around the colonies, outside the bottom part of the six-well plate (T1). Then, each NCR colony was isolated by a cloning ring, then transferred and cultured in a new six-well plate (T2) in presence of serum-free dKSFM. At confluence, keratinocytes were detached with trypsin–ethylenediaminetetraacetic acid (trypsin-EDTA; Cat. No. BE17-161E; Lonza) and expanded into T25 flasks in serum-free dKSFM. Primary cultures were grown for 5 weeks, which was considered an ideal culture period to allow the expansion and growth of pure keratinocyte colonies.

2.4 | Immunofluorescence assays

NCR primary keratinocytes were seeded (5×10^3 cells) on cover glasses in presence of DMEM F12/dKSFM (1:1 ratio) medium or 100% dKSFM medium at 37°C, 5% CO_2. After 24 hr, keratinocytes were fixed with 10% of neutral buffered formalin (Cat. No. 05-01005Q; Bio-Optica, Milan, Italy) for 7 min at room temperature, permeabilized in 0.1% Triton X-100 (Cat. No. 93420; Fluka Chemi AG, Buchs, Switzerland)/DPBS for 2 min and blocked using 1:20 normal goat serum (sc-2043; Santa Cruz Biotechnology, Inc., Dallas, TX) in 3% bovine serum albumin (Cat. No. A3294; Sigma-Aldrich, Milan, Italy)/DPBS for 30 min. Then, cells were stained with an anti-cytokeratin antibody (clone MNF116; Cat. No. M0821; Dako, Glostrup, Denmark)

at room temperature for 2 hr, followed by incubation with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (F5262; Sigma-Aldrich) at room temperature for 1 hr, in the dark. Nuclei were counter-stained with (4′,6-diamidino-2-phenylindole [DAPI]; Cat. No. D1306; Thermo Fisher Scientific). The colon cancer cell line HTC-116 (obtained from American Type Culture Collection [ATCC]; Cat. No. CCL-247) was used as a positive control, while human fibroblasts, MRC-5 (obtained from ATCC; Cat. No. CCL-171), were used as negative control for cytokeratin (CK) expression. Negative staining control was performed by omitting the primary antibody. Images were acquired with an Eclipse E-2000 fluorescence microscope (Nikon Instruments, Sesto Fiorentino, Firenze, Italy) equipped with a DXM 1200 F digital camera, using the ACT-1 software (Nikon Instruments, Sesto Fiorentino, Italy).

2.5 | Digital droplet polymerase chain reaction (ddPCR) analysis

Total RNA from MRC-5, HTC-116, and NCR-derived primary cultures was extracted with the RNeasy Plus Mini Kit (Cat. No. 74134; Qiagen, Hilden, Germany) according to manufacturer instructions. RNA quality and quantity were assessed by using a Nanodrop spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE). First-strand complementary DNA (cDNA) was synthesized from 50 ng total RNA by reverse transcription using ImProm-II Reverse Transcription System (Cat. No. A3800; Promega, Italy) according to manufacturer instructions. The expression of specific epithelial markers such as CK8, CK18, and CK20 and fibroblast markers, as α-smooth muscle actin protein (αSMA) and type I collagen (Col I) was determined using ddPCR (Bio-Rad, Milan, Italy). Primer nucleotide sequences for target genes are shown in Table 1. β-2-microglobulin (b2m) quantification was used to normalized messenger RNA (mRNA) levels of target genes. Gene expression assay for b2m was obtained from Bio-Rad (Cat. No. 10031258). To perform ddPCR, 22-μl reaction mixtures containing 0.2 μM primers, 11 μl of QX200 EvaGreen ddPCR Supermix (Cat. No. 1864033; Bio-Rad), 1 ng of cDNA, and RNase-free water, were prepared. Each reaction was loaded into a disposable droplet generator cartridge (Bio-Rad), wherein 20 μl of droplet generation oil for EvaGreen (Cat. No. 1864112; Bio-Rad) was added. The cartridge was then placed inside the QX200 droplet generator (Cat. No. 1864108; Bio-Rad). The ddPCR device generates ~17,000–20,000 droplets per sample and thus the PCR occurs as individual PCR reactions in droplets. When droplet generation was completed, droplets were transferred to a 96-well PCR plate (Bio-Rad). The plate was heat-sealed with foil and placed in a conventional thermal cycler. Thermal cycling conditions were as follows: 95°C for 5 min, then 40 cycles of 94°C for 30 s and 55°C or 60°C for 1 min, and three final steps at 4°C for 5 min, 90°C for 5 min, and a 4°C indefinite hold to enhance dye stabilization. A no template control was included in every assay. At the end of the PCR amplification protocol, the droplets were read individually with the QX200 Droplet Reader (Bio-Rad) and quantified with QuantaSoft droplet reader software (Bio-Rad). Positive droplet populations were separated from negative droplets and quantified automatically as copies/μl.

2.6 | Statistical analysis

Statistical analysis was performed by GraphPad Prism 6 Software (GraphPad Software, La Jolla, CA) using one-way analysis of variance and the unpaired *t* test to compare differences in expression between selected groups. Results were expressed as mean ± *SEM* of copies/μl of target gene normalized by b2m expression (Iyer et al., 2017) and pooled from four replica experiments. $p < 0.05$ were considered as statistically significant.

2.7 | Ethical statement

The Ethics Committee of the University-Hospital of Ferrara institutional review board approved this study. All patients gave written informed consent to participate in the present investigation in compliance with the Declaration of Helsinki.

3 | RESULTS

3.1 | Experimental design protocol

Our growth procedure for keratinocytes obtained from NCR mucosa biopsies, was developed in three different steps by using: (a) the Col II for tissue digestion; (b) cell cloning rings to isolate and purify primary normal keratinocyte colonies; (c) a defined keratinocyte growth medium. This cell medium does not allow contaminant

TABLE 1 Primers used for droplet digital PCR (ddPCR) analysis

Genes	Primer nucleotides (nt) sequences	Annealing temperature (°C)
Cytokeratin 8 (CK8)	F: 5′-ACCCTCAACAACAAGTTTGCCTCC-3′ R: 5′-TCCACTTGGTCTCCAGCATCTTGT-3′	60°C
Cytokeratin 18 (CK18)	F: 5′-ATCTTGGTGATGCCTTGGAC-3′ R: 5′-CCTGCTTCTGCTGGCTTAAT-3′	60°C
Cytokeratin 20 (CK20)	F: 5′-ACGCCAGAACAACGAATACC-3′ R: 5′-TTCAGATGACACGACCTTGC-3′	55°C
Type I collagen (Col I)	F: 5′-CAGCCGCTTCACCTACAGC-3′ R: 5′-TTTTGTATTCAATCACTGTCTTGCC-3′	60°C
α-Smooth muscle actin (αSMA)	F: 5′-GCGTGGCTATTCCTTCGTTA-3′ R: 5′-ATGAAGGATGGCTGGAACAG-3′	60°C

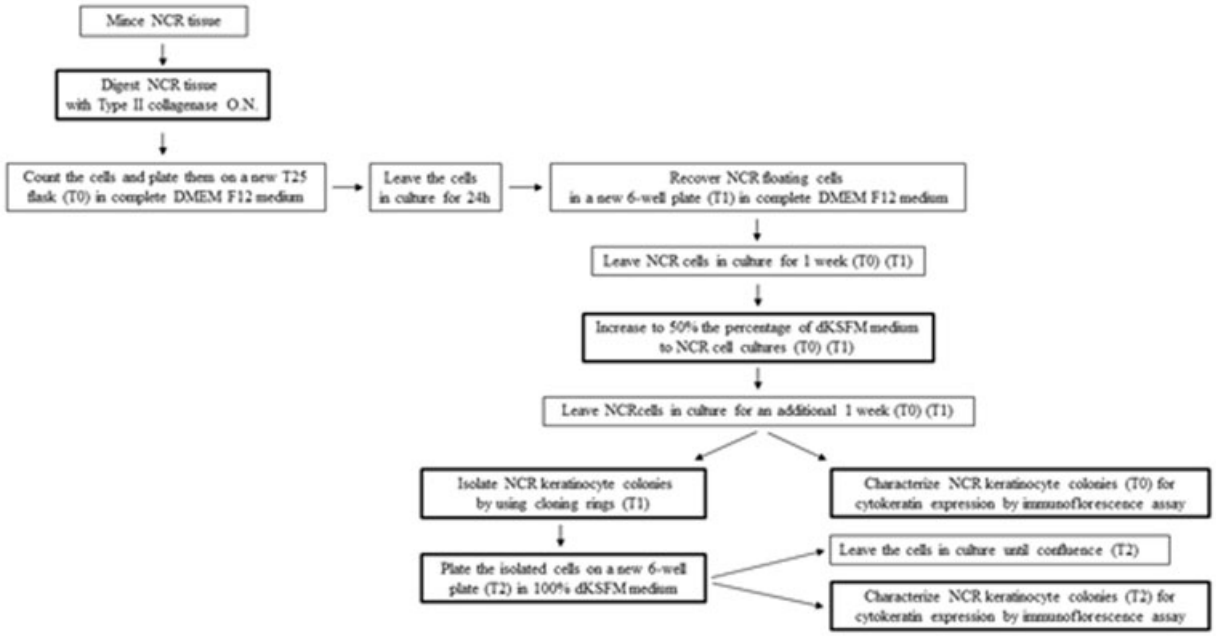

FIGURE 1 Flow diagram. The procedure for isolation and culture of human primary keratinocytes from normal colorectal mucosa biopsy is represented in this diagram

fibroblasts to grow, together with the keratinocytes maintained in culture for a long-term period.

Our protocol steps for preparing and culturing human primary keratinocytes from colonic mucosa, are simple, rapid, feasible, reproducible, and reliable (Figure 1).

In brief, surgically resected small fragments of human NCR were minced and digested with Col II overnight. Then, the digestive solution was removed, whereas the resulting cell suspension was counted, plated on a T25 cell culture plastic flask and incubated in complete DMEM F12 cell medium. A range of approximately 10^4–10^5 cells were then isolated from each NCR biopsy. Subsequently, 8–24 hr later the majority of seeded cells were attached to the plastic vessel. Floating rounded cells were recovered before feeding the attached cells with fresh medium, and plated on a six-well plate in complete DMEM F12 medium for 48 hr. The medium was changed twice weekly. The recovery procedure allows cells endowed with slow attachment capability to be rescued later, therefore enables an increased amount of viable cells, which may further form primary keratinocyte colonies. NCR-derived primary cultures were maintained in complete DMEM F12 medium for 1 week. During this period, the primary cultures were characterized by a heterogeneous mixture of keratinocytes and high proliferative fibroblasts. To isolate a keratinocyte cell population, inhibiting at the same time the fibroblast overgrowth, at Day 7 of culture, NCR-derived primary cultures were kept in an increasing percentage of dKSFM. In particular, primary keratinocytes were cultured in DMEM F12/dKSFM (1:1 ratio) containing 5% FBS for 1 week. Two weeks after plating, most of the initially attached cells gave rise to colonies of condensed cells showing the typical cobblestone morphology (Deveney et al., 1996). These epithelial cell monolayers were then isolated using the cloning rings and then expanded in culture by the dKSFM medium to obtain homogeneous primary keratinocyte cultures, without fibroblasts. The epithelial nature of these cells was established by evaluating the expression of specific markers, such as epithelial CKs (Karantza, 2011). At Day 10–14 of culture, cells grown in dKSFM medium showed multiple colonies exhibiting the epithelial morphology and specific markers.

It is well established that cell cultures represent the most useful model of study for investigating the in vitro process of carcinogenesis. In this context, it is useful to recall that cellular and molecular studies on keratinocytes have been poorly reported. To this purpose, our protocol which was set up to isolate and grow keratinocyte colonies from NCR mucosa provides a significant and useful culture system to investigate

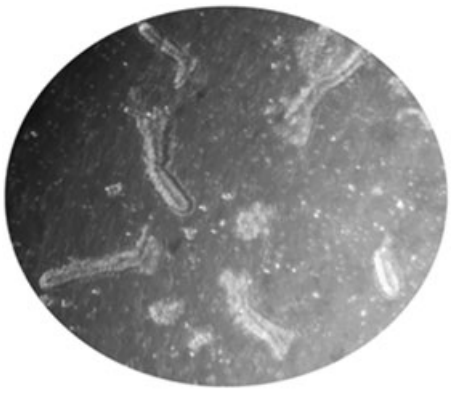

FIGURE 2 Crypts-derived biopsy. The representative image of crypts obtained by mechanical dissociation of human normal adult colorectal mucosa specimen is shown after processing the surgical specimen

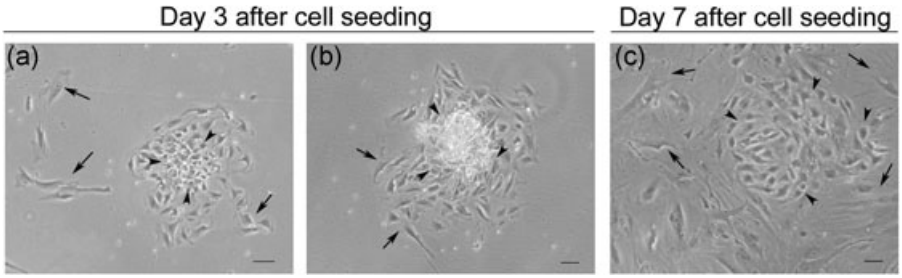

FIGURE 3 Cultured human primary colonic epithelial cells visualized on the inverted microscope Photographs were taken with a digital camera. (a,b) A few cells migrated out from the tissue fragment at Day 3 with scattered fibroblasts (arrows) surrounding the keratinocytes (arrowheads). (c) During the first week of culture, epithelial cells grew rapidly generating multiple colonies, which were visible at Day 7, surrounded by fibroblasts. Scale bar = 50 μm

the cellular and molecular mechanisms that lead to colorectal carcinoma process, as well as other colorectal pathologies.

3.2 | Establishment of human primary keratinocyte cultures from NCR mucosa

In the first step of our innovative protocol, clumps of epithelial cells were mechanically generated by a gentle scraping of NCR specimens. Microscope analyses showed in clumps some fibroblasts still attached to them, together with the presence of fragmented crypts (Figure 2). To maximize the yield of individual colonic epithelial cell, NCR tissue specimens underwent a collagenase digestion. The application of this procedure gave rise to colonies in the range of 50–100 colonies/NCR biopsy.

In our growth conditions, such as the medium and isolation procedures used at different stages, cell cultures derived from NCR tissue showed different morphological characteristics. Specifically at Stage I: NCR cells, cultured for 2–3 days in complete DMEM F12, mostly showed single cells that began to spread and migrate out of the digested tissue (Figure 3a,b). At Day 7, heterogeneous cell populations were still present in primary cultures. Cells were characterized by small areas of confluent epithelial-like cell monolayers surrounded by fibroblasts (Figure 3c). To avoid the massive fibroblast outgrowth and, therefore, the possibility to contaminate the primary cultures, cells were grown at Day 7 in presence of dKSFM medium, till confluence as described below.

Stage II. Cell populations were cultured in presence of DMEM F12/dKSFM (1:1 ratio), with 5% FBS, for further 7 days. At Day 14, NCR-derived primary cell culture showed about 20% of the fibroblast contamination, while keratinocyte colonies began to grow (Figure 4a–c[1]). As shown in Figure 4b, keratinocytes characterized by a uniform size had a typical epithelial "cobblestone" morphology.

Then, keratinocyte cells were isolated using the cloning rings. In addition, this procedure allowed to inhibit completely the fibroblast

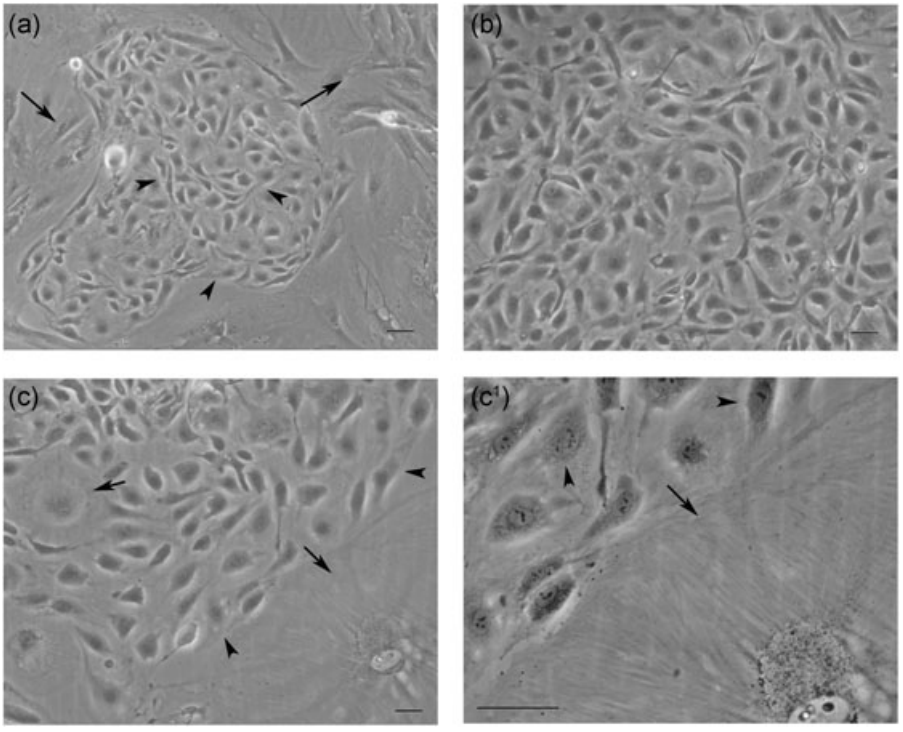

FIGURE 4 Representative images of human primary epithelial colonies expanded at Day 14. Photographs were taken by a digital camera mounted on an inverted microscope. Images show the typical cobblestone morphology of keratinocytes (arrowheads) and spindle shape fibroblasts (arrows) at different enlargements: (a–c) ×10 and (c[1]) ×20. Scale bar = 50 μm

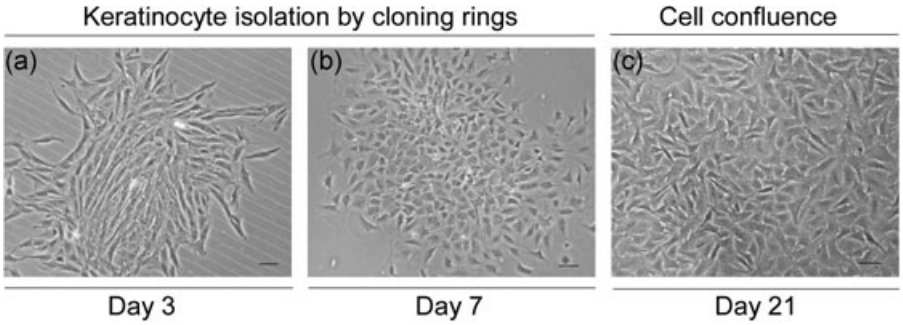

FIGURE 5 Human epithelial colonies isolated manually with cloning rings. (a,b) The morphological appearance of proliferating keratinocytes displays a uniform aspect, growing in colonies with defined edges. (c) At confluence, keratinocytes grew together closely forming a compact monolayer without fibroblast contamination. Scale bar = 50 μm

overgrowth and consequently the contamination of primary keratinocyte colonies by this connective component. To favor the expansion of isolated and purified epithelial cells, each colony was grown independently in a single well in presence of serum-free dKSFM medium.

Stage III. At this stage, the cell culture was characterized by the appearance of multiple keratinocyte colonies (Figure 5). These cells, which rapidly expanded in the plastic vessel, exhibited an epithelioid morphology, as expected for the keratinocyte shape. Conversely, the fibroblast population was definitively eliminated (Figure 5a,b). At Day 21, the keratinocyte monolayers reached the confluence, acquiring at the same time a marked epithelial morphology (Figure 5c).

3.3 | NCR-derived primary keratinocytes characterization by immunofluorescence

To confirm the epithelial nature of NCR-derived primary keratinocytes, the expression of a pancytokeratin marker was investigated by immunofluorescence. The pancytokeratin antibody used is a blend of CKs expressed in most, if not all, epithelial tissues and it detects an epitope common to CKs 5, 6, 8, 17, and 19. As shown in Figure 6a–c, the human keratinocyte colonies gave a positive reaction for pancytokeratin marker since Day 14 of culture. This positivity became stronger at Day 21 and remained stable up to confluence (Figure 6d–o).

The detection of CK staining in colon cancer cell line HTC-116 and the negative immunoreactivity in normal human fibroblast MRC-5, corroborated the epithelial origin of NCR-derived keratinocytes (Figure 7).

3.4 | NCR-derived primary keratinocytes characterization by ddPCR

To distinguish fibroblasts from keratinocytes in NCR-derived primary cells, at every stage of culture, we assessed the expression of epithelial- and intestine-specific markers, such as CK8, CK18 (Fujisaki & Shimoda, 1993; Liu, Zhang, Zhou, Qin, & Shen, 2010), and CK20 (Chougule et al., 2012; Helenius, Antman, Asghar, Nyström, & Toivola, 2016; Mohammadi, Saidijam, Kaki, Etemadi, & Shabab Yadegarazari, 2016) and the expression of fibroblast-specific markers such as Col I and αSMA (Milara et al., 2012; Wang et al., 2017; Watson et al., 2012), using the innovative PCR technique, the ddPCR.

In agreement with data published before, where the established real-time PCR technique was used (Castellanos-Gonzalez, Cabada, Nichols, Gomez, & White, 2013; Chougule et al., 2012; Liu et al., 2010), in our experiments, the expression of the above markers at mRNA level was investigated by ddPCR. It should be noted that this innovative ddPCR technique has greater accuracy and higher sensitivity than other available PCR systems.

As shown in Figure 8, NCR-derived primary cells showed an increase in the expression of specific epithelial markers, such as CK8, CK18, and CK20, during the different stages of culture. Specifically, they revealed low mRNA levels of CKs when cells are maintained in DMEM F12 medium with 10% FBS (F12). Then, the expression of CKs became higher in cells grown in presence of serum-free dKSFM medium (dKSFM 100%).

Specifically, CK8 and 18 had significantly higher mRNA levels in dKSFM 100% compared with F12 condition. Moreover, cells grown in dKSFM 100% exhibited a CK8 expression similar to HTC-116 cells, used as positive control for CK expression.

Conversely, NCR-derived primary cells showed a decrease in the expression of fibroblastic markers, Col I and αSMA, throughout the culture. NCR-derived primary cells expressed high Col I and αSMA mRNA levels at the first stage of culture (F12), then, when primary keratinocyte cultures became homogeneous with very low fibroblast contamination, Col I and αSMA expression significantly lowered compared with the early culture stages (F12 and dKSFM 50%). ddPCR data are in agreement with the results obtained using the immunofluorescence technique.

4 | DISCUSSION

The cell growth procedure, described herein, provides evidence that long-term culture of human primary keratinocytes from NCR mucosa can be obtained. Our NCR keratinocyte cell culture was set up and developed in three key steps by employing (a) the Col II for tissue

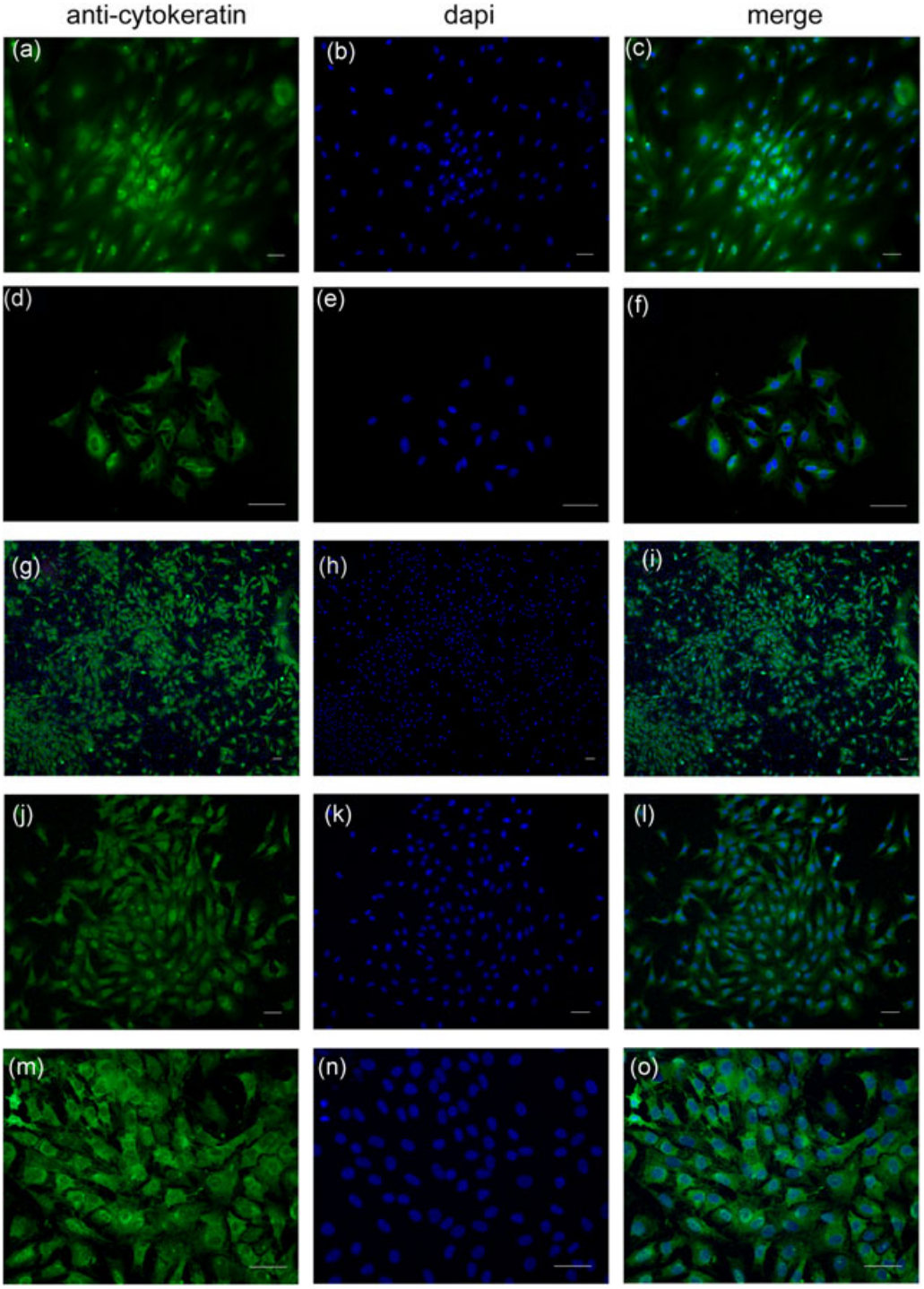

FIGURE 6 Human colorectal keratinocytes were characterized by their epithelial morphology and expression of the anti-cytokeratin marker. (a–c) Fluorescence staining showed the expression of specific epithelial keratins 5, 6, 8, 17, e 19 (green) in the keratinocytes cultured in DMEM F12/dKSFM (1:1 ratio), but not in the fibroblasts that are present at the Stage I of the isolation process. (d–f) The fluorescence staining showed the presence of keratinocytes isolated with cloning rings, while fibroblasts are absent. (g–o) The fluorescence staining of expanded colony of keratinocytes in dKSFM medium, without fibroblast contamination. 4× (g–i); ×10 (j–l); ×20 (m–o). Scale bar = 50 μm. DMEM F12: Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 medium; dKSFM: defined keratinocyte serum-free basal medium [Color figure can be viewed at wileyonlinelibrary.com]

digestion, (b) cloning rings to isolate and purify primary keratinocyte colonies and, (c) a defined keratinocyte growth medium to eliminate fibroblasts from the primary culture, thus maintaining keratinocytes in long-term culture.

Compared with previous colonic keratinocyte culture methods (Liu et al., 2010; Perreault & Beaulieu, 1996) our protocol enables to obtain NCR-derived keratinocyte colonies, which can be expanded at highly proliferative rates throughout different simple and rapid

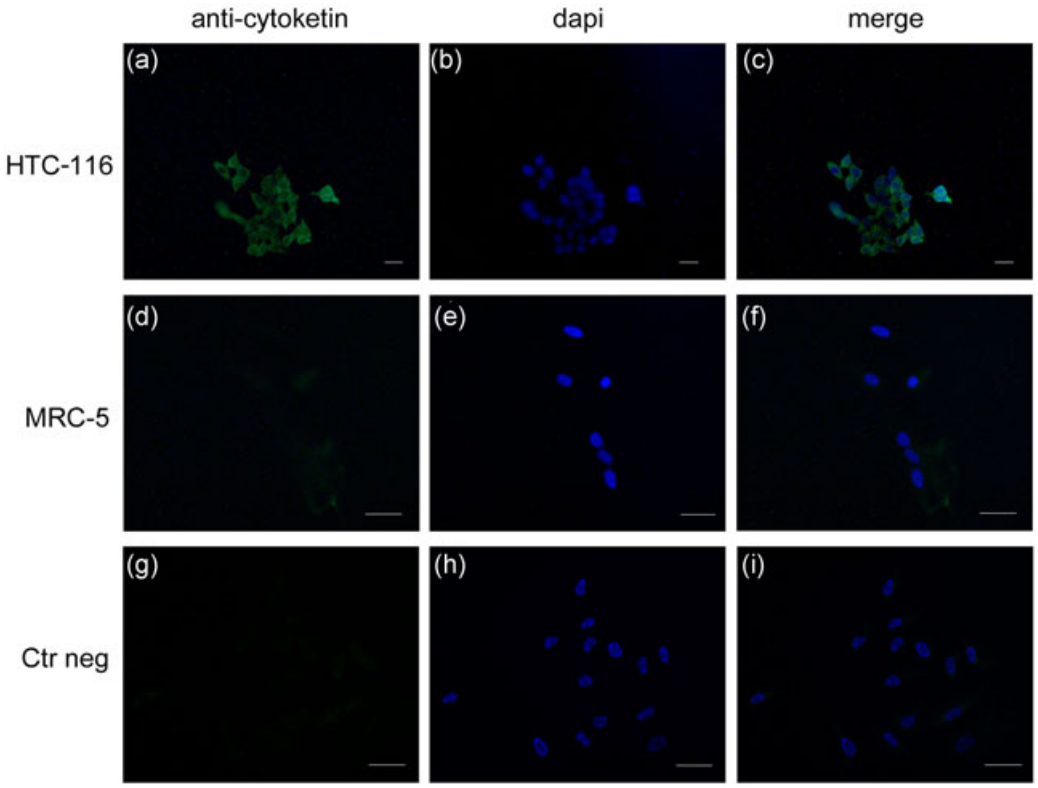

FIGURE 7 Controls for the immunofluorescence assay. (a–c) Fluorescence staining of colon cancer cell line HTC-116, used as positive control. (d–f) Normal human fibroblast, MRC-5 used as negative control for cytokeratin expression. (g–i) The negative staining control performed by omitting the primary antibody. Scale bar = 50 μm

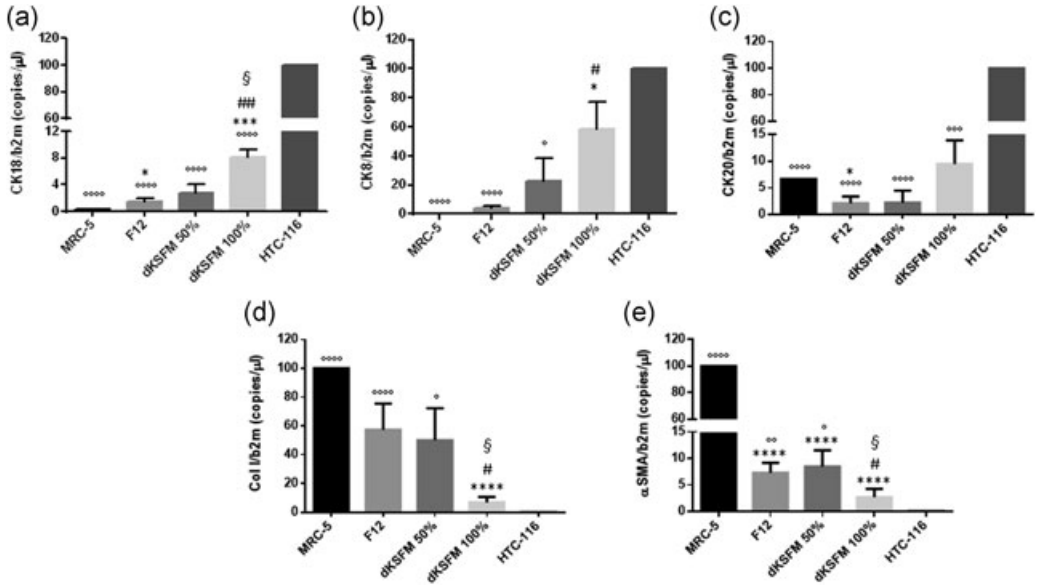

FIGURE 8 Human colorectal keratinocytes were identified by epithelial- and fibroblast-specific markers at the mRNA level by ddPCR analysis. (a–c) ddPCR showed that cytokeratins 8 (CK8), 18 (CK18), and 20 (CK20) had high mRNA levels in NCR-derived primary cells maintained in serum-free dKSFM medium (dKSFM 100%). (d,e) On the contrary, Col I, and αSMA were expressed at low mRNA levels in NCR-derived primary cells maintained in dKSFM 100%. Normal human fibroblast MRC-5 and epithelial colon cancer cell line HTC-116 were the positive control of Col I/αSMA and CK8/18/20 expression, respectively. All data are reported as mean ± *SEM* of copies/μl of target gene normalized by b2m expression and pooled from four replica experiments. Statistical analysis was performed using one-way ANOVA and the unpaired *t* test. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ versus MRC-5; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus F12; $^{\S}p < 0.05$ versus dKSFM 50%; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$, $^{\circ\circ\circ\circ}p < 0.0001$ versus HTC-116. ANOVA: analysis of variance; b2m: β-2-microglobulin; Col I: type I collagen; ddPCR: droplet digital polymerase chain reaction; dKSFM: defined keratinocyte serum-free basal medium; mRNA: messenger RNA; NCR: normal colorectal; αSMA: α smooth muscle actin; *SEM*: standard error of mean

TABLE 2 Troubleshooting table

Limitations	Possible reasons	Solutions
Contamination of normal colorectal (NCR) tissue biopsy by infection agents	Equipment was not sterilized Possible environmental contamination Possible endogenous contamination	Make sure all the equipment is autoclaved Work in sterile conditions, under laminar flow biohazard hood Use of antibiotics, large spectrum
Low cell yield in primary culture	Cutting with tweezers, scissors, and a disposable blade is incomplete Collagenase digestion of NCR tissue fragment is incomplete	Ascertain absence of tissue clusters Digestion takes longer in collagenase type II solution (up to 24 hr)

passages with no sign of differentiation and without fibroblast contamination (Figure 5).

To date, two main methods were used to set up primary human colon epithelial cell cultures. (a) The first method is based on the use of replication-inactivated murine 3T3 embryonic fibroblasts, as feeder cells (Rasmussen et al., 2013). Feeder cells, inactivated by mitomycin C or γ-irradiation, secrete soluble factors into the culture medium, fostering the deposit of extracellular matrix molecules on the feeder layer surface. This extracellular matrix film facilitates the cell attachment and growth of cocultured keratinocytes (Alitalo, Kuismanen, Myllyla, Kiistala, & Asko-Seljavaara Vaheri, 1982). (b) The second method employs digestive enzymes, such as collagenase (Lichti et al., 2008; Gesell & Luk, 1993), dispase (Vidrich et al., 1988), trypsin-EDTA or thermolysin (Yuan et al., 2015), alone or in combination, to dissociate colonic mucosal cells, as well as epidermis-derived keratinocytes (Jensen, Driskell, & Watt, 2010; Perreault & Beaulieu, 1996).

These methods show several limitations. Indeed, some treatments have not been effective in yielding a sufficient amount of cells to form epithelial cell colonies (Deveney et al., 1996; Vidrich et al., 1988). An important factor currently preventing the establishment of successful cultures of human adult colonic epithelial cells is due to the limited quality and quantity of tissue biopsies available from surgical resections. To overcome this problem, some authors have used fetal intestine biopsies. The fetal specimens enable cell lines, which are characterized by the persistent renewal of intestinal cells, to be set-up and maintained for a long period of time (Liu et al., 2010; Quaroni & Beaulieu, 1997). It is worth noting that fetal cells have different characteristics compared to adult differentiated cells.

Our innovative cell culture protocol employs the Col II to digest small fragments of NCR mucosa (10–20 mm^3) from adults. Our procedure allows to obtain a significant number of viable epithelial cells (about 10^4–10^5 cells/NCR biopsy) which could form 50–100 primary keratinocyte colonies.

It is noteworthy that primary cultures, although deriving from a heterogeneous cell mixture composed of keratinocytes and fibroblasts (Figure 3), give rise by selection and isolation to pure keratinocyte cell colonies. Indeed, cloning rings and dKSFM, allowed us to obtain isolated human primary keratinocytes from NCR mucosa (Figures 4 and 5). The success in achieving culturing human primary keratinocytes was demonstrated by the cell morphology and the expression of epithelial CK marker. As shown in Figure 6, NCR keratinocyte cultures gave a clear positive reaction for CK maintained throughout the entire culture period. In addition, the high expression of CKs 8, 18, and 20 mRNA levels in NCR-derived primary cells maintained in serum-free dKSFM medium, confirmed the epithelial origin of these cells (Figure 8).

It is well established that in previous methods most cell cultures were limited to short term duration (Lichti et al., 2008; Vidrich et al., 1988) and characterized by the tendency to become committed to terminal differentiation in a few days (Rasmussen et al., 2013). Our innovative procedure circumvent these limitations. In our protocol, the use of a defined keratinocyte growth medium (a) allows the primary culture to enrich in viable keratinocytes, (b) favors the cell growth and (c) the long-term maintenance of keratinocytes in culture (at least 5 weeks).

As described in Table 2, our protocol presents some limitations that, however can be easily solved. The major limitation is related to NCR tissue biopsy. Indeed, it is not always possible to obtain a primary keratinocyte culture because of endogenous contamination with infectious agents of the specimen. In other circumstances, potential sources of contamination could be derived from equipment or environmental contamination. In these cases, basic precautionary procedures as keeping the surgical specimen in cold tissue culture medium and the sterile conditions should be sufficient to avoid the sample contamination. Another problem can be the low cell yield in primary culture. The optimization of the enzymatic digestion step should eliminate this limitation.

Overall, this investigation reports, for the first time, an effective protocol for the setting up, isolation and culture of human primary keratinocytes from NCR mucosa without feeder layers and fibroblast contaminations. Our protocol provides a valuable, simple and useful tool for studying NCR keratinocytes, investigating at cellular and molecular levels the alterations that trigger the onset and progression of colorectal carcinoma and other colon-related diseases. In addition, this new protocol for keratinocyte cultures can be used to study the metabolism and drug assays (Asarat, Vasiljevic, Apostolopoulos, & Donkor, 2015; Yamaura, Chapron, Wang, Himmelfarb, & Thummel, 2016). Moreover, human colorectal mucosa-derived primary keratinocytes can be used to assess the effects of growth factors and other molecules on membrane receptors, enzymatic activities, transport, and secretory functions (Matsumoto et al., 2014) as well as the mechanisms of interactions of intestinal pathogens with the mucosa surface (Fréour et al.,

2009). They represent the ideal model of study to investigate cellular, molecular and functional alterations that occur in the colorectal epithelium and that give rise to inflammatory diseases as well as intestinal cancers.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

F. M. designed the study. C. F. collected and provided the clinical specimens. E. T., M. R., I. B., S. P., E. M., M. R. I., J. C. R., and P. R. set up the research, collected and analysed data, and generated the figures. E. T., M. R., M. T., and F. M. cowrote the paper. All authors read and approved the final manuscript.

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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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