Proliferation, Cell Cycle Exit, and Onset of Terminal Differentiation in Cultured Keratinocytes: Pre-Programmed Pathways in Control of C-Myc and Notch1 Prevail Over Extracellular Calcium Signals

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So far it was reported that a switch from low to high extracellular calcium induces growth arrest and terminal differentiation in cultured human and mouse keratinocytes. We had observed that both canine and mouse keratinocytes proliferate in high (1.8 mM, respectively, 1.2 mM) or low (0.09 and 0.06 mM) calcium-containing medium. In-depth analysis of this phenomenon revealed, as reported here, that the switch between proliferation and terminal differentiation occurred irrespective of calcium conditions when the canine and murine keratinocytes reach confluency. The ''confluency switch'' coincided with transcriptional upregulation of cell cycle inhibitors $p21^{WAF1}$ and $p27^{KIP1}$ as well as proteins marking onset of terminal differentiation. It was further accompanied by downregulation and nuclear clearance of c-Myc, and conversely activation of Notch1, which are shown to be critical determinants of this process. Together, this study demonstrates that even in the absence of and similar to their *in vivo* environment, cultured canine and mouse keratinocytes follow a pre-defined differentiation program. This program is in control of c-Myc and Notch1 and does not require complementary signals for onset of terminal differentiation except those given by cell–cell contact. Once triggered, completion of the terminal differentiation process depends on elevated extracellular calcium to stabilize intercellular junctions and components of the cornified envelope.

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A fine balance between proliferation and differentiation underlies epidermal renewal. Over recent years, evidence has accumulated that the differentiation process is initiated when epidermal stem cells exit their compartment to generate so-called transient amplifying cells (Watt, 2001; Fuchs *et al*, 2004). These stem cell daughters undergo a limited number of rapid mitotic divisions, subsequently exit the cell cycle, become quiescent, and eventually commit to terminal epidermal differentiation. The proto-oncogene c-Myc is upregulated in dividing transient amplifying cells and after dimerization with Max functions as an important transcription factor (Arnold and Watt, 2001). c-Myc has recently emerged as a key player in driving the exit of stem cells into the epidermal, transient amplifying compartment (Arnold and Watt, 2001; Waikel *et al*, 2001). The transient amplifying cells in turn require low c-Myc levels to commit to terminal differentiation (Pelengaris *et al*, 1999; Waikel *et al*, 1999). Abrogating proliferation and supporting terminal epidermal differentiation further relies on activation of the cell-surface receptor Notch1 as was demonstrated by keratinocyte-specific Notch1-deficient mice (Rangarajan *et al*, 2001; Nicolas *et al*, 2003). Although only little is known about the cross-talk between Notch and c-Myc, the cell contact-dependent mechanism leading to Notch activation was studied in great detail (Mumm and Kopan, 2000; Baron, 2003). After binding of ligands exposed on neighboring cells such as Delta1 or Jagged, the Notch receptor is cleaved by a γ secretase complex; the resulting intracellular domain of Notch (NICD) translocates to the nucleus and activates transcription of target genes.

When epidermal keratinocytes from mouse, rat, and human are taken in culture, proliferation can be abrogated and terminal differentiation initiated by elevation of the calcium concentration in the culture medium (Hennings *et al*, 1980a, b; Yuspa *et al*, 1989; Pillai *et al*, 1990; Bikle *et al*, 2001; Mochizuki *et al*, 2001; Tu *et al*, 2001). Calcium is therefore often regarded as being both sufficient and indispensable for these processes, in particular, in mouse keratinocytes. The concept of calcium as being indispensable for the terminal differentiation process, however, remained puzzling. It was reported many years ago that mouse keratinocytes grown in suspension and also human keratinocytes express terminal differentiation markers in low calcium-containing medium (Watt and Green, 1982; Drozdoff and Pledger, 1993). The intercellular adhesion molecule desmoglein (Dsg)3 was also detected under low calcium conditions (Kulesz-Martin *et al*, 1989, 1990). Moreover, supplementing 1.2 mM calcium-containing medium with additional growth factors supported long-term proliferation of mouse keratinocytes (Morris *et al*, 1987). In contrast to mouse and human keratinocytes, little is known about the effect of calcium on cultured canine keratinocytes. Interestingly, however, these cells are usually expanded in

Abbreviations: Dsg, desmoglein; GFP, green fluorescent protein

medium containing elevated levels of extracellular calcium (1.8 mM) (Wilkinson *et al*, 1987; Suter *et al*, 1991, 1997).

In culture, proliferating human and mouse keratinocytes are characterized by an active cell cycle resulting in cellular division under the control of cyclins such as cyclin D1, cyclin-dependent kinases as well as their inhibitors (Santini *et al*, 2001; Weinberg and Denning, 2002). The cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p27^{KIP1}$ are involved in the G1/S checkpoint control and, through inactivation of cyclins, will drive the cells into G0 and eventually into terminal differentiation. It should be emphasized that growth arrest does not automatically result in onset of terminal differentiation. Therefore terminal differentiation must be assessed by means of upregulation of specific markers at both the transcriptional and protein level (Watt, 1989).

Biochemical markers accompanying terminal differentiation can be separated in "early markers" (keratins 1 and 10, Dsg3 and 1, involucrin), and "late markers", which, in the epidermis are expressed in the granular layer and beyond (like loricrin, filaggrin) (Dlugosz and Yuspa, 1993). During early steps of terminal differentiation, the cells reinforce intercellular adhesion that is mediated by adherens junctions and desmosomes in a calcium-dependent manner (Green and Gaudry, 2000; Fuchs and Raghavan, 2002; Jamora and Fuchs, 2002). Strong cell–cell adhesion is indispensable for late terminal differentiation, where involucrin, filaggrin, and loricrin are cross-linked to membrane proteins via transglutaminase to form the thick insoluble cornified envelope (Kasturi *et al*, 1993; Koch *et al*, 2000).

So far, a comprehensive survey in keratinocytes on the consequences of low *versus* elevated extracellular calcium for expression of recently identified proliferation markers, those driving cell cycle exit, onset, and progression of terminal differentiation markers, has not been reported. This hampers a clear definition of the calcium-regulatory ability during different phases of epidermal keratinocyte differentiation. Moreover, parameters of cell confluency and hence signals generated by ample cell–cell contact were often not taken into account in previous studies as the calcium switch mostly was introduced when cells reached confluency. Importantly, signaling molecules that regulate cell growth and differentiation *in vivo*, including c-Myc and Notch1, were not assessed so far as potential signals alternative to extracellular calcium.

These open questions were addressed here by rigorously monitoring the effects of low (up to 0.09 mM) and elevated calcium (1.2 and 1.8 mM) on proliferation, cell cycle exit, onset, and achievement of terminal differentiation in relation to confluency. This was done for keratinocytes known to proliferate easily in elevated calcium (canine keratinocytes) *versus* those believed to be most sensitive to elevated calcium conditions (mouse keratinocytes). This comprehensive survey now reveals that confluency is key in driving proliferating keratinocytes into terminal differentiation and involves the calcium-independent and reciprocal regulation of two critical determinants: c-Myc and Notch1. It was only after onset of terminal differentiation that extracellular calcium was required to sustain upregulation and/or stabilization of terminal differentiation markers and intercellular adhesion molecules.

Results

Proliferation of canine keratinocytes in low *versus* high calcium-containing media Canine keratinocytes were plated at the same density in high (1.8 mM) or low (0.06 mM) calcium-containing medium with or without serum, and assessed for morphological changes (Fig 1*A*) and growth rates (Fig 1*B*).

Growth rates in high calcium concentrations exceeded those in low concentrations and proliferation was best in media containing serum. Despite these variations, keratinocytes made ample cell–cell contacts in all cultures around day 2 and invariably reached 100% confluency at day 3 and latest day 4 (Fig 1*A* and *B*). Synchronicity was achieved because of differences in cell size. In high calcium medium, cells were small and cobble-stoned whereas remaining flat and spread-out in low calcium medium (Fig 1*A*). Post-confluency, keratinocytes in all media continued to multiply for one to four additional cell cycles and became more tightly packed. After that time point proliferation ceased as seen by the entry of the growth curves into a plateau (Fig 1*B*). It is notable that post-confluent cells more frequently detached from the monolayer in low calcium media. Cultures grown in 0.09 mM had an intermediate growth rate between that in 1.8 and 0.06 mM calcium (data not shown).

The striking observation from this analysis was that irrespective of the calcium conditions and morphology the canine keratinocytes had a similar fate; they proliferated until they reached confluency and then underwent one to four

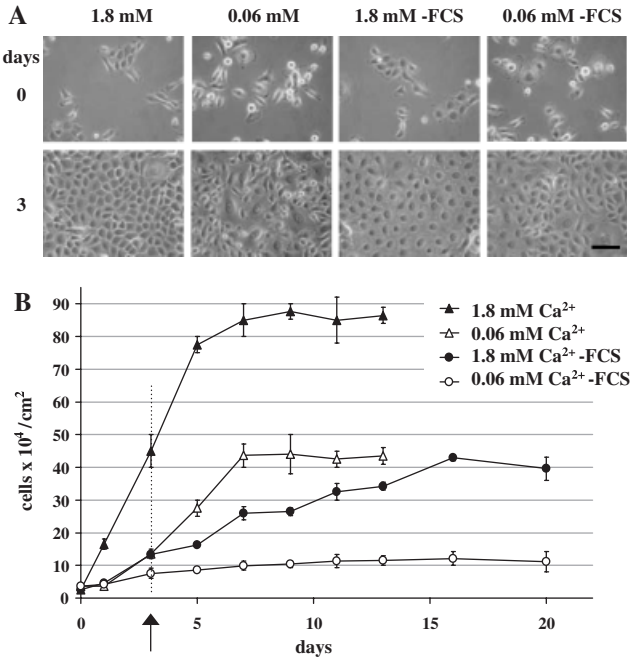


Figure 1
Morphology and proliferation rate of canine keratinocytes in high and low calcium-containing medium. (*A*) The change in morphology at the indicated days post-seeding is shown by phase contrast microscopy in cells plated in 1.8 or 0.06 mM calcium-containing media with or without serum (−FCS). *Scale bar*: 80 μm. (*B*) Graphs depict proliferation rates of the same cell cultures as shown in (*A*) in media as indicated. Note that 100% confluency was reached under all conditions around day 3 after seeding (*arrow*). Bars indicate the mean of duplicates. FCS, fetal calf serum.

additional cellular divisions before they became growth arrested.

Expression of proliferation and terminal differentiation markers in canine keratinocytes To investigate whether the different culture conditions allowed canine keratinocytes to exit the cell cycle and commit to terminal differentiation, we studied the steady-state level of a selected set of markers for which antibodies recognizing canine proteins exist.

The exit from the cell cycle was assessed by defining the expression of $p21^{WAF1}$ and $p27^{KIP1}$, two cyclin-dependent kinase inhibitors. Desmosomal cadherins Dsg3 and 1 were chosen, which, respectively, characterize early and advanced terminal differentiation in human and mouse keratinocytes. Involucrin and loricrin, two products of the cornified envelope, were also addressed. These collective markers were investigated by western blot analysis of cell lysates harvested over a period of 15 d from attached canine keratinocyte cultures grown in 1.8, 0.09, or 0.06 mM calcium with or without serum.

Proliferating keratinocytes (day 1; see Fig 1) had a low steady-state level of cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p27^{KIP1}$ that increased around day 3 when cells made ample cell contact (Fig 2*A* shows 1.8 and 0.06 mM calcium without serum). Interestingly, results for cells grown in low calcium were indistinguishable from those cultured in high calcium-containing medium.

In the same lysates, the increase in proteins driving cell cycle exit was followed by upregulation of the steady-state level of terminal differentiation markers and adhesion molecules (Fig 2*A*). At day 3, Dsg1, involucrin, and loricrin were readily detectable and further substantially increased after day 10 in high calcium-containing medium. Importantly, a similar fate of these proteins was observed in low calcium medium with the only exception that the steady-state levels started lower and remained lower. The level of Dsg3 only slightly changed in high calcium at day 15 and remained at a low level in low calcium medium. The results obtained with cells cultured in the presence of serum reproduced the same pattern of cell cycle exit and terminal differentiation marker expression (data not shown).

Commitment of keratinocytes to terminal differentiation involves increased expression of specific proteins because of transcriptional regulation. Among the regulated genes are *Dsg1* and *involucrin* as was shown for human keratinocytes (Pasdar *et al*, 1995; Balasubramanian *et al*, 2000). To address regulation of these genes, we performed real-time PCR on total RNA isolated from the same cultures as used for protein analyses.

Two days post-confluency the level of Dsg1 mRNA increased simultaneously under both low and high calcium conditions whereas the increase in involucrin mRNA in high calcium medium preceded that in low calcium medium (Fig 2*B*). Over the period of 15 d investigated, the mRNA of Dsg1 increased 27 times in high calcium-containing medium and 14 times under low calcium conditions. It is noteworthy that at the beginning of the experiment, the level of Dsg1 mRNA was five times lower in the cells adapted to low calcium medium as compared with the ones grown in high calcium medium (Fig 2*B*, *insets*). mRNA encoding involucrin increased 300 times in 1.8 mM extracellular calcium and 13 times in 0.06 mM calcium. Surprisingly, the level at day 1 was five times higher in 0.06 mM calcium than that of cells grown in 1.8 mM calcium. The opposite was seen at the protein level (Fig 2*A*), pointing toward independent transcriptional and translational control mechanisms for involucrin in cultured canine keratinocytes. Consistent with the protein expression pattern, Dsg3 mRNA levels were not substantially upregulated over time (Fig 2*A*).

These results demonstrate that even serum-starved canine keratinocytes proliferate until they make ample cell contact and then exit the cell cycle and commit to terminal differentiation independent of an extracellular calcium signal. In contrast to the proliferative phase, accumulation of high levels of terminal differentiation markers was dependent on high extracellular calcium.

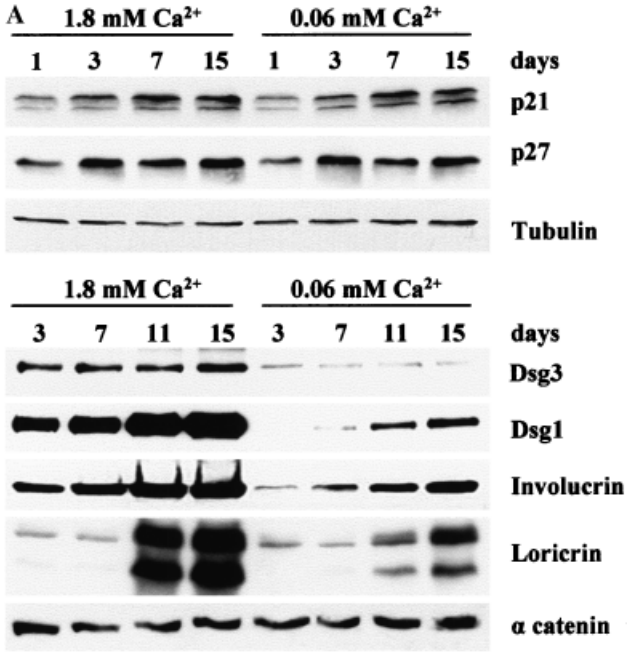
Requirement for calcium to stabilize intercellular adhesion molecules in canine keratinocytes In human keratinocytes, adhesion molecules are displayed at the plasma membrane in the absence of high extracellular calcium but fail to be stabilized (Demlehner *et al*, 1995). This phenomenon was assessed in the canine keratinocytes. The presence of surface-exposed desmosomal protein Dsg3 and associated plakoglobin as well as the adherens junction protein E-cadherin and associated β-catenin was investigated by double label immunofluorescence microscopy (Fig 3; shows 1.8 and 0.09 mM calcium).

Under high calcium condition, adhesion components were stabilized at the plasma membrane as expected (Hennings *et al*, 1980a; Caldelari *et al*, 2000). Cultures in low calcium also displayed adhesion components at points of cell–cell contact, although to a lesser extent and in a less continuous pattern. Concurrently, in low calcium medium cytoplasmic staining for β-catenin and plakoglobin was substantial and protein-filled vesicles were often observed (Fig 3, *arrows*). Consistent with the low steady-state level of Dsg1 protein and mRNA under low calcium conditions, staining was rarely detected at the plasma membrane (data not shown).

The collective results on the analyses of canine keratinocytes show that high calcium and serum in the culture medium provides a growth advantage. Irrespective of the proliferation rate and calcium conditions, however, canine keratinocytes reach confluency and continue for one to four mitotic divisions before undergoing growth arrest and committing to terminal differentiation. This indicates that these processes do not rely on additional extracellular calcium signals. In contrast, past the onset of terminal differentiation, extracellular calcium appears essential for rapid and sustained upregulation and stabilization of the differentiation-dependent proteins and mRNA.

Proliferation and terminal differentiation of mouse keratinocytes in low *versus* high calcium-containing media The finding that the exit from the cell cycle and onset of terminal differentiation in cultured canine keratinocytes occurred independent of extracellular calcium stimuli was intriguing and prompted us to investigate this phenomenon in mouse keratinocytes.

According to the literature, mouse keratinocytes are grown until confluency in serum-free low calcium medium (below 0.1 mM) and a switch to high calcium medium



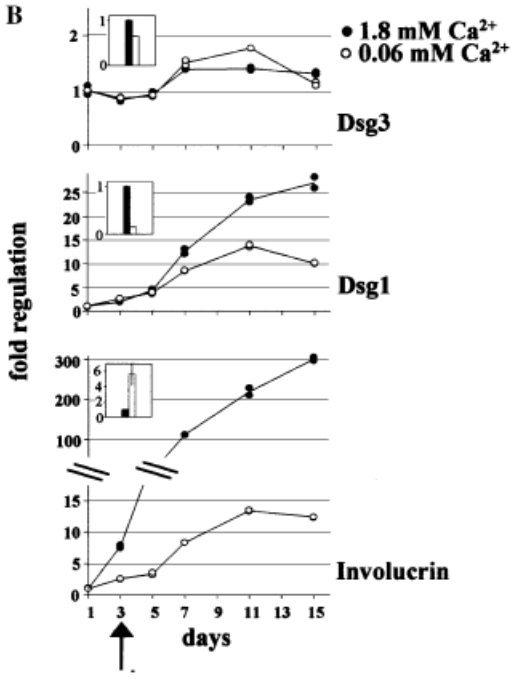


Figure 2
Expression of cell cycle exit and differentiation markers in canine keratinocytes. (*A*) Lysates from cell cultures grown in 1.8 or 0.06 mM calcium-containing medium without serum (selected time points are shown) were analyzed by western blotting using the indicated antibodies. The same protein samples were used for all blots. Acidic tubulin (carried out on the same blot as $p21^{WAF1}$) and α-catenin (caried out on the same blot as desmoglein (Dsg)1) were used as controls for equal loading. (*B*) Graphs show the fold regulation of steady-state levels of indicated mRNA as determined by quantitative real-time PCR. The same cultures as in (*A*) are shown. Relative levels of mRNA are presented. For better evaluation, each sample was compared with day 1 of each culture condition. *Insets*: relative level of mRNA in the low calcium sample at day 1 as compared with the high calcium sample at day 1. Bars indicate the mean of duplicates.

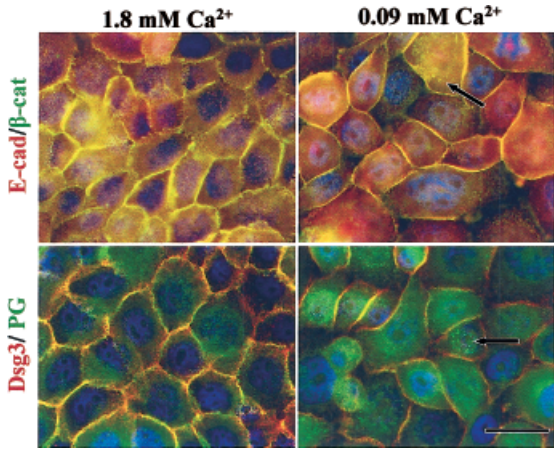


Figure 3
Immunofluorescence analysis of adherens junction and desmosomal components at the plasma membrane of canine keratinocytes under low and high calcium conditions without serum. The merge of staining obtained by double-labeling immunofluorescence microscopy of confluent cultured canine keratinocytes in 1.8 mM (day 5) and 0.09 mM calcium (day 7) is shown for E-cadherin/α-catenin and desmoglein (Dsg)3/plakoglobin, respectively. Arrows indicate cytoplasmic vesicles. *Scale bar*: 50 μm.

(above 1 mM) is introduced to trigger terminal differentiation (Yuspa *et al*, 1989; Caldelari *et al*, 2001). In our study, morphology and growth behavior were therefore compared between (i) mouse keratinocytes grown in 0.09 mM calcium-containing medium, (ii) cells switched to 1.29 mM calcium 12 h after seeding, and (iii) those switched shortly after confluency on day 4 (Fig 4). The early calcium switch was used to evaluate the effect of high extracellular calcium on proliferation and cell cycle exit.

In line with common knowledge (Hennings *et al*, 1980b; Hennings and Holbrook, 1983; Yuspa *et al*, 1989), the switch to high calcium provoked an immediate and profound change in the morphology of the mouse keratinocytes (Fig 4*A*). Cells changed from angular to flat and polygonal. Despite these striking changes, the mouse keratinocytes in high calcium-containing medium continued to proliferate at a rate comparable to those in low calcium medium and all cultures reached confluency around day 3 (Fig 4). As observed for the canine keratinocytes, mouse cells in either medium then performed about four additional mitotic divisions and ceased to multiply around day 7 as seen by the formation of a growth plateau (Fig 4*B*).

In conclusion, under the conditions used here, mouse keratinocytes proliferate and exit the cell cycle irrespective of the extracellular calcium concentration. These results not only demonstrate that the early calcium switch failed to stall mouse keratinocyte proliferation, but further suggest that the keratinocytes are not dependent on an extracellular calcium signal but on cell contacts to become quiescent. This was also confirmed by re-plating cells 1 d after introducing the calcium switch, i.e. prior to forming ample cell–cell contacts. The passaged cells readily attached to the culture flask and could be propagated over several passages if confluency was prevented (data not shown).

Expression of proliferation and terminal differentiation markers in mouse keratinocytes Cell cycle exit and commitment to terminal differentiation was further addressed in

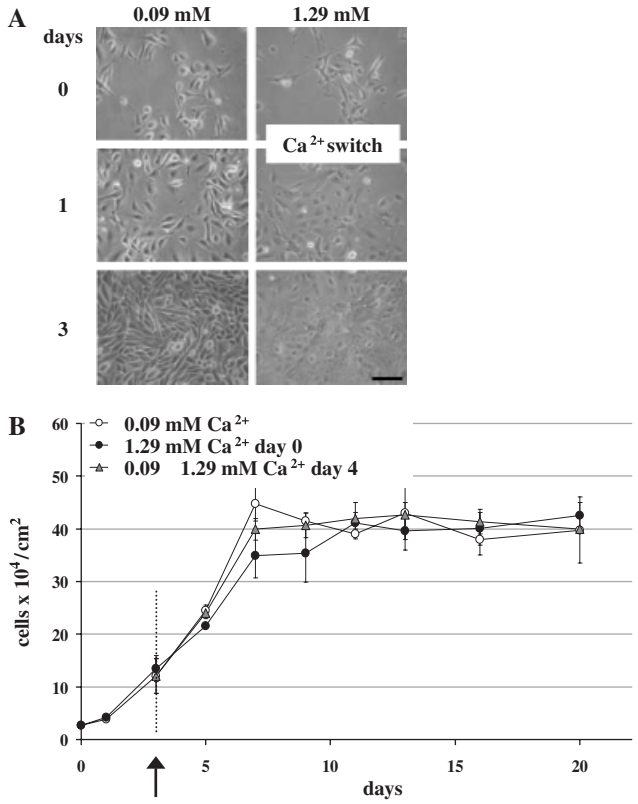


Figure 4
Morphology and proliferation rate of mouse keratinocytes in high and low calcium-containing medium. (*A*) A change in morphology at the indicated days post-seeding is observed by phase contrast microscopy in cells cultured in 0.09 mM calcium-containing medium and those switched to 1.29 mM 12 h post-seeding. *Scale bar*: 80 μm. (*B*) Graphs show proliferation rates of the cell cultures shown in (*A*). Irrespective of culture conditions, 100% confluency was reached around day 3 after seeding (*arrow*) and a plateau was reached on day 7. Bars indicate the mean of duplicates.

the mouse keratinocytes grown in low and switched to high calcium medium 12 h post-seeding. Cell cycle and differentiation markers were investigated at both protein and mRNA steady-state levels (Fig 5*A* and *B*).

Under both calcium conditions protein levels of $p21^{WAF1}$ were high at day 1 after seeding, which is likely because of the substantial level of growth factors in the media (Weinberg and Denning, 2002). On the mRNA level, however, $p21^{WAF1}$ started to increase at day 2 in cells grown in high calcium and from day 3 onwards in cells grown in low calcium medium. $p27^{KIP1}$ mRNA followed the increase of $p21^{WAF1}$ with some delay, and its protein level increased when $p21^{WAF1}$ decreased (around day 7). The latter is

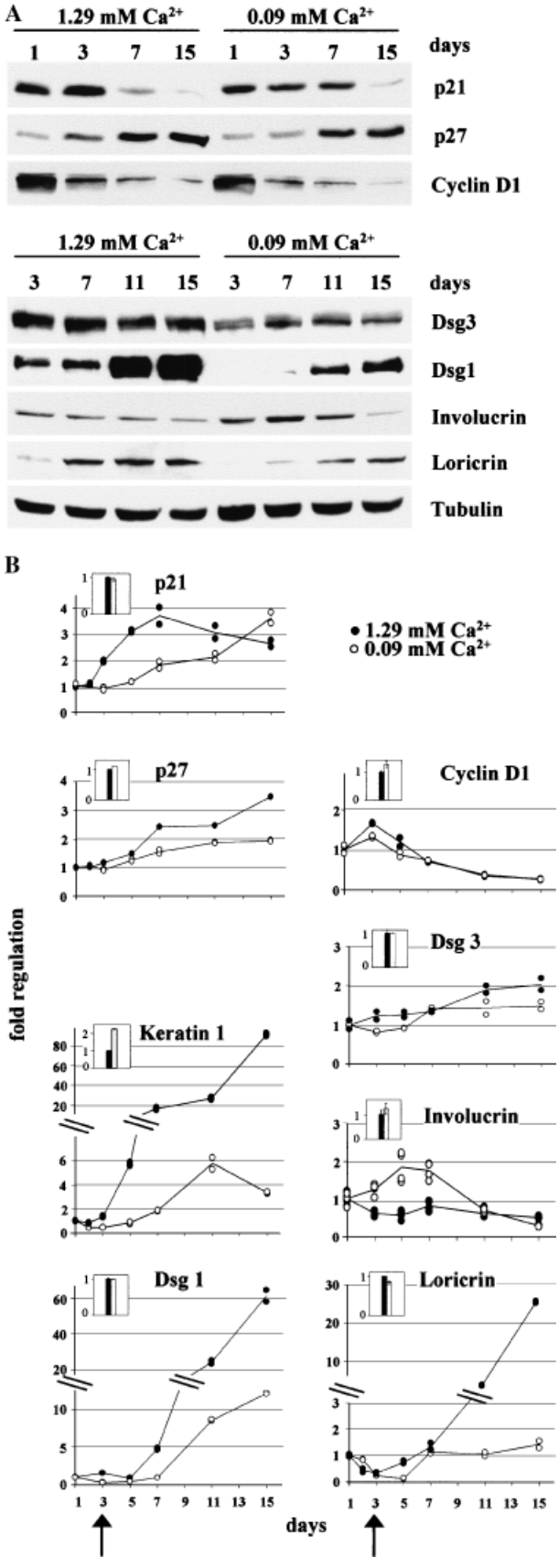


Figure 5
Expression of cell cycle exit and differentiation markers in mouse keratinocytes. (*A*) Western blot analysis with the indicated antibodies was performed on lysates from cell cultures grown in 0.09 mM or switch to 1.29 mM calcium 12 h after seeding. The same protein samples were used for all blots. Acidic tubulin (carried out on the same blot as involucrin) and α-catenin (data not shown) were used as loading controls. (*B*) Graphs show the fold regulation of steady-state levels of indicated mRNA as determined by real-time quantitative PCR on the same cultures used in (*A*). Relative levels of mRNA are presented. For better evaluation, each sample was compared with that of day 1 of each culture condition. *Insets*: relative level of mRNA in low calcium samples as compared with high calcium samples at day 1. Bars indicate the mean of duplicates.

consistent with the idea that $p27^{KIP1}$ is responsible for maintaining differentiated cells in a post-mitotic state (Weinberg and Denning, 2002). Sustained levels of cyclin inhibitors further correlated with the rapid decay of cyclin D1 mRNA and protein as soon as cells started to make ample contacts under both low and high calcium conditions.

As seen with canine keratinocytes, the expression pattern of cell cycle markers clearly indicated that initiation of cell cycle exit in mouse keratinocytes was not dependent on high extracellular calcium but on confluency. Also consistent with the canine keratinocytes, mRNA or protein level of terminal differentiation markers Dsg1, keratin 1, and loricrin were substantially upregulated under both calcium conditions between days 3 and 5, with levels being higher in high calcium-containing medium. Additionally, Dsg3 only moderately increased at the mRNA but not the protein level. The surprising finding was that the steady-state levels of involucrin protein and mRNA increased to some extent under low calcium but not under high calcium conditions as will be further discussed below.

Stabilization of desmosomal and adherens junction markers was also investigated under high and low calcium conditions in the mouse keratinocyte cultures (Fig 6). Results were similar to those obtained for canine cells, i.e. these structures were less well organized and stabilized under low calcium conditions, in particular, with regard to Dsg1. It is noteworthy, however, that even under low calcium conditions substantial amounts of intercellular components were present at the plasma membrane.

As observed with canine cells, our collective results on mouse keratinocytes demonstrate that the switch from proliferation to cell cycle exit and onset of terminal differentiation was not dependent on extracellular calcium signals. This finding was supported by the profiles of the proliferation curves that were highly comparable in either media. Conversely, calcium was crucial to substantially upregulate and/or stabilize differentiation-dependent mRNA and proteins. Although these findings appear to contrast with common knowledge, it should be kept in mind that most laboratories, including ours, normally introduce the calcium switch when the cells are confluent. Under the latter conditions, the contact-induced exit from the cell cycle and onset of terminal differentiation described here are overlapping and indistinguishable from the calcium-induced events.

c-Myc and Notch1 accompany mouse and canine keratinocyte differentiation This study so far reports that the effect of confluency prevails over a switch in extracellular calcium to initiate cell cycle arrest and terminal differentiation in cultured keratinocytes. This raised the question of whether signals related to proliferation and cell–cell contact identified *in vivo* also prevailed over calcium in cell culture.

Based on current knowledge (Arnold and Watt, 2001; Waikel *et al*, 2001), we tested whether c-Myc de-regulation preceded initiation of cell cycle exit and onset of terminal differentiation. C-Myc expression was addressed at the mRNA and protein level using real-time PCR and western blot analysis, respectively, in mouse keratinocytes grown in low and high calcium-containing medium (Fig 7*A* and *B*). Interestingly, whereas most markers we have investigated

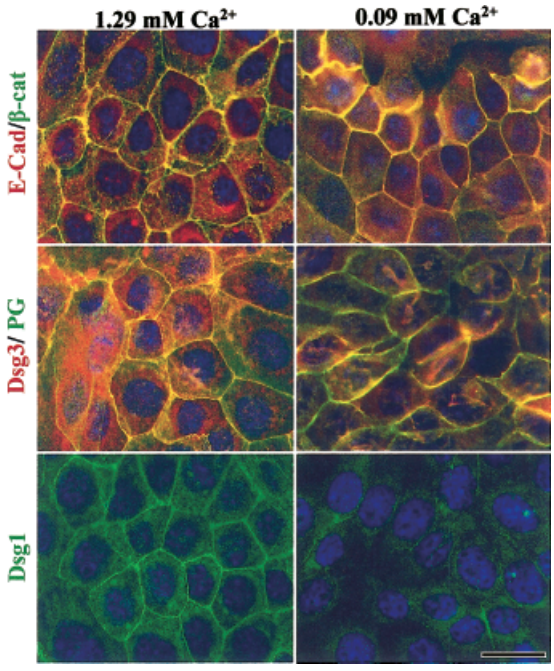


Figure 6
Immunofluorescence analysis of adherens junction and desmosomal components at the plasma membrane of mouse keratinocytes under low and high calcium conditions. The merge of staining obtained by double-labeling immunofluorescence microscopy of keratinocytes cultured in 1.29 and 0.09 mM calcium (day 5) is shown for E-cadherin/α-catenin and desmoglein (Dsg)3/plakoglobin, respectively. Dsg1/2 staining was carried out on parallel cultures assessed by single-labeling immunofluorescence (*bottom panels*). *Scale bar*: 50 μm.

were upregulated as cells became confluent, c-Myc mRNA increased like cyclin D1 immediately after seeding and decreased at confluence after day 3 (Fig 7*A*; compare with Fig 4*B*). The protein steady-state level followed a similar pattern (Fig 7*B*). The fold upregulation of c-Myc mRNA was similar under both calcium conditions. In contrast, the protein level was higher in cells grown in low compared with high calcium medium, indicating transcriptional and post-transcriptional regulation.

As a transcription factor, activated c-Myc is expected to localize to the nucleus. To address subcellular localization of c-Myc, immunofluorescence microscopy was performed on the canine and murine keratinocytes during the proliferative state and the subsequent exit from the cell cycle (Fig 7*C*). Strong nuclear staining for c-Myc was characteristic for proliferating canine and murine keratinocytes. Staining disappeared in confluent canine cells whereas it remained perinuclear in confluent mouse keratinocytes. These events occurred independent of the extracellular calcium concentration.

Downregulation of c-Myc at confluency can potentially be achieved through activation of the Notch signalling pathway, which is known to be triggered by cell contacts and is also directly involved in keratinocyte differentiation (Rangarajan *et al*, 2001). Therefore, we assessed Notch1 expression and activation with respect to confluency by real-time PCR, western blot analysis, and immunofluorescence microscopy in mouse and canine keratinocyte grown in high or low calcium-containing medium (Fig 8). Concurrently with c-Myc downregulation, the mRNA level (Fig 8*A*)

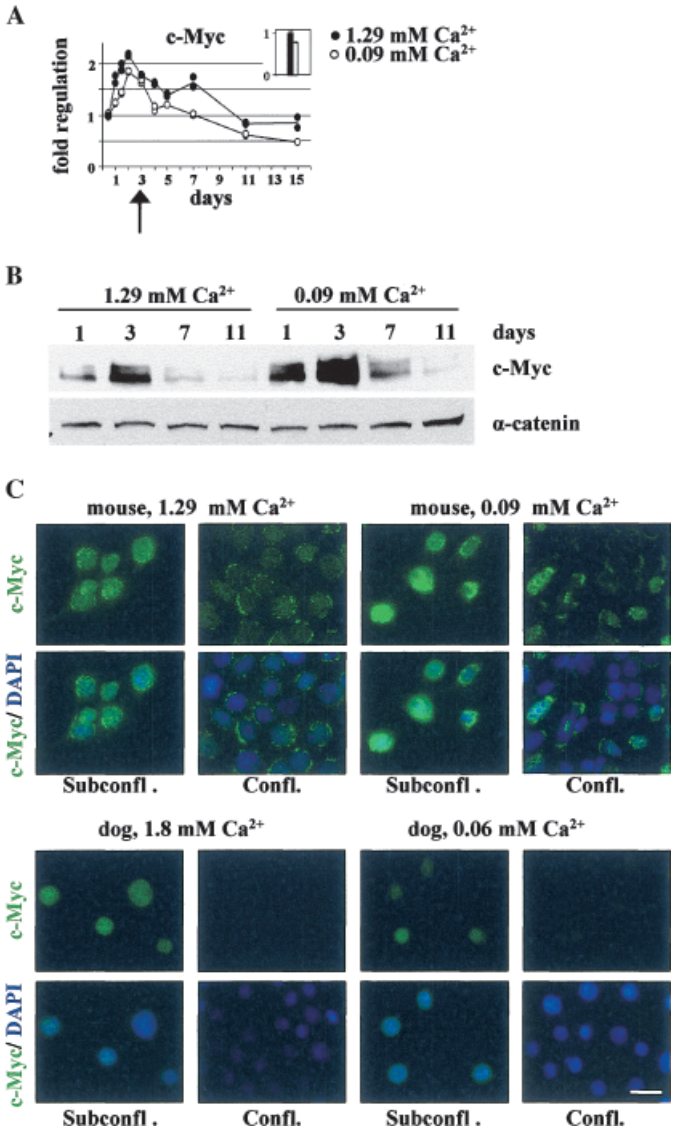


Figure 7
Expression of c-Myc in murine and canine keratinocytes. (*A*) Graph shows fold regulation of the steady-state level of murine c-Myc mRNA as determined by real-time PCR. Relative levels of mRNA are presented. Each sample was compared with day 1 of each culture condition. *Insets*: relative level of mRNA in low calcium samples as compared with high calcium samples at day 0.5. Bars indicate the mean of duplicates. (*B*) Western blot analysis was performed on the samples used for Fig 4*A*. Note that the level of c-Myc increased during proliferation, with a maximum on day 3, and decreased after confluency. α-Catenin was used as a loading control on the same blot. (*C*) Using immunofluorescence microscopy, nuclear accumulation of c-Myc was seen in subconfluent but not in confluent canine and murine keratinocyte cultures grown under the indicated calcium conditions. Lower panels show counter-staining with DAPI. It is noteworthy that more than 90% of all cells showed nuclear c-Myc under subconfluent conditions (day 1). Experimental procedures and photographic exposures were kept constant. *Scale bar*: 50 μm.

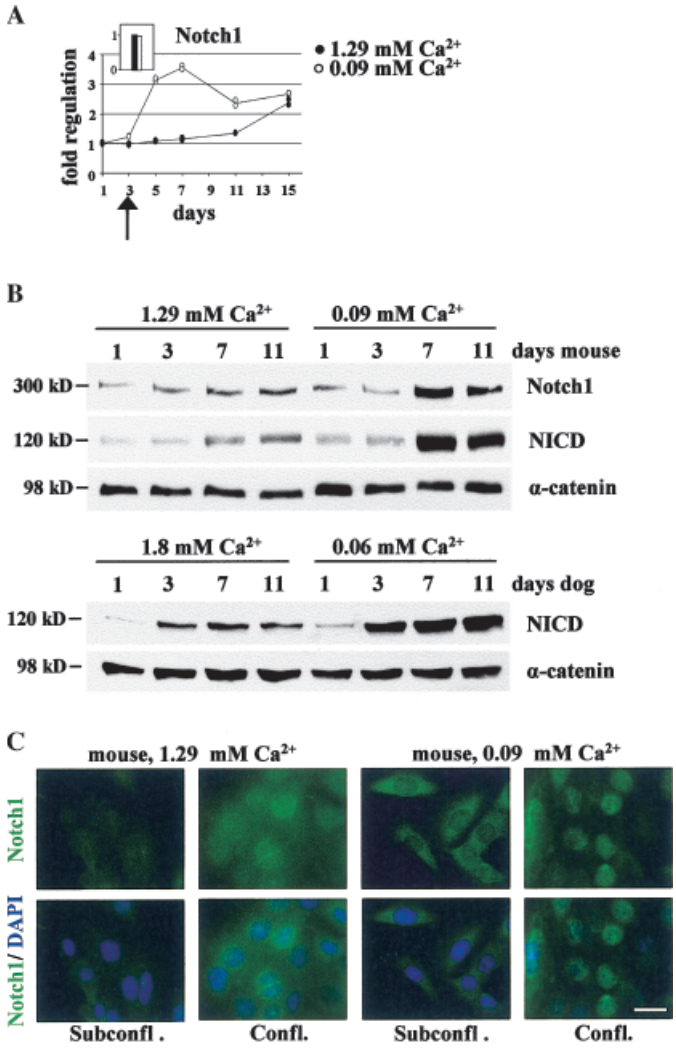


Figure 8
Expression of Notch1 in murine and canine keratinocytes. (*A*) Graph shows fold regulation of the steady-state levels of murine Notch1 mRNA as determined by real-time PCR. Relative levels of mRNA are presented. Each sample was compared with day 1 of each culture condition. *Insets*: relative level of mRNA in low calcium samples from day 1 as compared with high calcium samples at day 1. Bars indicate mean of duplicates. (*B*) Western blot analysis was carried out on the same samples as used for Figs 2*A*, 5*A*, and 7*A*. The levels of active, cleaved (NICD) and the non-active, non-cleaved Notch1 (long exposure) increased in confluent cells, predominantly under low calcium conditions. α-Catenin was used as loading control. (*C*) Using immunofluorescence microscopy on murine cultures Notch1 accumulated in the nucleus in confluent cultures. Experimental procedures and photographic exposures were held constant. *Scale bar*: 50 μm.

and the protein level of cleaved (active) Notch1 increased after the mouse keratinocytes had reached confluency and persisted at a high level (Fig 8*B*; NICD). The uncleaved, non-active protein followed a similar regulation pattern. Importantly, levels were substantially higher in low calcium than in high calcium medium, under which condition the increase of Notch1 mRNA was only evident around day 11. The protein level of activated Notch1 in canine cells was similar to that in mouse cells, except that its upregulation already occurred at day 3. As seen by immunofluorescence microscopy, Notch1 showed diffuse faint cytoplasmic staining in the subconfluent cells whereas it became nuclear in confluent cultures (Fig 8*C*).

c-Myc and Notch1 are critical determinants of the proliferation switch So far we have observed that onset of terminal differentiation, including regulation of c-Myc and Notch1 proceeded independent of calcium conditions. To define the relevance of c-Myc inhibition and Notch1 activation for onset of terminal differentiation in proliferative cells under low calcium conditions, we used two novel

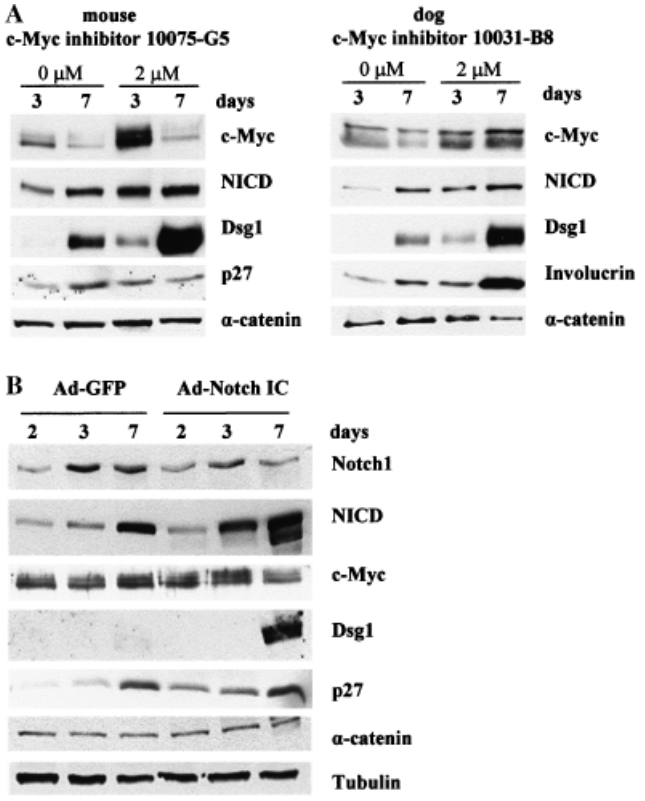


Figure 9
Inhibition of c-Myc and activation of Notch1. (*A*) Lysates of murine and canine cultures exposed to the indicated Myc/Max inhibitors 12 h post-seeding were analyzed by western blot analyses after 3 and 7 d. Note that in the presence of the inhibitors Notch1 was activated and desmoglein (Dsg)1 increased. α-Catenin was used as loading control on the same blot as used for analyses of c-Myc and p27 (mouse) or Notch1 (dog). (*B*) Lysates of murine and canine cultures infected 24 h post-seeding with adenovirus expressing constitutive active Notch (Ad-Notch NIC) or green fluorescent protein (GFP) as a control were analyzed by western blotting up to 7 d post-seeding. Notch expression led to an increase in the level of Dsg1 as compared with the control and slight decrease in c-Myc. Loading controls were α-catenin carried out on the same blot as Notch1 and Dsg1, and acidic tubulin was carried out on the same blot as c-Myc.

c-Myc inhibitors 10075-G5 and 10031-B8 (preventing the interaction of Myc with its activator Max; Yin *et al*, 2003) and expressed a constitutively active Notch1 (Rangarajan *et al*, 2001).

Twelve hours post-seeding, murine and canine cells were incubated with varying concentrations of the c-Myc inhibitors in low calcium medium, and proliferation and terminal differentiation markers were assessed by western blot analysis up to 7 d. The initial observation was that c-Myc inhibitor concentrations as low as 5 μM led to an immediate growth arrest of both cell types and at higher concentrations to cell death (data not shown). Already concentrations up to 2 μM cells provoked premature terminal differentiation. This was seen by a slightly more rapid increase of $p27^{KIP1}$ at day 3 followed by substantially higher Dsg1 and/or involucrin levels at day 7 (Fig 9*A*). Strikingly, these changes were also accompanied by premature activation of Notch1 as seen by more substantial accumulation of its intercellular domain (NICD). This was at least as prominent in c-Myc inhibited cells at day 3 than at day 7 in control cells when they become growth arrested (Figs 1*B* and 4*B*).

To test whether Notch1 activation had consequences similar to c-Myc inhibition, we ectopically expressed a constitutively active NICD or control green fluorescent protein (GFP) using recombinant adenovirus (Ad-Notch IC, Rangarajan *et al*, 2001; Ad-GFP). Mouse keratinocytes were infected 1 d post-seeding to achieve comparable expression levels of NICD during the proliferative phase than seen at day 7 in control cells (Fig 9*B*). Cells were harvested over 7 d, i.e. prior to and at growth arrest, and lysates were assessed by western blotting. Consistent with earlier findings, NICD expression led to some increase in the level of $p27^{KIP1}$ (Rangarajan *et al*, 2001). Concurrently, Dsg1 accumulated whereas c-Myc started to decline in Ad-Notch IC-infected cells in contrast to Ad-GFP-infected cells, which at day 7, had only little Dsg1 and still elevated c-Myc levels.

Together, these results demonstrate that under the conditions used here, c-Myc and Notch1 have opposing effects, appear to be mutually exclusive, and are both critical determinants in the switch from proliferation to terminal differentiation in keratinocytes cultured in low calcium medium.

Discussion

In contrast to what was reported for mouse keratinocytes (Hennings *et al*, 1980a), elevated extracellular calcium failed to stall proliferation in mouse and canine keratinocyte cultures under the conditions used here. Canine keratinocytes proliferated even better in elevated extracellular calcium, a phenomenon already described many years ago for post-confluent human keratinocytes (Milstone, 1987). Interestingly, despite this difference, only *subtle* changes in expression of the investigated proliferation and terminal differentiation markers were observed between the canine and murine cell cultures. We will therefore restrict the discussion to the common features of these two types of cultures.

In contrast to elevated extracellular calcium, confluency triggered cell cycle exit of murine and canine keratinocytes as supported by reciprocal regulation of markers such as cyclin D1, $p21^{WAF1}$, and $p27^{KIP1}$ (Fig 10). Furthermore, downregulation of c-Myc and upregulation of Notch1, which are shown to be critical determinants of this process, occurred in both cultures and were more prominent under low calcium conditions.

Good evidence exists that Notch1 activation is sufficient to trigger cell cycle exit *in vivo* in mouse and human epidermal keratinocytes (Rangarajan *et al*, 2001; Nickoloff *et al*, 2002; Nicolas *et al*, 2003). Notch activation typically requires cell–cell contact because Notch-receptor ligands such as Delta or Jagged are not soluble but cell surface anchored molecules (Mumm and Kopan, 2000; Baron, 2003). Our results are in line with these reports as they show activation and nuclear localization of Notch1 in post-confluent cells in the absence of complementary signals such as calcium. Moreover, expression of activated Notch1 prior to confluency accelerated terminal differentiation, emphasizing the key role of Notch1 during that process.

Interestingly, premature Notch1 activation coincided with a more rapid decay of the transcription factor c-Myc.

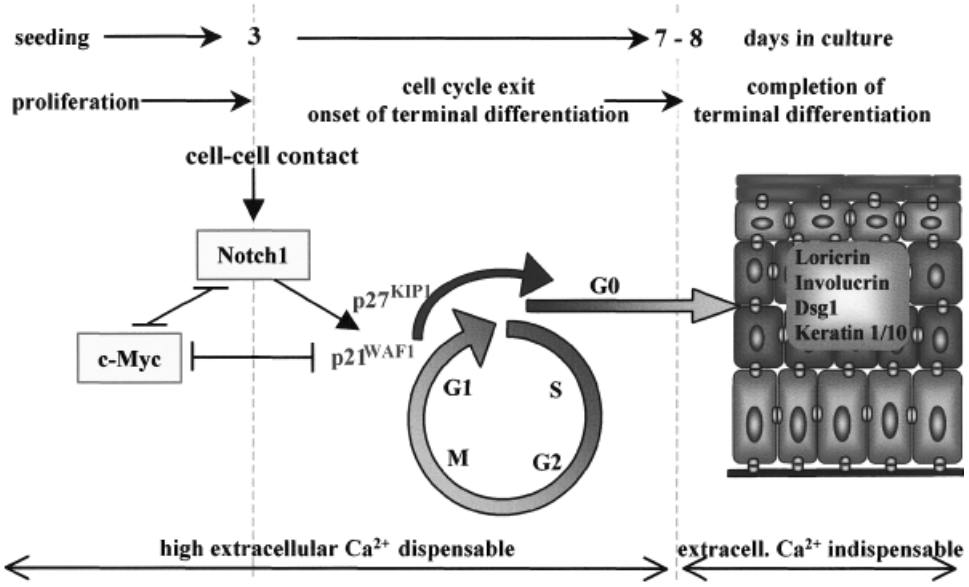


Figure 10
Diagram presenting the findings of this study on the involvement of high extracellular calcium, c-Myc, and Notch1 in proliferation, cell cycle exit, onset, and achievement of terminal differentiation in murine and canine keratinocyte cultures. In both cultures high extracellular calcium fails to stall proliferation, and the exit from the cell cycle is invariably triggered at confluency. At that time point Notch1 is activated and c-Myc inhibited in a calcium-independent fashion. These two factors are key determinants in this process and in control of $p21^{WAF1}$ and $p27^{KIP1}$. When the cell cycle inhibitors accumulate, cells exit the cell cycle (G0) and become quiescent. Concurrently, expression of terminal differentiation markers is induced, which is more substantial in the presence of high extracellular calcium. This demonstrates that once initiated, calcium is indispensable for completion of the terminal differentiation process.

Although so far not reported for keratinocytes, the suppression of c-Myc by Notch1 is conceivable. Notch1 family members drive the proliferation switch via transcriptional upregulation of $p21^{WAF1}$ and $p27^{KIP1}$ (Rangarajan *et al*, 2001; Nickoloff *et al*, 2002; Nicolas *et al*, 2003), which are potent suppressors of c-Myc (Horiguchi-Yamada *et al*, 2002). Interestingly, the inverse, namely suppression of $p21^{WAF1}$ and $p27^{KIP1}$ by c-Myc, has also been reported (Claassen and Hann, 2000; Fernandez *et al*, 2003; Sancho *et al*, 2003). Consistently, in this study, c-Myc inhibition by drugs during the proliferative phase was sufficient to induce growth arrest and onset of terminal differentiation. This phenomenon was demonstrated in cultured keratinocytes, and emphasizes that c-Myc inhibition, like Notch1 activation, is critical in the switch between proliferation and terminal differentiation. Accordingly, c-Myc was downregulated at confluency whereas mRNA encoding $p21^{WAF1}$ increased concurrently with Notch1 activation and Notch1 mRNA accumulation.

c-Myc inhibition during proliferation also triggered Notch1 activation. Together with the fact that constitutively active Notch1 led to a more rapid decay of c-Myc, these data suggest that during keratinocytes proliferation and onset of terminal differentiation c-Myc and Notch1 are key regulators that are mutually exclusive and presumably under control of a common mechanism triggered by cell confluency. We therefore suggest that also in cultured keratinocytes, cell to cell signaling pathways activated by confluency and in control of Notch1 activation and c-Myc inhibition trigger cell cycle exit followed by transcriptional upregulation of $p21^{WAF1}$ and $p27^{KIP1}$ (Fig 10). More in-depth analysis of the exact pathways activated through confluency as well as of the factors in control c-Myc will now be required.

In line with cell–cell contacts being sufficient in driving exit from the cell cycle and subsequent onset of terminal differentiation, upregulation of mRNA and protein for terminal differentiation markers like keratin 1, Dsg1, and loricrin occurred in cells under low calcium conditions. It is noteworthy that similar results have been reported for human keratinocytes (Poumay and Pittelkow, 1995). Also, involucrin was upregulated in low calcium medium in the mouse and canine cells.

A curious observation was that involucrin protein failed to increase in mouse keratinocytes under high calcium conditions although the relative level of involucrin mRNA was high (as judged by the c_t values obtained from real-time PCR). As it is currently accepted that involucrin is upregulated when mouse keratinocytes commit to terminal differentiation, this result might be in part explained by the fact that involucrin is more soluble in cells held in low extracellular calcium, whereas it is integrated into the cornified envelope and mostly insoluble in the lysis buffer used here when cells are grown in high calcium medium. Although this phenomenon requires further investigations, the important point to be made is that involucrin was specifically upregulated in the mouse keratinocytes under low calcium conditions.

As noted, more cells detached from the monolayer culture in low extracellular calcium medium. In contrast, dead scales only formed in elevated extracellular calcium. This demonstrates that the onset of terminal differentiation occurs in low calcium medium but fails to be completed in a monolayer culture without elevated extracellular calcium. The failure to stabilize intercellular adhesion structures under low calcium conditions might be one of the major reasons for this phenomenon. This is consistent with the concept that assembly of the cornified envelope is initiated and the terminal differentiation process is enhanced through stabilization of intercellular adhesion by calcium (Koch *et al*, 2000). It also confirms the requirement for extracellular calcium once the terminal differentiation process is initiated (Fig 10).

An indispensable role for calcium in late terminal differentiation is further compatible with the *in vivo* situation. In human and mouse epidermis, the calcium gradient is only built up as the keratinocytes in the epidermis move outward to the granular layer (Menon *et al*, 1985; Elias *et al*, 2002). Consistently, transgenic mice expressing a truncated form of the calcium receptor in the epidermis that prevents

transmission of the extracellular calcium signal only showed ultrastructural changes in the terminally differentiated keratinocytes of the granular layer (Komuves *et al*, 2002). Despite lack of ultrastructural changes in the deep epidermis of these mice, however, more numerous proliferating cells were seen in the basal and also suprabasal layers (Komuves *et al*, 2002). This further suggests that calcium receptor-mediated signalling can provide a complementary signal that expedites cell cycle exit. Such a possibility is compatible with the slower upregulation of terminal differentiation markers that we observed in mouse and canine keratinocytes under low calcium conditions.

The possibility that calcium is dispensable but can speed up the exit from the cell cycle once a confluency-induced signal is provided also explains the discrepancy between our results and the earlier findings by Hennings *et al* (1980b). In the latter study, it was found that elevation of calcium in the medium abrogated cell growth during the proliferative phase of mouse keratinocytes. As a matter of fact, cells were seeded at four times higher density in that study than in ours, allowing for immediate cell–cell contact. The effects of cell–cell contact, confluency, and calcium were therefore overlapping as can be judged from the phase contrast pictures of cell cultures (Hennings *et al*, 1980b). Nonetheless, the growth-inhibitory effect of elevated calcium was more pronounced in the latter study than in ours. This might be because of differences in the growth-promoting ability of a medium containing fetal calf serum (FCS) used by Hennings and collaborators and that of a serum-free medium containing keratinocyte-specific growth factors used in this study. Although interesting, more in-depth analysis of this phenomenon would exceed the scope of this study.

Based on our data, we conclude that in cultured canine and mouse keratinocytes elevation of extracellular calcium is important to bring an initiated terminal differentiation process to completion. In contrast, confluency-induced signals prevail over extracellular calcium signals in the switch from proliferation to terminal differentiation for which c-Myc inhibition, Notch1 activation, and subsequent upregulation of $p21^{WAF1}$ and $p27^{KIP1}$ mRNA are crucial determinants similarly as *in vivo*. This suggests that the keratinocytes under study are committed precursors, which preserve their capacity to differentiate along the epidermal lineage even in the absence of their *in vivo* environment.

Materials and Methods

Cell cultures Skin biopsies were obtained from a healthy beagle dog and wild-type C57BL6 mice, and keratinocyte cultures were established as previously described (Wilkinson *et al*, 1987; Suter *et al*, 1991; Caldelari *et al*, 2000). Canine cells were expanded in Dulbecco's modified Eagle's medium (without calcium, 21068-028, Invitrogen, Carlsbad, California) supplemented with $CaCl_2$ (1.8, 0.09, or 0.06 mM), 1% antibiotic/antimycotic (15240-062, Invitrogen), and 1% L-glutamine (W060080, Bioconcept, Allschwil, Switzerland), 10^{-10} M choleratoxin (CT, C3012, Sigma, Deisenhofen, Germany), 10 ng per mL epidermal growth factor (EGF, b 100-15, Bioconcept), and 1.5%–5% FCS (10270-106, Invitrogen). Mouse cells were expanded in serum-free, fully supplemented CnT-02 (CELLnTEC, Bern, Switzerland) containing 0.09 mM $CaCl_2$. This medium was designed to specifically support long-term growth of progenitor cells. The calcium concentrations in the media were routinely checked by photometry using a chemistry analyzer (Hitchi 912, Roche Diagnostics, Basel, Switzerland). Both cell types were grown in a humidified incubator at 34°C under 5% CO_2. Experiments were repeated with cells from different passages (5–50), always giving identical results. Figures are shown for murine cells from passages 23 to 37 and canine cells passages 19 to 50. It is noteworthy that in spite of identical results between different passage numbers, the murine cells changed from diploid to mostly tetraploid over time. At passage 5, no tertraploid cells were observed whereas most cells were tetraploid and some polyploid at passage 50. The reason for genome multiplication so far is unclear. But lack of major consequences of progressive tetraploidy for keratinocyte terminal differentiation as seen during our study is compatible with the fact that tetraploid mouse keratinocytes in culture retain their ability to differentiate (Bruegel Sanchez *et al*, 2004). This in turn is in line with the normal development of all organs except the brain in tetraploid mouse embryos (Henery *et al*, 1992).

For all experiments, keratinocytes were plated at 3×10^4 cells per cm^2. Canine cells, which attach to the culture device independent of calcium concentrations, were directly seeded in 1.8 mM (stated as high calcium), 0.09, or 0.06 mM (both stated as low calcium) calcium-containing medium. To serum-starve cells, FCS, EGF, and CT were omitted 24 h post-seeding. Mouse keratinocyte cultures were always initiated in CnT-02 containing 0.09 mM calcium and half of the cultures switched to CnT-02 adjusted to 1.29 mM $CaCl_2$ 12 h post-seeding or at day 4. The time points are given in days with the start point 12 h after seeding (day 0). To avoid fluctuations, part of the medium was replaced daily by fresh medium. Calcium concentrations and serum conditions were chosen with respect to the most commonly used conditions for keratinocyte differentiation published in the literature (Hennings *et al*, 1980b; Wilkinson *et al*, 1987; Suter *et al*, 1991; Caldelari *et al*, 2000).

To inhibit c-Myc, cells were incubated 12 h post-seeding with small molecule inhibitors of the Myc–Max interaction. Inhibitors tested at several concentrations were the molecules 10031-B8 and 10075-G5 (Calbiochem, San Diego, California) (Yin *et al*, 2003). Notch activation was mimicked by adenovirus-mediated expression of Notch1-NICD (Ad-Notch1 IC, a kind gift from F. Radke, Ludwig Institute for Cancer Research, Epalinges, Switzerland) (Nicolas *et al*, 2003) or the Ad-GFP as control. Mouse cells were infected 24 h post-seeding with approximately 100 MOI of adenovirus diluted in 0.09 mM calcium CnT-02. Twenty-four hours later, comparable infection efficiency was monitored by visualizing green fluorescence and cells harvested up to 7 d. Experiments were carried out two times. The ethical committee of the University of Bern, Vetsuisse Faculty and the Swiss Office for biosafety approved all the described studies.

Proliferation assay Canine and mouse keratinocytes were seeded into 8.8 cm^2 dishes and growth curves were established by counting cell numbers per cm^2 at alternate days after seeding using a hamocytometer. Viability was determined by trypan blue exclusion. Experiments were run two times for murine cells and three times for canine cells in duplicates.

Quantitative real-time PCR Total RNA was isolated daily using the RNeasy Mini Kit (74104, Qiagen Sciences, Maryland, Florida) according to the manufacturer's instructions. Complementary DNA were generated by reverse transcription using 1.58 μg of total RNA, random primers (C1181, Promega, Madison, Wisconsin), and reverse transcriptase (M368B, Promega) in a Progene temperature cycler following the manufacturer's instructions. Primers for canine Dsg1, 3, involucrin, and murine c-Myc, Notch1, $p21^{WAF1}$, cyclin D1, keratin 1, Dsg1, 3, involucrin, loricrin, and cyclophillin were designed using the Primer Express program ABI Prism (AB Applied Biosystems, Foster City, California) (Table S1, primer sequences) and custom made (Microsynth, Balgach, Switzerland). A standard 18S ribosomal RNA control reagents kit (4308329, Applied Biosystems) was used to normalize canine mRNA. Mouse mRNA was

normalized with cyclophillin mRNA. Primer pairs were validated by titration to obtain a gradient of linearity between −3.2 and −3.6. Moreover, the melting temperature profile was assessed to ensure that only one product was amplified and to exclude primer–dimer formation. The assay was carried out in 25 μL containing Syber Green PCR Master Mix (4309155, Applied Biosystems) according to the manufacturer's protocol and was carried out using the ABI Prism 7700 Sequence Detector System (ABI/Perkin Elmer, Foster City, California). The final RNA quantification was carried out by the comparative C_T method and reported as the n-fold difference relative to the chosen calibrator (day 1 after seeding). Analyses were caried out in duplicate of two or three independent experiments.

Western blot analysis Cultures were washed with PBS + to discard floating cells and then directly scraped in RIPA lysis buffer (containing 1% Triton and 0.1% SDS) and lysates rotated for 1 h at 4°C, centrifuged for 10 min at 14,000 × *g* at 4°C. Six times sample buffer was added to the supernatant and boiled for 2 min prior to freezing. Protein concentration was measured and equal amounts (in average 25 μg) were separated on SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuel BioScience, Dassel, Germany) using a semi-dry electro-blotting apparatus (Biorad). The membrane was blocked with 5% milk and incubated with antibodies according to the manufacturer's protocol. The blots were developed with an enhanced chemiluminescence's kit (SuperSignal, 34080, Pierce, Rockford, Illinois).

Primary antibodies used for canine proteins were $p21^{WAF1}$ (sc-397, Santa Cruz Biotechnology, Santa Cruz, California), $p27^{KIP1}$ (610241, BD Biosciences, Franklin Lakes, New Jersey), Dsg1/2 (DG3.10, Progen, Heidelberg, Germany), Dsg3 (DesmoG3abr, RDI, Flanders, New Jersey), involucrin (0.1 F established in our laboratory; Suter *et al*, 1987), loricrin (PRB-145P, Babco, Richmond, California), Notch1 (sc-6014, Santa Cruz Biotechnology), tubulin (T9028, Sigma), and α-catenin (71-1200, Zymed Laboratories, South San Francisco, California). The antibodies that differed in the analysis of murine lysates were $p21^{WAF1}$ (556431, BD Biosciences), cyclin D1 (556470, BD Biosciences), Dsg3 (3114, a kind gift from J. R. Stanley, University of Pennsylvania, Philadelphia), involucrin (ERLI 3, a kind gift from F. Watt, Imperial Cancer Research Fund, London), and c-Myc (06-340; Upstate Biotechnology, Lake Placid, New York). Secondary antibodies were anti-mouse IgG (P0260, Dako, Glostrup, Denmark) and anti-rabbit IgG (7074, Cell Signalling Technology, Beverly, Massachusetts).

Immunfluoresence microscopy For immunfluoresence analysis mouse and dog cells were seeded in eight-well chamber slides (Lab-teks 177445, Nalge Nunc International, Naperville, Illinois). Surface-exposed E-cadherin and Dsg3 were assessed by exposure of non-permeabilized cells to antibodies for 1 h at 4°C prior to fixation/permeabilization with 100% methanol and incubation with β-catenin or plakoglobin as previously described (Caldelari *et al*, 2001). Antibodies used were E-cadherin (DECMA, a kind gift from R. Kemler, Max-Planck-Institute for Immunology, Freiburg-Zähringen, Germany), β-catenin (610153, BD Biosciences), Dsg3 (human pemphigus vulgaris IgG; Caldelari *et al*, 2001), Dsg1/2 (DG3.10, Progen), plakoglobin (610253, BD Biosciences), and c-Myc (551102, BD Biosciences) for canine cells. For murine cells c-Myc (06.340, Upstate Biotechnology) was used after 20 min fixation with 4% PFA, followed by 20 min permeabilization with 0.5% Triton. The same procedure was used to detect Notch1 (sc-6014, Santa Cruz Biotechnology) in murine and canine cells. Nuclei were counterstained for 20 s with Hoechst 33258 (B-2883, Sigma) diluted 1:3000.

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID23655/JID23655.htm
Table S1. Primer sequences used for real-time PCR

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Tacrolimus enhances TGF beta-related generation of Langerhans cells

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Correction to: Abstract 290, *J Invest Dermatol* 2006; **126** (Suppl 3): s51.

In Abstract 290 of *J Invest Dermatol* **126** (Suppl 3), there is an error in the author names and affiliations. The correct authors and affiliations for this abstract are: Bartlomiej Kwiek[1,2], Jean-Pierre Allam[2], Andrzej Langner[1], Thomas Bieber[2], Natalija Novak[2]

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Proliferation, Cell Cycle Exit, and Onset of Terminal Differentiation in Cultured Keratinocytes: Pre-Programmed Pathways in Control of C-Myc and Notch1 Prevail Over Extracellular Calcium Signals

Journal of Investigative Dermatology (2006) **126,** 2734. doi:10.1038/sj.jid.5700631

Correction to: *Journal of Investigative Dermatology* (2005) **124**, 1014–1025.

With respect to this article, the authors would like to draw attention to the use of c-Myc inhibitors presented in Figure 9a. In repeats of these experiments, the increase in terminal differentiation markers was not always seen to the same extent in canine cells, and in mouse cells it was either weak or absent. One of the reasons for the discrepant result with respect to the published data is that the blot with Dsg1 shown for mouse lysates erroneously was from canine lysates which show a greater difference in differentiation marker expression in response to c-Myc inhibition. The authors deeply regret this error. However, this does not explain the variable results for other proteins. The authors presume that the window in which the c-Myc inhibitors are effective in keratinocytes is narrow because endogenous c-Myc inhibition triggered by the terminal differentiation process overlaps with drug-induced inhibition as soon as cell contacts start to form.

Seasonal and Latitudinal Impact of Polymorphic Light Eruption on Quality of Life

Journal of Investigative Dermatology (2006) **126,** 2734. doi:10.1038/sj.jid.5700618

Correction to: *Journal of Investigative Dermatology* (2006) **126**, 1648–1651. doi:10.1038/sj.jid.5700306

In the article by Ling *et al.*, an error was introduced in the pagination stage of the publication. Although the online publication of this article is correct, the Supplementary Material listed in the printed version is incorrect, and should read as indicated below. The publisher regrets the error.

SUPPLEMENTARY MATERIAL

Table S1. Study participants.
Figure S1. Correlation between DLQI and visual analogue score for global assessment of life quality, for all locations.
Figure S2. Maximum ambient UVR levels at study centers during quality of life assessment weeks.
Supplementary Text. Materials and Methods, Supplementary References.
