Stem Cell Research (2015) 14, 224–237

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Derivation of keratinocytes from chicken embryonic stem cells: Establishment and characterization of differentiated proliferative cell populations

Mathilde Couteaudier[a], Laëtitia Trapp-Fragnet[a], Nicolas Auger[a], Katia Courvoisier[a], Bertrand Pain[b], Caroline Denesvre[a], Jean-François Vautherot[a,*]

[a] INRA, UMR 1282, Infectious Diseases and Public Health, ISP, Biova Team, Centre INRA de Tours, F-37380 Nouzilly, France
[b] INRA, USC 1361, INSERM U846, Université Lyon 1, U846 S, Institut Cellules Souches et Cerveau, F-69500 Bron, France

Received 18 July 2014; received in revised form 16 December 2014; accepted 12 January 2015
Available online 17 January 2015

Abstract A common challenge in avian cell biology is the generation of differentiated cell-lines, especially in the keratinocyte lineage. Only a few avian cell-lines are available and very few of them show an interesting differentiation profile. During the last decade, mammalian embryonic stem cell-lines were shown to differentiate into almost all lineages, including keratinocytes. Although chicken embryonic stem cells had been obtained in the 1990s, few differentiation studies toward the ectodermal lineage were reported. Consequently, we explored the differentiation of chicken embryonic stem cells toward the keratinocyte lineage by using a combination of stromal induction, ascorbic acid, BMP4 and chicken serum. During the induction period, we observed a downregulation of pluripotency markers and an upregulation of epidermal markers. Three homogenous cell populations were derived, which were morphologically similar to chicken primary keratinocytes, displaying intracellular lipid droplets in almost every pavimentous cell. These cells could be serially passaged without alteration of their morphology and showed gene and protein expression profiles of epidermal markers similar to chicken primary keratinocytes. These cells represent an alternative to the isolation of chicken primary keratinocytes, being less cumbersome to handle and reducing the number of experimental animals used for the preparation of primary cells.

Introduction

The skin is the first barrier protecting vertebrates against an external environment assault including the entrance of various pathogens. Avian skin, structured in the dermis separated from the epidermis by a basal membrane, is similar to mammalian skin, except for the presence of feathers and

* Corresponding author. Fax: +33 2 47 42 77 74.
E-mail addresses: mathilde.couteaudier@hotmail.fr (M. Couteaudier), Laetitia.Fragnet@tours.inra.fr (L. Trapp-Fragnet), bertrand.pain@inserm.fr (B. Pain), Caroline.Denesvre@tours.inra.fr (C. Denesvre), Jean-Francois.Vautherot@tours.inra.fr (J.-F. Vautherot).

http://dx.doi.org/10.1016/j.scr.2015.01.002
1873-5061/

scales in place of hairs and the lack of sebaceous glands (Hodges, 1974; Lucas and Stettenheim, 1972; Matoltsy, 1969; Spearman and Hardy, 1985; Stettenheim, 2000). The feather follicle, an epidermis invagination (Lucas and Stettenheim, 1972), gives rise to the feather through a complex morphogenesis and allows the renewal of feather after molting (Yu et al., 2002, 2004; Yue et al., 2005). The avian epidermis is composed of keratinocytes at different levels of differentiation that evolve progressively from the basal layer to the cornified layer creating a squamous epithelium. These avian keratinocytes evolve progressively from the basal layer to the cornified layer by differentiation. During this keratinization process, the keratinocytes undergo important structural and physiological modifications (Lucas and Stettenheim, 1972; Matoltsy, 1969; Spearman and Hardy, 1985). Avian keratinocytes differ from mammalian keratinocytes by their capacity to accumulate lipid droplets in their cytoplasm (Menon et al., 1981; Vanhoutteghem et al., 2004) and to synthesize specific β-keratins (Gregg et al., 1984; Vanhoutteghem et al., 2004).

The generation of mammalian keratinocyte cell-lines has been reported, with the example of the human HaCaT cell-line (Boukamp et al., 1988) or the murine COCA cell-line (Segrelles et al., 2011), but to date, only chicken primary keratinocytes (CPK) with limited potential for serial passages have been described (Vanhoutteghem et al., 2004). In avian cell biology, there is a need for the generation of differentiated cell-lines of specified origin by using reproducible protocols. To date, only fibroblasts that have limited potential for further differentiation (Himly et al., 1998) or tumor cells with altered phenotype (Kawaguchi et al., 1987) or retrovirus-transformed cell-lines are available (Baba et al., 1985; Ness et al., 1987). The routine isolation of differentiated cell-lines, such as the chicken lung epithelial cell-line CLEC213 (Esnault et al., 2011) may not be routinely reproduced in all laboratories. Avian primary cells are most commonly used for avian cell studies; however significant disadvantages are inherent to their use, such as a complex protocol to set up in a laboratory, or a lifetime limited to the first few passages.

During the last decade, significant advances have been made regarding the differentiation of mammalian embryonic stem (ES) cells to the dermo-epidermal lineage (Coraux et al., 2003; Iuchi et al., 2006; Ji et al., 2006) by using bone morphogenetic protein 4 (BMP4) and retinoic acid induction (Guenou et al., 2009; Metallo et al., 2008). Moreover, functional basal keratinocytes obtained after this differentiation could be successfully used for the reconstruction of a pluristratified epidermis because of the high proliferative capacity and long-term culture of these embryonic stem cell derived keratinocytes *in vitro* (Guenou et al., 2009). In the *Gallus* genus, chicken embryonic stem (cES) cells have been established from the long term culture of early blastodermal cells taken from a Stage X–XII (Eyal-Giladi and Kochav, 1976) pregastrulating embryo (Pain et al., 1996). Most published reports on differentiation of cES cells have been related to differentiation toward the neural lineage, while only a few studies have been reported for the differentiation toward the ectodermal lineage (Boast and Stern, 2013; Pain et al., 1996). To our knowledge, differentiation of avian ES cells into keratinocytes has not been performed yet.

In the present work, we demonstrated for the first time the potential of cES cells to differentiate into keratinocytes. After examination of several protocols describing the derivation of keratinocytes from mammalian ES cells (Aberdam, 2004; Coraux et al., 2003; Green et al., 2003; Iuchi et al., 2006; Metallo et al., 2008), we adapted the protocol of Guenou et al. (2009) to the cES cells. Indeed, Guenou et al. (2009) described the isolation of proliferating clones of differentiated keratinocytes, starting from a two dimensional culture rather than from embryoid bodies (Metallo et al., 2008) and leading to the isolation of fully differentiated keratinocytes, unlike other protocols making use of transforming genes (Iuchi et al., 2006). Consequently, we differentiated the cES cells toward cells of the epithelial lineage by induction with BMP4 and ascorbic acid (Guenou et al., 2009) in the presence of chicken serum (Vanhoutteghem et al., 2004). Chicken embryonic stem cell-derived keratinocytes (K-cES) obtained could be serially passaged up to 20 passages and showed an expression profile of epidermal differentiation markers similar to primary keratinocytes.

Materials and methods

Media

FAD medium was essentially as described (Guenou et al., 2009), composed of a 3:1 mixture of Dulbecco's modified Eagle's medium (DMEM — Invitrogen)/Ham's F12 nutrient mixture (Invitrogen) supplemented with 10% fetal calf serum (Pan Biotech), 10% chicken serum (Invitrogen) (Vanhoutteghem et al., 2004), 1% L-Glutamine (Lonza), 5 μg/ml insulin–transferrin–selenium (3572 K, Invitrogen), 0.5 μg/ml hydrocortisone (H0396, Sigma), 10^{-10} mol/l cholera toxin (C8052, Sigma), 1.37 ng/ml triiodothyronine (R4642, Sigma), 24 μg/ml adenine (A9795, Sigma), and 10 ng/ml recombinant human epidermal growth factor (EGF) (236-EG-200, R&D System).

Preparation of culture dishes and feeder cells

Mouse fibroblasts 3T3-J2F, kindly provided by Dr Djian (Vanhoutteghem et al., 2004) were routinely cultivated in DMEM with 10% fetal calf serum in gelatin coated flasks. Feeder layers were obtained by seeding 2.5×10^4 3T3-J2F cells/cm^2, which had been treated with 10 μg/ml mitomycin C (Sigma).

Culture of chicken primary keratinocytes

Chicken primary keratinocytes were prepared from newborn white leghorn chicks WL B13/B13 sacrificed by cervical dislocation. This procedure was carried out in accordance with the French legislation for animal experiments and ethics stating (Art. R-214-89) that, in this case, organ (skin) sampling does not require a governmental authorization. Cell suspensions were prepared and cultivated essentially as described by Vanhoutteghem et al. (2004) except that the dissociation step was performed in phosphate buffer saline (PBS) solution, pH 7.4, with 0.25% trypsin (Gibco — 15090-046) for 45 min. Primary keratinocytes were cultivated in modified FAD medium based on a 1:1 mixture of DMEM/Ham's F12 with the additives described previously at 37 °C in 5% CO_2.

Differentiation of cES cells into epidermal cells

Chicken ES cells were prepared and maintained as described (Aubel and Pain, 2013; Pain et al., 1996). The differentiation protocol of cES cells was adapted from Guenou et al. for human ES cells (Guenou et al., 2009) with the adjunction of chicken serum in the differentiation medium (Vanhoutteghem et al., 2004). The cES cells were seeded on 3T3-J2F feeder cells in FAD medium supplemented with 0.5 nmol/l human recombinant BMP4 (314-BP-010, R&D System) and 0.3 mmol/l ascorbic acid (A4403, Sigma), and medium was changed every two days. Five independent experiments were performed. The differentiation step lasted 30 to 40 days until colonies of cells with an epithelial morphology had appeared. The progress in differentiation of the cES cells was monitored every 2 days at the beginning of the experiment and every 10 days from day 10, by following the expression of marker genes in real time RT-qPCR or immunofluorescence microscopy (see below). Clusters of K-cES cells at the end of the differentiation were isolated and cultivated on plates coated with 5 $\mu g/cm^2$ rat tail collagen type I (Sigma), in FAD medium at 37 °C in 5% CO_2.

Oligonucleotides

Oligonucleotides targeting the selected genes were designed with Primer 3 software (Rozen and Skaletsky, 2000) from the chicken genome and purchased from Eurogentec (Seraing, Belgium). Primers and corresponding genes are listed in Fig. S1.

RT-qPCR analysis

Skin fragments from 28 day-old White Leghorn chicken (sacrificed by cervical dislocation) or separated epidermis and dermis from newborn White Leghorn chicks (WL B13/B13, sacrificed by cervical dislocation) were stored in RNA later solution (AM7021, Invitrogen) at −20 °C before extraction. Tissue samples were ground with glass beads in a mixer mill (MM200 — Retsch) in lysis buffer (RLT buffer of RNeasy mini kit, Qiagen). Cell monolayers were trypsinized, pelleted, resuspended in RLT buffer and stored at −80 °C before extraction. Total RNA from cells or grinded tissues was extracted with the RNeasy mini kit (Qiagen) according to the manufacturer's protocol. A supplemental step of genomic DNA lysis with DNase I digestion was added as described in the manufacturer's instructions. Two micrograms of RNA was reverse transcribed using the superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) with oligo$(dT)_{20}$. The resulting cDNA was analyzed by real-time RT-PCR performed using iQ SYBR Green SuperMix kit (Biorad), according to the manufacturer's instructions on a Chromo-4 system (Biorad). The Delta Ct method was used to compare gene expression of differentiated cells by using the chicken ribosomal gene RPS17 as internal reference and normalizing the results to the cES cells (Lavial et al., 2007).

Statistical analysis

Quantitative RT-PCR results were obtained from at least three independent experiments which had been repeated three times. Quantitative RT-PCR results were represented by the mean of triplicates for each independent experiment. Statistical analysis of RT-qPCR results was performed by Kruskal–Wallis one-way ANOVA test followed by two-tailed Mann–Whitney test. Analyses were done by using the software GraphPad Prism 5 (*$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$ for statistically significant differences).

Antibodies

Antibodies to cytokeratin type II (1h5), mitochondria (4C7) and CD44 (1D10) were purchased from the Developmental Studies Hybridoma Bank (Iowa University, USA). The other antibodies were kindly provided by Pr Danielle Dhouailly for αK-11E10 (keratin 14) (Collomb et al., 2013), by Dr Furuse for anti-occludin (Furuse et al., 1993) and by Dr Philippe Djian for anti-involucrin (Vanhoutteghem et al., 2008).

Immunofluorescence analysis by microscopy

Cells were fixed in a 4% paraformaldehyde solution in PBS at room temperature for 20 min, permeabilized with 0.5% Triton X100 (Sigma) in PBS for 5 min, washed once in PBS containing 0.1% Triton X100 (PBST) and blocked with bovine serum albumin (BSA fraction V – PAA – GE Healthcare) at 1% in PBST 20 min at room temperature. Cells were incubated with αK-11E10 (1:50), 1h5 (1:200), 4C7 (1:100), 1D10 (1:50) or with anti-involucrin (1:200) antibodies for 1 h at room temperature. After three washes in PBST, Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen) for αK-11E10, 1H5, and 4C7 or Alexa Fluor® 488 or 594 goat anti-rabbit IgG (Invitrogen) for involucrin, both diluted at 1:2000 in PBST-1% BSA, were added and incubated at room temperature for 1 h. For intracellular neutral lipid droplet staining, 1 μg/ml Nile red solution (Invitrogen) in PBST-1% BSA was added to the secondary antibody and incubated for 1 h at room temperature in the dark. The cells were then washed 3 times with PBST, and nuclei were stained with a 1:2000 dilution of Hoechst 33342 (Molecular Probes) in PBST for 5 min at room temperature. After washing PBS, coverslips were mounted on a drop of Vectashield (Vector Laboratories). Observations of cells were performed with an Axiovert 200 M inverted epi-fluorescence microscope equipped with a Plan-Apochromat 20×/0.8 and an EC Plan-Neofluar 40×/1.3 oil/Dic objectives both compatible with the ApoTome system (Zeiss). Images were obtained with a CCD Axiocam MRm camera (Zeiss) using the Axiovision software (Zeiss). Visualization of Nile red staining was performed with a LSM 700 Zeiss Confocal microscope by using the Zen software (Zeiss). The 3D reconstruction was performed from a 60 image stack acquired on a TCS SP8 Confocal microscope equipped with a Plan Apo 63×/1.2 water objective (Leica), by using the 3D module of LAS X software (Leica).

K-cES cells culture and proliferation

K-cES cell populations were maintained in culture in FAD medium. At confluence, K-cES cell populations were passaged, which consisted in dissociating the cells by trypsinization (see above) and seeding the cell suspension in a collagen I coated dish at a splitting ratio of 1 to 4.

Doubling times for K-cES cells were calculated over 20 consecutive passages. For this, every 3 days, K-cES cells were trypsinized, counted and seeded at a density of 1.10^6 cells/T25 flask. The doubling time were obtained using the formula DT = ln2 * t / (ln C1 − ln C0), where t is the culture duration, C1 is the number of cells at the end of the culture and C0 is the number of seeded cells.

Cell cycle analysis

One million of harvested cells were washed twice in PBS, fixed in 70% cold ethanol and stored for two hours at 4 °C. Cell suspensions were washed twice, resuspended in PBS with 500 μg/ml ribonuclease A (Sigma), and incubated for 1 h at 37 °C. After a filtration through a 30 μm nylon mesh, DNA was stained with 10 μg/ml propidium iodide (Invitrogen) for 20 min in the dark at room temperature. Cells were subjected to flow cytometry using a MoFlo high-speed cell sorter (DakoCytomation A/S, Fort Collins, CO, USA) as described (Trapp-Fragnet et al., 2014) and the profiles were analyzed using the MultiCycle AV software (Phoenix Flow Systems, California, USA).

Transmission electron microscopy (TEM)

Cells were fixed in the culture dish for 1 h at 4 °C in the TRUMP solution composed of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 (McDowell and Trump, 1976). Cells were removed with a cell scraper, pelleted, resuspended in TRUMP solution, and processed as previously described for TEM (Patient et al., 2007). Ultrathin sections stained with 5% uranyl acetate plus 5% lead citrate and placed on EM grids were examined with a JEOL 1011 microscope (JEOL, Tokyo, Japan).

Isolation of corneocytes in culture

Squamous cells in the supernatant of confluent K-cES cells culture were harvested and treated as described (Sun and Green, 1976; Vanhoutteghem et al., 2004). After centrifugation at 3000 g, corneocytes in the pellet were resuspended in PBS and observed under phase contrast microscopy on the Axiovert 200 M microscope with the 10×/0.25 CP-Achromat objective. Images were acquired as described above.

Results

Differentiation of cES cells

The differentiation of cES cells was induced essentially as described by Guenou et al. (2009) except that 10% of chicken serum was added to the FAD medium, as the adjunction of chicken serum was described as essential for chicken primary keratinocyte cultures (Vanhoutteghem et al., 2004). The first criterion for the determination of the differentiation was the morphological changes observed over time in cES cells during the induction period (Fig. 1). From days 1 to 6, the cES cells formed tight and compact colonies of small rounded cells arranged in clusters into the 3T3-J2F fibroblasts monolayer, remaining morphologically similar to primary ES cells (Aubel and Pain, 2013). Subtle changes occurred after 8 to 12 days of induction as evidenced by an increase in the size of the colonies resulting from changes in the proliferation rate and in the cell morphology. After 20 days of induction, a change in cell morphology could be clearly observed as the cells became more pavimentous, with a reduced nucleocytoplasmic ratio. Cell clusters continued to expand from days 20 to 30, forming structured colonies of homogenous large cells. At the end of induction period (D40), colonies of differentiated cells showed a cobblestone morphology similar to chicken primary keratinocytes (CPK) (Fig. 1), with an intracellular lipid droplet, as described by Vanhoutteghem et al. (2004).

Progression of the expression of pluripotency and epidermal markers during differentiation

Even if the gene expression of different cell markers in the chicken skin had not been thoroughly investigated, we hypothesized that the dynamic of epidermis marker appearance in chicken was similar to that observed in mammals. Therefore, we designed our primers to amplify cDNA encoding differentiation markers, homologs to those described in mammalian keratinocytes. Along the differentiation period, we compared the marker gene expression of differentiating cES cells to CPK after normalizing to undifferentiated cES cells, by focusing on a few representative markers of the different layers of the skin. Monitoring of gene expression by RT-qPCR was either reproduced from Lavial et al. for OCT4/cPOUV and NANOG (Lavial et al., 2007) or developed and validated for keratins 5 (KRT5), 14 (KRT14), 10 (KRT10) and involucrin (INV). All the statistical analyses were performed by comparing the data for differentiating cells with those from cES cells at day 0. A statistically significant ($P < 0.0001$) progressive downregulation of cPOUV and NANOG genes was observed from day 10 on, which reached a maximum at day 30 (NANOG) to 40 (cPOUV) (Fig. 2). Gene expression of either cPOUV or NANOG was undetectable in CPK. Gene expression of KRT5 and KRT14, markers for proliferating keratinocytes located in the epidermis basal layer, increased progressively and significantly ($P < 0.0001$) from day 8 until day 40 to ultimately reach, for KRT5, the level of KRT5 expression in CPK. KRT10 and INV genes, described as markers of differentiated keratinocytes of the epidermis suprabasal layer, were also detected at a statistically significant level from day 8 and day 10 on, respectively. KRT10 gene expression appeared to be higher in cES differentiated cells at the end of induction than in CPK (Fig. 2).

Keratin expression in differentiated cells could be monitored by staining with the αK-11E10 monoclonal antibody (Collomb et al., 2013), an antibody which detects the KRT14 in the chicken skin (Fig. S2). No staining of the cES cells with αK-11E10 could be observed at day 0, but as early as 10 days post-plating, treated cells showed specific staining associated with clusters of differentiated cells (Fig. 3). At 20 days post-induction, a heterogeneous staining was observed, suggesting that, within a cluster, cells could be at different stages of differentiation. Then, αK-11E10 staining increased over time and at late time of

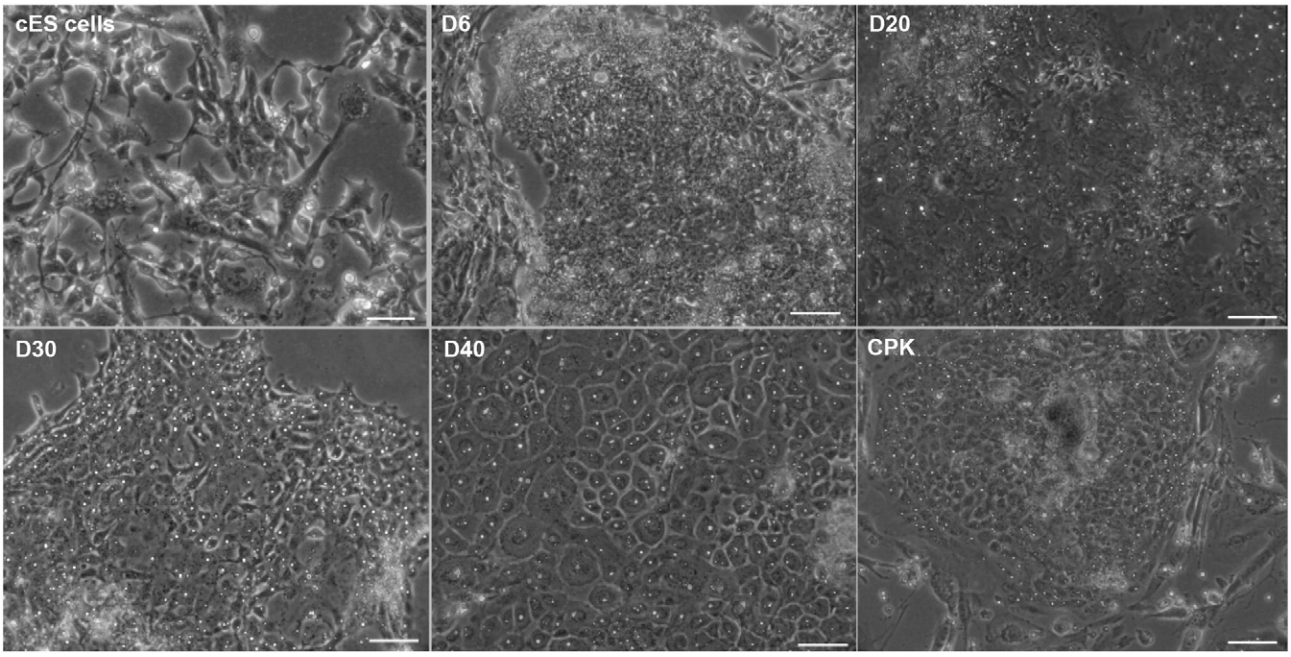

Figure 1 cES cells undergo dramatic morphological changes during the differentiation period. Analyses of cell changes using microscopy were performed at days six (D6), twenty (D20), thirty (30) and forty (D40) of induction. Stem cell morphology of the cES cells was progressively lost over time and pavimentous cell colonies were established by D40. At D40, colonies of K-cES cells were similar in morphology to CPK. The scale bars represent 100 μm.

differentiation, some islets of pavimentous cells did not stain with αK-11E10 possibly indicating a higher degree of differentiation in the keratinocyte lineage. We also used an anti-occludin antibody, specific for a membrane protein localized at tight junction, to follow the progression of cell morphology during the differentiation period (Fig. 3). Occludin staining was detected in the cytoplasm of differentiating cells until day 20. From day 30 on, we observed a change in the dynamics of occludin expression, as the protein localized to the tight junction of pavimentous cells. During the differentiation period, we observed a concomitant change in morphological characteristics and gene expression of epidermal markers indicating a differentiation of the cES cells toward a keratinocyte lineage.

Selection and isolation of differentiated clusters of cells

Induction with BMP4 and ascorbic acid was relieved after day 40, and for each of our five independent experiments, about twenty colonies of differentiated cells were isolated on the basis of cell morphology. Finally, we isolated and cultivated three homogenous populations of cells, K-cES-K1, -KP2 and -K8 cells exhibiting a typical epithelial cobblestone morphology similar to the morphology of CPK (Fig. 4A). K-cES-K1, -KP2 and -K8 cells actively proliferated up to 20 serial passages showing no detectable changes in their morphology, as illustrated in the comparison between cells at passage 12 and passage 32 (Fig. 4A). The three cell populations presented a doubling time of ~2 days (Fig. 4B) and could be frozen and thawed at will. Cell cycle analyses were performed on 50% and 100% confluent K-cES-K1, -KP2 and -K8 cells (passage 12) (Figs. 4C and S3). At 50% confluence, all K-cES cells showed the typical pattern of DNA content of cycling and proliferating cells, presenting a profile of dividing cells with 40% of cells in S phase (Fig. 4C). At 100% confluence, K-cES-K1 cells showed a cell-cycle arrest in G0/G1 phase whereas confluent K-cES-K8 cells showed a profile of cycling and proliferative cells, almost identical to their cell cycle profile at 50% confluence. K-cES-KP2 cells showed a profile which was intermediate between K-cES-K1 and -K8 cells, with an increase in the number of cells in G0/G1 phase and a decrease in the number of cells in S phase.

Morphological markers of keratinocytes

Chicken keratinocytes accumulate important quantities of lipids in intracytoplasmic droplets (Vanhoutteghem et al., 2004). Lipid droplets in K-cES cells were stained with Nile red, specific for neutral lipid staining (Fig. 5). Large lipid droplets, often close to the nucleus, were visible in almost every cell in K-cES-K1, -KP2 and -K8 cells (Figs. 5A–C). We further studied the K-cES cell morphology by analyzing in TEM ultrathin sections of cells grown on monolayer at 80% confluence (Figs. 5D–G). We observed squamous keratinocytes with their large lipid droplets, around the nucleus (Fig. 5D). Desmosomes (Figs. 5E–G) and tight junctions (Fig. 5E) could also be identified. Their presence attested that K-cES cells at confluence establish cell-specific junctions of epithelia as mammalian keratinocytes. Keratin tonofilaments were also observed in almost every cell and were associated with desmosomes, connecting them to the cytoskeleton (Fig. 5G).

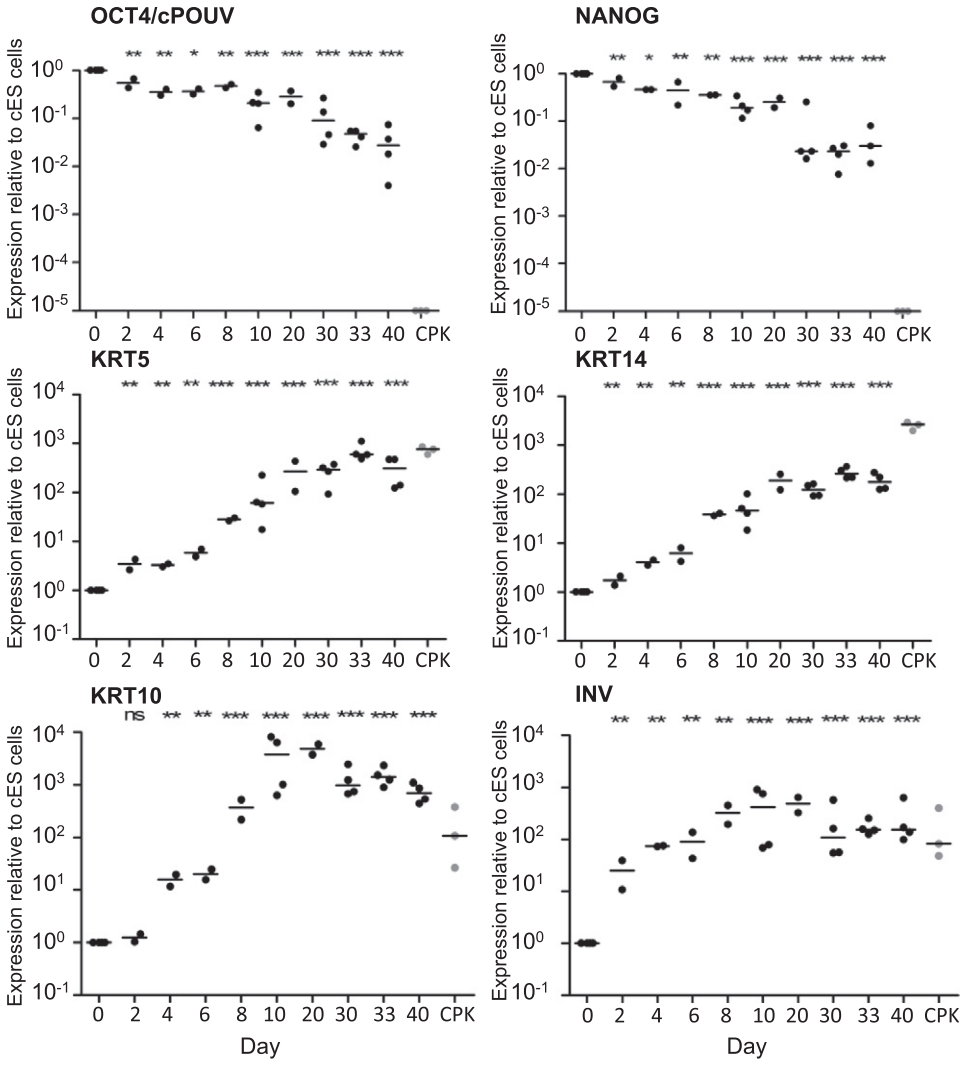

Figure 2 Evolution of the expression of pluripotency and epidermal markers during the differentiation period. OCT4/cPOUV, NANOG, KRT5, KRT14, KRT10 and INV expressions were analyzed in differentiating cES cells over a 40 day period. Four independent measures are presented in each plot and one dot is the average of a triplicate measure on one sample. Statistical analysis was performed by Kruskal–Wallis one-way ANOVA test followed by two-tailed Mann–Whitney test, compared with cES cells at day 0 for each time and each markers (*$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$ for statistically significant differences). Bars correspond to the median value.

Characterization of gene and protein expression in K-cES cells

For the characterization of K-cES cells, we compared the expression profile of a number of epidermal markers in K-cES cells, CPK, epidermis and dermis layers of new-born chicken skin, and adult chicken skin (Fig. 6). For all the markers tested, the differences of gene expression in K-cES cells *versus* cES cells were statistically significant ($P < 0.001$). The pluripotency markers, OCT4/cPOUV and NANOG, were significantly ($P < 0.001$) down-regulated in K-cES cells (Fig. 6A). KRT5 and KRT14, markers of the proliferating keratinocytes of the epidermis basal layer, were expressed at high level in K-cES-K1, -KP2 and -K8 cells as well as in CPK and new-born chick epidermis, with individual variations that were statistically significant or not, depending on the cell type (Fig. 6B). Expression of KRT5 or KRT14 was found to be low in the new-born chick dermis, while being intermediate between the dermis and epidermis layers in adult skin (Fig. 6B). Markers of the intermediate layer of the epidermis, transglutaminase 5 (TG5) and desmoglein 2 (DSG2), were detected at high level in the three populations of K-cES cells as in CPK, adult skin and new-born chick epidermis (with no significant differences), whereas they were not expressed in the new-born chick dermis (Fig. 6C). Desmoglein 1 (DSG1), a marker of the transitional layer, was expressed at high levels in K-cES-KP2 and K8 cells, as well as in CPK, the epidermis (no statistically significant differences), and in the skin (Fig. 6D). Intriguingly, DSG1 expression was lower in K-cES-K1 cells than in others K-cES cells, almost at the same level as in the dermis. KRT10 gene was expressed at very

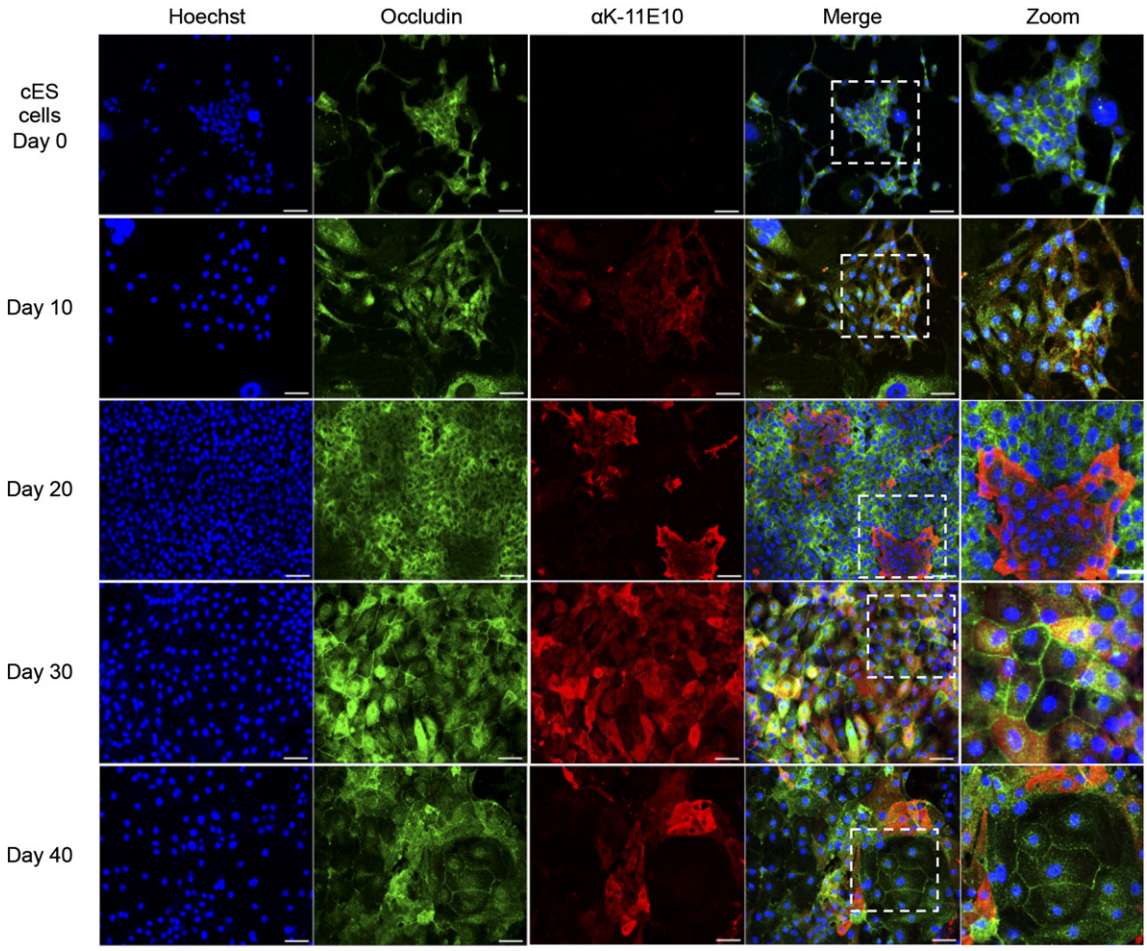

Figure 3 Changes in keratin expression during the differentiation period by immunofluorescence microscopy. Cells were fixed at day 0, 10, 20, 30 and 40, permeabilized and stained with an anti-occludin antibody (green) for tight junctions and the αK-11E10 antibody (red). Nuclei were stained with Hoechst 33342 dye (blue). Scale bars represent 50 μm. Expanded views of the boxed region in the 4th column are presented in the 5th column.

high levels in the epidermis and in the skin ($P < 0.001$), at a lower, but comparable level in K-cES-K1, -KP2 cells, CPK (no significant differences) and in the dermis, whereas it was hardly detected in K-cES-K8 cells (Fig. 6D). A marker for the cornified layer of the epidermis, Small Proline-Rich protein (SPRR9) gene was expressed at high level in the skin and at a lower, but significantly different level in CPK and in the epidermis and was almost undetectable in K-cES and in the dermis (Fig. 6E). Involucrin was present in all samples except in the dermis and cES cells (Fig. 6E). Finally in K-cES cells, the profile of expression of marker genes for the basal, intermediate and transitional layers was very similar to the expression profile of these genes in our gold standard, CPK.

At the level of protein expression, only a few antibodies are available for markers in the *Gallus* genus. Staining with αK-11E10 and 1h5 antibodies in K-cES-K1 cells (Fig. 7A) and in -KP2 and -K8 cells (data not shown) showed intracellular filament networks indicative of keratin staining. Involucrin was detected in the cytosol of all of K-cES-K1 cells by immunofluorescence (Fig. 7A) and in K-cES-K8 and -KP2 cells (data not shown). Our results on protein expression were in good accordance with the gene expression results and all data confirmed the phenotype characteristics of K-cES cells.

K-cES cells undergo terminal differentiation in culture

K-cES cells cultivated in monolayer tended to pile up to form patches of multilayered cells at different level of differentiation. The 3D-reconstruction of a pile of K-cES-K1 cells stained by anti-involucrin and anti-CD44 antibodies (Fig. 7B) showed differentiation indicative of stratification in culture. Interestingly, the involucrin staining was restricted to the upper cells of the pile, localizing at the membrane of the keratinocytes (Fig. 7B). Consequently, we checked for the presence of corneocytes in the supernatant of confluent monolayers of K-cES cells. Detergent-insoluble cell envelopes were obtained for K-cES-K1, -KP2 and -K8 cells (Fig. 7C) attesting that these keratinocytes could undergo terminal differentiation, forming corneocytes in culture, as described for CPK (Vanhoutteghem et al., 2004).

Discussion

A common challenge in avian cell biology in the avian species is the generation of differentiated cell-lines, which may be used to study either organ construction or interaction of

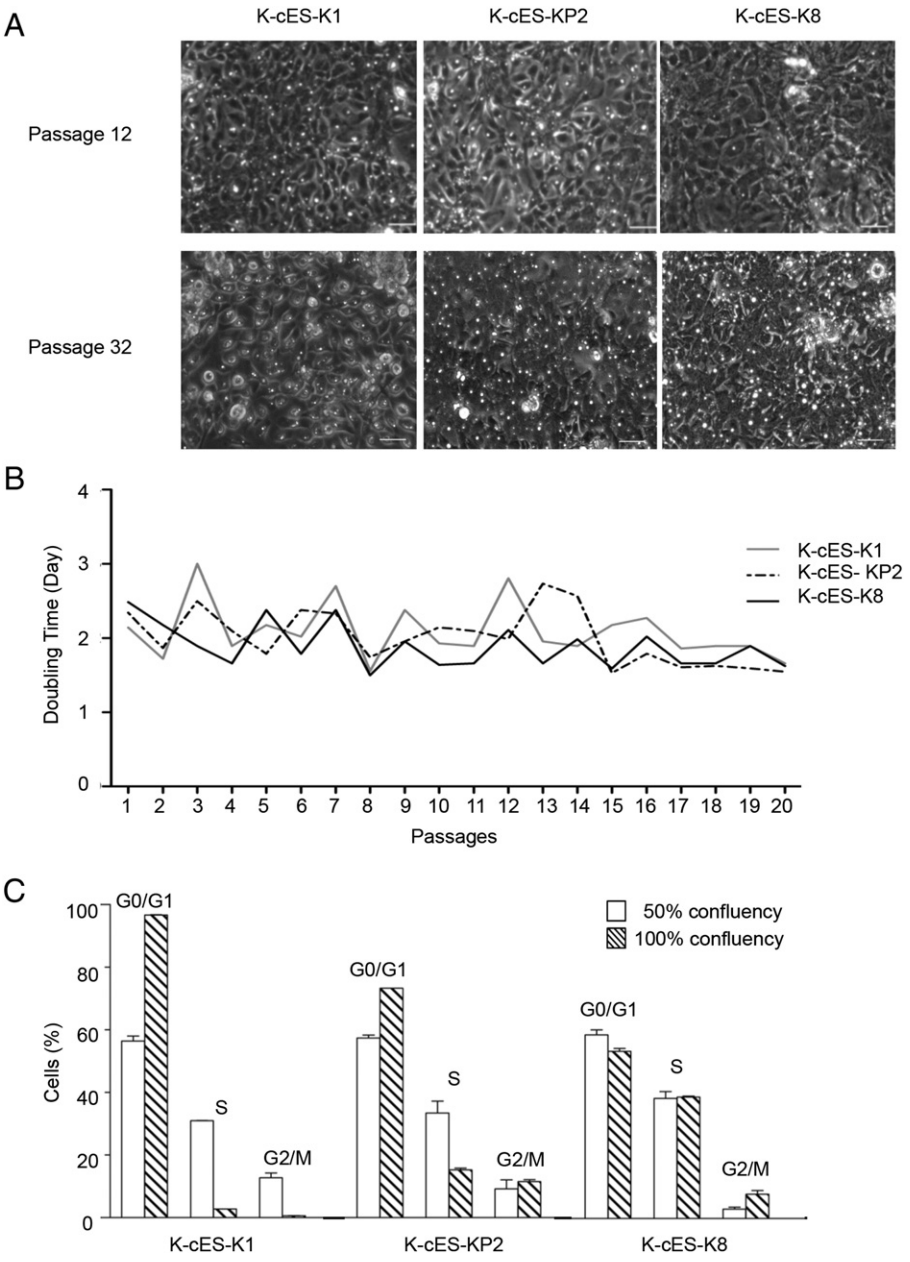

Figure 4 Characterization of K-cES cell populations in culture. (A) Phase-contrast micrograph of confluent homogeneous K-cES cells with keratinocyte-like morphology, at passage 12 and at passage 32. Scale bars represent 50 μm. (B) Population doubling time of K-cES cells. K-cES cells (passage 7) were passaged twenty times every 3 days with a seeding of 1 million cells/T25 flasks. (C) Representative cell cycle profiles at 50% confluence and 100% confluence. Cell cycle analyses were performed by flow cytometry.

pathogens with differentiated cells. During the last decade, the developments in ES cell biology together with the identification of the major signaling molecules have open the way for the selection of differentiated keratinocytes from human or murine ES cells (Aberdam, 2004; Coraux et al., 2003; Green et al., 2003; Guenou et al., 2009; Iuchi et al., 2006; Metallo et al., 2008). The differentiation process is based on the induction of ES cells with a combination of specific growth factors, signaling molecules and chemicals together with the exposure to extracellular matrix molecules. In order to derive chicken keratinocytes from chicken ES cells, we based our selection process on a protocol describing the derivation of keratinocytes retaining their proliferative potential from several human ES cell-lines (Green et al., 2003; Guenou et al., 2009; Iuchi et al., 2006). Here we describe the use of a combination of BMP4/ascorbic acid during a 30 to 40 day period for the induction of differentiation in cES cells seeded on 3T3-J2F cells, a feeder layer routinely used to support keratinocyte differentiation (Rheinwald and Green, 1975). In the combination of inducers selected, BMP4 appears to be a key molecule in all protocols leading to the isolation of keratinocytes in two-dimensional culture (Coraux et al., 2003; Guenou et al., 2009; Metallo et al., 2008). In this study, we used a human recombinant BMP4 to differentiate cES cells as the genes of BMPs are highly conserved (95% identity) between avian and mammalian

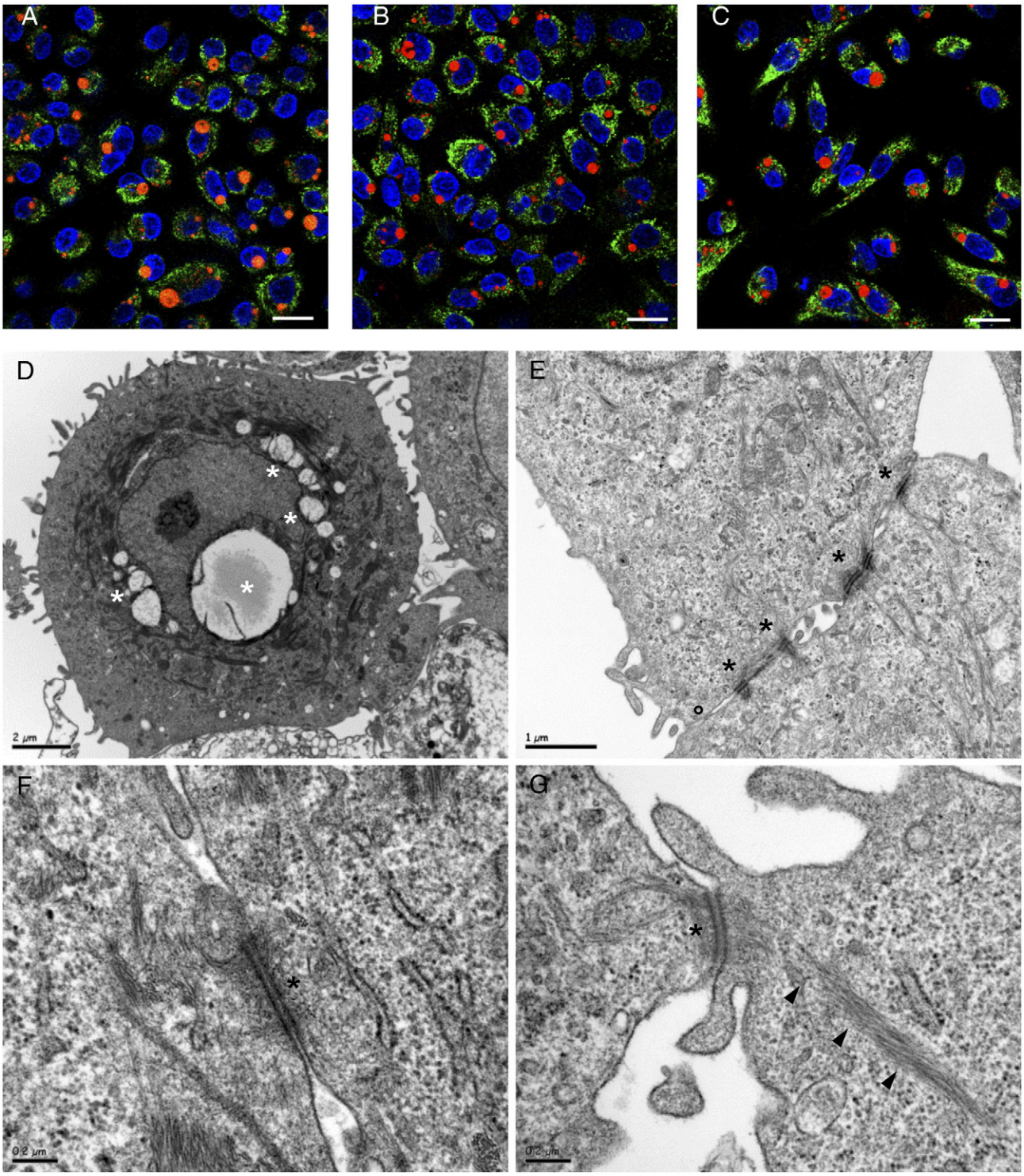

Figure 5 K-cES cells display morphological characteristics of primary keratinocytes. (A–C) Intracellular lipid droplets in K-cES-K1 (A), -KP2 (B) and -K8 (C) cells. Lipid droplets in K-cES cells were stained with Nile red, mitochondria with 4C7 antibody (green), and nuclei counterstained with Hoechst 33342. Pictures were captured with a LSM700 Zeiss Confocal microscope. Scale bars represent 20 µm. (D–G) K-cES cells examined by transmission electron microscopy. (D) Morphology of K-cES-K1 cells with lipid droplets (white stars) close to the nucleus (magnification 10,000×). (E–G) Desmosome junctions (black stars) and tight junctions (black ring) in confluent K-cES-KP2 cells (E) (magnification 25,000×), in K-cES-K1 cells (F) (magnification 80,000×), and in K-cES-K8 cells (G) (magnification 80,000×). Black arrows indicate keratin filaments tethered to the plasma membrane through the desmosomal structure, also named tonofilaments.

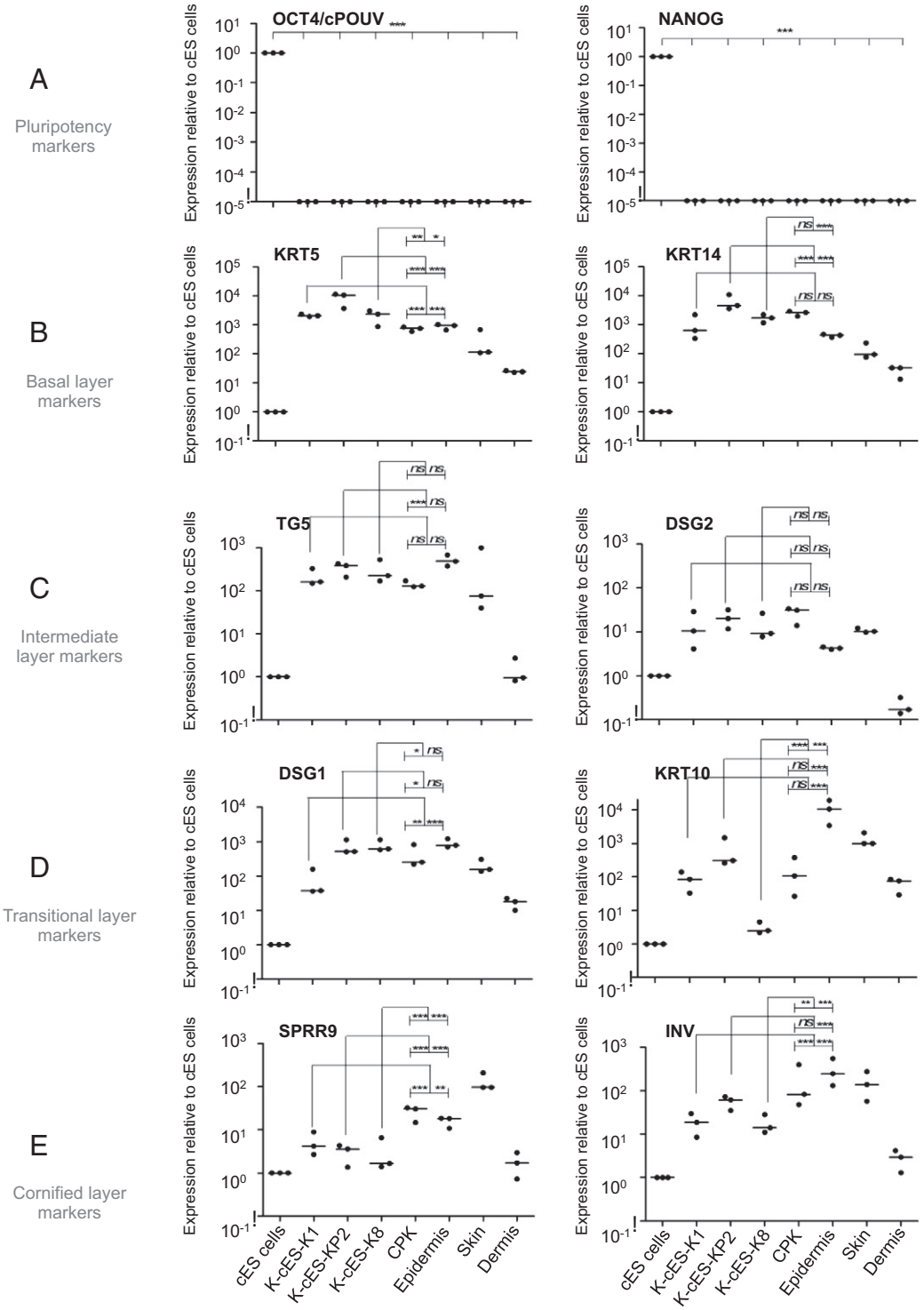

Figure 6 Epidermal marker expression profiles in K-cES cells. Marker genes OCT4/cPOUV and NANOG (A), KRT5 and KRT14 (B), TG5 and DSG2 (C), DSG1 and KRT10 (D), and SPRR9 and INV (E) were analyzed by RT-qPCR in K-cES-K1, -K8 and -KP2 cells. Plots represent three independent measures, one dot is the average of a triplicate of one sample. Statistical analysis was performed by Kruskal–Wallis one-way ANOVA test ($P < 0.0001$) followed by two-tailed Mann–Whitney test. The comparison of K-cES cells with CPK and epidermis is indicated for all markers (*$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$ for statistically significant differences). Bars correspond to the median value.

species and recombinant human proteins have proved to be efficient in the induction of differentiation in chicken cells (Kim et al., 2013). BMP4 is known to maintain epithelial commitment (Stottmann et al., 2006) and inhibit the development of neural precursors (Gambaro et al., 2006); of interest for our project, BMP4 was also specifically shown

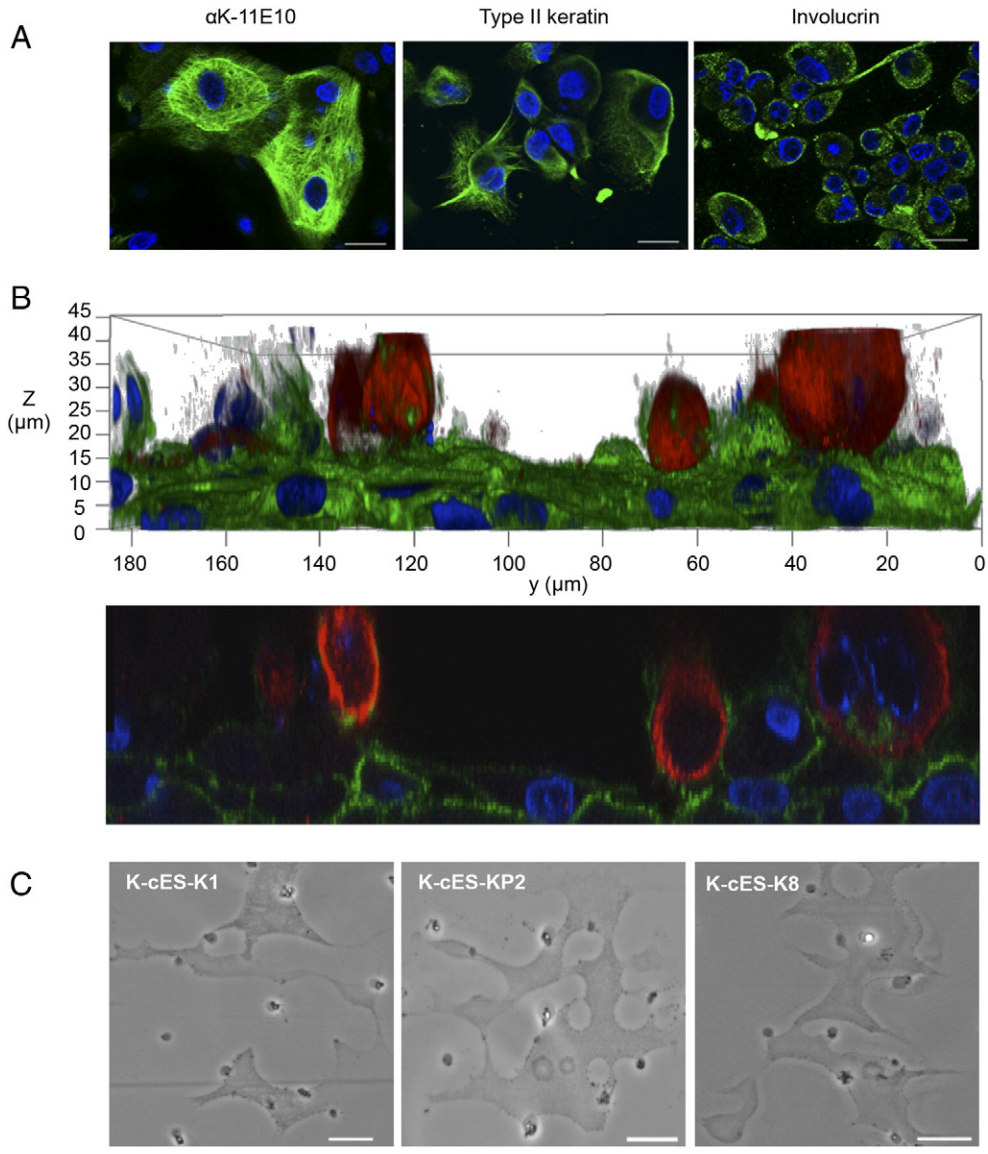

Figure 7 Protein expression in K-cES cells and terminal differentiation. (A) Protein expression in K-cES-K1 cells analyzed by fluorescence microscopy. αK-11E10, type II keratins and involucrin expression (green) in fixed and permeabilized cells. Nuclei were stained with Hoechst 33342 dye (blue). Scale bars represent 50 μm. (B) 3-Dimension reconstructions of K-cES-K1 cells spontaneously differentiating in culture, stained with anti-involucrin (red) antibody and anti-CD44 antibody (green). Nuclei were stained with Hoechst 33342 dye (blue). K-cES cells showing relocalization of involucrin to the cell membrane are in the upper layer of the cell culture. (C) Spontaneous terminal differentiation of keratinocytes into corneocytes of K-cES cells. Corneocytes obtained from squamous cells floating in the culture medium harvested by centrifugation and heated at 100 °C in a 2% SDS–2% 2ME solution show conserved morphology with corneocytes from CPK.

to induce the formation of early epidermal progenitors from ES cells (Harvey et al., 2010). Ascorbic acid, among other properties, promotes keratinocyte survival (Boyce et al., 2002) and chicken serum was specifically described as having a concentration-dependent growth promoting activity on chicken keratinocytes (Vanhoutteghem et al., 2004). During the induction period, colonies of pavimentous cells progressively appeared (approximately 20 colonies per 60 mm dish) which, by days 30 to 40, were morphologically indistinguishable from CPK. Furthermore we could isolate and serially passage three K-cES cell populations from five independent experiments leading to a rather satisfactory yield of spontaneous establishment of new cell-lines with a defined differentiated phenotype. Considering the frequency of isolation of replicating clones, this relatively low frequency has also been described in other species (Metallo et al., 2008) and could also be linked to the known difficulty of establishing spontaneous chicken cell-lines.

The monitoring of the differentiation over time during the induction period, as well as the characterization of gene expression profiles of K-cES cells was dependent on the delineation of pertinent marker genes. These genes are well characterized in mammalian species, with the KRT5/KRT14 representative of proliferating keratinocytes of the basal layer, KRT1, INV and KRT10 as markers for the supra basal layers (Candi et al., 2005). However, with the

exception of INV, the expression of these genes as markers of keratinocyte differentiation has not been thoroughly studied in chicken epithelial cells. When considering the expression of the first cytokeratin marker to appear in mammalian epithelial cell differentiation, KRT8, we detected a discrepancy when comparing the gene expression profiles of chicken *vs* human differentiating ES cells, as KRT8 expression showed little variation in cES cells differentiating toward keratinocytes (Fig. S4). However, recent transcriptome analyses of gene expression in cES cells showed that KRT8 is the third highly expressed gene in these cells (Jean et al., 2015). We herein show that the expression profile for KRT5/KRT14 along the differentiation period is similar to what was described for the keratinocyte differentiation of several human ES cells (Guenou et al., 2009), except that gene expression reached a plateau between days 20 to 30 in chicken cells whereas the expression of their homologs in differentiating human ES cells continued to increase until day 40. Interestingly INV and KRT10, markers of the suprabasal layer keratinocytes, were also upregulated significantly from day 10 and day 8 respectively. In our K-cES cells, these markers appeared at the same time, earlier than in the ES cell derived human keratinocytes. In reconstituted skin, the immunodetection of both proteins shows their association with the spinous/granulous or cornified (INV positive) layers (Chavez-Munoz et al., 2013; Guenou et al., 2009). As KRT10 is supposed to replace KRT5 intermediate filaments during differentiation of human skin keratinocytes (Candi et al., 2005), one cannot exclude an early upregulation of KRT10 in differentiating keratinocytes, before they reach the following stages that lead to their final cornification. We also noticed in differentiating K-cES patches of cells showing a heterogeneous staining with our anti-cytokeratin antibody associated with minor morphological differences; this lead us to hypothesize that cells within a K-cES cell population could reach higher levels of differentiation than others, as obviously seen in mouse ES cell-derived keratinocytes (Yoshida et al., 2011). The presence of keratinocytes reaching a higher level of differentiation in the colonies would thus explain the upregulation of KRT10. Similarly involucrin is known to be synthesized as a soluble protein at an early stage of differentiation in cultured human keratinocytes (Watt and Green, 1981), being cross-linked by transglutaminases at late stages of differentiation (cornification) (Rice and Green, 1977, 1979; Vanhoutteghem et al., 2008).

During the induction period, several colonies of cells were observed showing a pavimentous morphology with an obvious change over time. The three stable cell populations obtained, K-cES-K1, -KP2 and -K8 cells, could be maintained in culture up to twenty passages, without alteration of their phenotype, and could be frozen and thawed at will, as described for keratinocytes derived from human ES cells (Guenou et al., 2009; Metallo et al., 2008). These three K-cES cell populations have a major interest in terms of practical usability, having both efficient proliferation along the 20 serial passages and a doubling time of 2 days. Cell-cycle studies identified differences between morphologically similar cell populations, as K-cES-K1 cells stopped proliferating upon reaching a high cell density in culture, whereas -K8 and -KP2 cells continued to proliferate, suggesting they were less affected by contact-inhibition of cell proliferation. It should be noted that K-cES-K8 cells do not show "overproliferation" as is seen in transformed cell-lines which form clumps in cell culture (McClatchey and Yap, 2012). Whether this aspect in KcES-K1 cell *versus* -K8 cell multiplication reflects a difference in differentiation remains to be elucidated as, with the exception of KRT10, both cell-lines show a very similar gene expression profile with the markers tested. These results will be taken into consideration when designing experiments on keratinocyte differentiation at an air-to-liquid interphase, to assess whether an improved proliferation potential might be advantageous or deleterious for the reconstitution of a squamous epithelium *in vitro*.

In all K-cES cell populations, we report an upregulation of marker genes (KRT5/KRT14; DSG-1 & -2 and TG5), which are, in mammalian keratinocytes, known to be specific of the basal and upper layer keratinocytes of the epidermis (Candi et al., 2005). This profile is very similar to what was described for keratinocytes derived from human or murine ES cells (Guenou et al., 2009; Metallo et al., 2008; Yoshida et al., 2011), and also to the expression profile of these markers in CPK, the epidermis and skin. Regarding the markers of the *Stratum corneum*, we showed that SPRR9 gene may be considered as a *bona fide* marker of corneocytes, being expressed in the skin, epidermis and CPK. Interestingly, although we could observe some spontaneous terminal differentiation of K-cES cells described for CPK (Vanhoutteghem et al., 2004), we showed a low level of activation of the SPRR9 gene in K-cES cells. This might be due to the low level of cornification events in the culture of K-cES cells. Involucrin was one of the very few markers for which we could follow the gene and protein expression. We confirm that involucrin expression is associated to keratinocyte commitment and terminal differentiation in chicken keratinocytes. As in human keratinocytes (Banks-Schlegel and Green, 1981; Eckert et al., 1993; Rice and Green, 1977, 1979), involucrin is expressed at the early stages of differentiation in chicken keratinocytes, in which, upon differentiation, involucrin is relocalized to the outer membrane of K-cES cells apparently undergoing cornification. Our initial criterion, the selection of cells with a cobblestone morphology, proved to be a reliable criterion to select keratinocytes derived from cES cells. K-cES cell populations all displayed the typical morphology of chicken keratinocytes, with large intracellular lipid droplet, desmosomes and tight junctions with abundant (keratin) tonofilaments (Vanhoutteghem et al., 2004). As in CPK, corneocytes could be observed in our cell culture system; however optimization of cell culture conditions will have to be conducted toward the generation of pluristratified reconstituted epidermis, possibly at the air-to-liquid interphase as reported for keratinocytes derived from mammalian ES cells (Aberdam, 2004; Chavez-Munoz et al., 2013; Coraux et al., 2003; Guenou et al., 2009; Lamb and Ambler, 2013; Metallo et al., 2010; Yoshida et al., 2011).

In this study we demonstrate the feasibility of the selection of highly differentiated keratinocytes from chicken ES cells. Our study also contributes to define a panel of marker genes for keratinocyte differentiation in the chicken and to lay the foundations for the monitoring of the differentiation of chicken ES cells to K-cES cells on the basis of the upregulation of these genes. The use of K-cES cell populations may afford a new model to further investigate squamous epithelium reconstitution in avian species.

The K-cES cells may also serve as a pertinent cellular model to study avian pathogens which spread in association with exfoliating skin keratinocytes. In all vertebrates, skin keratinocytes contribute to the edification of an active barrier against pathogens, however a number of them evolved to adapt to the keratinocyte differentiation pathways in order to disseminate in the environment. In chicken, Marek's disease virus (MDV) is shed from the epidermis and feather follicle keratinocytes which are a major replication site of MDV (Calnek and Hitchner, 1969; Couteaudier and Denesvre, 2014), from which infectious viral particles are released into the environment (Calnek and Hitchner, 1969; Calnek et al., 1970; Nazerian and Witter, 1970). To date, no *in vitro* cell system has reproduced the cellular environment favoring the complete morphogenesis of MDV in feather follicle keratinocytes. Currently, chicken embryonic skin cells are routinely used *in vitro* to study MDV (Dorange et al., 2000); however these monolayers are mainly composed of fibroblastic cells. On the other hand CPK cultivation (Vanhoutteghem et al., 2004) remains a complex protocol to set up for routine virological studies. Preliminary tests showed that K-cES cells are susceptible to MDV infection and support its replication (data not shown). The K-cES cells will provide an alternative to the preparation of primary cells (Dorange et al., 2000; Vanhoutteghem et al., 2004), expressing a large number of marker genes and allowing a reduction in the number of experimental animals sacrificed. Further efforts will also be devoted to the reconstruction of an *in vitro* stratified epidermis, which in turn should provide a "chicken skin model" to study *ex vivo* MDV replication. This should eventually help us to understand how viral particles are spread from the keratinocytes to the environment and to build new strategies to reduce MDV dissemination and infectious pressure.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.01.002.

Acknowledgments

We are grateful to D. Dhouailly, M. Furuse and P. Djian for providing respectively, the αK-11E10, the anti-occludin and the anti-involucrin antibodies. We are also very grateful to Yves Le Vern for the cytometry experiments, Sonia Georgeault for her expert technical assistance in the preparation and examination of TEM samples, and Gilles Lepape for his advices on statistical analyses. We thank Keith Jarosinski for his dedicated help in proofreading and style adjustments and S. Trapp for his comments on the manuscript. M. Couteaudier was supported by a fellowship of the Region Centre. Jean-François Vautherot and Bertrand Pain were supported through the French National Research Agency (ANR, 315) for MADISPREAD, an ERA-NET EMIDA program.

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