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Mechanism of Reversion of Calcium-Induced Differentiation in Keratinocytes

A Dissertation Presented

by

Azadeh Jadali

to

The Graduate School

in Partial Fulfillment of the

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in

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(Immunology and Pathology)

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Abstract of the Dissertation

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2010

Terminal differentiation defines cells that permanently exit the cell cycle in the process of specialization. Under some circumstances, however, this process is reversed as demonstrated by the ability of differentiated keratinocytes to form hair bearing skin epithelia when transplanted onto a suitable environment. To gain insights into the mechanism of reversion of keratinocyte differentiation, a well characterized culture of mouse keratinocytes, in which calcium (Ca^{2+}) concentrations above 0.1 mM induce differentiation was used. A low Ca^{2+} switch in differentiated cultures triggered culture-wide morphological and biochemical changes indicated by re-initiation of proliferation, de-repression of cyclinD1, and acquisition of basal cell- like characteristics. Retroviral labeling of differentiated cultures ruled against the possibility of expansion of a small population of Ca^{2+}-resistant keratinocytes following the low Ca^{2+} switch.

The use of selective inhibitors of signaling pathways, suggested a requirement for protein kinase D (PKD) and mitogen activated protein kinases (MAPK). Inhibition of keratinocyte reversion by Goedecke 6979, an inhibitor of PKD and protein kinase C (PKC)-α, but not with

GF10923X, a general inhibitor of PKCs, suggested PKC-independent PKD activation. PKD phosphorylation/ activation followed complex kinetics with a biphasic transient phosphorylation within the first 6 hrs followed by a sustained and progressive phosphorylation beginning at 24 hrs after the low Ca^{2+} switch. Despite expression of all three PKD isoforms in keratinocytes, specific knockdown of PKD1 by RNA interference or over-expression of dominant negative form of PKD1 blocked re-initiation of proliferation and morphological reversion in differentiated cultures, indicating an essential and non-redundant role for PKD1 in this process. Furthermore, analysis of ERK1/2 activity in reverted cultures indicated a biphasic pattern of ERK1/2 phosphorylation consistent with that of PKD. Suppression of ERK1/2 phosphorylation in keratinocytes over-expressing a dominant negative form of PKD directly correlated with the inhibition of DNA synthesis and supported a role for PKD-mediated ERK activation.

These data demonstrate an essential and non-redundant role for PKD1 in reversion of differentiation in keratinocytes, partly through sustained activation of the ERK-MAPK pathway. These findings imply a critical role for PKD in conditions such as wound healing and tumor formation where the normal differentiation process may be reversed.

I dedicate my dissertation to my family for all their help, support, and love throughout the years. My mom, in particular, was instrumental in me pursuing a higher education. From a young age she instilled in me the value of hard work and perseverance. Her encouragement and support helped me realize that I can do anything I dream of.

TABLE OF CONTENTS

LIST OF FIGURES …………………………………………………………........................x

LIST OF TABLES……………………………………………………………………..xiii

ABBREVIATIONS ………………………………………………………………………xiv

CHAPTER I. INTRODUCTION.……………………………………………………………1

I.1. Epidermis Structure and Function.……………………………………… …………....1

I.2. Regulation of Epidermal Proliferation and Differentiation ……………………………...5

I.3. De-differentiation.…………………………………………………………………10

I.4. Calcium-Induced Differentiation- A Model to Study to KC Growth and Differentiation in Culture……………………………………………………………………13

I.5. Protein Kinase C and Regulation of KC Differentiation………………………………..16

I.6. Protein Kinase D Signaling……………………………………………..................19

I.7. ERK-MAPK Pathway Signaling……………………………………………………..23

I.8. Goal of Dissertation………………………………………………………………..26

CHAPTER II. MATERIALS AND METHODS……………………………………………..27

II.1. Primary Cell Preparation…………………………………………………….........27

II.2. Proliferation Assays………………………………………………………………...28

II.3. mRNA Expression Analysis……………………………………………...............29

II.4. Genomic DNA Analysis………..31

II.5. Western Blot Analysis………………………………………………………….....32

II.6. Construction of Retroviral Vectors……………………………………………….....33

II.7. Generation of Recombinant Retroviruses……………………………………………...33

II.8. Titration of Recombinant Retroviruses……………………………………….......34

II.9. Retroviral Transduction………………………………………………………......34

II.10. Flow Cytometric Analysis…………………………………………………….....35

II.11. Immunohistochemistry of Cultured KCs……………………………………......36

II.12. Immunofluorescent Staining of Tissue Sections………………………………......36

II.13. Chemicals………………………………………………………………………..37

II.14. Antibodies………………………………………………………………..……...38

II.15. Statistics……………………………………………………………………….......38

CHAPTER III. RESULTS……………………………………………….............................40

III.1. A Culture Model to Study Reversal of Keratinocyte Differentiation……….……...........40

III.1.1. Reversal of Ca^{2+}-induced differentiation in response to a low Ca^{2+} switch in primary cultures of mKCs……………………………………………………......40

III.1.2. Contamination of epidermal cultures with melanocytes…………………………43

III.1.3. The extent of the reversibility of Ca^{2+}-induced KC differentiation……………...47

III.1.4. Biochemical characterization of keratinocyte cultures in response to the low Ca^{2+}switch…………………………………………………………………………..49

III.1.5. Immunohistochemical analysis of early and late differentiation markers in differentiated and reverted cultures……………………………………………...51

III.1.6. Differentiation-resistant KCs do not contribute to the repopulation of differentiated cultures in response to low Ca^{2+}conditions……………………………......53

III.1.7.Quiescent KCs do not contribute to the repopulation of differentiated cultures

in response to the low Ca^{2+}switch……………………………………………………………55

III.1.8. Induction of a mitogenic response in response to the low Ca^{2+} switch………...57

III.1.9. Conclusion of III.1…………………………………………………………...59

III.2. Mechanism of Re-initiation of Proliferation in Differentiated KCs………..………...60

III.2.1. Identification of signaling pathways activated by the low Ca^{2+} switch…………60

III.2.2. Role of PKD in Re-initiation of Proliferation in Differentiated KCs……..…..64

III.2.2.1. PKD is the target of Go6976 inhibition of re-initiation of proliferation

in KCs……..…………………………………………………………...……64

III.2.2.2. PKD1 expression is up-regulated in differentiated mouse KCs...………66

III.2.2.3. Kinetics of PKD1 activation in response to the low Ca^{2+} switch in

differentiated cultures of KCs…………………………………………………...68

III.2.2.4. Sustained PKD1 activation is required for re-initiation of proliferation

in differentiated KCs……………………………………………………………70

III.2.2.5. PKD1 is essential for re-initiation of proliferation in differentiated KC

cultures………………………………………………………………………..72

III.2.2.6. The unique and non-redundant role of PKD1 in reversion of KC

differentiation…………………………………………………………………75

III.2.2.7. PKD1 kinase activity is required for reversion of differentiated

KCs……………………………………………………………………………...77

III.2.2.8. Conclusion of III.2.2…………………………………………………..79

III.2.3. Role of ERK1/2 in re-initiation of proliferation in differentiated KCs………...80

III.2.3.1. Activation of ERK- MAPK pathway in response to the low Ca^{2+}

switch…………………………………………………………………………80

III.2.3.2. Specific role of ERK1 in reversion of KC differentiation…………….....83

III.2.3.3. PKD1- mediated activation of ERK1/2 in response to the low Ca^{2+} switch………………………………………………………………………85

III.2.3.4. Analysis of ERK1/2 expression and activity in PKD1-deficient KCs…...87

III.2.3.5. Conclusion of III.2.3……………………………………………………..91

III.3. Mechanism of PKD1 Activation……………..…………………………………………92

III.3.1. PKC-independent activation of PKD1 in response to the low Ca^{2+} switch………....92

III.3.2. Role of RhoA-ROCK-nPKC-SFK signaling pathway PKD1 activation………..…..95

III.3.3. Conclusion of III.3………………………………………………………………100

CHAPTER IV. DISCUSSION ……………………………………………………………101

CHAPTER V. FUTURE DIRECTIONS………………………………………………………114

REFERENCES ………………………………………………………………......................117

List of Figures

Figure 1. Schematic representation of epidermal structure and protein expression………………4

Figure 2. Schematic representation of the structure of PKD isoforms…………………………..22

Figure 3. Reversal of differentiated morphology and re-initiation of DNA synthesis in cultures exposed to low Ca^{2+} conditions……………………………….……………………………42

Figure 4. Expansion of contaminating melanocytes in reverted epidermal cultures isolated from FVB mice……………………………………………………………………………..45

Figure 5. Reversal of Ca^{2+}-induced differentiation in epidermal cultures devoid of melanocytes……………………………………………………………………………………46

Figure 6. The extent of the reversibility of Ca^{2+}-induced differentiation………………………..48

Figure 7. Analysis of proliferation and differentiation markers during KC reversion..…………50

Figure 8. Immunohistochemical analysis of differentiation markers in differentiated and reverted KC cultures……………………………………………………………………..52

Figure 9. Differentiation-resistant KCs do not account for repopulation of differentiated cultures in response to the low Ca^{2+} switch……………………………………………………...54

Figure 10. Quiescent undifferentiated KCs do not account for repopulation of reverted cultures………………………………………………………………………………………...56

Figure 11. De-repression of cyclinD1 in response to the low Ca^{2+} switch……………………...58

Figure 12. Go6976 and U0126 inhibit re-initiation of proliferation in differentiated KCs.……..63

Figure 13. PKD is the target of Go6976 inhibition of re-initiation of proliferation in differentiated KCs…………………….…………………………………………………….65

Figure 14. Up-regulation of total PKD levels in differentiated KCs…………………………….67

Figure 15. Kinetics of PKD1 activation in response to the low Ca^{2+} switch in differentiated cultures of mKCs…………………………………………………………………………...69

Figure 16. Sustained PKD1 activation is required for mitogenic response in reverted cultures……………………………………………………………………………………...71

Figure 17. PKD1 depletion inhibits re-initiation of proliferative response in differentiated keratinocytes exposed to low Ca^{2+}conditions……………………………………………………74

Figure 18. Expression of PKD isoforms in mouse keratinocytes………………………………..76

Figure 19. Over-expression of dominant negative form of PKD1 inhibits re-initiation of proliferation and morphological reversionin differentiated KCs…………………...……………78

Figure 20. Kinetics of ERK1/2 activation in response to the low Ca^{2+} switch…………………..82

Figure 21. ERK1 depletion does not inhibit re-initiation of proliferation in differentiated KCs………………………………………………………………………………………………84

Figure 22. Involvement of PKD1 in activation of ERK-MAPK pathway during KC reversion…………………………………………………………………………………......86

Figure 23. CRE-mediated recombination and subsequent PKD1 depletion in PKD1 conditional knockout mouse KCs……………..……………………………………………89

Figure 24. Analysis of ERK1/2 expression and activation in PKD1-deficient reverted mKCs……………………………………………………………………………………………90

Figure 25. PKC-independent activation of PKD1 in differentiated KCs in response to lowering Ca^{2+}…………………………………………………………………………………94

Figure 26. PKCη does not mediate PKD1 activation in reverted cultures…...…………………97

Figure 27. Inhibition of RhoA-Rock signaling pathway potentiates re-initiation of proliferation in differentiated KCs………………………………………………….……..…..98

Figure 28. Src tyrosine kinases are not responsible for PKD1 activation in response to the low Ca^{2+} switch in differentiated KCs…………………………………………………………...99

List of Tables

Table 1. List of Primers……………………………………………………………………….39

Table 2. Summary of the effects of pharmacological inhibitors of signaling pathways on morphological reversion in response to the low Ca^{2+} switch………………………..………..62

Abbreviations

AD	Acidic domain
APD	Alanine/proline rich domain
BCA	Bicinchoninic acid
BCS	Bovine calf serum
βME	βeta-mercaptoethanol
bp	Base pairs
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
C	Celsius
Ca^{2+}	Calcium
Ca^{2+}_o	Extracellular calcium
Ca^{2+}_i	Intracellular calcium
CaR	Calcium sensing receptor
CD	Cysteine rich zinc finger domain
D	Differentiated
DAB	3,3' Diaminobenzidine
DAG	Diacylglycerol
DME	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DN	Dominant negative
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor

ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FIL	Filaggrin
GFP	Green fluorescent protein
GPCR	G protein coupled receptor
HBSS	Hanks balanced salt solution
HD	Hydrophobic domain
HPV	Human papillomavirus
Hr	Hours
HRP	Horse radish peroxidase
IHC	Immunohistochemistry
IKKα	IκB kinase α
INV	Involucrin
IP	Inositol phosphate
IP3	Inositol 1,4,5 triphosphate
JNK	c-Jun NH2-terminal kinase
K	Keratin
KC	Keratinocyte
KC-SFM	Keratinocyte-serum free media
KD	Kinase domain
KO	Knockout
Lys	Lysine
LOR	Loricrin

MAPK	Mitogen activated protein kinase
Min	Minutes
MOI	Multiplicity of infection
O/N	Over night
P	Proliferative
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-Tween
PE	Phycoerythrin
PFA	Paraformaldehyde
PGK	Phosphoglycerate kinase
PH	Pleckstrin homology domain
PKC	Protein kinase C
PKD	Protein kinase D
PLC	Phospholipase C
PS	Pseudo substrate
P/S	Penicillin/ streptomycin
PTH	Parathormone
qRT-PCR	Quantitative real time reverse transcriptase- polymerase chain reaction
R	Reverted
RIN-1	Ras and Rab interactor 1
RT	Room temp
SCC	Squamous cell carcinoma

SDS	Sodium dodecyl sulfate
SER	Serine
SFK	Src family kinase
shRNA	Short hairpin-ribonucleic acid
siRNA	Small interfering RNA
TPA	12-O-tetradecanoylphorbol-13-acetate
Tyr	Tyrosine
VEGF	Vascular endothelial growth factor
WT	Wild type

Chapter I. Introduction

Skin is the largest organ of the body and is composed of epidermis, the underlying dermis, and the basement membrane that separates the two. The epidermis is the outer most layer of the skin and acts as a barrier protecting the body from external agents and the environment and from loss of moisture. It also regulates body temperature and is part of the immune system.

I.1. Epidermal Structure and Function

The epidermis is a stratified squamous epithelium primarily composed of keratinocytes (KCs; cells that express specialized intermediate filaments called keratins) and consists of four layers, basal, spinous, granular and cornified, as depicted in Figure 1. Each layer is defined by position, morphology and the state of differentiation. The first and innermost layer is the basal layer. Cells in this layer are attached to the basement membrane, are mitotically active, and are characterized by expression of specific basal-cell associated protein markers such as Keratin (K)5 and K14, p63, integrins $\alpha6\beta4$ and $\alpha3\beta1$ (Fuchs, 2007). The cells in the basal layer are attached to the basement membrane by hemidesmosomes and focal adhesions which are 2 types of cell adhesion complexes, composed of integrins (Fuchs, 2007; Watt, 2002). Hemidesmosomes contain $\alpha6\beta4$ integrins which connect intracellularly to the keratin intermediate filaments to confer mechanical strength (Watt, 2002; Wilhelmsen et al., 2006). Focal adhesions contain $\alpha3\beta1$ integrins which adhere to actin and the microtubule network (Fuchs, 2007; Watt, 2002). Both hemidesmosomes and focal adhesions adhere extracellularly to laminin 5, the major extracellular matrix (ECM) ligand for the basement membrane (Turksen and Troy, 1998; Watt, 2002; Wilhelmsen et al., 2006).

Commitment to differentiation begins when basal cells, given the proper stimuli, exit the cell cycle, down regulate expression of integrins, detach from the basement membrane and move upward (HALL and WATT, 1989; Watt, 2002). As the cells move from the basal layer they alter their keratin expression from K5 and K14 to K1 and K10. This alteration in keratin expression is considered the most reliable indication that KCs have committed to differentiation (Fuchs and Green, 1980). The first of the suprabasal layers is the spinous layer. In this layer, KCs undergo extensive morphological changes indicated by their spinous shape, reflected by their K1/ K10 intermediate filament bundles connected to the robust desmosomes. These connections provide a solid, integrated mechanical framework across and within the epidermal sheets (Coulombe and Wong, 2004). Other than K1 and K10, suprabsal KCs can also express K6, K16 and K17 but only during wound healing (Ekanayake-Mudiyanselage et al., 1998). The keratin network in the epidermis not only plays an important role in mediating remodeling of the cytoskeleton for migration, it been shown to be involved in cell proliferation and differentiation (Coulombe and Wong, 2004; Kim et al., 2006; Koch and Roop, 2004). As the cells move upward they begin to express cornified envelope precursors such as involucrin. The 3rd layer is the granular layer, characterized by the presence of electron-dense keratohyalin granules which are large granules filled with profilaggrin (the precursor of filaggrin; another cornified envelope protein). Additionally, cornified envelope proteins, rich in glutamine and lysine residues, like loricrin, cornifin, sciellin, and transglutaminase are synthesized and deposited under the plasma membrane of the granular cells. The final stages of differentiation and the formation of the 4th layer, the cornified layer, begin with aggregation of keratin filaments into tight bundles (by filaggrin) to form large macrofibillar cables which promote the collapse of KCs into a flattened shape. Furthermore, the cornified layer is characterized by nuclear disintegration, destruction

of cellular organelles, and formation of the cornified envelope (Proksch et al., 2008; Segre, 2006; Simon et al., 1994; Turksen and Troy, 1998; Watt and Green, 1981). The cornified envelope is a tough lipid/ protein polymer structure that is formed under the plasma membrane of corneocytes (KCs of the cornified layer rich in protein) and consists of a protein envelope and a lipid envelope. It is a product of covalent cross-links of envelope precursor proteins by Ca^{2+}-dependent transglutaminase and attachment of lipid envelope, a plasma membrane-like structure that replaces the plasma membrane on the external aspect of corneocytes (Madison, 2003; Proksch et al., 2008). Together these profound changes in structure results in formation of an impermeable barrier that is eventually shed from skin.

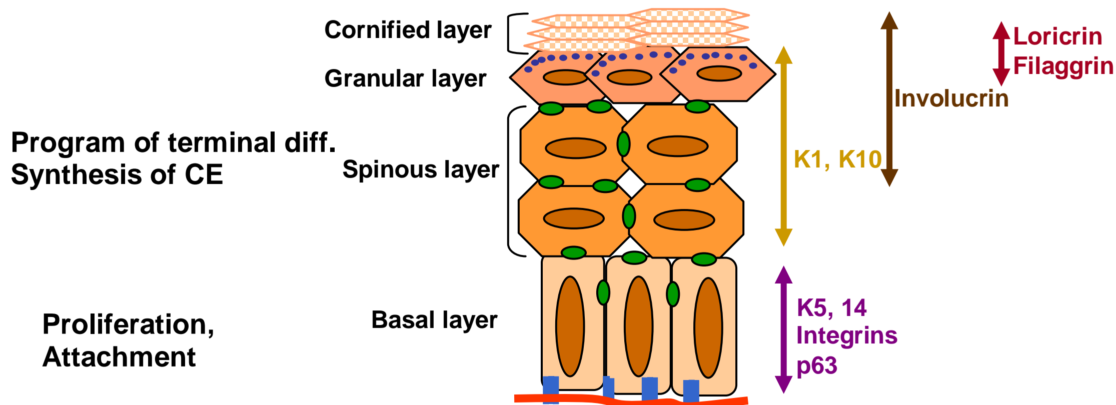


Figure 1. Schematic representation of epidermal structure and protein expression. Epidermis is a multilayered tissue which undergoes a vertical program of differentiation with a proliferative compartment in the basal layer and a differentiated compartment in the suprabasal layers (spinous, granular and cornified layer). Cells in the basal layer are attached to the basement membrane (red line) by hemidesmosomes and focal adhesions (blue lines). KCs adhere to each other through adhesion junctions and desmosomes (green ovals). In the basal layer makers like K5, K14, integrins and p63 are expressed. As KCs detach from the basement membrane and move upward they start the program of terminal differentiation, express K1 and K10, and synthesize various precursors of the cornified envelope.

I.2. Regulation of Epidermal Proliferation and Differentiation

Epidermal homeostasis is maintained through a fine balance between replication of stem cells and progenitors, followed by differentiation. Disruption, alteration and imbalance of the normal proliferation and differentiation of the epidermis can lead to many skin disorders as well as cancer (HALL and WATT, 1989; Potten and Morris, 1988). There are major signaling pathways that regulate epidermal proliferation and differentiation. Of those p63, a homolog of p53, plays an essential role in epidermal development and along with its interaction with other signaling pathways is a key regulator in epidermal growth and differentiation.

p63 is expressed as 2 spliced variants ΔNp63 and TAp63 (Yang et al., 1998) and is required for epidermal lineage commitment, epidermal proliferation and differentiation, barrier formation, cell adhesion and adhesion to basement membrane (Koster, 2010). Two independent studies have demonstrated that deletion of p63 from the germ line of mice results in severe developmental abnormalities. These mice are born with translucent skin and lack appendages including teeth, hair follicles and mammary glands (Mills et al., 1999; Yang et al., 1999). Furthermore, the epidermis of p63 deficient mice does not stratify and exists as a disorganized single layer lacking expression of K5 and K14. These mice die within hours after birth due to a lack of proper barrier formation (Mills et al., 1999; Yang et al., 1999).

However, despite the similarities in the phenotype seen in these mice, two different conclusions have been drawn. One group has suggested that in the absence of p63 skin development is stopped at early stages without starting a pluristratification program; hence p63 is required for the commitment of immature ectoderm to epidermal lineage (Mills et al., 1999). On the other hand, the other group saw patches of skin in these mice that expressed markers of differentiation including involucrin, filaggrin and loricrin. Therefore, they concluded that p63

does not influence the differentiation process, but impairs the proliferation potential of stem cells (Yang et al., 1999).

Additional studies looking at the function of p63 in the skin have also generated contradictory results in terms of p63 function. Koster speculates that the contradictory results in p63 function may be due to differences in p63 expression levels and/or its interactions with different cofactors, as well as which population of KCs in the basal layer are being studied for p63 function. She suggests that p63 maintains proliferation in early transit amplifying cells while it induces cell cycle exit in mature transit amplifying cells (Koster, 2010). Furthermore, studies have also demonstrated that ΔNp63 and TAp63 antagonize each other's function (Yang et al., 2002). Therefore, others have suggested that the differences seen in one group versus the other may be due to antagonistic function of the 2 isoforms of p63 and/or due to differences in models used to study the role of p63 in skin (Candi et al., 2008).

One of the many functions of p63 includes its ability to induce IκB kinase α (IKKα), an important mediator of cell cycle exit during KC differentiation (Candi et al., 2007; Koster et al., 2007; Marinari et al., 2009). Ikkα$^{-/-}$ mice display a similar phenotype to p63$^{-/-}$ mice, including translucent skin, shortened limbs and truncated tail and snout, and die soon after birth due to barrier defects and dehydration (Descargues et al., 2008) suggesting that IKKα may be a downstream target of p63. Studies have demonstrated that TAp63 can transactivate IKKα directly by binding to a p53-like consensus sequence on the IKKα promoter or indirectly via up-regulation of Ets-1 and GATA-3 transcription factors that can also bind to the IKKα promoter, (Candi et al., 2006; Gu et al., 2004) which have been shown to be important mediators of hair and skin development (Kaufman et al., 2003). Additionally, ΔNp63 can indirectly result in transactivation of IKKα though GATA3 induction (Candi et al., 2006).

IKKα, one of the 2 catalytic subunits of the IKK complex, is required for NFκB activation and like p63 plays an important role in normal epidermal development (Descargues et al., 2008; Sil et al., 2004). However, the regulation of epidermal proliferation and differentiation by IKKα is not dependent on NFκB activation. The major function of IKKα in the epidermis is induction of cell cycle arrest (Hu et al., 2001). Studies have demonstrated that IKKα functions as a cofactor to Smad2/3 in a Smad4-independent TGFβ signaling pathway resulting in induction of Oval1 and Mad1 which are negative regulators of Myc (Descargues et al., 2008). The c-myc proto-oncogene is a well studied transcription factor known to regulate cell cycle progression through the G1-S phase and is considered to regulate KC proliferation and differentiation based on the timing and intensity of its activity (Watt et al., 2008). Therefore, IKKα mediates KC proliferation and differentiation by inducing several myc antagonists to drive cell cycle arrest and KC differentiation (Descargues et al., 2008).

Another important signaling pathway that is involved in KC proliferation and differentiation is the Notch pathway. In KCs, Notch signaling is essential to promote differentiation and inhibit tumorgenesis (Dotto, 2009). Conditional KC-specific deletion of Notch1 results in hyperplastic epidermis, up-regulation of integrins in the suprabasal layers, and improper expression of keratins in the basal and suprabasal layer of the epidermis (Lefort and Dotto, 2004). Furthermore, studies have demonstrated that in KCs, activation of Notch1 alone can result in cell cycle arrest and differentiation through induction of p21, K1, K10 and involucrin expression (Rangarajan et al., 2001). Notch1 is also in direct crosstalk with p63. p63 and Notch1 mainly function as antagonists of each other to maintain epidermal homeostasis (Nguyen et al., 2006). While p63 is tied to epidermal cell fate determination and KC proliferation, Notch1 inhibits proliferation and promotes KC differentiation (Koster, 2010; Lefort

and Dotto, 2004). Notch1 represses p63 expression by down regulation of interferon-responsive factors like IRF7 and IRF3, which bind to the p63 promoter to induce expression of p63 (Dotto, 2009; Nguyen et al., 2006). Additionally, studies suggest that Notch1 activation can result in NFκB activation which in turn has been shown to bind to the promoter of p63 to suppress its expression (Dotto, 2009; Nickoloff et al., 2002). In turn, p63 has also been shown to regulate Notch1. Increased p63 expression has been shown to offset Notch1-dependent transcription by negatively targeting HES1 and p21 which promotes differentiation (Dotto, 2009; Moriyama et al., 2008). In few cases, p63 directly regulates Notch1 transcription by binding to the Notch1 promoter and inhibiting Notch1 expression (Yugawa et al., 2007). Interestingly, in the early stages of differentiation, p63 and Notch1 function synergistically where p63 can induce expression of JAG1 and JAG2 in neighboring cells to synchronize differentiation in suprabasal KCs and signal for the later stages of differentiation to occur (Dotto, 2009; Sasaki et al., 2002).

Epidermal growth factor receptor (EGFR) signaling is another signaling pathway critical in regulation of epidermal homeostasis (Jost et al., 2000). It is one of the most studied determinants of epidermal proliferation. EGFR is highly expressed in the basal layer and functions as a built-in mechanism to maintain self renewal, while at the same time suppressing differentiation. Inhibition of EGFR/ERK signaling in proliferative KCs results in differentiation, while sustained activation of EGFR/ ERK signaling (in cases where they would normally be down-regulated) results in suppression of differentiation (Jost et al., 2000; Zenz and Wagner, 2006). Recent studies have demonstrated that EGFR mediates suppression of differentiation through down regulation of Notch1 expression and activity. The underlining mechanism involves transcriptional down regulation of p53 by the EGFR effector c-Jun.

Suppression of p53, in turn, results in suppression of Notch1 gene expression and subsequent inhibition of differentiation (Kolev et al., 2008). Regulation of Notch1 and p53 expression by EGFR demonstrates another cross interacting pathways involved in regulating KC proliferation and differentiation.

Together, these data demonstrate the complexity of underlying mechanisms involved in regulating KC proliferation and differentiation. Individual and cross interactions among these and other signaling pathways play an important role in epidermal homeostasis.

I.3. De-Differentiation

The epidermis is a continuously renewing tissue undergoing constant regeneration. Cell kinetics and fate-mapping studies in epidermis have demonstrated the existence of a hierarchy from stem cells to transit amplifying cells (committed progenitors) and to terminally differentiated cells (Ghazizadeh and Taichman, 2001; Hume and Potten, 1982; Morris et al., 2004). The primary source of cell renewal in normal epidermis are stem cells (Blanpain et al., 2007; Potten and Booth, 2002). Recent studies, however, have demonstrated that there is some plasticity between stem cells and their progeny. They have shown that not only stem cells, but transit amplifying cells, as well as early differentiating cells have the potential to regenerate a fully stratified epidermis in response to proper stimuli when transplanted in an *in vivo* environment (Li et al., 2004). However, while a degree of plasticity exists between stem cell and progenitors in the basal layer, commitment to differentiation is believed to be irreversible (Li et al., 2004; Niemann et al., 2002; Potten and Loeffler, 1990).

De-differentiation is defined as the process of moving from a more differentiated state to a less differentiated state and regaining proliferative ability (Finney et al., 1987) and has been observed in many systems. In response to certain stimuli, differentiated cells in Drosophila and other lower vertebrates have been shown to de-differentiate from a differentiated state to a progenitor or stem cell-like state to participate in tissue regeneration (Brawley and Matunis, 2004; Guha et al., 2008; Kai and Spradling, 2004; Laube et al., 2006; Nakagawa et al., 2007). In mammals, de-differentiation is more controversial; however, it has been described in slow regenerating tissues like the liver, pancreas, and lung. In response to injury or oxidants, Clara cells of the lung de-differentiate, self renew and give rise to different cell types (Rawlins and Hogan, 2006). In the liver, tissue regeneration involves the proliferation of differentiated

hepatocytes and in some cases the interlobular bile ducts (Alison et al., 2004), not stem cells. In the adult pancreas, as well as after pancreatectomy, it is not stem cells but rather pre-existing β cells that are the source of new β cells in the pancreas (Dor et al., 2004).

In the epidermis, although terminal differentiation is believed to be irreversible, de-differentiation has been seen during wound healing and in many diseases of the skin where normal proliferation and differentiation is perturbed, as well as in many cancers. For example, during wound healing, the epidermis needs to regenerate the barrier rapidly, and therefore some degree of de-differentiation is observed in differentiated cells at the wound margin including the lateral migration of suprabasal cells at the wound margin (Garlick and Taichman, 1994; Mansbridge and Knapp, 1987; Usui et al., 2005), atypical expression of integrins by suprabasal KCs at the leading edge of wound (Hertle et al., 1992), and phenotypic changes and redistribution of K10, K16 and K17 to the basal layer of the re-epithelialized wound site (Paladini et al., 1996; Usui et al., 2005). In human papillomavirus (HPV), the virus targets post mitotic cells of the suprabasal layer and re-initiates DNA synthesis in these cells. This leads to acanthosis and parakeratosis as well as up regulation of the expression of cell cycle regulators like cyclin D1 and cyclin E (Crish et al., 2000). Additional studies have demonstrated that regulators of the cell cycle play a crucial role in the reversal of commitment to terminal differentiation (Pajalunga et al., 2007). Terminal differentiation is characterized by the series of events by which cells exit the cell cycle and gain specific specialized function. Inhibition of cyclin dependent kinase inhibitors has been shown to allow post mitotic, differentiated cells to overcome growth arrest and re-enter the cell cycle (Pajalunga et al., 2007). In the skin, p21 and p27 (both cyclin dependent kinase inhibitors) play an important role in regulating proliferation and differentiation (Kolly et al., 2005; Topley et al., 1999). Upon differentiation, p21 levels

increase, most likely due to the role p21 plays in differentiation-associated growth arrest (Missero et al., 1996). Furthermore, studies using p21 knockout mice have demonstrated that in KCs loss of p21 promotes increased proliferation and reversible commitment to KC differentiation (Topley et al., 1999).

Recent work in our lab has demonstrated that when differentiated cultures of mouse KCs, were grafted onto suitable sites *in vivo,* they were not only able to re-initiate proliferation, but were able to reform a multilineage, hair bearing epidermis (Mannik et al., 2010). Genetic labeling and lineage tracing using an INV driven CRE/lox reporter system verified that the new tissue was formed by the transplanted differentiated KCs (Mannik et al., 2010). This regeneration implies that signals from the microenvironment induce de-differentiation of differentiated KCs. However, what these signals are and the mechanism(s) by which they relay these signals are not known.

I.4. Calcium-Induced KC Differentiation- A Model to Study KC Growth and Differentiation in Culture

Primary cultures of mouse KCs have been used extensively to study the process of differentiation. In culture, KC proliferation and differentiation can be regulated by multiple factors including calcium (Ca^{2+}), pharmacological agents such as TPA (12-O-tetradecanoylphorbol-13-acetate- a phorbol ester), or agents such as vitamin D3 and retinoic acid (Bikle et al., 2004; Dotto, 1999; Fisher et al., 1995). Of these factors, Ca^{2+}-induced differentiation in primary cultures of mouse KCs has been extensively studied and characterized.

In cultures grown under low Ca^{2+} conditions (0.05 mM), a population of undifferentiated, basal cell-like cells is produced (Hennings et al., 1980). Raising Ca^{2+} levels above 0.1 mM in the medium triggers culture-wide terminal differentiation in a manner that closely resembles the *in vivo* event including growth arrest, cytoskeletal changes, desmosome formation, stratification, and cornification (Kolly et al., 2005; Yuspa et al., 1989). Within hours after switching to high Ca^{2+} KCs change from making K5 and K14 to making K1 and K10 (Yuspa et al., 1989). Within 1 day after increasing Ca^{2+}, the majority of KCs in culture undergo growth arrest (Missero et al., 1996) and subsequently, within 1-2 days, involucrin, loricrin and other cornified envelope precursors are cross-linked into the cornified envelope by the Ca^{2+}-sensitive transglutaminase I (Hohl et al., 1991; Rice and Green, 1979; Thacher and Rice, 1985). Additionally, increasing Ca^{2+} leads to a rapid re-distribution of desmoplakin, cadherins and catenins from the cytosol to the plasma membrane to form intercellular contacts (Braga et al., 1995; Yuspa et al., 1989).

The relevance of Ca^{2+}-induced differentiation in culture as compared to the *in vivo* condition is demonstrated by the distinctive Ca^{2+} gradient exhibited in the epidermis. Ion capture cytochemistry for Ca^{2+} localization has identified low levels of Ca^{2+} in the basal layer, increasing levels in the suprabasal layer, with the highest levels in the stratum granulosum (Bikle et al., 2004; Menon et al., 1985). The Ca^{2+} gradient functions to maintain differentiation function including permeability barrier homeostasis. The loss of Ca^{2+} gradient with or without disruption in epidermal barrier has been shown to modulate epidermal differentiation *in vivo* (Elias et al., 2002). Disruption in epidermal barrier, along with the loss of Ca^{2+} gradient, results in decreased Ca^{2+} levels, increased lamellar body secretion, increased proliferation and a decrease in expression of differentiation markers like involucrin, profilaggrin and loricrin (Bikle et al., 2004; Elias et al., 2002; Mauro et al., 1998). Therefore, extracellular calcium (Ca^{2+}_o) not only regulates KC differentiation in culture but plays an important role in regulating KC differentiation *in vivo*.

The increase in Ca^{2+}_o in culture leads to a biphasic increase in intracellular calcium (Ca^{2+}_i). The first phase is a short, acute phase which is followed by a longer, more sustained plateau of increased Ca^{2+}_i, which leads to activation of downstream targets and eventually leads to differentiation (Bikle et al., 2004). The response of KCs to the increase in Ca^{2+}_o resembles that of the parathyroid gland. In the parathyroid gland the Ca^{2+} sensing receptor (CaR), a G protein coupled receptor (GPCR) with a 7 transmembrane domain, regulates the secretion of parathormone (PTH) into the blood by responding to fluctuations in Ca^{2+}_o concentrations (Brown, 1991). CaR is also expressed in KCs (Bikle et al., 1996; Oda et al., 1998; Tu et al., 2007), where binding of Ca^{2+}_o to CaR activates the receptor and triggers downstream signaling pathways (Tu et al., 2001). Inactivation of CaR using an antisense cDNA has been shown to

inhibit Ca^{2+}_o-induced increase in Ca^{2+}_i, to suppress expression of differentiation markers like involucrin, transglutaminase and loricrin, and to inhibit E-cadherin-mediated cell adhesion, resulting in impaired differentiation (Tu et al., 2001; Tu et al., 2008; Tu et al., 2007). Together, these findings demonstrate the important role CaR plays in Ca^{2+}-induced KC differentiation.

Activation of CaR by Ca^{2+}_o stimulates phospholipase C (PLC) activation via Gq. Activated PLC converts PIP2 into inositol 1, 4, 5 triphosphate (IP3) and diacylglycerol (DAG) (Berridge, 1993; Jaken and Yuspa, 1988). IP3, in turn, induces Ca^{2+}_i release from internal stores and DAG, in turn, can activate the protein kinase C (PKC) pathway which signals to downstream targets (Bikle et al., 2001) and plays an important role in KC differentiation.

I.5. Protein Kinase C and Regulation of KC Differentiation.

The protein kinase C (PKC) family of serine threonine kinases is a large family of enzymes that are involved in KC proliferation and differentiation and can be divided into 3 groups. The classical PKCs (α, β, and γ isoforms) which can be activated by calcium and phorbol esters/ DAG, the novel PKCs (δ, ε, η, θ isoforms), which are Ca^{2+} -independent but can be activated by DAG/phorbol esters, and finally the atypical PKCs (ζ and λ/ι isoforms) that are activated independent of Ca^{2+} and DAG. KCs express PKC α, δ, ε, η, and ζ (Denning et al., 1995). Specific roles have been identified for each of these isoforms. Together, the various PKC associated functions and signaling pathways regulate skin physiology (Breitkreutz et al., 2007; Denning, 2004; Shen et al., 2001).

In *vivo*, PKCα is predominantly expressed in the spinous and granular layers. PKCα activation induces growth arrest and transition from spinous to granular layer, whereas inhibiting PKCα activity or blocking its synthesis with antisense oligonucleotides leads to loss of granular markers and revival of spinous layer markers (Breitkreutz et al., 2007; Jerome-Morais et al., 2009). Over-expression of PKCα in transgenic mice does not directly affect the epidermis and epidermal differentiation, but causes a severe inflammatory response and increased epidermal thickening (Wang and Smart, 1999).

PKCδ is predominantly expressed throughout the epidermis, but slightly more in the basal and lower spinous layer (Denning et al., 1996). In the basal layer, activated PKCδ localizes to hemidesmosomes and phosphorylates α6 integrin leading to the dissociation and internalization of the α6β4 integrin and disintegration of hemidesmosomes. This loss of adhesion allows for cells to either migrate upward into the suprabasal layers and differentiate, or migrate alongside the ECM during wound healing (Alt et al., 2004; Alt et al., 2001; Gimond et

al., 1995). Mice over-expressing PKCδ display increased resistance to tumor formation (Aziz et al., 2006; Reddig et al., 1999) and conversely in mice depleted of PKCδ tumorgenesis is enhanced partly due to the suppression of apoptosis (Humphries et al., 2006; Lu et al., 1997).

PKCε is expressed in basal KCs. Mice over-expressing PKCε are characterized by epidermal hyperproliferation and skin ulcers after 4 months of age. When tumor formation was induced, papilloma formation was reduced, while progression to SCCs was enhanced and the resulting SCCs were highly metastatic (Jansen et al., 2001; Reddig et al., 2000). Furthermore, PKCε knockout mice have demonstrated the critical role for PKCε-mediated phosphorylation of Stat3 and may be a key component in the mechanism by which PKCε imparts sensitivity to UVR-induced development of SCCs (Aziz et al., 2007). These results demonstrate the importance of PKCε in normal skin development and tumorgenesis.

PKCη expression is restricted to the granular layer. PKCη activation is crucial for the regulation of KC differentiation, where by over-expression of PKCη, not only leads to an increase in levels and activity of transglutaminase I, it is sufficient to induce growth arrest and morphological changes mimicking those of KC differentiation (Kashiwagi et al., 2002; Ueda et al., 1996). The latter is through its interaction with Fyn, a member of the Src family of kinases, which is required for normal epidermal differentiation (Cabodi et al., 2000). Interaction of PKCη and Fyn is necessary for Fyn activation and inhibition of proliferation through a Fyn-dependent down regulation of EGFR activity and function (Cabodi et al., 2000; Calautti et al., 1995). PKCη knockout mice demonstrate increased susceptibility to tumor formation in response to TPA (Chida et al., 2003). Furthermore, in response to injury, wound healing and in particular re-epithelialization was considerably delayed and impaired (Chida et al., 2003) in these mice. Together these data demonstrate that PKCη is a key regulator in maintenance of

epidermal architecture. Furthermore PKCη mediates epidermal homeostasis and plays an important role in tumor formation *in vivo*.

I.6. Protein Kinase D Signaling

Protein kinase D (PKD) is a serine threonine kinase classified as a distinct protein kinase family, which includes PKD1 (PKD/PKCμ), PKD2 and PKD3 (Rykx et al., 2003). PKD was originally categorized in the atypical PKC family of kinases but eventually removed due to its profound differences in its structure and substrate specificity. Figure 2 demonstrates the schematic structure of PKD1, 2, and 3. PKD structure is divided into the N-terminal regulatory domain and the C-terminal kinase or catalytic domain (KD). The PKD1 regulatory domain consists of an alanine/ proline rich domain (APD) also called the hydrophobic domain (HD), 2 cysteine rich zinc finger domains (CDI and CDII), an acidic domain (AD), and a pleckstrin homology domain (PH) and the C-terminal kinase or catalytic domain (KD) (Rozengurt et al., 2005; Rykx et al., 2003). A similar modular structure is found in PKD2 and PKD3 as depicted in Figure 2. The regulatory domain of PKD acts as an inhibitor of its kinase activity where deletion of this domain results in full activation of PKD (Vertommen et al., 2000). Additionally, individual domains within the regulatory domain seem to have inhibitory effects as well. Deletion or mutations of the PH domain, as well as deletion of the cysteine rich domain, results in a constitutively active form of PKD. This suggests an autoregulatory role for these domains to help maintain PKD in an inactive catalytic state (Iglesias and Rozengurt, 1998; Iglesias and Rozengurt, 1999; Waldron et al., 1999).

PKD1 can be activated by several mechanisms. In an inactive state PKD1, localizes mainly to the cytosol and to a lesser extent to the golgi and mitochondria. Upon activation, PKD1 can be translocated to the plasma membrane, the golgi apparatus, and the nucleus (Rozengurt et al., 2005). Activation of PLC (by GPCR or receptor tyrosine kinases) and release of DAG has been shown to regulate PKD1 activity. DAG mediates localization by binding to

its CDI domain and translocating it to the plasma membrane for phosphorylation and subsequent activation by PKCs. Activation of PKCε, η, and θ, as well as PKCδ, have all been shown to result in PKD1 activation in a direct or indirect manner (Doppler and Storz, 2007; Rozengurt et al., 2005; Wang, 2006). At the plasma membrane, nPKCs interact with the PH domain of PKD1, thereby releasing PKD1 auto-inhibition and transphosphorylate PKD1 at $Ser^{744/748}$ in the kinase activation loop, resulting in PKD1 activation (Wang, 2006). PKC-dependent transphosphorylation of PKD1 at Ser^{744} and Ser^{748} are believed to play a crucial role in PKD1 activation, as shown by mutagenesis studies (Iglesias et al., 1998). However, recent work by Jacamo and colleagues has demonstrated that phosphorylation at Ser^{744} is PKC-dependent while Ser^{748} phosphorylation is mediated by autophosphorylation (Jacamo et al., 2008). Following $Ser^{744/748}$ phosphorylation, Ser^{916}, located in the c-terminus of PKD1, is autophosphorylated and plays an important role in regulating the conformation of PKD1 (Rykx et al., 2003; Wang, 2006). PKD1 can also be activated independent of PKCs. Tyrosine phosphorylation of PKD1 in the PH domain, interaction of Gβγ subunits with PH domain, and proteolytic cleavage by caspases have been shown to lead to PKC-independent PKD1 activation (Endo et al., 2000; Jacamo et al., 2008; Jamora et al., 1999).

PKD1 has been implicated in a variety of cellular processes including cell proliferation, apoptosis, oxidative stress response, immune response, cell motility and invasion, and Golgi membrane trafficking (Jaggi et al., 2007). Of particular interest is the role of PKD1 in proliferation. In a variety of cells including KCs, T-cells, endothelial cells, fibroblasts, and pancreatic cells PKD1 has been shown to have a direct role in induction of proliferation. In pancreatic tumor cells, over-expression of PKD1 leads to up-regulation of anti-apoptotic proteins (such as survivin) and an increase in telomerase activity (Trauzold et al., 2003). In the human

pancreatic carcinoma cell line, PANC-1, PKC dependent PKD1 activation leads to DNA synthesis (Guha et al., 2002). In endothelial cells, VEGF (vascular endothelial growth factor) induces PKCα-dependent PKD1 activation, resulting in ERK signaling and endothelial proliferation (Wong and Jin, 2005). In Swiss 3T3 cells, increase in the duration of MAPK-ERK-RSK signaling induces proliferation and DNA synthesis due to an increase in PKD1 activity (Sinnett-Smith et al., 2009; Sinnett-Smith et al., 2004; Zhukova et al., 2001). In addition, it has been demonstrated that PKD1 activation results in phosphorylation of Ras effecter protein RIN1 at ser351. This results in blocking RIN1's interaction with Ras, allowing Ras to interact and phosphorylate Raf, leading to up-regulation of the Ras-Raf-MEK-ERK signaling pathway (Wang, 2006; Wang et al., 2002). And finally, although there is not much known about the role of PKD1 in the skin, a pro-proliferative, anti-differentiation role for PKD1 has been suggested in the epidermis (Bollag et al., 2004). In addition, studies have shown that proliferation in normal and neoplastic mouse epidermis and in culture is enhanced in response to PKD1 expression and activation (Rennecke et al., 1999; Ristich et al., 2006).

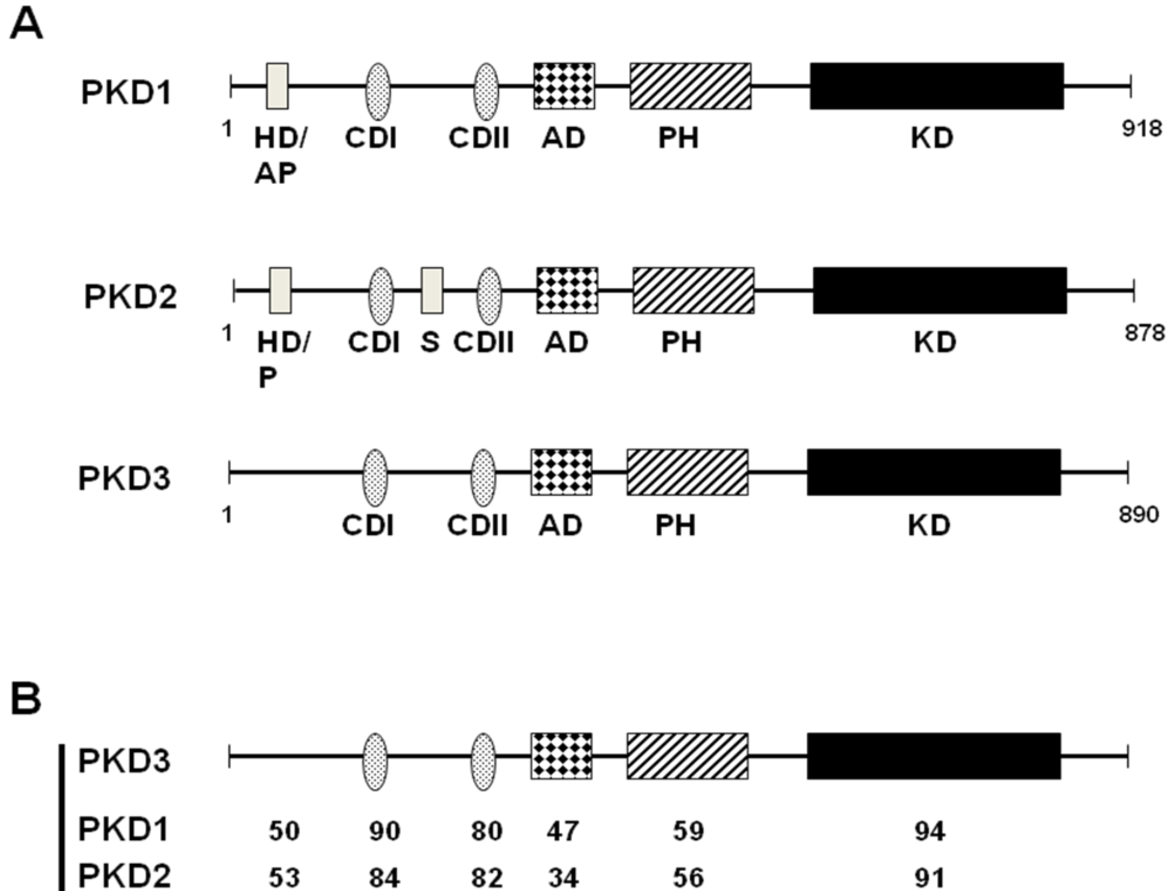


Figure 2. Schematic representation of the structure of PKD isoforms.
A) Schematic diagram of the structure of PKD family members (PKD1, PKD2, and PKD3). Numbers indicate amino acid position. **B)** The percent of amino acid sequence identity between PKD1, 2 and 3.
Abbreviations: APD, alanine/ proline rich domain; P, proline rich domain; HD, hydrophobic domain; CDI and CDII, cysteine rich zinc finger domains I and II; S, serine domain; AD, acidic domain; PH, pleckstrin homology domain; KD, kinase domain.

I.7. ERK1/2-MAPK Signaling

The mitogen activated protein kinases (MAPK) are a family of serine threonine kinases that can be activated in response to various extracellular stimuli and can lead to regulation of many cellular responses such as proliferation and differentiation, cell survival and apoptosis and transformation (Shaul and Seger, 2007). The mammalian MAPK family consists of 3 major cascades: the ERK (extracellular signal-regulated kinase), JNK (c-Jun NH2-terminal kinase), and p38, with each of these enzymes consisting of multiple isoforms (Dhillon et al., 2007; Schaeffer and Weber, 1999). Each of these cascades contains at least 3 tiers of protein kinases including a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K), and a MAPK which are considered the core of each cascade. Activation of each cascade involves phosphorylation and activation of MAP2K by MAP3K, which in turn phosphorylates and activates MAPK (Pearson et al., 2001). Activation of the MAPK, which are the terminal serine threonine kinases, leads to phosphorylation of various substrates including transcription factor such as Elk-1, c-Jun, ATF2, p53, c-myc and Stat1/3 (Roberts and Der, 2007) which are involved in many cellular processes. The phosphorylation cascade of MAPK allows for a more efficient regulation that leads to repression, enhancement and amplification of signals via a distinct mechanism (Pearson et al., 2001). The different components of the MAPK pathway have been intensely studies over the years, with the majority of emphasis placed on studying ERK-MAPK signaling because of the critical role they play in regulating proliferation, differentiation and survival, as well as their role in cancer progression.

The ERK signaling pathway is activated by growth factor-stimulated cell surface receptors like Ras GTPases (Malumbres and Barbacid, 2003). Activation of the ERK cascade begins with binding of ligands to their specific receptor and activation of small GTPase Ras

mediated by the receptor tyrosine kinase Grb2-SOS signaling. Activation of Ras leads to recruitment of one of Raf isoforms (MAPKKK) such as A-Raf, B-Raf, or c-Raf (Raf-1) to the cell surface for activation. Activated Raf phosphorylates and activates MEK1/2 (MAPKK) which in turn activates ERK1/p44 and ERK2/p42 (MAPK) by phosphorylation at 2 sites of tyrosine and threonine in the kinase activation loop (Roberts and Der, 2007; Shaul and Seger, 2007). ERK1/2 are ubiquitously expressed serine threonine kinases that phosphorylate and regulate a vast array of substrates with over 160 substrates discovered so far (Roberts and Der, 2007; Yoon and Seger, 2006).

In the skin, ERK-MAPK signaling appears to promote proliferation in the basal layer and inhibit normal differentiation in the suprabasal layer. Activation of H-Ras, Raf, or MEK1 in the skin have been shown to induce hyperplasia characterized by hyperproliferation and reduced differentiation (Scholl et al., 2004; Tarutani et al., 2003). Expression of dominant negative Ras in the basal layer leads to hypoplasia and death, while inhibition of Ras in the suprabasal layer has no effect (Dajee et al., 2002). Additionally, other studies have shown while epidermal specific individual knockout of MEK1 and MEK2 has no effect on epidermal development, MEK1/2 double knockout leads to epidermal hypoproliferation, hypoplasia, barrier defects, and prenatal death (Scholl et al., 2007). And finally, induction of Ras and MAPK hyperactivity is seen in spontaneous squamous cell carcinoma (Tarutani et al., 2003).

Among the many biological processes that ERK1/2 are involved in, one of the most crucial is cell cycle progression in epithelial and non-epithelial cells. (Chambard et al., 2007; Dumesic et al., 2009; Meloche and Pouyssegur, 2007; Roovers and Assoian, 2000). In fibroblasts, sustained ERK1/2 activity is required for G1/S phase transition, where ERK1/2 up-regulates and stabilizes cyclinD1, stabilizes the cyclinD1/cdk4 complex, and down regulates

anti-proliferative genes like Jund1 and Sox6. Additionally, loss of ERK1/2 activity leads to down regulation of cyclinD1 and G1/S phase growth arrest (Cheng et al., 1999; Lavoie et al., 1996; Meloche and Pouyssegur, 2007; Yamamoto et al., 2006). In the epidermis, knock down of ERK1 and 2 individually had no effect on epidermal proliferation and differentiation, while simultaneous knock down of ERK1/2 inhibited cell division, down-regulated cyclinB1 and c-Fos, and leads to G2/M phase arrest as well as resulting in hypoplasia and hyperproliferation without effecting differentiation (Dumesic et al., 2009). These findings, therefore, suggest a differential role for ERK1/2 in the epidermis as regulators of G2/M phase progression and demonstrated the importance of ERK-MAPK signaling in mediating pro-proliferative and pro-survival signals in the skin.

I.8. Goal of Dissertation

The aim of the present study was to first establish an *in vitro* model of KC de-differentiation and then use this model to delineate the mechanism by which differentiated KCs re-initiate proliferation and de-differentiate. Interestingly, in a series of experiments that will be discussed below, I found that lowering Ca^{2+} concentrations in differentiated cultures of mouse KCs resulted in re-initiation of DNA synthesis and morphological reversion of differentiated KCs. Analysis of these cultures demonstrated a crucial role for PKD1 in this process.

Chapter II. Materials and Methods

II.1. Primary Cell Preparation:

Epidermal cells were isolated from 1-2 day pups from FVB (Taconic Laboratories, James Town, NY) and B6.cg-KitW-sh/HNirJaeBsmJ mice (Jackson laboratories, Bar Harbor, ME), as well as C57BL/6 PKD$^{loxP/loxP}$ cKO mice (gift from Dr. Eric Olson). To isolate epidermal cells, newborn pups were first euthanized by placing them on ice for approximately 3 hours to induce hypothermia. Euthanized pups were then washed with iodine, rinsed with water and then submerged in 70% ethanol. Pups were removed from the ethanol and allowed to completely dry in a sterile dish. Using sterile techniques the limbs and tail of all pups were removed at the joint using scissors. Next, using a scalpel, a cut was made in the dorsal skin from the tail to the nose leaving the internal organs intact. The limb stumps were popped out and the skin was peeled off the pup from the tail over the head. Each skin was stretched flat on a sterile 15 cm dish with the dermis side facing down making sure the edges of the skin were not folded under. Finally, to allow for the separation of the dermis from epidermis, skin samples were floated on 25 mls of ice-cold 0.25% trypsin and placed at 4 ^{o}C overnight (O/N). The following morning, each skin was removed from trypsin and flattened on a dry surface of a new culture dish with epidermis down. Dermis was peeled away from the epidermis and the epidermis was placed in isolation media (HBSS (Invitrogen, Grand Island, NY) containing 4% FBS and P/S) on ice to neutralize the trypsin. Once all of the epidermis were collected, they were transferred to a 100 ml beaker containing 10 mls of fresh isolation media, on ice, and minced about 200 times into fine fragments using scissors. Next, 20 mls of media was added and the fragments were triturated through a 5 ml pipette 30-40 times and then filtered through a sterile 100 μm mesh nylon filter (Spectrum Laboratories Inc, Ca, USA). Cells were spun at 260 Xg for 10 minutes

(min) and the cell pellet was then resuspended in keratinocyte-serum free media (KC-SFM)) (Cat #-10725, Invitrogen, Grand Island, NY) containing P/S and 0.3 mM $CaCl_2$ to culture immediately or cryopreserved. For cryopreservation, cells were resuspended in KCM-SFM media containing 0.3 mM Ca^{2+} , 10% DMSO and 10% chealated FBS.

To culture KCs, tissue culture dishes were first coated with collagen solution (HBSS, 100 mg/ml BSA, 1 mM Hepes-pH 6.5, 0.03 mg/ml collagen (purified bovine collagen sol, Advanced BioMatrix Purecol) and incubated at 37 ºC for 15 min (2 mls of collagen solution was added to a 10 cm tissue culture dish). Collagen solution was completely removed and epidermal preparations (on average 1 skin was used to seed 2-3 10 cm dishes) were plated in collagen coated dishes in KC-SFM containing 0.3 mM Ca^{2+} for 6-8 hours (hrs) to allow attachment. Media was then changed to growth media containing 0.05 mM Ca^{2+} to allow for optimum KC proliferation. To induce differentiation, KCs were grown to confluence and exposed to 1.2 mM Ca^{2+} for 3 days unless indicated otherwise. Differentiated cultures were induced to dedifferentiate by lowering Ca^{2+} concentrations to 0.05 mM for 1 to 7 days (low Ca^{2+} switch). In all conditions media was changed every other day.

II.2. Proliferation Assays:

For BrdU labeling index, KCs grown on collagen coated permanox chamber slides (Lab-Tek Chamber slide system, Rochester, NY) were pulsed with 10 µM BrdU for 6 hrs. Cells were fixed in 2 % paraformaldehyde (PFA) solution for 10min at 4 ^{o}C, washed in PBS and permeablized in 0.5% triton-100 in PBS for 10 min at room temperature (RT). Next, cells were incubated with 0.5 mg/ml pepsin in 0.1 N HCL for 3 min at RT to disrupt chromatin and placed

in 1% BSA-PBS for 1 hr to block non-specific binding. Cells were then incubated with 1:500 anti-BrdU (BD Pharmingen, San Jose, CA) antibody in 0.2% BSA-PBS for at least one hour at RT. Slides were washed and incubated with 1:1000 dilution of Alexa 594-conjugated anti mouse antibody (A-11005, Molecular Probes Inc. Eugene, OR) in 0.2% BSA-PBS for 1 hr in the dark. Cells were washed and mounted with Vectashield mounting media containing DAPI (Vector Laboratories, Burlingame, CA). Pictures were taken from random areas of the slides using a fluorescent microscope and the number of BrdU-positive cells in a total of 1000 nuclei was determined and plotted as the percent of BrdU-labeled cells.

For thymidine incorporation, cells were grown in 24-well dishes. KCs were pulsed with 2.5 μCi [methyl-^{3}H] - thymidine (Specific activity=6.7 Ci/mMole, Perkin Elmer) per well for 1 hr or 16 hrs. Cells were trypsinized and each well of a 24 well dish was split into 3 wells of a 96 well dish and harvested using a semi-automatic harvester (Skatron Instrument., Lier, Norway). In parallel, one well of 24 well dish was trypsinized and counted for each condition to determine cell number. ^{3}H-Thymidine incorporation was measured using a scintillation counter and normalized for cell number.

II.3. mRNA Expression Analysis:

Total RNA was isolated from mouse epidermal cultures using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. RNA samples were then quantified and 1 μg RNA was analyzed on a 1% agarose gel to confirm RNA integrity. Relative mRNA

levels were measured by quantitative RT-PCR (qRT-PCR) or semi-quantitative RT-PCR (semi-qRT-PCR) using PGK as a house keeping gene.

For qRT-PCR, 1 μg of total RNA was first reverse transcribed using the Reverse Transcription QuantiTect kit (Qiagen, Valencia, CA). 1 μl of cDNA was amplified using Syber green Taq polymerase (QuantiTect kit, Qiagen, Valencia, CA) under the following conditions: 95 ^{o}C 15 min, 40 cycles of 95 ^{o}C 15 sec, 55 ^{o}C 30 sec, 72 ^{o}C 30 sec, 76 ^{o}C 10 sec in the Opticon2 Real Time System (MJ research, Watertown, MA) and analyzed using the Opticon Monitor analysis software. All samples were run in triplicate and no-template controls were included in each run. The RNA levels of the target genes were normalized against PGK-1 transcript levels and the comparative C_T ($2^{-\Delta\Delta CT}$) method was used for calculating relative mRNA expression. The PCR efficiencies, as determined by assaying serial dilutions of RNA, were approximately equal for the target genes and the housekeeping genes (Primers are described in Table 1).

For semi-quantitative RT-PCR, one step RT-PCR (reverse transcription followed by PCR) was carried out using 0.25 μg of total RNA using the one step RT-PCR kit (Qiagen, Valencia, CA) under the following conditions: 50 ºC 30 min, 95 ºC 15 min; 24-40 cycles of 94 ºC 30 sec, 55 ºC 30 sec, 72 ºC 1 min; and final extension at72 ºC 5 min.
PKD transcript levels following PKD1 knock down were analyzed by semi-qRT-PCR at 32 cycles for PKD1, 28 cycles for PKD2 and PKD3, and 24 cycle s for PGK. All samples were analyzed by 2 % agarose electrophoresis with ethidium bromide staining.

II.4. Genomic DNA Analysis:

Genomic DNA was isolated from the tail of C57BL/6 $PKD^{loxP/loxP}$ cKO mice by cutting about 1 cm of mouse tail using scissors. Tails were placed in a 1.5 ml centrifuge tube containing 500 µl lysis buffer (100mM Tris pH 8.0, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 µg/ml proteinase K) and rocked at 55 ^{o}C overnight. The next morning, the digested tissue was centrifuged for 10 min at 14000 Xg to pellet hair and debris. Supernatant was transferred to a new tube and equal volume (~500 µl) of isoproponal was added. The mixture was shaken vigorously to spool out DNA. With a sterile micropipette tip DNA was spooled out and transferred to a new 1.5 ml tube. The DNA was then allowed to dry for the remaining alcohol to evaporate. Finally, the genomic DNA was dissolved in 50-100 µl TE-pH 8.0 (10 mM Tris, 1 mM EDTA) rocking overnight at RT before being quantified.

Genomic DNA was isolated from cultured cells using the Allprep DNA/RNA mini kit and Qiashredder (Qiagen, Valencia, CA) according to the manufacturer's protocol.

To genotype and to confirm the presence of floxed region in PKD1 floxed mice, 200 ng of genomic DNA was amplified under the following conditions: 94 ºC 5 min; 40 cycles of 94 ºC 1 min, 58 ºC 1 min, 72 ºC 1 min; and final extension at 72 ºC for 10 min using primers described in Table 1.

To verify CRE-mediated recombination of PKD1 alleles, DNA isolated from CRE-transduced cultures were analyzed by semi-quantitative PCR using PKD^{loxP} primers. Samples were collected after 28 cycles for PKD1 and 24 cycles for PGK as control. All samples were analyzed by 2 % agarose electrophoresis with ethidium bromide staining.

II.5. Western Blot Analysis:

Cells were lysed in lysis buffer containing 50 mM TrisHCl-pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, 5 mM sodium pyrophosphate, 10% glycerol and Protease Inhibitors (Sigma-Aldrich, St. Louis, MO) for 20 min on ice. Lysates were then centrifuged for 10 min at maximum speed at 4 ^{o}C to pellet the cell debris. Protein lysates were then quantified using the Bicinchoninic Acid (BCA) protein assay kit (Pierce Thermo Scientific, Rockford, IL) according to manufacturer instructions. Equal volume of 2X SDS sample buffer (125 mM TrisHCl-pH 6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue, and 0.1% βME) was added to 30 µg of protein lysate and boiled for 5 min at 95 ^{o}C. The samples were then separated on a 10% SDS-PAGE, transferred to a 0.2 µm nitrocellulose membrane (BioRad, Hercules, CA), and blocked for 1hr in 5% nonfat dried milk in PBS containing 0.1% Tween20 (PBST). Membranes were incubated with primary antibody in either 1% milk-PBST or 5% BSA-PBST overnight at 4 ^{o}C, washed and incubated with HRP-conjugated secondary antibodies in 5% milk-PBST for 1-2 hrs. Blots were washed extensively in PBST and immunoreactive bands were detected using a chemiluminescence detection system (Pierce ECL, Thermo Fisher Scientific, Rockford, IL) after exposure to HyBlot CL film (Denville Scientific, Metuchen, NJ) or Amersham Hyperfilm (Amersham Bioscience). In some experiments, membranes were stripped using Restore Western blot stripping buffer (Pierce Thermo Scientific, Rockford, IL) and reprobed with a different antibody.

Immunoblots were quantified using the UN-SCAN-IT software (Silk Scientific Inc., Orem, Utah). PKD1 activity was quantified by dividing the signal from pPKD1-Ser^{916} by the signal from total PKD1 (sc-935). ERK1 and ERK2 activity was measured by dividing the signals for pERK1 by ERK1 and pERK2 by ERK2.

II.6. Construction of Retroviral Vectors:

Short hairpins targeting mouse PKD1, ERK1, and scrambled were designed using GeneScript siRNA target finder or by modifications of published siRNA sequences. Short hairpin oligonucleotides were first phosphorylated and annealed by setting up a phosphorylation/ annealing reaction containing 20 μM forward and reverse oligos, T4 polynucleotide kinase buffer, 10 mM ATP, and 10 U of T4 polynucleotide Kinase. The reaction was incubated at 37 ºC for 30 min followed by 95 ºC for 2 min and then slowly cooled to RT. Finally, the phosphorylated/annealed shRNA were subcloned into pSUPERretro.puro vector (Brummelkamp et al., 2002) (a gift from Dr. R Agami).

II.7. Generation of Recombinant Retroviruses:

Retroviral vectors were transfected into 293GPGs, a retroviral packaging cell line, using a calcium phosphate co-precipitation protocol to generate vesicular somatitis virus (VSVG)-pseudotyped recombinant retroviruses (Burns et al., 1993; Pear et al., 1993). The day before transfection, $5x10^6$ 293GPG cells were plated in 10 cm dishes in DMEM (Cat # 11965, Invitrogen, Grand Island, NY) containing 1 mM Hepes, 1 mM Glutamine, 10% FBS, 1 μg/ml tetracycline, 2 μg/ml puromycin, and 0.3 mg/ml G418). The following morning cells were transfected as follow: To a 15 ml polystyrene tube 0.75 mls of HBS (0.5 M Hepes/NaOH-pH 7.1, 5 M NaCl, 0.5 M KCl, 1 M dextrose, and 1 M $Na_2HPO_4.2H_2O$) and 20 μg plasmid DNA (viral vector) was added. While vortexing the DNA/HBS solution, equal volume of $CaCl_2$ was added drop wise and allowed to precipitate at room temp for 5 min. Precipitates were added drop wise to culture media in presence of 25 μM chloroquine and incubated for 8-10 hrs. Media was

removed and replaced with tet free collection media (DMEM (Cat # 11965, Invitrogen, Grand Island, NY) containing 10% FBS, 1 mM P/S and 1 mM Hepes). Virus containing media was collected starting 24-48 hrs post transfection and filtered through a 0.45 μM pore size filters and stored at 80 ºC.

II.8. Titration of Recombinant Retroviruses:

To assay viral titer, $5x10^4$ 3T3 cells were plated per well of a 6-well dish, in DMEM (Cat# 11885, Invitrogen, Grand Island, NY) containing 10% BCS, P/S, and Hepes. The next day, 3T3s were transduced with 100, 20, or 5 μl of each virus plus 8 μg/ml polybrene. Cells were incubated with virus for 3 hrs, while rocking every 30 min. After 3 hrs, virus-containing media was replaced with fresh media. Two days later, transduced cells from each well were passed at a final dilution of 10^{-3} and 10^{-4} in selection media containing 1 μg/ml puromycin. In 8-12 days, when colonies were large enough to count, cultures were washed with PBS, fixed for 5 min in methanol and stained with 0.5% crystal violet for 15 min. To determine the viral titer the number of colonies was multiplied by the dilution factor and divided by the volume of viral supernatant (mls) used.

II.9. Retroviral Transduction:

KCs were transduced at multiplicity of infection (MOI) of 2 in the presence of 8 μg/ml polybrene at three days post-seeding when cultures were highly proliferative as described above. Under these conditions between 60-80% of cells were routinely transduced (Lu and Ghazizadeh,

2005). Cultures transduced with viruses encoding shRNA were selected in 2 μg/ml puromycin starting at 36 hrs post-transduction.

In some experiments, to genetically label Ca^{2+}-resistant proliferating KCs, differentiated cultures were transduced with recombinant retroviruses encoding GFP (LZRS-GFP) at MOI of 2 for 2 consecutive days staring at 36 hrs after the high Ca^{2+} switch as described above.

II.10. Flow Cytometric Analysis:

For analysis of GFP-labeled cells, KCs were trypsinized with 0.14% trypsin, washed 2X with 1% BSA-PBS, resuspended in 500 μl of 1% BSA-PBS and analyzed by flow cytometry analysis. When cells were not analyzed immediately, they were fixed in 2% PFA and stored at 4 ºC (in the dark) until analyzed.

For flow cytometric analysis of melanocytes, epidermal cultures were trypsinized, counted, and $5x10^5$ cells were placed in a tube. Cells were washed in PBS and blocked in PBS containing 1% BSA, 5% goat serum and 0.5% sodium azide for 30 min on ice. Cells were incubated with 1:1000 anti CD117 (ckit) antibody (ebioscience Inc, San Diego, CA) or isotype control in 1% BSA/ 1% goat serum-PBS for 1 hr rocking at RT. Cells were washed 3X with 1% BSA-PBS and incubated with PE conjugated anti-rat antibody (0.5 μl for 10^6 cells, Imgenex) in the dark in the same solution for 30 min. Finally, cells were washed and resuspended in 0.5 ml 1% BSA-PBS for analysis or fixed in 2% PFA at 4 ^oC for later analysis.

II.11. Immunohistochemistry of Cultured KCs:

For IHC staining of KCs for differentiation markers, cells grown on permanox chamber slides were fixed in 2% PFA for 10 min at 4 ^oC. KCs were then rinsed in PBS and treated with 0.1 % H_2O_2 for 5 min at RT to quench endogenous peroxidase activity. Cells were permeablized with 0.2% TritonX-100 for 5 min at RT and blocked with 1% BSA in PBS for 30 min to 1 hr before incubation with primary antibody diluted in 0.2 % BSA-PBS at RT for 1 hr. Cells were washed and incubated with biotinylated secondary antibody (LinkI-1:10, Biogenex, San Ramon CA) in 0.2% BSA-PBS for 30 min followed by incubation with streptavidin conjugated tertiary (LinkII/ Label-1:10, Biogenex, San Ramon CA) for 30 min, in 0.2% BSA-PBS. Cells were incubated with 3,3' Diaminobenzidine (DAB) substrate (BD Pharmingen, San Jose, CA) until color appeared. Finally, cells were washed and mounted with Aquamont. Pictures were taken from random areas on the slide using a phase contrast microscope.

II.12. Immunofluorescent Staining of Tissue Sections:

Reconstituted skin sections were harvested and fixed in 4% PFA for 30 min at 4 ºC before embedding in OCT compound (Tissue-Tek, Torrance, CA). Frozen sections were hydrated, permeablized in 0.2% Triton X-100 for 10 min at RT, washed, and blocked in 4% milk in PBS for 1 hr at RT before being stained with a primary antibody against PKD1 (1:500, SC-935) in 0.5% milk for 1 hr at RT. Sections were washed with PBS and incubated with biotin-labeled anti-rabbit IgG (1:500) for 30-45 min at RT, followed by incubation with Alexa 594 conjugated streptavidin (1:1000, Molecular Probes, Eugene, OR) for 30 min at RT. Slides

were mounted with Vectashield mounting media containing DAPI (Vector laboratories, Burlingame, CA) and examined by a fluorescent microscope.

II.13. Chemicals:

The following chemicals and inhibitors were purchased from LC laboratories (Woburn, MA) Phorbol-12- myristate-13-acetate (TPA), Go6976 (inhibitor of classical PKC/ PKD), U0126 (inhibitor of MEK1/2), SB203580 (inhibitor of p38), LY294002 (inhibitor of PI3K), tryphostin AG490 (inhibitor of EGFR phosphorylation and Jak kinase, rapamycin (inhibitor of mTOR), Genistein (general tyrosine kinase inhibitor), and GF109203X (inhibitor of PKC). Y27632 (inhibitor of ROCK) was purchased from Ascent Scientific (Prinston, NJ) and PP2 (Src kinase inhibitor), PP3 and PKCη PS were purchased from Calboichem (La Jolla, CA).

Unless otherwise indicated, proliferative KCs were treated for a period of 8 hrs with varying concentrations of the different inhibitors at 3-4 days post-seeding when cells were highly proliferative. Differentiated KCs were treated with the inhibitors at the time of low Ca^{2+} switch to study the effects on reversion, or in some cases, were treated with the inhibitors one hour prior to the low Ca^{2+} switch and then again at the time of low Ca^{2+} switch when media was changed. In either case, inhibitors were added every time media was changed until assayed, unless indicated otherwise.

II.14. Antibodies:

Antibodies used against p63 (1:1000, SC-8431), PKD1 (1:1000, SC-935), ERK1 (1:1000, SC-94-G) and β-actin (1:1000, SC-1615) as well as anti-rabbit and anti-goat HRP conjugated antibodies (1:5000 sc-2317 and sc-2020 respectively) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for Keratin 10 (1:500, PRB-159P), Keratin 14 (1:1000, PRB-155P), loricrin (1:1000, PRB-145P), and involucrin (1:1000, PRB-140C) were from Covance (Berkeley, CA). Antibodies against PKD1 (1:1000, CS-2052), phosphorylated(p)PKD1-Ser^{916} (1:1000, CS-2051), pPKD1-$Ser^{744/748}$ (1:1000, CS-2054), pERK1/2 (1:1000, CS-9101), PKCα (1:1000, CS-2056) and pPKCα (1:1000, CS-9375) were from Cell Signaling Technologies (Danver, MA). Antibody against pPKD1-Ser^{748} (1:1000, ab17945-50) was purchased from Abcam (Cambridge, MA) and anti- cyclinD1 antibody (1:1000, 555442) was from BD Biosciences (San Jose, CA). HRP-conjugated anti-mouse antibody (1:5000, 32430) was from Pierce Thermo Scientific (Rockford, IL).

II.15. Statistical Analysis:

Statistical differences between means were measured by using the Student t-test or the one-way ANOVA and the Turkey's HSD post hoc comparison using the SPSS 17 software (SPSS, Chicago, IL). Values of $p<0.05$ were considered to have a significant difference.

Table 1. List of Primers.
List of primers used for quantitative and semi-quantitative RT-PCR, PCR and knock down of various genes.

Gene	Sequence	Expected size	Location in mRNA
p63	F 5'-CCTTATGAGCCACCACAGGT-3' R 5'-TGCATCGAGGTCTGTTTCTG-3'	497	583-602 1079-1060
K1	F 5'-GCAGCAGGTAGACACCACAA-3' R 5'-TTCTCTGCGTTGGTCCTCTT-3'	201	669-688 869-850
K10	F 5'-TGGTTACGGAGGTTTTGGAG-3' R 5'-GCATGGTCACCTTCTCGTTT-3'	227	192-211 418-399
Involucrin	F 5'-AGCCTCTGCCTTCTCCCTCC-3' R 5'-CTGCTGGTGCTCACACTTTTG-3'	279	1-14 258-278
Filaggrin	F 5'-TCAGTCCAGTGACAGCCAAG-3' R 5'-GACTCCTCCTCGCTGTGTTC-3'	426	669-688 1094-1075
Loricrin	F 5'-CGGATCGTCCCAACAGTATC-3' R 5'-CACCTCCACAGCTACCACCT-3'	340	807-826 1147-1128
PGK	F 5'-ATGAGATGATTATTGGTGGTGGA-3' R 5'-GGCTGACTTTATCCTCCGTGTTC-3'	477	822-844 1298-1276
PKD1	F 5'-CACTGTGACCTCAAGCCAGA-3' R 5'-CCAACAGACCACATGTCCAG-3'	200	2128-2147 2327-2308
PKD2	F 5'-AGAGTGCTCTCCATGCCAGT-3' R 5'-GACAGCGGGATTTCCTTGTA-3'	265	1070-1089 1334-1315
PKD3	F 5'-AATGTGCAGGGTCAAAGTCC-3' R 5'-CCCCTACTGCCATCACTGTT-3'	276	792-811 1067-1048
PKD^{loxP}	F 5'-GCCCACAGCTATTGTTCCTAA-3' R 5'-GGATAAAGTGATCAAGCAGCA-3' (between exon 11-12)	151	261215-261235 261366-261346
PKD^{KO}	F 5'-GAGCTTCACTTGGAATGACAC-3' R 5'-CCTTTAATCTGAGCACTTGG-3' (between exon 11-12 and between exon 14-15)	5346 (if not recomb.)	261257-261277 266602-266583
siRNA oligos			
PKD1shRNA1	5'-ATGCTGTGGGGGCTGGTAC-3'	-	490-506
PKD1shRNA2	5'-GAAGGAGATTTCTCATGAA-3'	-	2433-2451
C-shRNA	5'-GCAGTGGAGTTAGAAGGAGAA-3'	-	581-601
ERK1-shRNA1	5'-GAAACTACCTGCAGTCTCT-3'	-	836-854
ERK1-shRNA2	5'-GCCATGAGAGATGTTTACA-3'	-	343-364
Scrambled shRNA (no-target)	5'-TGTAGGACTTACAGAACGT-3'	-	-

Chapter III. Results

III.1. A Culture Model to Study Reversal of KC Differentiation

III.1.1. Reversal of Ca^{2+}-induced differentiation in response to a low Ca^{2+} switch in primary cultures of mKCs.

Since Ca^{2+} plays an important role in KC differentiation, to study the reversibility of commitment to differentiation, the response of differentiated KCs to a low Ca^{2+} switch was examined over a period of time. Mouse KCs were isolated from the skin of new born FVB mice and cultured in media containing 0.05 mM Ca^{2+} (low Ca^{2+} conditions; assigned here as proliferating cultures; P). As depicted in Figure 3A, when cultures reached confluency, Ca^{2+} concentrations were increased to 1.2 mM for 3 days (high Ca^{2+} conditions; designated as D3) to induce differentiation. Subsequently, Ca^{2+} concentrations were switched back to 0.05 mM (assigned here as reverted cultures; R) and cultures were analyzed periodically for morphological changes and proliferation rate. As expected, increasing Ca^{2+} concentrations induced a drastic change in cellular morphology, i.e. from small, angular cells to large, flat and polygonal shaped cells (Fig. 3B-D3). Interestingly, decreasing Ca^{2+} in differentiated cultures resulted in heterogeneous changes in morphology within 2 to 4 days post low Ca^{2+} switch. While about 30% of cells detached from culture, the majority of cells reverted from a differentiated phenotype toward a proliferative, basal cell-like phenotype (Fig. 3B-R2 and R4). By day 7 cultures were fully repopulated with the basal cell-like KCs (Fig. 3B-R7), suggesting re-initiation of proliferation in the reverted cultures.

Analysis of DNA synthesis in KCs cultures in response to fluctuations in Ca^{2+}_o by either BrdU labeling or ^{3}H-thymidine incorporation demonstrated induction of growth arrest in more

than 97% of differentiated KCs. Following the low Ca^{2+} switch however, there was a significant and steady increase in proliferation rate as early as 2 days after the low Ca^{2+} switch (R2). By R7, the rate of proliferation was similar to that of proliferating cultures (P) (Fig. 3C).

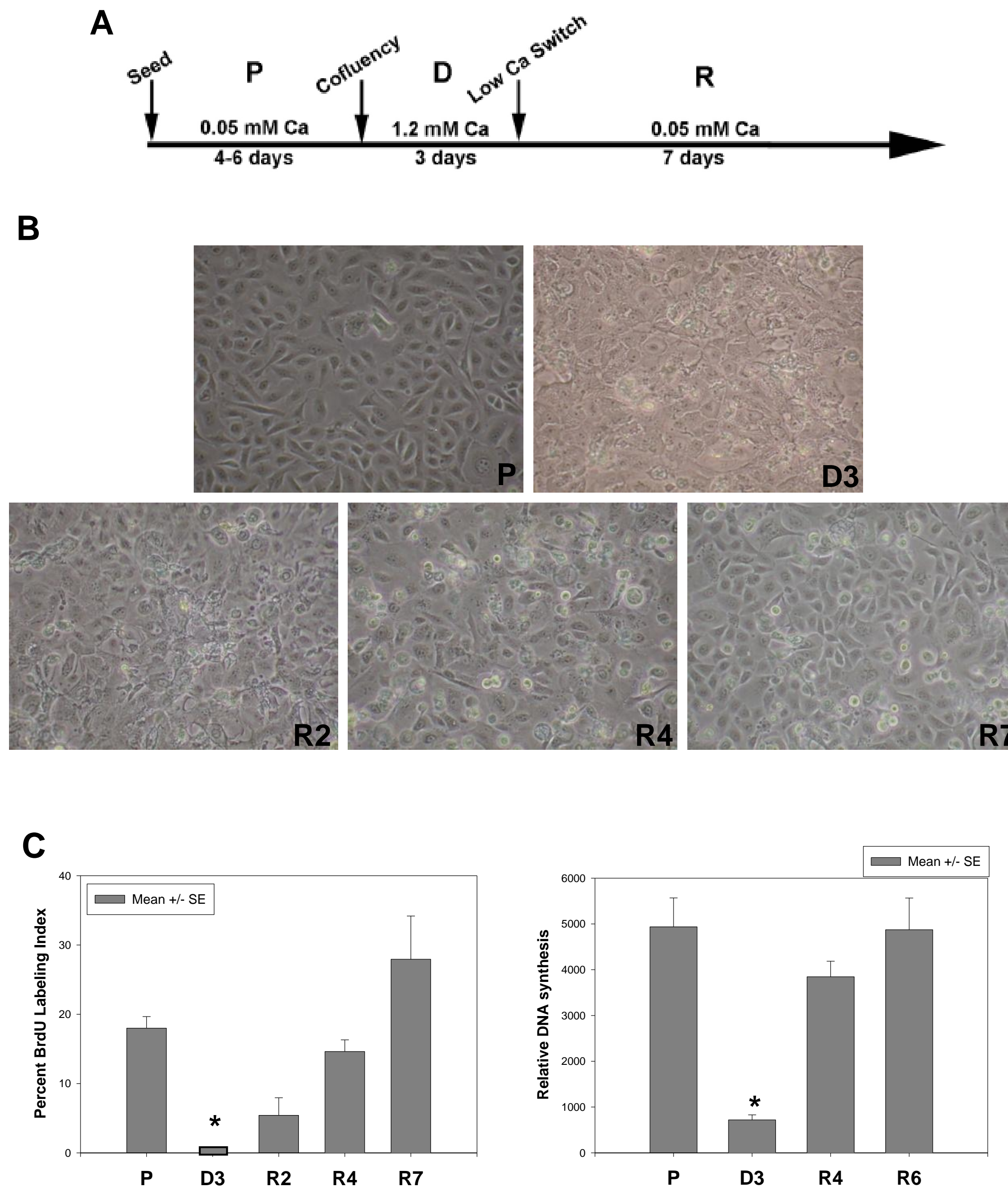


Figure 3. Reversal of differentiated morphology and re-initiation of DNA synthesis in cultures exposed to low Ca^{2+} conditions.
A) Schematic representation of KC culture conditions used in the study. **B)** Phase contrast images demonstrating morphology of FVB KCs grown in proliferative (P), 3 day differentiated (D3), and reverted cultures at 2 (R2), 4 (R4), and 7 days (R7) post low Ca^{2+} switch. **C)** Proliferation rates of various culture conditions demonstrated by BrdU labeling index (6 hr pulse) and ^{3}H-Thymidine incorporation (16 hrs pulse). For both BrdU labeling and ^{3}H-Thymidine incorporation in proliferative (P) cultures cells were pulsed 3 to 4 days post seeding when cells were highly proliferative, 3 days after Ca^{2+}-induced differentiation (D3), and 2, 4 and 7 days following the low Ca^{2+} switch for reverted cultures. *P value < 0.01 when comparing D3 to P or R. Values are expressed as mean +/- SE.

III.1.2. Contamination of epidermal cultures with melanocytes.

To confirm that cells in reverted cultures were indeed KCs, cultures exposed to low calcium conditions for 5 days (R5) were immunostained for K14 (a marker of KCs) and analyzed by immunohistochemistry. The staining demonstrated that while the majority of cells in culture were positive for K14 expression (Fig. 4A) some cells did not express K14 (indicated by arrows in Fig. 4A). Primary epidermal cultures normally consist of 3-5% melanocytes (Lu and Ghazizadeh, 2005). Immunohistochemical analysis of reverted cultures with an antibody against cKit (a marker for melanocytes) confirmed the identity of the K14-negative cells as melanocytes (Fig. 4A, open arrows). To quantify the extent of melanocyte contamination, proliferative, 3 day differentiated, and cultures reverted for 7 days (P, D3, and R7 respectively) were immunostained for cKit and analyzed by flow cytometry. These data indicated that both the proliferative and differentiated cultures contained approximately 7% melanocytes which increased to 36% in R7 cultures (Fig. 4B).

To restrict our analysis to KCs and to determine whether the melanocyte contamination may have contributed to changes observed in culture, epidermal cells were isolated from c-Kit$^{w/sh}$ mice which are deficient in melanocytes (Cable et al., 1995) and grown as described above. Similar to what was observed in FVB mice, when differentiated epidermal cultures were exposed to low Ca^{2+} conditions, cells regained basal cell-like morphology (Fig. 5A) and were able to overcome growth arrest shown by both BrdU labeling and ^{3}H-thymidine incorporation (Fig. 5B). These data demonstrated that morphological changes as well as re-initiation of proliferation in culture were attributed to KCs and were not affected by melanocytes. Similar results were observed using KCs isolated from a third strain of mice (C57BL/6) indicating that the

reversibility of commitment to differentiation is not strain dependent. To simplify our analysis however, for the remaining experiments KCs were isolated from c-Kit$^{w/sh}$ mice.

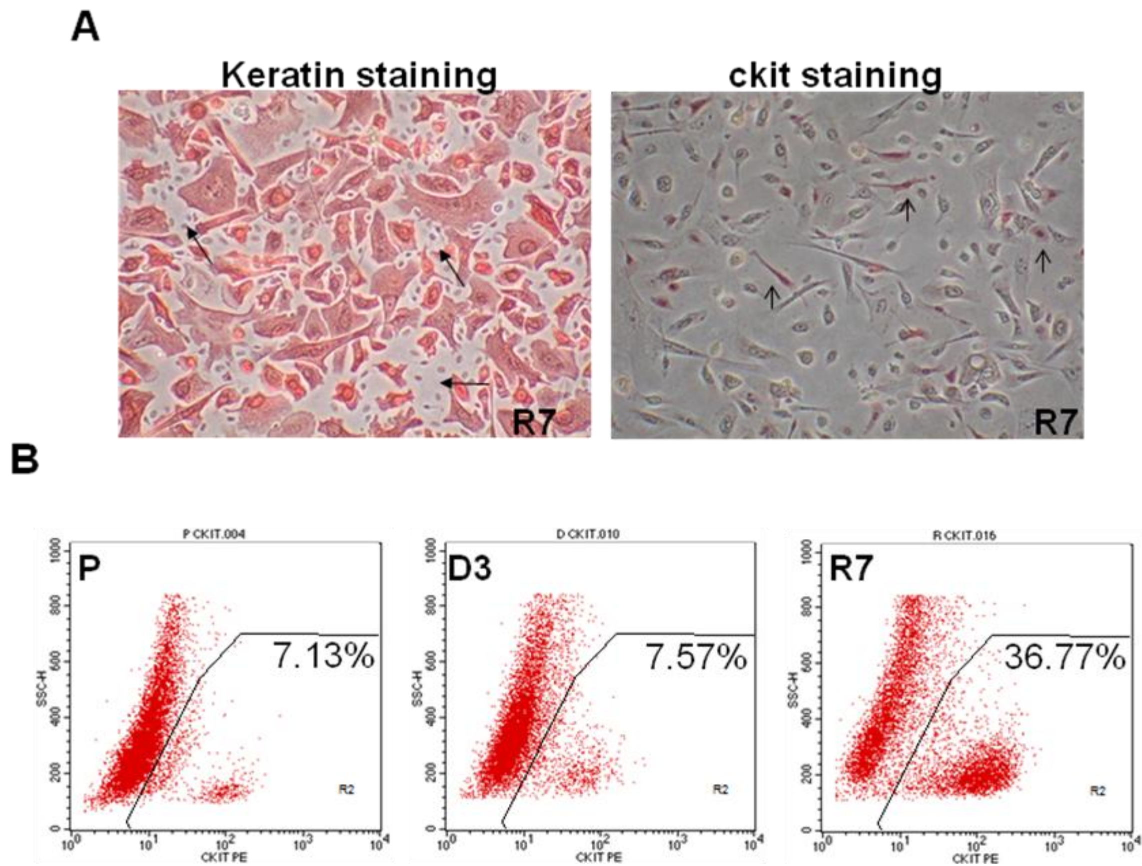


Figure 4. Persistence of melanocytes in reverted epidermal cultures isolated from FVB mice.

A) Immunostaining of 7 day reverted cultures with K14 and ckit indicates the presence of KCs and melanocytes, respectively. **B)** Proliferative (P), Differentiated (D3) and reverted (R7) KCs were stained for ckit and analyzed by flow cytometry. The percent of ckit positive cells are indicated on the top right corner of the graphs.

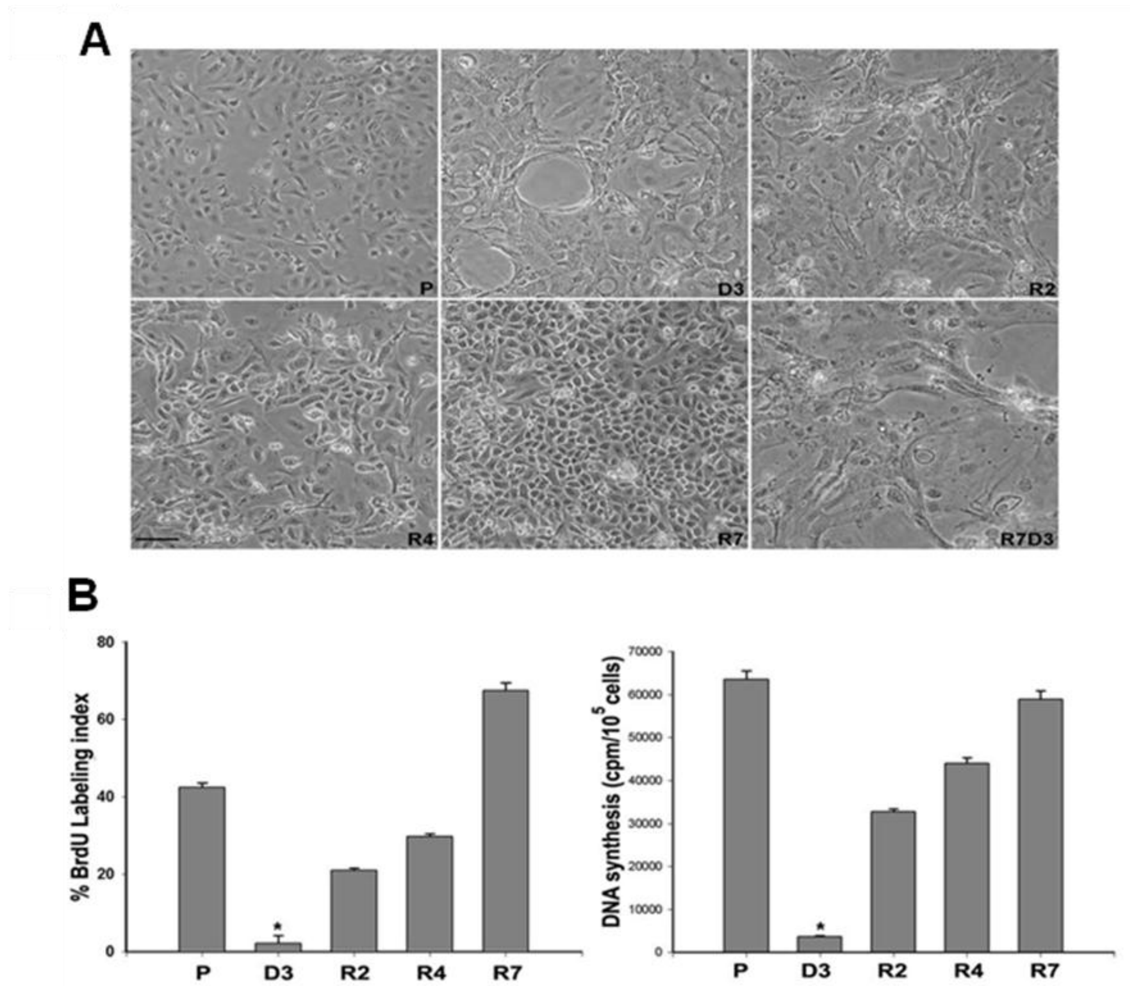
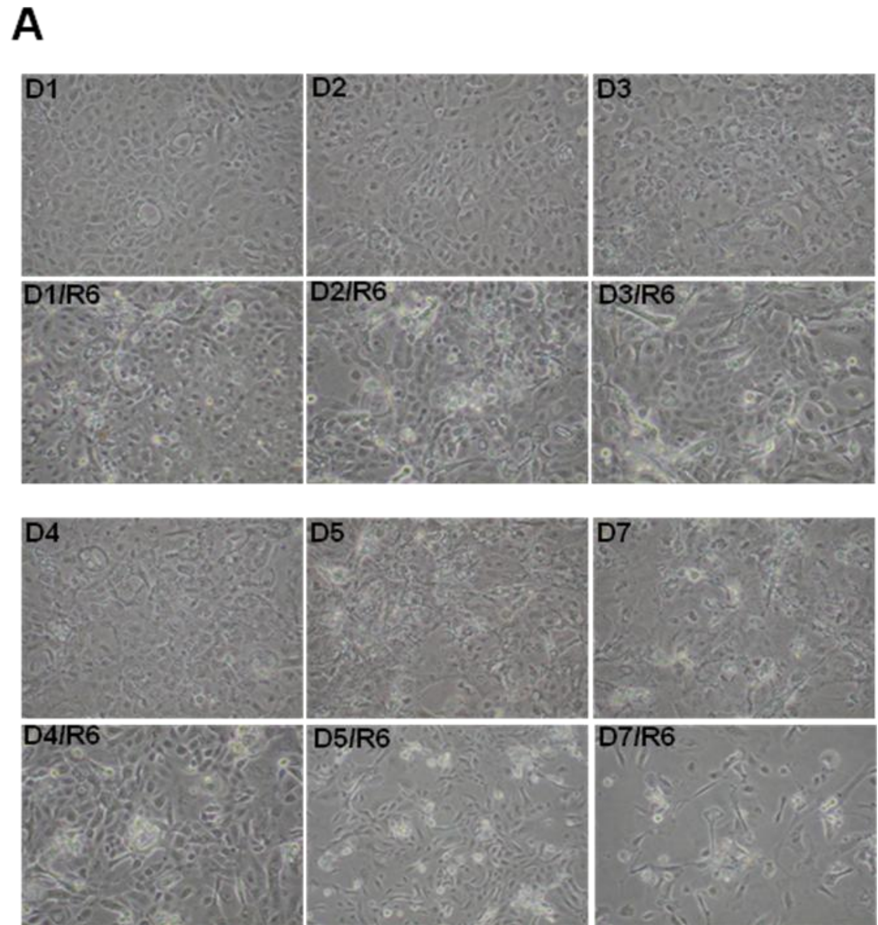


Figure 5. Reversal of Ca^{2+}-induced differentiation in epidermal cultures devoid of melanocytes.

A) Phase contrast images of mKCs isolated from c-Kit$^{w/sh}$ mice in P, D3, R2, R4 and R7, as well as R7 cultures induced to differentiate for a second time for 3 days (R7D3) showing morphological changes in response to Ca^{2+} fluctuations. **B)** Proliferation rate of KC cultures in response to fluctuations in Ca^{2+} measured by BrdU labeling or by ^{3}H-Thymidine incorporation. *P value < 0.001 when comparing D3 to P or R. Values are expressed as mean +/- SD of 3 independent experiments.

III.1.3. The extent of the reversibility of Ca^{2+}-induced KC differentiation.

To determine if the extent of time KCs were maintained in high Ca^{2+} had any effect on their response following the low Ca^{2+} switch, KCs were maintained in high Ca^{2+} for various periods of time before switching to low Ca^{2+}conditions. Analysis of DNA synthesis at various days after Ca^{2+}-induced differentiation demonstrated a progressive decrease in proliferation rate, reaching maximum growth arrest by 3 days (Fig. 6B-D3-black bar). Interestingly, in response to the low Ca^{2+} switch, KC cultures differentiated for up to 5 days retained their ability to re-initiate DNA synthesis and resume a proliferative phenotype (Fig. 6A and B-D5-graybar) but with slightly less efficiency. However, after 7 days in high Ca^{2+}, differentiated KCs lost their ability to re-initiate DNA synthesis. These data indicated that although increasing Ca^{2+}_o resulted in striking morphological changes and growth arrest within 3 days, the commitment of the majority of KCs to differentiation was reversible for a longer period of time. However, the ability to overcome growth arrest and de-differentiate was eventually lost.



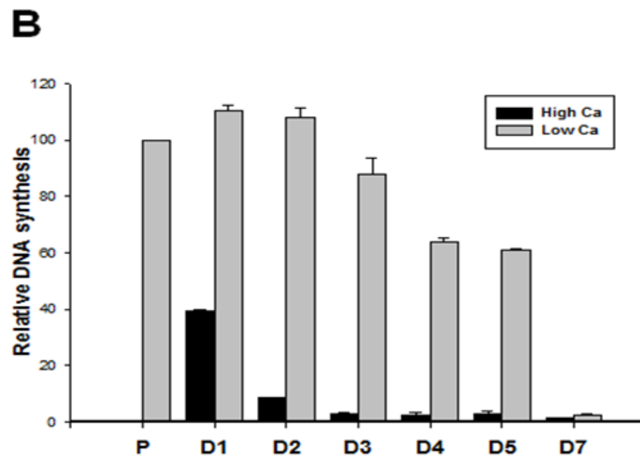


Figure 6. The extent of the reversibility of Ca^{2+}-induced differentiation.
A) Phase contrast photographs of cultures of mKCs differentiated for 1 to 7 days (D1-D7) demonstrating morphology before (Differentiated) or after switching to low Ca^{2+} for 6 days (R6).
B) Relative DNA synthesis measured by ^{3}H-Thymidine incorporation of KCs induced to differentiate for 1 to 7 days before (black bars) or after switching to low Ca^{2+} for 5 days (gray bars). Values are expressed as mean +/- SE.

III.1.4. Biochemical characterization of KC cultures in response to the low Ca^{2+} switch.

Analysis of epidermal markers of proliferation and differentiation by quantitative real time RT-PCR and western blot analysis indicated down-regulation of p63, a KC proliferation marker (Parsa et al., 1999), and up-regulation of differentiation markers K10, involucrin, filaggrin, and loricrin following Ca^{2+}-induced differentiation (Fig. 7A and B) (Dale et al., 1983; Mehrel et al., 1990; Sizemore et al., 1993; Watt, 1983). Within 4 days post low Ca^{2+} switch, when reversion to the proliferative phenotype is more homogeneous, this pattern was reversed for all markers with the exception of involucrin. Involucrin expression (both transcript and protein levels) remained high even after 7 days in low Ca^{2+} conditions when roughly the entire culture displayed basal cell-like morphology and had high proliferative indices (Figures 7A and B).

Furthermore, reverted cultures retained their ability to differentiate when exposed to high Ca^{2+}conditions for a second time as demonstrated by the reappearance of the large flat cells (Fig. 5A-R7D3), down-regulation of p63 and up-regulation of loricrin levels (Fig. 7C). Therefore, although KCs in reverted cultures were not transcriptionally equivalent to the original KCs in proliferating cultures, their capacity to respond to Ca^{2+}-induced differentiation had not changed.

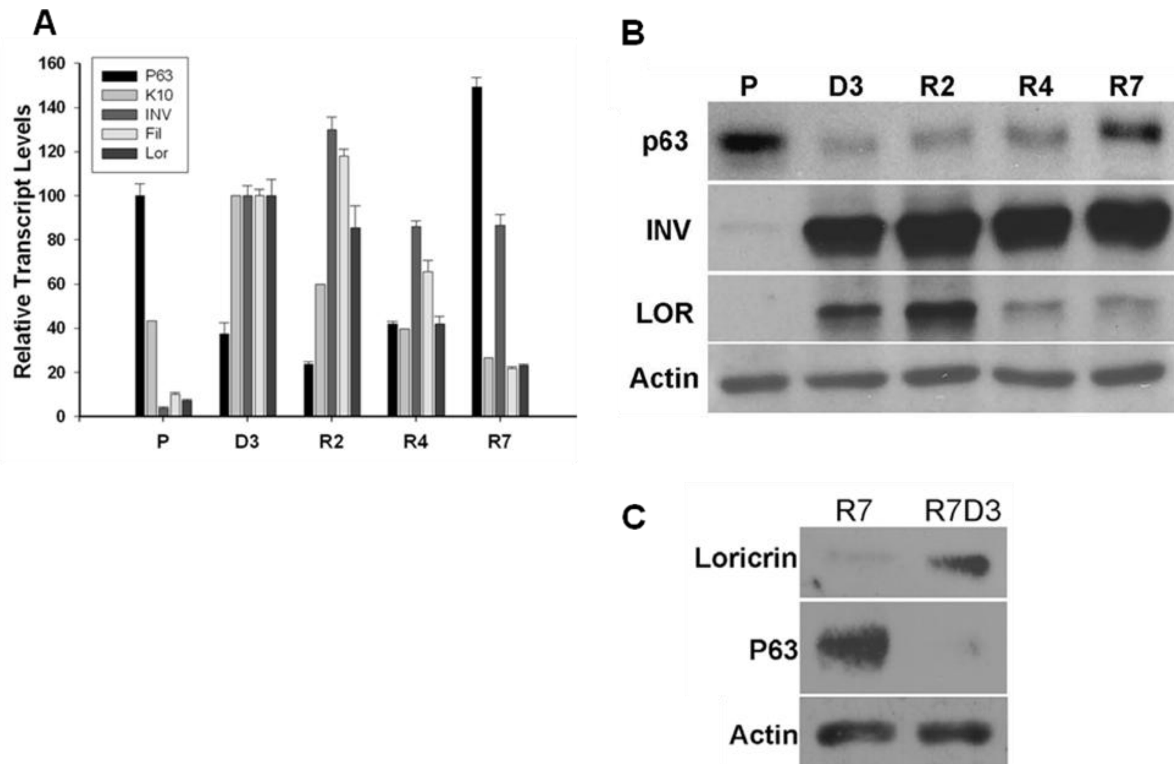


Figure 7. Analysis of proliferation and differentiation markers during KC reversion. RNA and protein lysates were collected for proliferative (P), 3 day differentiated (D3), and 2 (R2), 4 (R4), and 7 (R7) days following the low Ca^{2+} switch. **A)** Quantitative RT-PCR analysis of the transcript levels of p63, Keratin 10 (K10), Involucrin (INV), Filaggrin (FIL), and Loricrin (LOR). Transcript levels were normalized to phosphoglycerate kinase (PGK) levels. **B)** Western blot analysis using antibodies against p63, INV, LOR and actin (loading control). **C)** Western blot analysis of protein lysates prepared from reverted cultures at day 7 (R7) and 3 days after a subsequent Ca^{2+}-induced differentiation (R7D3) using antibodies against p63, LOR and actin. Result representative of 2 independent experiments.

III.1.5. Immunohistochemical analysis of early and late differentiation markers in differentiated and reverted cultures.

To gain insight into the proportion of cells that undergo Ca^{2+}-induced differentiation, and to determine what percent of KCs remained positive for differentiation markers after the subsequent low Ca^{2+} switch, differentiated and reverted cultures were immunostained for early (INV) and late (LOR) markers of differentiation and analyzed by immunohistochemistry. As shown in Figure 8, in response to exposure to high Ca^{2+} almost the entire culture was stained for INV (Fig. 8A). This was consistent with previous work in our lab using flow cytometry analysis (Mannik et al., 2010). However, despite the high levels of LOR detected in western blot analysis of differentiated cultures, only about 30 to 40% of KCs were stained with LOR (Fig. 8D). Upon lowering Ca^{2+} concentrations in differentiated cultures the number of LOR-expressing KCs were gradually decreased where by 4 days post low Ca^{2+} switch (R4) no LOR-expressing cells were detected (Fig. 8F, please note that nuclear staining is non-specific). Interestingly, similar to the western and RT-PCR results, INV expression levels did not change with lowering Ca^{2+} (Fig. 8A-C). By R4, the majority of the culture still remained positive for INV (Fig. 8C). These results indicated that although all cells had committed to differentiation (express INV), only 30-40% were terminally differentiated (expressed markers of late differentiation).

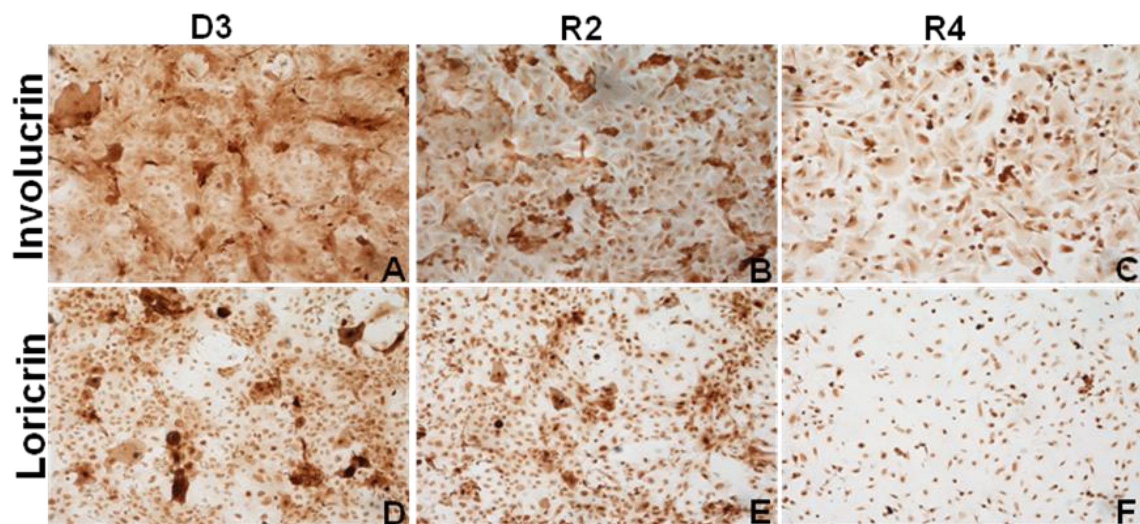


Figure 8. Immunohistochemical analysis of differentiation markers in differentiated and reverted KC cultures.

A-F) Phase contrast images of differentiated (D3), as well as reverted cultures at 2 and 4 days following the low Ca^{2+} switch (R2 and R4) immunostained with involucrin, as an early marker of differentiation or with loricrin, as a late marker of differentiation indicating the extent of KCs expressing these proteins. Results are representative of at least 3 independent experiments.

III.1.6. Differentiation-resistant KCs do not contribute to the repopulation of differentiated cultures in response to low Ca^{2+}conditions.

Morphological changes in the reverted culture and a relatively rapid repopulation of culture by proliferating cells were suggestive of de-differentiation. However, although more than 97% of KCs exit cell cycle upon increasing Ca^{2+} and express INV (Mannik 2010), 2-3% of KCs appear to be resistant to Ca^{2+}-induced growth arrest. Therefore, the possibility of a small population of proliferating KCs repopulating cultures upon low Ca^{2+} switch was investigated. To test this possibility, we took advantage of the property of retroviruses that can only infect replicating cells (Miller et al., 1990) to selectively label proliferating cells in differentiated cultures. Thus, differentiated cultures were transduced with a retroviral vector encoding GFP at a multiplicity of 2 for two consecutive days starting 36 hours after switching to high Ca^{2+}. The percentage of GFP-labeled cells was analyzed by flow cytometry either before or 7 days after switching to low Ca^{2+}. As a control, proliferating cultures of KCs were also transduced with GFP-encoding retroviral vector before differentiation and the subsequent low Ca^{2+} switch to demonstrate high efficiency and persistent labeling of proliferating cells. As shown in Figure 9, while about 90% of KCs in proliferating cultures (P-GFP and P-GFP-R7) were labeled with GFP, only about 3% of cells in differentiated cultures were transduced (D-GFP). These data confirmed the BrdU labeling indices, indicating the presence of a small population of proliferating cells in differentiated culture. The percentage of GFP-positive cells following exposure to low Ca^{2+}conditions for 7 days (D-GFP-R7), however, remained at 3%. A lack of a significant expansion of Ca^{2+}- resistant proliferative cells following exposure to low Ca^{2+} (D-GFP-R7) indicated that these cells could not have caused the repopulation of cultures by the proliferating KCs.

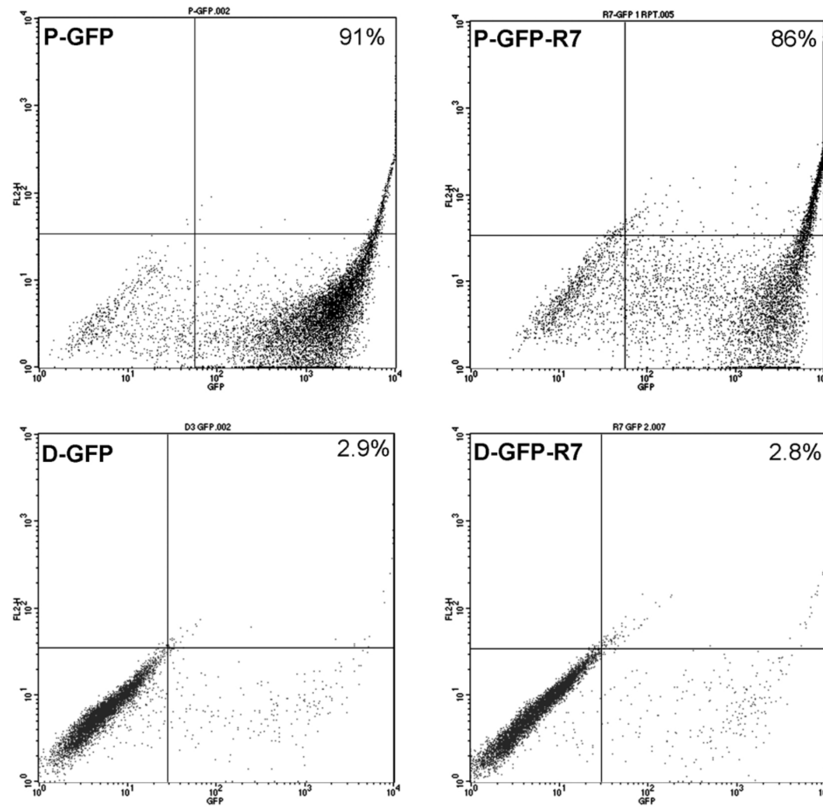


Figure 9. Differentiation-resistant KCs do not account for repopulation of differentiated cultures in response to the low Ca^{2+} switch.
Flow cytometric analysis of differentiated cultures of mKCs transduced with a retroviral vector encoding GFP for 2 consecutive days before (D-GFP), or after the low Ca^{2+} switch (D-GFP-R7). Proliferative KCs were transduced with GFP and analyzed 48 hrs post-transduction (P-GFP) or after differentiation and the subsequent exposure to low Ca^{2+} conditions (P-GFP-R7). The percent of GFP positive cells are indicated in the top right corner of the graphs. Results are representative of 3 independent experiments.

III.1.7. Quiescent KCs do not contribute to the repopulation of differentiated cultures in response to the low Ca^{2+}switch.

Another potential source of proliferating cells in reverted cultures may be attributed to quiescent cells in differentiated cultures. In culture, confluency usually induces quiescence but can be reversed by removal of contact inhibition when cells are sub-cultured (Coller et al., 2006). To examine this possibility, differentiated cultures were trypsinized and sub-cultured at 20,000 or 40,000 cells /cm^2 in low Ca^{2+} conditions. Cells were counted after one day to determine plating efficiency or after 4 days to measure growth. As a control, confluent, undifferentiated cultures were sub-cultured at 20,000 cells /cm^2. Our results indicated that while the majority of sub-cultured undifferentiated KCs attached and proliferated, differentiated KCs, at either density, were unable to attach and proliferate (Fig. 10). These data argued against a significant contribution from quiescent undifferentiated cells during reversion of differentiated cultures.

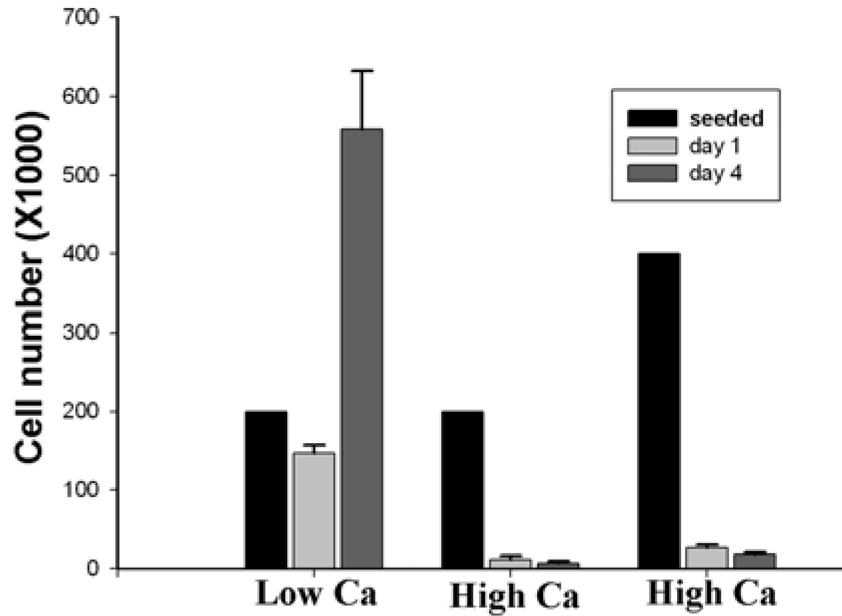


Figure 10. Quiescent undifferentiated KCs do not account for repopulation of reverted cultures.
Confluent cultures of KCs maintained in proliferative (low Ca^{2+} conditions) or differentiated (high Ca^{2+} conditions) conditions for 3 days were re-plated at 20,000 and 40,000 cells/ cm^2. Graph depicts the number of cells at the time of seeding, or at 1 (day 1), or 4 days (day 4) post plating. Values are expressed as mean +/- SE from 2 independent experiments.

III.1.8. Induction of a mitogenic response in response to the low Ca^{2+} switch.

The lack of repopulation of KC cultures in response to low Ca^{2+} by differentiation-resistant KCs or quiescent KCs suggested re-initiation of proliferation in differentiated KCs. To investigate the timing of cell cycle re-entry, cyclin D1 levels in response to changes in Ca^{2+} concentrations were examined. Cyclin D1 is up-regulated in the G1 phase of the cell cycle and regulates G1/ S transition (Sherr, 1995). As expected, cyclin D1 levels were high in proliferative cultures and completely repressed in differentiated cultures. In reverted cultures however, de-repression of cyclin D1 was evident as early as 3 hrs after the low Ca^{2+} switch and progressively increased to levels comparable to that of proliferative KCs within 4 days (Fig. 11). The fairly rapid re-emergence of cyclin D1 suggested activation of mitogenic signaling pathways prompted by the reduction of Ca^{2+}_o levels.

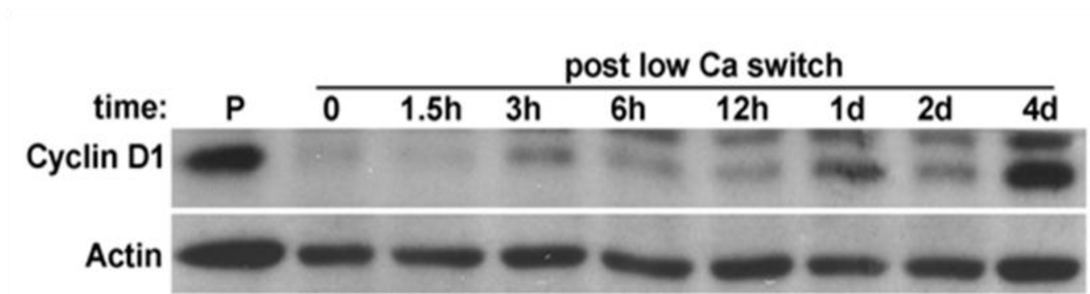


Figure 11. De-repression of cyclinD1 in response to the low Ca^{2+} switch.
Western blot analysis of KC protein lysates collected for P (proliferative), 3 day differentiated (time 0) and at indicated time points following the low Ca^{2+} switch using antibodies against cyclin D1 and actin (as loading control).

III.1.9. Conclusion of III.1.

In conclusion, these data demonstrated reprogramming of KCs in response to lowering Ca^{2+} concentrations, where by differentiated KCs de-differentiate to a less differentiated state as shown by de-repression of cyclinD1, re-initiation of DNA synthesis, and up-regulation of proliferation markers and down-regulation of differentiation markers.

III.2. Mechanism of Re-initiation of Proliferation in Differentiated KCs

III.2.1. Identification of signaling pathways activated by the low Ca^{2+} switch.

To gain insight into the mechanism of re-initiation of proliferation in differentiated KCs, a panel of chemical inhibitors of various signaling pathways involved in KC proliferation and differentiation were used to inhibit this process. Proliferative KCs grown in low Ca^{2+} were used as control to assess toxicity and effects of inhibitors on normal proliferation. Cultures were treated with inhibitors of the PI3Kinase pathway (LY29400), classical Protein kinase C (Go6976), p38 MAPK (SB203580), EGFR-Phosphorylation and Jak kinase (AG490), MEK1/2 (U0126), mTOR (rapamycin), or protein tyrosine kinases (Genistein) at various concentrations and the ability of differentiated KCs to resume proliferation was assessed (Table 2). Of the inhibitors, LY29400, SB203580, and rapamycin had no effect on either proliferation or re-initiation of proliferation. Treatment of proliferative cultures with AG490 and Genistein blocked proliferation and therefore could not be used to evaluate re-initiation of proliferation. Two inhibitors appeared to specifically block morphological reversion of differentiated KCs; Go6976 and U0126 (Fig. 12A) suggesting involvement of PKC and MAPK-ERK signaling pathways in this process.

To verify the inhibitory effects of Go6976 and U0126 on re-initiation of proliferation, proliferative or differentiated cultures of KCs (at the time of the low Ca^{2+} switch) were treated with increasing doses of Go6976 and U0126 and the proliferation rate was measured by ^{3}H-thymidine incorporation. Treatment of cells with Go6976 significantly inhibited re-initiation of proliferation at concentration ranging from 100 nM to 1 μM in a dose dependent manner (Fig. 12B-gray bars (R5)). At these concentrations there was no significant effect on

KC proliferation as they continued to grow in the presence of the inhibitor (Fig. 12B-black bars (P)). Treatment of cells with U0126 resulted in inhibition of proliferation and re-initiation of proliferation in a dose dependent manner. At concentrations of 1 μM and lower, U0126 had little effect on the proliferation rate of proliferating cultures, while it significantly inhibited re-initiation of proliferation and morphological reversion in differentiated cultures (Fig. 12C), suggesting involvement of MEK-ERK signaling in this process.

Table 2. Summary of the effects of pharmacological inhibitors of signaling pathways on morphological reversion in response to low Ca^{2+} switch.

KCs were treated with the indicated inhibitors at the time of the low Ca^{2+} switch. The effective doses of inhibitor that demonstrated an effect on KC proliferation and/ or reversion are indicated in parenthesis.

Inhibitor	Signaling Pathway	Effect on normal proliferation	Effect on re-initiation of proliferation in differentiated KCs
Rapamycin (<10 nM)	mTOR	No	No
Go6976 (>100 nM)	Classic PKCs (PKCαβγ) and PKD	No	Yes/ Inhibit
U0126 (1 μM)	MEK1/2	No	Yes/ Inhibit
AG490 (>10 μM)	EGFR phosphorylation and Jak Kinases	Yes/ Inhibit	ND
LY 294002 (< 20μM)	PI3K	No	No
SB203580 (<100 μM)	p38 and MAPK	No	No
Genistein (>10 μM)	Tyrosine Kinase	Yes/ Inhibit	ND

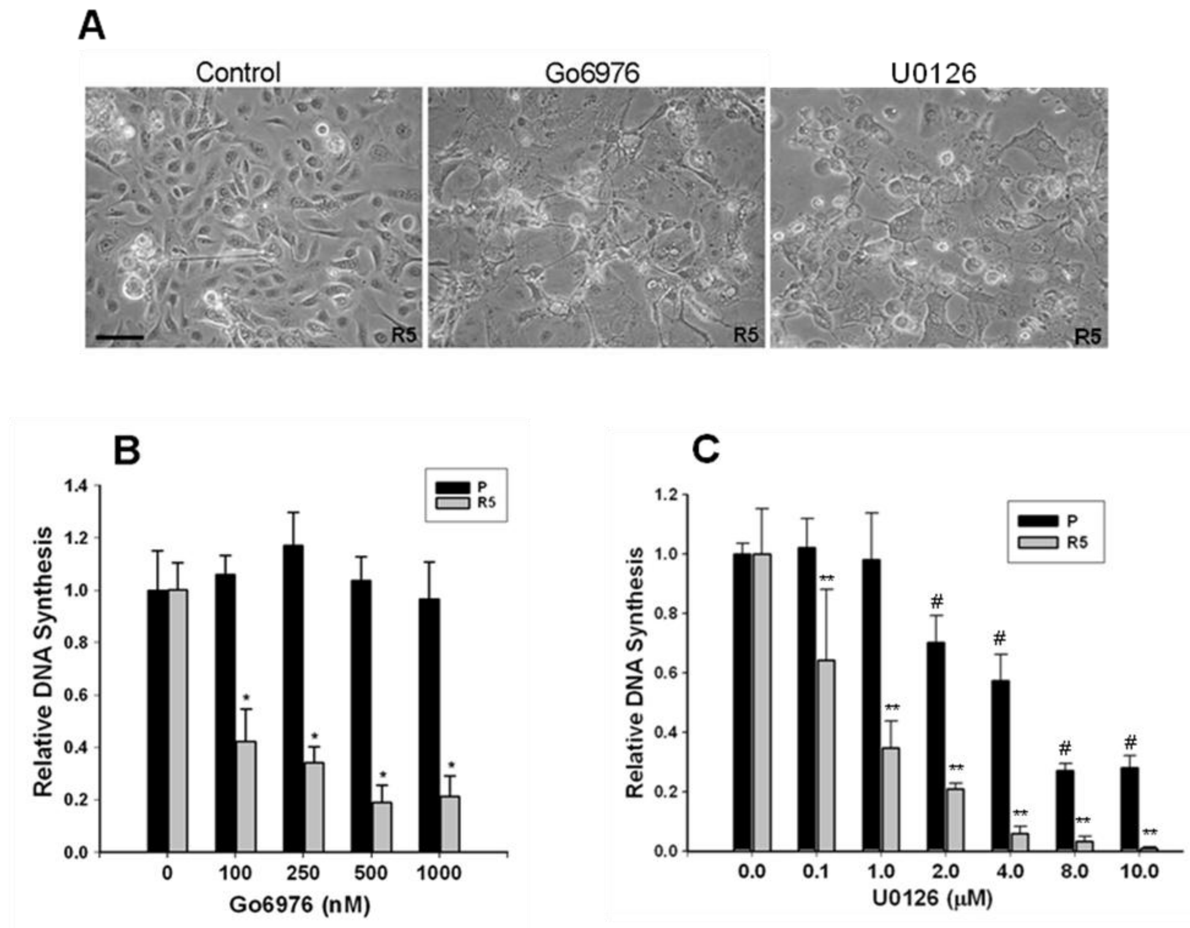


Figure 12. Go6976 and U0126 inhibit re-initiation of proliferation and morphological reversion in differentiated KCs.

A) Phase contrast images of R5 cultures treated with DMSO (control), 1 μM Go6976, and 4 μM U0126 at the time of the low Ca^{2+} switch. **B & C)** Relative DNA synthesis measured by ^{3}H-thymidine incorporation in proliferative (P-black bars) and 5 day reverted (R5- gray bars) cultures treated with varying concentrations of Go6976 (B) and U0126 (C). Values are expressed as mean +/- SD of 3 independent experiments. * P value < 0.01 in Fig B. ** P value < 0.00002 when comparing P and # P value < 0.01 when comparing R in Fig C.

III.2.2. Role of PKD in re-initiation of proliferation in differentiated KCs

III.2.2.1. PKD is the target of Go6976 inhibition of re-initiation of proliferation in KCs.

Of the calcium dependent PKCs that are targeted by Go6976, KCs only express PKCα (Denning et al., 1995). Although Go6976 is a selective inhibitor of PKCα (K_i=2.5 nM), at higher concentrations it has been shown to effectively inhibit PKD (K_i=20 nM). To determine which enzyme is the target of Go6976, the effect of GF 109203X (GF1; also known as Bisindolyleimide I), a general inhibitor of PKCs (K_i=10 nM for PKCα) that does not directly inhibit PKD (Zugaza et al., 1996) on re-initiation of proliferation was analyzed. As shown in Figure 13A and B contrary to the effects of Go6976, treatment with GF1, even at doses as high as 5 μM, had no significant effect on the rate of proliferation or morphological changes in reverted cultures. These results suggested a role for PKD in regulating re-initiation of proliferation in differentiated KCs.

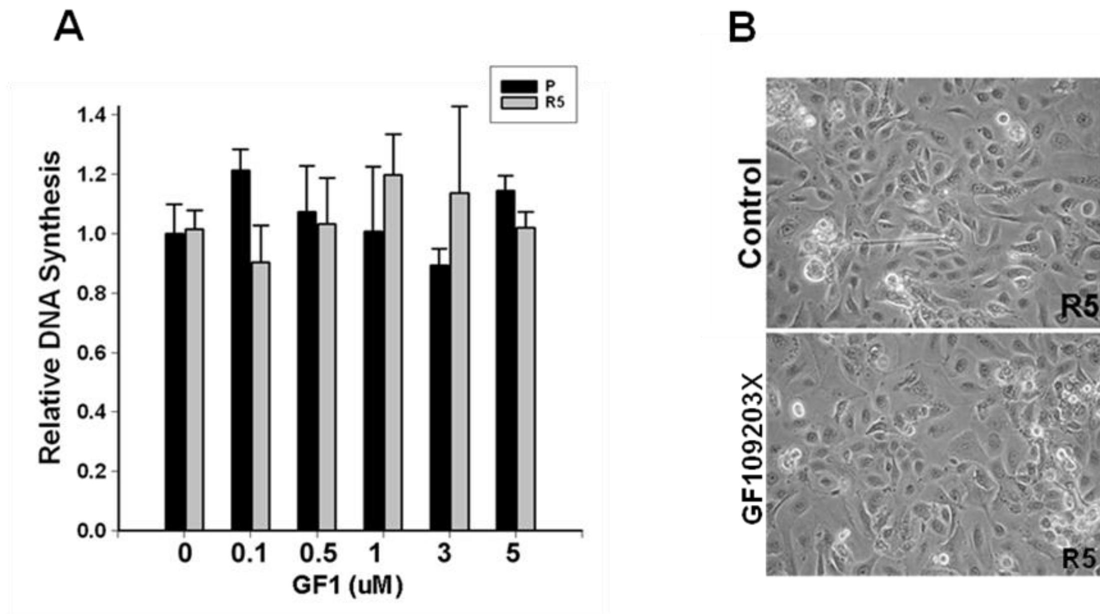


Figure 13. PKD is the target of Go6976 inhibition of re-initiation of proliferation in differentiated KC.
A) Relative DNA synthesis measured by ^{3}H-thymidine incorporation for proliferative (P-black bars) and reverted (R5-gray bars) KCs treated with varying concentrations of GF1. Values are expressed as mean +/- SD of 3 independent experiments. **B)** Phase contrast images of R5 cultures treated with DMSO (control) or 1 µM GF109203X.

III.2.2.2. PKD1 expression is up-regulated in differentiated mouse KCs.

PKD1 is a pro-proliferative enzyme that has been implicated in a variety of cellular processes (Jaggi et al., 2007). To assess the expression of PKD1 in normal proliferative and differentiated cultures of mKCs, PKD1 levels in these cultures was investigated by western blot analysis. Interestingly, contrary to previous reports where PKD1 levels were shown to decrease in response to Ca^{2+}-induced differentiation (Ernest Dodd et al., 2005; Rennecke et al., 1999), analysis of PKD1 levels in response to Ca^{2+}-induced differentiation using 2 different antibodies against PKD1 demonstrated a significant up-regulation of PKD1 levels (Fig. 14A). Consistent with KC cultures, immunostaining of mouse skin sections with anti-PKD1 antibody confirmed higher PKD1 levels in suprabasal layers of epidermis (Fig. 14B). Additionally, immunostaining of skin grafts expressing PKD1-GFP fusion protein verified the specificity of the antibody (Fig. 14B).

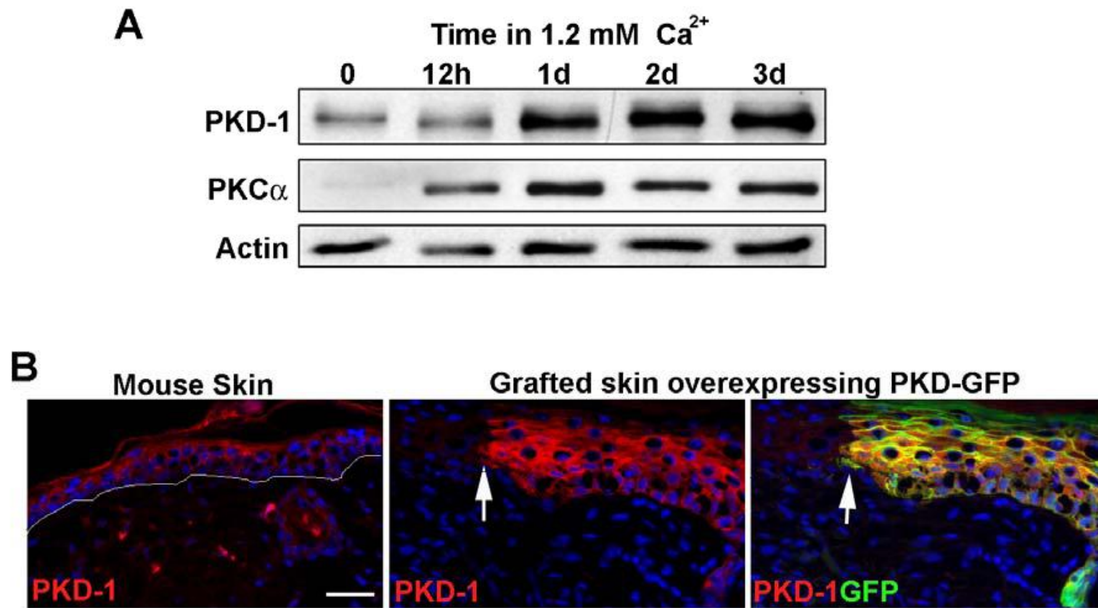


Figure 14. Up-regulation of total PKD1 levels in differentiated KCs.
A) Western blot analysis of total PKD1 and PKCα levels in proliferative (0), and differentiated cultures at 12hrs (12h) 1, 2, and 3 days after Ca^{2+}-induced differentiation (1d, 2d, 3d). Actin was used as a loading a control. **B)** Immunofluorescent staining for PKD1 in cryosections prepared from normal adult mouse skin or from skin regenerated from mKCs over-expressing hPKD1-GFP fusion protein. PKD1 staining is in red and GFP in green. The line shows the location of basement membrane and the arrow indicated the edge of the graft.

III.2.2.3. Kinetics of PKD1 activation in response to the low Ca^{2+} switch in differentiated cultures of KCs.

PKD is a signaling enzyme that is activated *in vivo* by a phosphorylation dependent mechanism (Zugaza et al., 1996). To determine the kinetics of PKD1 activation during re-initiation of proliferation, total and phosphorylated PKD1 levels were analyzed in response to Ca^{2+}-induced differentiation and at different time points following the subsequent switch to low Ca^{2+} conditions. As shown in Figure 15, PKD1 levels increased in response to Ca^{2+}-induced differentiation and remained elevated for at least 7 days after the low Ca^{2+} switch when all KCs resumed a proliferative state (Fig. 15A). Analysis of PKD1 activity however, showed a complex kinetics. In response to Ca^{2+}-induced differentiation, PKD1 remained predominantly inactive as shown by only a minor increase in PKD1 autophosphorylation despite the 6 fold increase in total PKD levels (Fig.15A). Exposure of differentiated KCs to the low Ca^{2+} conditions resulted in a rapid and transient PKD1 phosphorylation within the first 5 minutes (Fig. 15B). This was followed by a second transient increase in PKD1 phosphorylation (16 fold) by 3 hrs, which returned to basal levels by 12 hrs. Finally, a sustained and progressive increase in PKD1 activity was observed starting after 24 hrs and lasting until day 7 post reversion when cultures were at a highly proliferative state (Fig. 15A and 25A). In addition, PKD1 activity decreased only after a subsequent high Ca^{2+} switch to induce differentiation in R7 cultures (Fig. 15C). As expected, PKCα was phosphorylated in response to Ca^{2+}-induced differentiation and unlike PKD1, PKCα levels and activity remained unchanged during the first 2 days after low Ca^{2+} switch and gradually returned to pre-differentiation levels (Fig. 15A).

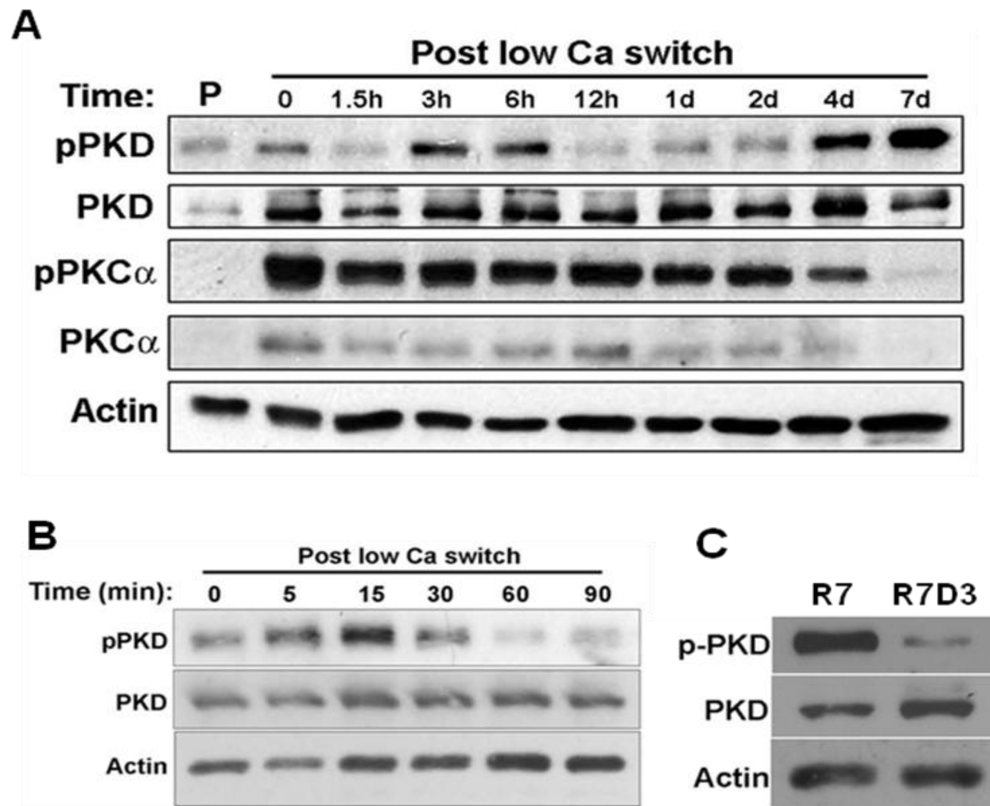


Figure 15. Kinetics of PKD1 activation in response to the low Ca^{2+} switch in differentiated cultures of mKCs.

A) Western blot analysis of protein lysates collected from proliferative, differentiated and reverted cultures at different time points following the low Ca^{2+} switch using antibodies against phosphorylated PKD1-Ser^{916} (pPKD), phospho-PKCα (pPKCα), and total PKD1 and PKCα. **B)** Shorter time course of PKD1 phosphorylation in response to the low Ca^{2+} switch using antibodies against phospho-PKD1-Ser^{916} and total PKD1. **C)** Analysis of PKD1 phosphorylation state in cultures reverted for 7 days (R7) and after Ca^{2+}-induced differentiation (R7D3) demonstrating down-regulation of PKD1 activity upon differentiation. Actin was used as loading control for all experiments.

III.2.2.4. Sustained PKD1 activation is required for re-initiation of proliferation in differentiated KCs.

To determine which phase of PKD1 activation was required for re-initiation of proliferation in response to the low Ca^{2+} switch, differentiated KCs were treated with 1 μM Go6976 either at the time of the low Ca^{2+} switch or at 1 or 10 hrs (after each of the early transient phases of PKD1 activation) following the subsequent low Ca^{2+} switch and analyzed by measuring DNA synthesis 5 days after reversion when cultures are expected to be highly proliferative. Interestingly, treatment of cells with Go6976 at all time points resulted in inhibition of proliferative response in reverted KCs (Fig. 16), indicating that the later, sustained phase of PKD1 activation was required for re-initiation of proliferation in differentiated cultures. This is consistent with studies demonstrating that sustained PKD1 activity is required for a mitogenic response in response to activation by GPCR agonists (Sinnett-Smith et al., 2009).

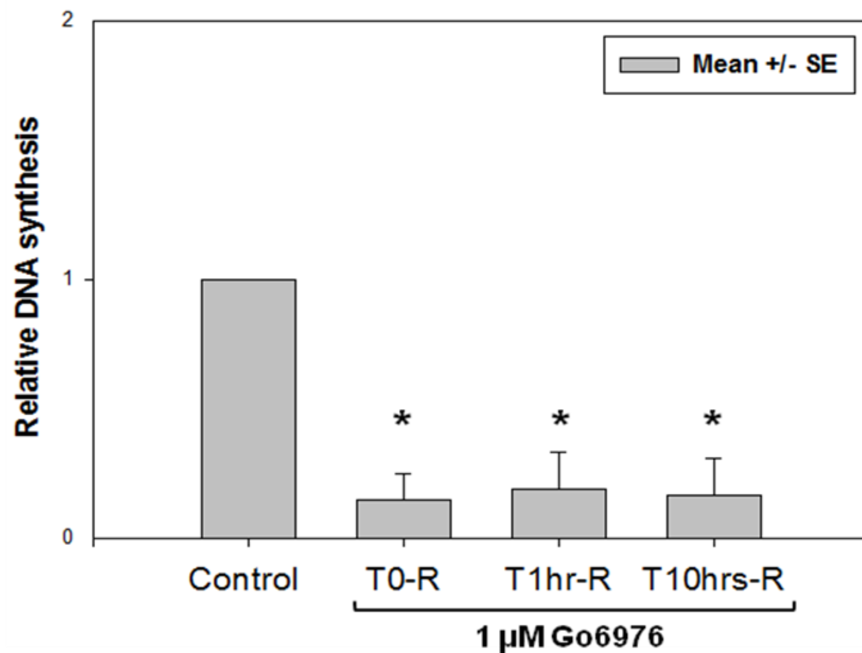


Figure 16. Sustained PKD1 activation is required for the mitogenic response in reverted cultures.
Relative DNA synthesis was measured by ^{3}H-thymidine incorporation (1 hr pulse) of 5 day reverted KCs treated with DMSO (control), 1 µM Go6976 at the time of the low Ca^{2+} switch (T0R), or 1 hr (T1hr-R) or 10 hrs (T10hr-R) after the low Ca^{2+} switch. *P value < 0.03 comparing control to each of the other conditions. Values are expressed as mean +/- SE of 2 independent experiments

III.2.2.5. PKD1 is essential for re-initiation of proliferation in differentiated KC cultures.

To demonstrate, in a more direct fashion, the role of PKD1 in re-initiation of proliferation in differentiated KCs small interfering RNA (siRNA) was used to deplete PKD1. Two different shRNA specific to PKD1 or a control shRNA were designed and cloned into the pSUPERretro.puro vector. Recombinant retroviruses were generated and used to transduce proliferative cultures of KCs. Transduced KCs were selected in puromycin, and grown to confluency. To examine the extent of PKD1 down regulation by shRNA, transduced cells were induced to differentiate for 3 days and analyzed by western blot using anti-PKD1 antibody. As demonstrated in Figure 17A, PKD1 levels in KCs expressing PKD1- specific shRNA were decreased to more than 80% of that of KCs expressing a control shRNA.

To determine whether the loss of PKD1 expression has any effect on re-initiation of proliferation, the proliferation rate in proliferative or reverted cultures expressing PKD1-specific or control shRNA were compared by ^{3}H-thymidine incorporation. In proliferative cultures there was no significant difference in the relative DNA synthesis between control and PKD1 depleted KCs suggesting that PKD1 does not play a role in normal proliferation of KCs (Fig. 17B- black bars). This was consistent with a lack of effect of Go6976 on normal proliferation of KCs (Fig 12B). In differentiated cultures exposed to the low Ca^{2+} switch, however, KCs transduced with PKD1 shRNA failed to re-initiate a proliferative program as indicated by a significant decrease in DNA synthesis (Fig. 17B- gray bars) and their inability to undergo morphological reversion (Fig. 17C-R6). PKD1-depleted KCs exposed to the low Ca^{2+} switch gradually detached and were lost from culture. By days 6 post low Ca^{2+} switch, very few cells remained in culture while the control cultures were confluent (Fig. 17C) It is unlikely that loss of cells in PKD1 depleted cultures was due to RNAi off-target effects because KCs expressing a

control shRNA were able to re-initiate proliferation similar to non–transduced cells (Fig. 17B and C) and KCs expressing 2 different PKD1-specific shRNA demonstrated the same results.

To verify that the inhibition of re-initiation of proliferation in these cells was due to a block in cell cycle re-entry and not due to cell loss, the effect of PKD1 depletion on cell cycle progression in reverted KC cultures was investigated by analysis of cyclin D1 levels at 2 days post low Ca^{2+} switch when KCs have not yet been lost from culture (Fig. 17C-R2). Western blot analysis demonstrated that cyclinD1 levels were highly repressed in PKD1 depleted KCs cultures as compared to that of control cultures (Fig 17D). These results indicated that repression of mitogenic signaling pathways is prompted by PKD1 depletion.

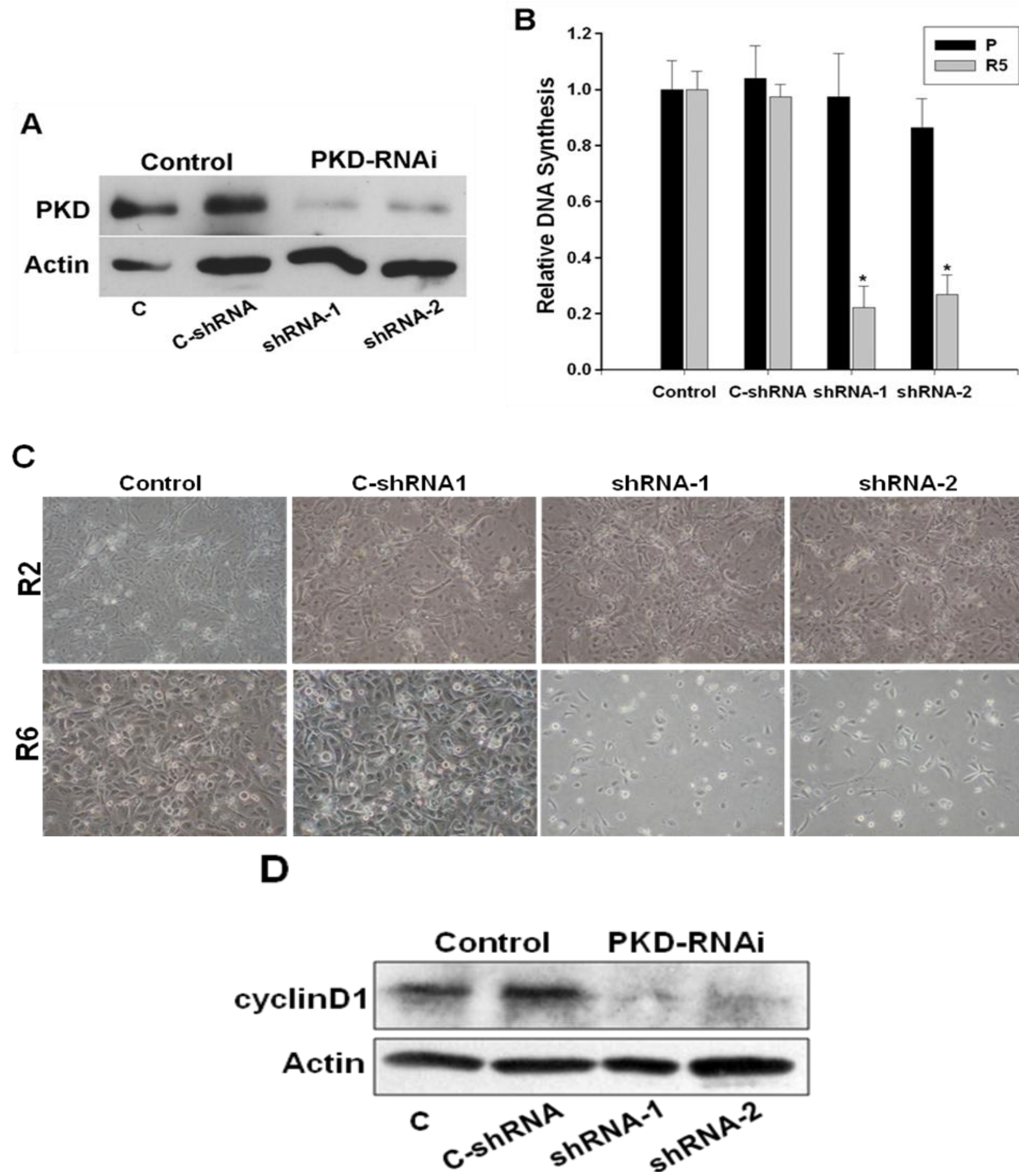


Figure 17. PKD1 depletion inhibits re-initiation of proliferative response in differentiated KCs exposed to low Ca^{2+} conditions.

A) Western blot analysis of KCs demonstrating efficient knockdown of PKD1 in differentiated (D3) cultures expressing 2 different shRNA targeting PKD1 (shRNA-1 and shRNA-2) and control shRNA (C-shRNA). **B)** Relative DNA synthesis measured by ^{3}H-thymidine incorporation for proliferative (P-black bars) and reverted (R5-gray bars) KCs expressing PKD1 specific shRNA. Values expressed as mean +/- SD of 4 independent experiments. *P value < 0.0001 comparing PKD1 knockdown groups with controls in reverted cultures. **C)** Phase contrast images of KCs transduced with PKD1 specific shRNA (shRNA-1 and shRNA-2) or control shRNA (C-shRNA) 2 or 6 days after the low Ca^{2+} switch (R2 and R6). **D)** Western blot analysis of KCs transduced with PKD1-shRNA at R2 using an antibody against cyclin D1, indicating repression of cyclin D1 in PKD1-depleted cultures as compared to the controls. Actin was used as a loading control.

III.2.2.6. The unique and non-redundant role of PKD1 in reversion of KC differentiation.

The PKD family of kinases consists of 3 PKD isoforms (PKD1, 2 and 3) that are highly related but can have distinct, non-redundant function (Rykx et al., 2003). Analysis of PKD1, PKD2 and PKD3 expression using semi-qRT-PCR demonstrated that all 3 isoforms were expressed in mKCs (Fig. 18A). To examine if loss of PKD1 has any effect on the expression levels of PKD 2 and PKD3, the transcript levels of all PKD isoforms were compared between KCs expressing PKD1-specific shRNA or a control shRNA. Analysis by semi-qRT-PCR verified PKD1 specific knockdown and demonstrated no effect on PKD2 and 3 transcript levels (Fig. 18B). Therefore, PKD1 plays a specific and non-redundant role in re-initiation of proliferation in differentiated cultures.

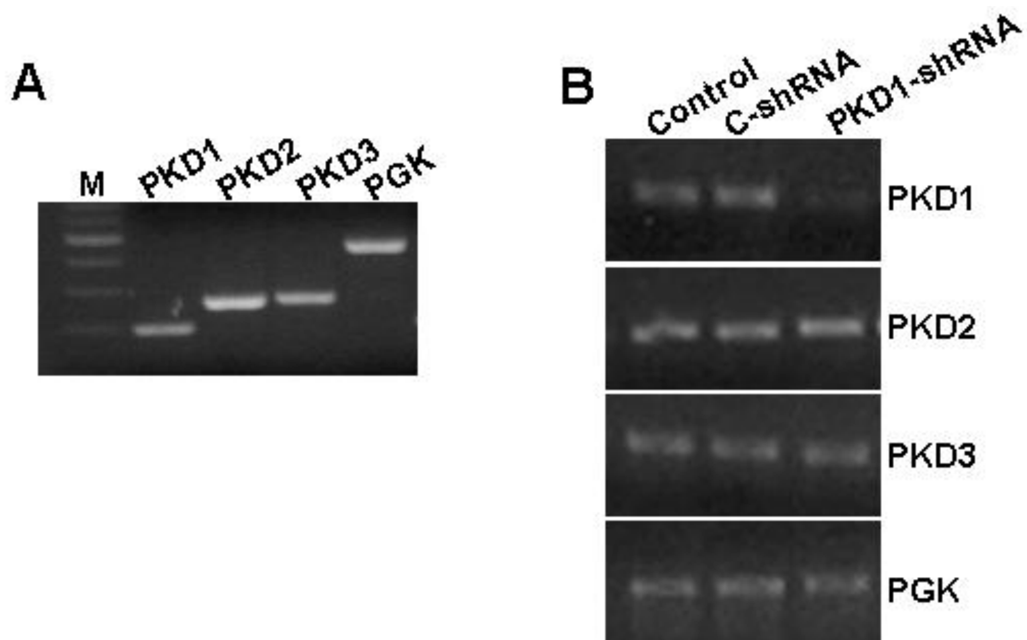
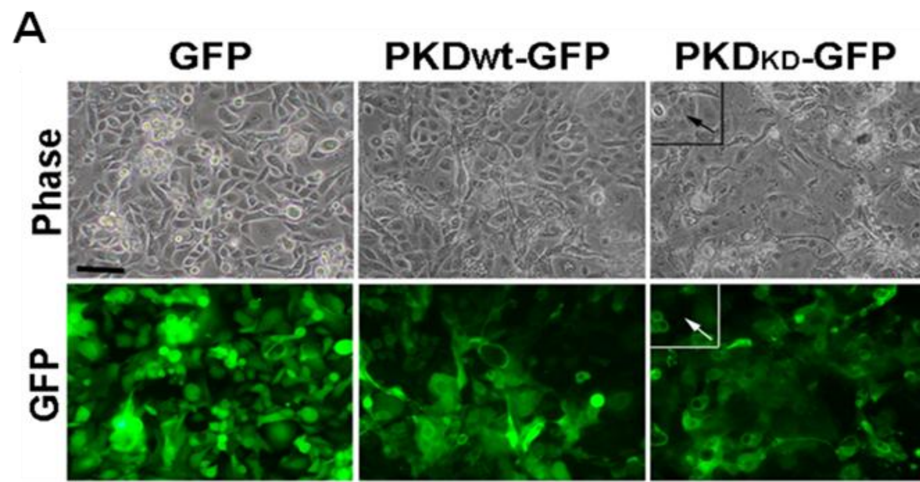


Figure 18. Expression of PKD isoforms in mouse KCs.
A) Semi-quantitative RT-PCR demonstrating expression of PKD1, 2 and 3 in mKCs using primers specific for each isoform. **B)** Semi-quantitative RT-PCR showing PKD1, 2 and 3 transcript levels in KCs expressing PKD1-specific shRNA or control shRNA. Phosphoglycerate kinase (PGK) was used as housekeeping gene.

III.2.2.7. PKD1 kinase activity is required for reversion of differentiated KCs.

To confirm that the observed effects of PKD1 depletion were due to the loss of PKD1 kinase activity, a dominant-negative form of PKD1 was used to block this process. Mutating Lys^{612} to Try in the kinase domain of PKD1 has been shown to render this enzyme kinase-deficient and to function as a dominant negative form (Hausser et al., 2002). Proliferating KCs were transduced with retroviral vectors encoding wild type PKD1 (PKD_{WT} GFP) or kinase deficient PKD1 (PKD_{KD} GFP) fused to GFP and analyzed for morphological reversion and DNA synthesis. KCs transduced with a retroviral vector encoding GFP were used as control. Over-expression of dominant negative PKD1 had no effect on normal proliferation as proliferation rates in these cultures were comparable with that of control cultures (Fig. 19B-black bars). When reverted, however, there was a significant inhibition of DNA synthesis in cultures in cultures expressing PKD_{KD} GFP (Fig. 19B-gray bars). Additionally, as shown in Figure 19A, upon exposure of differentiated cultures to the low Ca^{2+} conditions, unlike KCs expressing GFP or PKD_{WT} GFP, PKD_{KD} GFP- expressing KCs (labeled with GFP) failed to undergo morphological reversion, while non-transduced KCs in the same culture resumed a proliferative morphology, indicating cell-autonomous effects of PKD1 inhibition (Fig. 19A-arrows in insert).



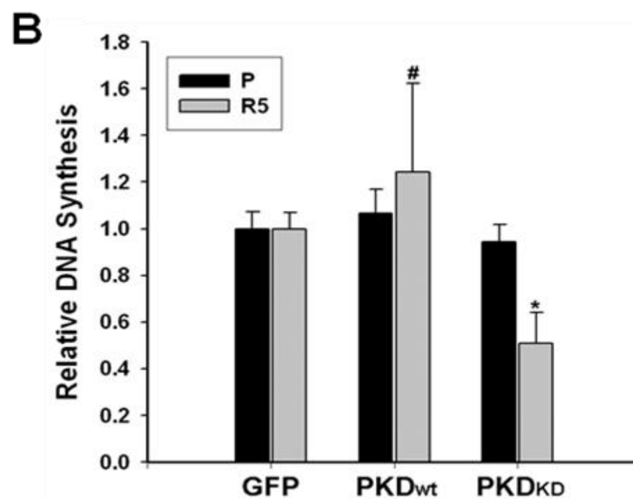


Figure 19. Over-expression of dominant negative form of PKD1 inhibits re-initiation of proliferation and morphological reversion in differentiated KCs.

A) Phase contrast and GFP fluorescent images of reverted cultures at 5 days post low Ca^{2+} switch expressing GFP (as control), wild type (PKD_{WT}-GFP), or kinase deficient (PKD_{KD}-GFP) forms of PKD1 fused with GFP. Arrow in the inserts demonstrates the morphology in non-transduced KCs in the same culture. **B)** Relative DNA synthesis measured by ^{3}H-thymidine incorporation for proliferative (P-black bars) and reverted (R5-gray bars) KCs expressing GFP, PKD_{WT}-GFP, or PKD_{KD}-GFP. Values are expressed as mean +/- SD of 3 independent experiments. *P value< 0.001 when comparing R5-PKD_{KD} to R5-GFP and #P= 0.042 when comparing R5-PKD_{WT}-GFP to R5-GFP.

III.2.2.8. Conclusion of III.2.2.

In conclusion, although PKD1 activity is not needed for normal proliferation and differentiation in mKC cultures, it plays an essential and non-redundant role in re-initiation of proliferation in differentiated KCs in response to the low Ca^{2+} switch.

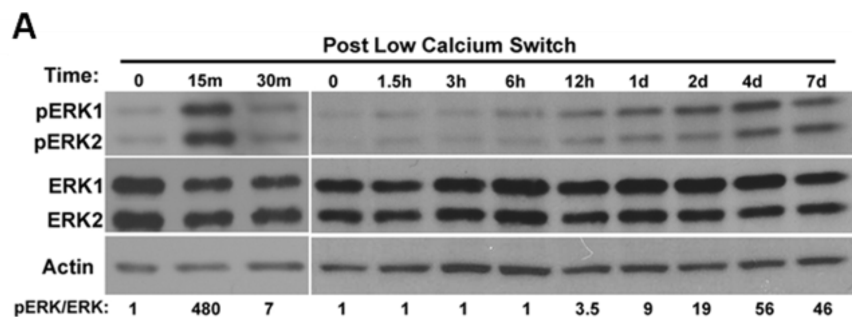
III.2.3. Role of ERK1/2 in re-initiation of proliferation in differentiated KCs

III.2.3.1. Activation of ERK- MAPK pathway in response to the low Ca^{2+} switch.

As previously stated, U0126 at concentrations below 1 μM significantly blocked re-initiation of proliferation in differentiated cultures, while having little effect on DNA synthesis in proliferating cultures (Fig. 12A and C) suggesting a potential role of MEK/ERK signaling in KC reversion.

The ERK-MAPK pathway is conserved in mammals and has been shown to be critical for epidermal homeostasis (Scholl et al., 2007). ERK proteins are directly activated by phosphorylation by the dual-specificity ERK kinase (or MEK) but the specific biological outcome of ERK activation (e.g., proliferation or differentiation) is dependent on the intensity and duration of its activity (Marshall, 1995). In quiescent fibroblasts, re-initiation of DNA synthesis has been associated with persistent ERK1/2 activation, while transient ERK1/2 activation cannot induce proliferation (Sinnett-Smith et al., 2009; Sinnett-Smith et al., 2004). To analyze ERK1/2 activation in response to the low Ca^{2+} switch, ERK1/2 phosphorylation was assessed in reverted cultures at various time points following the low Ca^{2+} switch. Similar to PKD1 activation, ERK1/2 activation followed a complex kinetics with a transient activation of ERK1/2 within the first 30 minutes followed by a gradual and more sustained ERK1/2 activation (Fig. 20A). Interestingly, the kinetics of ERK1 and ERK2 activation were different. While both ERK1 and ERK2 followed similar kinetics of activation during the transient ERK activation, ERK1 activation predominated the sustained phase of ERK activation (Fig. 20A and B). These findings are consistent with the differential role of ERK1 and ERK2 in mouse

epidermis demonstrating that significance of ERK1 in epidermal homeostasis and tumor development in skin (Bourcier et al., 2006).



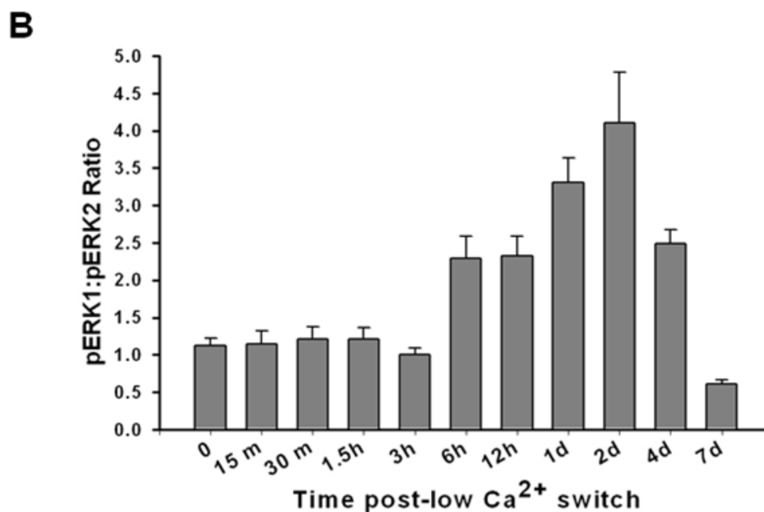


Figure 20. Kinetics of ERK1/2 activation in response to the low the Ca^{2+} switch.
A) Western blot analysis demonstrating the kinetics of ERK1/2 activation in response to the low Ca^{2+} switch using antibodies against pERK1/2, total ERK1/2 and actin (as loading control). The relative levels of phosphorylated ERK1 and ERK2 to total ERK1/2 are indicated at the bottom of the panel. **B)** The ratio of pERK1 to pERK2 was determined by densitometric scanning of ERK1 and ERK2 bands. Values are expressed as mean +/- SD from 3 independent experiments.

III.2.3.2. Specific role of ERK1 in reversion of KC differentiation.

Because ERK1 was the predominant phosphorylated species in response to the low Ca^{2+} switch, the effect of ERK1 knock down by RNAi on reversion of differentiated KCs were investigated. KCs were transduced with retroviral vectors encoding ERK1-specific shRNA or control shRNA. The knock down efficiency of ERK1 using 2 different short hairpins (ERK1-shRNA1 and ERK1-shRNA2) was more than 95% (Fig. 21A). Despite the significant depletion of ERK1 there was no effect on DNA synthesis in either proliferating or reverted cultures (Fig. 21B- P and R4). Western blot analysis of phosphorylated ERK1/2 levels indicated a significant increase in ERK2 phosphorylation in ERK1 depleted KCs, suggesting a compensatory role for ERK2 (Fig. 21A). This compensation by ERK2 when ERK1 was depleted may explain the lack of effect of ERK1 depletion on KC reversion (Fig. 21A and B).

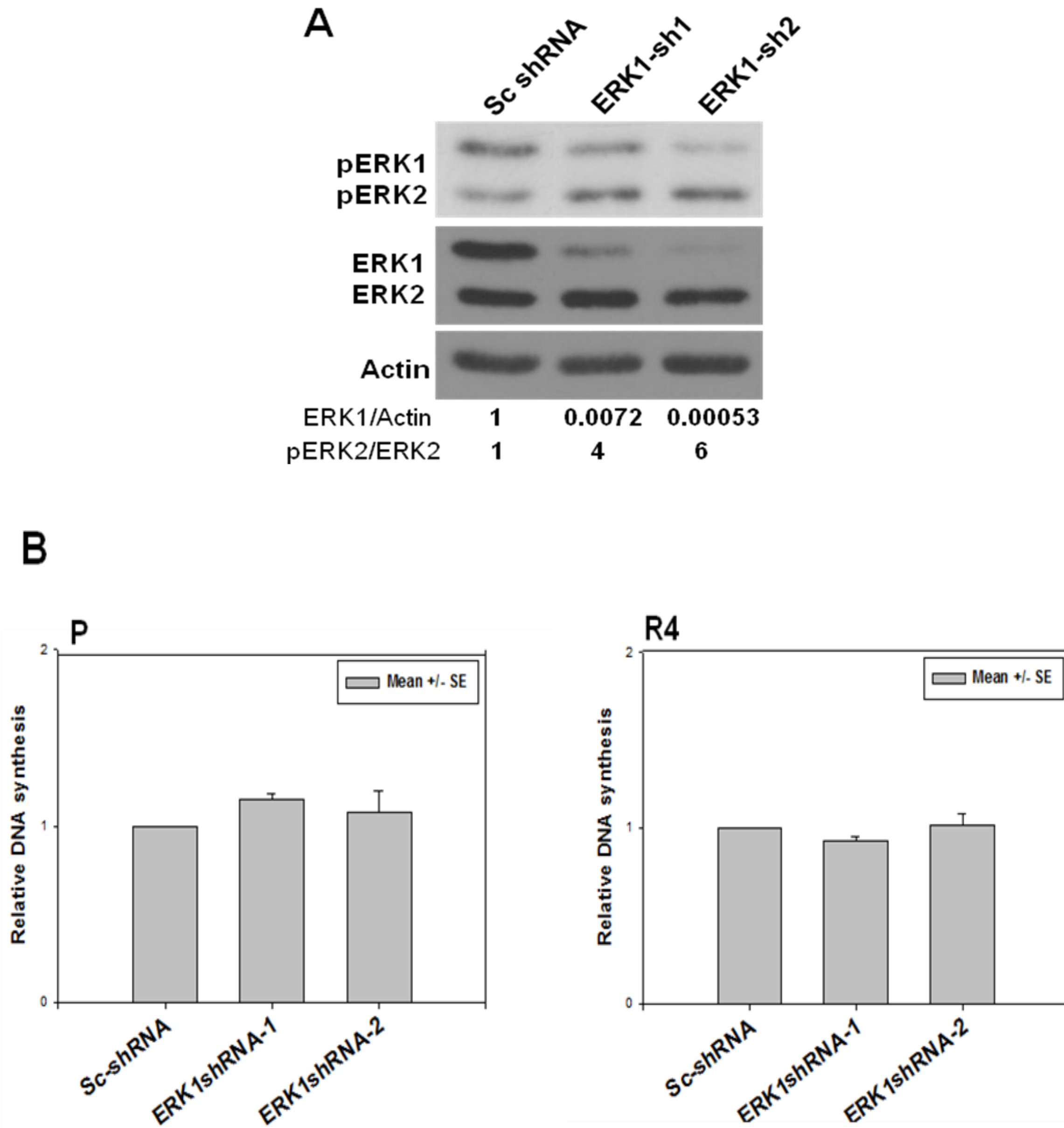


Figure 21. ERK1 depletion does not inhibit re-initiation of proliferation in differentiated KCs.
A) Western blot analysis of total and phospho-ERK1/2 in 2 day reverted KC cultures in KCs transduced with 2 different shRNA targeting ERK1 (ERK1shRNA1 and ERK1-shRNA2), or scrambled shRNA (Sc-shRNA). Actin was used as a loading control. The relative levels of ERK1 knock down and activated ERK2 are indicated in the bottom of the panel. **B)** Relative DNA synthesis measured by ^{3}H-thymidine incorporation (1hr pulse) for proliferative (P) and 4 day reverted (R4) KCs expressing scrambled or 2 different ERK1-specific shRNA. Values expressed as mean +/-SE of 3 independent experiments.

III.2.3.3. PKD1- mediated activation of ERK1/2 in response to the low Ca^{2+} switch.

PKD1 has been shown to potentiate the mitogenic responses of Swiss 3T3 cells to GPCR by increasing the duration of ERK1/2 activation (Sinnett-Smith et al., 2004). To determine if PKD1 activation induces ERK1/2 activity, the levels of phosphorylated ERK1/2 in KC cultures expressing GFP, PKD_{WT} GFP, PKD_{KD} GFP or PKD1-specific shRNA were analyzed at 48 hrs after the low Ca^{2+} switch during the sustained phase of ERK activation. As shown in Figure 22A, phospho-ERK1/2 levels were enhanced in cells expressing the wild type PKD1 and suppressed in cultures expressing the dominant negative form of PKD1, parallel to changes in proliferation rates of these cultures (Fig. 19B). Analysis of ERK1/2 phosphorylation in KCs depleted of PKD1 by shRNA, however, demonstrated a significant decrease in total ERK levels and therefore could not be used for this analysis (Fig. 22B).

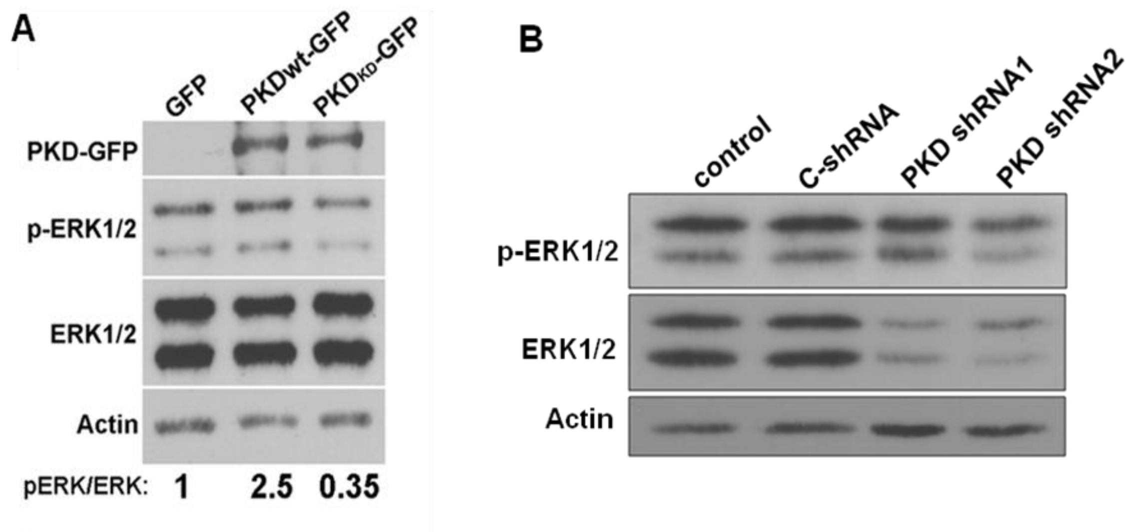


Figure 22. Involvement of PKD1 in activation of ERK-MAPK pathway during KC reversion.

A) Western blot analysis of 2 day reverted KC cultures expressing wild type (PKD_{WT}-GFP) and dominant negative (PKD_{KD}-GFP) PKD1 using antibodies indicated at the left side of the panel demonstrates inhibition of ERK1/2 phosphorylation in cultures over-expressing dominant negative PKD1. The relative levels of total activated ERK is indicated in the bottom of the panel. **B)** Western blot analysis of 2 day reverted KC cultures expressing 2 different PKD1 specific shRNAs (PKD shRNA1 and PKD shRNA2) and a control shRNA (C-shRNA) for pERK1/2 and total ERK1/2 demonstrate reduction in both phosphorylated and total ERK1/2 levels.

III.2.3.4. Analysis of ERK1/2 expression and activity in PKD1-deficient KCs.

To further investigate the potential effect of PKD1 on ERK expression and a role for PKD1 in mediating ERK1/2 activation in response to the low Ca^{2+} switch, I took advantage of the availability of PKD1 conditional knockout mice to deplete PKD1 in KCs. In these mice, exon 12-14 of PKD1 are floxed by the loxP sites (exons 13 and 14 encode the N-term region of the kinase domain of PKD1 including its ATP binding site) (Fig. 23A). Expression of CRE and subsequent recombination are expected to remove exons 12, 13 and 14 of PKD1, resulting in the loss of PKD1 expression due to instability of the mRNA and rapid degradation the protein (Fielitz et al., 2008). Mice were obtained from Dr. Olson's laboratory and genotyped to verify the presence of loxP sites.

To validate CRE-mediated recombination of PKD1 alleles, KCs isolated from PKD1 floxed mice were transduced with retroviral vectors encoding CRE-GFP or GFP alone (as control), genomic DNA was isolated, and analyzed by PCR using 2 different sets of primers. The first set of primers (primers designated a and d in Fig. 23A) were designed to generate a 359 bp fragment when the PKD1 floxed region is recombined. If no recombination occurred, no product would be detected due to the large size of the amplicon. In the second set (designated primer b and c), the reverse primer is located within the floxed region of PKD1, therefore recombination would result in the loss of the primer binding site and loss of amplicon. As shown in Figure 23B and C, CRE-mediated recombination was confirmed by using either methods as indicated by generation of a 359 bp fragment (Fig. 23B) and loss of amplicon (Fig. 23C) in the CRE-transduced KCs. CRE-mediated loss of PKD1 expression was confirmed by semi-qRT-PCR and western blot analysis. As shown in Figure 23D and E there was a

significant reduction in PKD1 expression in CRE-transduced KCs when compared to GFP-transduced KCs.

To investigate the potential effect of PKD1 on ERK1/2 expression and to confirm PKD1-mediated ERK1/2 activation in response to the low Ca^{2+} switch, PKD1 floxed KCs were transduced with GFP or GFP-CRE and analyzed for PKD1 and ERK1/2 expression and activity by western blot analysis. As shown in Figure 24, there was no change in ERK1/2 levels in PKD1 null mice (Fig. 24). This suggested that the reduction seen in total ERK1/2 levels in KCs transduced with PKD1-shRNA were as a result of an off target effect of the shRNA (Fig 22B). Moreover, PKD1 depletion had no effect on ERK1/2 activity and did not result in a decrease in ERK1/2 phosphorylation, despite the effects seen in KCs transduced with PKD_{KD} GFP (dominant negative form of PKD1) (Fig 22A). The lack of reduction in ERK1/2 activation in PKD1-depleted KCs in which reversion was completely inhibited suggested that ERK1/2 activation in response to the low Ca^{2+} switch was not sufficient to re-initiate proliferation in differentiated KCs.

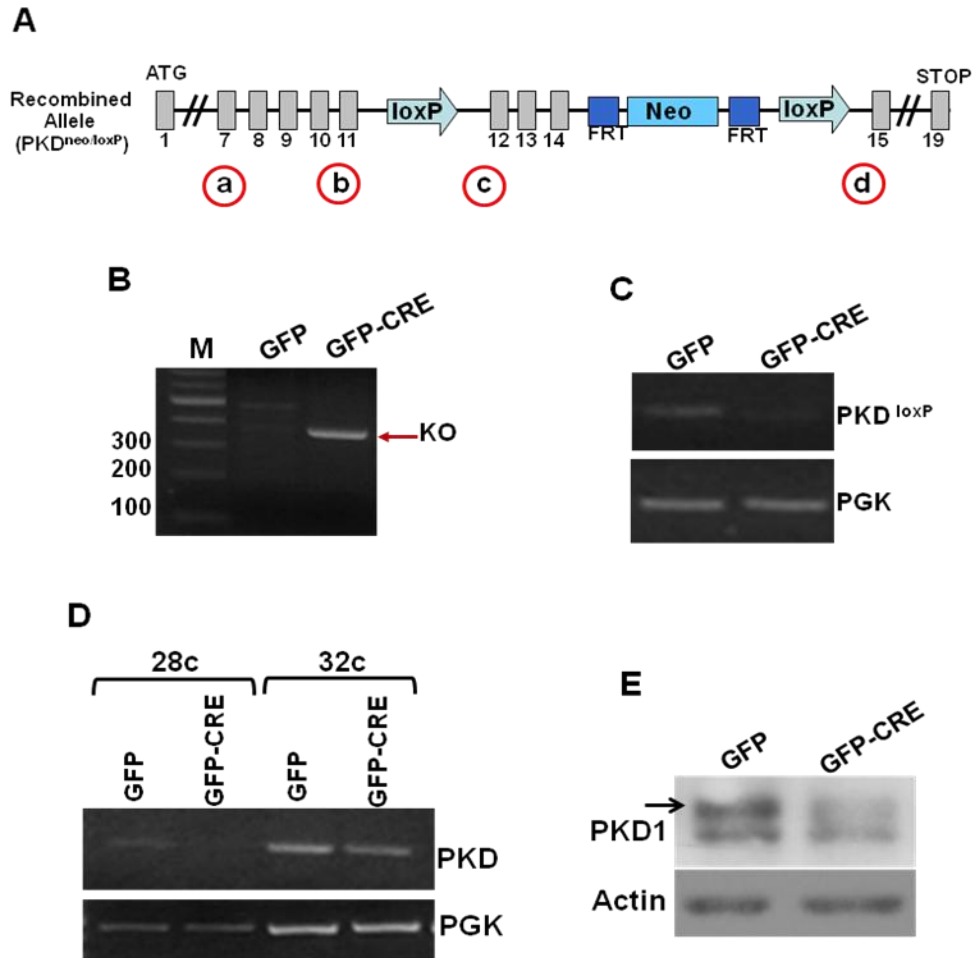


Figure 23. CRE-mediated recombination and subsequent PKD1 depletion in PKD1 conditional knockout mouse KC cultures.

A) Schematic diagram indicating the position of primers used for genotyping and for assessment of CRE-mediated recombination. Primers b and c (named PKD^{loxP}) were used to distinguish between WT-PKD1 (WT) and PKD1-loxP (loxP) PCR products as well as in determining CRE-mediated PKD1 recombination. In addition, primers a and d (named PKD^{KO}) were used to further validate PKD1 knockout (KO). **B)** PCR analysis of differentiated KCs transduced with retroviral vector encoding GFP or GFP-CRE. PKD^{KO} primers produced a 359 bp fragment (KO) in GFP-CRE transduced KCs, while KCs transduced with GFP alone showed no fragment due to the large size of the non-recombined PKD1 region. **C)** Semi-qPCR of differentiated KCs described in B using PKD^{loxP} primers at 28 cycles verified recombination of the PKD1 floxed region. **D)** Semi-qRT-PCR demonstrating PKD1 depletion in PKD1 floxed KCs transduced with GFP-CRE at 28 and 32 cycles, using PKD1 specific primers. PGK was used as housekeeping gene. **E)** Western blot analysis demonstrating PKD1 depletion in differentiated PKD1 floxed mKCs transduced with GFP-CRE using antibodies against PKD (cs-2052) and actin (as control). Arrow points to PKD1.

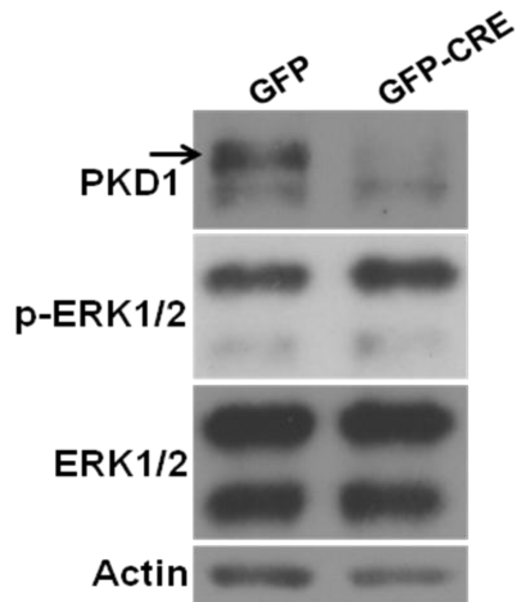


Figure 24. Analysis of ERK1/2 expression and activation in PKD1-deficient reverted mKCs.
Western blot analysis of PKD1 (cs-2052), ERK1/2 and pERK1/2 in cultures of PKD1 floxed mKCs transduced with GFP-CRE or GFP (as control) and reverted for 2 days. Actin was used as a loading control.

III.2.3.5. Conclusion of III.2.3.

In conclusion, these data demonstrate that inhibition of MEK1/2 at concentrations that did not block KC proliferation, blocked reversion. Additionally, ERK1/2 was phosphorylated in response to lowering Ca^{2+} with a predominant phosphorylation of ERK1 during the sustained phase of ERK1/2 activity. ERK1/2 activity was suppressed by over-expression of the dominant negative form of PKD1, but not by PKD1 deletion, where de-differentiation was blocked. Furthermore, these findings suggest that ERK1/2 activation may be necessary but not sufficient in inducing re-initiation of proliferation in differentiated KCs.

III.3. Mechanism of PKD1 Activation

III.3.1. PKC-independent activation of PKD1 in response to the low Ca^{2+} switch.

PKD1 activation is predominantly mediated by PKC-dependent phosphorylation at Ser^{744} and Ser^{748} in the activation loop followed by autophosphorylation at Ser^{916} (Wang 2006). It was therefore surprising to find that GF1, which has been shown to inhibit PKC-dependent activation of PKD1, had no effect on re-initiation of proliferation or reversion of differentiated KCs in response to the low Ca^{2+} switch (Lemonnier et al., 2004; Sinnett-Smith et al., 2009; Zugaza et al., 1996). Activation of PKD1 by phorbol esters has been shown to be PKC-dependent and is inhibited by GF1 (Zugaza et al., 1996). To verify the effective concentration of GF1 in inhibiting PKC-dependent PKD1 activation in mKCs, proliferative cultures of KCs were treated with increasing concentration of GF1 for 1 hr prior to addition of 100 nM TPA and analyzed for changes in PKD1 phosphorylation at Ser^{744} in the activation loop. As shown in Figure 25A, TPA treatment of mKC induced a rapid and intense increase in PKD1 phosphorylation. Pre-treatment of KCs with GF1 resulted in inhibition of PKD1 phosphorylation in a dose dependent manner with a complete inhibition at concentrations above 1 μM. A lack of effect of GF1 in inhibiting KC reversion suggested PKC-independent PKD1 activation.

Recent work by Jacamo et. al. have demonstrated that PKD1 is transphosphorylated by PKCs at Ser^{744}, while Ser^{748} is predominantly autophosphorylated (Jacamo et al., 2008). Therefore, we used site specific antibodies to detect the phosphorylation state of Ser^{744} and Ser^{748} in response to fluctuations in Ca^{2+} conditions. While Ser^{748} was phosphorylated with similar kinetics to Ser^{916}, a prominent signal was not detected for Ser^{744} (Fig. 25B). Treatment of

proliferative KCs with TPA induced similar levels of PKD1 phosphorylation on all 3 Ser residues (Fig. 25C) disputing against the differential affinity of these site-specific antibodies. The low signal levels observed for Ser^{744} is most likely a result of the cross reactivity of Ser^{744} antibody with Ser^{748} which was included in the peptide used to generate the antibody (Jacamo et al., 2008).

In conclusion, the inability of GF1 to inhibit re-initiation of proliferation in differentiated cultures and the lack of significant levels of phosphorylation at Ser^{744} in the activation loop of PKD1 indicated PKC-independent activation of PKD1 in response to the low Ca^{2+} switch in differentiated cultures.

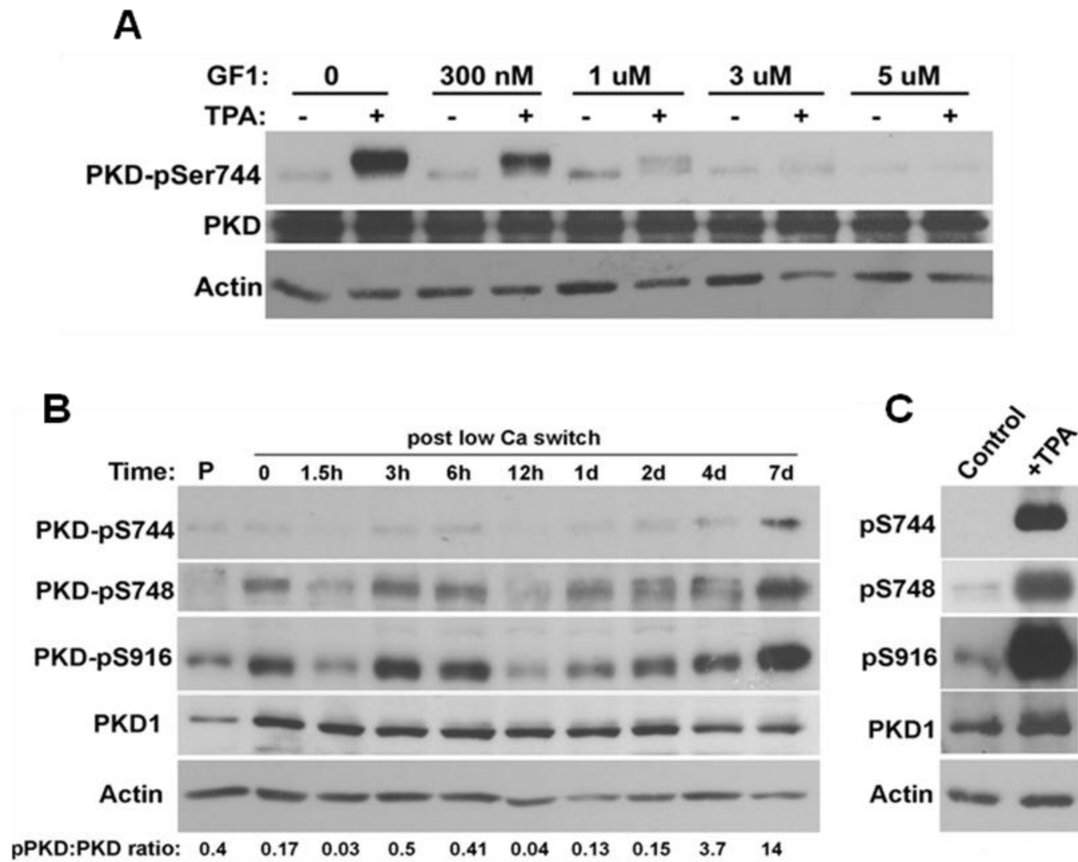


Figure 25. PKC-independent activation of PKD1 in differentiated KCs in response to lowering Ca^{2+}.

A) Western blot analysis of pPKD-Ser^{744} and PKD1 in proliferative KCs treated with or without varying concentration of GF1 (ranging from 300 nM to 5 μM) for 1 hr prior to stimulation with 100 nM TPA for 10 minutes. Results indicate the potency of GF1 in blocking PKC-dependent PKD1 activation in a dose dependent manner. A representative of 2 independent experiments is shown. **B)** Protein lysates collected for P, D3 (time 0) and indicated times after the low Ca^{2+} switch using antibodies against pPKD-Ser^{744}, pPKD-Ser^{748}, pPKD-Ser^{916} and PKD1. The ratio of pPKD-Ser^{916} to total PKD1 is shown at the bottom of the panel. Actin was used as a loading control. **B)** Proliferative cultures treated with 100 nM TPA for 10 min to induce PKC-dependent PKD1 phosphorylation demonstrated comparable affinity of these site specific antibodies.

III.3.2. Role of RhoA-ROCK-nPKC-SFK signaling pathway in PKD1 activation.

A recent study has demonstrated that EDTA- mediated depletion of Ca^{2+} results in loss of cell-cell contact and leads to PKD-mediated activation of NFκB through the RhoA-ROCK-nPKC-Src family kinase (SFK) signaling pathway (Cowell et al., 2009). To determine whether the same pathway was responsible for PKD1 activation in our system, I examined the effects of chemical inhibitors of PKCη, Rock, or SFK on KC de-differentiation. To determine whether PKCη plays a role in PKD1 activation, differentiated KC were treated with varying concentrations of a specific inhibitor of PKCη (pseudo substrate inhibitor; PS) 1 hr before the low Ca^{2+} switch and again at the time of the low Ca^{2+} switch when media was changed and the ability of cells to re-initiate proliferation was measured by ^{3}H-Thymidine incorporation. The PKCη PS has been shown to inhibit PKCη activity at concentrations ranging from 5 to 30μ M (Cabodi et al., 2000; Suzuki et al., 2009). As shown in Figure 26, at concentrations of 2 or 10 μM the PKCη PS had no significant effect on DNA synthesis. Treatment of KC cultures with 50 μM PKCη PS was toxic to cells and could not be used for analysis. These data demonstrated that PKCη could not be involved in activation of PKD1.

Interestingly, treatment of differentiated KCs with varying concentrations of Y27632, a ROCK inhibitor, which was shown to inhibit EDTA-mediated PKD1 activation (Cowell et al., 2009), not only did not inhibit re-initiation of proliferation but resulted in a dose dependent acceleration of DNA synthesis in reverted cultures (Fig 27A and B).

To investigate the role of SFK in de-differentiation, differentiated KCs were treated with increasing concentrations of PP2 (an inhibitor of the Src family of tyrosine kinases) as well as Genistein (a general tyrosine kinase inhibitor) 1 hr before the low Ca^{2+} switch and again at the time of the low Ca^{2+} switch when media was changed and the ability of cells to re-initiate

proliferation was measured by ^{3}H-Thymidine incorporation. As a control, cells were treated with PP3 (a control inhibitor for PP2). Additionally, proliferative KCs were treated with the same inhibitors to assess toxicity and their effect on normal proliferation. Treatment of proliferative cultures with Genistein at concentrations ranging from 10 to 100 μM blocked proliferation and therefore could not be used to evaluate re-initiation of proliferation (data not show). As expected, analysis of DNA synthesis and morphological reversion demonstrated that treatment of differentiated KCs with PP3 did not impair proliferation or re-initiation of proliferation in differentiated cultures (Fig. 28A and B-R5). Treatment with PP2 (K_i=5 nM for inhibiting Src) at concentrations ranging from 0.1 to 3 μM had no effect on re-initiation of proliferation or morphological reversion and at 1 μM, actually resulted in an acceleration of reversion (Fig. 28A and B-R5). At a higher dosage of PP2 (10 μM), differentiated KCs detached and were lost from culture (Fig. 28A and B-R5). Furthermore, proliferative KCs treated with 10 μM of PP2 demonstrated a significant reduction in proliferation (Fig. 28B-P). It is worth noting that higher concentration, PP2 is also an inhibitor of EGFR (K_i=480 nM) and this effect on KC proliferation and reversion may be related to EGFR inhibition (Fig. 28A and B-R5). In conjunction with these results, treatment of proliferative cultures with lower doses of PP2 had no effect on re-initiation (Fig. 28B-P). These results suggested that Src tyrosine kinases were not responsible for PKD1 activation in response to low Ca^{2+} switch.

Overall, these data suggested that the RhoA-ROCK-PKCη-SFK signaling pathway does not appear to play a role in activation of PKD1 in response to the low Ca^{2+} switch in differentiated KC cultures and that other signaling pathway are involved in PKD1 activation.

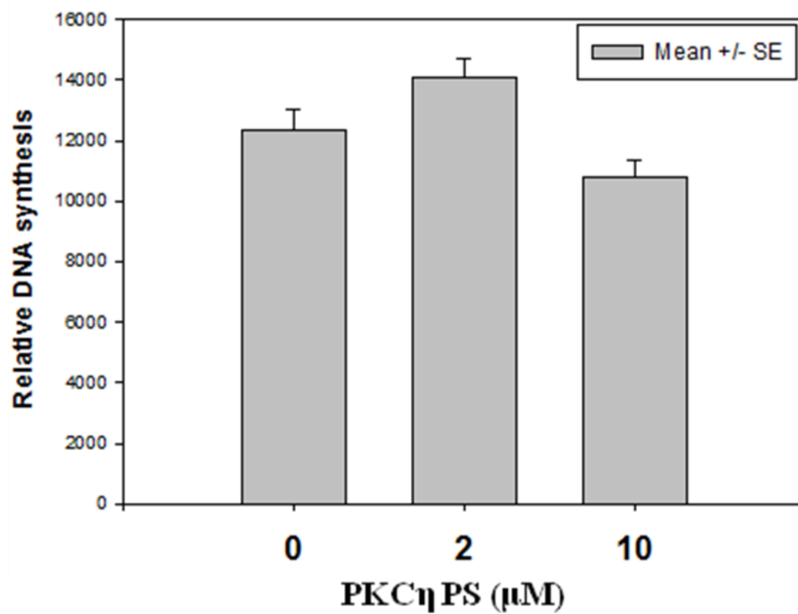


Figure 26. PKCη does not mediate PKD1 activation in reverted cultures.
Relative DNA synthesis measured by ^{3}H-thymidine incorporation (16 hr pulse) in 5 day reverted KC cultures treated with varying concentrations of PKCη PS argues against a role for PKCη in re-initiation of DNA synthesis in response to low Ca^{2+} switch. Values are expressed as mean +/- SE.

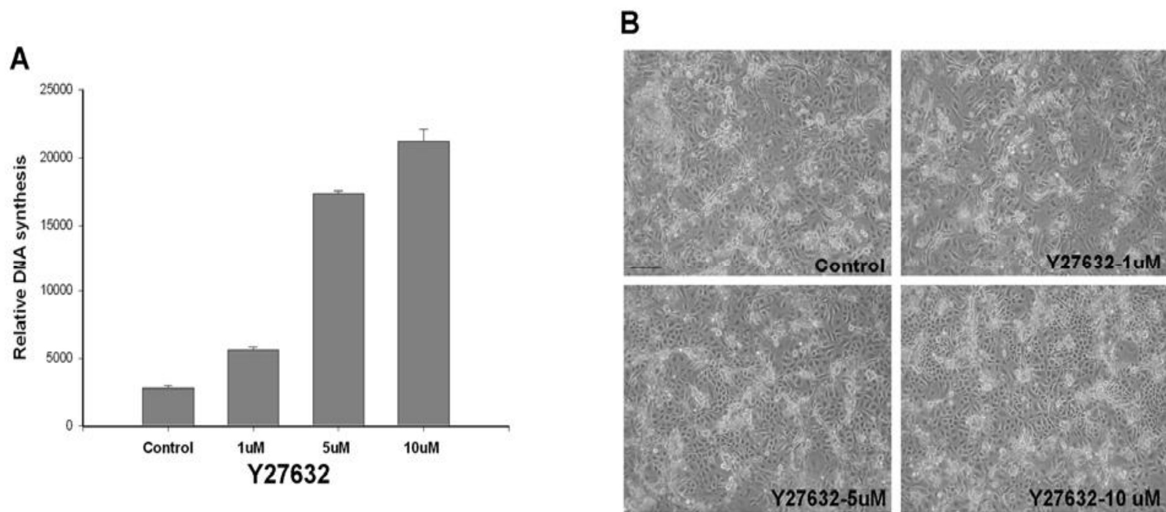


Figure 27. Inhibition of RhoA-Rock signaling pathway potentiates re-initiation of proliferation in differentiated KCs.
Differentiated KCs were treated with DMSO (as control) or increasing concentration of Y27632 at the time of the low Ca^{2+} switch. **A)** Relative DNA synthesis measured by ^{3}H-thymidine incorporation (with 1hr pulse) in 4 day reverted cultures indicates a dose dependent enhancement of DNA synthesis in response to ROCK inhibition. Values are expressed as mean +/- SE. **B)** Phase contrast images of reverted KC cultures as described in A. Results are representative of 3 independent experiments.

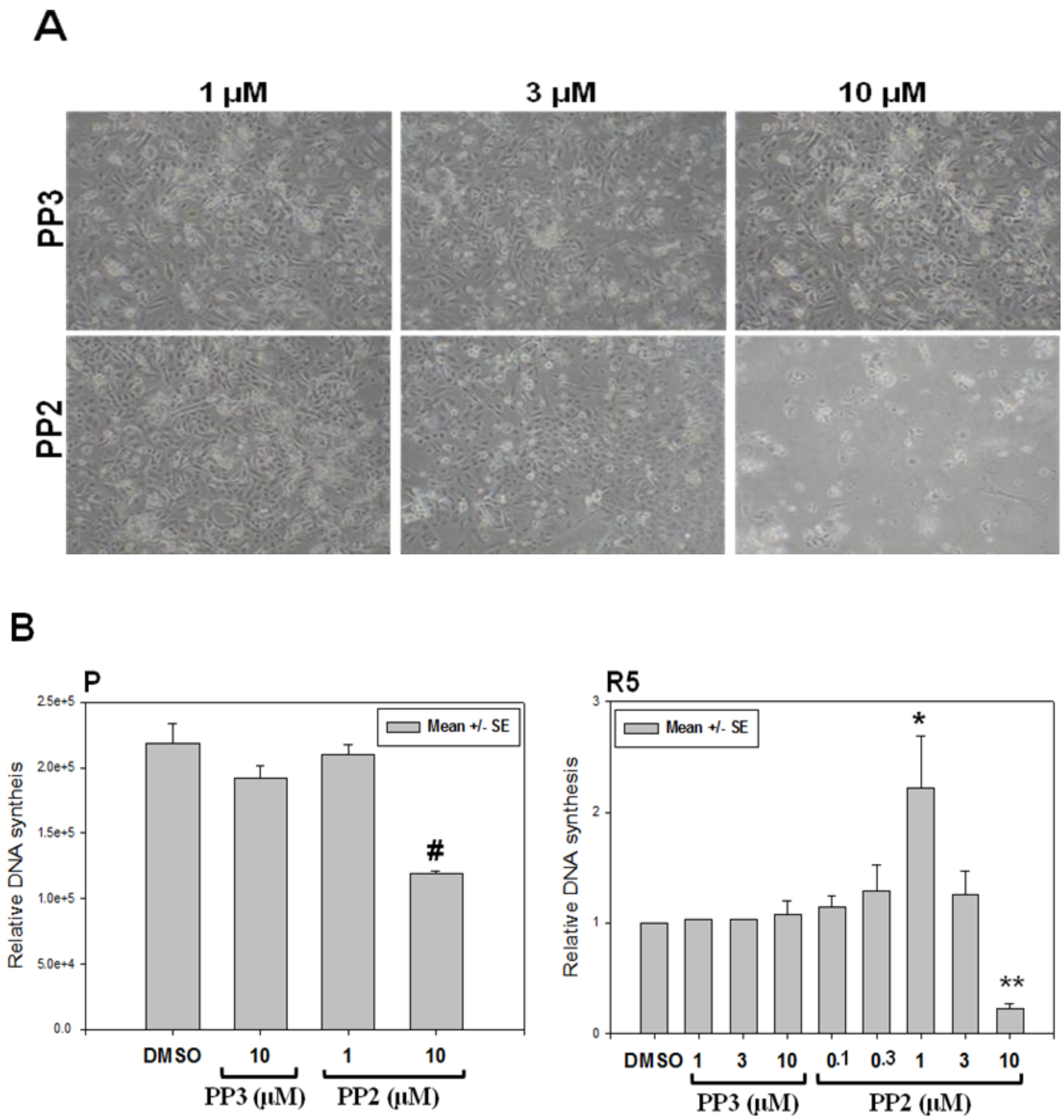


Figure 28. Src tyrosine kinases are not responsible for PKD1 activation in response to the low Ca^{2+} switch in differentiated KCs.

A) Phase contrast images of 5 day reverted cultures treated with varying concentrations of PP2 and PP3. **B)** Relative DNA synthesis measured by ^{3}H-thymidine incorporation (16 hrs pulse) in proliferative (P) and 5 day reverted (R5) KC cultures. Values are expressed as mean +/- SE from 5 independent experiments. *P value < 0.05 comparing DMSO to 1 µM PP2 in R5, ** P value < 0.0001 comparing DMSO to 10 µM PP2 in R5 and # P value < 0.02 comparing DMSO to 10 µM PP2 in P.

III.3.3. Conclusion of III.3.

In conclusion these data indicated PKC-independent activation of PKD1. Furthermore, the lack of inhibition of re-initiation of proliferation in differentiated KCs, using inhibitors of the RhoA-ROCK-nPKC-SFK signaling pathway suggested that this signaling pathway did not play a role in PKD1 activation.

Chapter IV. Discussion

Although commitment to epidermal differentiation is believed to be irreversible, recent studies have demonstrated that during tissue regeneration differentiated KCs retain the ability to de-differentiated and participate in reforming a fully functional skin when transplanted onto a suitable site *in vivo* (Mannik et al., 2010). The main objective of this study was to delineate the mechanism of re-initiation of proliferative response in differentiated KCs. As Ca^{2+}_o is a potent inducer of KC differentiation in culture, I examined the reversibility of commitment to Ca^{2+}-induced differentiation by modulating Ca^{2+} levels in primary cultures of mouse KCs and thus developed a culture model to study KC de-differentiation *in vitro*. I have shown that lowering Ca^{2+} concentration in differentiated cultures of mKCs triggered re-entry to cell cycle and reversion to a proliferative state, and identified PKD1 as a major regulator of this process.

Primary cultures of mouse KCs are a well established model to study epidermal differentiation through modulation of Ca^{2+}_o (Elias et al., 2002; Yuspa et al., 1989) Increasing Ca^{2+} concentration in cultures of mouse KCs to 1.2 mM for 3 days resulted in growth arrest in more than 97% of KCs and expression of differentiation markers such as keratins 1 and 10, INV, loricrin and filaggrin, consistent with earlier findings (Dotto, 1999; Hennings et al., 1980; Mannik et al., 2010). Interestingly, when these differentiated cultures were switched back to 0.05 mM Ca^{2+}, a divergent response was observed. In response to the low Ca^{2+} switch, some KCs were detached and lost from culture while others reverted to a proliferative phenotype. This reversion was independent of mouse strain as similar responses were observed in FVB or C57Bl/6 mice. The divergent response to the low Ca^{2+} switch is similar to the response of KC cultures following treatment with phorbol esters like TPA. Treatment of KC cultures with TPA induces a heterogeneous response resulting in differentiation and loss of one population and

proliferation and expansion of another (Yuspa et al., 1982). The response to TPA was postulated to be related to differences in maturation potential of basal KCs *in vivo* and *in vitro* (Yuspa et al., 1982). The differences in KCs response to the low Ca^{2+} switch can be explained in a similar fashion. When Ca^{2+} concentrations were increased in proliferating cultures, almost all KCs (more than 90%) started the differentiation program as demonstrated by growth arrest and expression of early differentiation markers like INV, but not all cells completed the differentiation program as indicated by expression of LOR, a marker of late differentiation, in only 30-40 % of cells. Furthermore, although KCs underwent maximum growth arrest within 3 days in high Ca^{2+}, KCs differentiated up to 5 days still retained their ability to overcome growth arrest and resumed a proliferative phenotype, while by 7 days in high Ca^{2+} they were no longer able to re-initiate DNA synthesis indicating that the ability to overcome growth arrest and de-differentiate is eventually lost. Therefore, I speculate that KCs at different stages of differentiation may respond differently to the low Ca^{2+} switch. KCs in the early stages of differentiation re-initiate DNA synthesis and revert to a proliferative phenotype, while KCs at the later stage of differentiation are shed and lost from culture. These findings are consistent with *in vivo* studies where analysis of the early events during tissue regeneration by differentiated KCs demonstrated involvement of INV-expressing KCs but not cells that were in the later stages of differentiation (Mannik et al., 2010). Hence, during the earlier stages of differentiation like in the spinous layer, before KCs express markers of the granular layer, commitment to differentiation is reversible as previously speculated (Morasso and Tomic-Canic, 2005). Currently, the process of differentiation in epidermis is defined as terminal differentiation which is initiated when proliferative KCs in the basal layer undergo “irreversible” growth arrest, switch expression of K5 and K14 to K1 and K10, down-regulate integrin expression and detach from

the basement membrane (Fuchs and Green, 1980). However, based on the work demonstrated here, and consistent with the *in vivo* studies of this laboratory (Mannik, et. al., 2010), the word “terminal differentiation” should be reserved only for later stages of KC differentiation (expressing LOR; granular layer) where KCs lose their ability to overcome growth arrest and de-differentiate.

Since about 3% of KCs in differentiated cultures were labeled with BrdU, the possible contribution of this small population of Ca^{2+}-resistant proliferating cells to the robust repopulation of reverted cultures was initially considered. However, retroviral labeling of Ca^{2+}-resistant proliferating KCs and a lack of expansion of this labeled population did not support this hypothesis. If these labeled cells were responsible for the rapid repopulation of reverted cultures, a significant increase in the percentage of the labeled KCs following the subsequent low Ca^{2+} switch would have been expected. Another possible source for the proliferative KCs found in reverted cultures was from quiescent, undifferentiated cells which could be activated upon loss of contact inhibition following the low Ca^{2+} switch and cell loss. My inability to sub-culture differentiated cultures, however, when confluent proliferating cultures were easily sub-cultured, disputed against this possibility. Furthermore, the rapid reappearance of cyclin D1 suggested activation of mitogenic signaling pathways prompted by the reduction of Ca^{2+}_o levels. Therefore, the more reasonable explanation for repopulation of differentiated cultures with proliferating cells was re-initiation of DNA synthesis and reprogramming in the majority of growth-arrested, differentiated KCs.

The low Ca^{2+} switch induced a gradual decrease in differentiation markers including K10, filaggrin, and LOR and an increase of proliferative markers including p63 and cyclinD1, indicating a reversion to a proliferative program. It is noteworthy that levels of INV and PKD1

were not reversed and remained high up to seven days after the low Ca^{2+} switch. Although this indicated that the source of proliferating KCs in reverted cultures were cells that were committed to differentiation, the basis for this continued expression is unclear. The persistence of INV expression may be due to an epigenetic imprinting of the INV locus that is not removed upon de-differentiation. Moreover, it is possible that additional signal(s) may be required for suppression of INV to predifferentiation levels. Indeed, this is consistent to previous observations in our laboratory demonstrating INV expression in the basal layer of epidermis regenerated from differentiated KCs up to 7 days after cell transplantation. At this stage, although epidermis is multilayered, late differentiation markers are not expressed. By 10 days, (3 days later) however, expression of differentiation markers including that of INV is normalized (Mannik et al., 2010). Therefore, it is possible that other signals, not present in culture, are required to suppress INV expression in the basal layer or longer periods of time is required for normalization of INV expression in culture.

Using a panel of inhibitors to identify the molecular mechanism involved in re-initiation of proliferation, we identified Go6976, an inhibitor of classical PKCs and PKD in blocking KC reversion in a dose dependent manner starting at 100 nM. At lower concentrations, Go6976 is a selective inhibitor of PKCα (K_i=2.5 nM) and at higher concentrations it has been shown to effectively inhibit PKDs (K_i=20 nM). The inhibition of reversion with Go6976 at higher concentrations and a lack of inhibitory effect by GF109203X, a specific inhibitor of PKCs, identified PKD as a target of Go6976, and suggested a role for PKD in de-differentiation of KC cultures.

Although a pro-proliferative and anti-differentiation role for PKD1 has recently been suggested in KCs (Bollag 2004), the exact role of PKD1 in epidermis remains to be defined.

My studies showed that PKD1 is predominantly expressed in differentiated mouse KCs both in culture and in intact skin. These findings differ from previous studies showing a reduction in PKD1 levels in Ca^{2+}-induced differentiated cultures (Ernest Dodd et al., 2005; Rennecke et al., 1999) and may be related to differences in mouse strain, culture conditions, or the lot of anti-PKD1 antibody used and its cross reactivity to other PKD isoforms. Analysis of the levels of autophosphorylated PKD1 (active form) however, indicated a 4 fold higher phospho/total PKD1 ratio in proliferating KCs when compared to differentiated cells, consistent with its pro-proliferative role (Ernest Dodd et al., 2005). Interestingly, despite a 6 fold increase in PKD1 levels in differentiated KCs, PKD1 transcript levels remained unchanged upon differentiation (data not shown), suggesting regulation of PKD1 levels by post-translational modifications. It is worth noting that inhibition of PKD1 had no significant effect on normal proliferation and differentiation of KCs in culture, suggesting that changes in PKD1 levels are not the predominant driver in KC proliferation and differentiation. More likely, it is the PKD1 activity that enables its pro-proliferative function. Clearly, further studies are needed to delineate the mechanisms regulating PKD1 during normal proliferation and differentiation of KCs.

PKD1 activation following the low Ca^{2+} switch followed a complex kinetics. The transient phosphorylation of PKD1 within the first 30 mins was consistent with recent studies demonstrating a rapid activation of PKD1 following EDTA-mediated depletion of Ca^{2+} (Cowell et al., 2009). Furthermore, PKD1 activation in response to GPCR agonists follows a biphasic kinetics consisting of a rapid, transient PKC-dependent PKD1 phosphorylation, followed by a later, sustained PKC-independent PKD1 phosphorylation (Jacamo et al., 2008; Sinnett-Smith et al., 2009). Although it is not clear whether the early phases of PKD1 activation in my system

were PKC-dependent, they do follow similar kinetics. PKD1 has been shown to be involved a vast array of biological activities in various cell types (Jaggi et al., 2007). The kinetics of PKD1 activation observed here may be either due to the dynamic changes in temporal activation of different signaling pathways involved in PKD1 activation or as a result of the response of different sub-populations of KCs. Most likely, in response to the low Ca^{2+} switch, the transient early activation of PKD1 is the result of receptor-mediated signaling, while the persistent and progressive activation likely reflects the higher baseline activity of PKD1 in proliferating KCs. Further analysis in the kinetics of PKD1 activation showed that inhibition of PKD1 activity after the transient period of activation was sufficient to block reversion indicating that the sustained and progressive PKD1 activation, not the early transient phosphorylation is required for mitogenic activity and KC reversion. This is consistent with the sustained PKD1 activation in 3T3 cells, which is shown to be required for ERK-MAPK mitogenic response and progression to DNA synthesis (Sinnett-Smith et al., 2009).

Although an essential role for PKD1 was demonstrated during KC reversion, pharmacological inhibition of PKD1 by Go6976 or siRNA-mediated depletion of PKD1 did not result in a complete inhibition of re-initiation of proliferation. When differentiated KCs were treated with Go6976 approximately 20% of KCs still retained the ability to re-initiate DNA synthesis despite increasing levels of the inhibitor. It is possible that Go6976 may not result in complete inhibition of PKD1 activity or Go6976 may activate other signaling pathways that induce proliferation. For example, treatment of cells with Go6976 has been shown to enhance ERK1/2 activation through activation of EGFR (Shah et al., 2005). In KCs transduced with PKD1-specific siRNA, despite 80% knock down of PKD1, a lack of complete inhibition of

re-initiation of proliferation was observed. This may be due to the activation of residual PKD1 following knock down by siRNA.

In response to the low Ca^{2+} switch, ERK1/2 activity followed a complex pattern of phosphorylation with an early, transient phosphorylation within the first 30 minutes followed by a sustained, progressive activation starting at 12 hrs post low Ca^{2+} switch. The pattern of ERK1/2 activation was similar to the kinetics of PKD1 activation and followed PKD1 phosphorylation closely. These results suggested PKD1-mediated activation of ERK1/2 to potentiate a mitogenic response. This is consistent with studies where ERK1/2 has been shown to mediate cell cycle progression via mechanisms like up-regulation of cyclinD1 expression (Lavoie et al., 1996; Weber et al., 1997) as well as studies where PKD1 has been shown to increases the duration of ERK1/2 activation to potentiate the mitogenic responses in Swiss 3T3 cells (Sinnett-Smith et al., 2009; Sinnett-Smith et al., 2004).

Interestingly, during the sustained activation phase, phosphorylation of ERK1 and ERK2 followed different kinetics with ERK1 being the predominant phosphorylated species. Although, ERK1 and ERK2 functions are mostly redundant, in some cases they also have unique roles (Brahma and Dalby, 2007). In the skin, $ERK1^{-/-}$ mice develop cutaneous lesions spontaneously, show an increase in the thickness of the epidermis and are more resistant to chemically-induced carcinogenesis; thus highlighting the significance of ERK1 in epidermal homeostasis and tumor development in skin (Bourcier et al., 2006). However, in my studies specific knock down of ERK1 showed no effect on reversion due to compensation by ERK2 activation and therefore the role of ERK1 in re-initiation of proliferative response could not be determined. This is consistent with compensatory roles of ERK1/2 demonstrated in previous studies. For example, in fibroblasts, ERK1 knockout has been shown to result in an increase in

ERK2 activation, while over-expression of WT-ERK1 resulted in inhibition of ERK2 phosphorylation (Vantaggiato et al., 2006). In epidermis, individual knock down of ERK1 and ERK2 had no phenotype, whereas knock down of both ERK1 and ERK2 simultaneously resulted in epidermal hypoplasia, hypoproliferation and cell cycle arrest (Dumesic et al., 2009). The differences in the role of ERK1 in epidermis observed in the aforementioned studies may be related either to the differences between human and mouse epidermis or the use of knock down rather than knock out of the gene. Residual activity of ERK1 in knock down studies may be sufficient to mediate its function. This is consistent with my observation indicating minimal expression of total ERK1/2 in proliferating KC despite its high levels of activity and phosphorylation (data not shown).

Although over-expression of dominant negative form of PKD1 suppressed ERK1/2 phosphorylation, the depletion of PKD1 by siRNA or conditional knockout did not have similar effects. These contradictory results may be related to the activity of other PKD isoforms expressed in KCs. Despite a unique and essential role for PKD1 in KC reversion, some of the functions of PKD1 including those related to ERK activity may be redundant. PKD isoforms are similar in structure and contain a highly homologous kinase domain (Rykx et al., 2003). Mutating Lys^{612} to Try in the kinase domain of PKD1 has been shown to render this enzyme kinase-deficient and to function as a dominant negative form (Hausser et al., 2002). Over-expression of this mutated form of PKD1 may inhibit or compete with the activity of all 3 isoforms, hence suppressing PKD1-mediated ERK1/2 activation. On the contrary, PKD 2 or 3 remain active in PKD1-depleted KCs mediating ERK1/2 activation. Regardless, the lack of reduction in ERK1/2 activation in PKD1-depleted KCs in which reversion was completely

inhibited suggested that ERK1/2 activation in response to the low Ca^{2+} switch was not sufficient to re-initiate proliferation in differentiated KCs.

Previous works have identified Ras-binding protein, RIN1, as a direct substrate of PKD1, whereby PKD1 phosphorylates RIN1, freeing Ras and resulting in up-regulation of the Ras/Raf/MEK/ERK signaling pathway (Wang et al., 2002). However, due to the unavailability of an antibody against phospho-RIN1, this link could not be demonstrated. Clearly, further work is needed to determine a direct link between PKD1 and ERK1/2 activation.

My data has indicated that in response to the low Ca^{2+} switch, PKD1 was activated in a PKC-independent manner. Treatment of differentiated KCs with GF1 did not have any effect on reversion of differentiated KCs, while it did inhibit TPA-mediated activation of PKD1. Additionally, enhanced phosphorylation of PKD1 at Ser^{748} which is autophosphorylated, but low phosphorylation at Ser^{744} which is a direct target of PKCs (Jacamo et al., 2008) was supportive of PKC-independent activation. In Swiss 3T3 cells the mitogenic response to Gq-coupled receptor agonists have also been shown to be attributed to PKC-independent sustained PKD1 activation (Sinnett-Smith et al., 2009). PKD1 activation is involved in the release of the auto-regulatory PH domain (Iglesias and Rozengurt, 1998). This can be achieved in a PKC-dependent or independent manner, through direct interactions between the PH domain and regulatory proteins like Gβγ subunits, by phosphorylation of Ser or Tyr residues in the regulatory or catalytic domain of PKD, or by proteolytic cleavage of PKD (Endo et al., 2000; Jamora et al., 1999; Waldron et al., 1999; Waldron and Rozengurt, 2003).

Treatment of Hela cells with EDTA had recently been shown to induce PKD1 activation, resulting in NFκB activation and cell survival (Cowell et al., 2009). A role for the RhoA-Rock-nPKC-SFK pathway in this process has been identified. However, this pathway

did not appear to have a role in Ca^{2+}-mediated activation of PKD1 in KCs. First, unlike many cell types in which NFκB promotes cell survival and growth, in KCs, activation of NFκB inhibits cell cycle progression (Seitz et al., 2000). Second, pharmacological inhibitors of this pathway either had no effect or potentiated reversion of differentiated KCs. These included a lack of effect or a dose dependent enhancement of DNA synthesis in differentiated KCs treated with PKCη or the Rock inhibitor, respectively. In addition, treatment of differentiated KCs with PP2, an inhibitor of Src family of kinases (K_i=5 nM) (Grossi et al., 2005; McMullan et al., 2003), at concentrations between 0.1 μM to 3 μM either potentiated or had no effect on KC reversion. At higher concentrations (10 μM) treatment with PP2 resulted in cell death and detachment, similar to the effect of the EGFR inhibitor, AG490, and consistent with studies that demonstrated increased cell loss and apoptosis of KCs in presence of an EGFR inhibitor (Rodeck et al., 1997). Since at higher doses PP2 also inhibits EGFR (K_i= 480 nM), the latter effects are likely due to inhibition of EGFR pathway which is known to be crucial for KC survival (de Giorgi et al., 2007; Jost et al., 2000).

Depletion of Ca^{2+} is known to disrupt Ca^{2+}-dependent adherence junctions. In the epidermis, ablation of α-catenin, an adherence junction protein, has been shown to activate the Ras/Raf/MEK/ERK signaling pathway resulting in stimulation of a mitogenic response (Vasioukhin et al., 2001). However, whether PKD plays a role in this process has not been investigated. It is worth mentioning that the CaR has been shown to be necessary in Ca^{2+}-mediated E-cadherin signaling and KC differentiation in response to elevated Ca^{2+}_o (Tu et al., 2008). However, unlike KCs, increase in Ca^{2+}_o levels in other cell types induces activation of the CaR/EGFR/ERK pathway which results in cell proliferation (Hobson et al., 2003; Saidak et al., 2009). In my studies, the rapid and transient phosphorylation of PKD1 and ERK1/2

within the first 30 minutes following the low Ca^{2+} switch is similar to the activation of the MAPK pathway following CaR activation (Kifor et al., 2001). Although in KCs, activation of CaR has been studied in response to elevation in Ca^{2+}_o levels (Tu et al., 2001), in the parathyroid gland CaR regulates the secretion of parathormone (PTH) into the blood by responding to fluctuations in Ca^{2+}_o concentrations (Brown, 1991) and not just increase in Ca^{2+}_o. It would be interesting to investigate whether CaR is activated in response to depletion of Ca^{2+}_o.

De-differentiation is characterized by the loss of differentiation markers and regaining proliferative capacity, and best describes my observations. Although de-differentiation is an important and common phenomenon in Drosophila and lower vertebrates, de-differentiation in mammals in rare and more controversial. However, de-differentiation has been observed in many cancers as well in slow renewing organs like the liver, pancreas and lung during tissue regeneration (Alison et al., 2004; Dor et al., 2004; Rawlins and Hogan, 2006) as well as during epidermal regeneration in the skin (Mannik et al., 2010). Interestingly, a recent study comparing the regenerative potential of muscles in lower invertebrates like newt to mammals has suggested that the lack of regeneration in mammalian myocytes is due to the presence of a tumor suppressor, ARF, that is absent in lower invertebrates (Pajcini et al., 2010). They demonstrated that transient inactivation of Rb and ARF in post-mitotic, differentiated mammalian muscle cells resulted in de-differentiation, demonstrated by cell cycle re-entry, loss of differentiation properties, and the ability to re-differentiate and fuse into myofibers when transplanted *in vivo* (Pajcini et al., 2010). Their work is similar to my studies in that in both systems de-differentiation involved activation of mitogenic signals to allow for cell cycle re-entry. In the cell cycle, ARF indirectly mediates Rb activity by inhibiting cyclinD1, to block cell cycle progression. Therefore, inhibition of Rb or ARF allows for derepression of cyclinD1 and cell

cycle progression (Sherr, 2001). It is possible that in my system transient inactivation of Rb and ARF would also result in KC reversion. It is worth noting that in both systems, only early differentiated muscles or KCs retained the ability to de-differentiate, while muscles or KCs at the later stages of differentiation lost their regenerative potential.

My studies were restricted to mouse KCs mainly because primary cultures of mouse KCs currently provide the best model to study the process of KC differentiation. Modulations in Ca^{2+}_o concentrations have identified Ca^{2+} as a major regulator of proliferation and differentiation in mouse KC cultures (Hennings et al., 1980; Yuspa et al., 1989). Because there are major differences between culture conditions and sensitivity to Ca^{2+}-induced differentiation between human and mouse KCs, I have not been able to extend my studies to cultures of human KCs. In cultures of human KCs, proliferation and differentiation is more dependent on cell density and not as tightly regulated by Ca^{2+} (Boyce and Ham, 1983; Wille et al., 1984). Indeed, I have found that Ca^{2+}-induced differentiation of human KCs for 3 days induced almost complete growth arrest, although only 30-40% of cells expressed INV. This suggested that the growth arrest is likely the result of contact inhibition rather than differentiation. Clearly a better culture model to induce more extensive differentiation will be required to study de-differentiation in human KCs.

In conclusion, the results presented here demonstrate that commitment to Ca^{2+}-induced differentiation in primary cultures of KCs is a reversible process. Lowering Ca^{2+} levels results in sustained activation of PKD1 in a PKC-independent manner, sustained ERK1/2 activity, re-initiation of DNA synthesis and morphological reversion of differentiated KCs from a differentiated phenotype to a proliferative phenotype. These findings suggest a crucial role for PKD1 in circumstances like tumor progression and wound healing where the normal

differentiation process may be reversed (Mansbridge and Knapp, 1987; Martin, 1997; Owens and Watt, 2003).

Chapter V. Future Directions

At the completion of the presented studies on the mechanism of KC de-differentiation, several questions remain to be addressed including: 1) What induces PKD1 activation? 2) What are the downstream targets of PKD1 activation? 3) Is PKD1 playing a role during de-differentiation and tissue regeneration *in vivo* and 4) What is the role of PKD1 in normal epidermis and during stress responses *in vivo*?

PKD1 has been shown to be activated by GPCR agonists (Jacamo et al., 2008; Wang, 2006). Additionally, in a variety of cell types, stimulation of GPCR induces EGFR phosphorylation (Gschwind et al., 2001). A recent study has demonstrated PKC-independent sustained activation of PKD1 via Gq-coupled receptor through a EGFR-dependent pathway in fibroblasts (Sinnett-Smith et al., 2009). These findings suggest a role for EGFR in PKD1 activation in response to the low Ca^{2+} switch. To determine if EGFR is required for PKD1 activation in mouse KCs, first the kinetics of EGFR activation in response to decreasing Ca^{2+} would need to be analyzed. Furthermore, the effect of chemical inhibitors of EGFR on PKD1 phosphorylation/activation needs to be determined.

While EGFR might be playing a role in PKD1 activation, another possible activator of PKD1 that cannot be ignored is the CaR. In my system, KC differentiation and reversion involves changes in Ca^{2+}_o. The CaR is a GPCR that is activated in response to fluctuations in Ca^{2+}_o (Brown, 1991; Tu et al., 2001). In my studies, the rapid and transient phosphorylation of PKD1 and ERK1/2 within the first 30 minutes post low Ca^{2+} switch is similar to the activation of the MAPK pathway following CaR activation (Kifor et al., 2001). Therefore, the role of CaR in KC de-differentiation needs to be investigated by transiently knocking down CaR expression

using a CaR antisense cDNA (since it has already been shown to be effective). Because CaR is required for Ca^{2+}-induced differentiation, a regulated expression of antisense will be desirable.

PKD1 is an important modulator of many signaling pathways (Wang, 2006) by phosphorylating specific substrates. One of the direct targets of PKD1 is RIN1. RIN1 normally acts as a negative regulator of Ras by binding to Ras and competing with Raf-1 interaction with Ras. PKD1-mediated phosphorylation of RIN1 at Ser351 (required for binding to 14-3-3), activates Ras resulting in up-regulation of the Ras-Raf-MEK1/2-ERK1/2 pathway (Han et al., 1997; Wang et al., 2002). Whether RIN1 is phosphorylated in response to the low Ca^{2+} switch needs to be determined.

Another direct target of PKD are class IIa HDACs (HDAC 4, 5, 7, 9). All 4 HDACs contain the PKD substrate phosphorylation consensus sequence (Wang, 2006; Yang and Gregoire, 2005). Studies have demonstrated phosphorylation of HDAC5 and HDAC7 and subsequent nuclear export, in response to PKD1 activation, resulting in changes in transcriptional regulation (Vega et al., 2004; Wang, 2006). Furthermore, in my earlier studies I found that treatment of differentiated KCs with Trichostatin A (TSA), an inhibitor of class I and II HDACs, one day before the low Ca^{2+} switch induced a rapid change in morphology and 5-7 fold increase in proliferation rate. By 48 hours after reversion, TSA treated cultures were fully reverted to the same levels achieved by 6-7 days in non-treated or DMSO-treated cultures (data not shown). Further studies are required to investigate a role for histone acetylation in KC de-differentiation which may be mediated through PKD1 phosphorylation of HDACs.

Finally, the most important question that needs to be addressed is the role of PKD1 in de-differentiation during tissue regeneration. Our lab is currently in the process of making conditional knockout of PKD1 targeted to epidermis by crossing $PKD^{loxP/loxP}$ mice with

K14-CRE mice. Analysis of regenerative potential of differentiated KCs isolated from these mice or their ability to respond to wound healing or tumor development would shed light in the role of PKD1 in epidermal adaptive responses.

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