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THE KERATINOCYTE GROWTH-DIFFERENTIATION SWITCH

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1. ABSTRACT

Growth/differentiation control of normal epithelial cells has been relatively understudied, because of the complexities involved in their cultivation and characterization. The present review is focused on progress in this area using the mouse primary keratinocyte system. This system reproduces under well defined culture conditions the switch between epithelial cell growth and differentiation which occurs *in vivo*, and enables the study of cells derived from wild type mice as well as mice with specific genetic alterations.

2. INTRODUCTION

The epidermis is characterized by a polarized pattern of epithelial growth and differentiation, with a single basal layer of proliferating keratinocytes and multiple overlying differentiated layers. Mouse primary keratinocytes cultured in medium at low calcium concentrations (0.05 mM; low calcium medium) exhibit many properties of basal epidermal cells, and proliferate indefinitely with no contact inhibition and continuous shedding into the medium (1). The shed keratinocytes express many if not all markers characteristic of the upper epidermal layers, and are irreversibly committed to differentiation (2, 3). In fact, these cells are still biochemically and transcriptionally active, but cannot be made to reattach and resume proliferation.

Addition of calcium to these cultures induces differentiation of the attached keratinocytes, triggering changes which more closely resemble those occurring *in vivo*, including establishment of close intercellular contacts (i.e. adherens junction and desmosome formation), stratification and corneification (1). By 24 hours of calcium treatment, >95% of attached keratinocytes become arrested in the G1 phase of the cell cycle (4). Induction of growth arrest is coupled with expression of biochemical markers of differentiation (keratin 1 and 10; involucrin; loricrin; filaggrin; epidermal transglutaminase). However, irreversible committment to differentiation occurs only at a later time, in that after 1 day of calcium exposure, still >50% of attached cells can resume proliferation if they are switched back to medium at low calcium concentrations (our unpublished observations).

Besides calcium, growth/differentiation of primary keratinocyte cultures can be controlled by pharmacological means. In particular, treatment with the phorbol ester TPA (12-0-tetradecanoylphorbol-13-acetate) induces growth arrest as well as expression of a set of differentiation markers such as involucrin, loricrin and filaggrin. However, expression of other markers induced by calcium, such as keratin 1 and 10, are not induced by TPA but are actually inhibited (5). TPA treatment also fails to induce any of the important changes in cell structure and adhesion which occur in response to calcium, but causes keratinocytes to "round up" and detach from the dish (6). As mentioned, keratinocytes which spontaneoulsy detach from the dish in cultures under low calcium conditions, exhibit many of the biochemical properties of terminally differentiated cells, even if none of the associated structural changes.

A similar biochemical program of differentiation is also induced by bringing keratinocytes artificially into suspension (7, 2).

Important questions that can be answered using this system are 1) which are the signal(s) that trigger the switch between keratinocyte growth and differentiation; 2) which are the intermediate biochemical pathways that are responsible for transduction of the differentiation signal; 3) which are the primary transcription and cell cycle regulatory events that are triggered by these signaling pathways.

3. SIGNALS WHICH CONTROL THE SWITCH BETWEEN KERATINOCYTE GROWTH AND DIFFERENTIATION

As mentioned, increased extracellular calcium elicits a rather complete differentiation program, inducing not only biochemical markers but also many of the structural changes which occur *in vivo*. An important question is whether extracellular calcium serves as a trigger for keratinocyte differentiation not only in culture, but also in the

intact skin. Consistent with this latter possibility is the fact that an increased gradient of calcium concentrations has been reported to exist *in vivo*, from the basal to upper epidermal layers. In particular, ion-capture cytochemistry (Oxalate-pyroantimonate technique) has indicated that extracellular calcium concentrations are significantly increased in the mid to upper granular layers (8), and a steady increase of calcium towards the corneified layer has been observed by particle probe methods (electron probe and proton probe X-ray microanalysis) (9).

Other calcium-responsive cells, such as parathyroid cells, express on their surface a low affinity transmembrane calcium receptor (10). Binding of calcium and/or other divalent or trivalent cations to this receptor triggers downtream signalling pathways which include phospholipase C activation and increase of intracellular calcium. Functional evidence indicates that a similar calcium-sensor mechanism may also be operative in keratinocytes (11), and an RT-PCR product with a sequence very similar if not identical to the parathyroid calcium receptor has been cloned from cultured keratinocytes (12). Thus, activation of a specific calcium-receptor may serve as a trigger for keratinocyte differentiation both *in vitro* and *in vivo*, with expression of this receptor - and sensitivity to calcium - possibly increasing as cells become committed to differentiate.

As an alternative to these possibilities, calcium may trigger keratinocyte differentiation through more indirect mechanisms, such as by promoting close cell cell contacts. As already mentioned, one of the earliest and most important changes associated with calcium-induced keratinocyte differentiation is establishment of close intercellular contacts, through adherens junction and desmosome formation (13,14). These contacts exert a deep influence on the organization of the submembranous cytoskeleton. An attractive but as yet little explored possibility is that, both *in vitro* and *in vivo*, increased cell cell adhesion serves as a primary trigger leading to keratinocyte growth arrest and differentiation. In this case, the differentiation-inducing capability of calcium would be limited to *in vitro* conditions where calcium concentrations are artificially modulated, while, *in vivo*, calcium may not be limiting, and other factors controlling cell adhesion could be involved.

Besides promoting cell adhesion, Increased extracellular calcium induces stratification, i.e. sliding of cells over each other with concomitant loss of adhesion to the substrate (6). This could be a third mechanism by which calcium induces differentiation, consistent with the fact that spontaneous or artificially-induced detachment of keratinocytes from the matrix is also associated with the induction of biochemical markers of differentiation, as well as growth arrest (2,3,7). *In vivo*, migration of keratinocytes from the basal to the immediately adjacent spinous layer could also serve as an important trigger for later differentiation events. Keratinocyte attachment to the underlying extracellular matrix is mediated, at least in part, by integrin-based focal adhesions. Besides attachment, these structures mediate cell spreading and migration and may contribute significantly to control of the switch between keratinocyte growth and differentiation (15).

In conclusion, the well ordered program of keratinocyte differentiation is probably controlled by several signals, which may function in both a parallel and complementary fashion. An extracellular calcium sensing mechanism, increase in cell-cell adhesion and detachment from the basement membrane could all be involved.

4. INTERMEDIATE SIGNALLING PATHWAYS WHICH ARE RESPONSIBLE FOR TRANSDUCTION OFTHE DIFFERENTIATION SIGNAL

The several, relatively independent signals which are involved in control of keratinocyte differentiation, may trigger common signaling pathways or, alternatively, may activate some specific pathways, which converge further downstream. Biochemical evidence suggests that both situations apply. In particular, the immediate/early signalling pathways triggered by calcium and TPA have been characterized in detail, they overlap only partially, and have a substantially different time course of activation.

As discussed, increased extracellular calcium acts at the keratinocyte cell surface, favoring processes such as cell-cell and cell-substrate interactions, and/or triggering a direct calcium-receptor mechanism. Intracellularly, phospholipase C (PLC) activation has been reported to occur within 30 seconds to 1 minute of calcium treatment, through an as yet undefined mechanism (16). Phosphatidyl inositol breakdown results in inositol 3 phosphate production, which in turn may trigger the very early increase of intracellular calcium concentrations (17), likely by mobilization from intracellular stores. A direct influx of extracellular calcium through voltage--independent channels has also been reported (18), but it may occur as a relatively late event, which could account for the second and sustained increase in intracellular calcium levels. Increased intracellular calcium could in turn activate a number of calcium-dependent pathways, such calcium/caldmodulin-dependent kinases and phosphatases (19). Surprisingly little is know about activation of these pathways in calcium-induced keratinocyte differentiation. Ongoing work in our laboratory suggests that the calcium-dependent phosphatase calcineurin (PP2B) may be involved. Pyk2, a novel tyrosine kinase of the Fak family directly activated by calcium has been recently reported to be induced in neuronal differentiation (20). However, no such activation has been found to occur in differentiating keratinocytes (our unpublished observations).

The other product of phosphatidyl inositol breakdown, diacyl glycerol (DAG), is also increased at very early times of calcium treatment (16). DAG is known to function as a direct activator of protein kinase C, and this family of kinases is strongly activated by TPA, the other potent - if only partial - inducer of keratinocyte differentiation. Several isoforms of PKC exist, which differ substantially in their requirements for activation, exhibit separate subcellular distributions, and may phosphorylate rather different substrates (21). Involvement of the PKC-alpha isoform in calcium-induced differentiation is indicated by the fact that treatment of primary mouse keratinocytes with PKC-alpha antisense oligonucleotides abrogates induction of late differentiation markers such as loricrin, filaggrin and SPR-1 (22). Induction of these same markers is also reduced in keratinocytes where the PKC-alpha, -delta and -epsilon isoforms are pharmacologically downmodulated (23). These three isoforms also translocate to the cell particulate fraction upon calcium induced differentiation (24). A role for the PKC-eta isoform is suggested by the observation that cholesterol sulfate, a potent but non-specific activator of this isoform, functions

as a strong inducer of late differentiation marker expression (25). Cholesterol sulfate itself accumulates in the granular layer of the epidermis, suggesting that even *in vivo* this molecule may serve as an important inducer of keratinocyte differentiation through modulation of PKC function (26).

Besides playing a primary role in keratinocyte differentiation, the various PKC isoforms are themselves modulated during the differentiation process, with total levels of PKC-alpha being decreased and those of PKC-epsilon and PKC-eta increased in calcium-treated keratinocytes (25). The mechanism(s) responsible for this differential regulation of PKC isoforms remain to be established.

Thus, intracellular calcium-, PLC- and protein kinase C-dependent pathways seem to play an important role in control of keratinocyte differentiation. However, the specific contributions of any of these pathways remain to be elucidated.

5. TYROSINE PHOSPHORYLATION: KEY TO REGULATION OF KERATINOCYTE DIFFERENTIATION

As has been shown in other unrelated differentiation and systems (27), induction of tyrosine phosphorylation occurs as an early and specific event in keratinocyte differentiation in response to both calcium and TPA (28). Specific tyrosine kinase inhibitors (Genistein, Herbimycin A) were shown to block the response to both agents (28), indicating that induction of tyrosine phosphorylation is not merely coincidental with the differentiation process, but plays a necessary regulatory function. Tyrosine phosphorylation of a single ~60 kD protein was found to occur within 5' of calcium treatment, while tyrosine phosphorylation of a second protein of ~80-85 kD was induced by both calcium and TPA. The p60 protein was identified as "p62", a tyrosine phosphorylated protein which associates the Ras-GTPase Activating Protein (29) and may be similar, but probably not identical (30), to an adaptor protein found in association with *ras*-GAP in other types of cells (31,32). In keratinocytes, p62 tyrosine phosphorylation is induced specifically in response to increased extracellular calcium, and not to increased intracellular calcium levels (by calcium ionophores) nor TPA exposure, nor treatment with a mitogen such as EGF or a growth inhibitory agent such as TGF-ß (11,29). Unlike p62, *ras*-GAP itself does not become tyrosine phosphorylated, but translocates to the membrane as early as 10' after exposure to calcium (but not TPA) (29). In this context, endogenous *ras* proteins do not appear to be significantly activated, suggesting that the p62/ras-GAP pathway may fulfill some unrelated function in these cells.

In parallel with these changes, at least two distinct tyrosine kinase activities were found to be induced at the onset of keratinocyte differentiation (6). One of these activities, immunoprecipitated by anti-phosphotyrosine antibodies, is specifically increased by calcium and a number of other divalent cations from the outside of the cell, suggesting that an extracellular cation-sensor mechanism is involved. Induction of this kinase activity occurs within minutes of calcium exposure (6), and correlates with the rapid tyrosine phosphorylation of p62 (11,29). This activity was not identified with any of the known tyrosine kinases that were examined. Syk, a novel epithelial-specific tyrosine kinase, is also activated at very early times of calcium-induced keratinocyte differentiation, but does not appear to be the kinase directly responsible for p62 phosphorylation (33).

A second tyrosine kinase activity, induced in response to both calcium and TPA, was identified as Fyn, a tyrosine kinase of the Src family (6). Fyn activation occurs a few hours after calcium-induced differentiation, but already within 5 minutes of TPA treatment. None of the other Src-family members appears to be activated after either calcium or TPA treatment, nor is Fak, a non-integral membrane tyrosine kinase closely associated with Src-kinases at sites of focal adhesion (our unpublished observations). The functional significance of Fyn kinase activation was investigated by examining the growth/differentiation behavior of keratinocytes derived from mice with a homozygous disruption of the *fyn* gene (6). Cultured keratinocytes from *fyn*-, *yes*- or *src*-mutant mice reach confluence at approximately the same time as wild type controls, indicating that the absence of any of these kinases has no severe effects on keratinocyte growth under basal culture conditions. However, after calcium treatment, *fyn*- but not *src*- or *yes*-negative keratinocytes show defective stratification, and assume a much larger size than their wild type counterparts. Accentuated morphological differences were also found after TPA exposure; unlike wild type cells, the mutant keratinocytes did not assume the typical spindle morphology induced by TPA, and failed to detach from the dish after 24 hours treatment (6). Biochemically, the *fyn* deficient keratinocytes expressed specific differentiation markers (filaggrin, loricrin) to a much lesser extent than control or *yes* knockout keratinocytes, in response to either calcium or TPA.

Cortactin, an 80-85kD substrate of Src and Fyn kinases which localizes with actin at cell adhesion sites (34), was found to be increasingly tyrosine phosphorylated after exposure to both calcium and TPA, with a time course similar to that of Fyn activation (6). Interestingly, tyrosine phosphorylation of the 80-85 kD proteins is strongly decreased in keratinocytes derived from *fyn* but not *yes* or *src* knockouts, indicating that Fyn may be the kinase which is mainly responsible for phosphorylation of this group of proteins.

An important question raised by these studies is what is the physiological function(s) of tyrosine kinase activation - and of Fyn in particular - in keratinocyte differentiation, and what are the specific downstream signaling pathways. Also, the skin of *fyn* knockout mice shows decreased expression of differentiation markers at birth, but looks otherwise normal (6). Thus, additional mechanisms must operate *in vivo* , which compensate for lack of Fyn function.

6. TRANSCRIPTION AND CELL CYCLE REGULATORY EVENTS CONNECTED WITH THE ONSET OF KERATINOCYTE DIFFERENTIATION

As discussed above, in keratinocyte differentiation at least two distinct signaling pathways exist. One is

specifically triggered by increased extracellular calcium (such as p62 tyrosine phosphorylation), the other is activated in response to both calcium and TPA (associated with Fyn kinase activation). An important question is how these pathways are linked to transcription and cell cycle regulation, and whether, at the transcriptional level, the response to calcium and TPA remains partially distinct.

Expression of the cyclin dependent kinase (CDK) inhibitor p21WAF1/Cip1, and the related p27Kip1 molecule, increases within 4 hours of calcium-induced differentiation (4, 35). The induction of these CDK inhibitors is the earliest detectable modification in cell cycle regulatory molecules, and could account, at least in part, for calcium-induced growth arrest (50% inhibition of DNA synthesis by 8-12 hours of calcium treatment) (4). Like p62 tyrosine phosphorylation, p21 expression increases specifically in response to extracellular calcium and is not induced by artificially raised intracellular calcium levels, nor by TPA treatment (35, 36). Induction of p21 can be explained by transcriptional activation of the p21 promoter, which is unlinked from p53 but dependent on p300, a transcriptional coactivator which is decreasingly phosphorylated by 6-12 hours of calcium treatment (35).

A number of signal transduction pathways may converge to induce p21 expression by activation of its promoter. In particular, tyrosine phosphorylation is a critical step in the activation of the STAT family of transcription factors, which regulate inducibility of the p21 promoter in response to EGF or IFN-gamma (37). p21 promoter activity studies revealed that the minimal region induced in keratinocyte differentiation consists of a stretch of 78bp close to and including the TATA box (36). This minimal sequence does not contain any STAT recognition sites, ruling out direct involvement of STATs in this context. Similarly, this sequence does not contain any E box binding sites, consistent with functional studies which indicated that MyoD family factors, which are implicated in p21 control during myoblasts differentiation (38), do not play a similar role in differentiating keratinocytes. Similar considerations apply to another important group of E box binding transcription factors, the myc family, which have been implicated in other aspects of keratinocyte differentiation (39,40 and refs. therein).

The minimal differentiation-responsive region of the p21 promoter is characterized by a series of GC rich repeats. This GC region alone, although essential, is not sufficient for promoter activation, and continuity with the adjacent TATA box is required (36). The GC rich region is concomitantly bound by the transcription factors Sp1 and Sp3. A few nucleotides insertion in the GC rich region dramatically decreases transcription factor binding as well as promoter activity and inducibility upon differentiation. The overexpression of either Sp1 or Sp3 restores the basal activity of the mutated promoter, but only Sp3 can restore its inducibility, implicating this latter factor as an important determinant of p21 control in differentiation (36). In other systems, Sp1 functions as a transcriptional activator, whereas Sp3 can either activate or repress promoter activity, depending on the individual promoter and cell line (41, 42). The activation or repression of different promoters by Sp3 may therefore depend on subtle differences between GC boxes and an interplay with other transcription factors, possibly cell type specific. Expression level and DNA binding activity of Sp3 (and Sp1) do not appear to change in keratinocyte differentiation. Other important regulatory factors which could bind the minimal region of the p21 promoter and with which Sp3 may functionally interact include E2F family members (43). Intriguingly, expression of a specific member of this family, E2F 5, has been found to be significantly increased in differentiating keratinocytes of the upper epidermal layers (44). The possibility that modulation of GC-binding factors extends beyond p21 control in keratinocyte differentiation is supported by the fact that a functionally important GC-rich region similar to that present in the p21 promoter has been found in the promoters of genes for the keratinocyte differentiation markers SPRP and transglutaminase (45, 46). Regulation of these promoters by the concomitant action of Sp1 and Ets-related factors has been reported (45,46), and an epithelial-specific Ets factor has been identified which may be involved in control of these promoters (47, 48).

The p21 promoter can be induced in the HaCaT human keratinocyte cell line by TGF-ß treatment (49), through the same Sp1/Sp3 binding site required for induction in mouse keratinocyte differentiation. In contrast to HaCaT cells, no induction of the p21 promoter occurs after TGF-ß treatment of mouse primary keratinocytes (36). In spite of these differences, which may be due to the use of cells of human versus mouse origin, and/or primary cells versus an established cell line, the possibility that TGF-ß and differentiation signaling pathways converge at the point of p21 promoter induction is an exciting one, which will deserve further attention.

Regulation of keratinocyte differentiation marker genes is another area of active investigation. These genes are controlled to a significant extent at the transcriptional level, and a number of transcription factors have been implicated in their regulation (50). Of particular importance appear to be AP-1 family factors. A common feature of the promoter region of most if not all differentiation-related genes is the presence of AP-1 binding sites, and modulation of AP-1 activity has been found to occur in keratinocytes in response to both calcium and TPA. AP-1 factors consist of homo- or hetero-dimers of jun (c-jun, junB, junD) and fos ((Fra-1, Fra-2, c-fos, fosB) family members, and, depending on the dimer composition, these factors can function as either activators or suppressors of transcription (51). The AP-1 binding sites of different promoters are not identical and may bind different AP-1 homo- and hetero-dimers, which in turn could account for their different regulation. A number of markers, such loricrin, filaggrin and involucrin, are induced by both calcium and TPA, while others, such as keratins 1 and 10, are induced by calcium but suppressed by TPA. This differential regulation of keratinocyte marker expression is paralled, and could be at least in part explained, by differential regulation of individual AP-1 components (52).

Besides changes in subunit composition, modulation of AP-1 activity can be achieved by phosphorylation or dephosphorylation of individual jun or fos family members at either activating or inactivating sites (53). Very little information is available about possible

changes in the phosphorylation state of individual AP-1 family members which may be associated with the keratinocyte differentiation process. This would seem particularly relevant, since these factors are well known substrates of kinases of the MAPK family (53), and activation of these kinases is a likely outcome of early signalling events triggered with keratinocyte differentiation. In particular, we have found that treatment of keratinocytes with TPA induces JNK and ERK1/2 kinase activitities. JNK is known to phosphorylate jun and directly modulate its transcriptional activity. ERK1/2 is likely to modify AP-1 activity more indirectly, by enhancing SRF-dependent transcription and thus increasing levels of fos expression (53) In contrast to TPA, increased extracellular calcium leads to no JNK activation and very little if any ERK1/2 activation (our unpublished observations). These findings are consistent with an early observation that no induction of c-fos expression occurs after treatment of keratinocytes with calcium, while it is readily observed with TPA (54). Thus, the signaling pathways triggered by calcium and TPA remain distinct even at the level of transcriptional regulation.

Transcription factors may not only be involved in positive control of differentiation, but also function as negative regulators. An interesting case is that of Whn, a keratinocyte-specific transcription factor of the forkhead family, the mutation of which is responsible for the nude mouse phenotype (55, 56). *Whn* was found to code for a repressor of genes related to keratinocyte differentiation, such as involucrin and transglutaminase (57). Rather than binding directly to the promoter of these genes, Whn is likely to exert more indirect effects, by interfering with the pathway(s) which lead to differentiation. Interestingly, the repressive function of *Whn* appears to be specific for the differentiation-related genes, as it had no effect on induction of p21. In parallel with this specificity, Whn-deficient keratinocytes (i.e. derived from nude mice) exibit a markedly increased sensitivity to the differentiating effects of TPA while their sensitivity to calcium appears normal (57). Identification of the genes directly regulated by *Whn* will be required for an elucidation of the mechanism(s) underlying this specificity of effects.

Another and quite surprising example of negative modulator of keratinocyte differentiation is that of p21 itself. As extensively discussed before, this gene is induced very early in differentiation and may be involved in cell cycle arrest which accompany this process (4, 35). However, a more complex role of p21 in differentiation is suggested by the fact that, in epithelial and nervous tissues, expression of this CKI is induced in postmitotic cells immediately adjacent to the proliferative compartment, but is decreased in cells further along the differentiation pathway (58-61). This suggests that p21 could participate in a negative feedback mechanism which ensures that epithelial cell differentiation proceeds in a sequential and well ordered manner. In fact, in parallel with the *in vivo* expression data, the p21 protein is specifically downmodulated in terminally differentiated primary keratinocytes, and this occurs by a proteasome-dependent pathway (3). Forced p21 expression in these cells, via recombinant adenovirus infection, inhibits terminal differentiation marker expression at both protein and mRNA levels (3). These inhibitory effects on differentiation are specific to intact p21, as they are not observed when overexpressing various p21 mutants, or the unrelated CKI p16INK4a, although all these molecules exert similar growth inhibition. Taken together, these findings reveal a so far unexpected mode of p21 regulation and function in differentiation, with this CKI playing an inhibitory role at late stages of this process, which is not simply amenable to cell cycle arrest. The intriguing possibility exists that p21 functions as a bridge between signaling complexes such as cyclin/CDKs and other multiprotein apparatuses, such as the transcription machinery involved in differentiation.

7. REFERENCES

1. Hennings, H., D. Michael, C. Cheng, P. Steinert, K. Holbrook and S. H. Yuspa: Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* 19, 245-254 (1980)

2. Li, L., T. Tennenbaum and S. H. Yuspa: Suspension-induced murine keratinocyte differentiation is mediated by calcium. *J Invest Dermatol* 106, 254-260 (1996)

3. Di Cunto F., G. Topley, E. Calautti, J. Hsiao, L. Ong, P.K. Seth, and G.P. Dotto: Inhibitory function of $p21^{Cip1/WAF1}$ in differentiation of primary mouse keratinocytes independent of cell cycle control. *Science* in press (1998)

4. Missero, C., F. Di Cunto, H. Kiyokawa, A. Koff and G. P. Dotto: The absence of p21Cip1/WAF1 alters keratinocyte growth and differentiation and promotes ras-tumor progression. *Genes Dev* 10, 3065-3075 (1996)

5. Dlugosz, A. A. and S. H. Yuspa: Coordinate changes in gene expression which mark the spinous to granular cell transition in epidermis are regulated by protein kinase. *J Cell Biol* 120, 217-225 (1993)

6. Calautti, E., C. Missero, P. L. Stein, R. M. Ezzell and G. P. Dotto: fyn tyrosine kinase is involved in keratinocyte differentiation control. *Genes Dev* 9, 2279-91 (1995)

7. Watt, F. M., P. W. Jordan and C. H. O'Neill: Cell shape controls terminal differentiation of human epidermal keratinocytes. *Proc Natl Acad Sci USA* 85, 5576-5580 (1988)

8. Menon, G. K., P. M. Elias, S. H. Lee and K. R. Feingold: Localization of calcium in murine epidermis following disruption and repair of the permeability barrier. *Cell Tissue Res* 270, 503-512 (1992)

9. Forslind, B., M. Lindberg, G. M. Roomans, J. Pallon and Y. Werner-Linde: Aspects on the physiology of human skin: studies using particle probe analysis. *Microsc Res Tech* 38, 373-386 (1997)

10. Hebert, S. C. and E. M. Brown: The extracellular calcium receptor. *Curr Opin Cell Biol* 7, 484-492 (1995)

11. Filvaroff, E., E. Calautti, M. Reiss and G. P. Dotto: Functional evidence for an extracellular calcium receptor

mechanism triggering tyrosine kinase activation associated with mouse keratinocyte differentiation. *J Biol Chem* 269, 21735-40 (1994)

12. Bikie, D., A. Ratnam, T. Mauro, J. Harris and S. Pillai: Changes in calcium responsiveness and handling during keratinocyte differentiation. *J. Clin. Invest.* 97, 1085-1093 (1996)

13. O'Keefe, E. J., R. A. Briggaman and B. Herman: Calcium-induced assembly of adherens junctions in keratinocytes. *J Cell Biol* 105, 807-17 (1987)

14. Lewis, J. E., P. J. Jensen and M. J. Wheelock: Cadherin function is required for human keratinocytes to assemble desmosomes and stratify in response to calcium. *J Invest Dermatol* 102, 870-7 (1994)

15. Giancotti, F. G.: Integrin signaling: specificity and control of cell survival and cell cycle progression. *Current Opinion in Cell Biology* 9, 691-700 (1997)

16. Jaken, S. and S. H. Yuspa: Early signals for keratinocyte differentiation : role of Ca2+-mediated inositol lipid metabolism in normal and neoplastic epidermal cells. *Carcinogenesis* 9, 1033-1038 (1988)

17. Kruszewski, F. H., H. Hennings, S. H. Yuspa and R. W. Tucker: Regulation of intracellular free calcium in normal murine keratinocytes. *Am J Physiol* 261, C767-773 (1991)

18. Reiss, M., L. R. Lipsey and Z. L. Zhou: Extracellular calcium-dependent regulation of transmembrane calcium fluxes in murine keratinocytes. *J Cell Physiol* 147, 281-291 (1991)

19. Yokokura H., O. Terada, Y. Naito, R. Sugita and H. Hidaka : Cascade activation of the calmodulin kinase family. *Adv Second Messenger Phosphoprotein Res* 31:151-157 (1997)

20. Lev, S., H. Moreno, R. Martinez, P. Canoll, E. Peles, J. M. Musacchio, G. D. Plowman, B. Rudy and J. Schlessinger: Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions. *Nature* 376, 737-745 (1995)

21. Ohno, S., Y. Akita, A. Hata, S. Osada, K. Kubo, Y. Konno, K. Akimoto, K. Mizuno, T. Saido and T. Kuroki: Structural and functional diversities of a family of signal transducing protein kinases, protein kinase C family; two distinct classes of PKC, conventional cPKC and novel nPKC. *Adv Enzyme Regul* 31, 287-30 (1991)

22. Lee, Y. S., A. A. Dlugosz, R. McKay, N. M. Dean and S. H. Yuspa: Definition by specific antisense oligonucleotides of a role for protein kinase C alpha in expression of differentiation markers in normal and neoplastic mouse epidermal keratinocytes. *Mol Carcinog* 18, 44-53 (1997)

23. Stanwell, C., M. F. Denning, S. E. Rutberg, C. Cheng, S. H. Yuspa and A. A. Dlugosz: Staurosporine induces a sequential program of mouse keratinocyte terminal differentiation through activation of PKC isozymes. *J Invest Dermatol* 106, 482-489 (1996)

24. Denning, M. F., A. A. Dlugosz, E. K. Williams, Z. Szallasi, P. M. Blumberg and S. H. Yuspa: Specific protein kinase C isozymes mediate the induction of keratinocyte differentiation markers by calcium. *Cell Growth Differ* 6, 149-157 (1995)

25. Denning, M. F., M. G. Kazanietz, P. M. Blumberg and S. H. Yuspa: Cholesterol sulfate activates multiple protein kinase C isoenzymes and induces granular cell differentiation in cultured murine keratinocytes. *Cell Growth Differ* 6, 1619-1626 (1995)

26. Osada, S., Y. Hashimoto, S. Nomura, Y. Kohno, O. Chida, O. Tajima, K. Kubo, K. Akimoto, H. Koizumi, Y. Kitamura, K. Suzuki, S. Ohno and T. Kuroki: Predominant Expression of nPKC, a Ca2+-independent isoform of protein kinase C in Ephitelial tissues, in association with epithelial differentiation. *Cell Growth & Differentiation* 4, 167-175. (1993)

27. Pazin, M. J. and L. T. Williams: Triggering signaling cascades by receptor tyrosine kinases. *Trends in Biochem Sci* 374-378. (1992)

28. Filvaroff, E., D. F. Stern and G. P. Dotto: Tyrosine phosphorylation is an early and specific event involved in primary keratinocyte differentiation. *Mol Cell Biol* 10, 1164-1173 (1990)

29. Filvaroff, E., E. Calautti, F. Mc Cormick and G. P. Dotto: Specific changes of Ras GTPase Activating Protein (GAP) and a GAP-associated p62 protein during calcium-induced keratinocyte differentiation. *Mol Cell Biol* 12, 5319-5328 (1992)

30. Medema, J. P., A. M. de Vries-Smits and J. L. Bos: Calcium induces tyrosine phosphorylation of a novel p120 GAP-associated protein of 65 kDa. *Oncogene* 11, 757-762 (1995)

31. Carpino, N., D. Wisniewski, A. Strife, D. Marshak, R. Kobayashi, B. Stillman and B. Clarkson: p62(dok): a constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells. *Cell* 88, 197-204 (1997)

32. Yamanashi, Y. and D. Baltimore: Identification of the Abl- and rasGAP-associated 62 kDa protein as a docking protein, Dok. *Cell* 88, 205-11 (1997)

33. Vasioukhin, V. and A. L. Tyner: A role for the epithelial-cell-specific tyrosine kinase Sik during keratinocyte differentiation. *Proc Natl Acad Sci USA* 94, 14477-14482 (1997)

34. Wu, H. and J. Parsons: Cortactin, an 80/85 Kilodalton pp60src substrate, is a filamentous actin-binding protein enriched in the cell cortex. *J Cell Biol* 120, 1417-1426 (1993)

35. Missero, C., E. Calautti, R. Eckner, J. Chin, L. Tsai, D. M. Livingston and G. P. Dotto: Involvement of the cell-cycle inhibitor Cip1/WAF1 and the E1A- associated p300 protein in terminal differentiation. *Proc Natl Acad Sci USA* 92, 5451-5455 (1995)

36. Prowse, D., L. Bolgan, A. Molnár and G. P. Dotto: Involvement of the Sp3 transcription factor in induction of p21Cip/WAF1 in keratinocyte differentiaion. *J Biol Chem* 272, 1308-1314 (1997)

37. Look, D. C., M. R. Pelletier, R. M. Tidwell, W. T. Roswit and M. J. Holtzman: Stat1 depends on transcriptional synergy with Sp1. *J Biol Chem* 270, 30264-30267 (1995)

38. Halevy, O., B. G. Novitch, D. B. Spicer, S. X. Skapek, J. Rhee, G. J. Hannon, D. Beach and A. B. Lassar: Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* 267, 1018-1021 (1995)

39. Vastrik, I., A. Kaipainen, T. L. Penttila, A. Lymboussakis, R. Alitalo, M. Parvinen and K. Alitalo: Expression of the mad gene during cell differentiation in vivo and its inhibition of cell growth in vitro. *J Cell Biol* 128, 1197-1208 (1995)

40. Hurlin, P. J., K. P. Foley, D. E. Ayer, R. N. Eisenman, D. Hanahan and J. M. Arbeit: Regulation of Myc and Mad during epidermal differentiation and HPV-associated tumorigenesis. *Oncogene* 11, 2487-2501 (1995)

41. Hagen, G., S. Muller, M. Beato and G. Suske: Sp1-mediated transcriptional activation is repressed by Sp3. *Embo* 13, 3843-3851 (1994)

42. Udvadia, A. J., D. J. Templeton and J. M. Horowitz: Functional interactions between the retinoblastoma (Rb) protein and Sp- family members: superactivation by Rb requires amino acids necessary for growth suppression. *Proc Natl Acad Sci USA* 92, 3953-3957 (1995)

43. Hiyama, H., Iavarone, A., LaBaer, J. and S.A. Reeves: Regulated ectopic expression of cyclin D1 induces transcriptional activation of the cdk inhibitor p21 gene without altering cell cycle progression. *Oncogene* 14, 2533-2542 (1997)

44. Dagnino, L., C. J. Fry, S. M. Bartley, P. Farnham, B. L. Gallie and R. A. Phillips: Expression patterns of the E2F family of transcription factors during murine epithelial development. *Cell Growth Differ* 8, 553-563 (1997)

45. Fischer, D. F., S. Gibbs, P. Van De Putte and C. Backendorf: Interdependent transcription control elements regulate the expression of the SPRR2A gene during keratinocyte terminal differentiation. *Mol Cell Biol* 16, 5365-5374 (1996)

46. Lee, J. H., S. I. Jang, J. M. Yang, N. G. Markova and P. M. Steinert: The proximal promoter of the human transglutaminase 3 gene. Stratified squamous epithelial-specific expression in cultured cells is mediated by binding of Sp1 and ets transcription factors to a proximal promoter element. *J Biol Chem* 271, 4561-4568 (1996)

47. Andreoli, J. M., S. Jang, E. Chung, C. M. Coticchia, P. M. Steinert and N. G. Markova: The expression of a novel, epithelium-specific ets transcription factor is restricted to the most differentiated layers in the epidermis. *Nucleic Acids Res* 25, 4287-4295 (1997)

48. Oettgen, P., R. M. Alani, M. A. Barcinski, L. Brown, Y. Akbarali, J. Boltax, C. Kunsch, K. Munger and T. A. Libermann: Isolation and characterization of a novel epithelium-specific transcription factor, ESE-1, a member of the ets family. *Mol Cell Biol* 17, 4419-4433 (1997)

49. Datto, M. B., Y. Yu and X. F. Wang: Functional analysis of the transforming growth factor beta responsive elements in the WAF1/Cip1/p21 promoter. *J Biol Chem* 270, 28623-28628 (1995)

50. Eckert, R., J. Crish, E. Banks and J. Welter: The epidermis: genes on-genes off. *J Invest Derm* 109, 501-509 (1997)

51. Whitmarsh, A. J. and R. J. Davis: transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol. Med* 74, 589-607 (1996)

52. Rutberg, S., E. Saez, S. Lo, S. I. Jang, N. Markova, B. Spiegelman and S. H. Yuspa: Opposing activities of c-Fos and Fra-2 on AP-1 regulated transcriptional activity in mouse keratinocytes induced todifferentiate by calcium and phorbol esters. *Oncogene* 15, 1337-1346 (1997)

53. Treisman, R.: regulation of transcription by MAP kinase cascades. *Current Opinion Cell Biol.* 8, 205-215 (1996)

54. Dotto, G. P., M. Z. Gilman, M. Maruyama and R. A. Weinberg: c-myc and c-fos expression in differentiating mouse primary keratinocytes. *EMBO* 2853-2857 (1986)

55. Nehls, M., D. Pfeifer, M. Schorpp, H. Hedrich and T. Boehm: New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* 103-107 (1994)

56. Segre, J., J. Nemhauser, B. Taylor, A. Nadeau and E. Lander: Positional cloning of the nude Locus: genetic, physical, and transcription maps of the region and mutations in the mouse and rat. *Genomics* 28, 549-559 (1995)

57. Brissette, J., J. Li, J. Kamimura, D. Lee and G. P. Dotto: The product of the mouse *nude* locus, *Whn*, regulates the balance between epithelial cell growth and differentiation. *Genes & Development* 10, 2212-2221 (1996)

58. Deiry, W. S., T. Tokino, T. Waldman, J. D. Oliner, V. E. Velculescu, M. Burrell, D. E. Hill, E. Healy, J. L. Rees, S. R. Hamilton, W. K. Kenneth and B. Vogelstin: Topological control of p21WAF1/CIP1 expression in normal and neoplastic tissues. *Cancer Res* 55, 2910-2919 (1995)

59. Gartel, A. L., M. S. Serfas, M. Gartel, E. Goufman, G. S. Wu, W. S. El-Deiry and A. L. Tyner: p21 (WAF1/CIP1) expression is induced in newly nondividing cells in diverse epithelia and during differentiation of the Caco-2 intestinal cell line. *Exp Cell Res* 227, 171-181. (1996)

60. Ponten, F., B. Berne, Z. P. Ren, M. Nister and J. Ponten: Ultraviolet light induces expression of p53 and p21 in human skin: effect of sunscreen

61. Delalle, I. and V. S. Caviness: Temporal and spatial expression of p21Cip1/WAF1 in the developing murine neocortical cerebral wall. *Soc Neuroscience Abstracts* 22, 254 (1996)

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