In vitro culture conditions to study keratinocyte differentiation using the HaCaT cell line

Adeline F. Deyrieux · Van G. Wilson

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Abstract In vitro models to study the process of keratinocyte differentiation have been hindered by the stringent culture requirements and limitations imposed by the inherent properties of the cells. Primary keratinocytes only have a finite life span, while transformed cell lines exhibit many phenotypic features not found in normal cells. The spontaneously immortalized HaCaT cell line has been a widely employed keratinocyte model due to its ease of propagation and near normal phenotype, but protocols for differentiation and gene delivery into HaCaT cells vary widely in the literature. Here we report culture conditions for maintaining HaCaT cells in a basal-like state, for efficient differentiation of these cells, and for delivery of transgenes by transfection or adenoviral infection. This technological report will provide guidance to a large audience of scientists interested in investigating mechanisms of differentiation and skin morphogenesis.

Keywords HaCaT · Differentiation · Keratinocyte · Transfection · Infection · Calcium

Introduction

Understanding the transcriptional signals and regulatory pathways that take place during the process of keratinocyte differentiation will offer a great potential for treating skin diseases such as genetic abnormalities, infections, and skin cancer. Thus, there is a need to develop and characterize reliable in vitro models that closely mimic the pathways that lead to skin formation.

The skin is composed of stratified layers of keratinocytes consisting of a replicative basal layer and several suprabasal differentiated layers. Expression of keratin markers K5 and K14 is restricted to the basal layer, while K1, K10, and involucrin indicate a differentiated phenotype. Although several factors can trigger basal keratinocyte to differentiate, calcium is the most physiological agent and induces differentiation in vitro and in vivo in a similar manner. In vivo, basal keratinocyte are exposed to a low calcium concentration while differentiated keratinocytes are maintained by the presence of an increasing calcium concentration gradient (Menon et al. 1985). Sensing a high calcium concentration induces basal keratinocytes to exit the cell cycle and to commit to terminal differentiation.

Primary keratinocytes cultured in vitro at a low calcium concentration retain a basal phenotype, while addition of calcium >0.1 mM triggers their differentiation (Hennings et al. 1980). Unfortunately, primary cells have two major drawbacks. First, they require

A. F. Deyrieux · V. G. Wilson (✉)
Department of Microbial & Molecular Pathogenesis,
College of Medicine, Texas A&M Health Science Center,
College Station, TX 77843-1114, USA
e-mail: wilson@medicine.tamhsc.edu

supplementary growth factors to survive in vitro, and second, once induced for differentiation they rapidly die and do not allow long-term investigation of differentiation signals. In contrast, the naturally immortalized human HaCaT cell line can be grown in traditional media and can be maintained in culture for long periods of time. Exogenous oncogenic or mitotic genes are not responsible for the HaCaT immortalization phenotype (Boukamp et al. 1988), though the p53 gene is known to have UV-specific mutations in both alleles (Lehman et al. 1993). Importantly, normal morphogenesis and differentiation features are retained in the HaCaT cultures. For example, when HaCaT cells are grafted onto SCID mice, or grown in organotypic cultures, normal keratinization and stratification occurs, demonstrating that HaCaT cells retain all the functional differentiation properties of normal keratinocytes (Boukamp et al. 1988). Furthermore, HaCaT cells in culture can revert back and forth between a differentiated and a basal state upon changes in calcium concentration in the medium and express differentiation markers appropriate to the particular state. Therefore, HaCaT cells have many attractive features for an in vitro keratinocyte differentiation model, but conditions for their growth and transfection vary widely in the literature. Here we present conditions for consistent differentiation and for efficient gene delivery into this cell line.

Materials and methods

Cell culture and media

HaCaT cells were cultured in calcium-free DMEM (HyClone # SH3031901), with 10% chelexed FBS (GEMINI # 100–106), 4 mM L-Glutamine (HyClone # SH30034.01), and supplemented with calcium chloride at 0.03 mM or 2.8 mM final concentration. We noted that HaCaT cells maintained in this low calcium medium did not require addition of trypsin inhibitor for the cells to re-attach after EDTA-trypsin treatment. FBS was calcium-depleted by incubation with Chelex 100 resin (BioRad # 142–2832) for 1 h at 4 °C according to the BioRad protocol. The Chelex was subsequently removed using a 50 mL Millipore 0.22 µm filter unit system (Millipore # SCGP00525).

Transfection

Transfections with Lipofectamine 2000 were performed according to the manufacturer's protocol with little modification. Liposome and DNA were incubated in DMEM without Ca^{2+} and FBS. Standard 6-well tissue culture plates were used for all experiments. The liposome-DNA mixture was added to wells containing HaCaT cells in 500 µL of DMEM without Ca^{2+} and FBS. 2 mL of high or low calcium media was added to each well 4.5 h after transfection. Fresh medium was added the next day, and 48 h post-transfection the cells were visualized by phase contrast and fluorescence microscopy at magnification of 400X using an Olympus IX70 microscope. Images were captured digitally using a Qcolor3 camera (Olympus). Liposome toxicity on HaCaT cells was determined by counting the average number of cells attached to the plate in three different fields before transfection versus the average number of cells attached to the plate 24 h after transfection. Transfection efficiency was determined by counting the fluorescent versus non-fluorescent cells in three fields for each well.

Virus production and infection

For adenoviral (Ad) infection of HaCaT cells in 6-well plates, the medium was removed and the Ad-GPF virus was added in 2 mL of DMEM-10% chelexed FBS without Ca^{2+} , and with or without polybrene as indicated in the figures. Cells were incubated with virus for 3 h at 37 °C, then the medium was removed and 2 mL of fresh medium with high or low calcium concentration was added. Cells were visualized 48 h post-infection by phase contrast and fluorescent microscopy. Viral titer was assessed on 293A cells using the limiting dilution method described in the Qbiogene Adenovirus Manual (version 1.4). 293A cells were plated at 1×10^6 cells/well on 6-well plates prior to infection for titration.

Quantitative RT-PCR

RNA was extracted using the RNAqueous kit (AM-BION # 1912). RNA concentration was measured using a spectrophotometer and aliquots of 5 ng per µL were stored at –80 °C until use. The reactions for

Q-PCR contained 0.2 μ M stocks of LUX involucrin primers (designed with the Invitrogen custom primer software), 20 units of RNase OUT, 2 μ L of SuperScript III kit (Invitrogen # 12574-026) and 1 μ L of Rox dye (Invitrogen # 1261066) with 50 ng of RNA in a 50 μ L final volume. The LUX β -actin primer set (Invitrogen #101H-02) was used as the internal control and the involucrin forward (5'GA-AGCACCACAAAGGGAGAAGTATTGC[-FAM]TC-3') and reverse (5'CCACTGCACCTCCTGCTTCT-3') primers overlap exon-exon junctions, therefore only amplifying the cDNAs. The PCR reaction conditions were as follows: 20 min at 42 °C for the reverse transcription step, followed by 90 s at 94 °C to activate the Platinum Taq, and then 40 cycles of amplification (94 °C for 30 s; 60 °C for 30 s; 72 °C for 60 s). The Q-PCR plates were read with an ABI 7500 Real time PCR instrument and detection of the FAM or JOE label was recorded during the 72 °C step. Results are graphed as the fold increase of the relative quantitative values (RQ) where $RQ = 2^{(-\Delta\Delta Ct)}$. The data shown are the average from four independent RNA preparations collected in separate experiments.

Western blot and densitometry

Total cell extracts were prepared by adding a 1:1 (V/V) mixture of RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 15 mM MgCl₂, 1% NP40, 0.1% SDS, 1 mM DTT, 1:200 protease inhibitor cocktail [Sigma #P8340], 10 mM NEM) and 4 $\times$ SB (100 mM Tris pH 6.8, 20% glycerol, 8% SDS, 0.02% Bromophenol blue, 4% Beta-mercaptoethanol) directly to adhered cells, and the lysate was collected by pipeting. Samples were heated at 95 °C for 5 min and sonicated for 30 s at power 3 using a Misonix sonicator 3000. Samples were electrophoresed on 10% polyacrylamide SDS-gels, transferred onto Immobilon-P membranes (Millipore # IPVH00010), blocked for 15 min to 2 h with 3% non-fat milk in TTBS (150 mM NaCl, 50 mM Tris-Cl, pH 7.4, 0.005% Tween 20), and incubated for 1 h with the following antibodies: α -tubulin (1:15,000; Santa Cruz Biotechnology # Sc-5286) or involucrin (1:1,000; LabVision # MS-126-P0). Then the membranes were incubated with Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) at 1:10,000 for 1 h. The membranes

were subsequently rinsed in TTBS, treated with the Western Lightning Chemiluminescence reagent (PerkinElmer # NL102), and then visualized with X-ray film.

Results and discussion

To establish an effective and reliable model for studying keratinocyte differentiation in vitro, HaCaT cells were transferred from conventional DMEM medium to a low calcium concentration media [0.03 mM Ca²⁺]. Cells were monitored for morphology, as well as for K1 and involucrin gene expression. By 72 h in low calcium medium, the cells had lost their tight cell junctions, were less compacted, and showed modified morphology (Fig. 1a). After 3 weeks of culture in the low calcium conditions, the HaCaT cells became spindle shaped with an absence of cell to cell tight junctions (Fig. 1a), similar to primary cell maintained in low calcium. Furthermore, after 3 weeks of culture we were unable to detect K1 expression by western blot (data not shown). While involucrin remained detectable, it was at greatly reduced levels compared to cells maintained in high calcium medium (data not shown). Overall, these data indicate that in 0.03 mM calcium medium that HaCaT cells were able to revert back to a basal-like state both morphologically and biochemically. HaCaT cells could be maintained for long periods in the low calcium conditions and remained stably in this basal state.

To determine if the stable basal cells could be induced to differentiate, HaCaT cells were switched from low calcium medium to medium with 2.8 mM calcium. Within 4 h after calcium exposure the basal cells exhibited a more cuboidal shape with close packing suggestive of cell–cell tight junction formation (Fig. 1a). This observation mimics the rapid morphological response of primary cells to calcium (Yuspa et al. 1988) and re-enforces the concept that manipulation of HaCaT cell culture conditions represents an authentic and convenient model for differentiation. Moreover, western blot analyses indicated that by 72–96 h post calcium induction HaCaT cells exhibited de novo K1 expression (Fig. 1b). Similarly, by quantitative real time PCR we showed that calcium exposure led to a rapid, 5-fold up regulation of involucrin mRNA by 48 h

Fig. 1 HaCaT differentiation model: **(a)** Phase contrast images showing the timing of morphological changes in HaCaT cells in response to different calcium concentrations in the medium as indicated (see text for details). Phase contrast images were taken at the indicated times. **(b)** Detection of K1 and tubulin by immunoblot. HaCaT cells in low calcium medium (0.03 mM calcium) were induced to differentiate by replacement of the

original medium with high calcium medium (2.8 mM calcium). Protein samples were collected every 24 h for immunoblot analysis. **(c)** Detection of involucrin by quantitative RT-PCR. HaCaT were induced to differentiate as in panel (B) and RNA samples were collected every 48 h. Involucrin was detected as described in Materials and methods

post induction, reaching a 10-fold average increase by 96–144 h (Fig. 1c). These results indicated that HaCaT were now expressing a differentiated phenotype and are consistent with the conclusion that HaCaT cells are relatively normal in their response to physiological transduction signals which lead to keratinocyte differentiation.

For long-term culture, we noted that HaCaT cells needed to be maintained at less than 85% confluency in order to preserve either their basal or differentiated states. At high confluency, cells in low calcium medium began to differentiate, started expressing K1, and reverted to a more cuboidal shape with higher cell-to-cell packing (data not shown). Inversely, when HaCaT cells are maintained in high calcium medium, high confluency induced suppression of K1 expression (Capone et al. 2000). Thus, it is important to monitor K1 expression carefully during long term culture to ensure proper HaCaT state.

Next we wanted to establish the best conditions for gene delivery to HaCaT cells in order to study the

influence of specific pathway during, before and/or after the process of HaCaT differentiation. In general, keratinocytes are among the hardest cell type to transfect which limits transient gene delivery for overexpression studies. We tested the efficiency of both transfection and Ad infection on HaCaT cells maintained in low or high calcium medium. Lipofectamine 2000 was evaluated for toxicity and effectiveness at different degrees of cell confluency (Figs. 2, 3). In low calcium transfection studies, 4 μ L of Lipofectamine 2000 in 35 mm wells resulted in fairly low toxicity, with toxicity increasing significantly at higher amounts (Fig. 2a, b). Consequently, 4 μ L/well was used for all subsequent studies. Low calcium maintained HaCaT cells exhibited transfection efficiency close to 50% at cell confluencies less than 50%, with decreasing efficiency as confluency increased (Fig. 2a, c). Interestingly, the use of 4 μ L of Lipofectamine also gave the lowest toxicity and the best transfection efficiency for HaCaT cells maintained in high calcium medium, though the best

Fig. 2 Transfection of HaCaT cells maintained in low (0.03 mM) and high (2.8 mM) calcium media. **(a)** Graphical results are shown for the effect of confluency on transfection efficiency using Lipofectamine 2000 and on toxicity as a function of amount of Lipofectamine used. HaCaT cells maintained in low calcium medium were seeded at various cell numbers in 6-well dishes 24 h prior to transfection to obtain the indicated confluencies at the time of transfection. Cells were maintained in low calcium medium throughout the experiment. Transfection efficiency and toxicity were assessed

at 48 h post-transfection. Each point is the average of 2 or 3 repeats. **(b)** Representative phase contrast and fluorescence images of low calcium HaCaT cells seeded at 4.5×10^5 cells/well. Images were taken at 48 h after transfection. The ratio of Lipofectamine 2000 to DNA is each sample set is as indicated. **(c)** As in **(b)** except that the cells were plated at 8.5×10^5 cells/well prior to transfection. **(d)** As in **(a)** except that the cells were maintained in high calcium (2.8 mM) medium throughout. **(e)** As in **(b)** except the cells were in high calcium medium

transfection efficiency obtained averaged about 30% (Fig. 2d). Increasing the liposome volume or cell confluency did not significantly improve or impact the transfection efficiency for HaCaT cells in high calcium medium (Fig. 2d, e). One additional transfection reagent, TransIT (Mirus Corp.), was also tested, but we were unable to obtain more than 10% transfection efficiency in any of the condition reported above (data not shown).

As an alternative to transfection, we also tested the efficiency of gene delivery using the viral vector Ad5. Surprisingly, we noticed that HaCaT cells in low

(Fig. 3a) or high (not shown) calcium media could only be transduced at <5% with an MOI of 150. Increasing the MOI up to 500 did not improve transduction efficiency (data not shown). However, we report here that addition of polybrene dramatically improved the transduction efficiency (Fig. 3a). Addition of 6–8 $\mu\text{g}/\text{mL}$ of polybrene was enough to enhance transduction efficiency to 80–90% (Fig. 3a), and 8 $\mu\text{g}/\text{mL}$ of polybrene was chosen as the working concentration. In fact, the use of polybrene at this concentration enhanced infection over a wide range of MOIs, and no significant differences in infection

Fig. 3 Adenoviral infection of HaCaT cells. **(a)** Infection efficiency as a function of polybrene concentration. HaCaT cells were maintained in 0.03 mM calcium medium and were infected at an MOI of 150, in the absence or presence of polybrene as indicated. Each data point corresponds to the average of 2 or 3 repeats. **(b)** Infection efficiency and toxicity

as a function of MOI. Infections were performed as in **(a)** at the indicated MOIs. Each point corresponds to the average of 2 or 3 repeats. **(c)** Phase contrast and fluorescence images of low calcium HaCaT cells 48 h after infection at an MOI 150. **(d)** Phase contrast and fluorescence images of high calcium HaCaT cells 48 h after infection at MOI 20 or 300

efficiency were observed for MOIs of 20, 150 or 300 when polybrene was present (Fig. 3b, c, d). Additionally, no significant differences in transduction efficiency were noted between high and low calcium cell using those conditions (Fig. 3c, d). The efficacy of polybrene to transduce HaCaT cell line was also recently reported by Jacobson et al (Jacobsen et al. 2006), though they only examined HaCaT cells grown in standard DMEM medium. When grown in standard DMEM, HaCaT cells are not in the fully basal phenotype and express certain markers of differentiation. Our work extends the previous study by examining HaCaT cultures in both the basal and differentiated phenotype, and optimizing infection conditions for both phenotypic states.

In conclusion, we show that it is possible to obtain and maintain HaCaT cells in a basal-like state by depleting the calcium from the medium. Subsequent differentiation can be achieved by culturing the cells in medium with a higher calcium concentration. We

describe the phenotypic changes taking place and the time frame required for basal HaCaT cells to differentiate once induced with calcium. We also demonstrate effective de-differentiation of HaCaT cells when cultures are returned to low calcium medium. In addition, we established and standardized optimum conditions for HaCaT cell transfection and infection in either the basal or differentiated state. These advances will facilitate use of the HaCaT line as an inexpensive and technically simple keratinocyte culture model to study differentiation. As proof of this concept, we have recently utilized this model system to study the role of sumoylation in keratinocyte differentiation (Deyrieux et al. 2007).

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