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The Role of the EGF Receptor and Its Ligands in Differentiation and 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) Toxicity

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THE ROLE OF THE EGF RECEPTOR AND ITS LIGANDS IN DIFFERENTIATION AND 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN (TCDD) TOXICITY

by

Christina Marie Campion

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Dedication

This dissertation is dedicated to my parents Ed & Mary Ellen Campion and to Ioana Corrigan who supported me every step along the way and in memory of Ian Kinney who could not.

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ABSTRACT

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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a highly lipophilic polyaromatic hydrocarbon and a persistent environmental contaminant. In the human epidermis, TCDD causes enhanced proliferation and altered, accelerated differentiation. However, epidermal differentiation, mediated by calcium (Ca), opposes and attenuates proliferative signaling, driven by the epidermal growth factor receptor (EGFR). Using a monolayer cell culture model, confluent NHEKs were treated with high Ca (1.8 mM) or TCDD (10 nM). EGFR down-regulation, signaling, and proliferation were then examined to assess the proliferative capacity of differentiating skin versus pathophysiological, TCDD-mediated differentiation. We found that while Ca and TCDD caused marked (~50% and ~30%) loss of [^{125}I]-EGF binding, the effects of this down-regulation differed. ERK activity decreased 45% in Ca-treated cells and increased 300% in TCDD-treated cells. Utilizing ligand-specific ELISAs to interrogate cell-secreted EGFR ligands which might drive ERK activity, we found that Ca increased transforming growth factor-alpha (TGF-α) secretion, while TCDD increased TGF-α and EREG secretion relative to basal cells. Amphiregulin (AREG) increased with time in all treatments. Inhibiting ligand secretion with the MMP inhibitor, batimastat, or action with ligand-specific neutralizing antibodies in TCDD-treated cells reduced the increased ERK activity to basal levels but could not rescue ERK activity in Ca-treated cells. Similarly, in TCDD-treated NHEKs, neutralizing AREG or EREG reversed EGFR down-regulation, and removal of TGF-α further reduced biotin-EGF binding. In Ca-treated cells, neutralizing EGFR ligands had no effect on EGFR down-regulation. We then investigated how these treatments

influenced proliferative capacity of cell populations through dsDNA quantitation and EdU labelling. We found that while Ca-treatment led to ~15% fewer cells than basal they still retained an equal (~5%) amount of EGFR-dependent proliferating cells. Conversely, TCDD-treatment led to equal cell numbers relative to basal cells, both of which were reduced following ligand neutralization. However, TCDD-treatment led to a significant increase in the population of proliferating cells (~8%) relative to basal. Taken together, our data suggests that in Ca-treated cells, EGFR down-regulation is ligand-independent and correlated with a loss of proliferative signaling capacity while TCDD-treated cells down-regulate the EGFR in a ligand-dependent manner, and that TGF-α retains a population of surface-associated receptors which may drive increased proliferation.

PREFACE

This dissertation is presented in four chapters including two manuscripts. Chapter One introduces the current research and frames the experimental question and approach. Chapter Four ties together results and conclusions from Chapters Two and Three. The second chapter, entitled "Calcium causes ligand-independent EGFR down-regulation," contains the manuscript which is in preparation for submission to the *Journal of Cell Science*. Chapter Three entitled "Role of EGF Receptor ligands in TCDD-induced EGFR down-regulation and cellular proliferation" is published in *Chemico-Biological Interactions* volume 253, 23 April 2016. Both manuscripts are written in the format which aligns with the guidelines set forth by their respective journals. Chapters One, Four, and the Appendix are referenced in accordance to the guidelines of the journal *Endocrinology*.

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List of Abbreviations

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EGFR	Epidermal growth factor receptor	1
PI3K	Phosphatidylinositol-3 kinase	1
PLCγ	Phospholipase Cγ	1
RTK	Receptor tyrosine Kinase	1
Ca	Calcium	1
EGF	Epidermal growth factor	1
NHEK	Normal human epidermal keratinocytes	2
AhR	Aryl hydrocarbon receptor	2
TGF-α	Transforming growth factor-α	2
EREG	Epiregulin	2
AREG	Amphiregulin	2
EPA	Environmental Protection Agency	3
CYP1A	cytochrome P450 1A1	4
NIOSH	National Institute for Occupational Safety and Health	4
PAH	Polyaromatic hydrocarbon	5
PCB`	Polychlorinated biphenyl	5
PCDF	Polychlorinated dibenzo-furan	5
OH-TriCDD	2,3,7-trichloro-8-hydroxydibenzo-p-dioxin	9
OH-TCDD	1,3,7,8-tetrachloro-2-hydroxydibenzo-p-dioxin	9

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CYP1A2	cytochrome P450 1A2	9
bHLH/PAS	basic helix-loop-helix transcription factor / Per-Arnt-Sim domain	10
Hsp90	Heat shock protein 90	10
ARNT	Aryl hydrocarbon receptor nuclear translocator	10
XRE	Xenobiotic response element	10
ERα	Estrogen Receptor α	11
ERE	Estrogen response element	11
E_2	Estradiol	11
iXRE	Inhibitory XRE	11
TSS	Transcription start site	12
CYP1B1	Cytochrome P450 1B1	12
ROS	Reactive oxygen species	12
Sod1	Superoxide dismutase 1	12
Flg	Filaggrin	12
P450SCC	P450 cholesterol side chain cleavage enzyme	13
IL#	Interleukin (1, 10, etc)	13
NF-kβ	nuclear factor kappa-light-chain-enhancer of activated B cells	13
LPS	Lipopolysaccharide	14
7-DHC	7-Dehydroxycholesterol	19
25(OH)D_3	25-hydroxyvitamin D_3	19

		Page
$1,25\text{-}(OH)_2D_3$	1,25-dihydroxy-vitamin D	19
UVB	Ultraviolet B	19
ECM	Extracellular matrix	20
TAC	Transit-amplifying cells	22
K#	Keratin 1, 10, 14, etc	22
TJ	Tight junction	22
Evpl	Envoplakin	23
Ppl	Periplakin	23
Dsg1	Desmoglein 1	23
CaR	Ca receptor	23
CE	Cornified envelopes	23
PKC	Protein kinase C	23
Tgm1	Transglutaminase-1	23
Ivl	Involucrin	23
Lor	Loricrin	24
SPRR	Small proline-rich protein	24
DP	Desmoplakin	27
ER	Endoplasmic reticulum	27
PIP_2	phosphatidylinositol 4,5-bisphosphate	27
IP_3	inositol triphosphate	27
DAG	Diacyl glyceride	27
MMP	Matrix metalloproteinase	28

		Page
TEWL	Transepithelial water loss	30
CTD	C-terminal domain	30
PTB	Phosphotyrosine binding domain	31
ERK	extracellular signal-related kinase	33
PKA	Protein kinase A	33
SHC	src homology 2 containing containing transforming protein 1	37
Grb2	cytoplasmic growth factor receptor bound protein 2	37
SOS	son of sevenless homolog 1	37
JNK	c-Jun amino terminal kinase	38
MAPKKK	MAP kinase kinase kinase (also MAP3K)	38
MAPKK	MAP kinase kinase (also MAP2K)	38
MAPK	Mitogen-activated protein kinase	38
FGF	Fibroblast-derived growth factor	38
MEK1/2	MAPK/ERK kinase ½	39
SCC	Squamous cell carcinoma	40
EMT	Epithelial-mesynchymal transition	41
BTC	Betacellulin	45
HB-EGF	Heparin binding EGF-like growth factor	45
EPGN	Epigen	45
NRG#	Neuregulin (1-4)	45
NMB	No measured binding	46

		Page
NC	No change	45
ADAM#	A disintegrin and metalloproteinase (17, 10, etc)	49
PLZF	promyelocytic leukemia zinc finger protein	51
TACE/ADAM17	Tumor necrosis factor-alpha converting enzyme	52
PMN	Polymorphonuclear neutrophils	54
TERT	Telomerase Reverse Transcriptase	58
DMEM	Dulbecco's minimal essential medium	62
F12	Ham's F12 medium	62
FBS	Fetal bovine serum	62
Ctx	Cholera toxin	62
ELISA	Enzyme-linked immunosorbent assay	64
bat	Batimastat	105
K-SFM	Keratinocyte serum-free medium	114
BPE	Bovine pituitary extract	114
BSA	Bovine serum albumin	114
PBS	Phosphate-buffered saline	115
PBS-T	Phosphate-buffered saline with 0.1% Triton X-100	116
PACE	Phospho-antibody cell-based ELISA	116
EdU	5-ethynyl-2-deoxyuridine	117
MoG	Mixture of Gaussian	118
TNE	Tris-NaCl-EDTA	118
AU	Arbitrary units	171

CHAPTER ONE

Introduction

Rationale for this Study

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) causes seemingly contradictory phenotypes of epidermal hyperproliferation and accelerated, though altered, keratinocyte differentiation (1-5). Previous findings from our lab show that this occurs through an epidermal growth factor receptor (EGFR)-dependent mechanism which can be attenuated by the use of an EGFR-inhibitor or an EGFR-specific neutralizing antibody (6). The literature is divided as to how this phenomenon occurs; several studies suggested that TCDD stimulates the synthesis and release of EGFR ligands (7-9) while others suggested that the EGFR is instead activated in a ligand-independent way, such as through transactivation by phosphatidylinositol-3 kinase (PI3K)- activated phospholipase Cγ (PLCγ) (10) or through the non-receptor tyrosine kinases (RTK) such as c-src (11, 12). However, work from several laboratories show that TCDD and calcium (Ca), the physiological modulator of keratinocyte differentiation, cause EGFR down-regulation from the cell surface (13-15). EGFR down-regulation, which would attenuate signaling seemingly contradicts the requirement for epidermal growth factor (EGF)-like ligands and EGFR activation in TCDD-induced proliferation (14). However, data supporting a hyperproliferative role of TCDD in keratinocytes has been equivocal, dependent upon endpoint, treatment protocol, and which cell lines have been used (13, 14, 16).

In this study, we sought to clarify the role of the EGFR and its ligands in mediating the TCDD-induced proliferation of normal human epidermal keratinocytes (NHEKs) by addressing the following questions:

1. What causes the down-regulation of EGFRs observed in Ca differentiated or TCDD-treated NHEKs?
2. Is the loss of EGFRs associated with a reduction in (or the loss of) EGFR signaling capacity?
3. What role do individual EGFR ligands play in EGFR down-regulation and proliferation in Ca- or TCDD-treated cells?

While the literature suggests that crosstalk exists between the aryl hydrocarbon receptor (AhR) which binds TCDD, other cellular signaling pathways, and the EGFR (12, 17, 18), our work shows that TCDD enhances the secretion of transforming growth factor-α (TGF-α) and epiregulin (EREG) while maintaining an environment of high amphiregulin (AREG). We further found that neutralization of all three ligands is required to reduce cell number but that neutralization of singular EGFR ligands have unique effects on loss of surface-associated EGFR and keratinocyte proliferation.

Literature Review

Human exposure to 2,3,7,8-tetrachloro-p-dioxin

TCDD is a ubiquitous environmental contaminant that is not produced intentionally but persists in the environment due to its lipophilicity and production as a byproduct in industrial and natural combustion processes in the presence of chlorinated waste products (19). TCDD is also a product of pulp bleaching in paper production and is thus found as a contaminant in paper products including, alarmingly, some feminine hygiene products and diapers at very low levels (20). TCDD is also created during the production of some herbicides (19). Agent Orange is the most prominent example, but it has also been produced in other milder pesticides (19)—though many of these have been

discontinued since by the Environmental Protection Agency (EPA). Due to heavy regulation of TCDD-producing industries, exposure to high levels of TCDD is uncommon (19) though lower level exposure is not.

TCDD is a highly toxic and lipophilic chemical which tends to concentrate and persist in the food chain, particularly in fish. It follows that the major source of exposure to TCDD and related compounds is dietary (**Table 1**). The average person carries a TCDD load of 32 parts per trillion in his or her fat in a steady state (21). Regardless, these levels, even in the most extreme situations are well below the United States Department of Agriculture's reference dose (20, 21). Nevertheless, its persistence in the body can prolong the effect of even a low dose of TCDD. In a study of Agent Orange-exposed Vietnam War veterans, TCDD was found to have a half-life of 7.1 years (95% c.i. of 5.2 – 8.6 years) in the human body--much longer than in most laboratory animals (22). However, TCDD half-life can be much shorter (1-3 years) in patients exposed to very high levels (>100,000 pg/g lipid weight) (23) and over 10 years for patients exposed at low levels (<50 pg/g lipid weight) (22). This phenomenon was observed following the poisoning of Viktor Yushchenko, but also observed in highly exposed individuals from European occupational or accidental exposures (23). Researchers hypothesized that this was due to a higher concentration of activating metabolizing enzymes in the skin or liver (23). By a similar rationale, a meta-analysis found that half-life could also be dramatically reduced in patients who were regular smokers, owing to the smoke's induction of cytochrome P450 1A1 (CYP1A1) (24). TCDD has also been shown to have a shorter half-life in individuals with a lower percentage of body fat, owing to a smaller reservoir for TCDD storage (25) , as well as for patients who are under 18 at time of

exposure due to rapid growth and turnover of lipid stores, breastfeeding women, and individuals on drugs used to mobilize fats (23, 24). However, weight loss post-exposure did not affect serum levels of TCDD (24).

The preceding information reveals how difficult it is to quantify TCDD's relative toxicity in the human body. The National Institute for Occupational Safety and Health (NIOSH) has not determined a relative exposure limit or an immediately danger to life and health limit, nor has Occupational Safety and Health Administration assigned a permissible exposure limit (19). However NIOSH notes TCDD to be an occupational carcinogen (19). Even in animals, the LD_{50} varies between 500 ng/kg body weight in guinea pigs to over 1.1 mg/kg body weight in Siberian hamsters (26). Within strains or even sexes of rats, fold-level differences can exist (26, 27) and studies in NHEKs *in vitro*

Table 1. Average dietary TCDD exposures (21)

Food	[TCDD] (pg/g)	Average daily TCDD intake pg/person/day
Ocean fish	500	8.6
Freshwater fish	70	--
Meat	35	6.6
Cheese	16	0.31
Milk	1.8	0.20

show an order of magnitude in individual variation of EC_{50} (0.45 nM – 1.41 nM) of TCDD in four donor cell lines (28). LD_{50} has not been determined in humans due to the paucity of lethal dose exposures to TCDD, though it appears to be on the higher end of the interspecies range presented above (23, 29). Still, regulatory bodies maintain that even low level exposures can be harmful. While the effect of chronic, low level exposure

to TCDD is difficult to parse, several large scale accidental or intentional exposures have brought TCDD into the public eye and helped elucidate the effects of TCDD on human health *in vivo*.

Large-scale exposures since World War II

Food contamination: Dioxins and other polyaromatic hydrocarbons (PAHs) bioaccumulate in fatty tissue in animals, but there have been several incidences over the past few decades involving excessive contamination from dioxins or other PAHs such as polychlorinated biphenyls (PCBs) or polychlorinated dibenzo-furans (PCDFs). The most famous case occurred in 1968 in the Japanese Kyūshū province. Rice bran oil was contaminated by PCBs, affecting 14,000 people, and over 400,000 birds who died of respiratory distress. In humans, a disease called Yusho disease was identified, marked by skin and eye lesions, irregular menstruation in women, weakened immune responses, and cognitive defects in children. In 1979, Taiwanese rice bran oil was similarly contaminated, causing many of the same symptoms and referred to as YuCheng illness (30). These, as with most environmental contaminations, were of a mixture of PCBs and PCDFs compared to laboratory based studies which tend to use TCDD (30). However, victims in the Yusho and Yucheng group have been closely monitored for the past four decades for outcomes on reproductive health, cancer incidence, skin manifestations of PCDF intoxication, and metabolic syndromes (31). One difference between the two exposures is that a coplanar PCB in the Yusho incident appears to be linked with thymic atrophy and decreased androgen metabolism, contributing to reproductive effects observed in exposed women (32). Women were also more strongly affected in the Yucheng cohort with increased risks of diabetes, chloracne, or hypertension compared to

a control population (33). As recently as 2008, a dioxin contamination of Irish pork caused a worldwide recall, though fortunately the European Union recognized this before it caused a global health crisis. About 10% of the population of Ireland was exposed to meat thought to have been contaminated through animal feed (34). It is too soon to see if chronic health problems have arisen from these exposures, but early data indicate TCDD has not been elevated in breast-milk of first time Irish mothers, though some increase in PDCFs was observed (35).

Seveso, Italy: On July 10, 1976, a factory in Seveso, Italy, 9 miles north of Milan exploded. The factory produced 2,4,5-trichlorophenol, an intermediate for hexochlorophene, an antibiotic used in toothpaste and soaps, and for 2,4,5-trichlorophenoxyacetic acid, an herbicide (36). TCDD was created unintentionally as a byproduct of this reaction and of the 6 tons of chemicals released. 1 kg of TCDD was distributed over an 18 km^2 area, in some areas as high as 100 ppm (36). Within days, 3,300 animals, mostly poultry and rabbits were found dead, and over the next two years 80,000 animals were slaughtered to prevent bioaccumulation within the food chain. Over 5,436 humans lived within an area with soil concentrations > 5 $\mu g/m^2$ (36). This represents the largest scale human exposure in a residential population to date and the cancer and disease incidences of these exposed populations has been extensively followed in the nearly 40 years since. (37).

Love Canal, New York: From 1942 until 1953, Love Canal, NY was used as a dumping ground for waste from the Hooker Electrochemical Company, the US military, and the city of Niagara Falls. Here, Hooker stored 55 gallon drums of industrial waste, including “caustics, alkalines, fatty acids and chlorinated hydrocarbons from the

manufacturing of dyes, perfumes, solvents for rubber and synthetic resins (38, 39)." After 1953, the canal was paved and sodded over, and sold to the Niagara Falls City School District for $1 (U.S.). They built a school and surrounding community on the site (39). Love Canal represents persistent exposure like Seveso. Twenty years later ground water testing showed the presence of dioxins at ~53 parts per billion as a result of seepage from the dump into the sewer. Further research showed an increased incidence of miscarriages and birth defects in children born in Love Canal (40, 41). Infants born to exposed mothers were also more likely to be preterm, at a low birth weight, and had a skewed male:female ratio (41). Children born and raised in Love Canal suffered increased incidences of epilepsy, cognitive disorders, eye problems, and skin rashes (38). Nearly 1/3 of the population showed high white blood cell counts and chromosomal damage, considered to indicate increased likelihood of leukemia (42) and after 30 years, one study found a slightly elevated incidence of bladder and kidney cancer in former Love Canal residents, though the sample size was too small to draw definitive conclusions. No increase in all-cause mortality was observed (43, 44).

Times Beach, Missouri: Times Beach, Missouri was a town of around 2000 people located 17 miles south of St. Louis and the site of another mass exposure to TCDD (45). In 1972, the City of Times Beach paid a subcontractor to pressure wash 2400 feet of dirt roads within the town. This subcontractor who owned a trucking and oil business, had also held contracts for waste disposal for the Northeastern Pharmaceutical and Chemical Company, Inc., a company that manufactured hexachlorophene from 2,4,5-trichlorophenol (the same chemical manufactured in Seveso). This subcontractor used the same trucks to carry dioxin contaminated wastes as he did to pressure wash the roads and

over the next four years, he sprayed an estimated 160,000 gallons of toxic waste on the roads (45). In 1983, dioxin concentrations on the roads were still measured at 0.3 ppm (46). Interestingly, the public health ramifications of Times Beach were minimal at best as no cases of chloracne, the hallmark condition of dioxin exposure were observed (47). In fact, a 1991 EPA report questioned whether the Times Beach evacuation had really been necessary (48) and in 1997, Times Beach was removed from the list of superfund sites needing cleanup (47). Further, soil samples as of November 2012 indicate that Route 66 State Park in Times Beach now poses no threat to park visitors or workers (47, 49).

Viktor Yuschenko's poisoning: The most public instance of dioxin in the news was the 2004 poisoning of Ukrainian presidential candidate Viktor Yushchenko. Yuschenko was admitted to hospital in early September 2004 with acute pancreatitis, and over the coming months developed a disfiguring skin condition, identified by doctors as chloracne (23). In December 2004, serum samples from Yushchenko confirmed a level of 108,000 pg/g lipid weight laboratory-grade TCDD-- more than 50,000 times that of the general population (23). Over the next three years, a team of doctors monitored Yushchenko's blood, urine, feces, skin, sweat, and material extracted from skin lesions for levels of TCDD and its metabolites, as 2,3,7-trichloro-8-hydroxydibenzo-p-dioxin (OH-TriCDD) and 1,3,7,8-tetrachloro-2-hydroxydibenzo-p-dioxin (OH-TCDD) wherein they were able to make several determinations about TCDD metabolism and secretion in humans (23). Doctors found that most TCDD was excreted fecally, a similar finding to observations in rodents (27). They also found that most excreted TCDD was unmetabolized, particularly in the skin and serum, though fecal samples contained the

highest concentration of metabolites (23). Further, Sorg *et al.* discovered a high concentration of TCDD in samples removed from TCDD-induced skin lesions, suggesting a new mechanism for TCDD elimination (23). In Yushchenko, researchers found TCDD to have a half-life of 15.4 months, much shorter than expected even with his high initial serum levels. Some of this accelerated metabolism was attributed to frequent surgery and biopsying as well as to pharmacological interventions which enhanced cytochrome P450 1A2 (CYP1A2) expression and dioxin metabolism, and mobilized lipid turnover, depleting the reservoir for dioxin, such as Olestra and Orlistat (23).

Molecular and genetic actions of TCDD

Epidermal hyperproliferative disorders are frequently caused by genetic and idiopathic factors, and chemicals have also been shown to replicate this disease phenotype. 2,3,7,8-tetrachlorodibenzo-p-dioxin (**Figure 1**) is the most potent congener in a class of polychlorinated dibenzodioxins (19) and binds to a receptor known as the AhR (50).

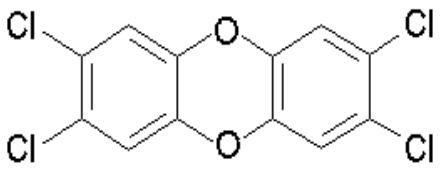

Figure 1. Structure of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (19)

The AhR is an ligand-activated transcription factor and serves as a basic helix-loop-helix transcription factor containing a Per-Arnt-Sim domain (bHLH/PAS) which binds polyaromatic and halogenated aromatic hydrocarbons (51). In its unliganded form, the AhR is cytosolically bound to several proteins including heat shock protein 90 (Hsp90) (50). Upon ligand binding (**Figure 2**), the nuclear localization signal on the AhR is exposed, allowing the AhR to migrate to the nucleus where it partners with ARNT, the aryl hydrocarbon receptor nuclear translocator (52). The AhR-ARNT heterodimer binds DNA at 5-7mer sites identified as xenobiotic response elements (XREs) with a consensus sequence of TnGCGTG (with n indicating any base) (53). Although this is the most clearly understood mechanism of AhR activation, very few genes have been confirmed to be controlled directly through an XRE. A computerized search of the human genome discovered 72,318 putative XREs and found further that XREs were more heavily concentrated in coding regions of DNA compared to non-coding regions, and were most concentrated within 1.5 kb of the transcription start site (TSS) (54). Canonical genes induced following AhR activation include cytochrome P450 1A1 and 1B1 (CYP1A1 and CYP1B1) (52, 54) both of which been confirmed as XRE-dependent in mouse, and CYP1A1 has been confirmed in rat (54-56). CYP1A1 and CYP1B1 are

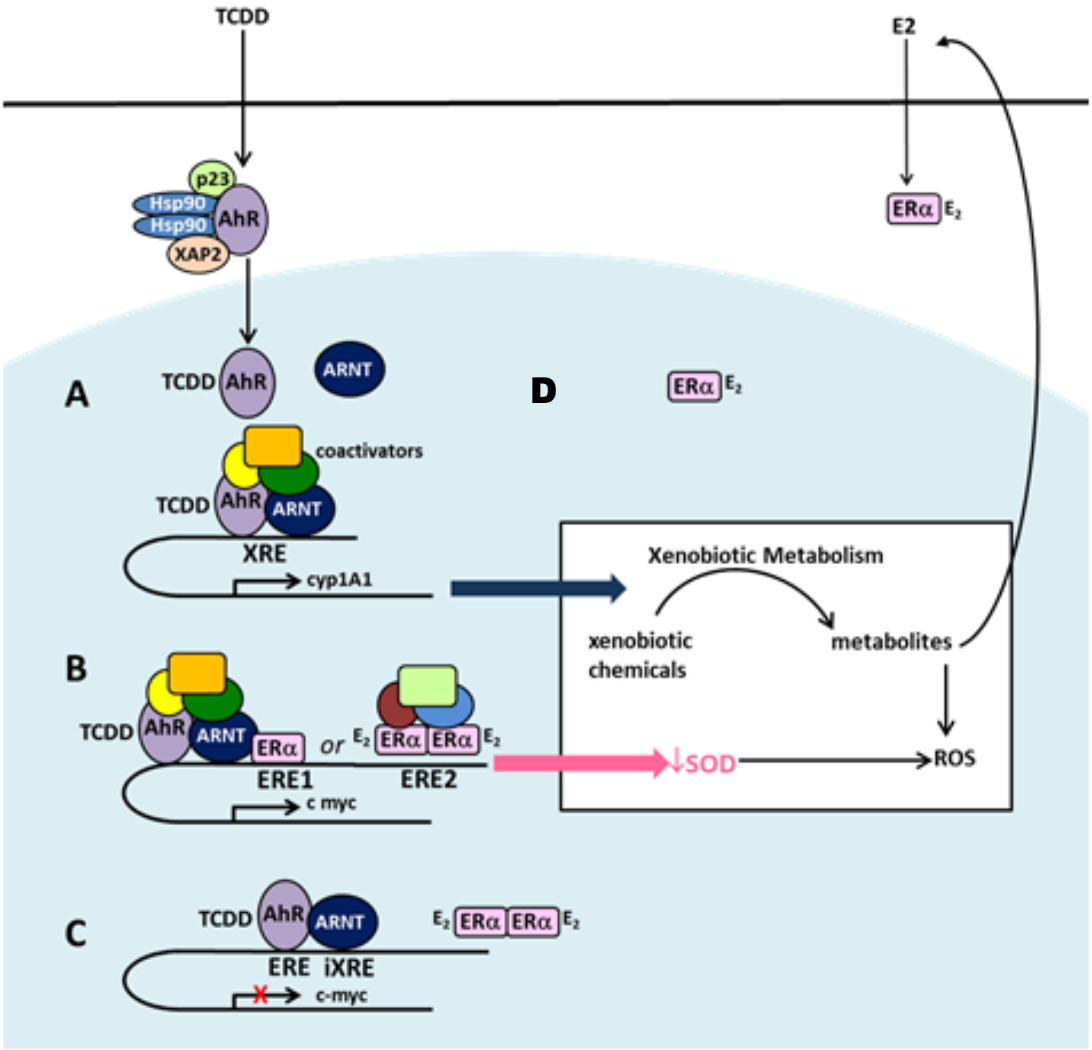

Figure 2. Canonical and non-canonical mechanisms of AhR-mediated activation A. TCDD-dependent activation of AhR through AhR/ARNT binding at an XRE sequence leads to upregulation of target genes (53, 63) **B.** TCDD-dependent estrogenic activity through AhR/ARNT/Estrogen Receptor α (ERα) at an estrogen response element (ERE) or estradiol (E2) dependent activation via E2 classical pathway lead to an upregulation of c-myc (64). **C.** TCDD-dependent anti-estrogenic activity through AhR/ARNT binding to inhibitory XRE (iXRE) sequences, blocking ERα homodimer binding to ERE sites leading to a downregulation of c-myc (64). **D.** Xenobiotic metabolism may create estrogenic metabolites, antiestrogenic metabolites, or ROS. Sod1 removes ROS from the cell. (18).

members of the cytochrome P450 protein family of monooxygenases that are involved in xenobiotic and drug metabolism, a logical target for the AhR (52). CYP1B1 in particular hydrolyzes PAHs and 17β-estradiol (57, 58). Genes involved in metabolism, lipid synthesis, and response to reactive oxygen species (ROS) are also directly regulated by the AhR including: aldehyde dehydrogenase 3 family member A1 (Aldh3a1), NAD(P)H dehydrogenase, quinone 1 (Nqo1), glutathione S-tranferase alpha 2 (GSTA2), UDP glucuronosyltransferase 1-6 precursor (Ugt1A6) were verified as XRE-mediated in rats (54, 59). Superoxide dismutase 1 (Sod1) converts superoxide radicals to molecular oxygen and hydrogen peroxide (H_2O_2) which can then be broken down by catalase. Catalase is critical in the cellular ROS response and has been shown to be XRE-mediated in humans and in rats, presumably to aid in neutralization of ROS generated as xenobiotic metabolites (60, 61). Nevertheless, XRE-mediated genes directly related to keratinocyte differentiation are still largely undescribed. In mice, the EGF receptor ligand EREG is regulated by an XRE, though this finding has not been replicated in humans (9). Finally, work from our lab has shown Filaggrin (Flg), a marker of keratinocyte differentiation, to be likewise regulated by an XRE 1400 bp upstream of its TSS (18).

Increasingly, there has been discussion of non-canonical mechanisms of action of the AhR, particularly those pertaining to its endocrine disrupting actions (**Figure 2B-C**). TCDD and many planar hydrocarbons in its class can have anti- or pro-estrogenic effects which may be tissue- specific (62). In porcine ovarian cells, TCDD was shown to affect estrogenic signaling and oocyte maturation at the level of steroidogenesis through inhibition of P450 cholesterol side chain cleavage enzyme (P450SCC) and aromatase (63), fundamental steps in estrogen synthesis. This finding was echoed in rainbow trout

where TCDD inhibited P450SCC and steroidogenic acute regulatory protein (stAR) (64). Aluru *et al.* also showed that TCDD-exposed trout produced less cortisol following an adrenocorticotrophic hormone (ACTH) challenge, suggesting TCDD exposure may decrease glucocorticoid production and diminish stress responses (64). The AhR can directly bind the ERα, and the AhR/ARNT/ERα can either signal through estrogen-regulated genes in the absence of E2 or bind inhibitory iXREs located near EREs, conformationally blocking the binding of ligand bound ERα (65, 66).

The AhR can function as a mediator of inflammation in several ways: directly, through XRE-mediated induction of inflammatory cytokines involved in Th17 cell differentiation, particularly (interleukin) IL-22, IL-23, and IL-1A, as well as the retinoid-related orphan receptor (RORα) (67, 68). This may also be due to the ability of AhR to bind nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (69, 70). This likely evolved as lipopolysaccharide (LPS) binds the AhR, however, pathologically, it leads to a hyperinflammatory feedforward loop that explains dioxin's role in persistent inflammation *in vivo* (70, 71). AhR binding to NF-kB also serves as a mechanism of cell cycle inhibition wherein AhR sequesters NF-kB, attenuating its role in DNA-synthesis (72). AhR can also inhibit cell cycle progression by sequestering pRb which preferentially binds to the liganded Ahr (66, 69). Finally, AhR can alter cellular sex steroid signaling by functioning as a ligand-dependent scaffolding molecule within cullin 4b E3 ubiquitin ligase complex which targets these hormones for degradation (73).

Overall, the AhR has a variety and wide ranging subset of cellular and tissue responses from hormonal, to inflammatory, to proliferative. While it is likely that some effects of TCDD on epidermal differentiation are mediated through canonical, XRE-

mediated routes, it is possible that cofactor competition, or non-canonical mechanisms are mediate TCDD-accelerated epidermal differentiation.

Health risks of TCDD exposure

As discussed above, population-wide acute or chronic exposures to TCDD have given researchers insight into target tissues for PAHs. TCDD intoxication produces or contributes to a variety of diseases in four major categories: 1) immunotoxic and inflammatory, 2) oncogenic, 3) reproductive and developmental, and 4) skin (74).

Immunological effects: The immune system is complicated and intrinsically tied to tissue homeostasis throughout the body, however there is evidence that the immune system is a site of toxicity for TCDD. Prolonged dioxin exposure causes thymic atrophy in rats (27), a response also recognized in YuCheng victims (32). It is suggested that an AhR-mediated loss of NF-kB signaling which is required for normal medullary thymic development is involved in this thymic atrophy (70, 75). Lacking a competent thymus, the patient has difficulty with T-Cell maturation, which TCDD further compounds. Individuals exposed to PAHs *in utero* show poor notch1-induced T cell differentiation, predisposing them to T-acute lymphocytic leukemia (T-ALL) (76). Further, TCDD promotes Th17 maturation in a seemingly direct, XRE-mediated manner (68) which is linked to increased inflammation and autoimmunity. Nude mice, which congenitally lack T cells, are less sensitive than their immunocompetent littermates (77). These changes in immune cell function cause an increased susceptibility to infection and a hypersensitivity to LPS (78). While it was originally thought that this enhanced susceptibility was specific to gram negative bacteria, it has also been shown to increase rates of infection, morbidity,

and mortality from gram positive bacteria (79), viruses (80, 81), parasites (82, 83), and even establishment of transplanted neoplasms (84).

Oncogenic effects: TCDD is classified as a known human carcinogen, (19). While victims of the 1976 Seveso disaster showed increased incidences of metabolic disorders and blood cancers (85), a meta-analysis of TCDD-exposed populations show an increased risk of cancer in general as opposed to a specific type (86, 86). However when these findings were applied to Agent Orange-exposed military veterans or smaller occupational groups, the all-cancer risk was not supported (87). In animal models, mice, rats, and hamsters exposed to TCDD reliably developed tumors in multiple organ systems (88). In fact, TCDD-induced tumor development in these rodent models increased in a dose-dependent manner (88).

Others suggest that TCDD functions not as a direct mutagen, but as a tumor promoter, leading to more tumors following tumor initiation studies (89, 90). Interspecies variability in sensitivity and LD_{50} as well the ethical inability to perform these same experiments in humans outside of cell culture leave these opposing hypotheses unanswered (26, 91). In female rats, the LD_{50} is twice that in males (45 µg/kg to 22 µg/kg, respectively) (88). This makes prediction of toxicity dose based upon exposure level imprecise. The toxic equivalency factor which quantitates the potency of a ligand is established based upon toxicity in male rats and fails to take into account interspecies or interstrain pharmacogenomic differences in rodents, but also population level variance in sensitivity for humans exposed to admixtures of PAHs in large or, even small, scales (92).

Where the evidence for TCDD-mediated oncogenesis is somewhat stronger is in the offspring of acutely TCDD-exposed parents. In rats exposed *in utero* exposure are predisposed offspring to mammary cancer by increasing the numbers of terminal end buds. Additionally, mice exposed to TCDD *in utero* are predispose to T-ALL as a result of desensitizing them to notch1-signaling which drives T cell development (76), and children of Agent Orange exposed veterans have increased incidences of rhabdomyosarcoma (93).

Reproductive and developmental effects: One of the most common fears regarding endocrine disrupting chemicals is that they will mimic estrogen in males, feminizing them. However endocrine disrupting chemicals can function in a variety of ways including : 1) inhibition of aromatase which converts testosterone to E2; 2) antagonism or agonism of the ERα which can be anti- or pro-estrogenic; 3) agonism or antagonism of the androgen receptor; 4) agonism of the AhR which can inhibit or activate ERα; 5) interaction with steroid binding proteins; and 6) altering hormone metabolism (94) . Effects have been seen on male reproductive health and hormones: PCB exposure in the YuCheng incident has led to decreased serum androgen and gonadal testosterone synthesis in male patients (32). TCDD exposure in rats leads to decreased epididymal sperm count through an apoptotic rather than anti-spermiogenesis mechanism (95, 96). Other works in rats and hamsters confirmed lowered sperm count and demonstrated decreased sex organ size and reduced semen quality, in the presence of normal levels of androgens. However, it is in the hypothalamic-pituitary-gonadal (HPG) axis of women where TCDD has its most marked effects.

TCDD can lower fertility in both males and females (97, 98). However, in women, TCDD also increases the risk involved in a pregnancy. Pregnant women suffer from higher incidences of miscarriage following TCDD exposure (41) and enhanced placental inflammation which, if insufficient to cause a miscarriage, can lead to preterm birth or incidences of endometriosis (99). TCDD can be detected in the breast milk of lactating mothers who have been exposed to high levels of TCDD (35). Further, twenty years after the Seveso incident, researchers observed a strongly elevated incidence of breast cancer in 900 women exposed to high levels of TCDD (100). However at thirty years, this was no longer significantly different than the population at large (37).

The risks of TCDD exposure *in utero* and in newborns are greater still. Following the Seveso incident, 30 pregnant women in Zone A nearest the site had induced abortions, and 4 women who had been pregnant during the incident spontaneously miscarried (100). Of these, no gross morphological, or obviously fetotoxic effects were observed, though the spontaneous abortions showed structural abnormalities (100). The small number of embryos, often incomplete structurally, is not sufficient to rule out a damaging effect of *in utero* exposure (100). More, observations from Love Canal support this hypothesis. What initially drew attention to the contamination in Love Canal was a higher rate of miscarriage, as well as a higher rate of birth defects (101), matching a finding previously observed in mice where *in utero* exposure caused cleft palate (102, 102) . Learning and cognitive disabilities were seen in children raised in Love Canal or exposed *in utero* to contaminated rice bran oil (31, 41). Children born in Love Canal were more likely to be low birth weight or premature (41). But these observations were all made in very small populations, making definitive conclusions difficult.

Dermatologic effects: The hallmark symptom of TCDD exposure in humans is chloracne (74). Chloracne is characterized by hyperproliferation and altered differentiation of the interfollicular epithelium as well involution and hyperkeratinization of the pilosebaceous unit which is comprised of the hair follicle and sebaceous gland (74). Comedones on the interfollicular epidermis contain a higher concentration of TCDD than unaffected skin, directly linking chloracne to the disease (23). Interestingly, chloracnegenesis seems inversely proportional to cancer, perhaps due to increased secretion of TCDD through dermal lesions leading to a shorter window of exposure. In a report studied twenty years after the Seveso incident, no individuals who developed chloracne shortly after the explosion subsequently developed cancers (85).

As chloracne is one of the most common morbidities of humans following TCDD exposure, the skin becomes an ideal model in which to study TCDD biology. TCDD causes a variety of effects on the skin, upregulating genes involved in epidermal differentiation, including 24/60 of the genes on the Epidermal Differentiation Complex, a region on chromosome 1q21 (18). In mice, TCDD exposure caused epidermal barrier to form one day earlier *in utero* in what has been shown to be a Flg-dependent manner (5). TCDD has been shown to upregulate Flg mRNA in an XRE-dependent manner (1) and this upregulation is blocked by stimulation of the EGFR (6). TCDD has been shown to accelerate barrier formation through the increased synthesis of barrier lipids (103). In addition, TCDD upregulates EGF-like ligands which could drive hyperproliferation (7, 9, 104).

The Structure of Human Skin

Skin is the body's largest organ (**Figure 3A**), and provides the first line of defense against external threats. It also plays an important regulatory role against desiccation (105). The epidermis, comprised of highly stratified squamous epithelial cells, provides a physical and watertight barrier (105). It is interspersed with tissue- specific macrophages, called Langerhans cells, sensory receptors called Merkel disks, and melanin-producing cells called melanocytes (106) which collectively provide a physical, biochemical, and adaptive barrier (107).

The epidermis also plays an important role in vitamin D synthesis following sun exposure (108). Sunlight causes the conversion of 7-dehydroxycholesterol (7-DHC) in the membrane of keratinocytes and dermal fibroblasts to to cholecalciferol (vitamin D_3). Cholecalciferol is hydroxylated in the liver to make 25-hydroxyvitamin D_3 ($25(OH)D_3$). $25(OH)D_3$ is further hydroxylated in the kidney by 1-alpha-hydroxylase to form 1,25-dihydroxy-vitamin D ($1,25\text{-}(OH)_2D_3$). This end product, calcitriol, regulates Ca intake enterically and the release of Ca from the mineralized matrix of bone. Melanocytes embedded in the epidermis counterbalance this vitamin D production by absorbing excess Ultraviolet B (UVB) and preventing 7-DHC activation (109). Skin color is dependent upon the inherited density and clustering of granular melanosomes in melanocytes (with caucasian skin having fewer often in clusters while darker skin has more diffusely distributed melanosomes (110)), UVB radiation also causes branching of dendritic shaped melanocytes and the increased trafficking of melanin-containing melanosomes, to decrease ultraviolet (UV) light-induced damage to DNA. This also causes a induced darkening of the skin, such as tanning or photoaging (108, 110)

The same cells that give rise to the epidermis also gives rise to the pilosebaceous units, comprised of hair follicles and sebaceous glands (106), or in hairless regions, sebaceous glands which cover all of the body except the soles of the feet and hands (111). The sebaceous gland secretes sebum, a waxy substance comprised of triglycerides, squalene, wax esters, and other lipid metabolites (112). Sebum serves to lubricate and further waterproof the epidermis as well as acidifying the skin to a pH between 4.5 and 6.2 which serves as a barrier against bacteria, viruses, and dermal contaminants (113). In high temperatures, sebum emulsifies sweat from eccrine sweat glands (also embedded in the epidermis) to further cool the body (114). Hair follicles and sebaceous glands are maintained by stem cells located in in the follicular bulge (115). Epidermal hair serves a thermoregulatory and sensory function.

Below the epidermis lies the dermis: a highly vascularized and innervated layer comprised of fibroblasts, adipocytes, and macrophages in an extracellular matrix (ECM) comprised of collagen, elastin, and a loose reticular extrafibrillar matrix consisting of proteoglycans and glycosaminyoglycans (105, 115). Within the dermis are blood vessels and capillary beds which provide oxygen to epidermal keratinocytes, and a variety of sensory neurons for pressure, temperature, and pain (115). Below the dermis lies the hypodermis, which stores subcutaneous fat that further stabilizes body temperature and can be broken down to provide energy (115).

The Structures of the Epidermis

Stratum Basale: Within the epidermis, a single layer of cuboidal basal cells sits in contact with the basement membrane of the dermis via hemi-desmosomal structures [**Figure 3B** and see (116)]. This contact provides cells access to the dermal ECM and

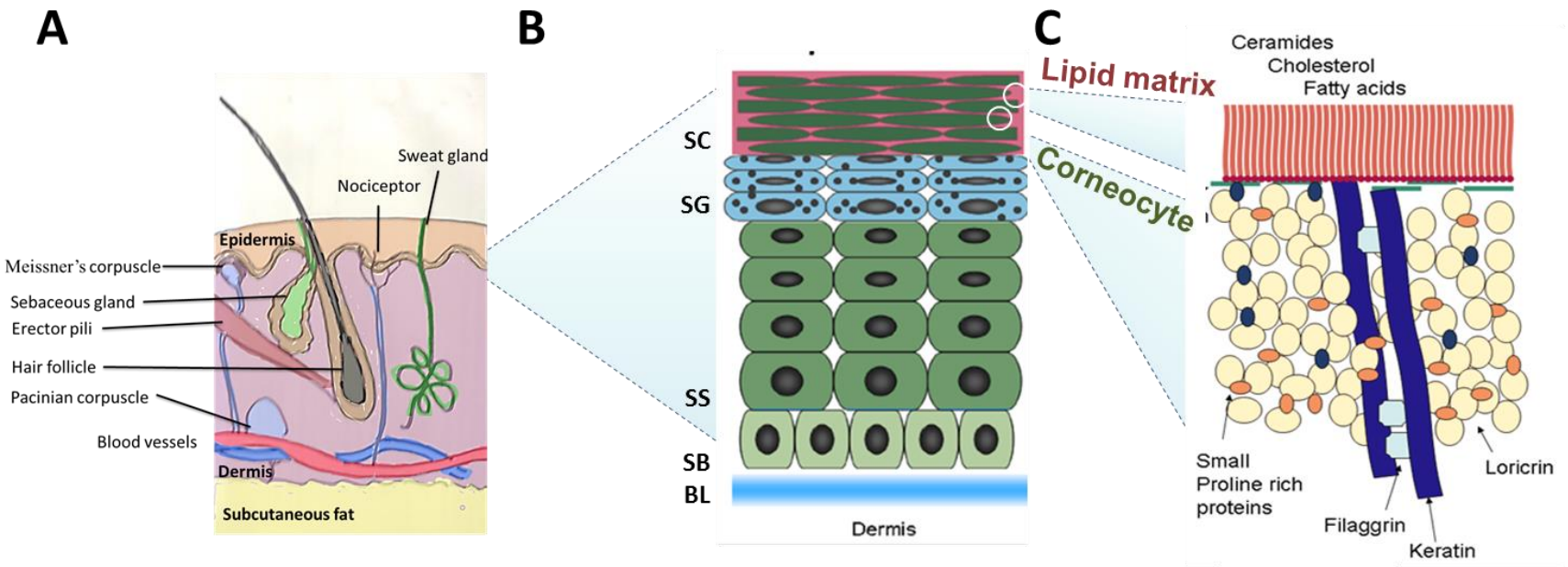

Figure 3. Human skin and accessory structures, the epidermis, and the cornified envelope. A) The human skin is derived from the mesoderm embryonically and is comprised of multiple layers. The superficial epidermis provides a defensive epidermal barrier and prevents desiccation. The dermis provides structural support to the epidermis as well as vascularization and innervation for pressure, temperature and pain. Below the dermis lies the subcutaneous layer which is primarily used for fat storage (115). **B)** The epidermis is comprised of keratinocytes and contains melanocytes, and skin-specific macrophages known as Langerhans Cells. As keratinocytes lose contact with the basal lamina (BL) and move from basal to suprabasal layers. they undergo terminal differentiation as they move from the stratum basale (SB) through the stratum spinosum (SS) and stratum granulosum (SG) towards the stratum corneum (SC) where they express layer-specific markers of differentiation (left). **C)** In the stratum corneum, cells extrude lamellar bodies containing ceramides, fatty acids and cholesterol outside the cell. The membrane of the mature, anucleate keratinocyte (corneocyte) has also undergone structural changes including the cross-linking of small proline-rich proteins (SPRR) to membrane proteins such as loricrin (Lor) and involucrin (Ivl). Corneocytes have also undergone keratinization where keratins 1 & 10 are crosslinked and bundled together by filaggrin (Flg).

also provides juxtacrine signals that maintain the basal cells in a proliferative, undifferentiated state (107). The stratum basale contains pockets of proliferative cells that asymmetrically divide to maintain the epidermal stem cell population and produce a population of transit-amplifying daughter cells (TACs) which migrate superficially into the suprabasal layers (117, 118). TACs are an undifferentiated population of cells transitioning between multipotent stem cells and differentiated cells (119). These daughter cells are already expressing markers of the earliest phases of keratinocyte differentiation, keratin-5 and -14 (K5, K14) and p63 (119).

Stratum Spinosum: Suprabasal keratinocytes become increasingly differentiated as they move towards the stratum corneum, the uppermost layer of the epidermis (107). As cells progress towards the epidermal surface through the stratum spinosum and granulosum to the stratum corneum (**Figure 3B**), they undergo profound morphological changes and consist of populations of cells transitioning between multipotent stem cells and differentiated cells. They begin to express markers of differentiation. In the stratum spinosum, cells have lost contact with the basal membrane and begin to increasingly rely upon on transitional desmosomal contact and tight junctions (TJs) with other keratinocytes for juxtacrine signaling and maintenance of differentiation status (105). TJ proteins such as claudins 1, 4, and 7, as well as occludins and zona occludens protein 1 (ZO1) are expressed in the stratum spinosum (105). These TJs serve to enhance epidermal barrier function in the case of disruption of the stratum corneum (120) and mice defective for occludins or claudins die from excessive water loss shortly after birth. In addition, aberrant expression of claudin-4 and occludin have been linked to epidermal diseases such as psoriasis vulgaris, ichthyosis, and lichen planus (121). Cells within the

stratum spinosum also change their protein expression. These proteins, including k1 and k10, envoplakin (Evpl), periplakin (Ppl), 14-3-3δ, and members of the S100 family of Ca^{2+} binding proteins [S100a7, S100a8, S100a9 and S100a10 (122-125)], are important in barrier structure and function and serve as markers of keratinocyte differentiation (**Table 2**).

Stratum granulosum: As cells progress into the stratum granulosum, cell morphology progresses towards a more squamous phenotype, exemplified by flat, scale-like cells, and an increased cytoplasm: nucleus ratio. High extracellular Ca (>1 mM) leads to the formation of keratin-rich cytoskeletons linked and stabilized at desmosomes through desmoglein 1 (Dsg1), a desmosomal cadherin (126, 127). Dsg1 inhibits EGFR signaling in the stratum granulosum and firmly commits cells to terminal differentiation (128). Higher extracellular Ca further raises intracellular Ca mediated by the Ca receptor (CaR) whose expression is regulated by 1,25-$(OH)_2D_3$ (123, 129). The CaR activates PLC signaling, particularly PLCβ and PLCγ1 which ultimately leads to the upregulation of several markers of differentiation and cornified envelope (CE) precursors such as transglutaminase 1 (Tgm1) and Flg (11). Increased intracellular Ca is required for the redistribution of signaling molecules such as protein kinase C (PKC)-α and calmodulin to the membrane (130), metalloproteinase and Tgm1 activation (131-133), and is correlated with the expression of markers of differentiation such as Flg (a protein that bundles keratins (1, 107, 134)) and two Tgm substrates involucrin (Ivl) and loricrin (Lor) (135).

Stratum Corneum: Within the stratum corneum (**Figure 3C**), terminally differentiated cells undergo nuclear degradation as part of a specialized program of cell death called cornification (136). While cornification leads to nuclear and DNA

degradation as is typically seen in apoptosis, it is a slower and Ca-dependent process wherein organelles are lysed but DNA is not fragmented. Further, cells remain metabolically active, though mitotitically inactive until eliminated by desquamation (137). Corneocytes (fully differentiated, anucleate cells of the stratum corneum).

Table 2. Epidermal markers of differentiation by layer

Epidermal Layer	Gene markers	Reference
Stratum corneum	Lor, Flg, SPRR, Ivl, Cnfn	(121)
Stratum granulosum	Ivl, Tgm3, Tgm5, Claudins, Desmogleins, Desmocollins, Cdsn	(105, 119)
Stratum spinosum	K1, K10, Tgm1, Evpl, Ppl	(105)
Stratum basale	K5, K14	(105, 118, 138)
Basal lamina	α6β4 Integrin, Lama5	(105, 118, 138)

Abbreviations: Loricrin (Lor), Filaggrin (Flg), Small proline-rich protine protein (SPRR), Involucrin (Ivl), Cornifelin (Cnfn), transglutaminase (Tgm), Corneodesmosin (Cdsn), Keratin (K),Laminin-5 (LAMA5)

undergo changes in membrane lipid composition and Tgm1-mediated cross-linking of membrane associated proteins, predominantly Lor, Ivl, and keratins to form a structure known as the CE (107, 137). Interestingly, extracellular Ca drops in the stratum corneum, an event that precedes the extrusion of lamellar bodies from the cytoplasm and formed in the stratum granulosum (139). The specialized lipids contained within these lamellar bodies help to form a watertight matrix in which a multi-layered physiochemical barrier of mitotically inactive, insoluble cells lie within a matrix of specialized membrane lipids similar to “bricks and mortar” in a wall (105).

The stratum corneum is continuously lost through desquamation allowing keratinocytes from the suprabasal layers to take their place (107). The stratum basale maintains this population of cells through the continued production of TACs from the epidermal stem cell population maintained therein, (115, 140). The average turnover of the normal epidermis occurs in 40-56 days *in vivo* in humans and 8-10 days in murine skin (141); However, this turnover time can be shortened with disruption to epidermal barrier as in the case of wound healing (142, 143).

The signals governing keratinocyte differentiation versus proliferation are tightly regulated through the interplay of mitogenic cell-basal laminar interactions and factors such as growth factors and inhibitory micro-environments with increased extracellular Ca, decreased EGFR signaling, and loss of basal-laminar interactions. Further, toxicological or pharmacological interventions can modify epidermal homeostasis. As such, altered proliferation and/or differentiation are implicated in many dermatological conditions, such as psoriasis (142, 144, 145). Epidermal turnover occurs every 28-45 days in the presence of increased inflammation leading to the development of characteristic thick, pruritic plaques and lesions (142). Desmosomal connections or cytoskeletal components are frequently altered or lost in common epidermal diseases (**Table 3**).

Ca as a physiological regulator of epidermal differentiation

As discussed above, Ca is an important modulator of epidermal differentiation. Within the epidermis, Ca is lowest in the stratum basale, increases steadily reaching its

Table 3. Skin diseases with disrupted barrier show cytoskeletal aberrations

Disease	Cause	Genes altered	Presentation	Reference
Atopic dermatitis	Genetic or autoimmune	Flg	Dry skin overall with patches of itchy, red, swollen, cracked skin. More common in children	(134, 151, 152)
Epidermolysis bullosa	Genetic	K5, K14	Fluid filled blistering following pressure	(153)
Harlequin ichthyosis	Genetic	ATP-binding cassette transporter 12 (ABCA12)	Severe hyperkeratosis and greatly accelerated epidermal proliferation. Reduced lipid transfer across the membrane leads to diamond shaped scales and cracked, exposed skin	(154)
Ichthyosis vulgaris	Genetic	Flg	Thickened, cracked skin forming "scales" that improve with age	(155-157)
Lamellar ichthyosis	Genetic	Tgm1	Extensive scaling of the skin with hyperkeratosis	(156, 157)
Pemphigus	Autoimmune	Dsg1, Dsg3	Epidermal acantholysis forming painful, non-pruritic blisters beginning in the mouth (pemphigus vulgaris) or the scalp, back, and arms (pemphigus foliaceus)	(153)
Psoriasis	Immune-mediated inflammatory	PSORS1-9 CDSN	Thickened epidermis and loss of stratum granulosum. Plaques covered with silvery scales with erythrymatous borders	(142, 158)

peak in the stratum granulosum and then dropping again within the stratum corneum (146, 147). As cells similarly progress from basal to suprabasal epidermal layers, high extracellular Ca can cause genomic and non-genomic effects (148) such as increases in expression of markers of differentiation [e.g. Ivl, Lor, and Tgm1 protein within hours (149)], and increased cornification at 1-2 days (150). High extracellular Ca has also been shown to have non-genomic effects on cellular homestasis such as redistribution of desmoplakin (DP), a desmosomal protein that anchors intermediate filaments in desmosomes to the membrane (141) and increases in cell-cell adhesions (15), both of which are important in establishing and maintaining epidermal barrier (115, 159, 160). Ca is also required for the activation of differentiation-related Tgm1 which crosslinks proteins in the CE (107) as well as for the activation of several metalloproteinases essential for migration through the epidermis (131, 133, 161). Ca increases the protein phosphotyrosine content in keratinocytes, activating the non-RTKs fyn (12) and src (11, 162). Through the use of signaling defective mutants of src and fyn, Xie *et al.* showed that these non-RTKs as well as PI3K can phosphorylate PLCγ1 which is required for keratinocyte differentiation in a PI3K-dependent manner (10, 11).

The cause of this epidermal Ca gradient is not entirely clear however it has been shown to be integral to the epidermal barrier formation and differentiation processes (139). Disruption of the epidermal barrier leads to disruption of the epidermal Ca gradient, and that re-establishment of this gradient precedes barrier restoration (139, 147). Further, diseases which disrupt the epidermal barrier, such as psoriasis, or experimentally induced essential fatty acid deficiency show corresponding loss of this Ca gradient (167, 168). *In utero,* the Ca gradient is established alongside fetal barrier formation (146).

However, mechanistically this barrier is poorly understood. The epidermis is replete with membrane-associated Ca transporters which could export Ca released from the endoplasmic reticulum (ER) to the extracellular compartment (169). Elias *et al.* showed through the artificial re-establishment of barriers in barrier-disrupted tissues exposed to cold that energy-free, passive mechanisms such as transcutaneous water flow were sufficient to establish this Ca gradient (139). In fact, studies in two-photon microscopy showed that the majority of this extracellular Ca originated in the intracellular compartment, having been released from the ER or Golgi into the cytoplasm (170).

Concentrations of extracellular Ca that promote epidermal differentiation are associated with increased intracellular Ca (163, 164) produced by Ca binding to the CaR and inducing a conformational change that leads to Gαq-dependent activation of PLCγ (165). PLCβ then cleaves phosphatidyl-inositol 4,5-bisphosphate (PIP_2) in thecellular membrane to form the second messengers inositol trisphosphate (IP_3) and diacyl-glycerol (DAG). (10). DAG activates PKC (166) and IP_3 releases Ca from intercellular stores (165). Intracellular calcium can also be elevated through the opening of Ca-gated outwardly rectifying chloride channels which depolarize the cell causing voltage-gated Ca channels in the ER to release Ca into the cytoplasm (234-236).

Elevated Ca causes genomic effects (125) such as increases in expression of markers of differentiation (149), and morphological changes such as cornification (150). High extracellular Ca also has non-genomic effects on cellular homestasis such as redistribution of DP, a desmosomal protein that anchors intermediate filaments in desmosomes to the membrane (140) and increased cell-cell adhesions (15), both of which are important in establishing and maintaining epidermal barrier function (115, 159, 160).

Ca is also required for the activation of differentiation-related Tgm1 which crosslinks proteins in the CE (107) and the activation of matrix metalloproteinases (MMPs) which cleave membrane-associated ligands and are required for cell motility (171).

The EGF Receptor: A target for regulation by TCDD and Ca

It would be easy to argue that TCDD builds upon the differentiation-inducing mechanisms similarly affected by increased intracellular Ca. However, keratinocyte commitment to differentiation is represented not only by genetic, structural, and morphological changes to the cell, but also by the attenuation of EGFR signaling, a common regulatory target for both Ca and TCDD. Within the epidermis, the EGFR is the major driver of epidermal proliferation (172). Studies have shown that EGFR signaling is strong in basal keratinocytes and is inhibited by Ca in suprabasal layers (173). This is accompanied by the decreased staining and [^{125}I]-EGF binding capacity observed in the suprabasal layers of the epidermis (174-176) and indicates that keratinocyte differentiation is dependent upon both enhanced extracellular Ca and attenuation of EGFR signaling (177, 178). Secretion of EGFR ligands, such as TGF-α, AREG, or EREG, are frequently enhanced in hyperproliferative skin disorders such as psoriasis or atopic dermatitis (145, 179, 180) and experimental overexpression of EGFR ligands can replicate psoriatic phenotypes (176, 181). Further, juxtacrine signaling by surface-associated EGFR ligands maintains differentiated keratinocyte phenotypes while MMP-mediated cleavage of these ligands drives keratinocyte migration in wound healing (182).

Work from our lab has shown that EGF, the prototypical ligand for the EGFR, upregulates nearly 3000 genes in keratinocytes, many involved in processes of epidermal disease (172). Further, pretreatment with EGF decreases the expression of markers of

keratinocyte differentiation *in vitro* and enhances transepithelial water loss (TEWL) indicative of impaired barrier function and dedifferentiation (172). Pre-treatment with EGF inhibited TCDD-induced increases in expression of markers of keratinocyte differentiation, suggesting EGFR signaling is important not only in promoting keratinocyte proliferation but in suppressing differentiation (6).

Structure and Function of the EGFR: The EGFR is a 170-kDa protein that is a member of the ErbB family of Type I RTKs in which each ErbB possesses different ligand affinities and subtly different downstream signaling events (183). All four ErbB receptors are structurally related and have three domains: an ecto-domain, a transmembrane domain, and a C-terminal intracellular tyrosine kinase domain (184). The receptors, while structurally similar, have very different sequences: the EGFR and ErbB2 are the most closely related with 49% identity (64% similarity). In contrast EGFR/ErbB3 have 37% identity (53% similarity) and EGFR/ErbB4 have 28% identity (49% similiarity) (184). The tyrosine kinase domain is the most conserved between receptors (39-81% identity) and the C-terminal domain (CTD) the area of lowest identity (12-30%) (184) (**Figure 4**).

Work in keratinocytes addressing ErbB signaling tends to focus on the EGFR. Keratinocytes also express ErbB2, and ErbB3 both *in vivo* and *in vitro* (185). The EGFR is expressed most strongly in the stratum basale and stratum spinosum where cells are actively proliferating, but is also present in the upper layers of the epidermis (185, 186). ErbB2 and ErbB3 are found in multiple cell lines and embryonic skin at low levels (185, 186). Stoll *et al.* identified ErbB2 in the cytoplasm of keratinocytes in the stratum basale, and on the surface of cells in the stratum granulosum and stratum corneum in sections of

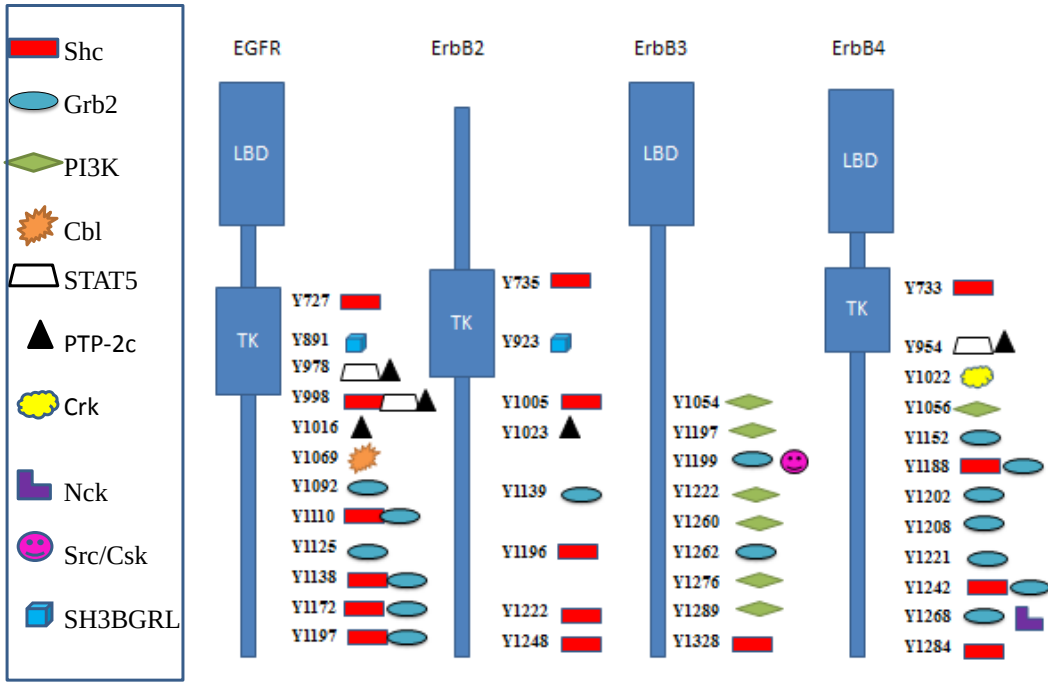

Figure 4. Phosphotyrosine sites on the ErbB receptors and their effector molecules. The scaffolding and adaptor molecules Shc and Grb2 can bind on multiple sites across all four receptors, while other signaling and effector molecules show far more specific receptor recognition. The E3 ubiquitin ligase Cbl which leads to receptor ubiquinylations only recognizes phosphorylated EGFR and PI3K binds on ly ErbB3 and ErbB4 . Src/Csk and STAT5 recognize ErbB3 and EGFR respectively. The adaptor proteins Crk and Nck recognize phosphotyrosine residues on ErbB4 and are thus not relevant to epidermal ErbB signaling in skin (186). SH3BGRL, an SH3/SH2 domain containing protein, and protein tyrosine phosphatase 2C (PTP-2c) bind EGFR and ErbB2. Figure adapted from (187). Abbreviations: Ligand-binding domain (LBD), Tyrosine kinase (TK)

human skin and A431 keratinocytes *in vitro* (185). However, in the MD-MBA breast cancer cell line which expresses higher levels of ErbB2, smaller, cuboidal "basal-like" cells expressed a mixture of surface and punctate intracellular ErbB2, suggesting that the relative amount of ErbB receptors present in a system can drive alterations in cellular localization and recycling of active ErbB receptor (185). Similar observations were made regarding EGFR *in vivo* or in EGFR-overexpressing A431 cells *in vitro* (185). ErbB2, which lacks a ligand binding domain but possesses a constitutively active tyrosine kinase domain, has been shown to enhance tumor promotion by phorbol esthers in murine epidermal overexpression models (187). Further, due to its suprabasal expression, ErbB1/ErbB2 heterodimers are thought to play a role in ligand-induced differentiation while signaling through ErbB1 homodimers functions to drive proliferation and migration (185). This may reflect the tendency of ErbB heterodimers to recycle causing a more persistant EGFR signal as opposed to the shorter signaling induced by EGFR homodimers. ErbB3's role in skin is poorly understood but it does appear to be important in melanocyte development, and plays a role in melanoma progression (188). ErbB4 has not been shown in the epidermis, keratinocyte monolayer or organ culture, or keratinocyte cell lines (185). In normal human keratinocytes in monolayer culture, ErbB2 and ErbB3 receptor numbers increase as cells become confluent, suggesting that ErbB2 and ErbB3 serve to maintain tissue structure and differentiation but also allow for a rapid response to wound regeneration wherein the loss of cell-cell contact changes EGFR signal from predominantly juxtacrine activation by membrane bound pro-EGFR ligands to paracrine activation by soluble ligands. (189).

Across the ErbB family, phosphorylation of tyrosine residues in the CTD recruits signaling and adaptor proteins to the receptors. This allows signaling molecules as well as scaffold and adaptor proteins to interact with receptors via their SH2 or phosphotyrosine-binding (PTB) domains (190). The low degree of sequence identity within the CTD of ErbB receptors, contributes to distinctive signaling activity associated with each ErbB receptor and ErbB receptor dimers (**Table 4**). The EGFR has several sites which associate with Grb2, Stat5, and Shc, as well as sites for c-Cbl, a ubiquitin ligase which target receptors for internalization (191). ErbB2 interacts primarily with Shc leading to Ras-dependent extracellular signal-related kinase (ERK) activation, but also protein kinase A (PKA) (192). In murine models and human skin cancers, elevated ErbB2 is strongly linked to tumor promotion and neoplasm, respectively (187, 192, 193) while ErbB3 and ErbB4 are the only members of the ErbB family that can interact with the p85 subunit of PI3K (191, 194).

Upwards of 20% of the EGFR protein is N-glycosylated, which targets it to the plasma membrane where it exists as an inactive monomer, or transiently, as a dimer (184). Localization of the EGFR to the membrane increases its effective concentration and makes dimerization more likely (195). However, evidence also exists to support the presence of pre-formed dimers which dissociate upon ligand binding (196). Though these preformed homo- and heterodimers are thought to be largely inactive (197), their existence allows for more rapid activation of ErbB receptors and a higher degree of phosphorylation of cellular ERK and Akt following EGF challenge (198). Over half of the EGFR found in caveolae exist in their unphosphorylated state, but the readily available pool of potential dimer partners and effector molecules enhances receptor

Table 4. Common ErbB phosphorylation sites.

EGFR				
Site	**Phosphorylated by**	**EGFR Recruits**	**Activates**	**Reference**
Y845	Src	Stat5, P38	Ras, Stat5	(211-213)
Y992	Auto/transphosphorylation	PLCγ, SHC	Stat3, PKC, Erk, Vav2	(212, 214) (215-217)
Y1045	Auto/transphosphorylation	c-cbl	Ubiquitinylation	(208, 208, 208, 218, 219)
Y1068	Auto/transphosphorylation	Grb2, JNK	Ras, Stat3, c-Jun, AP-1	(212, 218, 220, 221)
Y1086	Auto/transphosphorylation	Grb2, SH2	Stat3, MAPK, Pak1	(212, 216, 217)
Y1148		Shc	MAPK, Stat1, AP-1	(212, 215, 222-224)
Y1173	Autophosphorylation	Shc	MAPK, PLCγ, Shp2	(212, 215, 222-224)(225)
S1046/7	Cam Kinase II	P38	c-Cbl independent internalization	(225-227)
S1070	Cam Kinase II	c-Cbl	Internalization & ubiquitinylation	(209, 225, 226)
		ErbB2		
Site	**Phosphorylated by**	**ErbB2 Recruits**	**Activates**	**Reference**
Y877	Src		Stat3, PKA	(192)
Y1112	Auto/transphosphorylation	c-cbl	Ubiquitinylation & degradation	(228, 229)
Y1221/2	Auto/transphosphorylation		Shc, ErbB2	(190, 223, 230-232)
Y1248	Auto/transphosphorylation		ErbB2	(230, 231)
T686	PKC		Internalization	(233)
S1113	Calmodulin Kinase II (CaMK II)			(234)
		ErbB3		
Site	**Phosphorylated by**	**ErbB3 Recruits**	**Activates**	**Reference**
Y1289		PI3K	PI3K, Akt	(194)

Abbreviations: Mitogen activated protein kinase (MAPK), Y= tyrosine, S=serine, T=threonine, Calmodulin Kinase II (CaMK II)

responsiveness by facilitating dimerization and transactivation at the membrane rafts (199).

Upon ligand binding, receptor dimerization is induced by changes in conformation of the ligand-bound ectodomain and the exposure of the dimerization loop. While all 10 dimeric configurations of ErbB receptors are possible, some heterodimers are preferred (175, 200). ErbB2 lacks a ligand-binding domain, resulting in the constitutive exposure of its dimerization domain and making it the preferred dimerization partner for the all ErbBs (201). More, EGFR/ErbB2 heterodimers decrease EGFR degradation while EGFR homodimers are sorted more rapidly to the lysosome for receptor degradation (202, 203). Receptors dimerize and transphosphorylate one another (204) which allows signaling molecules, scaffolds, and signal inactivators to bind via SH2 and PTB domains.

Following dimerization, phosphorylation of two regions of the CTD targets the EGFR to clathrin-coated pits where it is rapidly internalized into endosomes (203, 205-207). From here, ligand either remains bound to the receptor and the ligand-receptor complex is degraded, or it is released and the receptor is recycled to the surface (**Figure 5**; 202). EGFR homodimer sorting to the lysosome is directed by multiple mono-ubiquitinylation (208, 209). While the process is not fully understood, receptor endocytosis appears to be cholesterol-dependent and concentrated at lipid rafts (202, 206). Clathrin-mediated endocytosis, conversely, leads to a higher rate of EGFR recycling to the surface prolonging the ability of EGFR to signal (210). This non-clathrin endocytosis drives receptor sorting to lysosomes and is more likely to attenuate EGFR signaling (184, 210).

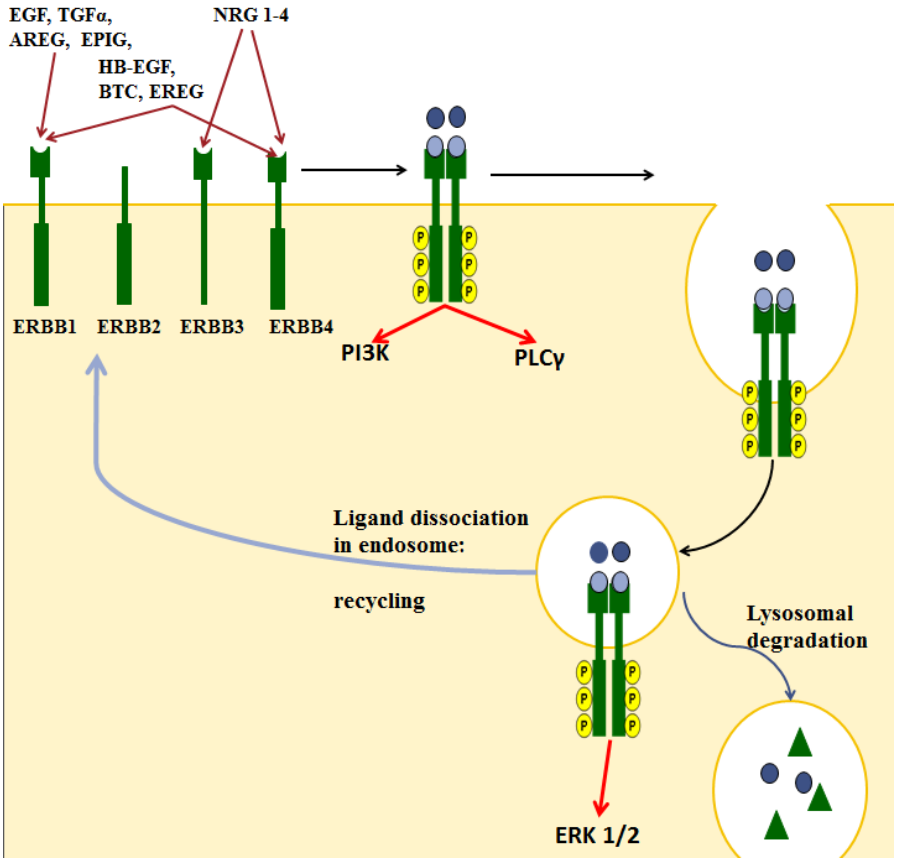

Figure 5. ErbB subtypes and ligand specificity, signaling, and trafficking. The top left portion of this figures shows the four ErbB receptor subtypes (ErbB1-4) and differential ligand specificities. ErbB2 lacks a ligand binding domain and ErbB3 lacks a tyrosine kinase domain (2, 184). Ligand binding promotes dimerization, phosphorylation, and ubiquitinylation of the ErbB dimer. This targets it for association with effectors promoting ErbB-mediated signaling as well as targeting the dimer for internalization in clathrin-coated pits (52, 118, 203, 234). In endosomes, ligand identity and dimer composition direct receptor recycling versus degradation (183).

Heterodimers of EGFR/ErbB2 or EGFR/ErbB3 tend to dissociate from the ligand regardless of ligand identity and are recycled back to the surface giving heterodimers a stronger and more persistent signaling capacity than EGFR homodimers (202). However, while receptor dimer pairs and method of endocytosis can dictate receptor degradation or recycling, ligand can also play a role. EGF causes downregulation of EGFR homodimers while other ligands, such as TGF-α, direct recycling of the receptor to the surface (205, 235).

Location-Dependent EGFR Signal Transduction and Down-regulation: EGFR signaling activates pathways involved in proliferation, cell survival and migration and its activity must be inhibited in the course of normal keratinocyte differentiation (175, 236). Although phosphorylated EGFRs in endosomes have lost access to ligands, these receptors can still signal, While EGFRs cannot associate with or activate PLCγ or PI3K whose substrates are located in the plasma membrane (199, 222), the receptors remain phosphorylated and associated with the scaffolding protein SHC (src homology 2 containing containing transforming protein 1), the adaptor protein Grb2 (cytoplasmic growth factor receptor bound protein 2), or the guanine nucleotide exchange factor SOS (son of sevenless homolog 1), all of which allow ERK signaling to be propagated (237).

These changes in location-dependent signal transduction can have profound effects on keratinocyte cell fate. EGFR signaling is canonically considered to be proliferative in keratinocytes, and ERK activation is also linked to keratinocyte proliferation and hyperproliferation in disease states (238-240). Previous studies have shown that PLCγ activation is required for keratinocyte differentiation (11) and that distribution of PLCγ is frequently altered in hyperproliferative skin conditions such as

psoriasis or seborrheic keratosis (241). PLCγ can be directly activated by EGFR-dependent phosphorylation and is also activated in a PI3K-dependent manner involving Ca-, c-src-, and cadherin-dependent mechanism (11, 242). PI3K sits upstream of PLCγ in keratinocytes but can also signal through Akt to promote keratinocyte differentiation and prevent cell death (242). As discussed previously, ErbB3 is the only ErbB receptor in keratinocytes with a binding site for PI3K, a pro-differentiation signaling cascade in keratinocytes (191). Thus through differentiation-induced changes in ErbB receptor complement that cause a suprabasal increase of ErbB3, the EGFR could switch from pro-proliferative signaling to pro-differentiation signaling, dependent upon context.

MAPK/ERK pathway: Five members of serine/threonine MAPKs have been classified to date: ERK 1/2, c-Jun amino terminal kinases (JNK), p38, ERK 3 and 4, and ERK5 (243). Each MAPK pathway consists of a MAPKK kinase (MAPKKK) that activates a dual specificity MAPK kinase (MAPKK) which in turn activates a mitogen-activated protein kinase (MAPK) by phosphorylation of tyrosine and threonine residues. The ERK signaling cascade is one of the primary mitogenic pathways in the epidermis, and a pathway that can be activated by all receptor tyrosine kinases. Activation of the ERK pathway is initiated by phosphorylation of growth factor receptors including those for EGF, platelet derived growth factor (PDGF), insulin-like growth factor 1(IGF1), fibroblast-derived growth factor (FGF), and dermally-secreted keratinocyte growth factor (KGF / FGF7) (244).

In the ERK 1/2 pathway (**Figure 6A**), EGF-like ligands activate the EGFR, inducing dimerization and transactivation of ErbB dimer pairs. The phosphorylation of tyrosines such as Y1068 and Y1086 on EGFR, recruit Grb2 via its SH2 domain (217,

220). The SH3 domains of Grb2 bind the guanine nucleotide exchange factor, SOS, which activates a membrane-associated GTPase, Ras (244). Activated Ras interacts with a downstream effector, Raf, the MAPKKK of the RAS-RAF-MEK-ERK pathway (243). Raf then phosphorylates the dual specificity kinases MAPK/ERK kinases (MEK), MEK1 and 2, which in turn activate ERK 1/2 (243).

Phosphorylated ERK that is not associated with scaffolding molecules can move into the nucleus where it is a major modulator of cellular proliferation and involved in entry into the G1 phase of cell cycle (245). Activated ERK phosphorylates over 80 downstream effectors including nuclear substrates and transcription factors such as AP-1, c-Myc, c-Fos, STAT3, and Pax6. ERK also phosphorylates cytoskeletal and membrane-associated proteins such as CD120A, Syk, and Calnexin which alter keratinocyte cytoskeletons to allow suprabasal migration (246). The localization of ERK signaling can also influence which interacting partners are able to interact with the receptors. With the loss of juxtacrine signaling in wounding, EGFR dimers can be internalized, and signal through ERK at the level of the endosome (237, 247). Conversely, the loss of cytosolic phosphorylated ERK through nuclear localization can attenuate signaling through cytosolic proteins maintaining suprabasal phenotype of keratinocytes in the case of an intact epithelium (238). Cytosolic ERK drives cytoskeletal changes and motility (248). Changes in ligand-stimulated EGFR activation following wounding and desmosomal interruption stimulate cytoskeletal changes, allowing for migration of keratinocytes across the wound (182, 240). ERK can also lead to activation of other proteins which lead to cytoskeletal or transcription factor phosphorylation such as Rsk2 and MSK1 (249).

In the epidermis, the ERK pathway plays an important role in regulating normal epidermal homeostasis, as well as keratinocyte proliferation and viability (238). Increases in ERK activity associated with activating mutations of Ras or Ras over-expression occur in the majority of human squamous cell carcinomas (SCC) and is a key player in the

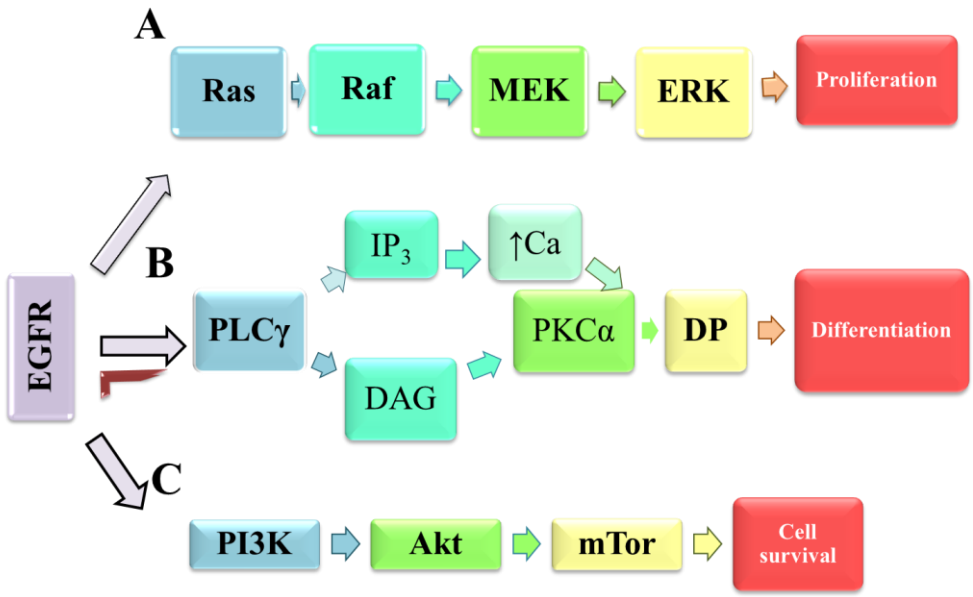

Figure 6. Signaling Pathways downstream of the EGFR. The EGFR upon activation will dimerize and is capable of interacting with several downstream pathways involved in cell proliferation, differentiation, and survival. Three prominent pathways are (A) RAS/RAF/ERK (B) PLCγ and (C) PI3K. PKC which is stimulated indirectly by EGFR activation also provides feedback inhibition to the EGFR

development of murine SCC models (238). In the epidermis, Ras is activated in basal layers to drive proliferation while the Ras protein is lost suprabasally (250). Within the normal epidermis, Ras overexpression or hyperactivation of the RAS-RAF-MEK-ERK

pathway in basal layers leads to epidermal hyper-proliferation and thickening, as well as impaired differentiation (250).

Phospholipase Cγ/Protein Kinase C: PLCγ is a membrane-associated enzyme that cleaves PIP_2 into IP_3 and DAG. While both function as second messengers interacting with downstream pathways, DAG remains membrane- associated while IP_3 diffuses into the cytosol where it interacts with IP_3 receptors in the ER and releases calcium into the cytosol. Ca, along with DAG activate classical PKCs (α, β, and γ) while DAG alone is required for activation of novel PKCs (ε, η, δ, and θ) (251). PKC is a serine/threonine kinase which has a number of substrates and is capable of autophosphorylation (252). PKC is thus activated by and linked to increased intracellular Ca and phosphorylates the EGFR on T654, which causes its downregulation (253, 254).

PKC (**Figure 6B**) is also linked to Ca-driven structural changes such as enhanced desmosome formation (252). PKC α is activated by DAG and Ca and phosphorylates DP, a required part of the desmosome which anchors intermediate filaments to desmosomal plaques (255). DP phosphorylation by PKCα is required for desmosomal formation (256), but loss of PKCα seems to be necessary for the dissolution of desmosomes preceding epithelial-mesynchymal transition (EMT) in wounding (257).

In the normal epidermis, membrane-associated PLCγ co-localizes with the EGFR only in the basal compartment. In contrast, suprabasal expression of PLCγ is common in many epidermal hyperproliferative diseases such as psoriasis, seborrheic keratosis, and the margins of second degree burns (241). However, PKC co-localizes to desmosomes in murine and human epidermis and serves to maintain epidermal barrier function in the intact epidermis (130, 250). The expression and activation of certain isoforms,

particularly PKCη, but also PKCα and δ, have been shown to be necessary for the expression of differentiation markers such as Lor, Ivl, SPRR-1, and Flg as well as the initiation of the differentiation process (258, 259).

Phosphoinositide-3 Kinase signaling: There are three classes of phosphoinositide-3 kinases (260). The most commonly studied are class I PI3Ks which are targets for activation by RTKs and G-protein coupled receptors (GPCRs) (260). Class I PI3Ks phosphorylate PIP_2 to PIP_3 at the cellular membrane while classes II and III are less well understood and thought to be involved in vesicular signaling (261, 262). Class I PI3Ks are heterodimeric molecules comprised of a 110 kDa catalytic subunit and an 85 kDa regulatory subunit containing a p110-binding region as well as two SH2 sites (260). Class I PI3Ks are activated by ErbB3 and ErbB4 (261).

Evidence shows that PI3K/Akt is activated by the EGFR or c-Src in early epidermal differentiation both *in vivo* and *in vitro* (185, 242). However, the role of PI3K in epidermal homeostasis is complicated and seems to be highly context dependent. In the best understood scenario, PI3K signaling promotes cell survival by stimulating Akt-driven inhibition of pro-apoptotic genes (242). Epidermal PI3K also activates PLCγ through the non-receptor kinases fyn and src, suggesting that PI3K is involved in epidermal differentiation (262). However the role of PI3K may not be so much of an on-off switch, as a context-dependent pro-survival and differentiating signal.

While PI3K deletions in mice cannot be studied as they are early embryonic lethal (263), mice lacking phosphatase and tensin homolog (PTEN) which inactivates PI3K signaling, display a “shaggy bear” phenotype characterized by wrinkly skin and fur symptomatic of hyperkeratosis, hyperproliferation, and hypergranulation. These data

suggest that an unregulated increase in PI3K promotes aberrant proliferation (263), a hypothesis supported by data in the HaCaT human keratinocyte cell line where PI3K overexpression leads to increased invasiveness and epidermal thickening in organotypic culture (263). In addition, constitutive activation of PI3K inhibits the expression of markers of differentiation while driving hyperproliferation, leading to disorganized, hyperplastic, and poorly differentiated tissue (263, 264).

This contextual signals driving differentiative and anti-apoptotic responses as opposed a proliferative, migratory role in the epidermis makes sense, within the context of wound healing and EGFR internalization. While both the p110 α and β catalytic subunits of PI3K are strongly expressed in the skin, the basal activation of Akt in normal skin is limited to the hair follicle sheath, the basal layer of neonatal murine epidermis, and extensively in the wounded epidermis where it stimulates cytoskeletal rearrangement to allow for EMT and keratinocyte wound infiltration (242, 263). Wounding leads to a rapid increase of phospho-Akt, particularly during the inflammatory phase of wound healing, but also leads to the upregulation of the p110 γ catalytic subunit of PI3K perhaps suggesting that enhanced Akt phosphorylation reflects an upstream increase in PI3K activity (263).

Although the role of PI3K is still emerging, it appears to inhibit apopostis while maintaining a degree of epidermal plasticity for the skin's healing response (242, 263). Stimulation of PI3K is tied to differentiation, both through location and ErbB receptor subtype. Due to its colocalization with membrane phospholipids, Class I PI3K is associated with signaling from the membrane (261). It clusters to TJ and desmosomal structures at the membrane and thus readily activates and is activated by ErbB receptors

colocalized to these same structures. Activation of PI3K by juxtracrine-activated EGFR at desmosomes can serve as a presence/absence indicator of cell-cell contact which is lost following desmosomal disruption. This leads to enhanced activation and altered PI3K signaling upon disruption or wounding (10, 260, 265). Further, only ErbB dimer pairs containing ErbB3 or ErbB4 can signal through PI3K (183). However, ErbB4 is not expressed in the epidermis suggesting that EGFR-ErbB3 dimers drive PI3K signaling in the epidermis (185).

EGFR Ligands

EGF and its family of ligands (**Table 5**) are formed as membrane bound-promolecules containing one or more EGF-like domains (176). Of the 11 ligands in the EGFR ligand family, the EGFR recognizes the most ligands including EGF, TGF-α, betacellulin (BTC), heparin binding EGF-like growth factor (HB-EGF), epiregulin (EREG), amphiregulin (AREG), and epigen (EPGN) (266). In addition, BTC, HB-EGF, and EREG bind to ErbB4 (175, 267). ErbB2 possesses no ligand-binding domain and thus recognizes no EGF-like ligands while neuregulins (NRG) 1-4 bind to ErbB3 and ErbB4 (268). Since EGFR signaling promotes proliferation, cell survival, and migration its signaling capacity must inhibited in the course of normal keratinocyte differentiation (175, 236). The epidermis contains EGFR, ErbB2, and ErbB3 which can recognize all EGFR ligands and signal through EGFR/ErbB2 and EGFR/ErbB3 heterodimers as well as EGFR/EGFR homodimers (197).

EGFR ligands in the skin auto-induce their own mRNA as well as cross-induce that of other EGFR ligands (279-281). For TGF-α, this occurs through a PKC-dependent stabilization of the TGF-α transcript that increases the half-life of its mRNA in primary

Table 5. Properties of EGFR ligands and their role in the epidermis.

Ligand	Size (kDa)	ErbB Bound (269)	EGFR Kd	TCDD-induced mRNA	Role in the epidermis	Reference
AREG	11.3	EGFR	4.2[4]	NC	Epidermal proliferation, wound healing, inflammatory response, over-expression causes psoriatic-like lesions	(211-213, 269)
BTC	9.0	EGFR, ErbB4	1.4[3]	NC	hair follicle development and hair cycle, but KO or over-expression models have no skin phenotype	(269)
EGF	6.2	EGFR	1.8[3]	NC	Used historically in skin literature, but not produced in the epidermis	(3, 175, 270)
EPGN	7.9	EGFR	2678[3]	+3.77-fold	Causes sebaceous hyperplasia *in utero*	(111, 271)
EREG	6.0	EGFR	UNKN	+2.02-fold	Loss of EREG causes chronic dermatitis-like phenotype	(272)
HB-EGF	9.7	EGFR, ErbB4	6.8[3]	NC	Involved in wound healing and keratinocyte migration	(182, 273)
TGF-α	5.2	EGFR	8.8[2]	+1.71-fold	Elevated in many hyperproliferative skin diseases, KO causes a wavy hair phenotype	(179, 270, 274, 275)

Abbreviations: No measured binding (NMB), No change (NC). [1]molecular weight of pro-ligand [2]solubilized full length EGFR in A431 cells [3]As competitive binding experiments for EGFR ligands are performed in a heterogeneous system, K_d have been calculated from IC_{50} utilizing the Cheng-Prusoff equation (276): $K_d = IC_{50}/(\frac{(1+[competitor])}{(K_{Dcompetitor})})$ for 50 pM [^{125}I]-EGF (277) [4]or 645 pM [^{125}I]-EGF (278).

keratinocytes (8, 282, 283). In other systems, such as the transformed colon epithelial LIM1215, auto-induction occurs through an EGFR-dependent transcriptional mechanism (282). In both cases, inhibition of the EGFR or PKC reverses the increase in TGF-α mRNA (282). Similar increases in BTC mRNA levels were observed in RIE-1 gastrointestinal cells, however increases in secreted AREG and HB-EGF were only partially explained by a transcriptional mechanism (283). AREG and HB-EGF were also found to be elevated in cell lysates and conditioned medium before other EGFR ligands suggesting a role in promoting synthesis of other EGFR ligands (283). This suggests that increased cleavage as opposed to, or in conjunction with, increased synthesis drives increase of some ligands.

EGFR ligands are released at different times, to different degrees, and have differential downstream effects following ErbB receptor activation (284). In keratinocytes, AREG is strongly proliferative, and in conjunction with HB-EGF, can account for around 70% of proliferation in monolayer keratinocytes grown in the absence of EGF (285). However, in mammary cell lines, AREG was the only assayed EGF-like ligand that failed to induce proliferation or increase ErbB3 phosphorylation (284). Other ligands, such as TGF-α, are not observed at a high level in normal skin, but are elevated in response to toxin exposure or epidermal disease (7, 274, 282). Overall, AREG, HB-EGF, TGF-α, and EREG are secreted by normal keratinocytes or upregulated in epidermal hyperproliferative diseases (196).

Competition assays show that EGFR ligands possess markedly different affinities for the EGFR. However, these data must be interpreted cautiously, as most assays have used [^{125}I]-EGF instead of iodinated forms of the ligand in question (270, 278). In

addition, each ligand possesses a different functional EC_{50} for proliferation, cell motility, and the EGFR signaling pathways they activate. While all EGF ligands in keratinocytes bind exclusively to the EGFR (176, 286), each ligand preferentially stabilizes differing ErbB dimer pairs which can have implications in endocytic sorting and receptor downregulation (176, 286). ErbB2, the preferential dimer partner for EGFR, raises the EGFR's affinity for TGF-α and HB-EGF (270), making ligand binding to EGFR/ErbB2 dimer pairs more likely than EGFR homodimers. Further, the presence of a heparin binding domain like that possessed by AREG and HB-EGF lowers the affinity of these ligands for the EGFR (288) though enhances acid-resistance to the low pH in lysosomes, directing receptors to degrade as opposed to TGF-α which dissociates at a low pH and leads the receptors to recycle (287).

EGFR ligands are produced and trafficked to the cell surface in a membrane-associated pro-form (171). From the membrane, ligands can signal directly with neighboring cells in a juxtacrine manner or can be cleaved by Ca-dependent metalloproteinases to interact with receptors on the same (autocrine) or neighboring (paracrine) cells (**Figure 7**) (171, 269). This provides a mechanism for changes in EGFR signaling following disruption of cell-cell contact in wounding, and as a mechanism of altering EGFR signaling in differentiation (182, 289). Juxtacrine signaling does not allow for receptor endocytosis as the membrane-tethered ligand prevents internalization (171). This keeps the EGFR at the surface where it can signal through PI3K and PLCγ (289, 290). Overall EGFR signaling represents a highly evolved system with a high degree of combinatorial complexity. In the epidermis, four ligands are secreted which can activate three ErbB receptors, producing five functional dimer pairs. These dimers can signal

persistently at the cell surface, or inside the cell, and can activate a number of different downstream signaling pathways . This allows for the temporal and spatial control of EGFR signaling that is critical for epidermal homeostasis but still not fully understood (291).

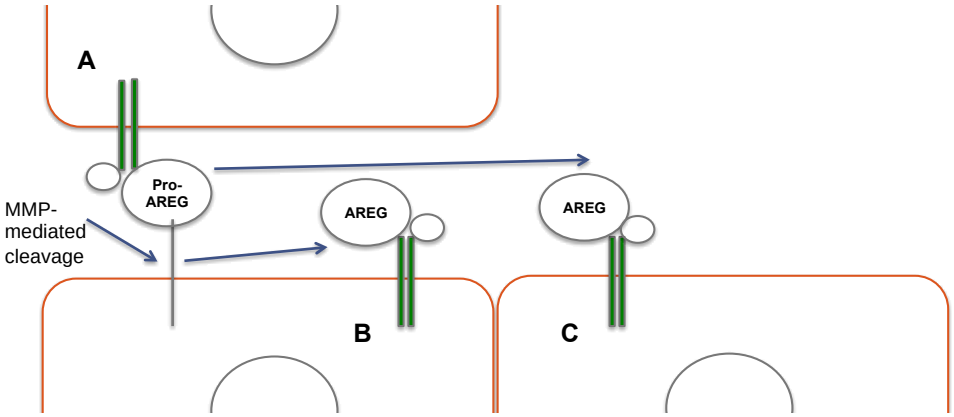

Figure 7. Mechanisms of EGFR activation for EGF-like ligands. Pro-AREG (or another EGF-like ligand) exists in a membrane-bound form wherein it can activate EGFRs on neighboring cells in a **(A**) Juxtacrine manner, or, following cleavage by MMPs or ADAMs, diffuse in its cleaved form to activate EGFRs in an **(B)** autocrine or **(C)** paracrine fashion. Adapted from (182)

EGF—the prototypical EGFR ligand: As the nomenclature suggests, EGF is the prototypical EGFR ligand and the first EGFR ligand identified (205, 292, 293). EGF has a high affinity for the EGFR and upon binding, induces dimerization and lysosomal degradation following internalization (204, 294). EGF is the best understood EGFR ligand and its frequent use has led to the preferential development and availability of tools specific to this ligand. These facts have led to a historical tendency to use EGF as an equivalent for other ligands in studying the EGFR in systems such as keratinocytes (7,

295). Choi *et al.* (8) identified TGF-α in conditioned medium and then used [I^{125}]-EGF to study TGF-α induced EGFR downregulation. However, differences in affinity, structure, and endosomal sorting of ligands and receptors create a false equivalency, particularly in the epidermis which does not produce soluble or membrane bound EGF (3, 176). Further, EGF binds to and down-regulates EGFRs differently than other ligands such as TGF-α which causes rapid receptor cycling, or AREG which causes slow recycling (287).

Amphiregulin (AREG): In humans, AREG expression is controlled by two copies of the AREG gene, AREG and AREGB which are found on chromosome 4q13.3 and flanked by BTC on the 3' end and EPGN on the 5' end (296). These genes produce a 1.4 kb, six exon product which is translated into a 252 amino acid pro-form of AREG (296). This pro-AREG contains five major domains, including a signal domain (aa 1-19), an N-terminal domain (aa 20-101), an EGF-like domain (aa 102-184), a transmembrane domain (aa 199-221) and a cytoplasmic tail (aa 222-252) (297). Pro-AREG is cleaved by a disintegrin and metalloproteinase (ADAM)17, ADAM15, and MMP1(298-301). Proteolysis results in a 98 amino acid soluble AREG with a molecular mass of 11.3 kDa. AREG is typically referred to as a low affinity ligand for the EGFR, even though its IC_{50} in competitive binding assays is within the same order of magnitude as most other ligands (278). This description appears to arise from the observation that AREG is far less potent than EGF in stimulating receptor phosphorylation or colony formation, in some cases requires 300 times as much ligand (270, 278). AREG, like EGF, remains associated with the EGFR in the late endosome. While it does not target the EGFR for lysosomal degradation, it does cause a slower recycling of the EGFR back to the cell surface (~30 minutes) compared to TGF-α (2-5 minutes) (287).

TCDD does not affect AREG production in the skin but does increase its production in murine ureters (104) and by tobacco smoke in human oral mucosal cells (302) . When experimentally overexpressed in murine skin, AREG caused a psoriatic phenotype characterized by epidermal thickening and severe, early-onset psoriasis when overexpression was targeted to suprabasal keratinocytes (181, 303). In the latter case, AREG overexpression was involved in the induction of synovitis, suggestive of a mechanism of psoriatic arthritis, a common co-morbidity to psoriasis (181, 304). In humans, AREG is an autocrine growth factor for keratinocytes, enhancing ErbB1-mediated ERK signaling and proliferation (145, 182, 240).

In cultured human keratinocytes, AREG mRNA expression was 19 times greater than any other EGFR ligand and soluble AREG was present at 7.5 times the concentration of any other EGFR ligand. AREG drives keratinocyte proliferation in an EGFR and CTD-dependent manner (182, 297, 305). Further, AREG is upregulated in human epidermal diseases such as psoriasis and squamous cell carcinomas of the epidermis, reinforcing its role in hyperproliferative conditions (306).

Heparin Binding EGF-like Growth Factor (HB-EGF): In humans, proHB-EGF is a 21 kDa protein first isolated from macrophages (289, 307) and biochemically characterized by Iwamoto (289, 307). Structurally similar to AREG, HB-EGF is found on the minus strand of chromosome 5q23 which encodes an 87 amino acid protein comprised of five exons (279). Interestingly, Pro-HB-EGF is unique among EGFR ligands in that it serves as a receptor for the diphtheria toxin. (308). Pro-HB-EGF is targeted to the cell membrane where it can signal in a growth-inhibitory juxtacrine manner at points of cell-cell contact. However, when cleaved by MMP-3, MMP-7 (309,

310) or MMP1 it becomes soluble and stimulates keratinocyte motility (298). HB-EGF is a low affinity ligand containing a heparin binding domain (311) and it is heavily O-glycosylated. Like AREG, HB-EGF possesses a heparin-binding domain that makes it acid resistant and less likely to dissociate in the late endosome (287). However, unlike AREG, HB-EGF leads to EGFR downregulation by targeting endocytosed receptors to the lysosome for degradation rather than slow recycling (203, 312)

Experimentally induced injuries lead to MMP-mediated cleavage of HB-EGF, producing its soluble form. Soluble HB-EGF stimulates not only proliferation like AREG, but is also involved in migration and EMT (182, 313). In human skin, pro-HB-EGF is responsible for cell-cycle inhibition and maintaining an epithelial phenotype (288). When HB-EGF is cleaved, the soluble HB-EGF enters the extracellular space and the CTD of HB-EGF enters the cytoplasmic where it alleviates transcriptional repression of cell cycle by promyelocytic leukemia zinc finger protein (PLZF). In fact, localization of the CTD correlates with cell cycle phase: cytoplasmic in early G1, nuclear in S phase (preceding late S phase transport of PLZF), and near centrosomes during cytokinesis. In this manner, cleaved HB-EGF serves not only to mitogenically signal to neighboring cells in autocrine/paracrine fashion following wounding, its CTD also drives proliferation in the secreting cell (314). Meanwhile prolonged juxtacrine signaling inhibits this CTD-driven cell cycle progression (314). HB-EGF also protects the cell from anoikis, a specialized form of apoptosis that occurs when cells lose contact with their basement membrane, allowing the cells to instead undergo cornification (171, 288). In normal and psoriatic skin, HB-EGF is expressed diffusely in all layers, though co-localization with ErbB3 increases in the stratum granulosum and stratum corneum, leading to an increased

activation of PI3K through EGFR-ErbB3 heterodimers, enhanced cell longevity, and epidermal thickening (145).

Transforming Growth Factor-Alpha (TGF- α): TGF-α is a high affinity EGFR ligand that causes rapid EGFR recycling (287). In the epidermis, TGF-α is expressed in the basal, spinous, and granular layers (275). The human TGFA gene is located on the minus arm of chromosome 2p13. The 106,914 base pair transcript is translated into a 160 amino acid, 17kDa protein comprised of 6 exons with tissue-dependent splice variants (274). TGF-α is cleaved by tumor necrosis factor-alpha converting enzyme (TACE/ADAM17), MMP1and ADAM10 (315) to create a 5.2 kDa soluble TGF-α (133, 315, 316). Alternately spliced products excluding exon 6, or part of exon 5 and 6 were elevated in conditioned medium from squamous cell carcinoma cells. These transcripts were cleaved as readily as wild type TGF-α, but led to enhanced proliferation in both cancerous and normal keratinocytes (274). TGF-α bound to EGFRs maintains epidermal signaling capacity by inducing rapid recycling to the cell surface (287).

In mice, loss of TGF-α creates a distinctive phenotype of wavy hairs growing from irregular hair follicles (317). Knockout mice also display decreased ear wound healing, but this result was not replicated in other wounding assays (318). Targeted overexpression of TGF-α causes spontaneous papillomas and epidermal hyperplasia reminiscent of psoriasis (319). In mice overexpressing TGF-α under a K1 promoter, mice exhibited increased incidences of epidermal tumorigenesis as well as enhanced sensitivity to tumor promoting phorbol esthers (320).

TGF-α was the first EGFR ligand whose secretion was reported to be enhanced in keratinocytes exposed to TCDD (7, 8). Elevated TGF-α is observed in TCDD exposed

tissues *in vivo*, including murine palates where leads to cleft palates and urogenitary tracts (3, 102). Pathophysiologically it is elevated in epidermal hyperproliferative disorders such as psoriasis and atopic dermatitis in humans (161, 179, 180).

Epiregulin (EREG): EREG is the last EGFR ligand found to be secreted by keratinocytes (196). Identified in 2000, EREG is located between EPGN and AREG on chromosome 4q13 (279). The 4.8 kb transcript is translated into a 19 kDa protein comprised of 169 amino acids (279, 287). EREG is cleaved by TACE and ADAM10 (316), and, like TGF-α, EREG causes rapid receptor cycling following endocytosis (287). In mice, the loss of EREG led to a late-onset, chronic dermatitis (321). Histological examination of these plaques and unaffected skin showed epidermal thickening and increased infiltration of macrophages into the skin (321). Shirasawa *et al.* posit that EREG's role in the skin is to mediate inflammatory response (321) as secreted and membrane-bound EREG regulate cytokine production by the keratinocyte and neighboring Langerhans cells (321). However, other work in keratinocytes supports a strongly mitogenic effect of EREG (322) and it is also upregulated in psoriatic plaques in humans, frequently accompanied by an increase in AREG and TGF-α (323). In addition, EREG is upregulated by TCDD in mice (8, 9, 104) which occurs via an XRE-dependent mechanism in murine keratinocytes. However, this promoter motif is not present in human cells (9).

Other EGFR Ligands: The remaining six EGFR ligands have limited to no known role in the epidermis. BTC is secreted from cells in the hair follicle (272, 281). While this can lead to induction of EGFR ligands in neighboring skin cells *in vivo* (281) and has been observed to be increase angiogenesis in wounded skin (272), it is not

observed in keratinocytes *in vitro* (272). EPGN similarly has no known role in the epidermis, but it has been shown to induce sebaceous hyperproliferation *in utero* (111). NRG 1-4 have not been observed in skin.

The role of growth factors and exogenous chemicals in epidermal wound healing

The epidermis has a high degree of plasticity, owing to the ability of skin to respond to damage. In the case of wounding, the epidermal barrier is disrupted, and importantly, keratinocyte desmosomes and TJs are lost, taking away desmosomally clustered juxtacrine signaling and leading to the enhanced secretion of growth factors that drive keratinocyte migration and healing (105, 324). The immediate response of the skin to wounding is not epidermal barrier repair, but rather, formation of a blood clot. Polymorphonuclear (PMN) neutrophils are attracted to the clot site and serve an antibacterial function, releasing antibacterial respiratory burst and phagocytizing debris (325). After the PMN fulfill their task, dermal and epidermal macrophages phagocytize them and other damaged or dead cells (325, 326). Over time, macrophages and monocytes in the wound decrease, allowing for the keratinocyte-driven proliferative stage of wound healing to begin (327).

As the inflammatory stage of wound healing ends, fibroblasts from the dermis migrate into the wound site, and re-establish the ECM. Macrophage-secreted cytokines recruit endothelial cells for angiogenesis (327). Once a solid layer of granulation tissue, comprised of new blood vessels, fibroblasts, inflammatory cells and the fresh ECM (328) has developed, the basal keratinocytes are directed by MMP-induced cleavage of growth factors to migrate across the injured epithelium (329). These EGFR ligands provide a strong proliferative pressure following the re-establishment of cell-cell contacts.

Differentiation and reformation of the epidermal barrier follow (329). However, it is the inherent plasticity of this system wherein EGFR ligands are capable of both maintaining differentiated state and driving dedifferentiation that is commonly exploited in hyperproliferative skin diseases.

Epidermal homeostasis and disease

While Ca drives differentiation in keratinocytes, EGFR signaling represents a powerful and complex proliferative stimulus which must be overcome in order to switch to a program of terminal differentiation. However, the assumption that Ca-induced differentiation progresses by silencing the proliferative signaling of the EGFR is too simplistic. Both EGFR and Ca activate PKC which drives differentiation and, in a negative feedback mechanism, silences the EGFR (253, 259). Ca is also involved in the activation of MMPs which cleave pro-EGF like ligands and increase autocrine and paracrine signaling (133, 240). However, there is interplay between EGFR phosphorylation, signaling, and Ca mediated signaling that is not well understood. One proposed mechanism of Ca/EGFR signaling cross-talk involves a desmosomal cadherin protein, Dsg1 (128). The desmoglein family of proteins Dsg2 and 3 are expressed in the stratum basale of the epidermis while Dsg1 is expressed suprabasally (330). Dsg1 is involved in normal cell adhesion, regulation of cell size, and morphological changes of differentiating keratinocytes (128) wherein they become broader, flatter, and more tightly associated with neighboring cells (107). Dsg1 also decreases ERK activity without affecting total protein content and induces markers of terminal differentiation (K10 and Lor) in a manner independent of its role in adhesion (128). These data suggest that Ca regulates keratinocyte differentiation through the inhibition of pro-proliferative ERK

signaling (173, 236). In addition, they are consistent with the ability of Ca to inhibit EGF-mediated induction of ERK signaling in cultured keratinocytes (177) and the reversible growth arrest and the expression of early markers of differentiation observed in keratinocytes treated with EGFR inhibitors (236).

In some ways, keratinocyte differentiation is the physiological opposite of proliferation. However, the reality is more nuanced. Nowhere is this interplay between differentiation and proliferation more apparent than in epidermal disease. While it is difficult to parse the hyperinflammatory or autoimmune aspects of chronic skin disorders such as psoriasis, ichthyoses, or atopic dermatitis, these diseases also contain an epidermal component wherein the epidermal barrier is compromised, frequently in the presence of epidermal thickening (142). In these diseases, alteration of keratin expression or disruption of differentiation-associated lipid synthesis lead to poorly cornified cells (134, 319). Autoimmune or genetic disruption of desmosomes, TJs, or other cell-cell contact mimics wounding in an otherwise intact epidermis (120, 153, 331).

As discussed above, the loss of cell-cell contact-dependent juxtacrine connections of EGFR ligands such as AREG, HB-EGF, and EREG leads to MMP-dependent cleavage of these ligands (182, 332) and a potential change in how an EGFR ligand signals. For instance, soluble HB-EGF contributes to wound healing and cellular migration while membrane-bound HB-EGF maintains a differentiated state and an intact barrier (332). However, increased amounts of EGFR ligands also drive epidermal proliferation which leads to epidermal thickening, a trait shared in many chronic epidermal diseases where EGFR or its ligands are frequently overexpressed in lesional skin (155). Further, keratinocytes in hyperproliferative skin disorders do not differentiate normally, and

accelerated migration from basal to suprabasal layers in the presence of mediators of inflammation leads to thicker plaques of poorly differentiated keratinocytes which further damage barrier function. In addition, genes associated with keratinocyte differentiation, such as Lor and Flg, are frequently down-regulated in psoriatic patients (152, 158, 333) in the presence of upregulated or otherwise heightened presence of EGFR ligands (176) .

Introduction to the Research Questions

NHEK as an *in vitro* model of epidermal homeostasis

TCDD has historically been studied in cells of various epidermal lineages (**Table 6**), systems far less complex than multihormone driven systems such as gonads, reproductive tracts or immune cells, and *in vivo* endpoints typically involving an epidermal component (4, 7). In skin, TCDD causes clear and easily interpreted endpoints in epidermal homeostasis and provides a defined platform for insight into AhR signaling and epidermal homeostasis. Well-established methods for monolayer and organotypic cell culture of primary keratinocytes in chemically-defined medium exist in which to study epidermal proliferation and differentiation (334). These are based upon initial work by Rheinwald and Green who determined minimal requirements for the growth and maintenance of primary keratinocytes (334). Further modifications to this method have led to a defined system wherein primary cell cultures of human keratinocytes derived from neonatal foreskins establish close intercellular contacts, stratify, cornify, and express markers of differentiation such as keratins 1 and 10, Ivl, Lor, Flg, and Tgm1 as in a normal epidermis (334, 335).

Table 6. Comparisons of keratinocyte cell lines

Cell Line	Source	Transformed	Passages	Differentiation Competent?	Known alterations	References
NHEK	Neonatal foreskin	Primary	5	Yes	Individual genomic differences	(239, 334)
N/TERT1	Neonatal foreskin	Yes, TERT	Immortal	Yes	Loss of p16INK TERT expression	(339)
HaCat	Adult male, distal periphery of melanoma	Yes, spont.	Immortal	Yes	Aneuploid P53 mutation Enhanced TERT	(340-342)
SCC-12F	60 yo male, Facial SCC	Yes, spont.	Immortal	Yes	From immunosuppressed transplant recipient	(343, 344)
A431	85 yo female, SCC	Yes, spont.	Immortal	Incomplete	P53 deletion Enhanced EGFR	(345)

Abbreviations: *TERT –telomerase reverse transcriptase*

In vitro, extracellular Ca mimics the gradient existing *in vivo* where Ca is low basally and higher in the granular/cornified layers (147, 336). After 24 hours in high extracellular Ca (between 1.2 and 2.0 mM) more than 95% of keratinocytes are arrested in G1 phase (337). Differences in physiological and pathophysiological proliferation or differentiation are not just molecular; marked changes in cellular morphology can be seen in less than a day (**Figure 8**). After 20 hours, cells treated with 1.8 mM Ca appear to be cornifying. They flatten into a larger, more differentiated phenotype and the borders of cells become less distinct as the NHEKs form a sheet. This morphology has been correlated with a suprabasal keratinocyte phenotype (13, 338). Conversely cells grown in the absence of TCDD or Ca, or cells treated with 100 ng/mL recombinant human EGF maintain clear borders. As time progresses, EGF-treated cells begin to stratify, then take on a fibroblast-like shape to migrate and fill this new layer. TCDD-treated NHEKs--also stratify, and take on an irregular appearance marked with vesicles and irregular borders. However, despite their appearance, TCDD does not induce apoptosis in NHEKs (4) and later work performed on these cells will show them to be equal if not more responsive to EGFR ligands than their basal medium-treated cohort. Nevertheless, these cells are clearly not behaving in fashions comparable to physiological models of proliferation (EGF) or differentiation (Ca) (4).

TCDD alters proliferation and differentiation

Both TCDD- and Ca-induced differentiation are associated with decreased [^{125}I]-EGF binding indicative of EGFR loss from the cell surface (13, 14). However, studies assessing TCDD and Ca effects on [^{125}I]-EGF binding have not examined the time-course of receptor loss, compared it to down-regulation produced by EGF, or defined the

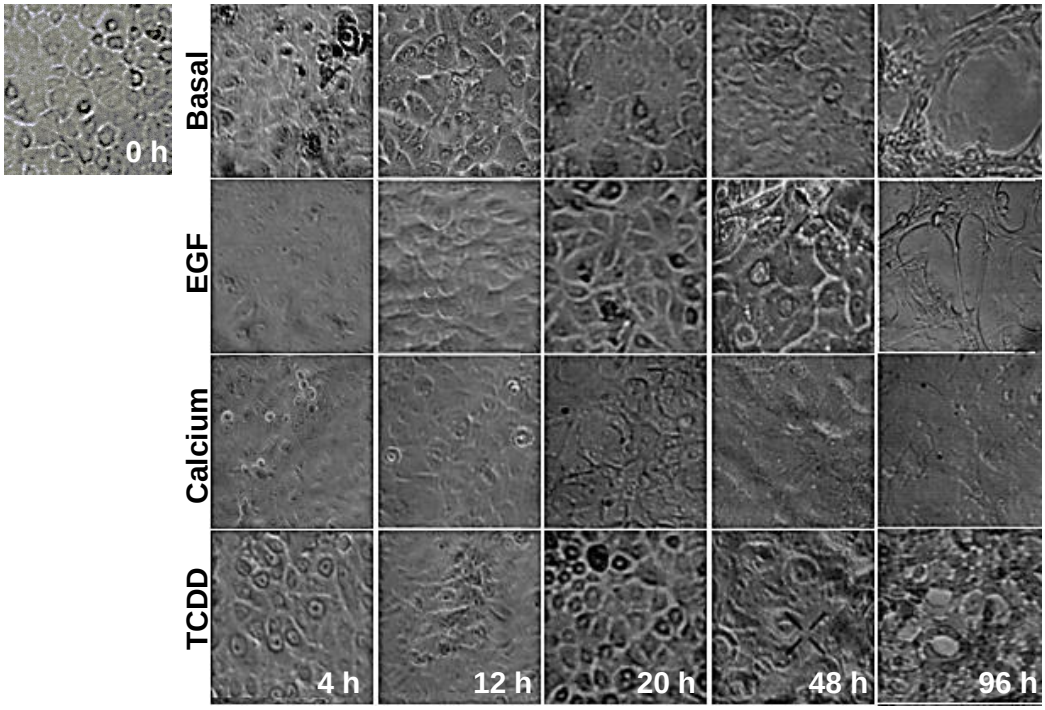

Figure 8. Confluent NHEKs following treatment with basal medium, EGF, Ca, or TCDD. EGF and TCDD induce stratification while TCDD and Ca induce cornification **.** Final magnification is 40x.

mechanism by which it occurs. In spite of loss of EGFRs from the cell surface, TCDD induces the expression of several EGFR ligands including TGF-α and EREG *in vivo* and *in vitro* (3, 7, 9), and promotes a hyperproliferative state *in vivo* (13, 74, 346). In keratinocyte cell culture, TCDD increases the secretion and production of several ligands, including TGF-α in humans and mice and EREG directly through an XRE upstream of the EREG TSS in mice (7-9). It is not clear that these increases in EGFR ligand secretion are shown to be directly responsible for alterations in proliferation. Additional data on TCDD's role in epidermal proliferation is equivocal and dependent upon growth and treatment protocols, endpoints, as well as cell lineage (**Table 7**). In mice, less responsive hairless (hr-/hr-) mice showed more hyperplasia than TCDD-sensitive mice which showed accelerated differentiation suggesting TCDD mimics Ca in driving differentiation and suppressing EGFR signaling (51). However human patients with chloracne present with epidermal thickening, an effect caused by unchecked proliferation or slowed epidermal turnover (74).

Although TCDD neither binds to nor directly modifies the EGFR (72), TCDD may alter cellular proliferation by mimicking some aspects of EGFR signaling (72). Mice exposed *in utero* to TCDD or EGF display similar phenotypes including premature eyelid opening, premature tooth eruption, decreases in body and thymic weight (348), and abnormal palatogenesis (102). However, TCDD decreases EGFR binding both *in vitro* and *in vivo* (14, 16, 348), a response that was less extensive in murine strains with lower sensitivity to the effects of TCDD (16). These data correlate TCDD responses with loss of binding and could result from alteration of EGFR synthesis, phosphorylation status, transactivation of the EGFR, or through production of an autocrine growth factor

Table 7. EGFR downregulation leads to differing endpoints depending on treatment condition

	Osborne 1986 (16)	Hudson 1985 (14)	Boonstra 1985 (347)	O'Keefe 1983 (15)	O'Keefe *et al.* 1982 (295)
Ca (mM) low/high	0.05 / 1.1	0.06 / 1.8	0.06 / 1.6	<0.01 / 1.1	0.09 / 1.8
EGF	8 ng/mL	8 ng/mL	10 ng/mL	10 ng/mL	10 ng/mL
TCDD	10 nM	100 nM	--	--	--
Cells	NHEK	SCC-12F	SCC-12F	NHEK	NHEK
Medium	75% DMEM/F12, penstrep, hydrocortisone, ctx, 5% FBS	DMEM	75% D/F, 5% FBS	75% DMEM/F12, hydrocortisone, transferrin, ctx	DMEM, hydrocortisone, transferrin, 5 ng/mL insulin
Pre-binding treatment	24 h ± Ca 0-72 h TCDD	24 h ± Ca 96 h ± TCDD 120 h ± EGF	72 h ± EGF	--	72 – 144 h EGF
[^{125}I]-EGF Incubation	30 min, 37^{o}C	30 min, 37^{o}C	2 h, RT	4 h, 4^{o}C	Preincubation on ice & 30 minutes, 4^{o}C
Results	TCDD ↓ EGFR binding, 2-fold ↑ CE, 51% ↓ DNA synthesis, 54% ↓ in basal cell number	Ca ↑ envelope competence, TCDD/Ca behaved like Ca rather than TCDD	Ca dependent binding loss is dose-dependent	5-fold increase in EGFR with no change in activity	Differentiated cells do not bind EGF, EGF causes 60% ↓ in [^{125}I]-EGF binding at 2 h.

Abbreviations: DMEM Dulbecco's minimal essential medium; F12 –Ham's F12 medium, FBS-fetal bovine serum, ctx—cholera toxin, 75% DMEM/F12: 75% DMEM, 25% Ham's F12

(8, 66). One suggested mechanism is that TCDD activates the EGFR in a non-canonical ligand-independent manner. TCDD increases PKC signaling and thus the activity of several non-RTKs, resulting in enhanced EGFR phosphorylation in the plasma membrane (349). For example, the non-RTK, c-src, is bound cytosolically in a complex with Hsp90 and the AhR, and released upon AhR ligand binding (350). When released from the AhR protein complex by AhR-ligand binding, c-src is activate and could phosphorylate and activate the EGFR, leading to a ligand-independent TCDD-induced proliferation (211, 351). Alternatively, TCDD-induced production of EGFR ligands could drive TCDD's proliferative response through a ligand-dependent receptor activation.

In addition to its proliferative effect, TCDD also accelerates differentiation as evidenced by assays of barrier function or quantitation of protein or mRNA for markers of differentiation (1, 5, 18). This occurs in the face of decreases in receptor number, suggesting that loss of receptor signaling is important, even though TCDD is increasing ligand production.

Experimental design

Both TCDD- and Ca-treated cells show a loss of [^{125}I]-EGF binding suggesting a down-regulation or relocalization of EGFRs (15). In NHEKs, exposure to high extracellular Ca causes concentration-dependent decreases in high affinity [^{125}I]-EGF binding (15). Likewise, TCDD treatment decreased EGF binding, EGF-induced DNA synthesis, and increased CE competence (13). Hudson *et al.* (14) found that this TCDD-induced repression of EGF signaling was enhanced in NHEKs grown in the presence of high (1.8 mM) extracellular Ca, while binding was less affected in low Ca which selects for basal, proliferating cells. However, down-regulation is not simply a means of

silencing receptor signaling; it can also alter signaling dynamics, serving as another layer of spatial and temporal control of complex networks of response to environmental cues (352).

To address how alterations in EGFR signaling modulate the proliferative capacity of NHEKs, we first explored to what degree EGFR down-regulation leads to a loss of signaling in Ca-induced keratinocyte differentiation and TCDD-induced hyperproliferation. In the second chapter of this work we sought to identify which ligands were produced in NHEKs grown in high Ca (1.8 mM) to define their role in physiological EGFR down-regulation. We further determined if EGFR down-regulation affected EGFR competence in activating ERK-signaling, and modified proliferative capacity. In the third chapter, we explored the impact of TCDD on EGFR down-regulation and cellular responses. To answer these questions we used ligand-specific enzyme-linked immunosorbent assays (ELISAs) to identify and quantify EGFR ligands secreted under conditions of high Ca or TCDD. We then interfered with ligand activity and measured the impact on Ca- or TCDD-induced proliferation, ERK activity, or EGFR down-regulation

While this work addresses the question of how Ca and TCDD induce EGFR down-regulation, it also shows that even under differentiating conditions, the EGFR still plays an active role in regulating keratinocyte function. It has also lays the groundwork for future studies examining the combined role of TCDD and Ca on epidermal proliferation and differentiation.

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CHAPTER TWO

Calcium causes ligand-independent EGFR down-regulation.

Summary Statement

Ca-induced EGFR down-regulation is EGFR ligand- independent. While TGF-α and AREG maintain a population of EGFRs which preserve a proliferative capacity in confluent NHEKs, loss of EGFR ligands is insufficient to reverse EGFR binding loss or alter signaling through ERK.

Abstract

Extracellular calcium (Ca) induces epidermal differentiation, regulating desmosome formation and inducing markers of keratinocyte differentiation. Previous work shows that Ca reduces EGFR by ~50%, suggesting that loss of pro-proliferative EGFR signals precede differentiation. In this study, we examined the effect of high (1.8 mM) extracellular Ca on [^{125}I]-EGF binding, EGFR ligand secretion, ERK activity, and proliferation in normal human epidermal keratinocytes. Ca caused a rapid and sustained decrease in [^{125}I]-EGF binding relative to basal cells. Secretion of the EGFR ligand amphiregulin (AREG) increased with time in culture, while transforming growth factor-α (TGF-α) secretion was time- and Ca-dependent. Inhibiting EGFR ligand secretion with the metalloproteinase inhibitor batimastat reversed EGFR down-regulation in Ca-treated cells, while neutralizing AREG, TGF-α or both did not. Ca reduced ERK activity in NHEKs, a response intensified by TGF-α neutralization. However, AREG neutralization had no effect on this decreased ERK activity. Ca-treated NHEKs had 15% fewer cells yet EdU labelling showed no significant difference in proliferative capacity.

Interestingly, the proliferative capacity did depend upon the presence EGFRs. Our data suggest that Ca-induced EGFR down-regulation is ligand-independent but that differentiated keratinocytes down-regulate EGFRs but require the continued presence of EGFRs to maintain a population of proliferating cells.

1. Introduction

Keratinocytes in the stratum basale of the epidermis are cuboidal, undifferentiated epithelial cells that asymmetrically divide and migrate superficially through the layers of the epidermis. As they progress upwards, cells undergo cytoskeletal changes that lead to adopting a squamous phenotype (Arnemann et al., 1993; Franke et al., 1987) and an increase in tight junctions and desmosomal contacts (Jensen and Proksch, 2009; O'Keefe et al., 1987). Differentiating keratinocytes undergo changes to their protein expression profile and the composition of their cellular membrane (Ng et al., 1996; Pillai et al., 1990), and extrude lamellar granules containing specialized lipids and fatty acids which contribute to epidermal barrier strength. This process, known as cornification, is integral in forming the epidermal barrier, which prevents transdermal water loss and infection (Candi et al., 2005).

Extracellular calcium (Ca) plays an important role in nearly all facets of epidermal differentiation. Within the epidermis, elevation of extracellular Ca increases the expression of differentiation markers (Rice and Green, 1979) and causes redistribution of the desmosomal protein desmoplakin and the formation of desmosomes (Godsel et al., 2005;O'Keefe et al., 1987). Ca also causes a rapid and sustained down-regulation of the epidermal growth factor receptor (EGFR) in a variety of cell lines (O'Keefe et al., 1982;O 'Keefe and Payne, 1983), suggesting that keratinocyte

differentiation requires the suppression of the pro-proliferative signaling associated with EGFR activation. In fact, Getsios *et al.* (Getsios et al., 2009) showed that the desmosomal protein desmoglein 1 (Dsg1) suppresses ERK signaling as an important step in promoting terminal differentiation of confluent keratinocytes. Finally, Ca activates matrix metalloproteinases (MMPs) which cleave membrane-bound ligands that aid in cell migration through the epidermis (Edwards et al., 2008; Franzke et al., 2012; Tallant et al., 2010) as well as transglutaminase 1, which crosslinks proteins in the cellular membrane to form the stratum corneum (Candi et al., 2005; Thacher and Rice, 1985).

The EGFR is a member of the ErbB family of receptor tyrosine kinases, which includes the EGFR, ErbB2, ErbB3, and ErbB4 (Prenzel et al., 2001) with EGFR, ErbB2, and ErbB3 being found in the epidermis (Stoll et al., 2001). The EGFR and its preferred dimerization partner, ErbB2 (Macdonald-Obermann et al., 2013; Schneider et al., 2008a;Xian et al., 1997), are found at the highest density in the stratum basale where keratinocytes are actively proliferating (Jost et al., 2001;Nanney et al., 1990). Keratinocytes express ErbB3 in suprabasal layers (Kiguchi et al., 2000; Piepkorn et al., 1994), suggesting that EGFR-ErbB3 dimers are involved in location-dependent differentiation (De Potter et al., 2001; Macdonald-Obermann et al., 2013; Piepkorn et al., 2003). Suppression of EGFR signaling is clearly important in promoting differentiation as, *in vitro,* treatment of confluent keratinocyte cultures with EGF promotes transepithelial water loss (TEWL) and down-regulate clusters of genes involved in ceramide biosynthesis, tight junction formation, and epidermal differentiation (Tran et al., 2012). In addition, the increased expression of the EGFR and its ligands, which is often observed in hyperproliferative diseases, is accompanied by impaired barrier function,

(Schneider et al., 2008b; Segre, 2006). Finally, microarray studies in our lab showed that EGF treatment upregulates genes involved in epidermal disease (Tran et al., 2012). Together these data show that EGFR activity plays an important role in determining keratinocyte differentiation status.

In spite of the importance of the EGFR in cellular proliferation, silencing the receptor can impede differentiation. EGFR signaling has been linked to activation of protein kinase C-alpha (PKCα) signaling in differentiating keratinocytes (Koese et al., 2013;Wallis et al., 2000) and studies in EGFR -/- mice showed hypophosphorylated PKC impedes post-natal barrier function and causes chronic dermatitis (Denning et al., 2000;Yang et al., 2003). In fact, murine genetic knockout of TGF-α causes decreased wound healing (Kim et al., 2001) and abnormal differentiation of hair follicles (Kim et al., 2001). Further, mice deficient in ADAM17 (an MMP that cleaves pro-EGFR ligands) do not develop competent epidermal barriers, similar to EGFR -/- mice (Kitajima, 2014). These data suggest that the EGFR is necessary for complete epidermal differentiation.

Although elevations in extracellular Ca down-regulate EGFRs in keratinocytes, the mechanism(s) involved is unclear. Typically, receptor down-regulation is ligand-mediated and assessed by measuring changes in [^{125}I]-EGF binding. However EGF is not secreted in the epidermis or its accessory structures. In fact, keratinocytes secrete a number of EGFR ligands including amphiregulin (AREG), epiregulin (EREG), heparin binding EGF-like growth factor (HB-EGF), and transforming growth factor-alpha (TGF-α) (Macdonald-Obermann et al., 2013;Schneider et al., 2008b). MMP-dependent autocrine secretion of AREG and TGF-α is important in regulating normal proliferation and epidermal thickening (Schneider et al., 2008a;Stoll et al., 2010), while HB-EGF

regulates wound healing and keratinocyte migration (Iwamoto and Mekada, 2000). In addition, each ligand has a distinct effect on EGFR activation as well as differing abilities to induce receptor degradation or recycling.

The impact of Ca on EGFR down-regulation, receptor signaling, ligand production, and proliferation/differentiation have been extensively studied, but using a variety of cell types, culture conditions and Ca concentrations. (Borowiec et al., 2013). These differences in culture conditions make it difficult to draw conclusions as to the effects of Ca on EGFR signaling and responsiveness. Because of the complexity of interpreting historical data across a wide range of cell lineages and experimental designs, we used a defined set of culture and experimental conditions to identify the mechanisms of Ca-induced differentiation and its impact on EGFR signaling in NHEKs. We show that elevated extracellular Ca (1.8 mM) rapidly decreases EGFR number and increases the production of TGF-α. However, despite increases in both AREG and TGF-α down-regulation of EGFRs is ligand-independent. In addition, EGFR down-regulation is associated with decreases in ERK activity and keratinocyte cell number that are not altered by interfering with AREG or TGF-α availability.

2. Results

EGFR down-regulation by Ca

Previous studies have shown that treating confluent NHEKs with high extracellular Ca causes the loss of cell-surface EGFRs (Choi et al., 1991;O'Keefe et al., 1982;Osborne and Greenlee, 1985). In order to determine how rapidly this response occurs and to compare it to ligand-induced down-regulation by EGF, we performed radioligand binding assays in NHEKs. EGF (100 ng/ml) rapidly reduced

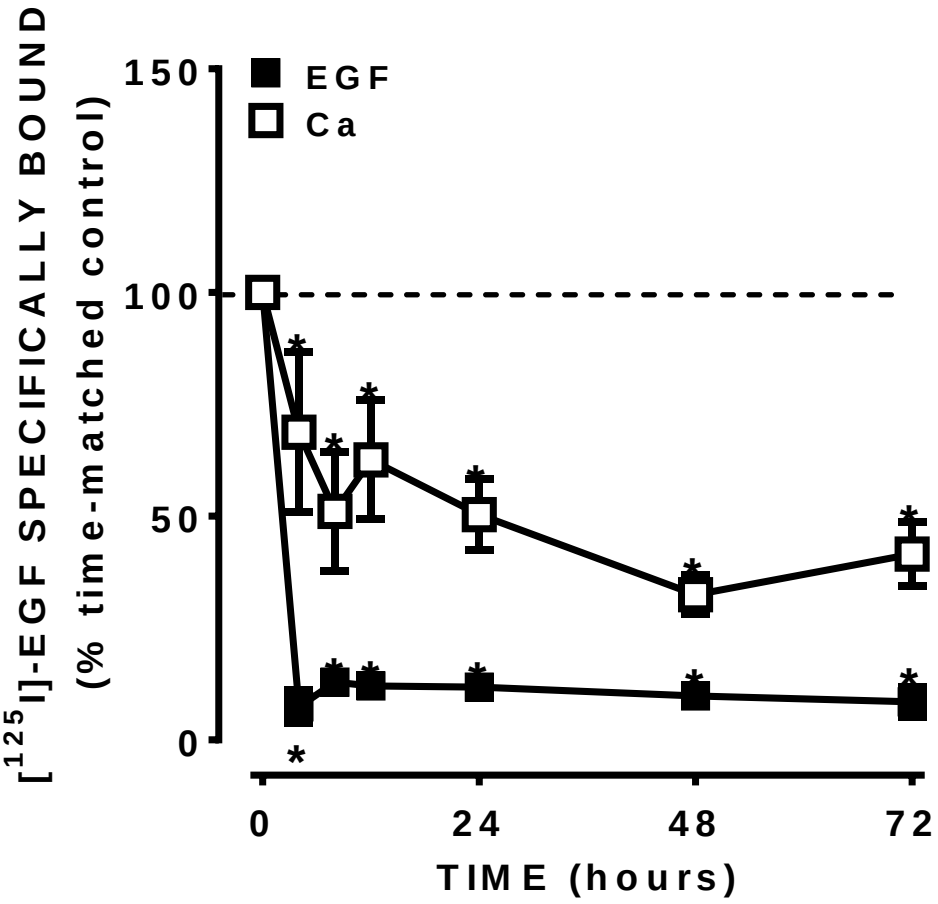

Figure 1: Calcium treatment down-regulates EGF receptors. NHEKs were grown as described in Materials and Methods and treated for the times indicated with EGF (100 ng/ml) or Ca (1.8 mM) then [^{125}I]-EGF binding was determined as described in the *Materials and Methods*. Data have been normalized to protein content and are expressed as the % time-matched control and reported as the means $\pm$ s.e.m. of three experiments assayed in triplicate. The dashed line indicates 100%. * indicates $p \leq 0.05$ for treatments compared to time-matched control.

[^{125}I]-EGF binding to ~10% of control values by 4 h, maintaining this significant ($p \leq 0.05$) level of inhibition for the duration of the experiment. Reductions in [^{125}I]-EGF binding took longer to reach maximal inhibition in Ca-treated cells, with significant effect on [^{125}I]-EGF binding from 4 hours onward ($p<0.05$) (**Fig. 1**). At 8 h, binding was reduced to 51% of control values and remained between 40-60% through 72 h.

Effect of Ca on EGFR ligand secretion

Keratinocytes secrete several EGFR ligands including AREG, EREG, HB-EGF, and TGF-α (Macdonald-Obermann et al., 2013; Schneider et al., 2008a). Our observation that Ca treatment mimics decreased [^{125}I]-EGF binding (Fig. 1) suggested that Ca induced the secretion of an EGFR ligand(s) that drives EGFR down- regulation. To assess the impact of Ca on EGFR ligand secretion, we assayed conditioned medium for the presence of the EGFR ligands TGF-α, AREG, and HB-EGF. These ligands were chosen based upon literature ties to epidermal disease, wounding, and homeostasis (Kennedy et al., 2013; Tran et al., 2012). TGF-α secretion was significantly enhanced by Ca treatment at 72 hours ($p<0.05$; Fig. 2A) going from 115 pg/ml in basal cells at 72 h to 425 pg/ml. Although AREG secretion increased with time in culture (Fig. 2B), Ca had no effect on its secretion. HB-EGF concentrations were below the detection limits of our ELISAs (**Appendix A**) consistent with the low levels of HB-EGF produced by NHEKs in culture (Piepkorn et al., 2003).

Role of EGFR ligands in EGFR down-regulation

The data presented in **Figure 2** show that NHEKs secrete significantly elevated levels of AREG at 72 h ($p \leq 0.05$) and that TGF-α is elevated in Ca-treated cells ($p \leq 0.05$). This suggests that one or both ligands causes the loss of [^{125}I]-EGF binding. To

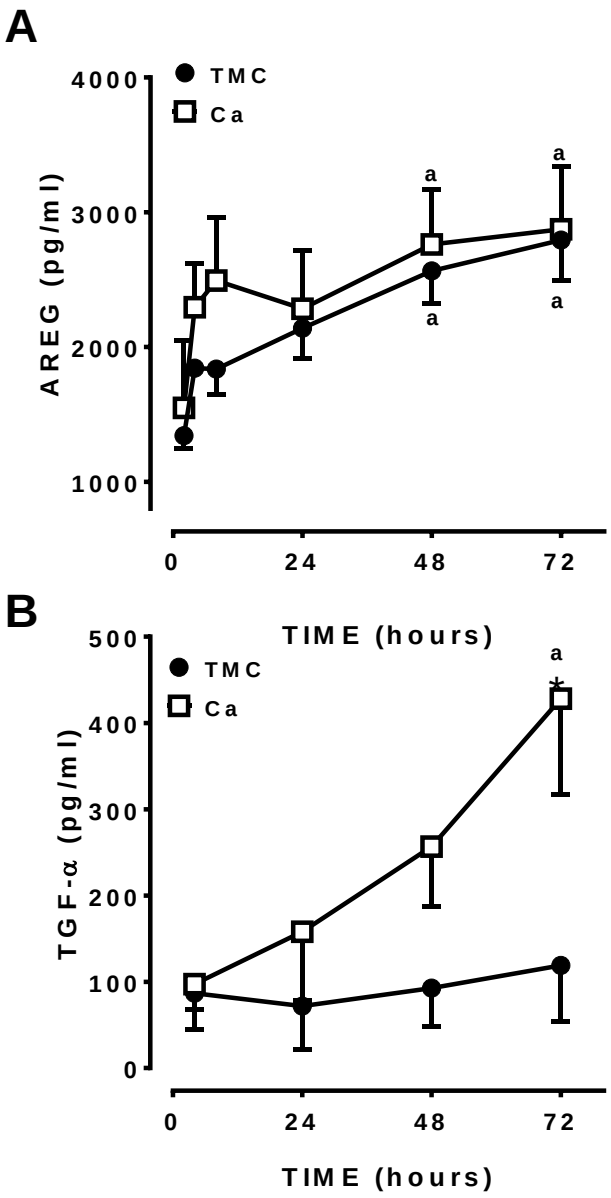

Figure 2. Calcium increases TGF-α but not AREG secretion. NHEKs were treated for increasing times with Ca (1.8 mM) and culture medium was collected. **(A)** AREG and **(B)** TGF-α content were determined by ELISA assay as described in *Materials and Methods*. Data are means $\pm$ s.e.m. of three experiments assayed in triplicate. (a) indicates $p \leq 0.05$ from Time 0. * indicates $p \leq 0.05$ for Ca-treated cells compared to time matched controls

assess the role of these ligands in mediating Ca-dependent EGFR down-regulation, NHEKs were treated with Ca in the presence or absence of batimastat, a broad spectrum metalloproteinase inhibitor (Rasmussen and McCann, 1997). Consistent with observed decreases in [^{125}I]-EGF binding (**Fig. 1**), treating NHEKs with Ca significantly reduced biotinylated EGF binding at 72 h ($p<0.05$; **Fig. 3**). Batimastat relieved receptor down-regulation in Ca-treated cells such that biotin-EGF binding was not significantly different from basal cells (**Fig. 3A**), indicating that that the shedding of a MMP-cleaved protein(s) relieves EGFR down-regulation. Batimastat was not able to reverse EGF-induced down-regulation (**Fig. 3A**), consistent with EGF addition to the medium rather than being released by the cells. To identify the ligand involved in EGFR down-regulation, NHEKs were treated with neutralizing antibodies for AREG or TGF-α alone or in combination. Surprisingly, neutralizing ligands alone or in combination had no significant effect on receptor down-regulation suggesting that Ca-induced decreases in biotin-EGF binding were ligand-independent. (**Fig. 3B**).

The Effect of Ca on ERK activity

The continued presence of EGFRs and the production of TGF-α and AREG in Ca-treated cells suggests that cells retain responsiveness to locally produced EGFR ligands. To determine if this is the case, cells were treated for 72 hours with Ca in the absence or presence of neutralizing antibodies for AREG (15 μg/ml) or TGF-α (5 μg/ml) alone or in combination. ERK activity was 55% lower in Ca-treated cells ($p<0.05$), consistent with the decrease in EGF binding observed in **Figures 1** and **2**. However, neutralizing AREG, TGF-α or both together had no significant effect on ERK activity in basal or

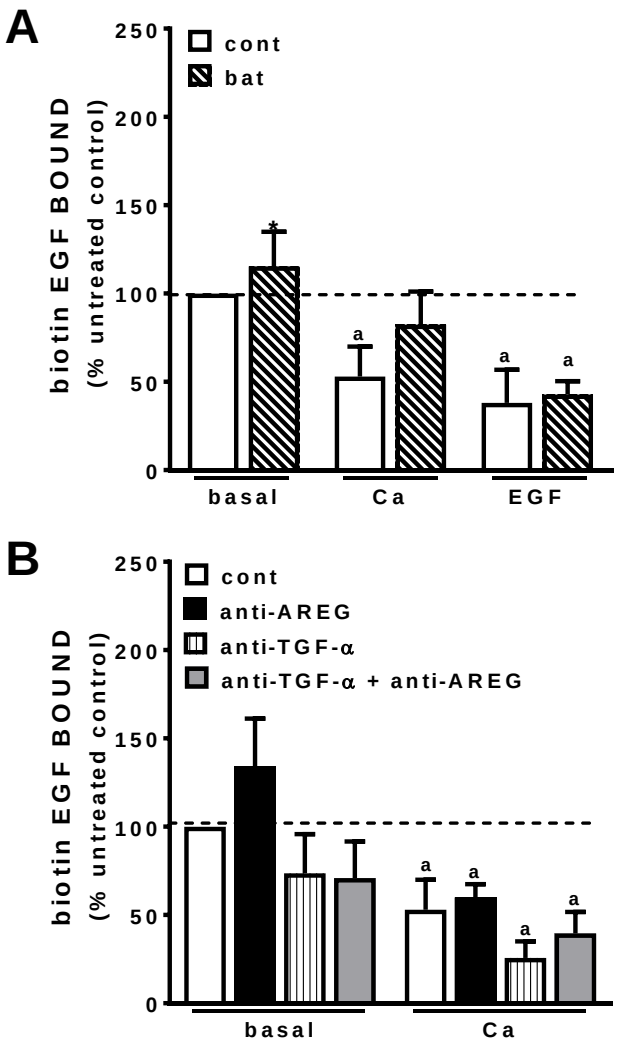

Figure 3. Effect of altering ligand availability on EGFR down-regulation. (**A**) NHEKs were treated with Ca (1.8 mM) for 72 h in the absence or presence of batimastat (bat; 3 μM) or **(B)** neutralizing antibodies for TGF-α (5 μg/ml) or AREG (15 μg/ml) alone or in combination. Biotin-EGF binding was then determined as described in *Materials and Methods.* Basal refers to K-SFM without Ca; control refers to treatments in the absence of batimastat or neutralizing antibodies. Data are means $\pm$ s.e.m. of six experiments assayed in triplicate, normalized to crystal violet staining, and reported as the % biotin-EGF bound in basal cells. (a) indicates significantly different from the basal control ($p \leq 0.05$), The dashed line indicates basal control normalized to 100%.

Ca-treated cells (**Fig. 4**), indicating that the local production of TGF-α and AREG was not maintaining ERK activity in these cells.

Role of EGFR ligands in regulating cell number

The continued presence of EGFRs and the production of both TGF-α and AREG could indicate that these ligands play a role in cell proliferation. To assess the effect of individual EGFR ligands on cell number, we utilized fluorescence-based double stranded DNA analysis. Treatment with Ca led to a significant 15.2% decrease in cell number relative to basal cells (**Fig. 5A**). Neutralizing AREG or TGF-α independently had no significant effect on cell number in basal or Ca-treated cells. Neutralizing both AREG and TGF-α significantly reduced cell number in basal cells ($p \leq 0.05$) but had no greater effect than Ca alone on cell number in Ca-treated cells (**Fig. 5A**). To address this EGFR-dependence, we measured the impact of interfering with EGFR activity on DNA synthesis using EdU labeling of actively proliferating cells treated for 72 h with Ca in the presence or absence of the EGFR inhibitor, PD153035 (300 nM). We first counted the total number of cells per field and found that it was not significantly different between basal and Ca- treated cells. PD153035 significantly reduced the number of EdU-labeled nuclei in basal and Ca-treated cells (**Fig. 5B, C**) indicating that DNA synthesis is dependent upon the continued presence of signaling-competent EGFRs.

3. Discussion

The epidermis is a continually renewing stratified epithelium that forms a protective barrier to the external environment. Proliferating cells in the basal layer migrate upwards and undergo terminal differentiation to replace cells that are lost to

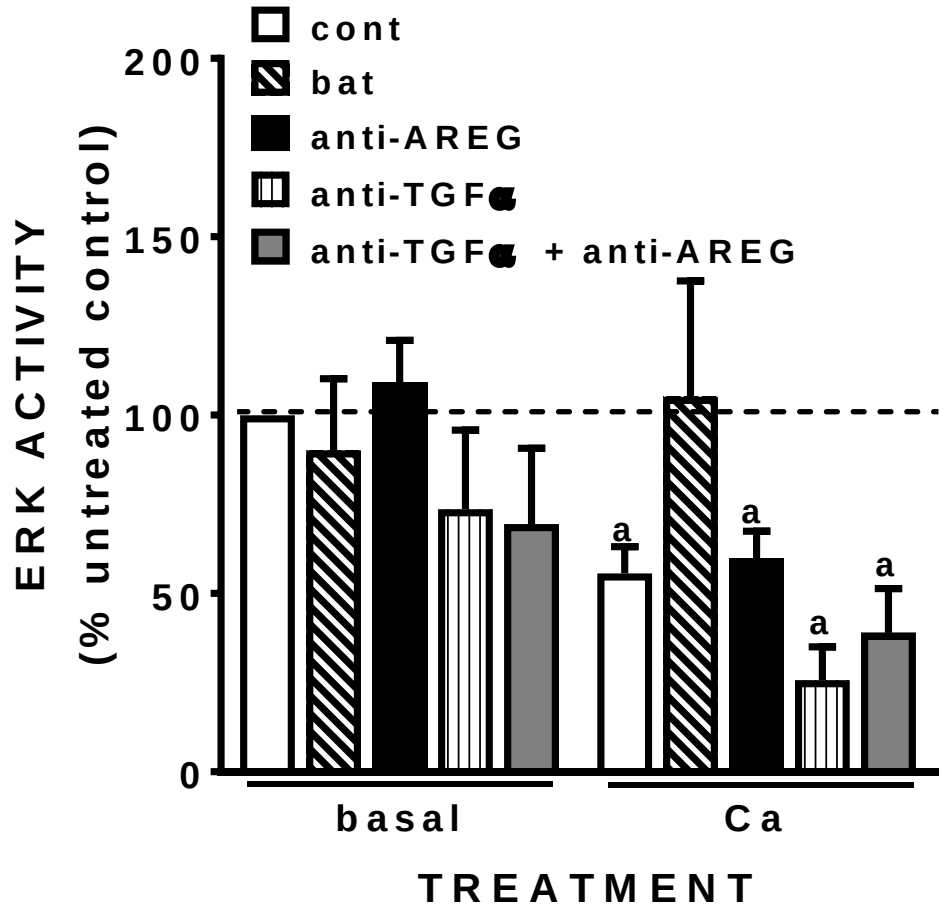

Figure 4. Effect of altering ligand availability on ERK activity. To assess the effect of secreted ligands on ERK activity, NHEKs were grown to confluence and treated for 72 h with 1.8 mM Ca in the presence or absence of batimastat (bat; 3 μM) or neutralizing antibodies for TGF-α (5 μg/ml) or AREG (15 μg/ml) alone or in combination. ERK activity was measured via PACE assay as described in *Materials and Methods*. Basal refers to K-SFM without Ca, control refers to treatments in the absence of inhibitors. Data are means ± s.e.m. of three experiments assayed in triplicate. The dashed line indicates the basal control normalized to 100%. (a) indicates significantly different from basal control.

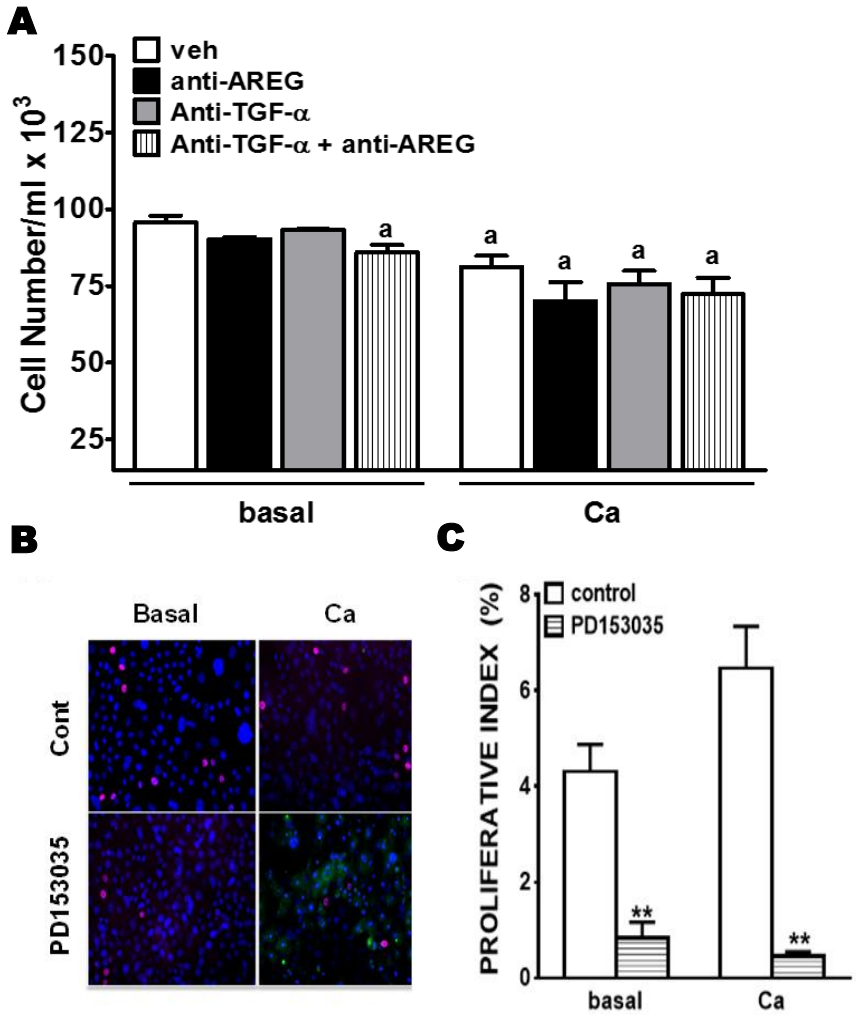

Figure 5. Effect of reducing EGFR ligand availability on cell number. In **(A)** NHEKs were grown in the presence or absence of neutralizing antibodies for TGF-α (5 μg/ml), AREG (15 μg/ml) or both. After treatment, cells were frozen and dsDNA labeled as described in *Materials and Methods.* Basal refers to K-SFM without Ca; vehicle refers to treatments in the absence of neutralizing antibodies. Data are means ± s.e.m. of three separate experiments assayed in tripicate and reported as percentage of basal vehicle. (a) indicates significantly different from basal control ($p \leq 0.05$). In (**B)** and **(C)**, NHEKs were grown to confluence in glass chamber slides and then treated for 72 h with Ca (1.8 mM) in the presence or absence of PD153035 (300 nM), an EGFR inhibitor. During the last 16 h they were incubated with 10 mM EdU and labeled as described in the *Materials and Methods*. **(B)** Representative confocal images of total (DAPI; 405 nm, blue) and EdU-staining nuclei (647 nm, pink) at 20x magnification. **(C)** Proliferative index of Edu positive nuclei per field (EdU labeled/DAPI labeled). Data are means ± s.e.m. from quantifications of five fields from three separate experiments. ** indicates significantly different from the within treatment control (≤ 0.01).

desquamation. Ca is an important modulator of this differentiation process (Eckert et al., 2004) with high extracellular Ca rapidly increasing the expression of differentiation markers such as involucrin, loricrin, and transglutaminase-1 (Rice and Green, 1979) and more slowly inducing cornification (Pillai et al., 1990). High extracellular Ca also down-regulates the EGFR (O'Keefe and Payne, 1983), a response that has been interpreted to show that differentiation requires suppression of epidermal EGFR signaling (Getsios et al., 2009; Jensen and Proksch, 2009; O'Keefe et al., 1982; O'Keefe and Payne, 1983). However, EGFR ligand production (**Fig. 2**) is maintained in the face of receptor down-regulation (**Fig. 1**) and differentiation (Campion et al., 2016; Rice and Green, 1978) leading us to wonder what role the ligands play in receptor down-regulation and the cellular consequences of receptor loss. In this study, we show that the Ca-induced down-regulation of EGFRs in NHEK is ligand-independent even though it is associated with constitutive production of AREG and increased TGF-α secretion. Further, it is associated with a reduction in ERK activity and cell number but the maintenance of a small pool of proliferating cells that require the continued presence of the EGFR .

Treatment of NHEKs with high (1.8 mM) extracellular Ca reduced [^{125}I]-EGF binding by 50% within 8 hours (**Fig. 1**), a response comparable to that observed by Osborne and by others (Hudson et al., 1985;Osborne and Greenlee, 1985). ERK activity decreased to a level comparable to EGFR down-regulation (**Fig. 4**) and led to a reduction of total cell number in Ca-treated cells at 72 h (**Fig. 5**). This would suggest that Ca-induced differentiation effectively "turns off" EGFR signaling in order to progress with epidermal differentiation.

However, this decrease in receptor number was accompanied by an increase in TGF-α secretion in Ca-treated cells (**Fig. 2B**) on a background of elevated AREG at 72 h (**Fig. 2A**). Denning *et al.* (Denning et al., 2000) showed similar effects of Ca on TGF-α mRNA, indicating that Ca regulates both the expression and production of this EGFR ligand. In addition, AREG secretion increased with time in culture in both basal and Ca-treated cells, presenting NHEKs with two EGFR ligands potentially capable of down-regulating the EGFR. Additionally, while there were fewer cells *in vitro*, we found that EGFR-dependent proliferation in Ca-treated cells was the same as in basal treated cells (**Fig. 5C**). These data, conversely, suggest that EGFRs in differentiating keratinocytes are providing a sufficient proliferative signal in at least a small population of cells, and provides a mechanism through the release of ligand as to how this may be occurring.

In fact, when basal or Ca-treated cells were treated with the metalloproteinase inhibitor batimastat, the EGFR down-regulation in Ca-treated cells was reversed (**Fig. 4**) and biotin-EGF binding in basal cells was enhanced (**Fig. 3A**). These data argue that there is an effect of a locally released ligand(s) on EGFR binding in both basal and Ca-treated cells. However, when we removed AREG from the culture medium with a neutralizing antibody, there was no change in biotin-EGF binding in basal or Ca-treated cells. While interfering with TGF-α appears to intensity EGFR down-regulation in Ca-treated cells, this change is not significantly different and neutralizing both ligands was similarly ineffective.

The ability of batimastat to reverse down-regulation while neutralizing antibodies could not would seem to argue that another EGFR ligand is involved EGFR down-regulation. HB-EGF, which down-regulates EGFRs (Stoll et al., 2001), could play this

role. However, HB-EGF is typically associated with wound healing and keratinocyte migration (Poumay and de Rouvroit, 2012) responses not activated in our cultures. More importantly, we could not detect HB-EGF in the medium of basal or Ca-treated cells by ELISA (**Appendix A**). Recent studies show that EREG is present in skin and drives keratinocyte proliferation (Macdonald-Obermann et al., 2013). However, when we neutralized EREG alone, or in combination with TGF-α and AREG, we could still not relieve EGFR downregulation (**Appendix A**). It is possible that a cryptic EGFR ligand down-regulates EGFRs in the skin, and there is evidence in literature that betacellulin plays a role in hair follicle development (Schneider et al., 2008b;Shirakata et al., 2010) and epigen has been shown to play a role in sebaceous gland development and pathology within the epidermis (Schafer et al., 2014). However, while *in vivo*, it is possible these ligands play a role, given their localized expression and secretion, it is less likely that they are affecting our system which models the interfollicular epidermis.

This leaves two possibilities: First, that batimastat has effects on EGFR levels independent of EGFR ligand secretion. However, batimastat mimics the MMP cleavage site in substrate (Brown, 1995) blocking actions of MMPs -1, -2, -3, -7, and 9 (Wang et al., 2005), and we could find no evidence in the literature for off-target effects on any signaling molecule that leads to differentiation. This leaves us with the possibility that EGFR down-regulation is ligand-independent. In fact, Ca activates phospholipase C independent of a receptor-ligand signaling event (Punnonen et al., 1993). This, in turn, would result in an increase in protein kinase C (PKC) activity. Since EGFR signaling activity is inhibited by PKC-dependent phosphorylation of T^{654} (van Baal et al., 2012) and PKC activation by phorbol esters reduced EGFR binding by 40-50% in in SV40

transformed keratinocytes (Xue et al., 1996), PLC-mediated and PKC-dependent signals might mediate EGFR down-regulation in Ca-treated keratinocytes. Further studies are required to identify the ligand-independent mechanisms that might result in EGFR down-regulation.

Receptor down-regulation is typically thought of as a means of reducing cellular responses to the continued presence of agonist (Mizuno *et al*, 2005). If TGF-α and AREG are not involved in receptor down-regulation, what other role might they play? Recent studies have shown that receptor down-regulation is an important mechanism for shaping ERK signaling dynamics and encoding specific cellular responses. In mammary epithelial cells, autocrine delivery of an EGFR ligand produced a persistent reduction in EGFR number that promoted cell migration in response to prolonged ERK signaling (Joslin et al., 2007). In contrast, acute elevations in ERK activity produced by exogenous EGF were not effective in promoting cell migration. In Ca-treated NHEKs, the reduction in EGFR binding was accompanied by a marked decrease in ERK activity, a response comparable to that reported by (Getsios et al., 2009;O'Keefe et al., 1982) and indicating that having fewer receptors at the cell surface lowered signaling competence. However, AREG and TGF-α were not maintaining ERK signaling in basal or Ca-treated cells, as neutralizing AREG and TGF-α, alone or in combination had no effect on ERK activity. These data argue that the local production of AREG and TGF-α is not involved in proliferation, a response typically associated with ERK activity, and we see that this is, in fact, true for Ca-treated cultures where cell number was significantly lower than that observed in basal cells and was not affected by neutralizing AREG and TGF-α either alone or in combination (**Fig 5A**). In fact, based on the decrease in ERK activity, the

15% reduction of total cell number in Ca-treated cells at 72 h (**Fig. 5**) was not unexpected and suggest that Ca-induced differentiation effectively "turns off" EGFR/ERK signaling in order to progress with epidermal differentiation. Interestingly, AREG and TGF-α do appear to be important in maintaining cell number in basal cells, as neutralizing both ligands significantly lowers cell number. This may not be surprising as the basal cell medium contains 0.09 mM Ca, a concentration which results in more rapid growth and greater NHEK saturation densities than that supported by 1.2 mM Ca (Micallef et al., 2009). Furthermore, EdU labeling of basal and Ca-treated cells indicated that both populations required a functional EGFR to maintain a pool of proliferating cells (**Fig. 5B,C**) that was comparable in both cell populations. Removal of EGFR ligands is likely not affecting cell number in Ca-treated cells as the majority of cells are growth-arrested and differentiated. In this case, the presence of the small population of proliferating cells and the maintenance of a reduced number of EGFR at the cell surface might be a mechanism for preserving EGFR ligand responsiveness in face of damage to the epithelium. The EGFR-dependent proliferation observed in **Fig 5C** could be due to trans-activation of the EGFR by a Ca-activated non-receptor tyrosine kinase such as pyk2 or src and there is evidence in literature which shows these proteins are activated during keratinocyte differentiation (Calautti et al., 2005; Gschwind et al., 2001; Xie et al., 2005).

Although we did not address this in our study, the last possible role of the ligands in post-confluent Ca-treated cells is that they play a role in keratinocyte differentiation. ADAM17-/- mice which do not produce soluble TGF-α show reduced barrier permeability and delayed differentiation, a phenotype shared by EGFR -/- mice (Franzke

et al., 2012). This finding is supported by the ability of exogenous TGF-α to rescue barrier formation (Franzke et al., 2012), and observations that TGF-α mRNA (Denning et al., 2000) and protein (**Fig 2**) increase in differentiating cells *in vitro.* Removal of EGFR ligands is likely not affecting cell number in Ca-treated cells as the majority of cells are growth-arrested and differentiated.

Taken together, these data suggest that TGF-α secretion is differentiation-associated rather than proliferative. In our work, we found neutralizing TGF-α caused noticeable though non-significant reduction in both biotin-EGF binding (**Fig 3B**) and ERK activation (**Fig 4**). This observation was similar to that in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated NHEKs grown in low (0.09 mM) Ca under identical experimental conditions (Campion et al., 2016), a condition which causes a differentiation-like phenotype in proliferative cells. This suggests that TGF-α, rather than driving EGFR down-regulation and silencing it, is maintaining a basal level of surface-associated EGFRs capable of responding to EGFR ligand in order to repair and maintain the epidermis.

In summary, our data show that Ca-induced EGFR down-regulation occurs through what appears to be a ligand-independent method. Ca induced EGFR down-regulation while ERK signaling and proliferation require ligands in basal cells. MMP-dependent secretion of both AREG and TGF-α drives ERK activation and maintains proliferation in basal keratinocytes. Ca induces TGF-α secretion while AREG production is associated with increasing time in culture and confluence regardless of treatment. However, while we show that Ca maintains a small population of EGFR dependent, proliferating cells, the predominant phenotype induced by Ca is differentiation. It is likely, however, that this

sub-population of proliferative cells provide a sufficient proliferative capacity to maintain and repair the epidermis. Further work should be undertaken to examine the extent of this role of as well as that of secreted EGFR ligands on keratinocyte differentiation.

4. Materials and Methods

Cell Culture

In all experiments, fifth passage NHEKs from neonatal foreskins (Lonza, Mapleton, IL) were plated at 5,000 cells/cm^2 in Costar 24- or 96-well cell culture dishes (Corning, Corning, NY). Cells were maintained in keratinocyte serum-free medium (K-SFM; Gibco Invitrogen, Carlsbad, CA) containing 0.09 mM Ca, 5 ng/ml recombinant human EGF, and 50 µg/ml bovine pituitary extract (BPE). The medium was changed every 48 h until confluence. At confluence, cells were changed to K-SFM without supplements (basal medium) for 48 h then transferred to basal medium containing high Ca (1.8 mM) as described in each figure legend. In some experiments, EGF (Bachem, Torrance, CA) was added to basal medium to serve as a positive control for ligand-induced EGFR down-regulation. For immunofluorescence, NHEKs were plated in glass chamber slides (BD Biosciences, San Jose, CA) that had been coated with fetal bovine serum. The medium was changed every 72 h until confluence. At confluence, cells were treated as described above and then subjected to experimental protocols for 72 h.

Binding of labeled EGF to intact cells

Time-dependent loss of cell-surface associated EGFRs was determined in NHEKs grown to confluence in 24-well dishes (Corning, Corning, NY) and treated for 8-72 h in Ca-containing medium. Following treatment, cells were washed with HEPES-buffered K-SFM. [^{125}I]-EGF/ml (specific activity 1128 Ci/mmol; Perkin Elmer, Waltham, MA) was

diluted in HEPES-buffered K-SFM with 0.1 % bovine serum albumin (BSA) and binding reactions were initiated by the addition of 200 µl of [^{125}I]-EGF (250,000 cpm/ml) to each well in the presence or absence of 1 µg excess unlabeled EGF. Binding reactions were carried out for 60 min at 37^{o}C and terminated by rapid washing with ice-cold phosphate-buffered saline (PBS). Monolayers were solubilized in 0.2N NaOH and aliquots were assayed for protein content using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA), and radioactivity by a Packard Cobra II Gamma counter (Perkin Elmer, San Jose, CA). Non-specific binding was subtracted from total counts and specific binding was normalized to protein content. Data are reported as the percentage of binding observed in time-matched controls and are the means $\pm$ s.e.m. of three separate binding experiments assayed in triplicate.

Measurement of AREG, HB-EGF, and TGF-α secretion by NHEKs

NHEK-conditioned medium was collected from basal or Ca-treated cells 4-72 h after treatment and stored frozen in aliquots at -80^{o}C until analyzed for AREG, HB-EGF, or TGF-α content using ELISA kits from Abcam (Cambridge, MA). Undiluted samples were added to the assay and AREG, HB-EGF, and TGF-α content were determined from standard curves. Data are reported in pg/ml and are the means $\pm$ s.e.m. of three experiments assayed in duplicate. HB-EGF was not detected in culture medium from either basal or Ca-treated NHEKs and was not assessed in the neutralization experiments.

Alteration of ligand activity

To investigate the role of each secreted ligand in the loss of EGF binding, cells were grown in 96-well cell culture dishes as described above in the presence or absence of 3 µM batimastat (Tocris Bioscience, Minneapolis, MN) or neutralizing antibodies for

AREG or TGF-α. To neutralize secreted ligands, antibodies to AREG (15 µg/ml), TGF-α (5 µg/ml) or both, were added to the medium. Medium was spiked with batimastat or neutralizing antibody after 36 h of treatment. The effect of interfering with EGFR ligand availability on cell-surface EGFRs was determined using biotinylated-EGF (Invitrogen, Carlsbad CA) in a modified protocol from DeWit *et al.* (, 2000). This assay was adopted to increase assay throughput and conserve antibody. After treating with Ca ± batimastat or neutralizing antibodies, plates were washed in ice-cold PBS and incubated for 1 h on ice with 10 ng/ml biotinylated EGF in the presence or absence of 1 µg/ml unlabeled EGF. Cells were fixed for 30 min at 37°C in 4% formaldehyde, washed once with PBS followed by two five-minute washes in PBS containing 50 mM glycine. Cells were blocked in 2% gelatin in PBS with 0.1% Triton X-100 (PBS-T) for 1 h at 37°C, then incubated for 1 h at 37°C in PBS-T containing 0.2% gelatin and streptavidin-HRP (1:500; Cell Signaling Technology, Danvers, MA). Plates were subjected to three five-min washes with PBS-T, and two five-min washes with PBS before developing with 1-Step Ultra TMB-ELISA Substrate (Thermo Fisher, Waltham, MA). Data were measured colorimetrically at 450 nm and normalized to crystal violet absorbance at 595 nm (as a measure of cell number) using a BioTek Synergy H1 microplate reader (Biotek Instruments, Inc, Winooski VT). Binding was reported as a percentage of that in cells grown in basal medium without inhibitors or antibodies.

Phosphoantibody Cell Based ELISA (PACE) and crystal violet staining

To test the effects of EGFR down-regulation on basal ERK activity, NHEKs grown in 96-well plates were treated for 72 h in the absence and presence of batimastat or EGFR ligand neutralizing antibodies as described above. At 72 h, basal ERK activity

was measured by PACE assay as described in Kramer *et al.* (, 2008). Briefly, treatments were terminated by rapid washing with ice cold PBS and cells were fixed in 4% formaldehyde in PBS, blocked, and incubated overnight with an activation-specific rabbit monoclonal antibody [phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204); #4370, Cell Signaling Technology, Danvers, MA]. The assays were developed by incubating with a HRP-conjugated goat anti-rabbit IgG and 1-Step Ultra TMB-ELISA Substrate and read at 450 nm. To normalize to cell number, fixed cells were washed and stained with 0.04% crystal violet (Sigma Aldrich, St. Louis, MO) (w/v) in 4% ethanol. Cells were lysed overnight in 10% SDS and lysates read at 595 nm following the protocol described by Kueng *et al.* (, 1989). Absorbance was read on a BioTek Synergy H1 microplate reader and data were expressed as a ratio of ERK (450 nm)/cell number (595 nm).

EdU Labeling of NHEKs

Cells grown in glass chamber slides were treated at confluence for 72 h with 1.8 mM Ca in the presence or absence of PD153035, an efficacious and selective EGFR inhibitor, as described above. Sixteen hours before the end of the experiment, cells were incubated with 10 μM 5-ethynyl-2´-deoxyuridine (EdU), fixed with 4% formaldehyde in PBS then permeabilized with 0.5% Triton-X 100 in PBS prior to incubation with the Click-It reaction cocktail containing copper sulfate ($CuSO_4$) and an AlexaFlour-conjugated azide that was crosslinked to cellular EdU as per manufacturer's protocols (ClickIt EdU Imaging kit; Invitrogen, Carlsbad, CA). Slides were then coverslipped using DAPI-containing mounting medium to stain nuclei. Cells were imaged at 20x magnification on Nikon A1 confocal microscope (Nikon, Inc, Melville, NY) at 647 nm for EdU-positive nuclei and 405 nm for total nuclei. Identical image capture parameters

were used for all images. EdU positive nuclei as well as total (DAPI-labeled) nuclei were counted using CellProfiler version 2.1.0 (Broad Institute, Cambridge, MA). Images were converted to gray scale then counted via "count objects" selecting for circularity and filtering using a Mixture of Gaussian (MoG) Global correction with standard threshold correction factors, and upper and lower bounds (1.0, 0.0, 1.0, respectively) (Carpenter et al., 2006). Five random fields on each of 3 slides were counted for all treatments and conditions.

Double-stranded DNA analysis

To support crystal violet data as an indicator of cell number, double stranded DNA was labelled using the FluoReporter blue fluorometric dsDNA Quantitation kit (Molecular Probes, Inc, Eugene, OR) following manufacturer's protocol (362). Briefly, cells were grown to confluence in black-walled and clear bottom plates and treated with 1.8 mM Ca $\pm$ 300 nM batimastat or neutralizing antibodies as described above. At 72 hours, medium was removed and plates were frozen at -80^{o}C to lyse cells. Plates were then thawed and rehydrated with Hoechst 33258 in Tris-NaCl-EDTA buffer and fluorescence was measured on a BioTek Synergy H1 microplate reader with excitation and emissions filters at 360 nm and 460 nm, respectively. Cell number in each treatment group was determined from a standard curve plated at the start of the experiment.

Data analyses

EGFR binding data and PACE assays were performed in three experiments utilizing triplicate wells. Data were analyzed via two-way ANOVA and significance ($p \leq 0.05$) was determined using a Bonferroni post-hoc test as performed in Prism GraphPad software version 5.0 (La Jolla, CA). TGF-α and AREG ELISAs were performed with

conditioned medium from three experiments and assayed in duplicate. EGFR ligand concentration was determined from standard curves and analyzed via one- or two-way ANOVA with a Bonferroni post-hoc test as appropriate.

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CHAPTER THREE

Role of EGF Receptor ligands in TCDD-induced EGFR down-regulation and cellular proliferation

Abstract

In cultures of normal human epidermal keratinocytes (NHEKs), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces the expression of the epidermal growth factor receptor ligands transforming growth factor-α (TGF-α) and epiregulin (EREG). TCDD also down-regulates EGF receptors (EGFR), suggesting that decreases in signaling contribute to the effects of TCDD. In this study, we treated post-confluent NHEKs with 10 nM TCDD and assessed its effects on EGFR binding, EGFR ligand secretion, basal ERK activity, and proliferation. TCDD caused time-dependent deceases in [^{125}I]-EGF binding to levels 78% of basal cell values at 72 h. Amphiregulin (AREG) levels increased with time in culture in basal and TCDD-treated cells, while TGF-α and epiregulin (EREG) secretion were stimulated by TCDD. Inhibiting EGFR ligand release with the metalloproteinase inhibitor batimastat prevented EGFR down-regulation. Neutralizing antibodies for AREG and EREG relieved receptor down-regulation while neutralizing TGF-α intensified EGFR down-regulation. Treating NHEKs with AREG or TGF-α caused rapid internalization of receptors with TGF-α promoting recycling within 90 min. EREG had limited effects on rapid internalization or recycling. TCDD treatment increased ERK activity, a response reduced by batimastat and the neutralization of all three ligands indicating that the EGFR and its ligands maintain ERK activity. All three EGFR ligands were required for the maintenance of total cell number in basal and

TCDD-treated cultures. The EGFR inhibitor PD153035 blocked basal and TCDD-induced increases in the number of cells labeled by 5-ethynyl-2´-deoxyuridine EdU), identifying an EGFR-dependent pool of proliferating cells that is larger in TCDD-treated cultures. Overall, these data indicate that TCDD-induced EGFR down-regulation in NHEKs is caused by AREG, TGF-α and EREG, while TGF-α enhances receptor recycling to maintain a pool of EGFR at the cell surface. These receptors are required for ERK activity, maintenance of total cell number, and stimulate stimulating the proliferation of a small subset cells.

1. Introduction

The epidermis is a stratified squamous epithelium that provides a barrier against the entrance of pathogens and foreign substances and protects the body from dehydration. Keratinocytes within the stratum basale are cuboidal, undifferentiated epithelial cells that divide and migrate superficially through the layers of the epidermis. Cells in the stratum basale maintain the proliferative capacity of the epidermis while the epidermal barrier is established as cells migrate outwardly and cornify [1]. As they progress upwards and differentiate, keratinocytes become more squamous-like [2,3], alter their membrane lipids, cross-link proteins and lose organelles such as mitochondria and nuclei [4-6]. The differentiation process is accompanied by decreases in epidermal growth factor receptor (EGFR) number at the cell surface (down-regulation), a reduction in EGFR signaling, and the attenuation of epidermal proliferative capacity [1,7-9], demonstrating the central role EGFRs play in the balance between proliferation in the basal layers and differentiation in the upper layers [10].

Keratinocytes express three members of the ErbB family of receptor tyrosine kinases, the EGFR (ErbB1), ErbB2, and ErbB3 (reviewed in [11]) with the EGFR

appearing to be the most important member mediating ligand effects in these cells [12]. The fourth family member, ErbB4, is not expressed in skin [12]. Each ErbB receptor possesses differing ligand affinities, as well as overlapping, but distinct downstream signaling cascades and distribution within the epidermis [13]. EGFR activation leads to receptor dimerization and phosphorylation of tyrosine residues in the receptor's C terminus which provides docking sites for scaffolding and signaling molecules. All ErbB receptors can form homo- or heterodimers but EGFR homodimers engage cellular endocytic machinery leading to EGFR internalization and degradation, while ErbB heterodimers recycle to the cell membrane [14,15]. The EGFR is expressed in all epidermal layers [12], but its density is greatest in the stratum basale where it drives proliferation in cells that have lost contact with the matrix [16,17].

Cells expressing EGFRs typically produce EGFR ligands that maintain cells in the absence of exogenous growth factors [18-21]. Normal keratinocytes produce amphiregulin (AREG), epiregulin (EREG), transforming growth factor-alpha (TGF-α) and heparin binding EGF-like growth factor (HB-EGF) [11]. Amphiregulin is produced in the largest quantities, consistent with its ability to induce the strongest autocrine stimulation to cell growth [22]. In contrast, TGF-α, HB-EGF, and epiregulin are expressed at very low levels [23]. Keratinocyte production of TGF-α [24,25] and EREG [26] can also be increased by treating cells with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a response consistent with the ability of TCDD to stimulate proliferation. However, TCDD-mediated increases in TGF-α and EREG are also accompanied by accelerated differentiation [27-30] and down-regulation of keratinocyte EGFRs [30-32], and studies in our lab have shown that inhibiting EGFR activation promotes the

differentiating effects of TCDD [28]. These data suggest that the loss of EGFR signaling mediates the accelerated differentiation of TCDD-treated keratinocytes.

Cellular responses to a ligand are determined by the number of receptors activated, and receptor down-regulation is a mechanism for modifying signaling dynamics to limit cellular responses [33,34]. The down-regulation of EGFRs observed as keratinocytes differentiate or in cells treated with TCDD may be an adaptive mechanism to maintain but redirect responsiveness in the face of continued agonist presence [35]. In mammary epithelial cells, Joslin *et al.* [36] showed that local production of EGFR ligands reduced EGFR number, but elevated basal ERK activity and enhanced cell migration to levels greater than that produced by exogenous EGF. Thus, rather than prevent EGFR signaling by receptor down-regulation, the local production of EGFR ligands by keratinocytes could fine tune EGFR signaling as receptor down-regulation is inversely proportional to receptor activity [37]. In this study, we used normal human epidermal keratinocytes (NHEKs) to study the mechanism involved in TCDD-induced EGFR down-regulation and the impact of down-regulation on EGFR signaling and cellular proliferation in post-confluent cultures. We show that TCDD increases the production of TGF-α and EREG, and that down-regulation of EGFRs is mediated by AREG, EREG, and TGF-α. In addition, we show that TGF-α promotes receptor recycling. These changes in receptor availability are associated with ligand-dependent elevations in ERK activity as well as an increase in a small pool of proliferating cells.

2. Materials and methods

2.1. Cell Culture

In all experiments, fifth passage NHEKs from neonatal foreskins (Lonza, Mapleton, IL) were plated at 5,000 cells/cm^2 in Costar 24- or 96-well cell culture dishes

(Corning, Corning, NY). Cells were maintained in keratinocyte serum-free medium (K-SFM; Gibco Invitrogen, Carlsbad, CA) containing 0.09 mM Ca, 5 ng/ml recombinant human EGF (EGF) and 50 µg/ml bovine pituitary extract. The medium was changed every 48 h until confluence. At confluence, cells were changed to K-SFM without supplements (basal medium) for 48 h then transferred to basal medium containing TCDD (10 nM) as described in each figure legend. In some experiments, recombinant human EGF (EGF; Bachem, Torrance, CA) was added to basal medium to serve as a positive control for ligand-induced EGFR down-regulation. For immunofluorescence, NHEKs were plated in glass chamber slides (BD Biosciences, San Jose, CA) that had been coated with fetal bovine serum. The medium was changed every 72 h until confluence, at which time cells were treated for 72 h as described above and then subjected to experimental protocols as described below.

2.2 EGFR ligand ELISAs

Culture medium was collected from basal and TCDD-treated cells 4-72 h after treatment and stored frozen in aliquots at -80^{o}C until analyzed for AREG, HB-EGF, or TGF-α content using ELISA kits from Abcam (Cambridge, MA) and EREG content using a kit from Antibodies-Online Inc. (Atlanta, GA). Undiluted samples were added to the assay and ligand content was interpolated from standard curves. Data are reported in pg/ml and are the means $\pm$ SEM of three experiments assayed in duplicate.

2.3 Phosphoantibody Cell Based ELISA (PACE) and crystal violet staining

To test the effects of EGFR down-regulation on ERK activity, NHEKs grown in 96-well plates were treated for 72 h in the absence and presence of batimastat or EGFR ligand neutralizing antibodies as described below. At 72 h, ERK activity was measured

by PACE assay as described by [38]. Briefly, treatments were terminated by rapid washing with ice-cold PBS and cells were fixed in 4% formaldehyde in PBS, blocked, and incubated overnight with an activation-specific rabbit monoclonal ERK antibody [phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204); Cell Signaling Technology, Danvers, MA]. The assays were developed by incubating with an HRP-conjugated goat anti-rabbit IgG and 1-Step Ultra TMB-ELISA substrate and read at 450 nm using a BioTek Synergy H1 microplate reader. To normalize to cell number, fixed cells were washed and stained with 0.04% crystal violet (Sigma Aldrich, St. Louis, MO) (w/v) in 4% ethanol following the protocol described in [39]. Cells were lysed overnight in 10% SDS and absorbance was measured at 595 nm. Data are expressed as a ratio of ERK (450 nm)/cell number (595 nm).

2.4. Interfering with ligand action

To investigate the role of each secreted ligand in EGFR down-regulation, ERK activation, and TCDD-induced proliferation, cells were grown in 96-well cell culture dishes in the presence or absence of 3 μM batimastat (Tocris Bioscience, Minneapolis, MN), a broad spectrum metalloproteinase (MMP) inhibitor [40], or neutralizing antibodies for TGF-α (5 μg/ml; R&D Systems, Minneapolis, MN), AREG (15 μg/ml; R&D Systems), or EREG (5 μg/ml; R&D Systems). The neutralizing capability of each antibody was verified by spiking basal medium with 0-30 ng/ml exogenous TGF-α,

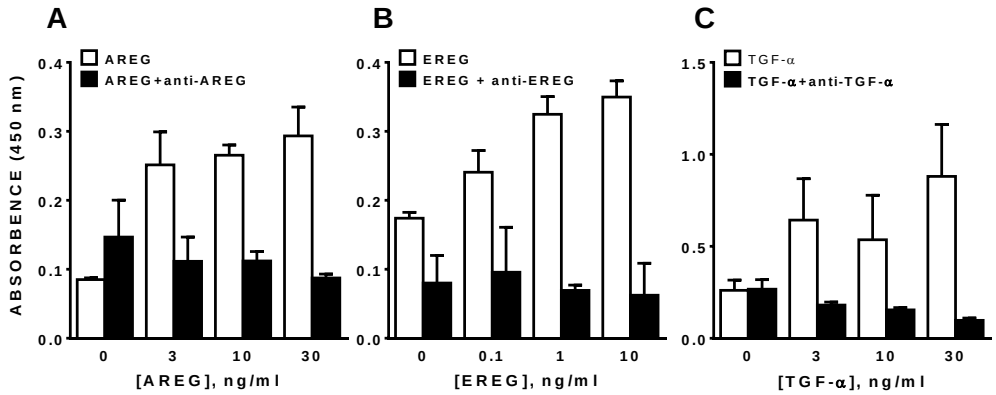

Fig. 1 Validating the effects of each neutralizing antibody. NHEKs were grown to confluence as described in the *Materials and Methods* section then challenged for five minutes with medium containing the indicated concentrations of exogenous **(A)** AREG, **(B)** EREG, or **(C)** TGF-α that had been pre-absorbed for 2 h at 37°C with neutralizing antibody for AREG (15 µg/ml), EREG (5 µg/ml), or TGF-α (5 µg/ml). ERK activation was then measured as described in the Methods. Data represent means ± SD of triplicate wells from one experiment.

AREG, or EREG and preincubating with or without the appropriate amount of neutralizing antibody. Plates were then stopped after five minutes, and ERK activation was measured using a PACE assay. Anti-TGF-α, anti-AREG, and anti-EREG all effectively neutralized high doses of their respective ligands (**Fig. 1**). Neutralizing antibodies were then added to binding assays, PACE assays and proliferation assays as described.

2.5 [^{125}I]-EGF Binding

Time-dependent loss of cell-surface EGFRs was determined in NHEKs grown to confluence in 24-well dishes (Corning, Corning, NY) and treated for 4-72 h in TCDD-containing medium. Following treatment, cells were washed three times with HEPES-buffered K-SFM. [^{125}I]-EGF/ml (specific activity 1128 Ci/mmol; Perkin Elmer,

Waltham, MA) was diluted in HEPES-buffered K-SFM with 0.1 % bovine serum albumin (BSA) and binding reactions were initiated by the addition of 200 µL of [^{125}I]-EGF (250,000 cpm/ml) to each well in the presence or absence of excess (1 µg/ml) unlabeled EGF. Binding reactions were carried out for 60 min at 37^{o}C and terminated by rapid washing with ice-cold phosphate-buffered saline (PBS). Monolayers were solubilized in 0.2N NaOH and aliquots were assayed for protein content using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA), and radioactivity by a Packard Cobra II Gamma counter (Perkin Elmer, San Jose, CA). Specific binding was determined by subtracting non-specific binding from total counts bound and normalized to protein content. Data are reported as the percentage of binding observed in time-matched controls and are means $\pm$ SEM of three separate binding experiments assayed in triplicate.

In order to conserve antibody and increase assay throughput while assessing the role of EGFR ligands in receptor down-regulation, cells were grown in 96-well dishes treated with EGF or TCDD for 72 h in the presence of batimastat or neutralizing antibodies as described above. Medium was spiked with batimastat or neutralizing antibody after 36 h of treatment. The effect of interfering with EGFR ligand availability on cell-surface EGFRs was determined using biotin-EGF (Invitrogen, Carlsbad CA) in a modified protocol from de Wit *et al.* [41]. Plates were washed in ice-cold PBS and incubated for 1 h on ice with 10 ng/ml biotin-EGF in the presence or absence of 1 µg/ml unlabeled EGF. Cells were fixed for 30 min at 37^{o}C in 4% formaldehyde, washed once with PBS, followed by two five-min washes in PBS containing 50 mM glycine. Cells were blocked in 2% gelatin in PBS with 0.1% Triton X-100 (PBS-T) for 1 h at 37^{o}C, then

incubated with streptavidin-HRP at a 1:500 dilution (Cell Signaling Technology, Danvers, MA) in 0.2% gelatin in PBS-T for 1 h at 37^{o}C. Plates were subjected to three five-min washes with PBS-T and two five-min washes with PBS before developing with 1-Step Ultra TMB-ELISA Substrate (Thermo Fisher, Waltham, MA). Absorbance at 450 nm was read on a BioTek Synergy H1 microplate reader and normalized to crystal violet absorbance at 595 nm. Data are reported as the percentage of binding in basal cells. To assess the ability of each ligand to down-regulate EGFRs, post-confluent NHEKs were exposed to EGF, TGF-α, amphiregulin, or epiregulin for 5-120 min using concentrations equal to the maximum amount of that ligand in the cultures at 3 days. Cells were then placed on ice and cell surface receptors were analyzed by measuring biotin-EGF binding as described above.

2.6 Double-stranded DNA analysis

As our previous study shows that TCDD treatment compromises mitochondrial function [42], analysis of changes in cell number was measured by double stranded DNA labeling using the FluoReporter blue fluorometric dsDNA Quantitation kit (Molecular Probes, Inc, Eugene, OR), following the manufacturer's instructions. Briefly, cells were grown to confluence in clear bottom black well plates (Corning Costar, Corning NY) and treated with 10 nM TCDD in the absence or presence of 3μM batimastat or neutralizing antibodies. At 72 hours, medium was removed from the cells, and the plates were frozen at -80^{o}C to lyse cells and release DNA. Plates were then thawed and rehydrated with Hoechst 33258 in Tris-NaCl-EDTA buffer and fluorescence was measured on a BioTek Synergy H1 microplate reader with excitation and emissions at 360 and 460 nm,

respectively. Cell number in each treatment group was determined from a standard curve plated at the start of the experiment.

2.7 EdU Labeling

At confluence, cells grown in glass chamber slides were treated for 72 h with 10 nM TCDD in the presence or absence 300 nM PD153035 (EMD Millipore, Billerica, MA), a highly selective EGFR inhibitor. Sixteen hours before the end of the experiment, cells were incubated with 10 µM 5-ethynyl-2´-deoxyuridine (EdU), fixed with 4% formaldehyde in PBS then permeabilized with 0.5% Triton-X 100 in PBS. Slides were incubated with the Click-It reaction cocktail containing copper sulfate and an AlexaFluor 647-conjugated azide that was crosslinked to cellular EdU according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Slides were coverslipped using DAPI-containing mounting medium to stain nuclei. Cells were imaged at 20x magnification on a Nikon A1 confocal microscope (Nikon, Inc, Melville, NY) at 647 nm for EdU-positive nuclei and 405 nm for total nuclei. Identical capture parameters were used for all images. EdU positive nuclei as well as total (DAPI-labeled) nuclei were counted using CellProfiler version 2.1.0 (Broad Institute, Cambridge, MA). Images were converted to gray scale then counted via "count objects" selecting for circularity and filtering using a Mixture of Gaussian (MoG) Global correction with standard threshold correction factors, and upper and lower bounds (1.0, 0.0, 1.0, respectively) [43]. The experiment was performed three times, with three slides per experiment. Five random fields on each of 3 slides were counted for all treatments and conditions.

2.8. Data analyses

EGFR binding data and PACE assays were performed in three experiments utilizing triplicate wells. Data were analyzed via two-way ANOVA and significance ($p \leq 0.05$) was determined using a Bonferroni post-hoc test as performed in Prism GraphPad software version 5.0 (La Jolla, CA). TGF-α, HB-EGF, AREG and EREG ELISAs were

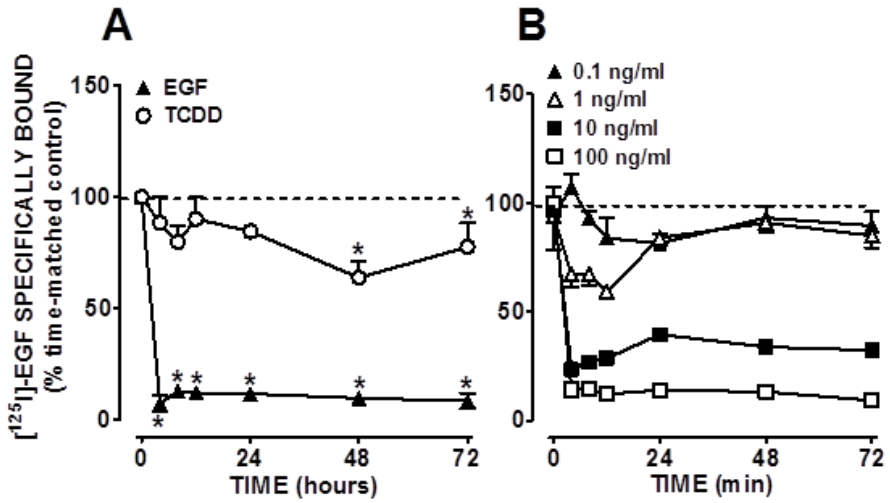

Fig. 2.TCDD decreases [^{125}I]-EGF binding. NHEKs were grown as described in the Material and methods section and **(A)** treated for the times indicated with EGF (100 ng/ml) or TCDD (10 nM), then [^{125}I]-EGF binding was determined. Data are reported as the means ± SEM of three experiments assayed in triplicate. * indicates $p \leq 0.05$ for treatment effect compared to time-matched control **(B)** NHEKs were treated for the times indicated with increasing concentrations of EGF (0.1-100 ng/ml), then [^{125}I]-EGF binding was determined. Data means ± SD of one experiment assayed in triplicate. The dashed line in (A) and (B) indicates 100%.

performed with culture medium collected from three experiments and assayed in duplicate. EGFR ligand concentrations were determined from standard curves and analyzed via one- or two-way ANOVA with a Bonferroni post-hoc test, as appropriate.

3. Results

3.1. EGFR down-regulation in TCDD-treated cells

Previous studies have shown that treating NHEKs for 1-4 days with TCDD causes the loss of cell-surface EGFRs ([30-32]). To determine how rapidly this response occurs and to compare it to ligand-induced down-regulation by EGF, we performed radioligand binding assays in NHEKs. EGF (100 ng/ml) rapidly reduced [^{125}I]-EGF binding to roughly 10% of control values by 4 h, maintaining this significant ($p \leq 0.05$) level of inhibition for the duration of the experiment (**Fig. 2A**). In contrast, TCDD treatment had a limited effect on [^{125}I]-EGF binding during the first 24 h but by 48 h, binding had decreased to 64% of control. At 72 h, binding rebounded to 78% of control values, but was still significantly lower than that in time-matched cells. In no case was the reduction in binding in TCDD-treated cells as rapid or as complete as that observed with EGF treatment. The decrease in [^{125}I]-EGF binding observed in EGF- or TCDD-treated cells was not the result of time-dependent decreases in basal cell receptor number, as binding did not change significantly with time in culture (**Fig. 3**).

To determine if lower doses of EGF could mimic the levels of down-regulation observed in TCDD-treated cells, NHEKs were treated with EGF (0.1-100 ng/ml) followed by the measurement of [^{125}I]-EGF binding. In cells treated with 0.1 ng/ml EGF, [^{125}I]-EGF binding was reduced to 90% of control within 24 h while 1 ng/ml EGF reduced binding to 60% of control within 12 h. By 72 h, [^{125}I]-EGF binding had returned to control levels in cells treated with either EGF concentration. Increasing the EGF dose to 10 or 100 ng/ml produced a more rapid, extensive, and persistent reduction in [^{125}I]-EGF binding, reaching ~40% of control levels by 72 h for 10 ng/ml and 12% of control

values for 100 ng/ml (**Fig. 2B**). These data show that the degree of receptor loss is dose-dependent and low concentrations of EGF remove fewer receptors from the cell surface.

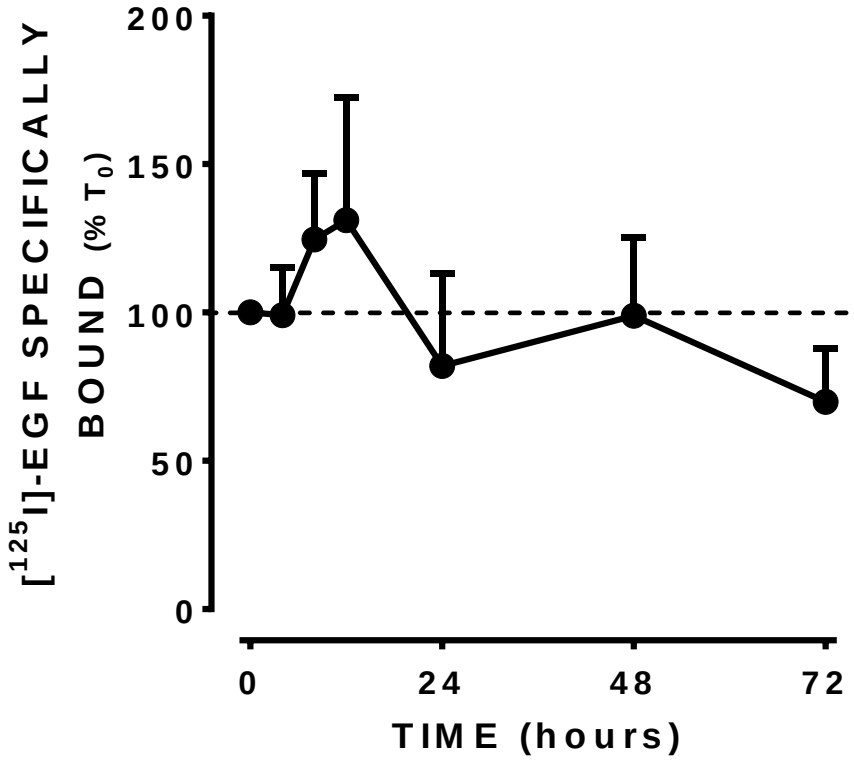

Fig 3. Changes in [^{125}I]-EGF binding with time in culture. Basal NHEKs were grown as described in the *Materials and Methods* section and total [^{125}I]-EGF binding was determined at each time point. Data are normalized to and reported as a % of total binding at T_0 and reflect the means $\pm$ SEM of three experiments assayed in triplicate.

3.2. Effect of TCDD on EGFR ligand secretion

Keratinocytes secrete several EGFR ligands including AREG, EREG, HB-EGF, and TGF-α (see Pastore *et al.* [11]) with TCDD reported to increase the expression and production of TGF-α [24,25], and AREG [44]. Our observation that TCDD treatment

decreases [^{125}I]-EGF binding (Fig. 1) suggested that the local production of an EGFR ligand(s) may drive receptor down-regulation. To assess the impact of TCDD on EGFR ligand secretion, we measured AREG, EREG, TGF-α, and HB-EGF in culture medium over the course of 72 hours. These ligands were chosen based upon literature, ties to epidermal disease and homeostasis, and evidence of TCDD-induced changes in mRNA expression by RT-PCR or microarray [42]. AREG secretion increased with time in culture. was comparable in both basal and TCDD-treated cells, and was inhibited by the broad-spectrum MMP inhibitor batimastat (**Fig. 4A,D**). EREG secretion was minimal (26 pg/ml) in basal cells, but at 72 h was present at a concentration of 718 pg/ml in culture medium from TCDD-treated cells (**Fig. 4B**). Like AREG, EREG production was inhibited by batimastat (**Fig. 4E**). Batimastat-sensitive TGF-α secretion by basal cells increased by 65 % at 72 hours and TCDD significantly enhanced TGF-α production over basal cells at 72 hours treatment (**Fig. 4C, F**), a response comparable to that reported by Choi *et al.* [24]. At 72 h, the concentration of TGF-α in the medium from basal cells was 119 pg/ml, versus 428 pg/ml in TCDD-treated cells. This value is comparable on a molar level to ~ 0.5 ng/ml EGF, a value between the low doses of EGF (0.1 and 1 ng/ml) that produce modest decreases in [^{125}I]-EGF binding in Fig. 1B. HB-EGF concentrations were below the detection limits of our ELISA (**data not shown**), consistent with the low levels of HB-EGF produced by NHEKs in culture [45] and its primary role in wound healing and keratinocyte migration [46]. Together, the data in Fig. 4 indicate TCDD modifies both the ligands produced and the concentrations available to the cells.

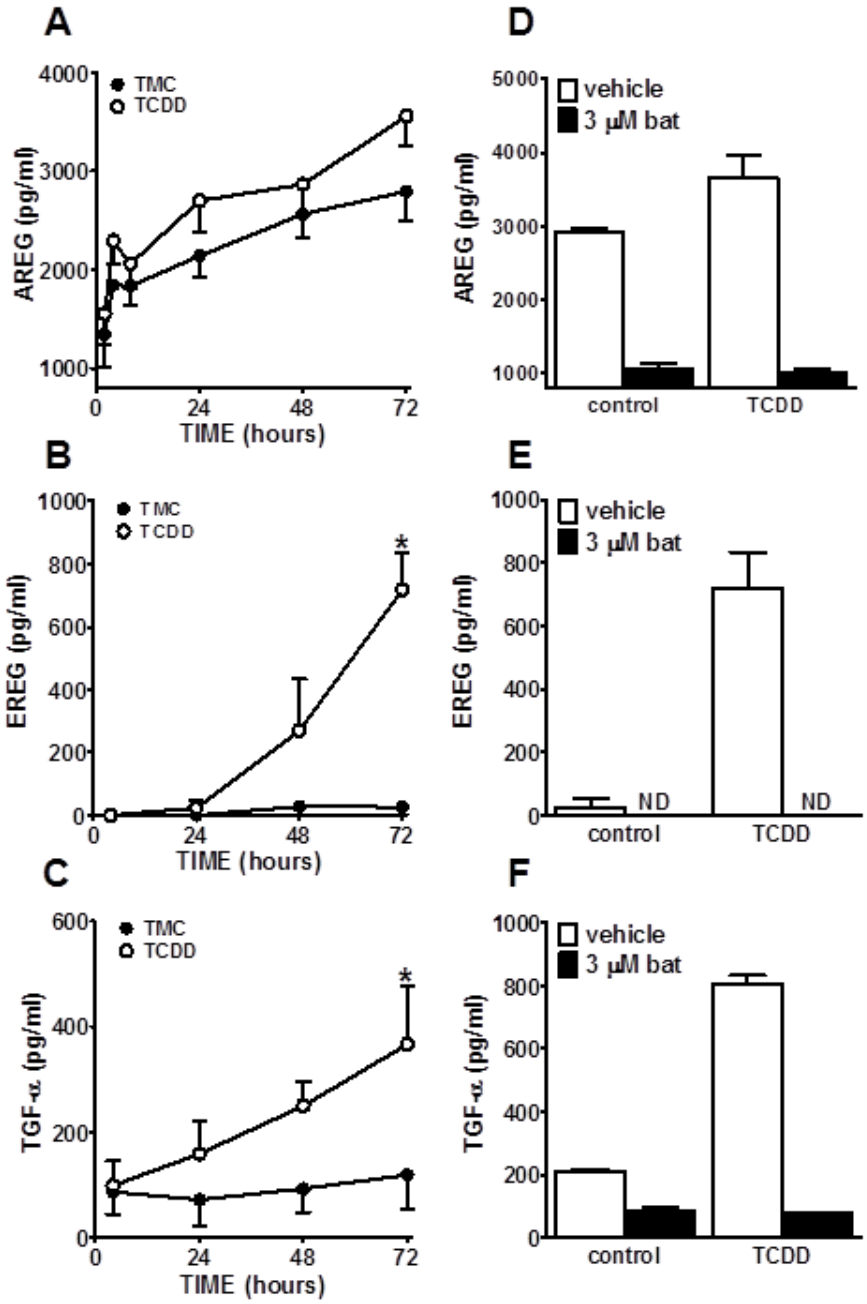

Fig. 4. Effect of TCDD on MMP-dependent EGFR ligand secretion. NHEKs were treated for 2-72 h with TCDD (10 nM) and the culture medium was collected for the measurement of EGFR ligands. **(A)** AREG, **(B)** EREG or **(C)** TGF-α content were determined by ELISA as described in Materials and methods section. Data are means ± SEM of three experiments assayed in duplicate. * indicates significantly different from time-matched control (TMC), $p \leq 0.05$. In D-F, cells were treated for 72 h with vehicle or TCDD ± 3 μM batimastat (bat). The culture medium was collected at 72 h for the measurement of **(D)** AREG, **(E)** EREG, or **(F)** TGF-α. ND indicates not detectable.

3.3 Role of EGFR ligands in EGFR down-regulation

The data presented in Fig. 4 show that NHEKs secrete AREG, EREG, and TGF-α, with TCDD enhancing the production of EREG and TGF-α. These data suggest that an EGFR ligand(s) causes the loss of [^{125}I]-EGF binding observed in TCDD-treated cells. To examine the role of these ligands in mediating TCDD-dependent EGFR down-regulation, NHEKs were treated with TCDD or EGF in the presence or absence of batimastat (3 μM) or neutralizing antibodies for AREG, EREG, or TGF-α, alone or in combination. Consistent with [^{125}I]-EGF binding (**Fig. 2A**), 10 nM TCDD and 10 ng/ml EGF significantly reduced biotin-EGF binding at 72 h with TCDD decreasing binding to 44±11% and EGF to 38±19% of basal cells (**Fig. 5A**). Batimastat relieved receptor down-regulation in TCDD-treated cells, indicating that the release of soluble EGFR ligands was driving receptor loss from the cell surface. As expected, batimastat had no effect on receptor down-regulation produced by the addition of exogenous EGF.

To examine the individual roles of the EGFR ligands in receptor down-regulation, NHEKs were treated with neutralizing antibodies for AREG, EREG, or TGF-α. TCDD treatment reduced biotin-EGF binding to 43 ± 7% of basal control values (**Fig. 5B**), and neutralizing AREG or EREG returned binding to levels that were not significantly different from basal control cells (67 ± 13% and 61 ± 10% of basal control levels, respectively). These data indicate that both AREG and EREG contribute to EGFR down-regulation in TCDD-treated cells. In contrast, neutralizing TGF-α in TCDD-treated cells significantly enhanced EGFR down-regulation (11 ± 1% of basal control levels), suggesting that TGF-α promotes receptor recycling rather than down-regulation. When TCDD-treated cells were incubated with all three neutralizing antibodies, biotin-EGF

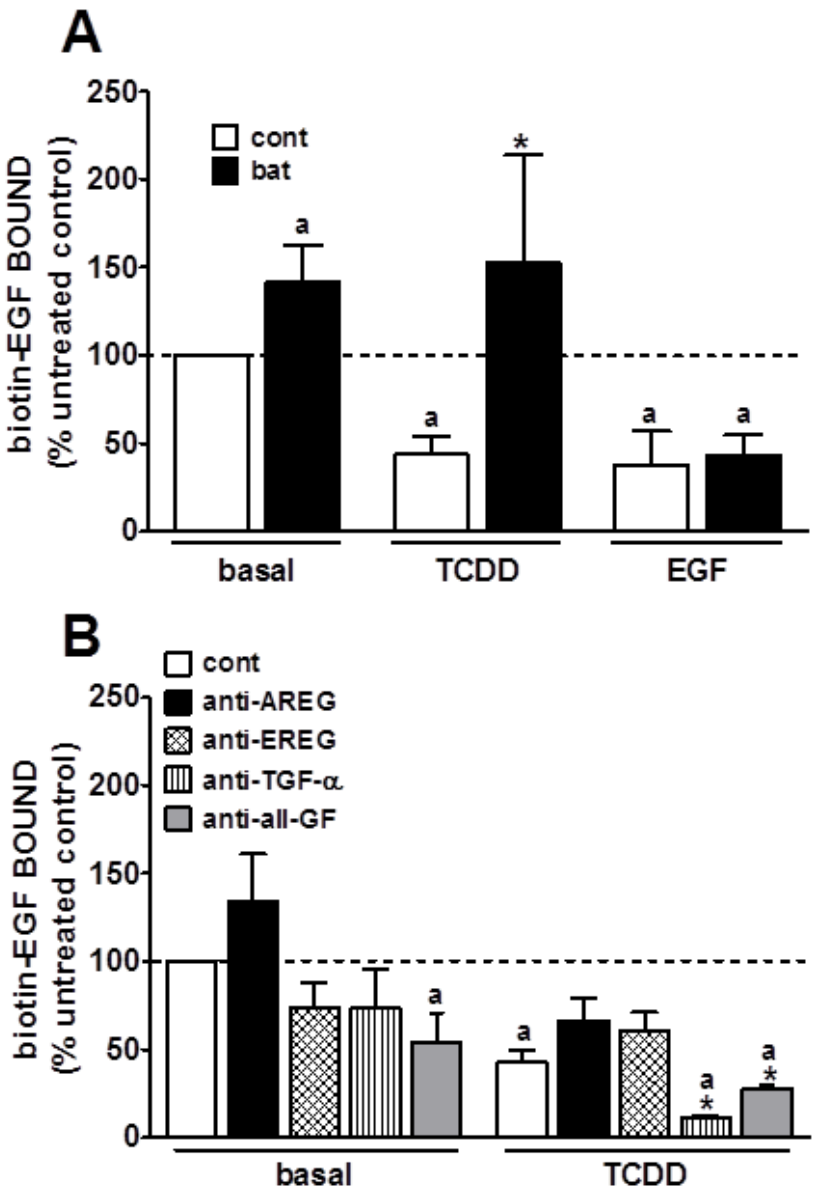

Fig. 5. Reducing EGFR ligand availability modifies EGFR down-regulation in response to TCDD. (A) NHEKs were treated with or without TCDD (10 nM) or EGF (10 ng/ml) for 72 h in the absence or presence of batimastat (bat; 3 μM) or **(B)** neutralizing antibodies for AREG (15 μg/ml), EREG (5 μg/ml), TGF-α (5 μg/ml), or all three growth factors (anti-all-GF) for 72 h. The measurement of biotin-EGF binding was then determined as described in the Material and methods section. Basal refers to medium (K-SFM) without TCDD; control refers to treatments in the absence of batimastat or neutralizing antibodies. Data are means $\pm$ SEM of six experiments assayed in triplicate, normalized to crystal violet staining, and reported as the % biotin-EGF bound in basal control cells. (a) indicates significantly different from the basal control at $p \leq 0.05$. * indicates significantly different from the within treatment control ($p \leq 0.05$).

binding remained at levels about 30 % of basal, indicating that the effect of neutralizing TGF-α predominates at 72 hours post-confluence.

The enhanced down-regulation of EGFRs when TGF-α was neutralized suggested that TGF-α may alter EGFR trafficking in TCDD-treated cells. Since the fate of an internalized receptor has a substantial impact on the duration of a receptor signal [36,47], we examined the effect of pretreating NHEKs with EGF, AREG, EREG or TGF-α on biotin-EGF binding. NHEKs were treated for 5-120 min with concentrations of exogenous EGFR ligands comparable to the levels found in conditioned medium at 72 h, then the amount of biotin-EGF binding was assessed. As observed in **Fig. 2**, treatment with EGF caused a rapid reduction in biotin-EGF binding to 11 $\pm$ 4% of T_0 values, a response that was sustained throughout the experiment (**Fig. 6**). TGF-α reduced binding to 87% of T_0 values within 10 min, while AREG reduced binding to 63% of T_0 values by 15 min. EREG did not affect biotin-EGF binding at any time point. These data are consistent with all three ligands producing effects on EGFR trafficking distinct from those of EGF, and suggest that one or all maintain a population of EGFRs at the cell surface.

3.4. Effect of EGFR ligands on ERK activity

Human keratinocytes maintain high steady-state levels of ERK activity due to the autocrine production of EGFR ligands [48] and the continued presence of EGFRs (**Fig 5B)** could be important in maintaining ERK activity. In fact, ERK activity was elevated in TCDD-treated cells as early as 24 h after the start of the treatment even in the face of a reduction in the number of EGFRs, and was significantly greater than time-matched controls at 72 h (**Fig. 7A**). To determine which EGFR ligand(s) enhanced ERK activity,

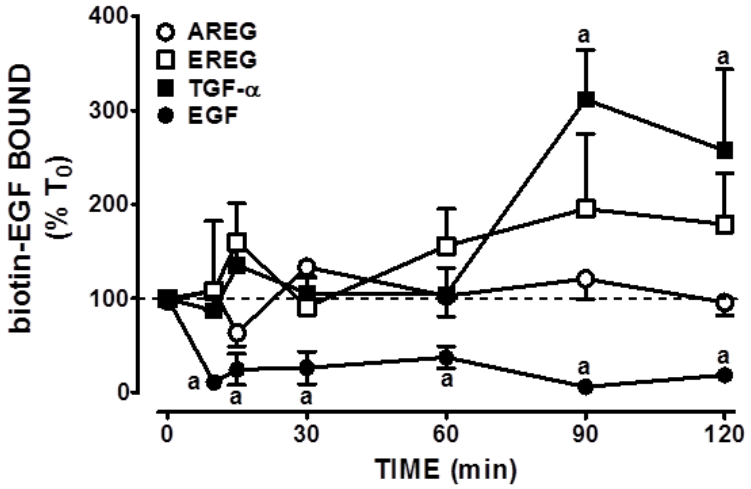

Fig. 6. EGFR ligands differentially alter EGFR recycling. NHEKs were treated for 0-120 min with 60 ng/ml EGF and concentrations of ligands present in culture medium at 72 h (3000 ng/ml AREG, 412 ng/ml TGF-α or 627 ng/ml EREG) then biotin-EGF binding was measured on ice to prevent internalization. The amount of biotin-EGF bound was then determined as described in the Materials and methods section. Data are means $\pm$ SEM of triplicate experiments. (a) indicates significantly different from T_0 ($p \leq 0.05$). The dashed line represents biotin-EGF binding at T_0.

we treated cells for 72 h with TCDD in the absence or presence of batimastat (3 μM) or neutralizing antibodies for AREG, EREG, or TGF-α alone, or in combination. Batimastat reduced ERK activity to 52% and 55% of control levels in both basal and TCDD-treated cells, respectively (**Fig. 7B**). These data indicate that the residual EGFRs present on the cell surface are sufficient to maintain EGFR-dependent ERK activity in response to locally secreted EGFR ligands in both basal and TCDD- treated cells. When we examined the roles of each EGFR ligand in maintaining ERK activity, neutralizing an individual ligand had no impact on the elevated ERK activity observed in TCDD-treated cells. However, when NHEKs were incubated with all three neutralizing antibodies,

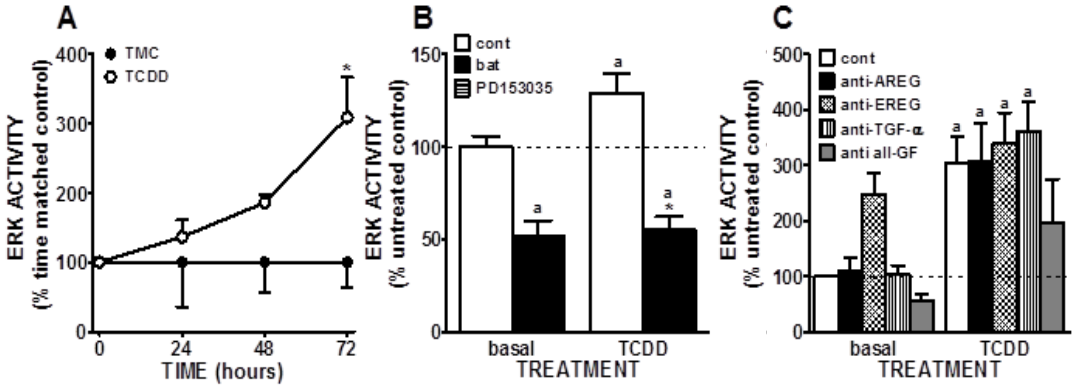

Fig. 7. TCDD-mediated increases in ERK activity are EGFR ligand-dependent. To assess the effect of TCDD on basal ERK activity over 72 h, NHEKs were grown for 0-72 h with or without 10 nM TCDD and **(A)** basal ERK activity was measured at the indicated times as described in the Materials and methods section. Data are reported as % time matched control and are means $\pm$ SEM of three experiments assayed in triplicate. * indicates significant difference from the time-matched control ($p \leq 0.05$) **(B)** To assess the role of secreted ligands on ERK activity, NHEKs were treated for 72 h with TCDD in the presence or absence of batimastat (bat; 3 μM) or **(C)** neutralizing antibodies for AREG (15 μg/ml), EREG (5 μg/ml), TGF-α (5 μg/ml), or all three growth factors (anti-all-GF). Basal refers to medium (K-SFM) without TCDD; control refers to treatments in the absence of inhibitors. Data are means $\pm$ SEM of three experiments assayed in triplicate. In **B, C,** * indicates significantly different from TCDD alone ($p \leq 0.05$) and (a) indicates significantly different from basal control ($p \leq 0.05$). The dashed line indicates the basal control normalized to 100%.

ERK activity was reduced to levels that were not significantly different from untreated basal cells (**Fig. 7C**). These data indicate that all three ligands are required to produce the increase in ERK activity observed in TCDD-treated cells.

3.5. Role of EGFR ligands in regulating cell number

The presence of AREG, EREG, and TGF-α in NHEK culture medium and the observation that all three ligands have an effect on EGFR down-regulation as well as maintenance of ERK activity led us to examine the role of each ligand plays in the proliferative response reported in TCDD-treated cells [25,26,49]. Cell numbers at 72 hours were not different in basal and TCDD-treated cells (95713 $\pm$ 2251 vs 94,360 $\pm$ 3175, respectively; **Fig. 8A**). These results are not surprising as these are post-confluent cultures. Neutralizing each individual ligand produced modest reductions in cell number, with greatest effect occurring in basal and TCDD-treated cells treated with the AREG antibody (a 6 % drop from 95,714 $\pm$ 2251 to 90,314 $\pm$ 650 cells in basal cultures and a 7 % drop from 94,380 $\pm$ 3175 to 88027 $\pm$ 1412 cells in TCDD-treated cultures). While the loss of individual ligands was not statistically significant, neutralizing all three ligands reduced cell number significantly in both basal (10.3%) and TCDD-treated (11.3%) cells. These data indicate that all three EGFR ligands play a role in maintaining cell number in post-confluent monolayers of basal and TCDD-treated cells.

Since TCDD produced no significant increase in cell number compared to basal cells, we wondered whether there might be a small population of cells proliferating in response to TCDD. To address this question, we measured DNA synthesis using EdU labeling in cells treated for 72 h with TCDD in the presence or absence of the EGFR inhibitor PD153035 (300 nM). Compared to basal cells, TCDD treatment significantly

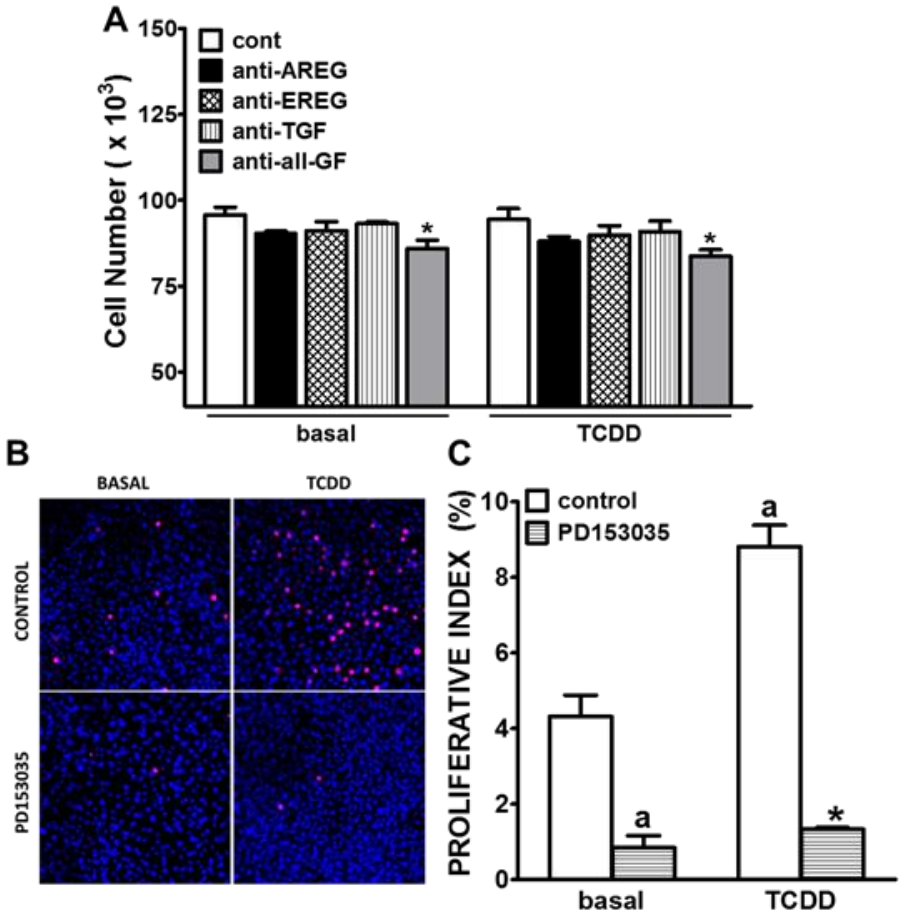

Fig. 8. Reducing ligand availability affects cell number and proliferation. (A) NHEKs were grown in the presence or absence of neutralizing antibodies for AREG (15 µg/ml), EREG (5 µg/ml), TGF-α (5 µg/ml) or all three growth factors (anti-all-GF) antibodies. After treatment cell number was measured as described in the Materials and methods section. Basal refers to medium (K-SFM) without TCDD; control refers to treatments in the absence of neutralizing antibodies. Data are means ± SEM of three separate experiments assayed in triplicate. * indicates significantly different from within treatment control. **(B)** and **(C)**, NHEKs were grown to confluence in glass chamber slides and then treated for 72 h with TCDD (10 nM) in the presence or absence of PD153035 (300 nM), an EGFR inhibitor. During the last 16 h cells were incubated with 10 µM EdU to label proliferating cells. **(B)** Representative confocal images of total (DAPI; blue) and EdU-staining nuclei (pink) at 20x magnification. Basal refers to medium (K-SFM) without TCDD; control refers to treatments in the absence of PD153035. **(C)** Proliferative index is determined as the percentage of the total cell population labeled by EdU [(EdU labeled/DAPI labeled) x100]. Data are means ± SEM from counting five fields from three separate experiments. * indicates significantly different from the within treatment control ($p \leq 0.05$). (a) indicates significantly different from basal control ($p<0.05$).

increased the percentage of the cell population that was proliferating (**Fig. 8B,C**). Inhibiting EGFR activity with PD153035 significantly reduced this percentage in both basal and TCDD-treated cells. The increased percentage of EdU positive nuclei in TCDD-treated cells in the face of a total cell population comparable to that of basal cultures, suggests that TCDD-treated cultures have a larger proliferative capacity than basal cells even in these post-confluent cultures.

4. Discussion

The epidermis regenerates constantly, requiring the concurrent expression of proliferative programming in the basal layer and differentiation programming in the suprabasal layers [16,17]. The EGFR and its ligands play critical roles in regulating the balance between proliferation and differentiation. EGFR number is greatest in the basal layer of the epidermis where its activation promotes proliferation and declines as keratinocytes migrate suprabasally and undergo terminal differentiation [16,17]. The EGFR and its ligands establish an autocrine loop in the skin with receptor activation stimulating cell proliferation in the basal layer, and with decreases in EGFR levels as the cells migrate suprabasally and differentiate [11,16,17]. The environmental toxin TCDD alters this autocrine loop by promoting EGFR down-regulation [30-32] and enhancing the production of receptor ligands [24-26]. These apparently opposing responses have been used to explain the proliferative effects of TCDD (increased EGFR ligand production) and its ability to accelerate keratinocyte differentiation (EGFR down-regulation and loss of signaling). However, *in vitro* studies have shown that the increase in ligand production occurs in the face of reduced receptor number, an effect that should alter the cells' ability to respond to the ligands. Since cellular responses to an agonist reflect the number of receptors activated [37], we were interested in understanding how a decrease

in receptor number accompanied by increased ligand production might mediate the effects of TCDD in NHEKs. Using post-confluent NHEKs, we show that TCDD increases the production of TGF-α and EREG, and that down-regulation of EGFRs is mediated by AREG, EREG and TGF-α. In addition, we show that TGF-α promotes receptor recycling. These changes in receptor availability are associated with an increase in steady-state ERK activity that is ligand-dependent, as well as an increase in a small pool of proliferating cells which maintains total cell number.

Basal NHEKs increase their AREG production while producing only small amounts TGF-α and virtually no EREG. Since basal cells are grown in the absence of added growth factors, these data indicate that AREG is the EGFR ligand maintaining these cells in the absence of exogenous growth factors [18-21]. These data are consistent with the observations of Piepkorn *et al.* [22] who showed that AREG is the most abundant and efficacious regulator of keratinocyte growth. TCDD-treated keratinocytes produced comparable amounts of AREG but had elevated production of both TGF-α and EREG, responses also observed by Choi *et al.* [24] and Patel *et al.* [26]. These changes in all three ligands occur at the same time that receptor numbers are declining, arguing that one or more of these ligands mediates EGFR down-regulation in TCDD-treated cells. When we inhibited EGFR ligand release with the MMP inhibitor batimastat, receptor binding increased not only in TCDD-treated cells but also in basal cells, arguing that a ligand(s) is modulating the number of cell surface receptors in both cell populations. Subsequent experiments with ligand-neutralizing antibodies showed that the elimination of all three ligands reduced receptor number in both basal and TCDD-treated cells. This was somewhat surprising as we expected the removal of all the ligands to relieve receptor

down-regulation. In fact, neutralizing either AREG or EREG in TCDD-treated cells partially restored biotin-EGF binding; however eliminating TGF-α intensified receptor loss, a response also observed in basal cells. Although AREG predominates in basal cells, at 72 hours, TGF-α is elevated in basal cells and markedly enhanced in TCDD-treated cells, suggesting that TGF-α may maintain a pool of EGFRs at the cell surface in both basal and TCDD-treated cells. This would be consistent with the ability of TGF-α to bias EGFRs to the cell membrane [47,50]. In fact, our observation that exogenous TGF-α increases EGFR binding at the cell surface supports this role of TGF-α, although these data must be interpreted cautiously as exogenous addition of ligands does not reflect the impacts of autocrine secretion of EGFR ligands [36]. TGF-α-induced increases in cell membrane-associated EGFRs has also been observed in prostate cancer cells where the ligand stabilizes EGFR mRNA and enhances the *de novo* synthesis of the receptor [51]. Vassar and Fuchs [52] showed that there were no major differences in EGFR levels in TGF-α-overexpressing mice compared to normal, demonstrating that TGF-α does not promote the loss of EGFRs. Together these results demonstrate that the combined effects of the three ligands is to maintain a reduced number of receptors on the cell surface by causing down-regulation (AREG, EREG, and TGF-α and promoting recycling (TGF-α).

Receptor down-regulation is typically thought of as a way to for cells to modify their responses to continued agonist presence. Differences in signaling dynamics (signal intensity, frequency, and duration) can encode specific cellular responses [34], as exemplified by studies from Joslin *et al*., [36] who showed that the autocrine production of EGFR ligands leads to a persistent reduction in EGFR number but a sustained

elevation in ERK activity that mediated an enhanced migratory response compared to that produced by exogenous EGF. Likewise, we show that the TCDD-induced down-regulation of EGFRs in NHEKs was accompanied by an elevation in ERK activity that was significantly greater than that in basal cells and maintained by the autocrine production of AREG, EREG, and TGF-α. The production of EGFR ligands also maintained ERK activity in basal cells, consistent with the findings of Iordanov *et al.*. [48]. In addition, these data indicate that TCDD alters an autocrine loop already present in keratinocytes by reducing receptor number with AREG, EREG, and TGF-α and maintaining cellular responsiveness to secreted ligands by TGF-α-induced receptor recycling.

Increases in ERK activity mediate a host of cellular responses including proliferation [53]. Both basal and TCDD-treated cells grow in the absence of exogenous growth factors, reflecting the ability of locally produced EGFR ligands to activate ERK and maintain total cell number. However, the total number of cells in basal- and TCDD-treated cultures were not significantly different. This result was not surprising as our monolayer cultures are post-confluent and more reflective of intact skin. Neutralizing individual ligands had modest impacts on cell number in basal or TCDD-treated cells, but in both basal and TCDD-treated cells the response was greatest in cells treated with AREG antibodies, consistent with AREG being the most abundant and efficacious regulator of keratinocyte growth [22]. Cell number was significantly reduced in basal and TCDD-treated cells when all three ligands were neutralized. Since NHEKs stratify in this culture system, our data suggest that the EGFR ligands are required for the homeostatic replacement of cells lost to sloughing during the culture period in both basal

and TCDD-treated cells. However, the complement and concentrations of EGFR ligands produced by basal and TCDD-treated cells was different, with TCDD causing a much larger increase in TGF-α production than observed in basal cells and stimulating the production of EREG. Since hyperproliferation is a well-described response to TCDD *in vitro* [25,26,49] and *in vivo* [44], we asked whether or not the TCDD could enhance the number of proliferating cells in the culture. EdU labeling of basal and TCDD-treated cells indicated that both populations required a functional EGFR to maintain a small pool of proliferating cells. In addition, TCDD-treated cells, which have a higher concentration of secreted TGF-α compared to control cells and produce EREG, show a greater percentage of EdU positive cells, indicating that these cultures have a greater proliferative capacity. These data support the idea that the hyperproliferative effect of TCDD in NHEKs is the result of producing a larger population of proliferative cells within a differentiating epidermis. Maintenance of this proliferative pool in basal cells reflects the ability of AREG to promote proliferation while the small amounts of TGF-α produce by these cells maintains receptor number and ligand responsiveness. AREG and TGF-α would serve the same purpose in TCDD-treated cells, but the enhanced production of TGF-α and EREG is responsible for the increased size of the proliferative pool of cells in these cultures.

In summary, our data indicate that TCDD alters an autocrine loop present in keratinocytes by enhancing the production of EGFR ligands and modifying EGFR trafficking to maintain the cells' responsiveness to locally produced growth factors. The alteration in EGFR number in TCDD-treated cells required AREG, EREG, and TGF-α and is accompanied by a TGF-α-mediated recycling of receptors that maintains a pool of

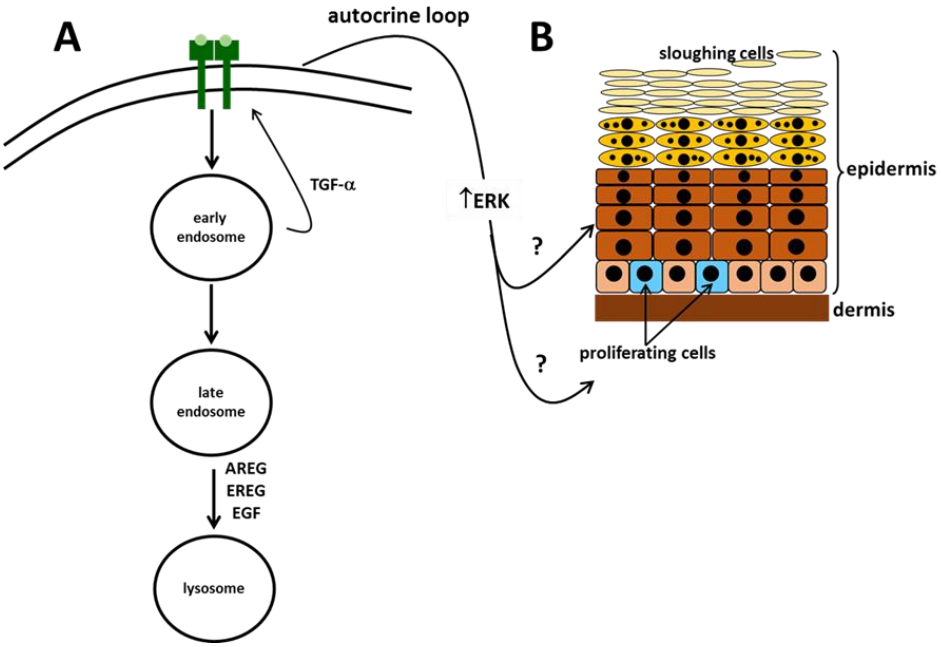

Fig. 9. TCDD alters the EGFR/ligand autocrine loop present in keratinocytes. (A). TCDD-induced EGFR internalization in NHEKs reflects the combined effects of AREG, EREG and TGF-α, while TGF-α promotes EGFR recycling. This receptor pool preserves the cell's ability to respond to the local production of AREG, EREG, and TGF-α leading to a sustained elevation in ERK activity compared to basal cells. **(B)** The enhanced production of TGF-α and EREG enhances the proliferation of a small subset cells in the basal or suprabasal layers of the skin that serve to maintain total cell number.

receptors at the cell surface. This receptor pool preserves the cell's ability to respond to the local effects of AREG, EREG, and TGF-α leading to a sustained elevation in basal ERK activity and the maintenance of a pool of proliferating cells that can quickly respond to disruption of the culture and replace cells lost to sloughing of the stratifying culture (**Fig. 9**).

5. Conclusion

TCDD treatment of NHEKs produces a ligand-dependent decrease in EGFR number that is limited by the ability of TGF-α to promote receptor recycling. These ligands and receptor changes enhance the activity of the autocrine loop that sustains the monolayer with TGF-α promoting EGFR recycling and upregulation as well as sustained levels of ERK activity that promote homeostatic replacement of cells during the culture period.

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CHAPTER FOUR

Conclusions and recommendations for future work

Concluding remarks

The role of EGFR in keratinocyte differentiation becomes increasingly complex as our understanding of receptor signaling dynamics and down-regulation evolves. But this work is not the first that argues EGFR plays a role in physiological or pathophysiological keratinocyte differentiation. Previous studies have shown that Ca-induced inactivation of EGFR signaling is a result of PKC-dependent receptor phosphorylation (1)—a process in which activated EGFR create a negative feedback loop which leads to EGFR silencing (1) and is observed in migrating and differentiating keratinocytes both *in vitro* and in the upper layers of the epidermis *in vivo* (2, 3).

TCDD similarly produces EGFR down-regulation and its signaling, a response which is correlated with keratinocyte differentiation. TCDD induces processes typically associated with keratinocyte differentiation, such as accelerated expression of markers o differentiation and lipid biosythetic genes in the epidermal differentiation complex (4-6) as well as increased functional markers of differentiation such as increases in percentage of cornified envelopes *in vitro* (4, 5, 7) and acceleration of epidermal barrier formation *in utero* (8, 9). These TCDD-induced phenotypes have been shown to be both inhibited by EGF and dependent upon the AhR (5, 6, 8). However, TCDD is also considered to be pro-proliferative, though these claims are more subject to experimental conditions endpoints (7, 10, 11) or suggest that TCDD functions in a proliferative capacity on subset of keratinocyte populations (12). However, the ability of TCDD to induce proliferation is

influenced by multivariate experimental cell culture conditions, timepoints, and cell lineages which complicate cross-study comparison which might otherwise suggest a mechanism.

While TCDD and Ca both induce keratinocyte differentiation, the nature of the changes in gene expression and barrier function are distinct. In this study, we address the ambiguity by examining Ca and TCDD effects in a single type of keratinocyte utilizing the same timepoints and downstream targets for EGFR regulation. What is perhaps most novel about this work is that, by bringing together and expanding upon experiments performed over the years in differing cell lines, culture conditions, and over varying time courses (**Table 1.7**), we are able to directly compare how Ca and TCDD-treatment alter EGFR signaling relative to one another and provide insight into mechanistic differences in between the two that can be applied to future work investigating TCDD-treatment in high Ca.

Loss of surface associated [^{125}I]-EGF binding observed in Ca- and TCDD-treated cells and the extent of this down-regulation differed. More importantly, the ability to reverse downregulation differed by treatment. In our work, Ca-treated cells still had 41% of the surface-associated EGFRs observed in basal cells (**Figure 2.1**), a number similar to observations in literature (13, 14), and this appears to be sufficient for EGFR-dependent barrier formation and wounding response. However, TCDD-treated cells maintained 78% of surface associated EGFRs suggesting a much less pervasive down-regulation (**Figure 3.2**). In both cases, the loss of EGFR was accompanied by elevated levels of EGFR ligands though the complement of secreted ligands differed. Denning *et al.* showed that Ca increased TGF-α mRNA nearly 30-fold--though did not look at protein (1). Our work

expands on this to show that TGF-α protein increased following not only treatment with high Ca (**Figure 2.2**) , but also TCDD in low Ca conditions (**Figure 3.4**) which had been suggested though not directly shown in keratinocytes (15, 16). In this work, Choi *et al.* (16) identified TGF-α in keratinocyte-conditioned medium but tested EGFR down-regulation through the use of EGF. Additionally, explaining this down-regulation of EGFR as entirely TGF-α-dependent fails to recognize the contribution of other EGFR ligands: AREG and EREG which were not investigated (or in EREG's case, known) at the time. We found that TCDD-treated cells secreted elevated levels of EREG (**Figure 3.4**) while AREG secretion increased in all cells, including basal cells over time in culture. While the presence of secreted ligand in basal cells provides a clear mechanism of EGFR activation, in differentiating cells, ligand secretion creates a seemingly paradoxical environment where surface associated EGFR is reduced in the presence of elevated receptor ligands.

This offers some insight into mechanisms of epidermal plasticity. Even within the differentiated, intact epidermis, an extracellular environment rich in proliferative ligands facilitates epidermal migration and re-epithelialization in response to wounding or UV damage (17-19) as well as promotes neoplasia and hyperproliferative disease states (3, 20). In the differentiated epidermis, EGFR occupancy by juxtacrine EGFR ligands is an important maintenance and anti-anoikos signal for the intact epidermis (21). Its disruption in wounding or disease leads to ligand shedding and the release of EGFR for ligand binding and internalization (22, 23). Soluble AREG leads to activation of the pro-proliferative ERK signaling pathway (22, 24) which is inhibited by Dsg1 at the intact desmosome (25). Without disruption, membrane-associated AREG prevents

internalization and signals through the anti-apoptotic PI3K at the cell membrane (26, 27) or the EGFR silencing PKC (21, 28, 29) which colocalizes to the desmosome (30).

Cleavage of membrane–associated pro-EGFR ligands, important in wound healing, maintains a proliferative capacity in confluent cell populations (31). In the differentiating epidermis, MMP-dependent EGFR-ligand shedding is critical to the maintenance of the epidermal barrier. ADAM17 -/- null mice develop barrier defects after birth and chronic dermatitis as adults and resemble mice with targeted epidermal knockout of EGFR (29). Similar to the aforementioned knockout phenotype, EGFR down-regulation within the differentiating epidermis is not complete. In fact, prolonged exposure to low level ligands is a mechanism for spatial and temporal regulation of receptor signaling (32).

It was found, when neutralizing these ligands, that TCDD-induced loss of surface associated EGFR was reversible following removal of AREG or EREG by neutralizing antibodies of ligand cleavage through the use of a broad spectrum MMP inhibitor (**Figure 3.6**). However, neutralization of ligands in any combination in Ca-treated cells showed EGFR binding loss to be ligand independent (**Figure 2.4**). Signaling capacity of cells under these two conditions proved to be different as well: ERK signaling in Ca-treated cells was ~55% that of basal cells at 72 hours, and neutralization of EGFR ligands was unable to restore it to basal levels (**Figure 2.3**). Conversely, TCDD-treated cells, with 22% fewer surface-associated EGFRs than the basal cells, had elevated ERK activity that was attenuated by the use of batimastat or neutralization of all three ligands (TGF-α, EREG, and AREG) (**Figure 3.7**).

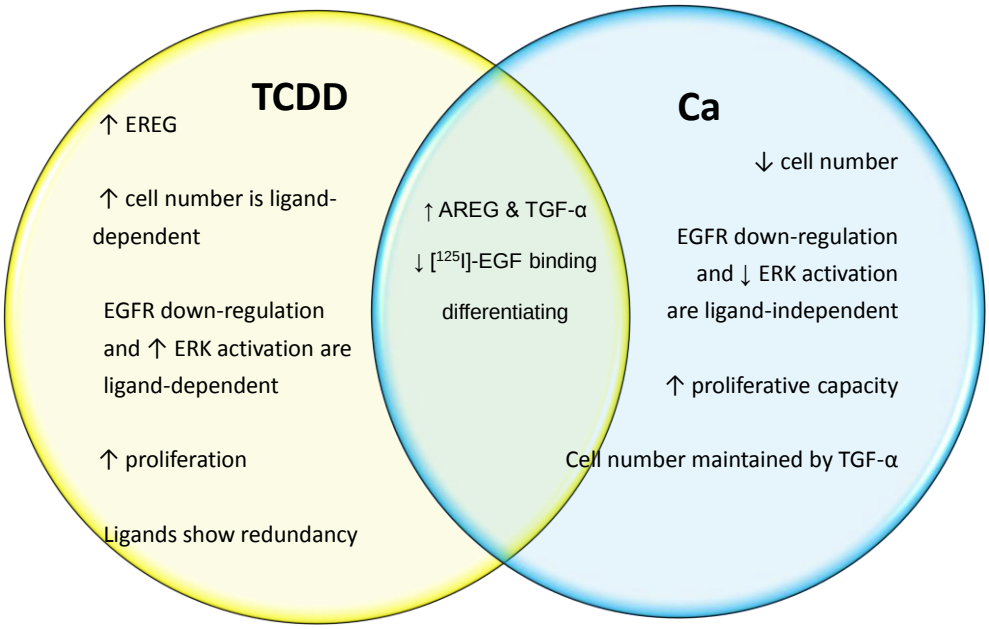

Figure 1. Comparison of endpoints for Ca- and TCDD-treated cells. Summarizing findings relating to EGFR down-regulation, EGF-like ligand secretion, basal ERK activation, and proliferation.

Finally, the impact of Ca and TCDD on cellular proliferation was also distinct. Ca-treated cells showed a decrease in total cell number (**Figure 2.5**), TCDD-treated cells had similar number to control cells (that was reduced in a ligand-manner (**Figure 3.8**) as well as showed a higher proliferative capacity as measured by EdU labelling. Still, neutralization of EGFR ligand secretion in all treatments led to a significant and marked decrease in proliferative capacity, suggesting that EGFR signaling, even in differentiating cells is an essential part of this system. The continued presence of the EGFR may allow Ca-dependent cells to maintain a proliferative population and in TCDD-treated cells, where EGFR down-regulation is ligand-dependent, explain how TCDD can simultaneously mimic portions of epidermal differentiation while simultaneously maintain proliferative-capacity.

Marked differences (**Figure 1**) in ability of ligands to account for changes in EGFR down-regulation, signaling, and proliferation observed following Ca- and TCDD-treatment suggest that Ca-induced alterations to EGFR localization and signaling are to a large degree, ligand-independent and therefore mechanistically distinct to the changes observed following TCDD exposure and therein may lie the difference in Ca- versus TCDD-induced keratinocyte differentiation. Cell number, while maintained by EGFR dependent proliferative capacity which maintained by a small portion of NHEKs is in both cases ligand-dependent, however, it the complement of ligands which impact how strongly this effect is.

Taken together, our data lead us to two important conclusions on Ca- and TCDD-induced differentiation.

1. That EGFR down-regulation observed in both Ca and TCDD-treated cells does not correlate with a loss of **proliferative capacity** though its effects on **ERK activation** and **cell number** differ.
2. Down-regulation observed in Ca- versus TCDD-treated cells is **mechanistically different**: neutralization of EGFR ligands leads to reversal of EGFR binding and altered ERK activation in TCDD-treated cells, while down-regulation in Ca-treated cells is ligand independent

However, the difference may not be so clearly defined as physiological versus pathophysiological differentiation. The difficulty of exploring the contradictory proliferative and differentiating role of TCDD in the epidermis through the use of monolayer keratinocyte culture of keratinocytes pidermally, Ca is not present in the dichotomous form of "high" or "low" within the epidermis. Instead, Ca represents a complex gradient with low Ca basally and higher Ca suprabasally (37, 38). Comparison of high Ca and TCDD compares cells under pro-differentiating and pro-proliferating, low Ca conditions respectively.

It is difficult therefore, to parse whether the ability of TCDD treated cells is due to the fact that cells in low Ca better replicate the effects of TCDD on basal cells compared to high Ca better representing the Suprabasal layers of the epidermis. This work performs the necessary examinations into the differing role of Ca and TCDD on EGFR down-regulation, and and signaling within the epidermis which paves the way for deeper explorations of the effect of TCDD in differentiating cells.

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APPENDIX A

Supplemental Figures for Chapter Two

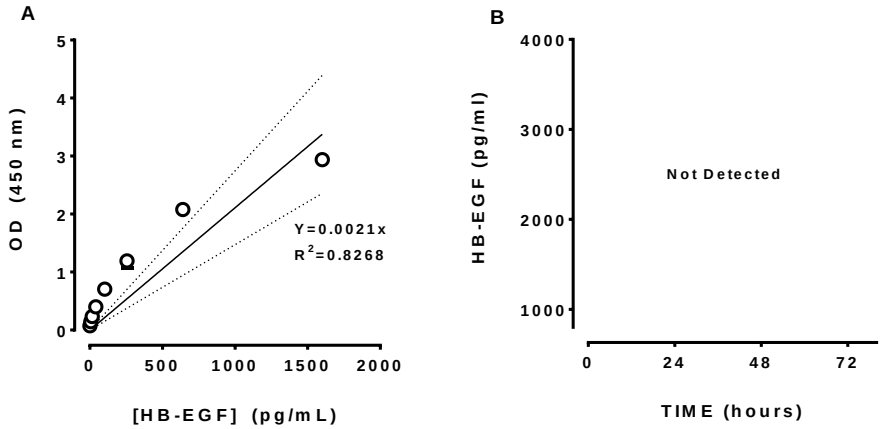

Fig. A1. HB-EGF in NHEK-conditioned medium is below detectable limits. NHEKs were treated for increasing times with Ca (1.8 mM) and culture medium was collected. HB-EGF (**A**) standard curve and (**B**) medium concentration were determined by ELISA assay. Standard curve represents means of one assayed in duplicate. In both control and Ca-treated NHEK-conditioned medium, HB-EGF concentration was below detectable levels in three experiments assayed in triplicate.

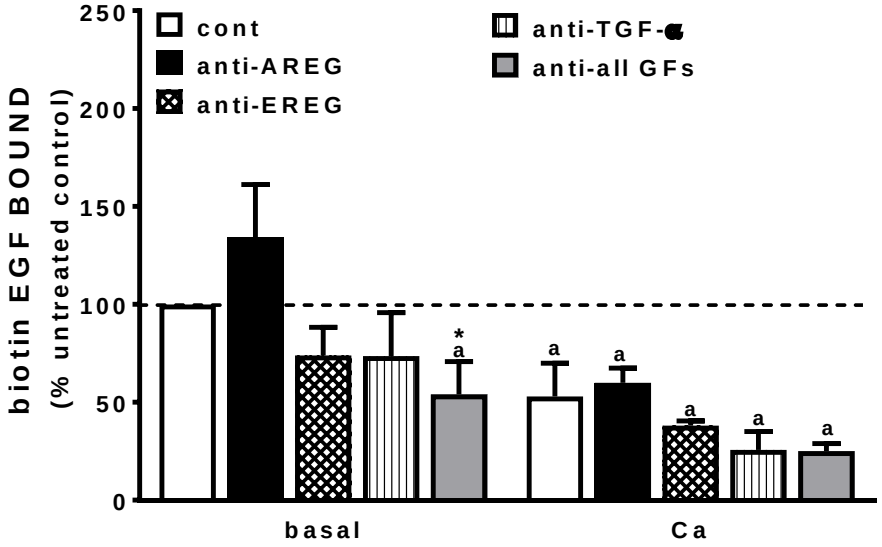

Fig. A2. Reducing EREG availability has no further effect on Ca-induced EGFR down-regulation. NHEKs were treated with or without Ca (1.8 mM) for 72 h in the absence or presence of neutralizing antibodies for AREG (15 µg/ml), EREG (5 µg/ml), TGF-α (5 µg/ml), or all three growth factors (anti-all-GF) for 72 h. The measurement of biotin-EGF binding was then determined as described in the Material and methods section. Basal refers to medium (K-SFM) without Ca; control refers to treatments in the absence of neutralizing antibodies. Data are means $\pm$ SEM of three experiments assayed in triplicate, normalized to crystal violet staining, and reported as the % biotin-EGF bound in basal control cells. (a) indicates significantly different from the basal control at p ($\leq$ 0.05). * indicates significantly different from the within treatment control (p $\leq$0.05) and indicates that anti-EREG together or separately do not have a significant effect on EGFR down-regulation in Ca treated cells beyond that of AREG and/or TGF-α.

APPENDIX B

Effects of co-treatment of TCDD in high calcium on EGFR signaling dynamics in NHEKs

Supplemental Methodology

Cells were grow and treated as described in the body of this Dissertation unless otherwise mentioned. Briefly, fifth passage NHEKs were grown to confluence, switched to basal K-SFM for 48 hours then treated with K-SFM with 1.8 mM Ca, 10 nM TCDD, or both 1.8 mM Ca and 10 nM TCDD. Neutralizing antibodies and inhibitors were used at the same concentrations described in text and figure legends.

Protocols for [^{125}I]-EGF binding, PACE assays, biotin-EGF binding, and EdU labelling were performed as addressed in the body of this work.

Determination of cell number

In lieu of double stranded DNA analysis, relative cell number was determined via crystal violet staining as described by Kueng et al (1989). Briefly, formaldahyde fixed cells were washed and stained with 0.04% crystal violet (Sigma Aldrich, St. Louis, MO) (w/v) in 4% ethanol (Kueng, et al., 1989; Kramer, et al., 2008) . Cells were lysed overnight in 10% SDS and lysates read at 595 nm.

Results

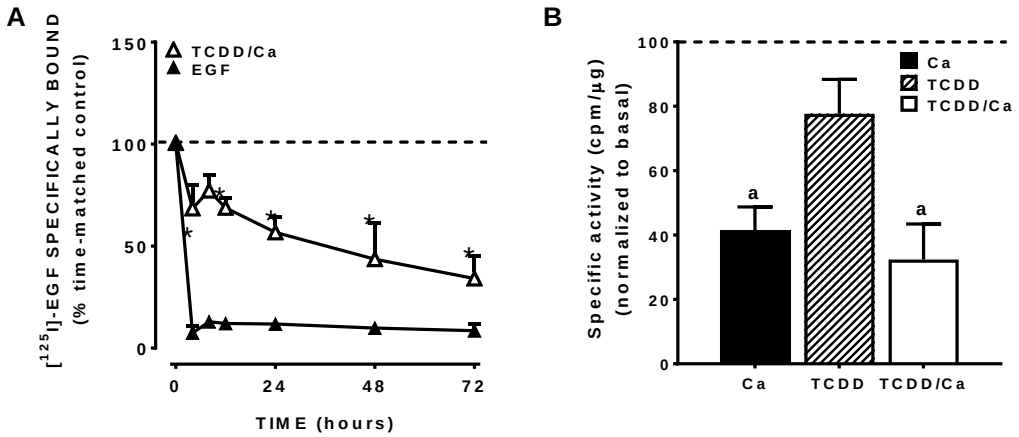

Figure 1. TCDD/Ca caused sustained [^{125}I]-EGF binding loss. NHEKs were grown as described in the body of this *Dissertation* then **(A)** treated for the times indicated with EGF (100 ng/ml) or TCDD/Ca (10 nM/1.8 mM) for 4-72 h then [^{125}I]-EGF binding was determined as described in . Data are expressed as the % time-matched control (TMC) and reported as the means $\pm$ SEM of three experiments assayed in triplicate. **(B)** TCDD/Ca co-treatment reduces [^{125}I]-EGF binding below beyond TCDD or Ca alone. NHEKs were treated for 72 h with 1.8 mM Ca, 10 nM TCDD, or TCDD/Ca then [^{125}I]-EGF binding was determined. Data are expressed as the % TMC and represent means $\pm$ SEM of three experiments assayed in triplicate. The dashed line in **(A)** and **(B)** indicates 100%. * indicates significantly different from time-matched control (TMC) ($p<0.05$), *a* indicates significantly different from TCDD ($p<0.05$), The dashed line indicates 100%.

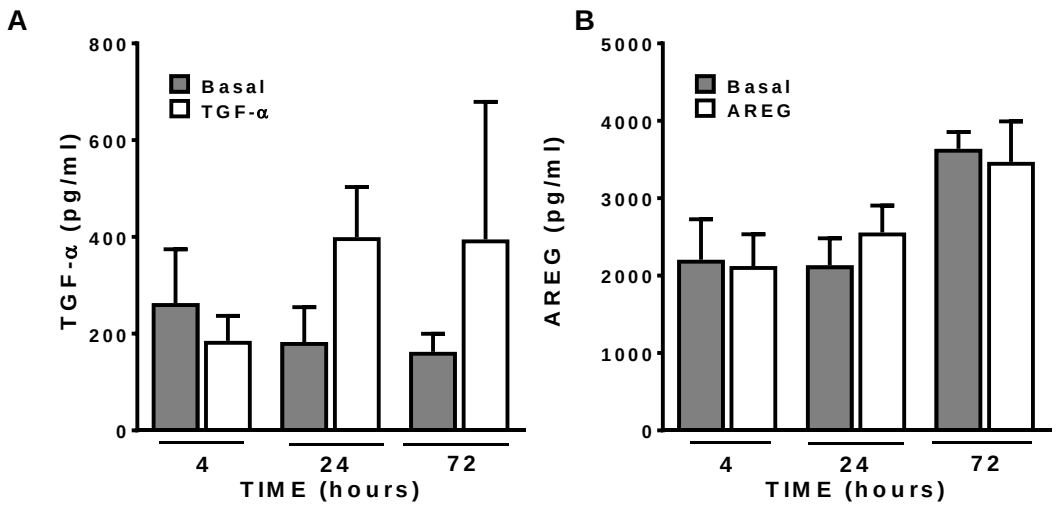

Figure 2. The effect of TCDD, Ca, and TCDD/Ca on TGF-α and AREG secretion. NHEKs were treated for 4, 24, or 72 h with TCDD/Ca (10 nM/1.8 mM), and conditioned medium was collected. **(A)** TGF-α or **(B)** AREG content were determined by ELISA as described in the body of the *Dissertation*. Data are means + SEM of three experiments assayed in duplicate. EREG has not been investigated in these cells.

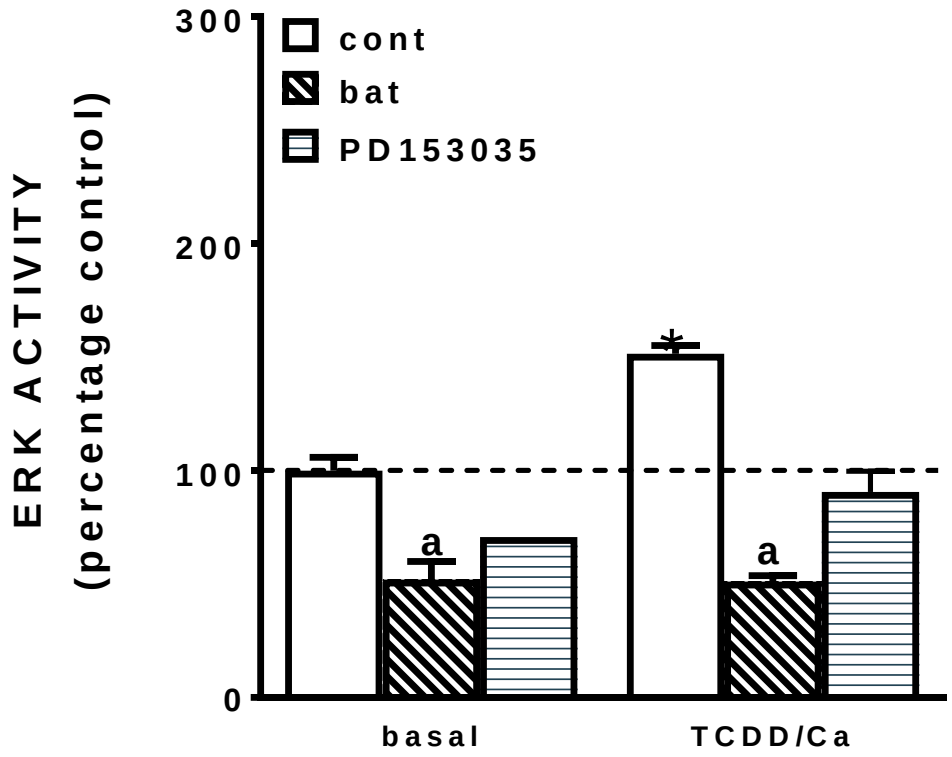

Figure 3. The effect of TCDD/Ca on ERK activation. NHEKs were treated for 72 h with TCDD/Ca (10 nM/1.8 mM) in the presence or absence of batimastat (bat; 3 μM) or the EGFR inhibitor, PD153035 (300 nM) and basal ERK activity was measured at 72 h via PACE assay as described in the body of this *Dissertation*. **(A)** TGF-α or **(B)** AREG content were determined by ELISA as described in the body of the *Dissertation*. Data are means + SEM of three experiments assayed in triplicate normalized to basal control cells, though PD153035 represents duplicate experiments.. *,** indicates significantly different from the within treatment control ($p<0.05$ and $p<0.01$, respectively). † indicates significantly different from the basal control ($p <0.05$). The dashed line indicates the basal control. Role of individual neutralizing antibodies has not been performed.

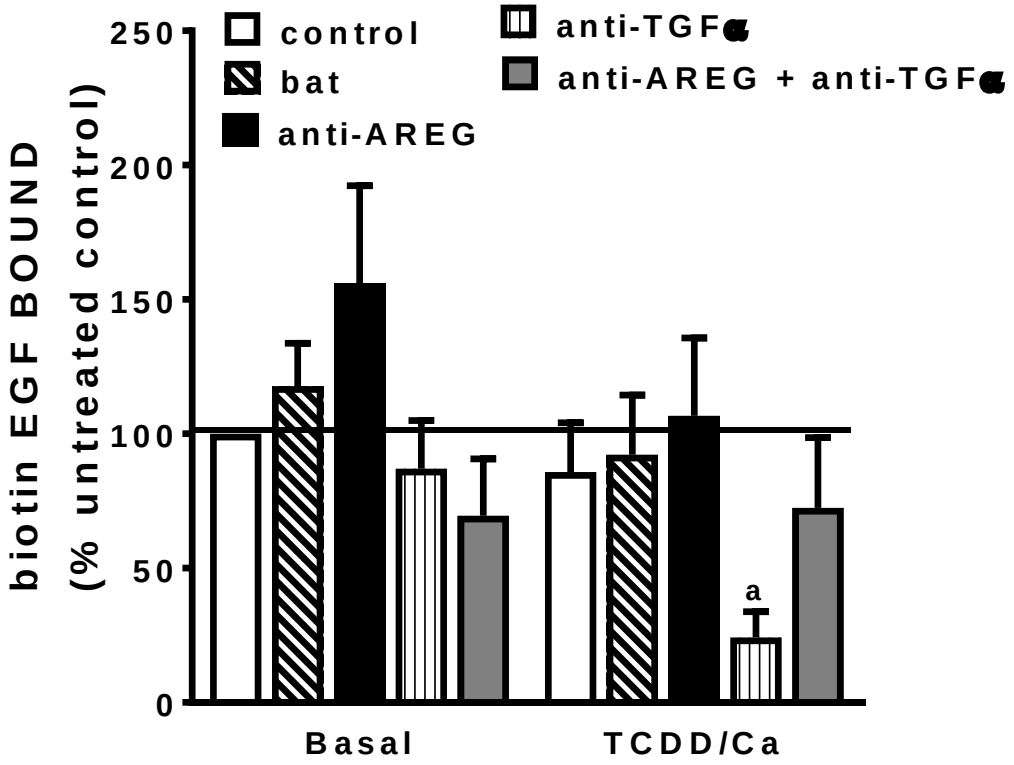

Figure 4. Reducing TGF-α availability modifies EGFR down-regulation. NHEKs were treated with or TCDD/Ca (10 nM/1.8 mM) for 72 h in the absence or presence of batimastat (bat; 3 μM) or neutralizing antibodies for TGF-α (5 μg/mL), AREG (15 μg/mL), or both for 72 h followed by the measurement of biotin-EGF binding as described in the body of this *Dissertation*. Basal refers to K-SFM without TCDD/Ca; control refers to treatments in the absence of batimastat or neutralizing antibodies. Data are means + SEM of six experiments assayed in triplicate, normalized to crystal violet staining, and reported as the % biotin-EGF bound in basal cells. a indicates significantly different from the within treatment control ($p < 0.05$). The dashed line indicates 100%.

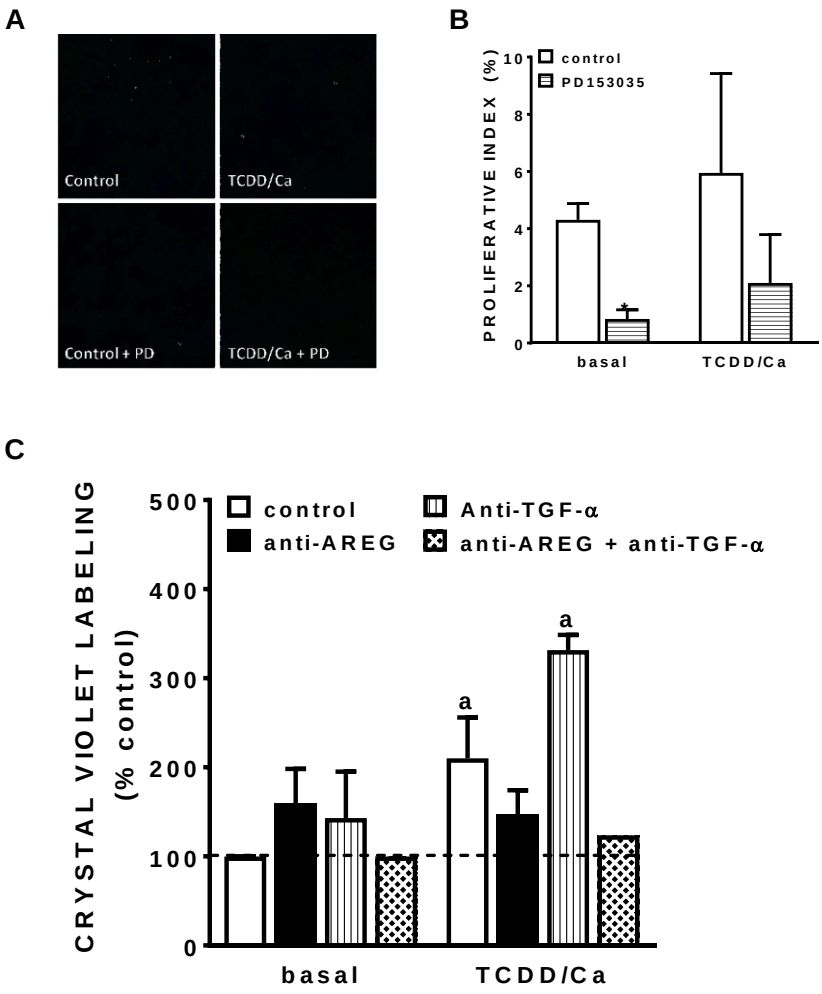

Figure 5: Inhibiting EGFR activity or reducing TGF-α availability have distinct effects on DNA synthesis and cell number. NHEKs were grown to confluence in glass chamber slides and then treated for 72 h with TCDD (10 nM), Ca (1.8 mM), or TCDD/Ca (10 nM/1.8 mM) in the presence or absence of PD153035 (300 nM), an EGFR inhibitor. During the last 16 h they were incubated with 10 mM EdU and labeled as described in the body of this *Dissertation*. **(A)** Representative confocal images of total (DAPI; 405 nm, blue) and EdU-staining nuclei (647 nm, pink) at 20x magnification. **(B)** Proliferative index of Edu positive nuclei per field (EdU labeled/DAPI labeled). Data are means + SEM from quantifications of five fields from three separate experiments**.** * indicates significantly different from the within treatment control ($p<0.05$ and $p< 0.01$ respectively). (C) NHEKs were grown to confluence and then treated for 72 h with TCDD, Ca, or TCDD/Ca in the presence or absence of neutralizing antibodies for TGF-α (5 μg/mL), AREG (15 μg/mL), or both. After treatment, cells were fixed in 4% formaldehyde and stained with 0.04% crystal violet as described in the *Supplemental Methods*. Data are means + SEM of three separate experiments assayed in triplicate and reported as percentage of time-matched control. a indicates significantly different from the basal control ($p<0.05$). The dashed line indicates 100%.

Table 1. Comparison with TCDD and Ca treatments

Characteristic	TCDD	Ca	TCDD/Ca
EGFR down-regulation	Ligand-dependent	Ligand-independent	Ligand-dependent (?)
EGFR ligand secretion	↑ TGF-α	↑ TGF-α ↑ EREG	↑ TGF-α *EREG?*
EGFR signaling at 72 h	Reduced Ligand-independent	Increased Ligand-dependent	Increased MMP-dependent
Cell number	Reduced	No change	Increased (crystal violet)
Proliferative Index	No Change	Enhanced	Indeterminate

APPENDIX C

Raybiotech™ sandwich ELISA-based arrays as a screen for NHEK-secreted ligands, changes in ErbB receptor complement, and ErbB phosphorylation At common sites

EGFR ligands in NHEK conditioned medium

Design and methology

Before pursuing whether EGFR down-regulation occurred through a ligand-dependent or independent mechanism, we first needed to know whether or not EGF-like ligands recognized by the EGFR were available at our experimental timepoints. In order to assess which if any EGFR ligands were available in NHEK-conditioned medium, we designed a custom ligand array from RayBiotech (Norcross, GA). Ligands were selected via TCDD-induced fold change in a keratinocytes microarray (1), literature search, and ligand availability. Literature was searched alongside Raybiotech antibody availability by utilizing PubMatrix (2), an NCBI literature mining tool. At the time, available ligands (n=292) were searched in pubmed against the terms: epidermis, keratinocyte, epidermal differentiation, skin, psoriasis chloracne, TCDD, epidermal disease, EGFR, calcium, and EGFR transactivation. Ligands were then ranked on a scale of 0-6 in each search wherein 0 was no results 1 $\leq$ 10 related articles, 2 $\leq$ 25 related articles, 3 $\leq$ 50 related articles, 4 $\leq$ 100 related articles, 5 $\leq$ 500 related articles, and 6 $\leq$ 1000. Within the top 100, we found TGF-α (ranked 5th), AREG (ranked 44th), HB-EGF (ranked 24th), and BTC (ranked 99th).

Capture antibodies for custom arrays were pre-dotted on nitrocellulose paper. Upon receipt, samples were incubated for 30 minutes at room temperature with proprietary blocking buffer before being incubated with 1 mL of cell culture conditioned medium for two hours at room temperature. Membranes were then washed five times with two proprietary washing buffers and incubated for one hour at room temperature with a mixture of biotin-conjugated anti-cytokine antibodies, washed again, and incubated for two hours with HRP-conjugated streptavidin. Membranes were then washed and developed via enhanced chemiluminescence on x-ray film. Film was scanned and spots quantitated using ImageJ software. Values are reported as arbitrary units (AU) that have been background subtracted and normalized to positive controls. Each membrane contained duplicate spots for each ligand and due to cost and the fact that this experiment served as a screening tool, each treatment x timepoint was performed on one membrane.

Identification of NHEK-secreted ligands

We found that TGF-α, HB-EGF, and AREG were secreted in cells under our treatment conditions (**Figure 1**). BTC had significant background even on the negative control membrane (incubated with basal K-SFM), suggesting it reacted to a component of the media. Regardless, no BTC effect was found. TGF-α increased 1.58-fold over basal in Ca treated cells, 2.49-fold in TCDD-treated cells, and 2.95-fold in TCDD/Ca-treated cells comparable to the 2.83-fold and 3.22-fold observed in Ca and TCDD-treated cells via ELISA (**Figure 1**). However, HB-EGF increased 1.97-fold, 2.58-fold, and 2.86-fold in Ca, TCDD, and TCDD/Ca-treated cells however was not detected in a ELISA for human HB-EGF with a lower detection limit of 6.55 pg/mL. AREG increased 1.18, 1.81, and

1.97-fold in Ca, TCDD, and TCDD-treated cells. EREG was not examined as it was not available at the time of this screen, however an ELISA assay became available after this initial screening and EREG was also analyzed. ERK activation or binding loss in TCDD-treated cells.

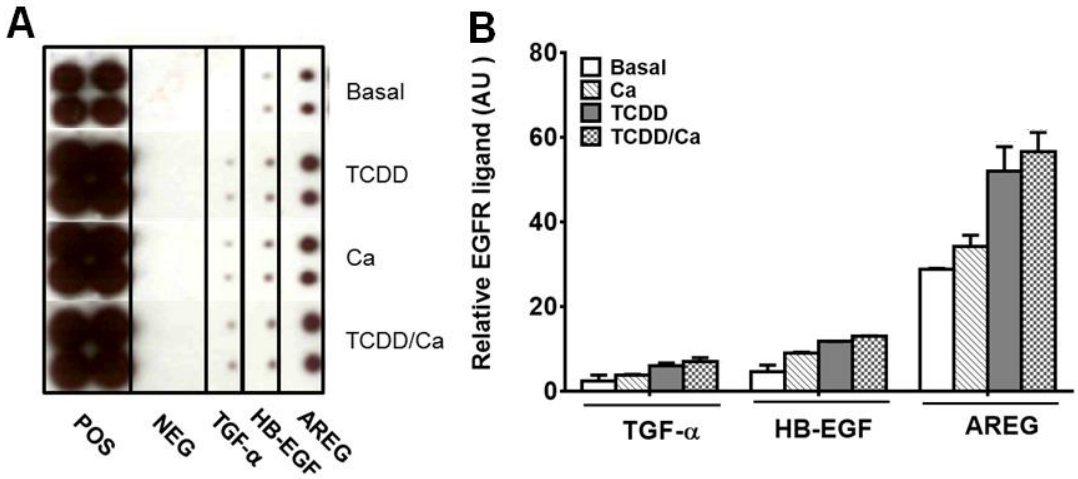

Figure 1. EGFR ligands increased in NHEK-conditioned medium. NHEKs were grown as described in *Methodology* TGF-α, HB-EGF, and AREG were assayed on a custom sandwich ELISA-based array. Antibody arrays were developed via (**A**) autoradiography and (**B**) quantitated via ImageJ. Data are representative of one experiment assayed in duplicate and background subtracted. Betacellulin (BTC) was also assayed but due to high non-specific binding on the control blot, we were unable to get any positive signal. ** indicates significantly different from basal cells ($p<0.01$).

Changes in phosphorylation of common sites on EGFR, ErbB2, and ErbB3 in NHEKs

Design and methology

To investigate how EGFR phosphorylation was altered, we first examined common EGFR phosphorylation sites (**Table 1**). Using another custom array from

Table 1. Fold change in phosphorylation of tyrosine, serine, and threonine residues on ErbB receptors at 72 hours

EGFR Phospho-site	Function	Basal (AU)	EGF (FC over basal)	Ca (FC over basal)	TCDD (FC over basal)	TCDD/Ca (FC over basal)
Y845	Phosphorylated by Src. Activates Ras and Stat5	48.92	ND	+2.15	-1.39	+3.63
Y992	Phosphorylated by EGFR/ErbB2. Activates STAT3, PKC, Erk, Vav2	31.11	ND	+1.46	-1.52	+9.64
Y1045	Phosphorylated by EGFR/ErbB2. Recruits c-Cbl for ubiquitinylation	31.67	-ND	+1.79	-2.71	+9.66
Y1068	Phosphorylated by EGFR/ErbB2 Ras, STAT3, c-Jun, AP-1	14.86	ND	ND	ND	ND
Y1086	Phosphorylated by ErbB receptors, Activates Ras, STAT3, c-Jun, and AP-1	ND	ND	ND	ND	ND
Y1148	Unknown phosphorylation mechanism. Activates MAPK, STAT1, and AP-1	30.27	ND	ND	ND	- 9.01
Y1173	Autophosphorylated. Activates MAPK, PLCγ, Shp2	25.92	ND	ND	ND	+3.09
S1046	Phosphorylated by Cam Kinase II. Causes c-Cbl-independent internalization/ubiquitinylation	22.02	ND	ND	ND	+5.62
S1070	Phosphorylated by Cam Kinase II. Causes c-Cbl-independent internalization/ubiquitinylation	ND	ND	ND	ND	ND
ErbB2 phospho-site	**Function**	**Basal**	**EGF**	**Ca**	**TCDD**	**TCDD/Ca**
Y887	Phosphorylated by Src Activates STAT3 and PKA	2.46	ND	ND	ND	ND
Y1112	Phosphorylated by EGFR/ErbB2. Recruits c-Cbl for ubiquitinylation	ND	ND	ND	ND	ND
Y1221	Shc, ErbB2	3.53	ND	ND	ND	ND
Y1248	Phosphorylated by unknown mechanism. Activates Shc and ErbB2	11.46	ND	+6.95	+3.61	ND
T686	Phosphorylated by PKC. Leads to internalization	ND	ND	ND	ND	ND
S1113	Phosphorylated by Cam Kinase II. Unknown function	ND	ND	ND	ND	ND
ErbB3 Phospho-site	**Function**	**Basal**	**EGF**	**Ca**	**TCDD**	**TCDD/Ca**
Y1299	Phosphorylated by an unknown mechanism. Activates PI3K	ND	ND	ND	ND	ND

Raybiotech, we assayed changes in the phosphorylation of thymine, serine and threonine residues.

Capture antibodies were pre-dotted on nitrocellulose paper for phosphosites on ErbB1, ErbB2, and ErbB3. Upon receipt, NHEKs grown for 72 hours in the presence of EGF (100 ng/mL), Ca (1.8 mM), TCDD (10 nM), or TCDD with 1.8 mM Ca (TCDD/Ca) were lysed in proprietary lysis buffer and quantitated using a Micro-BCA assay (Pierce Biotechnology, Rockford, IL) and equal amounts of protein were incubated for 2 hours at room temperature on membranes that had been pre-blocked for 30 minutes at room temperature with proprietary blocking buffer. Membranes were then washed five times with two proprietary washing buffers and incubated for one hour at room temperature with a mixture of biotin-conjugated anti-cytokine antibodies, washed again, and incubated for two hours with HRP-conjugated streptavidin. Membranes were then washed and developed via enhanced chemiluminescence on x-ray film.

Film was scanned and spots quantitated using ImageJ software. Values are reported as arbitrary units (AU) that have been background subtracted and normalized to positive control. Each membrane contained duplicate spots for each ligand and due to cost and the fact that this experiment served as a screening tool, each treatment x timepoint was performed one membrane.

Identification of ErbB phosphorylation sites

We found that most altered sites following Ca, TCDD, or TCDD/Ca were on ErbB1 (**Figure 2).**

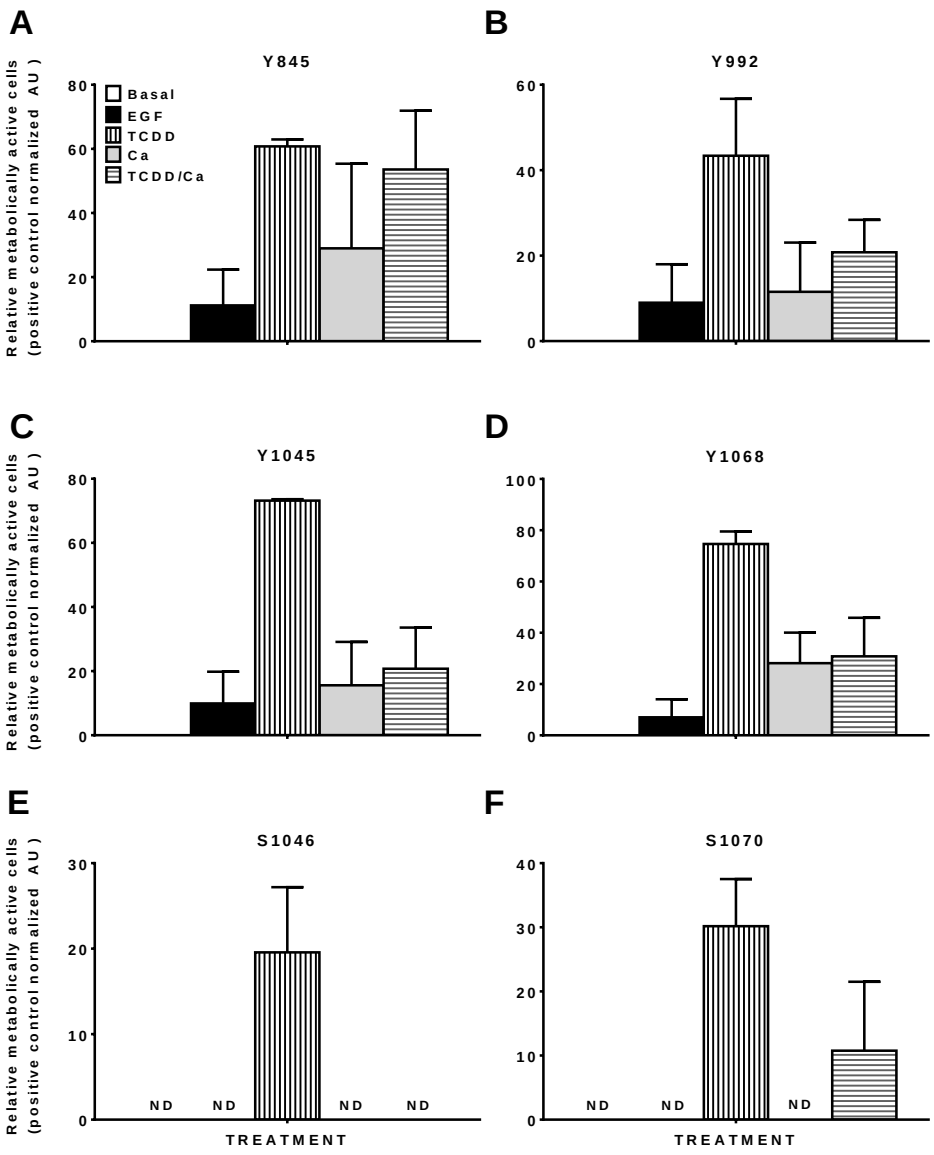

Figure 2. Phosphorylation sites on the EGFR at 72 hours. Keratinocytes were treated for 72 hours with EGF (100 ng/mL), Ca (1.8 mM), TCDD (10 nM), or TCDD/Ca. Not shown, Y1068, which had no detectable result. Data represent one experiment with duplicate spots. Data are background subtracted AU normalized to total EGFR protein and positive control.

References

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2. **Becker KG, Hosack DA, Dennis G,Jr, Lempicki RA, Bright TJ, Cheadle C, Engel J** 2003 PubMatrix: a tool for multiplex literature mining. BMC Bioinformatics 4:61