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DIETARY ENERGY BALANCE MODULATES GROWTH FACTOR SIGNALING DURING MULTISTAGE EPITHELIAL CARCINOGENESIS IN MOUSE SKIN

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by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

December 2010

Acknowledgements

I would like to thank my supervising professor, John DiGiovanni, for the support and countless opportunities provided during my tenure in his laboratory. The experience and knowledge acquired under your tutelage is invaluable and will provide the foundation for all of my future scientific endeavors. I would also like to thank the members of the DiGiovanni laboratory, especially Linda Beltran, Steve Carbajal, Okkyung Rho, Ronald Bozeman, Allyson Checkley, and Tarlochan Bhatt for not only the technical support that was provided, but also for the intellectual and personal conversations that enriched everyday spent in the laboratory. To my committee, Drs. Steve Hursting, Kimberly Kline, Linda deGraffenried, Susan Fischer and Karen Vasquez, I greatly appreciate the scientific input and support that you have provided, despite the infrequency of collective meetings. Finally, I would like to thank my family for the sacrifices that they have endured to make this possible. Without your love, understanding and endless support I would not be where I am today. Thank you for helping me make my dream a reality.

DIETARY ENERGY BALANCE MODULATES GROWTH FACTOR SIGNALING DURING MULTISTAGE EPITHELIAL CARCINOGENESIS IN MOUSE SKIN

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The University of Texas at Austin, 2010

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Energy balance refers to the relationship between energy intake and energy expenditure. Epidemiological studies have established a clear association between energy balance and cancer, however the underlying mechanisms are unclear. The objective of the current study was to evaluate the impact of caloric consumption on epithelial carcinogenesis and identify potential mechanisms of inhibition or enhancement. Using ICR female mice, we demonstrated that positive energy balance enhanced, while negative energy balance inhibited susceptibility to multistage carcinogenesis in mouse skin. We next evaluated diet-induced changes in the epidermal proliferative response. Calorie restriction (CR) significantly reduced epidermal hyperproliferation, in the presence and absence of tumor promotion, as compared to diet-induced obesity (DIO). Additional studies were conducted to determine the impact of dietary manipulation on TPA-induced growth factor signaling. CR reduced, while DIO

increased insulin like growth factor-1 receptor (IGF-1R) and epidermal growth factor receptor (EGFR) activation, which subsequently modulated signaling downstream to Akt and mTOR. These diet-induced changes in growth factor signaling were confirmed under steady-state conditions in multiple epithelial tissues (i.e., skin, liver and dorsolateral prostate) in multiple mouse strains (FVB/N, C57BL/6 and ICR). Further analyses demonstrated that caloric consumption directly correlated with levels of cell cycle progression related proteins and inversely correlated with levels of cell cycle inhibitory proteins. Genetic reduction of circulating IGF-1, liver IGF-1 deficient (LID) mouse model, inhibited two-stage skin carcinogenesis, reduced epidermal hyperproliferation and attenuated IGF-1R and EGFR growth factor signaling during tumor promotion, similar to CR, suggesting a potential for IGF-1R and EGFR crosstalk. Further studies, demonstrated that IGF-1 induced EGFR activation in cultured mouse keratinocytes, possibly due to IGF-1R and EGFR heterodimerization or IGF-1 induced changes in EGFR mRNA expression. *In vivo*, CR reduced, while DIO increased IGF-1R and EGFR association during tumor promotion. Furthermore, CR attenuated EGFR ligand mRNA expression both in the presence and absence of TPA treatment. Collectively, these findings suggest that dietary energy balance modulates epithelial carcinogenesis, at least in part due to diet-induced changes in levels of circulating IGF-1, which then modulate IGF-1R and EGFR crosstalk and downstream signaling to cell cycle related proteins, subsequently altering epidermal hyperproliferation.

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Chapter I

Introduction

Obesity, Energy Balance and Cancer

The prevalence of obesity, a risk factor for multiple cancers (1), has risen rapidly for the past several decades in the US (2). Estimates suggest that 14% of all cancer deaths in men and 20% of all cancer deaths in women are attributed to excess body weight, over a range of cancer types (1). Given the dramatic increase in obesity among children, the incidence of these obesity related cancers will continue to rise (3). Therefore, it is critical for research to be conducted that aims to identify potential mechanisms underlying the link between energy balance and cancer.

Diet-Induced Obesity (DIO) and Calorie Restriction (CR)

DIO enhances type-2 diabetes and several other chronic diseases in many experimental animal models, however its impact on carcinogenesis needs further characterization. In contrast, CR has consistently been shown to act as a potent inhibitor of carcinogenesis in animal models, regardless of mode of tumor induction (4, 5). This regimen restricts total energy intake (typically a 20%-40% reduction in carbohydrate and/or fat calories relative to *ad libitum*-fed controls), while maintaining isonutrient conditions, thus resulting in prevention of adult-

onset obesity (prevalent among the ad libitum-fed controls), lifespan extension, and suppression of cancer and other chronic diseases.

CR inhibits formation of spontaneous neoplasias (in knockout and transgenic mouse models) and suppresses radiation- and chemically-induced carcinogenesis in rodents. Two-stage skin carcinogenesis studies [7,12-dimethylbenz[a]anthracene (DMBA)-initiated, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-promoted], have demonstrated similar inhibitory effects of CR, findings that will be further detailed later in the Introduction and evaluated in Chapter 4 (6-9). The anticancer effects of CR are independent of species, tumor type, or mode of induction, suggesting that globally active circulating factors may be key mediators of the energy balance-cancer link. These circulatory proteins regulate many normal physiologic processes, including appetite regulation, energy expenditure, digestion, metabolism, and thermogenesis, but several of these proteins have also been shown to play a role in the regulation of tumorigenesis. Leptin, insulin-like growth factor (IGF)-1, insulin, glucocorticoids and several adipose-derived factors associated with inflammation have been identified as key mediators of the link between energy balance and cancer (see Figure 1.1 for review). The present study focuses on the role of IGF-1 in the modulation of epithelial carcinogenesis.

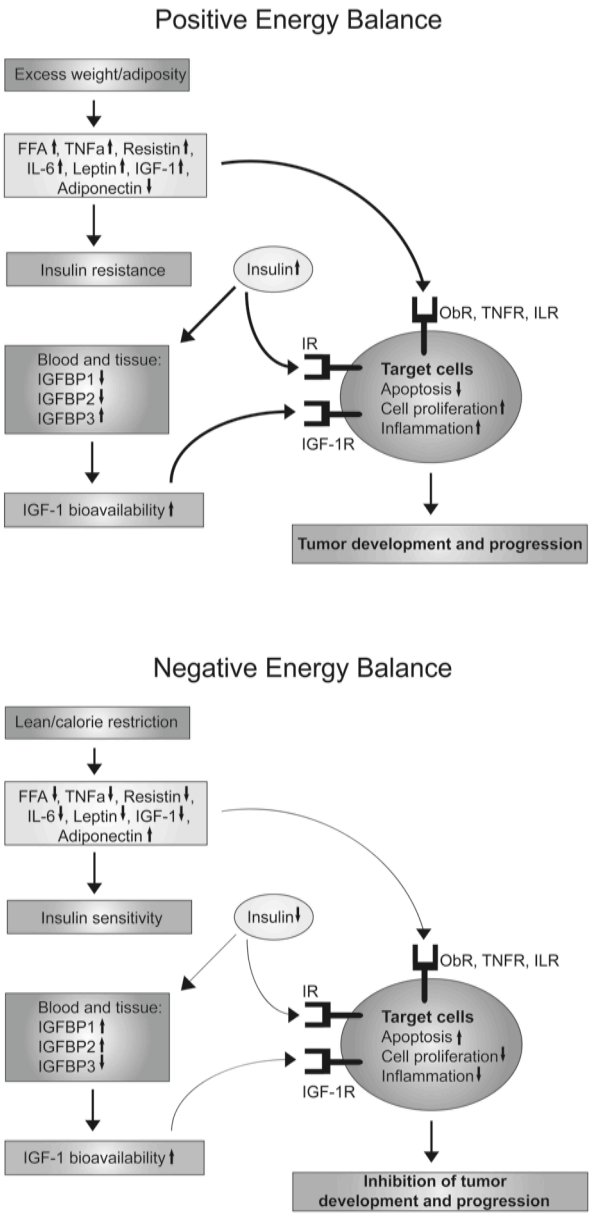

Figure 1.1. Summary of the effects of dietary energy balance on globally active circulatory proteins and the resulting cellular outcomes. Upper panel demonstrates that positive energy balance (obesity) increases levels of free fatty acids (FFA), TNF-α, resistin, IL-6, leptin, IGF-1 and insulin and reduces levels of adiponectin. These changes then result in increased signaling through corresponding receptors, leading to increased cell survival, proliferation and inflammation. Together, these effects lead to tumor development and progression. Lower panel demonstrates that CR elicits the opposite effects. Adapted from Calle et al. (10).

Mechanisms Associated with Energy Balance Effects on Carcinogenesis

Alterations in energy balance act broadly with respect to species, mode of induction, and tumor type. CR and obesity have been shown to differentially regulate the levels of a number of serum-related hormones and growth factors (5, 11), thus suggesting that globally active circulatory proteins may be the key mediators of the link between energy balance and cancer (again see Figure 1.1). A recent review identifies leptin, adiponectin, corticosterone, IGF-1 and insulin as the primary targets for these global effects of dietary energy balance on tumorigenesis (10). The rationale for these associations is provided in the following sections.

Leptin, an adipocyte derived hormone, regulates satiety and energy metabolism. CR consistently decreases, while obesity consistently increases leptin levels (12). Both epidemiological and animal studies are suggestive of an association between levels of circulating leptin and cancer risk, specifically for colorectal (13), prostate (14), endometrial (15) and breast cancers (16, 17). *In vivo*, leptin has been shown to promote angiogenesis and tumor invasion (18), while *in vitro,* leptin has been shown to stimulate cellular proliferation in preneoplastic and neoplastic cells, while having no effect on “normal” cells (19). Data suggests that leptin induces changes in intracellular signaling, which results in enhanced susceptibility to tumorigenesis. Specifically, leptin binds its transmembrane receptor (Ob-Rb), thus modulating activation of Jak-Stat and

MAPK signaling pathways, which have been shown to be critical regulators of carcinogenesis (for review see 20). Recent data suggests that these effects of leptin on tumorigenesis may be dependent on the ratio of leptin to adiponectin, as opposed to leptin levels alone (15, 21-23). Data from A-ZIP/F-1 mice, which lack adipose tissue and all associated adipokines, suggests that levels of leptin, however, may not be as critical of a regulator of tumorigenesis as levels of circulating IGF-1 (24, 25).

Adiponectin, another adipokine, regulates insulin sensitivity, as well as carbohydrate and lipid metabolism. Levels of adiponectin decrease in response to metabolic deficiencies, including type 2 diabetes, dyslipidemia, and extreme obesity. This reduction can be partially reversed by weight loss, although recent reports suggest that drastic weight changes, as achieved by severe CR or surgical intervention, are necessary to induce a significant increase in levels of adiponectin (26, 27). While the role of adiponectin in the modulation of tumorigenesis is not well characterized, recent data suggests an inverse correlation between levels of adiponectin and multiple cancer types, including both breast and colorectal cancers (28-34). Mechanistic studies suggest that the inhibitory effects of adiponectin are modulated, at least in part, due to activation of AMP-activated protein kinase (AMPK), which inhibits Akt and mTOR signaling, as well as reduced Stat signaling (30, 31). Again, recent data demonstrates that

many of these effects are mediated by leptin-adiponectin ratios, as opposed to levels of each hormone independent of the other (15, 21-23).

Corticosterone, an adrenal glucocorticoid hormone, has long been associated with the anticancer effects of CR. Severe CR (levels exceeding 30%) markedly increases corticosterone levels, while adrenalectomy reverses the inhibitory effects of CR on carcinogenesis (35-37). Glucocorticoid hormones have long been known to inhibit mouse skin tumor promotion, in the absence of dietary restriction (38). Several mechanisms have been proposed to account for the inhibitory effects of corticosterone, including inhibition of inflammation, induction of p27 (inhibition of proliferation), inhibition of protein kinase C, as well as reduced activation of Erk (35, 39-41). The ability of corticosterone to inhibit tumorigenesis, however, cannot be evaluated independent of changes in circulating IGF-1. Corticosterone supplementation not only inhibited mammary tumorigenesis, but also reduced levels of circulating IGF-1 in a dose dependent manner, again indicating a critical role for IGF-1 in the modulation of carcinogenesis (42).

The possible involvement of IGF-1 in cancer was first suspected when *in vitro* studies consistently showed that IGF-1 enhanced growth and proliferation of multiple cancer cell lines (19, 43-45). There is now abundant epidemiological evidence supporting the hypothesis that IGF-1 acts as a critical regulator of

several types of human cancer. Levels of circulating IGF-1 are primarily mediated by hepatic synthesis, which is regulated by growth hormone and insulin and influenced by nutritent intake and energy consumption. CR consistently reduces, while obesity consistently increases levels of circulating IGF-1. Many of the anticancer effects of CR have been shown to be mediated, at least in part, by levels of IGF-1. Specifically, restoration of IGF-1 levels in CR mice has been shown to ablate the anti-tumor effects of CR in multiple models (46-48). Furthermore, genetic reduction of circulating IGF-1 levels (liver IGF-1 deficient [LID] mouse model) inhibited tumorigenesis, in a manner similar to CR, using the two-stage skin carcinogenesis protocol (see Chapter III and 49). IGF-1 can act to enhance proliferation, cell growth and cell survival through both direct and indirect effects (50-53). IGF-1 can bind directly with the insulin-like growth factor 1 receptor (IGF-1R), resulting in increased activation of downstream signaling to Akt, mTOR, as well as other downstream effectors, or IGF-1 can act indirectly through other cancer-related molecules, such as the EGFR, which will be further discussed later in the Introduction.

Insulin resistance and chronic hyperinsulinemia increase risk for several cancers (10, 54), however it is unclear if these tumor-enhancing effects are regulated directly via increased insulin receptor signaling or alternatively due to indirect effects on IGF-1, estrogens, leptin or other hormones. Recent data suggests that crosstalk exists between the insulin receptor and multiple hormonal

pathways, making it difficult to ascertain the primary metabolic hormone regulating the effects of energy balance on cancer (55, 56). Furthermore, it has been well established that increased levels of insulin heighten both IGF-1 synthesis (hepatic) and IGF-1 bioavailability (reduces IGF-BP-1 production), which could further contribute to the observed enhancing effect of insulin on cellular growth, proliferation and survival (54). Consistent with the effects of energy balance on levels of IGF-1, CR reduces, while obesity increases levels of circulating insulin. Findings from the LID mouse model, however, suggests that insulin may not be the primary contributing factor to the effect of energy balance on tumorigenesis. Despite high levels of insulin, a 75% reduction in levels of circulating IGF-1 reduced susceptibility to two-stage skin carcinogenesis, similar to CR (again see Chapter III and 49).

Akt and mTOR: Downstream Effectors of IGF-1 and Insulin Signaling

It is well established that the PI3K/Akt/mTOR pathway is a critical mediator of the effects of IGF-1 on tumorigenesis (50, 51). This pathway is one of the most commonly altered pathways in human tumors, further demonstrating the importance of PI3K/Akt/mTOR signaling in human cancers (see Figure 1.2 and 57, 58-60). PI3K, which is activated by receptor tyrosine kinases (RTKs), including the IGF-1R and the insulin receptor (IR), and Ras, activates downstream effectors through production of phosphatidyl-inositol-3,4,5-

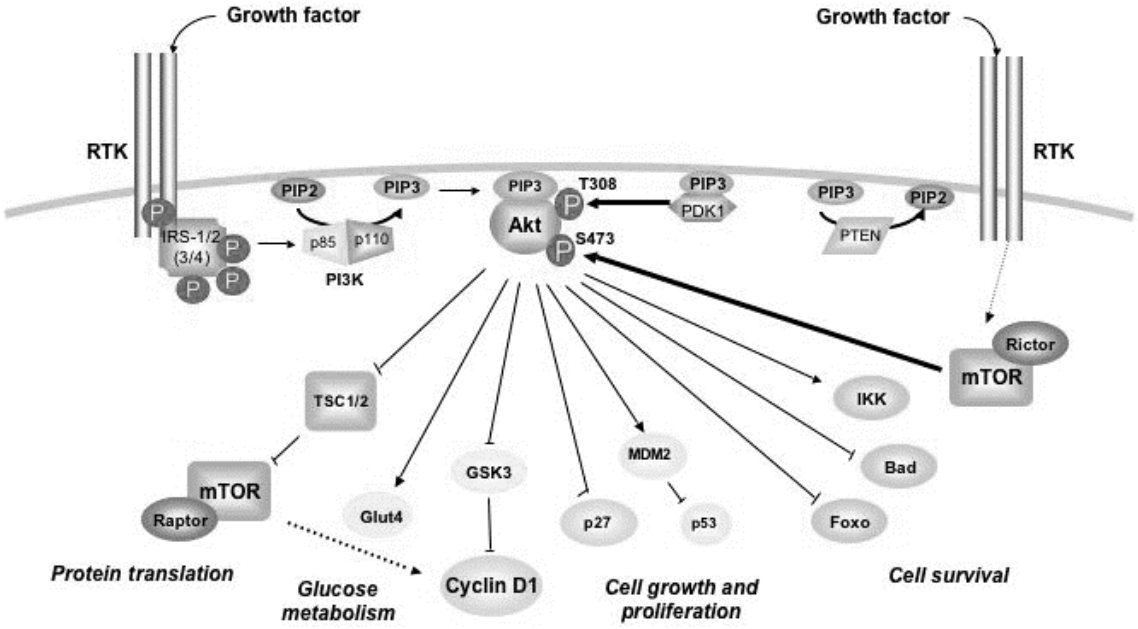

Figure 1.2. Overview of PI3K/Akt/mTOR signaling. Binding of growth factor to the receptor tyrosine kinase (RTK) phosphorylates scaffolding adaptor proteins, such as insulin receptor substrate (IRS), which then act as a docking site for phosphatidylinositol 3-kinase (PI3K), thus enabling activation via the receptor. Once activated, PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to produce phosphatidylinositol-3,4,5-trisphosphate (PIP3). This phosphorylation reaction can be reversed by PTEN. Akt and PDK1 bind PIP3 at the membrane. PDK1 then activates Akt via phosphorylation of the Thr308 residue found in the activation loop of the protein. Phosphorylation of Akt at Ser473 is hypothesized to be mediated by mTORC2 (mTOR-Rictor complex), which is activated via an unknown mechanism. Following activation, Akt regulates a number of cellular processes including: protein translation (mTORC1), glucose metabolism (Glut4 and GSK3), cell growth and proliferation (GSK3, cyclin D1, p27, p53), and cell survival (Bad, IKK, Foxo, p53). Adapted from Manning and Cantley, 2007 (61) and Crowell et al, 2007 (62).

trisphosphate (PIP3), a lipid second messenger, thus increasing cellular growth, proliferation, survival and metabolism (57, 59, 60).

Akt, a serine/threonine protein kinase, serves as the principal substrate of PIP3 and functions to regulate cellular survival, growth and proliferation through activation or inhibition of numerous downstream effectors. Akt enhances cell survival through its inhibitory actions on proapoptotic molecules such as Bad, p53 and FOXO family proteins (63-68) and regulates cellular proliferation and growth via increased cell cycle progression and heightened protein translation. These effects on cellular proliferation are primarily due to Akt-mediated inhibition of GSK3β, p27, and p53 (69-73), as well as induction of G1 to S phase cell cycle progression related proteins (cyclins D and E) (74, 75), while the effects on protein translation are due to enhanced mTOR activation and subsequent alterations in signaling to mTOR downstream substrates. Collectively these pathways form a sophisticated system that integrates cellular responses and environmental cues (51, 76).

mTOR, another highly conserved serine/threonine protein kinase, regulates cellular growth, proliferation, protein translation, and autophagy via phosphorylation of downstream targets, most notably S6K and 4E-BP1 (77). mTOR activation is not only regulated by growth factor and cytokine signaling, but it is also regulated by nutrient availability. These environmental and cellular

cues regulate mTOR phosphorylation through activation or repression of the tuberous sclerosis complex (TSC) (reviewed in 78, 79, 80). When active, TSC sequesters Rheb, a G-protein required for mTOR activation, thus inhibiting mTOR and its downstream effectors; however, when phosphorylated, TSC releases Rheb, allowing for a direct interaction with ATP, which can then lead to mTOR phosphorylation (81, 82). In the presence of growth factors, cytokines, or positive energy balance, such as that achieved during *ad libitum* feeding or consumption of a high-fat diet, TSC is inhibited and mTOR is activated, enabling heightened translation, growth, and proliferation. Conversely, nutrient deprivation conditions, such as those achieved during CR, inhibit mTOR, leading to cell growth arrest, inhibition of protein translation, and autophagy. AMPK and the upstream kinase, LKB1, function to repress mTOR in the presence of negative energy balance through activation of TSC. Low ATP/AMP ratios activate AMPK during nutrient depletion, and phosphorylation is maintained by LKB1 (reviewed in 83). Activation of this mTOR-repressive pathway not only functions to reduce cellular energy expenditure, but it also protects against stress-induced apoptosis. Nutrient availability regulates mTOR, yet no direct associations have been made between mTOR activation or repression, energy balance, and prevention of tumorigenesis. The effects of energy balance on Akt, mTOR and AMPK activation will be further discussed in Chapter II.

Interplay Between IGF-1 and Epidermal Growth Factor (EGF) Receptor Signaling

The EGFR has been shown to activate Akt and mTOR signaling, in a manner similar to both the IGF-1R and the IR. Data suggests that EGFR-mediated activation of Akt and mTOR may be modulated, at least in part, by IGF-1R and EGFR crosstalk. Numerous studies have established that crosstalk exists between these two receptors (reviewed in 84, 85) and several potential mechanisms have been proposed, including: i) IGF-1 induced ectodomain shedding of EGFR ligands; ii) IGF-1 induced transcription of EGFR and EGFR ligands; iii) and IGF-1R/EGFR heterodimerization (86-90). Additional data from mouse epidermis further supports this notion of IGF-1R/EGFR crosstalk. Overexpression of *IGF-1* in the epidermis (HK1.IGF-1) led to not only heighted IGF-1R phosphorylation, but also increased EGFR activation following TPA treatment. Taken together, these findings suggest a role for IGF-1R and EGFR crosstalk in the modulation of epithelial carcinogenesis, which will be further explored in relation to energy balance and IGF-1 in Chapter IV.

IGF-1R/Akt/mTOR Pathway and Multistage Epithelial Carcinogenesis in Mouse Skin

The two-stage skin carcinogenesis model, a well-established model for epithelial carcinogenesis, has been extensively utilized to evaluate the impact of modifying factors on tumor initiation, promotion and progression, as well as

identify potential mechanisms underlying tumorigenesis (91). Using this model system, data have accumulated that indicate a critical role for Akt signaling, which is induced by the IGF-1R, the EGFR or other growth factor receptors, in the modulation of epithelial carcinogenesis. In this regard, overexpression of *IGF-1* in the epidermis (i.e., BK5.IGF-1 transgenic mice) induced epidermal hyperproliferation, enhanced susceptibility to two-stage skin carcinogenesis, and induced spontaneous skin tumor formation (92, 93). Further characterization of the BK5.IGF-1 transgenic mice revealed upregulation of epidermal PI3K and Akt activities as well as upregulation of cell cycle regulatory proteins (92, 93). Inhibition of PI3K, using LY294002, inhibited these constitutive biochemical changes observed in the epidermis of BK5.IGF-1 transgenic mice, as well inhibited IGF-1–mediated skin tumor promotion in a dose-dependent manner. These data support the hypothesis that the PI3K/Akt signaling pathway is involved in regulating skin tumor promotion by IGF-1.

Additional studies have been conducted to further evaluate the role of Akt in the modulation of multistage carcinogenesis in mouse skin. Segrelles et al (94) reported sustained activation of epidermal Akt throughout two-stage skin carcinogenesis and more recent data published by this same group (95) and others (96, 97) confirmed the involvement of Akt-mediated cellular proliferation in mouse skin tumorigenesis. Furthermore, overexpression of *Akt* (BK5.Aktwt) or constitutively active Akt (BK5.Aktmyr) in the epidermis dramatically enhanced

susceptibility to two-stage skin carcinogenesis (98). This effect on tumor susceptibility may be due to an Akt-mediated increase in epidermal proliferation, which correlated with significant elevations of G1 to S phase cell cycle proteins, including cyclin D1 (99). In conjunction with these changes, a marked increase in signaling downstream of mTOR was observed suggesting that protein translation was also upregulated in an Akt-dependent manner. Collectively these data suggest an important role for Akt signaling to mTOR and other downstream substrates in the modulation of mouse skin tumorigenesis.

Dietary Energy Balance and Two-Stage Carcinogenesis in Mouse Skin

Multiple studies have examined the effect of dietary energy balance and fat consumption on tumorigenesis using the two-stage skin carcinogenesis model. CR has been shown to consistently reduce susceptibility to skin tumorigenesis, however these effects are limited to tumor promotion (6, 100, 101). Specifically, CR during tumor promotion leads to a significant reduction in tumor incidence, multiplicity, and papilloma size (6, 100, 101). Early studies suggested that these inhibitory effects of CR on tumorigenesis were due to elevated levels of circulating corticosterone. Birt and colleagues (7), as well as Pashko and Schwartz (37) reported that adrenalectomy reversed the inhibitory effects of 40% CR on two-stage skin carcinogenesis, while restoration of circulating corticosterone levels in adrenalectomized mice partially restored CR-mediated inhibition. Similar findings were reported by Thompson and colleagues

using the rat mammary model (36, 42, 102). Further data suggested additional potential inhibitory mechanisms, which included reduced Erk activation and inhibition of TPA-mediated AP-1 DNA binding (35, 39, 103). While these studies provided novel information regarding mechanisms underlying the inhibitory effects of CR on tumorigenesis, they utilized severe CR (40% CR), which has been shown to significantly increase levels of corticosterone, as compared to more mild CR regimens, as well as elevate the stress response. A more recent study, which utilized a mild CR regimen (20% CR) demonstrated a reduction in TPA-mediated activation of PI3K and Ras signaling following 20% CR (104). Data presented in Chapters II, III and IV support these findings and suggest that these inhibitory effects of CR on growth factor signaling are modulated, at least in part, due to reduced levels of circulating IGF-1.

Additional studies have been conducted to examine the impact of a high fat diet on two-stage skin carcinogenesis, although no clear conclusions can be drawn from the resulting data. *Ad libitum* consumption of a high-fat (46 Kcal% fat) diet had no effect on tumor multiplicity, tumor incidence or tumor progression, as compared to the *ad libitum* control (11 Kcal% fat), regardless of the presence or absence of diet-induced changes in body mass (101, 105). In contrast, isocaloric consumption of this same high-fat diet yielded significant differences in both tumor incidence and multiplicity, as compared to the *ad libitum* control, despite no significant difference in body mass distribution (106). Furthermore, studies

evaluating the effect of fat type on skin tumorigenesis in this model system yielded interesting results, but these studies kept the caloric density of the diets equivalent and therefore did not address the impact of weight or adiposity on tumor formation (107-109). The data presented in Chapters II and IV demonstrate the effect of positive energy balance on two-stage skin carcinogenesis and identify potential mechanisms underlying these effect, thus bridging the existing gap in knowledge.

Specific Aims

The overall goal of this project was to determine the impact of energy balance, across the spectrum of negative to positive, on epithelial carcinogenesis using the two-stage skin carcinogenesis protocol and to elucidate potential mechanisms underlying this energy balance-cancer link. The individual specific aims were as follows:

1) Determine the impact of positive and negative energy balance on steady-state growth factor signaling (Chapter II). We evaluated diet-induced changes in cellular signaling in multiple epithelial tissues (epidermis, liver, DL prostate) and multiple mouse strains (C57BL/6, FVB/N, ICR). Western blot analyses were performed to determine the effect of CR and DIO on IGF-1R and EGFR downstream signaling (e.g., Akt and mTOR), as well as AMPK activation.

2) Examine the impact of genetically reduced levels of circulating IGF-1 on phorbol-ester (TPA)-induced skin tumor promotion (Chapter III). In this aim, we determined the impact of genetically reduced levels of circulating IGF-1, in the absence of altered energy balance (LID mouse model), on TPA-induced epidermal proliferation and skin tumor promotion. We also examined the impact of reduced levels of circulating IGF-1 on both basal and TPA-induced epidermal growth factor signaling.

3) Determine the impact of dietary energy balance manipulation on two-stage skin carcinogenesis and identify potential underlying mechanisms (Chapter IV). We evaluated diet effects on TPA-induced epidermal proliferation and two-stage skin carcinogenesis, as well as determined the effect of energy balance on growth factor signaling and cell cycle progression. Furthermore, we identified mechanisms of crosstalk between the IGF-1R and the EGFR in keratinocytes and evaluated the effect of dietary manipulation on crosstalk between these two receptors.

Chapter II

Dietary energy balance modulates signaling through Akt/mTOR pathways in multiple epithelial tissues (110).

Introduction

The prevalence of obesity has dramatically increased over the past forty years in the US, with nearly two-thirds of adult Americans currently considered overweight and nearly one-third obese (2). The obesity rates in children and adolescents are rising alarmingly as well (2). Epidemiologic studies have established obesity as an important risk factor for several types of epithelial cancers. For example, the American Cancer Society conducted a large prospective study of the relationship between obesity and cancer and estimated that 20% of total cancer deaths in women and 14% of cancer deaths in men are attributable to excess body weight (1). Insights into the mechanisms underlying the increased cancer risk associated with obesity are urgently needed to develop new strategies for preventing and treating obesity-related cancers.

In experimental model systems, calorie restriction (CR), which induces negative energy balance and prevents or reverses obesity, is arguably the most potent dietary-based intervention for preventing cancer (5). CR has been shown to inhibit formation of spontaneous neoplasias in several knockout and transgenic mouse models, to suppress tumor growth in tumor transplant models,

and to inhibit radiation- and chemically-induced carcinogenesis in a variety of rodent cancer models (5, 9, 100, 101, 111, 112). In contrast, tumor development is generally enhanced in rodent models of diet-induced obesity (DIO) (111, 113-116). Despite the well-established anticancer effects of CR, no mechanism of inhibition has been clearly identified.

We and others have previously established that reductions in circulating insulin-like growth factor (IGF)-1 are associated with the anticancer effects of CR in specific model systems (5). In addition, with the exception of a short-term IGF-1 infusion study (47), restoration of circulating IGF-1 levels in mice on CR diets has been shown to ablate many of the anti-tumor effects of CR in multiple tumor models (46, 48). In contrast, DIO can lead to insulin resistance and increased circulating IGF-1 (111). We have also reported that A-Zip/F-1 mice, which lack white adipose tissue but are diabetic, display elevated IGF-1 levels, and (like obese mice) are highly susceptible to several types of epithelial cancers (24). Taken together, these data suggest a critical role for circulating levels of growth factors, such as IGF-1, in the regulation of dietary energy balance effects on carcinogenesis.

The possible involvement of IGF-1 in cancer was first suspected when *in vitro* studies consistently showed that IGF-1 enhanced the growth of a variety of cancer cell lines (43, 44). A role for IGF-1 in cancer was further confirmed when

human breast (117), colon (118), and lung tumors (119) were shown to overexpress IGF-1, the IGF-1 receptor (IGF-1R), or both. Additional epidemiological evidence identified an association between elevated circulating levels of IGF-1 and increased risk of several epithelial cancers in humans (120, 121). Increased signaling through the IGF-1R leads to enhanced suppression of apoptosis, increased mitogenesis and cell cycle progression (122, 123).

Evidence suggests that many of these IGF-1-related effects on cellular growth and metabolism involve signaling through the phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR pathway (50, 92, 93), one of the most commonly altered pathways in human tumors (57-60, 124). For example, overexpression of *IGF-1* in the epidermis of HK1.IGF-1 and BK5.IGF-1 transgenic mice led to a dramatic increase in sensitivity to tumor development using the two-stage skin carcinogenesis protocol, a well established model for epithelial carcinogenesis (92, 125). Thus, elevated tissue levels of IGF-1 and enhanced signaling through the IGF-1R led to enhanced susceptibility to tumorigenesis. The ability of elevated tissue IGF-1 levels to promote skin tumors is due, at least in part, to activation of the PI3K/Akt signaling pathway, which has been shown to regulate epithelial cell proliferation (93, 94, 99). When either wildtype or myristoylated mouse Akt was overexpressed in epidermal basal cells under control of the *BK5* promoter, susceptibility to two-stage skin carcinogenesis was further enhanced (99). Western blot analyses performed on protein lysates prepared from either

Akt transgenic mouse model demonstrated enhanced signaling through the Akt/mTOR pathways, with heightened activation of downstream effectors of both Akt and mTOR (99). A similar pattern of increased activation of Akt/mTOR signaling in the skin epidermis of the fatless but diabetic A-Zip/F-1 mice was associated with the increased skin and mammary tumor susceptibility observed in these mice (24). Collectively, these data further support the hypothesis that elevated IGF-1R signaling, and in particular activation of the Akt/mTOR pathways, may contribute to increased susceptibility to epithelial carcinogenesis. AMPK, which acts as a nutrient-dependent regulator of mTOR (83), may also be involved. During nutrient deprivation conditions, AMPK can be activated by upstream kinases and functions to repress activation of mTOR, thus reducing cellular energy expenditure (126-132).

In the present study, we utilized well-established dietary regimens to induce positive and negative energy balance in mice in order to further explore potential mechanisms underlying the energy balance and cancer link (111, 133-136). Biochemical analyses were performed on multiple epithelial tissues from three commonly used mouse strains to determine diet-induced changes in steady-state cell signaling. The results indicate that dietary energy balance manipulation modulates signaling through Akt and mTOR pathways in all three tissues examined (i.e., epidermis, liver, dorsolateral prostate). Furthermore, modulation of these signaling pathways appeared to be primarily mediated via alterations in

signaling through the IGF-1R and the epidermal growth factor receptor (EGFR). Finally, phosphorylation of AMPK in response to either a positive or negative energy balance appeared to be tissue-dependent. Overall, the current data identify Akt/mTOR signaling pathways as potential targets for cancer prevention and in particular for prevention of obesity-related cancers.

Materials and Methods

Chemicals and biologicals. Antibody against phospho-EGFR (Y1086) was purchased from AbCam (Cambridge, MA), while antibodies against phospho IGF-1/insulin receptor (IR) (Y1135/1136), phospho Akt (S473), Akt, phospho mTOR (S2448), mTOR, phospho-p70S6K (T389), p70S6K, phospho-4E-BP1 (T37/46), phospho S6 ribosomal (S235/236), phospho GSK-3β(S9), Cyclin D1, and phospho AMPK (T172) were all purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-β actin, as well as anti-rabbit and anti-mouse secondary antibodies were obtained from Sigma Chemicals Co. (St. Louis, MO). Antibodies against phospho IRS-1 (Y989), phospho IRS-1 (Y632), phospho IRS-1 (Y465), and phospho IRS-1 (Y941) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and combined to generate an anti-phospho IRS-1 antibody cocktail.

Animals. Male FVB/N and C57BL/6 mice (30 per genetic background, 3-4 weeks of age) were purchased from NCI (Frederick, MD) and singly housed for the duration of the experiment. Thirty-two female ICR mice (3-4 weeks of age) were purchased from Harlan Teklad (Indianapolis, IN) and group housed for the duration of the experiment.

Diet regimens. All diets were purchased in pellet form from Research Diets, Inc. (New Brunswick, NJ). Upon arrival, mice were placed on a 10Kcal% fat (control)

diet (AIN-76A semipurifed diet, fed *ad libitum*; diet D12450B) for a one-week equilibration period and then randomized into three dietary treatment groups (10 mice per group): a) control diet (10Kcal% fat), fed *ad libitum*; b) DIO (HF) diet (60Kcal% fat)(D12492), fed *ad libitum*; c) 30% CR diet (D03020702). These diets have been previously described (9). For the study conducted in ICR mice, female mice were maintained on the diets described above (8 mice per group) and an additional dietary treatment was introduced: 15% CR diet (D03020703). Mice receiving either CR diet were administered a daily aliquot equivalent to either 70% or 85% of the daily amount of total energy consumed by the control diet group. Both CR diets were adjusted to provide 100% of all vitamins, minerals, fatty acids, and amino acids relative to the control group. Under group housing conditions, mice receiving the 30% CR diet were placed in a cage divider system for two hours and allowed to consume their daily food allotment. Average body mass and food consumption was determined weekly for each dietary treatment group. With the exception of ICR mice, which received their diet regimens for 15 weeks, all mice were maintained on their diet regimens for 17 weeks. All groups were terminated by CO_2 asphyxiation and tissues and blood were collected, processed and stored as described below.

Preparation of protein lysate. Immediately after the mice were terminated the dorsal skin was shaved, and then a depilatory agent was applied for 30 seconds and then removed. The skin was excised and the epidermal tissue was scraped from the excised skin using a razor blade into prepared lysis buffer (0.5% Triton

X-100; 1% NP40; 10% glycerol; 50 mM HEPES, pH 7.5; 150 nM NaCl; 1mM EGTA; 1.5 mM $MgCl_2$; 10% Sigma inhibitor cocktail; 10% phosphatase inhibitor cocktail I; and 10% phosphatase inhibitor cocktail II) and homogenized using an 18-guage needle. Epidermal scrapings from all mice in each dietary group were pooled (10 mice per group for FVB/N and C57BL/6 and 8 mice per group for ICR). The liver and dorsolateral prostate were removed from FVB/N and C57BL/6 mice, frozen in liquid nitrogen, and then ground using a mortar and pestle. Once in powder form, liver and prostate tissues were homogenized using an 18-guage needle in the lysis buffer described above. Again, both liver and prostate tissues from 10 mice were pooled per dietary group. The epidermal, liver, and prostate homogenates were then centrifuged at 14,000 RPM for 15 minutes, and the supernatant was aliquoted for use for Western blot analysis.

Western blot analysis. For analysis of receptor tyrosine kinase activation and activation of Akt/mTOR signaling molecules, 100 μg of epidermal lysate was electrophoresed in 4-15% SDS polyacrylamide gels according to the method of Laemmli (137). The separated proteins were then electrophoretically transferred onto nitrocellulose membranes and blocked with 5% BSA in TBS with 1% Tween 20 (TTBS). Blots were then incubated overnight with the antibodies described above in 5% BSA in TTBS. Blots were washed with TTBS three times for 15 minutes each, and then incubated in anti-rabbit and anti-mouse secondary antibody in 5% BSA in TTBS for two hours. Blots were washed again with TTBS

three times for 15 minutes each, and then the protein bands were visualized by enhanced chemiluminescence [Pierce, Rockford, IL]. Protein quantification was then determined using an alpha imager system. Each blot was repeated, producing nearly identical results.

Serum IGF-1 analysis. Blood was collected by cardiac puncture immediately following $C0_2$ asphyxiation (10 mice per diet group for FVB/N and C57BL/6 and 8 mice per diet group for ICR), allowed to sit at room temperature for 2 hours, and then spun at 7,500 RPM for 7 minutes. Supernatant was then collected and spun again under the same conditions. The final supernatant was collected, flash frozen in liquid nitrogen, and stored at -80˚C until analysis. Total mouse serum IGF-1 concentration was then measured using a 25 μl sample with a radioimmunoassay (RIA) kit (Diagnostic Systems Laboratories, Inc. Webster, TX).

Results

Effect of dietary manipulation on body weight distribution, feed consumption, and serum IGF-1 levels in FVB/N and C57BL/6 male mice.

FVB/N and C57BL/6 male mice were randomized into three dietary treatment groups (10 per group): a) control (10Kcal% fat), b) DIO regimen (60Kcal% fat), and c) lean regimen (30% CR) and maintained on these diets for 17 weeks. As shown in Tables 2.1 and 2.2, there were no statistically significant differences in body mass among the three groups at the start of the study. The weight distribution of both the FVB/N and C57BL/6 on the CR diet began to diverge from the other two dietary groups within two weeks of diet commencement. In contrast, 8 weeks of experimental diet consumption was necessary to separate the control group from the DIO group in mice from either genetic background (data not shown). Following 17 weeks on diet, the average body mass of mice maintained on the CR diet was significantly reduced, while the average body mass of mice maintained on the DIO (HF) diet was significantly increased relative to FVB/N and C57BL/6 mice on the control diet (see again Tables 2.1 and 2.2) (Student's t-test, $p < 0.05$).

Average feed consumption was determined for each diet regimen across the 17-week experiment. As shown in Tables 2.1 and 2.2, mice maintained on the CR diet consumed 30% fewer grams of feed relative to the control in both FVB/N and C57BL/6 mice, corresponding to a 30% reduction in caloric intake.

Table 2.1. Weight distribution, food consumption and serum IGF-1 levels of FVB male mice maintained on a control, high fat and lean diet regimen (n=10/group).

Experimental diet	Mass (g) study start	Mass (g) study end	Average food consumption (g)	Serum IGF-1 (ng/mL) study end
10Kcal% fat	27.2 ± 0.4	41.9 ± 0.9[a]	26.8 ± 0.45[a]	742 ± 62.23[a]
60Kcal% fat	25.5 ± 0.3	46.7 ± 0.9[b]	24.2 ± 0.42[b]	987 ± 107.85[b]
30% CR	26.8 ± 0.3	26.8 ± 0.3[c]	19.0 ± 0.30[c]	440 ± 65.47[c]

The data represents mean ± SEM.
[a-c] Values are significantly different from each other for parameter given in column heading.

Table 2.2. Weight distribution, food consumption and serum IGF-1 levels of C57BL/6 male mice maintained on a control, high fat and lean diet regimen (n=10/group).

Experimental diet	Mass (g) study start	Mass (g) study end	Average food consumption (g)	Serum IGF-1 (ng/mL) study end
10Kcal% fat	17.3 ± 0.3	37.5 ± 0.8[a]	23.3 ± 0.43[a]	615 ± 52.25[a]
60Kcal% fat	17.7 ± 0.3	47.0 ± 0.8[b]	22.9 ± 0.88[a]	987 ± 92.53[b]
30% CR	16.9 ± 0.4	21.1 ± 0.3[c]	16.2 ± 0.37[b]	208 ± 32.38[c]

The data represents mean ± SEM.
[a-c] values are significantly different from each other for parameter given in column heading.

Although feed consumption was reduced in FVB/N and C57BL/6 mice maintained on the DIO (HF) regimen relative to the control groups (statistically significant reduction only occurred in FVB/N mice), total energy consumption (kcal) was significantly increased by greater than 20% in DIO mice of either genetic background (data not shown, Student's t-test, $p < 0.05$).

Serum analyses of total IGF-1 were performed to further characterize the effects of dietary manipulation on circulating IGF-1 levels. As shown in Tables 2.1 and 2.2, serum IGF-1 levels were significantly different among the diet groups in both FVB/N and C57BL/6 mice, with the greatest differences occurring between mice on the CR and DIO regimen. FVB/N mice on the CR diet exhibited a 55% reduction in total circulating IGF-1 levels relative to FVB/N mice on the DIO diet, while C57BL/6 mice on the CR diet exhibited a 79% reduction in total circulating IGF-1 levels relative to C57BL/6 mice on the DIO diet. These data, in combination with the weight distribution data, indicate that both FVB/N and C57BL/6 mice respond similarly to dietary energy balance manipulation.

Impact of dietary energy balance manipulation on activation of Akt and mTOR in multiple epithelial tissues. In an effort to explore the signaling pathways involved in the dietary energy balance effects on epithelial carcinogenesis, we performed Western blot analyses on protein lysates prepared

from pooled epidermal, hepatic, and DL prostate tissues collected from the FVB/N and C57BL/6 male mice maintained on the different diets as described above (tissue samples were pooled from 10 mice per diet group). As shown in Figure 2.1, CR reduced, whereas the DIO diet increased activation (i.e. as assessed by phosphorylation status) of both Akt and mTOR in all three epithelial tissues examined, relative to mice maintained on the control diet. These data demonstrate that dietary energy balance manipulation altered steady-state signaling to Akt and mTOR in the epidermis, liver and DL prostate.

Effect of dietary energy balance modulation on signaling downstream of Akt and mTOR in multiple epithelial tissues. In light of the observed effects of dietary energy balance modulation on steady-state activation of Akt and mTOR, we next examined several downstream signaling molecules. As shown in Figure 2.2, CR consistently led to decreased activation of downstream effectors of both Akt and mTOR (e.g., GSK-3β, p70S6K, and 4E-BP-1, respectively) in the epidermis, liver, and DL prostate, as compared to mice maintained on either a control or DIO diet. Cyclin D1 levels were also reduced in all three tissues by CR. CR was also shown to inhibit activation of S6 ribosomal protein in the epidermis and liver, however S6 ribosomal protein was not examined in prostate due to the limited amount of tissue available. Thus CR, relative to the control and DIO diets, consistently inhibited signaling downstream of Akt and mTOR,

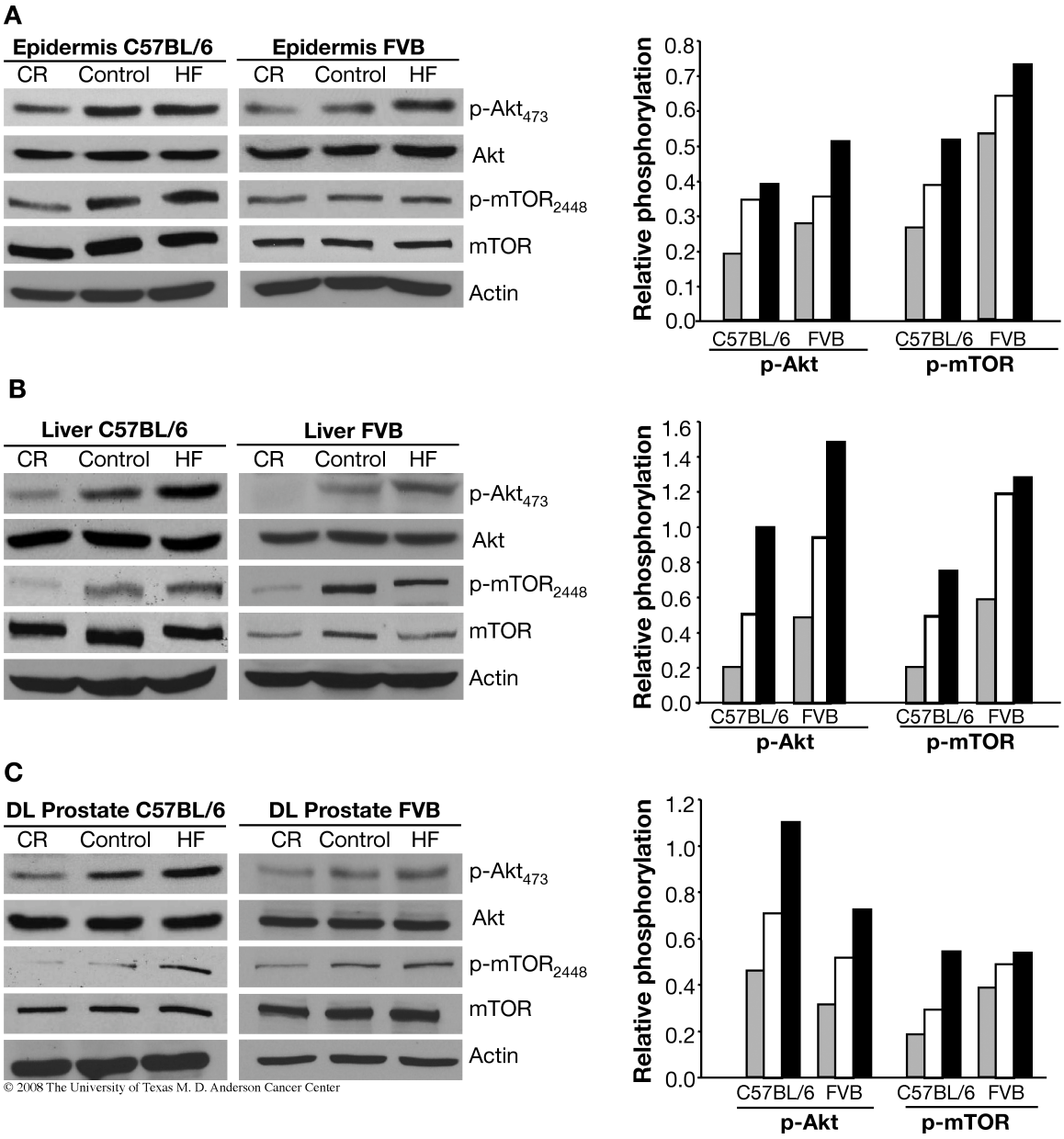

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Figure 2.1. Effect of dietary energy balance manipulation on the activation of Akt and mTOR in multiple epithelial tissues. Pooled protein lysates were prepared from epidermis, liver, and DL prostate excised from FVB/N and C57BL/6 mice maintained on the three dietary regimens for 17 weeks (10 per group). Western blot analyses were then conducted to examine activation of Akt and mTOR in various tissues as follows: A, epidermis; B, liver; C, DL prostate. Quantification was then performed: CR (grey bars), control (white bars), HF/DIO (black bars). Western blots for each pooled tissue sample were repeated with nearly identical results.

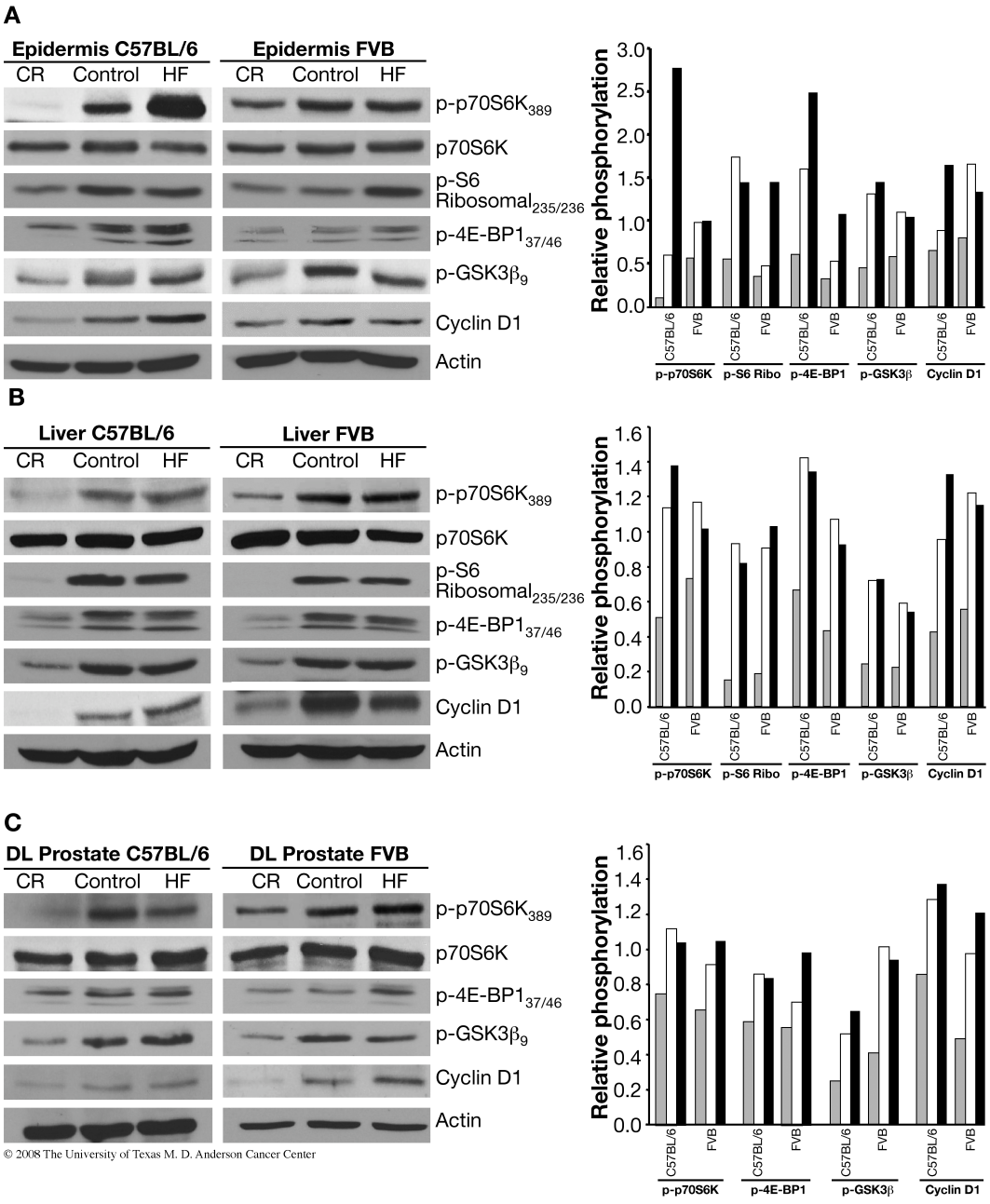

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Figure 2.2. Effect of dietary energy balance manipulation on the activation of Akt and mTOR downstream signaling in multiple epithelial tissues. Pooled protein lysates were prepared from epidermis, liver, and DL prostate excised from FVB/N and C57BL/6 mice maintained on the three dietary regimens for 17 weeks (10 per group). Western blot analyses were then conducted to examine activation of Akt and mTOR downstream signaling molecules in various tissues as follows: A, epidermis; B, liver; C, DL prostate. Quantification was then performed: CR (grey bars), control (white bars), HF/DIO (black bars). Western blots were repeated for each pooled tissue sample with nearly identical results.

independent of genetic background or epithelial tissue examined, suggesting CR functions to suppress Akt and mTOR signaling in multiple epithelial tissues.

While the tissues from mice on the CR diet consistently showed reduced signaling through Akt, mTOR and downstream molecules relative to tissues from mice on the control and DIO diets, differences between the latter two groups were less apparent in some cases. This lack of consistent differences between the control and DIO diet regimens may be due to the fact that both regimens appeared to induce positive energy balance and weight gain. The differences in body weight and adiposity are much greater between mice on the CR diet compared to control mice and mice on the DIO diets.

Role of AMPK in the regulation of mTOR signaling. To explore the role of AMPK in the regulation of mTOR signaling in tissues from mice on the various diets, we performed Western blot analyses to examine its activation status. As shown in Figure 2.3, activation of AMPK, as measured by phosphorylation at threonine 172, was similar in protein lysates from epidermis and prostate across all three diets. This was also true for both genetic backgrounds (i.e., FVB/N and C57BL/6). In contrast, phosphorylation of AMPK was elevated in protein lysates from liver of mice on the CR diet relative to mice on either the control or DIO diets. Again, this was true for either genetic background. These results suggest

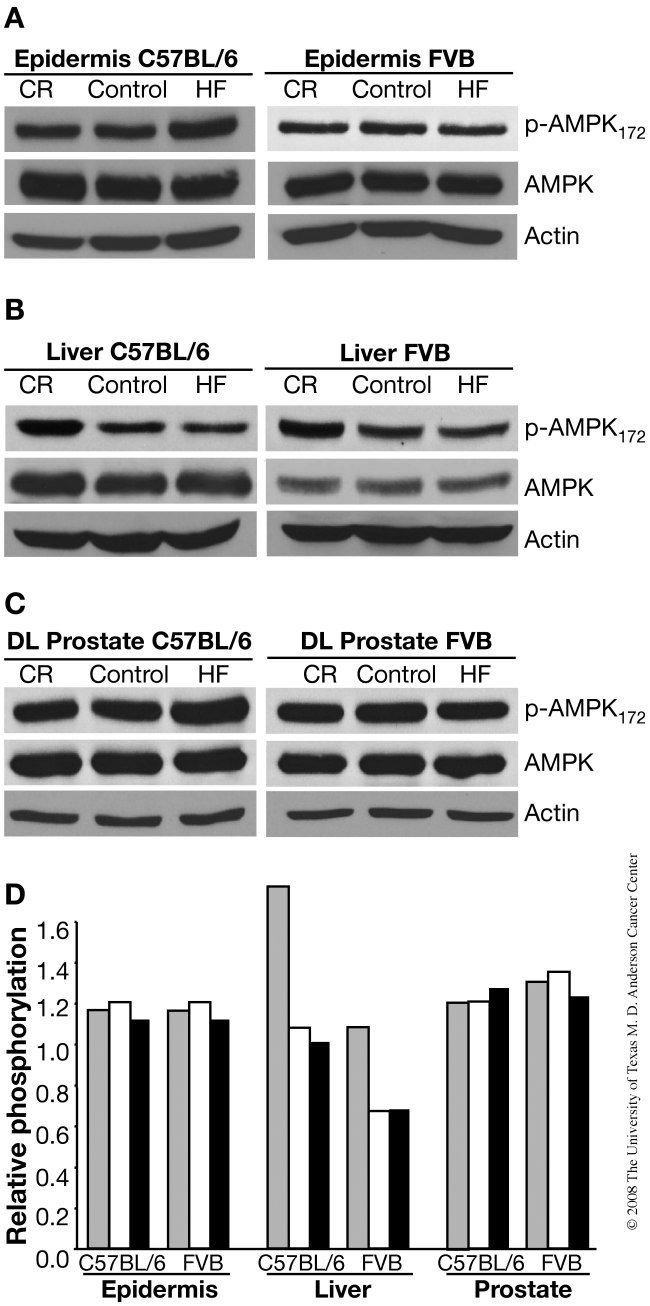

Figure 2.3. Impact of dietary energy balance manipulation on the activation of AMPK in multiple epithelial tissues. Pooled protein lysates were prepared from epidermis, liver, and DL prostate excised from FVB/N and C57BL/6 mice maintained on the three dietary regimens for 17 weeks (10 per group). Western blot analyses were then conducted to examine activation of AMPK in various tissues as follows: A, epidermis; B, liver; C, DL prostate. D, Quantification of AMPK activation in multiple tissues: CR (grey bars), control (white bars), HF/DIO (black bars). Western blots for each pooled tissue sample were repeated with nearly identical results.

that effects of dietary energy balance modulation on AMPK signaling may be tissue dependent. The lack of dietary energy effects on epidermal AMPK was confirmed in subsequent studies using ICR mice (see below).

Impact of dietary energy balance manipulation on activation of MAPK and c-Src. In light of the finding that dietary energy balance differentially modulated activation of Akt and mTOR, we also performed Western blot analyses to examine activation (i.e., phosphorylation) of both Erk1/2 and c-Src. As shown in Figure 2.4, CR reduced, while the DIO regimen enhanced phosphorylation of both of these signaling molecules in all three epithelial tissues.

Effects of dietary energy balance manipulation on EGFR, IGF-1R and Akt/mTOR signaling in the epidermis of female ICR mice. In an effort to further confirm the effect of dietary energy balance manipulation on signaling via the Akt and mTOR pathways, we randomized female ICR mice into four dietary treatment groups (8 mice per group): a) control (10Kcal% fat), b) DIO regimen (60Kcal% fat), c) 15% CR, and d) 30% CR. As shown in Table 2.3, the DIO (HF) diet significantly increased body mass, while both 15% and 30% CR significantly decreased body mass, relative to mice on the control diet, following fifteen weeks of dietary manipulation. Serum IGF-1 levels in ICR mice also correlated with body mass and caloric consumption. While differences in serum IGF-1 levels between the two CR diet groups and the control diet and DIO groups were

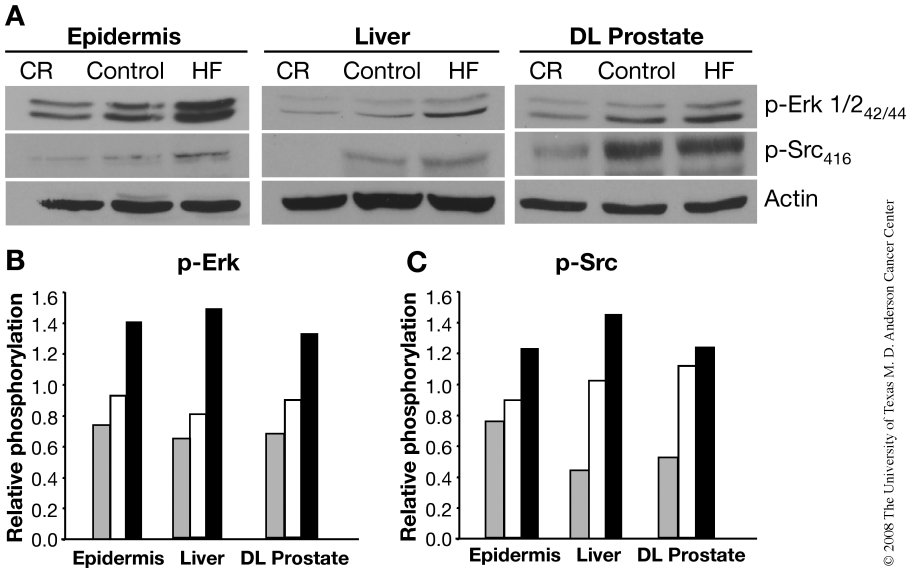

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Figure 2.4. Impact of dietary energy balance manipulation on activation of MAPK and c-Src. Pooled protein lysates were prepared from epidermis, liver and DL prostate excised from C57BL/6 mice maintained on the three dietary regimens for 17 weeks (10 per group). A, Western blot analyses were then conducted to examine the effect of dietary energy balance on activation of Erk1/2 and Src in epidermis, liver, and DL prostate. Quantification was then performed: CR (grey bars), control (white bars), HF/DIO (black bars). B, Quantification of Erk1/2 phosphorylation in multiple tissues. C, Quantification of c-Src phosphorylation in multiple tissues. Western blots for each pooled tissue sample were repeated with nearly identical results.

Table 2.3. Weight distribution, food consumption and serum IGF-1 levels of ICR female mice maintained on a control, high fat, normal and lean diet regimen (n=8/group).

Experimental diet	Mass (g) study start	Mass (g) study end	Average food consumption (g)	Serum IGF-1 (ng/mL) study end
10Kcal% fat	30.8 ± 0.50	40.3 ± 1.06[a]	25.6 ± 0.3[a]	786 ± 86.26[a]
60Kcal% fat	32.2 ± 0.56	55.9 ± 1.28[b]	31.5 ± 0.4[b]	917 ± 84.19[a]
15% CR	30.9 ± 1.07	34.9 ± 0.84[c]	21.5 ± 0.2[c]	542 ± 88.31[b]
30% CR	30.8 ± 0.66	23.3 ± 0.52[d]	18.1 ± 0.2[d]	311 ± 42.5[c]

The data represents mean ± SEM.
[a-d] values are significantly different from each other for parameter given in column heading.

significantly different from one another ($p<0.05$) the differences between the control and DIO groups were not ($p>0.05$). Thus, female ICR mice appeared to respond to dietary energy balance manipulation in a fashion similar to that observed in the male FVB/N and C57BL/6 mice.

To further examine differences in cellular signaling resulting from dietary manipulation, epidermal lysates were collected and pooled from 8 mice maintained on the various diets for 15 weeks and then analyzed by Western blot analysis. As shown in Figure 2.5, dietary energy balance manipulation differentially regulated activation and signaling through both the EGFR and IGF-1R. In this regard, CR (both at 30% and 15% CR) reduced steady-state activation (i.e., phosphorylation) of the EGFR, IGF-1R and IRS-1. In addition, CR (especially 30% CR) led to a significant reduction in activation of Akt, mTOR, and downstream effectors of mTOR (i.e., p70S6K and S6 ribosomal protein), as compared to the mice on the control or DIO (HF) diet. When activation of AMPK was examined, no effect of dietary energy balance manipulation was observed. These latter results are similar to those shown in Figure 2.3 for both FVB/N and C57BL/6. Together, these data suggest that dietary energy balance alters signaling in epidermis through both the EGFR and IGF-1R, which then leads to changes in Akt and mTOR signaling, and these changes are independent of AMPK.

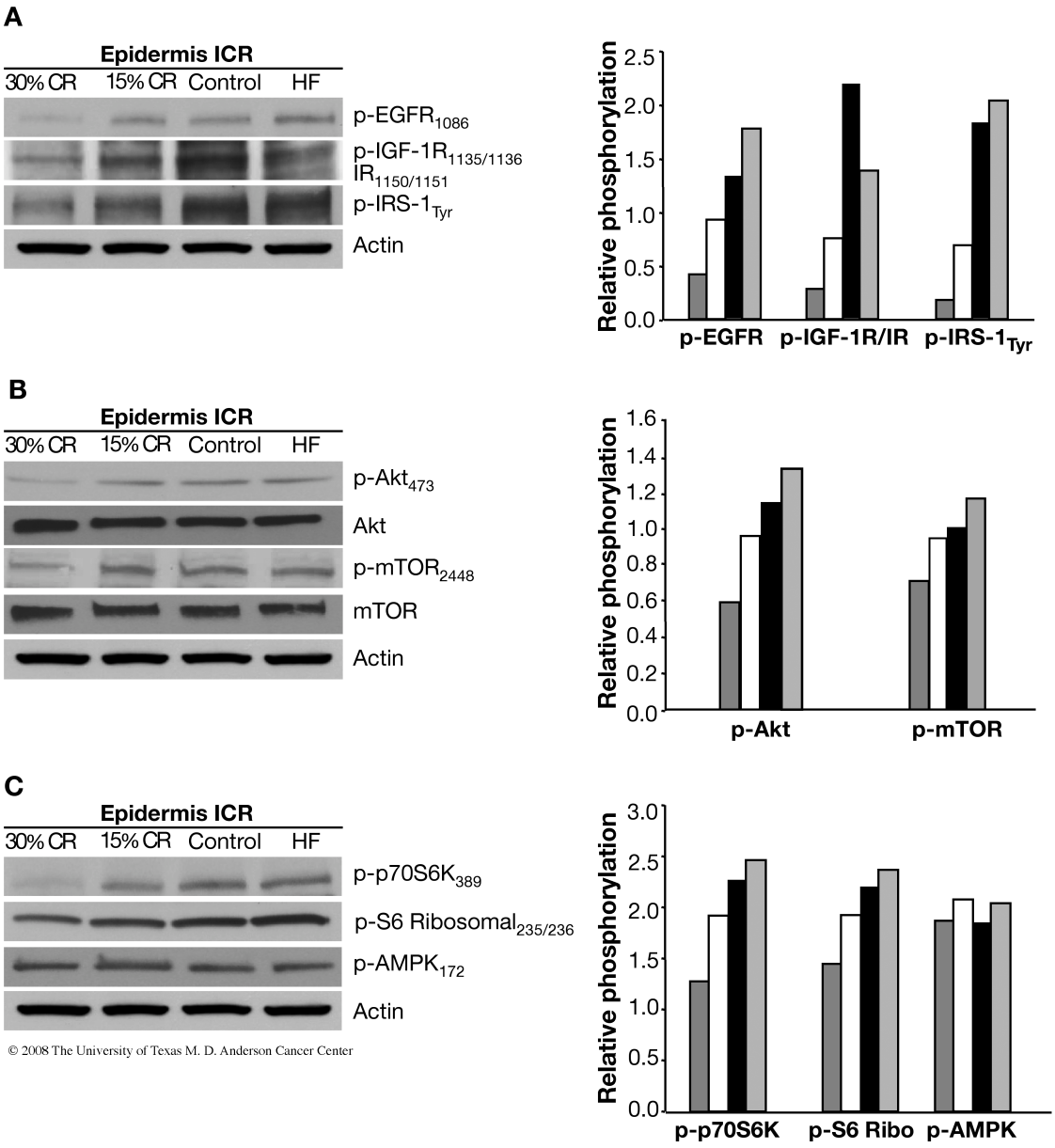

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Figure 2.5. Effect of dietary energy balance manipulation on the activation of epidermal signaling pathways in ICR female mice. Female ICR mice (8 per group) were maintained on the 30% CR (dark grey bars), 15% CR (white bars), control (black bars), and HF/DIO (light grey bars) diets for fifteen weeks. Pooled epidermal lysates were prepared following sacrifice for Western blot analysis. A, Western blot analysis and quantification of the effect of dietary energy balance modulation on epidermal IGF-1R and EGFR activation. B, Western blot analysis and quantification of the effect of dietary energy balance modulation on epidermal Akt and mTOR activation. C, Western blot analysis and quantification of the effect of dietary energy balance modulation on epidermal Akt and mTOR downstream signaling. Western blots for each pooled tissue sample were repeated with nearly identical results.

Discussion

The current study was designed to examine potential mechanisms involved in the effects of dietary energy balance on cellular signaling and epithelial carcinogenesis. Numerous studies have examined the dietary energy balance-cancer link, although few have provided a mechanistic explanation for the observed effects. We employed commonly used regimens for dietary manipulation to examine alterations in steady-state cellular signaling which occur in multiple epithelial tissues. Body weight distribution data generated in the current study paralleled that reported in recent publications, thus validating the model systems used in the current investigation (111, 133-136). Consistent with data published in earlier studies (111, 133), we also found that positive energy balance significantly increased, while negative energy balance significantly decreased, levels of circulating IGF-1 in FVB/N and C57BL/6 mice, relative to the respective controls. Similar effects of dietary energy balance on serum IGF-1 levels were observed in ICR mice maintained on the different diets. Thus, the effects of dietary energy balance on serum IGF-1 levels were independent of genetic background or gender. As noted in the Introduction, changes to globally active circulatory proteins, such as IGF-1, may mediate the effects of dietary energy balance on epithelial carcinogenesis (5, 116). In addition, we found significant effects on steady-state growth factor signaling pathways associated with dietary energy balance modulation. In particular, we found that negative energy balance decreased while positive energy balance increased signaling

through the IGF-1R and EGFR. Furthermore, downstream signaling via several pathways including Akt and mTOR was affected in a similar manner. Overall, the current data suggest that dietary energy balance across the spectrum from negative (CR) to positive (DIO) modulates major growth factor signaling pathways linked to tumor development and tumor progression in multiple epithelial tissues.

As noted in the Introduction, evidence in the literature suggests a role for circulating IGF-1 in modulating tumorigenesis (5, 116). Further evidence comes from using liver IGF-1 deficient mice (138-141). In this regard, deletion of the IGF-1 gene in hepatocytes leads to a 75% reduction in circulating IGF-1 levels (141), allowing for examination of the impact of reduced circulating IGF-1 on carcinogenesis in multiple tissues, in the absence of dietary manipulation. This LID mouse model has been used to study the effect of reduced circulating IGF-1 on mammary tumor development with both chemical induction and transgenic approaches (139). Additional studies were conducted to examine the effect of reduced circulating IGF-1 levels on growth and metastasis of Colon 38 adenocarcinoma cells following orthotopic transplantation (140). Both of these studies showed significant effects of reduced circulating IGF-1 on tumor growth (inhibition) although the underlying mechanism(s) for these effects were not explored. When serum IGF-1 levels were restored in LID mice using rh-IGF-1 supplementation, the inhibitory effects on colon cancer were abolished. More

recently, we have examined the effect of reductions in circulating IGF-1 on two-stage skin carcinogenesis, a well-established model for epithelial carcinogenesis (138). In these studies, LID mice were highly resistant to two-stage skin carcinogenesis. Mechanistic studies showed that LID mice had a reduced responsiveness to TPA-induced epidermal hyperplasia and epidermal proliferation. Furthermore, biochemical studies showed that LID mice exhibited reduced activation of both the IGF-1R and the EGFR as well as downstream signaling through Akt and mTOR following TPA treatment compared to wild type mice. These data suggested a possible mechanism whereby reduced circulating IGF-1 attenuated activation of Akt and mTOR thus reducing the response of epidermal cells to tumor promotion. Furthermore, these data suggested a possible mechanism whereby reduced circulating IGF-1 that occurs during CR might contribute to its ability to inhibit carcinogenesis in multiple tissues.

In an effort to determine if modulation of circulating IGF-1 levels through manipulation of dietary energy balance led to altered Akt/mTOR signaling, as suggested by the studies with LID mice, we examined the status of critical signaling molecules in the Akt and mTOR pathways in the epidermis, liver, and dorsolateral prostate from both FVB/N and C57BL/6 mice maintained on disparate dietary regimens. As shown in Figure 2.1, positive energy balance enhanced, while negative energy balance inhibited activation of both Akt and mTOR, regardless of tissue or genetic background. Furthermore, the inhibitory

effects of CR were confirmed when activation or expression of downstream effectors of both Akt and mTOR was examined. Of particular interest is the effect of dietary energy balance on Cyclin D1 levels across the three tissues. Cyclin D1 levels are known to be regulated downstream of both Akt and mTOR (142-145) as well as downstream of other signaling pathways activated by growth factor receptor signaling (146-148). As can be seen in Figure 2.2, phosphorylation of GSK-3β, which is immediately downstream of Akt, was modulated by dietary energy balance in all three tissues in a manner consistent with Akt phosphorylation status and Cyclin D1 levels. The importance of Cyclin D1 in epithelial carcinogenesis in mouse epidermis has been shown in a number of studies using transgenic mouse models (149-151) as well as Cyclin D1 knockout mice (152).

Further Western blot analyses were performed to determine if the effects of dietary energy balance on steady-state signaling to the mTOR pathway were controlled by AMPK, a known upstream nutrient-sensing regulator of mTOR signaling (83). As shown in Figure 2.3, AMPK activation was not affected by dietary manipulation in protein lysates from either the epidermis or dorsolateral prostate, however, CR led to activation of AMPK in the liver. Notably, these results in the liver differ from previously published data in which hepatic AMPK phosphorylation was found to be unchanged in C57BL/6 male mice in response to CR (153). There are several differences, however, between the study by

Gonzalez et al (153) and our current study as follows: i) mice were maintained on a 35% CR dietary regimen; ii) CR mice were fed on an alternate-day feeding regimen; and iii) food consumption of control mice was actually restricted by 10%. In our current study, dietary energy balance manipulation appeared to alter signaling to mTOR in epidermis and prostate in a manner independent of AMPK activation, although in liver, AMPK does appear to play a role. Further work will be necessary to explore how changes in dietary energy balance impact AMPK activity *in vivo* in specific tissues.

The data from our recent studies using LID mice suggested that reduced circulating IGF-1 levels affected signaling through both the IGF-1R and EGFR in epidermis of mice during tumor promotion (138). Therefore, Western blot analyses were performed on epidermal protein lysates from mice maintained on control, DIO and CR diets. For these studies, we used female ICR mice and also included a 15% CR group. Notably, we found that dietary energy balance affected signaling through the IGF-1R and EGFR in epidermis consistent with its effects on downstream signaling (see Figures 2.1A, 2.2A and 2.4). This effect on receptor tyrosine kinase (RTK) activation is strikingly similar to the effects seen in LID mice and may explain, in part, the mechanism by which dietary energy balance alters signaling to Akt and mTOR as well as other downstream signaling pathways (Erk1/2 and c-Src). Collectively, these data suggest that serum IGF-1 levels may regulate signaling through both the IGF-1R and EGFR possibly

through modulating crosstalk between these two cell surface RTKs. In support of this latter idea, we previously reported the development of HK1.IGF-1 transgenic mice where expression of *IGF-1* is targeted to epidermis using the *human keratin 1* (*HK1*) promoter (125, 154). Following treatment with TPA, there was a significant increase in EGFR activation in epidermis of HK1.IGF-1 transgenic mice compared to wild type mice. These data suggested that tissue levels of IGF-1 and presumably activation state of the IGF-1R could influence the overall activation level of the EGFR. A number of mechanisms have recently been proposed whereby crosstalk between the EGFR and IGF-1R may occur (88-90, 155, 156). Current experiments are exploring possible mechanisms whereby circulating IGF-1 levels, as modulated by dietary energy balance manipulation, influence crosstalk between the IGF-1R and the EGFR.

In conclusion, we have shown that dietary energy balance modulation alters signaling through the Akt and mTOR pathways in multiple epithelial tissues of mice, regardless of genetic background. The mechanism for the effect of dietary energy balance on signaling to the Akt and mTOR pathways appears, at least in part, to be mediated by changes in serum IGF-1 levels, which then alters signaling through the IGF-1R and EGFR. The role of AMPK in regulating mTOR signaling *in vivo* during energy balance modulation is less clear and may be highly tissue specific. Further work using *in vivo* model systems will be important in this regard. Earlier work reported by Birt and colleagues showed an

attenuation of TPA-induced AP-1 activation (35, 39, 103) and ERK activation in mice on 40% CR diets. We found that dietary energy balance also modulated steady state activation of both Erk1/2 and c-Src (Figure 2.4). Both of these signaling pathways are known to be downstream of RTKs such as the IGF-1R and EGFR (146-148). Finally, recently, Xie et al. (157) reported reduced PI3K and ras signaling in response to TPA in skin of SENCAR mice on CR diets, as compared to controls. In this study, phosphorylation of Akt in epidermis following TPA treatment was reduced by CR and to a greater extent by CR plus exercise. Collectively, these data together with the data presented in our current study supports the hypothesis that dietary energy balance modulates signaling downstream of cell surface receptors. A summary of our current results and its implication for epithelial carcinogenesis is shown in Figure 2.6. The observation that dietary energy balance manipulation leads to altered signaling through both Akt and mTOR in multiple epithelial tissues via modulation of cell surface RTK signaling is novel. Furthermore, the observation that levels of one or more critical cell cycle regulatory proteins (e.g., Cyclin D1) are directly modulated by dietary energy balance is also a novel finding. These findings provide the basis for future translational studies targeting the Akt/mTOR pathway via combinations of lifestyle (i.e., moderate calorie restriction regimens) and pharmacologic approaches for the prevention and control of obesity-related epithelial cancers in humans.

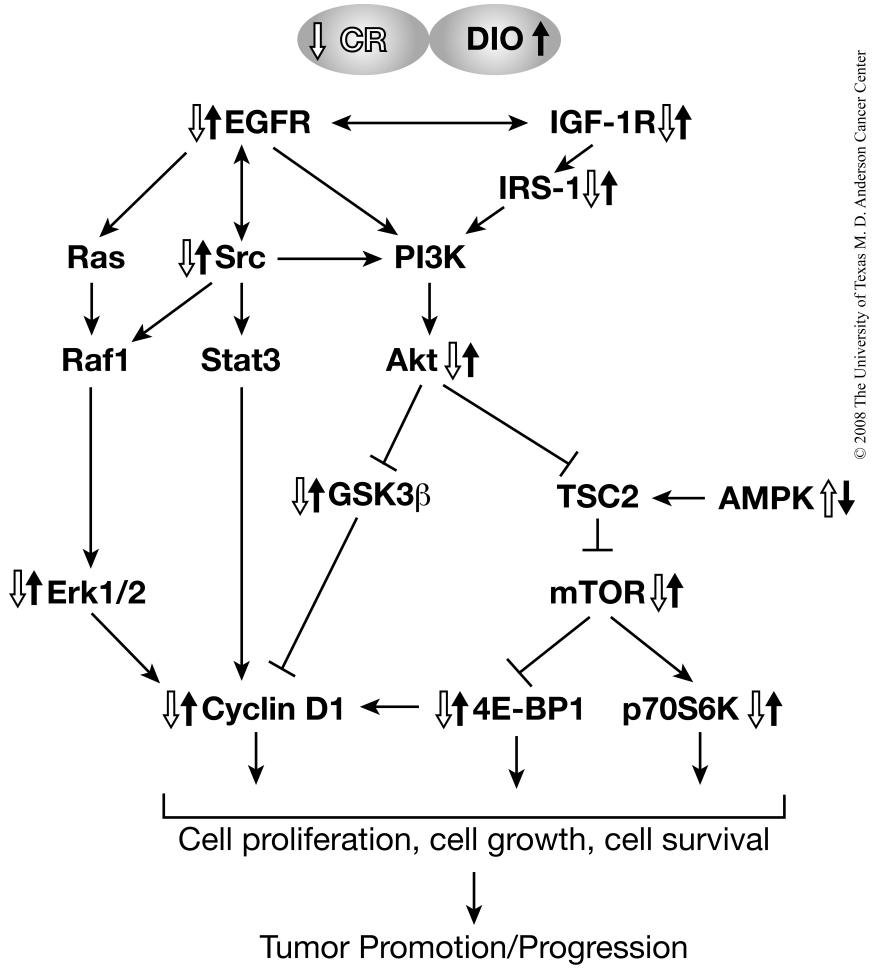

Figure 2.6. Proposed mechanism by which dietary energy balance modulates cellular signaling and epithelial carcinogenesis. Positive energy balance increases, while negative energy balance decreases levels of circulating IGF-1, resulting in differential activation of the IGF-1R. An alteration in signaling through the IGF-1R subsequently affects signaling through the EGFR, due to receptor cross-talk. As a result of this increased or decreased signaling to cell surface receptor tyrosine kinases, signaling through downstream pathways is differentially regulated. Data from the current study show that signaling downstream of Akt and mTOR is differentially modulated by dietary energy balance. In this regard, obesity (⬆) enhances, while CR (⇩) decreases activation of Akt, mTOR, and their downstream effectors. Of particular importance is the differential effect of dietary energy balance on Cyclin D1 expression. Signaling downstream of both Akt and mTOR has been shown to regulate Cyclin D1. Modulation of the Akt and mTOR signaling pathways, in combination with altered signaling through other growth factor signaling pathways, provides a mechanistic explanation for the effect of dietary energy balance on epithelial carcinogenesis.

Chapter III

Reduced levels of circulating IGF-1 inhibits susceptibility to two-stage skin carcinogenesis (49).

Introduction

Dietary energy balance refers to the balance between caloric intake and energy expenditure (9). Findings from both epidemiological and experimental studies suggest chronic positive energy balance, which can lead to obesity, heightens the risk of developing multiple cancers, while a state of negative energy balance, as induced by calorie restriction (CR), decreases these risks (9). While the obesity-cancer link remains poorly understood, the anticancer effects of CR have been more extensively examined. CR has been shown to inhibit formation of spontaneous neoplasia arising in several knockout and transgenic mouse models, suppress tumor growth in tumor transplant models, and inhibit radiation- and chemically-induced carcinogenesis in rodents (7-9, 158). A number of studies have examined the effect of negative energy balance on epithelial tumorigenesis using the two-stage skin carcinogenesis model. This model allows for examination of the effect of CR on both the initiation and promotion stages of tumorigenesis. Consistently, CR during the promotion phase was shown to significantly reduce tumor incidence, multiplicity, and papilloma size (100, 101, 158) . Despite the well-established anticancer effects of CR, no mechanism of inhibition has been clearly identified. Several studies

indicate changes in globally active circulatory factors, particularly IGF-1 or glucocorticoids, may underlie the inhibitory effects of CR (5). We and others have shown that CR reduces circulating IGF-1 levels, whereas restoration of circulating IGF-1 levels in CR animals abolishes many of the inhibitory effects observed with CR alone (46, 159). These experiments suggest a critical role for circulatory IGF-1 levels in regulation of epithelial tumor development and growth.

Previous research conducted in our lab has suggested a role for IGF-1 in skin carcinogenesis. In this regard, overexpression of *IGF-1* under control of the *bovine keratin 5* (*BK5*) promoter dramatically enhanced two-stage skin tumorigenesis (160). In addition, elevated expression of IGF-1 and activation of the IGF-1 receptor (IGF-1R) in keratinocytes led to spontaneous tumor promotion in previously initiated mice (93, 160). Furthermore, we reported upregulation of epidermal phosphoinositide-3 kinase (PI3K) and Akt activities as well as upregulation of cell cycle regulatory proteins in these BK5.IGF-1 transgenic mice (93, 160). Additional studies revealed that the PI3K inhibitor, LY294002, directly inhibited these constitutive biochemical changes observed in the epidermis of BK5.IGF-1 transgenic mice. Finally, topical application of LY294002 inhibited IGF-1-mediated skin tumor promotion in these mice in a dose-dependent manner. These data support the hypothesis that the PI3K/Akt signaling pathway is involved in regulating skin tumor promotion by IGF-1. Data published by Segrelles et al. (94) reported sustained activation of epidermal Akt throughout

two-stage carcinogenesis in mouse skin, supporting a role for activation of this pathway in the development of skin tumors in this model system. Additional data recently published by this same group and others further confirms the involvement of Akt-mediated cellular proliferation in mouse skin tumorigenesis (95-97). We have recently found that diverse tumor promoters lead to activation of epidermal Akt through a mechanism involving the epidermal growth factor receptor (EGFR) (161), providing additional support that Akt signaling plays a critical role in multistage skin carcinogenesis.

To further explore the role of PI3K/Akt signaling in epithelial carcinogenesis, we generated transgenic mice that overexpress either wild type mouse *Akt* or a constitutively active, myristoylated form of mouse Akt in epidermal basal cells under control of the *BK5* promoter. These mice showed enhanced susceptibility to two-stage skin tumorigenesis (162). Western blot analyses performed on protein lysates prepared from epidermis of TPA treated Akt transgenic mice demonstrated enhanced signaling through the PI3K/Akt/mTOR pathway, with heightened activation of signaling molecules found downstream of activated Akt and mTOR. In addition, we observed elevated levels of critical G1→S-phase cell cycle regulatory proteins in the epidermis of these Akt transgenic mice. Collectively, data generated from IGF-1 and Akt overexpressing transgenic mouse lines, as well as other studies, indicate that enhanced signaling through

the PI3K/Akt/mTOR pathway plays an important role in two-stage skin carcinogenesis, especially during the tumor promotion stage.

In the present study, we have utilized the liver deficient IGF-1 mice (LID mice) (141) as a model to replicate the reduction in circulating IGF-1 seen during CR. LID mice, through conditional deletion of the IGF-1 gene in hepatocytes, have an ~75% reduction in the level of circulating IGF-1 (141). Therefore, LID mice were used to examine the impact of reduced circulating IGF-1 on susceptibility to two-stage skin carcinogenesis and to skin tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Biochemical studies then examined the impact of reduced circulating IGF-1 on signaling downstream of both the EGFR and IGF-IR. The results indicate that reduced circulating IGF-1 levels dramatically influence susceptibility to two-stage skin carcinogenesis. In addition, reduced circulating IGF-1 modulates signaling through the Akt/mTOR pathway. This mechanism may explain, at least in part, the inhibitory effects of CR on skin carcinogenesis.

Materials and Methods

Chemicals and biologicals. TPA was purchased from Alexis Biochemicals (San Diego, CA). Bromodeoxyuridine (BrdU) was purchased from Sigma Chemicals Co. (St. Louis, MO), and 7,12-dimethylbenz[*a*]anthracene (DMBA) was obtained from Eastman Kodak Co. (Rochester, NY). The antibody against phospho EGFR (Y1086) was purchased from AbCam (Cambridge, MA), while antibodies against phospho IGF1-R /insulin receptor (IR; Y1135/1136), phospho Akt (S473), Akt total, phospho mTOR (S2448), mTOR total, phospho p70S6K (T389), phospho 4E-BP1 (T37/46), and phospho S6 ribosomal (S235/236) were all purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-beta actin, as well as anti-rabbit and anti-mouse secondary antibodies were obtained from Sigma Chemicals Co. (St. Louis MO). Antibodies against phospho IRS-1 (Y989), phospho IRS-1 (Y632), phospho IRS-1 (Y465), and phospho IRS-1 (Y941) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and combined to generate an anti-phospho IRS-1 antibody cocktail.

Animals. Age-matched LID mice (FVB/N genetic background) and wild type littermates were obtained from Taconic Transgenics (Germantown, NY) where a contract production colony was maintained. Mice were generated and genotyped as previously described (141, 163). Mice were weighed prior to randomization into experimental groups, and then again at the conclusion of the tumor induction experiment. Mice were fed *ad libitum* for the duration of the experiments.

Histological analysis. Dorsal skin samples were fixed in formalin and embedded in paraffin prior to sectioning. Four μm sections were cut and stained with hematoxylin and eosin. Mice received an intraperitoneal injection of BrdU (100 μg/g body weight) in 0.9% NaCl thirty minutes prior to sacrifice. For analysis of epidermal labeling index, paraffin sections were stained with anti-BrdU antibody as previously described (164). To examine the effects of TPA, the dorsal skin of LID and wild type littermates (nine per genotype; 6-8 weeks of age) was shaved, and then topically treated with either 0.2 ml acetone (vehicle), 3.4 nmol TPA, or 6.8 nmol TPA two days later. Mice were treated twice weekly for two weeks and sacrificed 48 hours after the last treatment. Thirty minutes prior to sacrifice, mice were injected with BrdU, as described above. The dorsal skin was removed, fixed in formalin, and processed as described above. Epidermal thickness and labeling index (LI) were then determined as previously described (165).

Two-stage carcinogenesis experiment. The backs of female LID and wild type littermates (88 per genotype; 6-8 weeks of age) were shaved two days prior to initiation. Twenty-eight mice of each genotype received a topical application of 0.2 ml acetone to the shaved dorsal skin, while the remaining mice were initiated with a topical application of 100 nmol DMBA in 0.2 ml acetone. Two weeks following initiation, TPA promotion was begun by topically applying either 6.8

nmol TPA or 13.6 nmol TPA in 0.2 ml acetone twice weekly, until papilloma multiplicity reached a plateau. LID and wild type mice received treatment regimens as follows: acetone/6.8 nmol TPA; DMBA/6.8 nmol TPA; DMBA/13.6 nmol TPA. Tumor incidence (per cent of tumor bearing mice) and multiplicity (papillomas per mouse) were recorded weekly throughout the experiment.

Preparation of epidermal lysates. An additional group of female LID and wild type littermates (thirty-five per genotype; 6-8 weeks of age) were dorsally shaved and treated with a single application of acetone vehicle (0.2 ml), 3.4 nmol TPA, or 6.8 nmol TPA two days later. Four, six, and eight hours following treatment, mice were sacrificed by cervical dislocation. A depilatory agent was applied to the dorsal skin (thirty seconds) and then removed. The skin was then excised and the epidermal layer removed by razor blade into prepared lysis buffer (0.5% Triton X-100; 1% NP40; 10% glycerol; 50 mM HEPES, pH 7.5; 150 nM NaCl; 1mM EGTA; 1.5 mM $MgCl_2$; 10% Sigma inhibitor cocktail; 10% phosphatase inhibitor cocktail I; and 10% phosphatase inhibitor cocktail II) and homogenized using an 18-guage needle. Epidermal scrapings from five mice were pooled to generate lysates. The lysates were then centrifuged at 14,000 RPM for 15 minutes and the supernatant aliquoted for Western blot analysis.

Western blot analysis. For analysis of receptor tyrosine kinase activation and activation of Akt/mTOR signaling molecules, 100 μg of epidermal lysate was

electrophoresed in 4-15% SDS polyacrylamide gels according to the method of Laemmli (166). The separated proteins were then electrophoretically transferred onto nitrocellulose membranes and blocked with 5% BSA in TBS with 1% Tween 20 (TTBS). Blots were then incubated overnight with the antibodies described above in 5% BSA in TTBS. Blots were washed with TTBS three times for 15 minutes each, and then incubated in anti-rabbit and anti-mouse secondary antibody in 5% BSA in TTBS for two hours. Blots were washed again with TTBS three times for 15 minutes each, and protein bands were visualized by enhanced chemiluminescence [Pierce, Rockford, IL]. Protein quantification was then determined using an alpha imager system. The relative density of each protein band, was normalized to the density of the corresponding beta actin band. In the case of p-Akt and p-mTOR, the phospho protein band density was additionally normalized to the total protein band density, thus normalizing phospho protein expression to total protein expression. All normalized values were then expressed as fold induction relative to the acetone LID control.

Serum analysis. LID and wild type mice (eight per genotype) were sacrificed at 6-8 weeks of age using CO_2 asphyxiation. Blood was collected following sacrifice using cardiac puncture, allowed to sit at room temperature for 2 h, and then spun at 7,500 RPM for 7 minutes. Supernatant was collected and spun again under the same conditions. The final supernatant was collected, flash frozen in liquid nitrogen, and then stored at -80ºC until use. Total mouse serum

IGF-1 concentration was then measured using 25 μl samples with a radioimmunoassay (RIA) kit (Diagnostic Systems Laboratories, Inc. Webster, TX).

Statistical analysis. To compare the effects of serum IGF-1 levels on tumor multiplicity, differences in the average number of papillomas per mouse were analyzed using the Mann-Whitney U test. This statistical test was also used to analyze mean differences in epidermal thickness and LI. For comparison of papilloma incidence (tumor incidence) the χ^2 test was utilized. Differences in mean serum IGF-1 levels were analyzed using the Student's t-test. SPSS 11.0 statistical software was used for all analyses. In all cases, significance was set at $p \leq 0.05$.

Results

Serum IGF-1 levels and body mass of LID mice. As previously described, conditional knockout of *igf-1* in the liver led to a significant reduction in circulating IGF-1 levels, with no impact on post-natal growth and development (141). In the current study, we analyzed both parameters at the beginning of the two-stage carcinogenesis experiment. No significant differences in body weight distribution between LID and wild type mice were observed (18.1 ± 0.15 gram average in LID mice versus 18.8 ± 0.15 gram average in wild type mice). Serum analyses were also performed to confirm the effects of targeted deletion in the liver on serum IGF-1 levels. Circulating levels of total IGF-1 were markedly lower in LID mice versus wild type mice (115 ± 22.95 vs 642 ± 41.16; $p < 0.05$, Student's t-test).

Impact of reduced circulating IGF-1 levels on two-stage skin carcinogenesis. To evaluate the impact of circulating IGF-1 levels on epithelial carcinogenesis, a two-stage skin carcinogenesis experiment was conducted using both LID and wild type mice. Groups of LID and wild type littermates were initiated with either acetone (vehicle) or 100 nmol DMBA and then two weeks later promoted topically with either 6.8 nmol or 13.6 nmol TPA given twice weekly. Based on previous published (17, 167) and unpublished studies from our laboratory, FVB mice do not develop skin tumors when initiated with DMBA alone at doses up to 400 nmol per mouse, thus these control groups were not included. Tumor promotion in all groups was continued for 21 weeks. The incidence of

papillomas (tumor incidence) and the average number of papillomas per mouse (tumor multiplicity) were determined weekly for each group. As shown in Fig. 3.1, LID mice initiated with DMBA and promoted with either dose of TPA, as compared to similarly treated wild type mice, had a significantly reduced tumor response ($p < 0.05$, Mann-Whitney U Test). In this regard, at week 21, a 68% inhibition of papilloma formation was observed in the 6.8 nmol TPA treatment group and a 55% inhibition was observed in the 13.6 nmol TPA treatment group, relative to the corresponding wild type littermates (Fig. 3.1A). Tumor volumes were measured in each group after 14 and 20 weeks of promotion with TPA to determine whether circulating IGF-1 levels affected growth of papillomas once formed, however, no significant differences were observed at either timepoint examined (data not shown). No papillomas developed in either group initiated with acetone followed by twice-weekly treatments with TPA (6.8 nmol). Tumor latency was correspondingly affected in both of the LID groups initiated with DMBA and promoted with TPA as compared to similarly treated wild type mice. The time to 50% tumor incidence in wild type versus LID mice promoted with 6.8 nmol TPA was 8.0 versus 13.0 weeks, whereas for mice promoted with 13.6 nmol TPA these values were 6.5 versus 9.0 weeks, respectively (Fig. 3.1B). Differences in tumor latency were statistically significant ($p < 0.05$, χ^2 test). These data demonstrate that circulating IGF-1 levels significantly affected susceptibility to chemically-induced epithelial carcinogenesis in mouse skin.

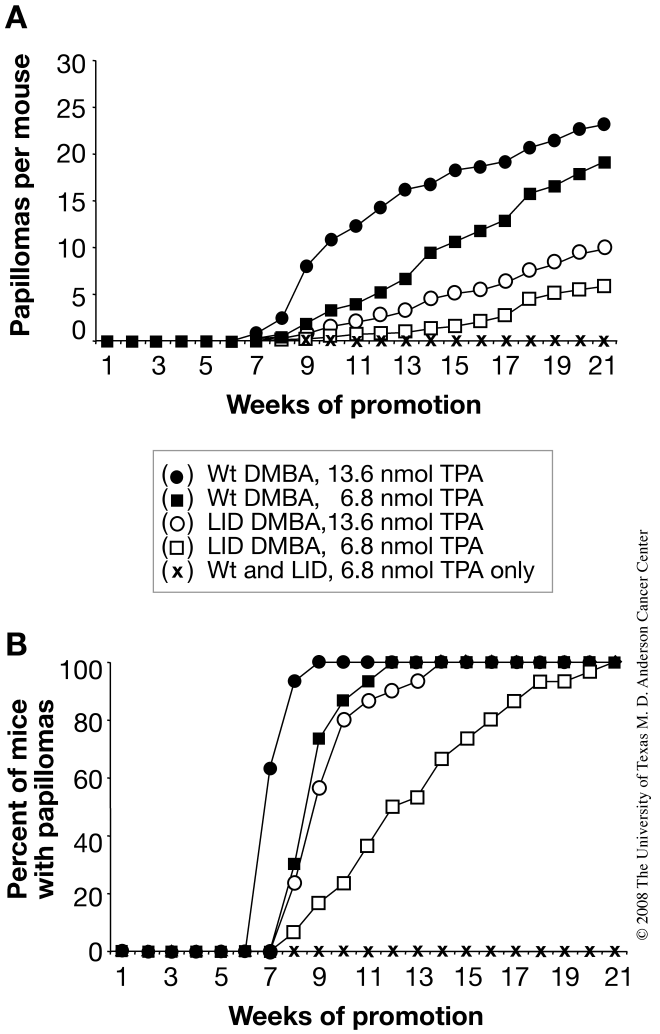

Figure 3.1. Impact of reduced circulating IGF-1 levels on two-stage skin carcinogenesis. Groups of female LID mice and wild type (Wt) littermates (28-30 mice per group) were initiated with either acetone or 100 nmol DMBA. Two weeks following initiation, mice were promoted with either 6.8 nmol TPA or 13.6 nmol TPA twice weekly for 21 weeks. A, Tumor multiplicity (papillomas per mouse). B, Tumor incidence. (●) Wt DMBA, 13.6 nmol TPA; (■) Wt DMBA, 6.8 nmol TPA; (○) LID DMBA, 13.6 nmol TPA; (□) LID DMBA, 6.8 nmol TPA; (X) Wt and LID, 6.8 nmol TPA only (i.e., no initiation). Note that neither Wt nor LID mice treated with TPA only developed any tumors and thus have been represented by the same symbol in the graphs. Differences in the average number of papillomas per mouse at 21 weeks between LID groups and corresponding wild-type littermate groups for each dose of TPA were statistically significant ($p < 0.05$, Mann Whitney U test).

Reduced serum IGF-1 and TPA-induced epidermal hyperproliferation. In light of the dramatic reduction in tumor development seen in LID mice undergoing two-stage skin carcinogenesis as shown in Fig. 3.1, we examined their responsiveness to TPA-induced epidermal hyperproliferation. For these experiments, groups of both LID mice and wild type littermates received four treatments with either vehicle, 3.4 nmol TPA, or 6.8 nmol TPA (given twice-weekly) over the course of two weeks. Forty-eight hours following the last treatment, whole skin sections were processed and then examined for epidermal hyperplasia (measured as epidermal thickness) and epidermal LI. Figures 3.2 and 3.3 show representative H&E stained sections and BrdU stained sections, respectively, for acetone, 3.4 nmol TPA and 6.8 nmol TPA treated groups. Visual inspection of these sections reveals that LID mice displayed reduced epidermal thickness and labeling index in both control (acetone) and TPA-treated groups compared to wild-type mice. Quantitative analyses of the effect of TPA on both epidermal thickness and epidermal LI are summarized in Fig. 3.4. LID mice exhibited statistically significant reductions in epidermal thickness both in the presence and absence of TPA treatment when compared to wild type littermates (Fig. 4A) ($p < 0.05$, Mann-Whitney U test). Examination of the LI showed similar reductions that were also statistically significant (Fig. 3.4B) ($p < 0.05$, Mann-Whitney U test). Thus, reduced circulating IGF-1 decreased epidermal proliferation in the absence of any treatment as well as following TPA treatment.

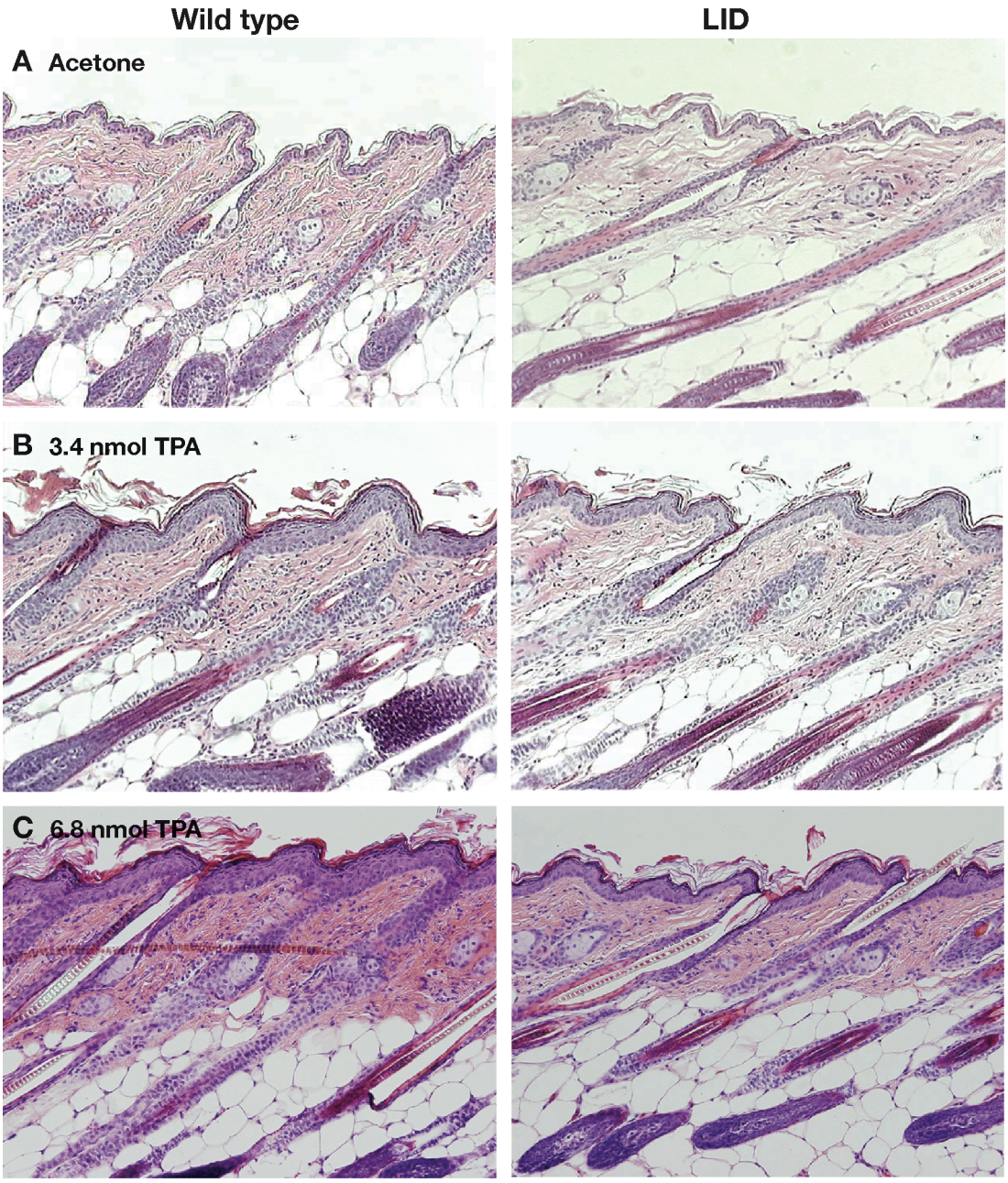

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Figure 3.2. Representative H&E stained sections of dorsal skin. Skin was collected from 8-week old LID mice and wild type littermates after multiple treatments with either acetone, 3.4 nmol TPA, or 6.8 nmol TPA. A, Wild type and LID mice after treatment with acetone; B, Wild type and LID mice after treatment with 3.4 nmol TPA (twice weekly for two weeks); C, Wild type and LID mice after treatment with 6.8 nmol TPA (twice weekly for two weeks).

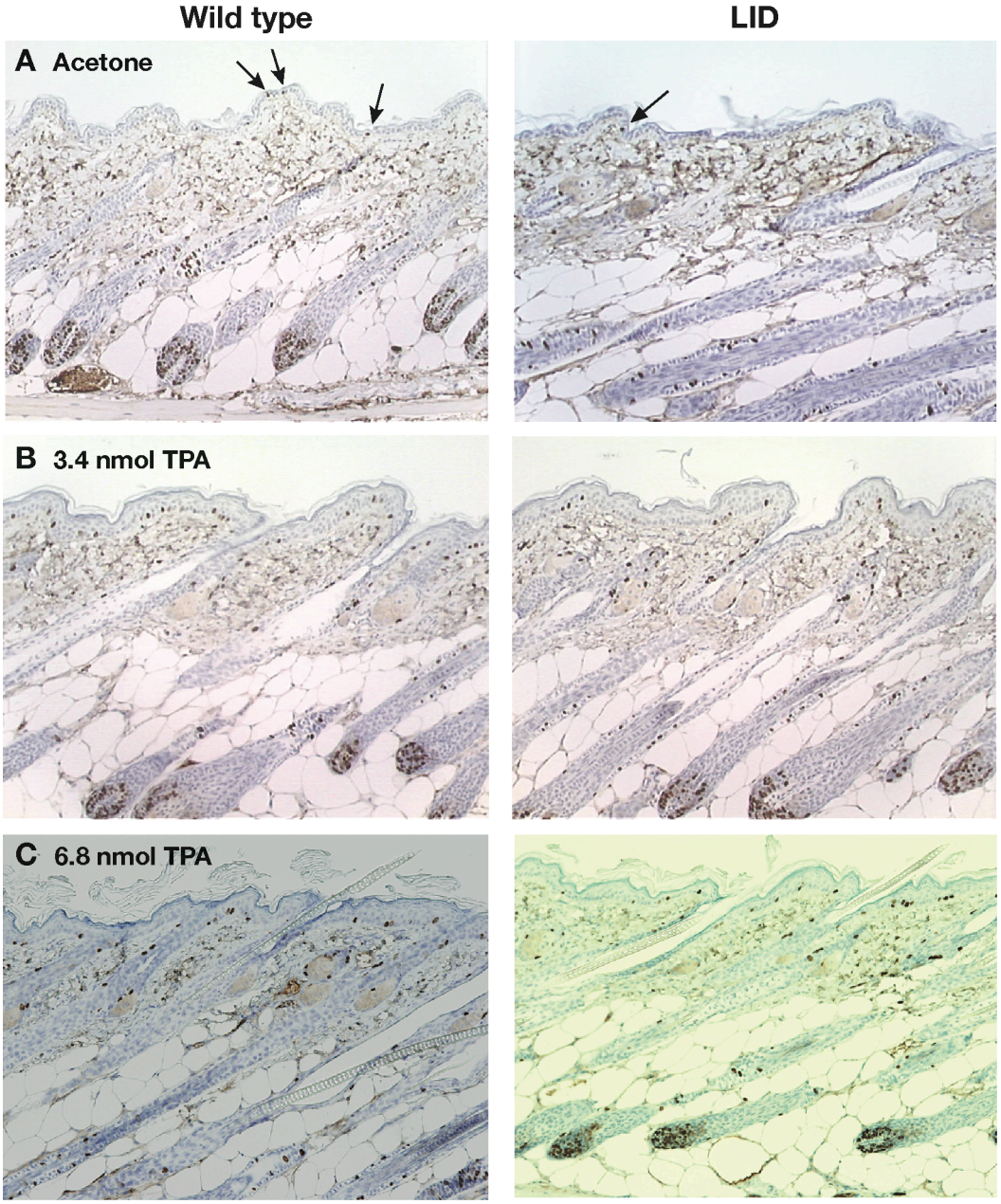

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Figure 3.3. Representative sections of staining for BrdU incorporation in dorsal skin. Skin was collected from 8-week old LID mice and wild type littermates after multiple treatments with either acetone, 3.4 nmol TPA, or 6.8 nmol TPA. A, Wild type and LID mice after treatment with acetone; B, Wild type and LID mice after treatment with 3.4 nmol TPA (twice weekly for two weeks); C, Wild type and LID mice following treatment with 6.8 nmol TPA. Arrows in A, (left and right panels) point to BrdU labeled epidermal cells.

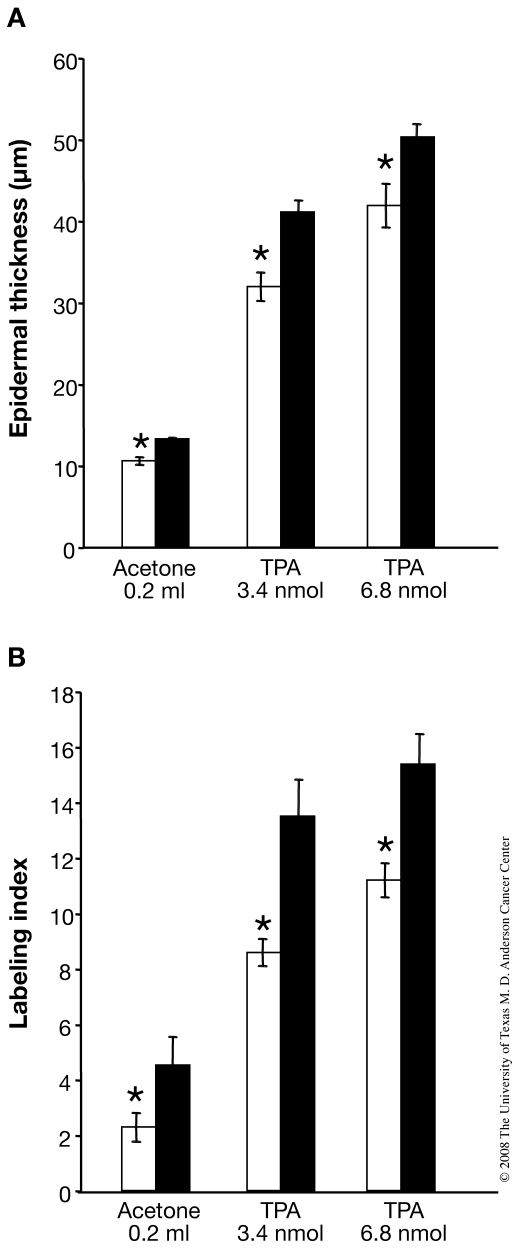

Figure 3.4. Quantitative evaluation of the effects of TPA on epidermal hyperplasia and labeling index in LID mice and wild type (Wt) littermates. A, Epidermal thickness (μm) measured in LID mice (white bars) and Wt littermates (black bars) treated with acetone, 3.4 nmol TPA, or 6.8 nmol TPA. B, Labeling index values (percent of cells stained positive for BrdU) measured in LID mice (white bars) and Wt littermates (black bars) treated with acetone, 3.4 nmol TPA, or 6.8 nmol TPA. Groups of three mice of each genotype received four treatments with either acetone, 3.4 nmol TPA, or 6.8 nmol TPA. Values represent the mean ± SEM. ∗, significantly different than corresponding values from wild-type littermate group ($p < 0.05$, Mann-Whitney U test).

Effect of reduced circulating IGF-1 levels on EGFR, IGF-IR and Akt/mTOR signaling in epidermis. To further explore potential mechanisms whereby a reduction in circulating IGF-1 leads to inhibition of epidermal proliferation and skin tumor promotion, experiments were conducted to evaluate alterations in epidermal signaling molecules in the presence and absence of TPA treatment. For these experiments, groups of LID and wild type mice were treated with a single dose of either acetone, 3.4 nmol TPA, or 6.8 nmol TPA and sacrificed at 4, 6, and 8 hours following treatment. To evaluate alterations in cellular signaling occurring in both vehicle treated and TPA-treated mice, epidermal lysates were prepared for Western blot analysis. As shown in Figs. 3.5 and 3.6, topical treatment of both wild type mice and LID mice with either 3.4 nmol or 6.8 nmol TPA led to rapid activation (i.e., phosphorylation) of the EGFR, IGF-1R/IR, IRS-1, Akt, mTOR and downstream effectors of mTOR (e.g., p70S6K, p-eIF4B, p-4E-BP1 and p-S6-ribosomal protein). These data are consistent with previously published data from our laboratory (125, 161, 162, 168). Notably, the level of activation as assessed by phosphorylation of all signaling molecules examined was reduced in LID mice following TPA treatment compared to wild type littermates (Fig. 3.5 A-C, Fig. 3.6 A-C). In contrast, comparison of the groups that received acetone alone revealed only minor differences in phosphorylation levels of EGFR, IGF-1R, IRS-1, Akt, mTOR, p70S6K, eIF4B, 4E-BP1, and S6 ribosomal protein in the epidermis of LID mice as compared to the wild type mice

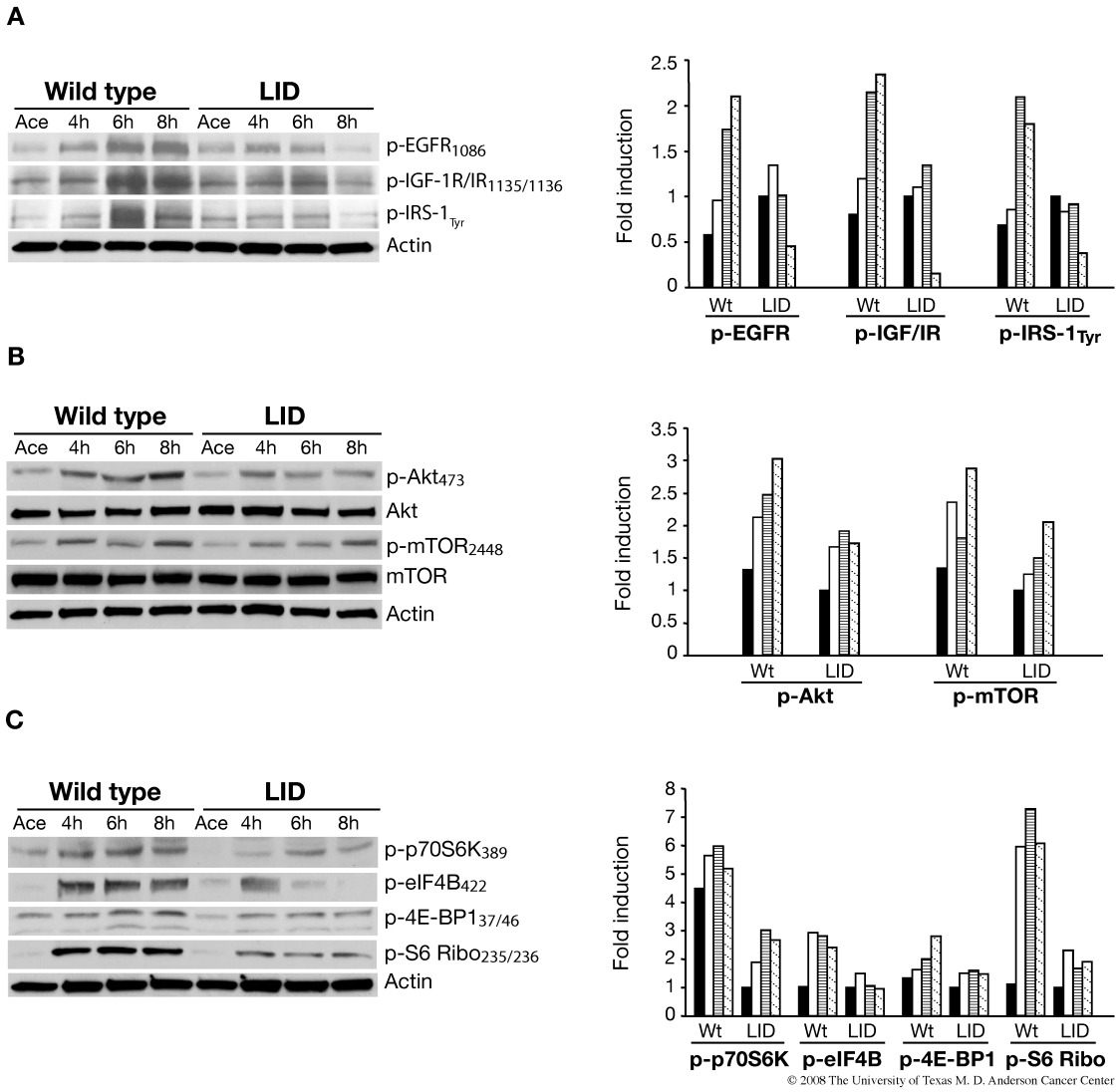

Figure 3.5. Effect of reduced circulating IGF-1 on basal and TPA-induced (3.4 nmol) epidermal signaling pathways in LID mice and wild type (Wt) littermates. Female LID mice and Wt littermates received a single treatment of either acetone or 3.4 nmol TPA. Mice treated with TPA were sacrificed at 4 h (white bars), 6 h (cross hatched bars), or 8 h (broken diagonal bars) following treatment (five mice per genotype at each timepoint), while mice treated with acetone (black bars) were sacrificed 4 h following treatment (again five mice per genotype). Pooled epidermal lysates were prepared for Western blot analysis as described in Materials and Methods. A, Western blot analysis and quantification of the effect of reduced circulating IGF-1 on EGFR and IGF-1R activation. B, Western blot analysis and quantification of the effect of reduced circulating IGF-1 on Akt/mTOR activation. C, Western blot analysis and quantification of the effect of reduced circulating IGF-1 on mTOR downstream signaling. Densitometric graphs represent the Western blots shown following normalization to the corresponding actin. The results shown are representative from one of two completely independent experiments giving nearly identical results.

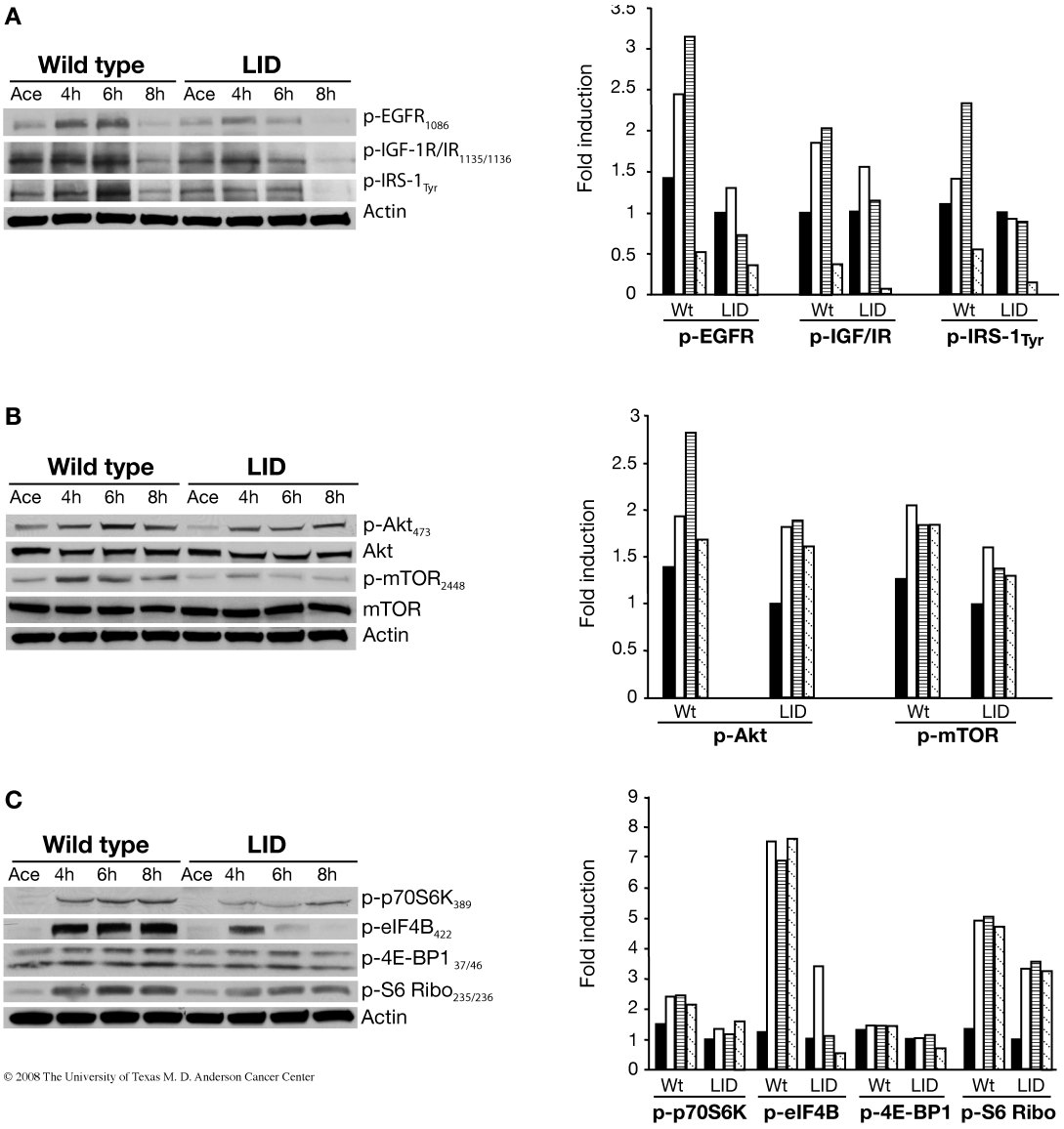

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Figure 3.6. Effect of reduced circulating IGF-1 on basal and TPA-induced (6.8 nmol) epidermal signaling pathways in LID mice and Wt littermates. Female LID mice and Wt littermates received a single treatment of either acetone or 6.8 nmol TPA. Mice treated with TPA were sacrificed at 4 h (white bars), 6 h (cross-hatched bars), or 8 h (broken diagonal bars) following treatment (five mice per genotype at each timepoint), while mice treated with acetone (black bars) were sacrificed 4 h following treatment (five mice per genotype). Pooled epidermal lysates were prepared for Western blot analysis as described in Materials and Methods. A, Western blot analysis and quantification of the effect of reduced circulating IGF-1 on EGFR and IGF-1R activation. B, Western blot analysis and quantification of the effect of reduced circulating IGF-1 on mTOR activation. C, Western blot analysis and quantification of the effect of reduced circulating IGF-1 on mTOR downstream signaling. Densitometric graphs represent the Western blots shown following normalization to the corresponding actin. The results shown are representative from one of two completely independent experiments giving nearly identical results.

(see again Fig. 3.5A-C; Fig. 3.6 A-C). Thus, reduced circulating IGF-1 levels appeared to attenuate signaling through the EGFR and IGF-1R following treatment with TPA. In particular, a marked reduction in signaling through both Akt and mTOR was observed. Note that nearly identical results were obtained in repeat experiments using both doses of TPA.

Discussion

The current study was designed to examine the impact of reduced circulating IGF-1 on epithelial carcinogenesis in a well-established model system. In this regard, LID mice exhibited a significantly reduced tumor response in terms of both incidence and multiplicity following two-stage skin carcinogenesis using DMBA as the initiator and TPA as the promoter. Histologic evaluation of dorsal skin from LID mice showed statistically significant reductions in epidermal thickness and LI compared to wild type littermate controls, both in the absence and presence of treatment with TPA. The reduction in epidermal proliferation observed in LID mice following TPA treatment may explain, in part, the significant inhibition of skin tumorigenesis seen in LID mice. As noted in the Introduction, previous work from our laboratory using transgenic mice where IGF-1 expression was elevated at the tissue level through targeted overexpression in skin keratinocytes showed that these mice have increased susceptibility to two-stage skin carcinogenesis (125, 160, 169). The current results clearly demonstrate that levels of circulating IGF-1 can also directly impact susceptibility to skin tumor promotion and two-stage skin carcinogenesis.

The LID mouse model has been used in previous studies to examine the effect of reduced circulating IGF-1 on mammary tumor development (both chemically-induced as well as tumors that develop in a transgenic model) (139). Furthermore, LID mice were used to study the effect of reduced circulating IGF-1

levels on growth and metastasis of Colon 38 adenocarcinoma cells following orthotopic transplantation (140). In both of these reports, reduced circulating IGF-1 levels had a significant inhibitory effect on tumor development. In the colon cancer study by Wu et al. (140), there was also a reduction in liver metastases in LID mice relative to wild type controls and these authors suggested that altered VEGF levels and differential vascularization might have played a role in some of the observed differences in tumor progression. When serum IGF-1 levels were restored in LID mice using rh-IGF-1 supplementation, the inhibitory effects on colon cancer were abolished. Although these studies suggested an important role for IGF-1 in mediating tumor growth and metastasis, neither study provided significant insight regarding the molecular mechanisms evoked as a consequence of physiological reduction of IGF-1 and its impact on tumor development.

In an effort to further elucidate mechanisms for the inhibitory effect of reduced circulating IGF-1 on epithelial carcinogenesis, Western blot analyses were performed using epidermal lysates from LID mice and wild type littermates. Previous studies from our laboratory have shown that activation of EGFR and erbB2 is an early event in mouse keratinocytes following exposure to tumor promoters, including TPA, either *in vivo* or *in vitro* (125, 161, 168, 170). Activation of the EGFR and erbB2 appears to be due to increased availability of EGFR ligands through a combination of ectodomain shedding and increased synthesis

(161, 171-173). Activation of EGFR and erbB2 in epidermis following TPA treatment leads to activation of downstream signaling pathways such as src (170), Stat3 (174) and Akt (161). Furthermore, following topical treatment with TPA the IGF-1R becomes activated, although upregulation of ligands (i.e., IGF-I or IGF-II) does not appear to account for this activation (175). As shown in Figures 5A and 6A, the EGFR, IGF-1R and IRS-1 were activated in epidermis early after topical treatment of LID and wild type mice with TPA. However, the level of activation of these receptors was reduced in LID mice compared to wild type littermates (~50% overall reduction in phosphorylation). These data suggest that reduced circulating IGF-1 levels influenced signaling through both the EGFR and IGF-1R following TPA treatment. In support of this idea, we previously reported the development of HKI.IGF-1 transgenic mice where expression of *IGF-1* is targeted to epidermis using the *human keratin 1* (*HK1*) promoter (125, 169). Following treatment with TPA, there was a significant increase in EGFR activation in epidermis of HKI.IGF-1 transgenic mice compared to wild type mice. These data suggested that tissue levels of IGF-1 and presumably activation state of the IGF-1R could influence activation of the EGFR. A number of mechanisms have recently been proposed whereby crosstalk between the EGFR and IGF-1R may occur (89, 90, 156, 176, 177). Current experiments are exploring possible mechanisms whereby reduced circulating IGF-1 levels may disrupt crosstalk between the IGF-1R and EGFR.

Further analysis of signaling downstream of the EGFR and IGF-1R showed reduced activation of Akt and mTOR and signaling downstream of mTOR in LID mice relative to wild type littermates following TPA treatment. As noted in the Introduction, TPA induces activation of Akt in mouse epidermis very early after topical treatment (161). Furthermore, Akt becomes constitutively activated in papillomas and remains constitutively activated throughout multistage carcinogenesis in mouse skin (94, 96, 97, 161). This activation of Akt has been shown to critically regulate proliferation throughout epithelial carcinogenesis (94, 162). In the present study, decreased signaling through the Akt and mTOR pathways in LID mice may account, in part, for the reduced susceptibility of LID mice to tumor promotion and two-stage skin carcinogenesis. This conclusion is supported by the following lines of evidence: i) the PI3K inhibitor LY294002 blocks IGF-1 mediated skin tumor promotion in BK5.IGF-1 transgenic mice (93); ii) Akt knockout mice display reduced sensitivity to two-stage skin carcinogenesis using the DMBA-TPA protocol (178); iii) overexpression of Akt sensitizes the epidermis to TPA-induced epidermal hyperplasia and skin tumor promotion (162); and iv) topical application of rapamycin effectively blocks TPA-induced activation of mTOR and epidermal hyperproliferation in wild type mice [B. Hammann, T. Moore and J. DiGiovanni, unpublished data].

Finally, LID mice were used in the current study to mimic the reduction of circulating IGF-1 induced by CR (5, 141). Although the effects of CR extend

beyond reductions in serum IGF-1 levels and include alterations in many serum hormones, adipokines and cytokines (5), the exact role of IGF-1 levels in the anti-carcinogenic effects of CR remain to be determined. The current data, taken together with previously published studies (139, 140) suggest that reduced circulating levels of IGF-1 can dramatically influence susceptibility to tumorigenesis in multiple epithelial tissues. Our study provides one plausible mechanism for this effect, namely, reduced signaling through the EGFR and IGF-1R and subsequent attenuation of downstream signaling through Akt and mTOR. Very recently, Xie et al. (104) reported reduced PI3K and ras signaling in response to TPA in skin of SENCAR mice on CR diets. In this study, phosphorylation of Akt in epidermis following TPA treatment was reduced by CR and to a greater extent by CR in addition to exercise. In preliminary experiments, we have found reduced signaling through the EGFR, IGF-1R, Akt and mTOR in epidermis of mice on CR diets following TPA treatment compared to mice on control diet [T. Moore and J. DiGiovanni, unpublished studies]. Collectively, these data support the hypothesis that reduced circulating IGF-1 during CR impacts cell signaling at the tissue level during tumor promotion thereby reducing susceptibility to two-stage skin carcinogenesis.

In conclusion, we have shown that reduced circulating IGF-1 (induced through genetic manipulation) in the absence of dietary energy restriction dramatically reduces susceptibility to two-stage skin carcinogenesis. The

mechanism for this effect appears to involve, at least in part, reduced epidermal proliferation in response to the tumor promoter TPA. This appears also to be due to reduced signaling through the EGFR and IGF-1R and manifested, at least in part, by reduced signaling through Akt and mTOR. The observation that a decrease in the circulating level of IGF-1 can impact signaling through cell surface receptors in a tissue such as skin is novel and provides further support that such changes contribute to the overall susceptibility to carcinogenesis.

Chapter IV

Dietary energy balance modulates susceptibility to skin tumor promotion through altered IGF-1R and EGFR crosstalk and downstream signaling.

Introduction

Energy balance refers to the relationship between caloric consumption and energy expenditure (9). Epidemiological and animal studies have established a direct correlation between positive energy balance (overweight, obesity) and the risk for developing or risk of mortality from multiple cancers (1, 10, 111, 113, 115, 179, 180). In contrast, negative energy balance (calorie restriction, CR) has been shown to consistently inhibit tumorigenesis, regardless of mode of tumor induction (i.e., spontaneous neoplasia in transgenic or knockout mouse models, chemically- or radiation-induced tumorigenesis, tumor transplant models) (7-9, 158). Studies have been conducted to evaluate the impact of high fat diet consumption and severe CR (40% CR) on two-stage skin carcinogenesis. This chemically-induced model for epithelial carcinogenesis enables evaluation of all stages of tumor development, including tumor initiation, promotion and progression (91). Forty percent CR consistently inhibited tumor promotion by TPA, however, no effects were observed on either tumor initiation or tumor progression (6, 100, 101). Studies that examined the impact of a high-fat diet (46 Kcal% fat), as compared to a low-fat, control diet (11 Kcal% fat), on two-stage

skin carcinogenesis yielded inconsistent results. *Ad libitum* consumption of the high-fat diet did not significantly increase tumor incidence, multiplicity or progression, regardless of the presence or absence of diet-induced changes in body mass (101, 105). In contrast, isocaloric consumption of a high-fat diet, as compared to control diet, led to a significant increase in both tumor incidence and multiplicity, but not progression, despite the inability of the high-fat diet to induce changes in body mass when isocalorically consumed (106). These findings suggest a role for energy balance in the modulation of two-stage skin carcinogenesis; however, the effect of diet-induced obesity (DIO) remains unclear.

Several mechanisms have been proposed to account for the inhibitory effects of CR on two-stage skin carcinogenesis. Early studies conducted by Birt and colleagues suggested an inhibitory effect of 40% CR on epithelial carcinogenesis due to heightened corticosterone levels (7, 181). Specifically, adrenalectomy of CR mice partially reversed the inhibitory effect of CR on two-stage skin carcinogenesis, while corticosterone supplementation of adrenalectomized mice reintroduced CR-mediated inhibition (7, 181). Further studies using 40% CR demonstrated a reduction in TPA-induced AP-1 activation, as well as a reduction in ERK phosphorylation in mouse epidermis following dietary energy restriction (35, 39, 103). While these studies provide a plausible link between increased levels of corticosterone and CR-mediated inhibition of

tumorigenesis, these effects may instead be related to an increase in stress response. In other model systems, moderate and mild CR has been shown to inhibit tumorigenesis, in a manner similar to 40% CR, however, these less severe CR regimens do not induce the dramatic increase in levels of circulating corticosterone observed with 40% CR. This inconsistency suggests that another mechanism is responsible for the effect of both negative and positive energy balance on two-stage skin carcinogenesis. Xie et. al. reported a reduction in TPA-mediated activation of PI3K and Ras signaling following 20% CR (104). These findings are supported by recent publications from our laboratory that show differential effects of dietary energy balance (CR and DIO) on activation and signaling downstream of the IGF-1R and the EGFR in mouse epidermis (110 and see Chapter II). Data from this study and others suggests that these effects of dietary energy balance on cellular signaling may be mediated by diet-induced changes in circulating IGF-1 levels (49, 110 and see Chapter III).

It has been well established that CR reduces, while DIO increases levels of circulating IGF-1 (5, 110, 111). Early studies demonstrated that IGF-1 supplementation in CR mice abolished the anti-tumor effects of CR (46-48). Furthermore, a genetic reduction in levels of circulating IGF-1 (comparable to those observed in CR mice), using the liver IGF-1 deficient (LID) mouse model, inhibited mammary tumor development and growth and metastasis of orthotopically transplanted Colon 38 adenocarcinoma cells, while restoration of

IGF-1 to the level observed in wildtype mice eliminated the inhibitory effects of genetic IGF-1 depletion on colon cancer (49, 139, 140). Additional studies using A-Zip/F-1 mice, which lack white adipose tissue but have elevated levels of IGF-1 (similar to DIO mice) demonstrated enhanced susceptibility to multiple epithelial cancers (24). Taken together, these findings suggest a critical role for levels of circulating IGF-1 in the regulation of dietary energy balance effects on tumorigenesis.

IGF-1 acts as ligand for the IGF-1R, which has been shown to regulate multiple cellular processes, including cellular growth, proliferation and survival (122, 123). Studies conducted in our laboratory using transgenic mouse models that overexpress *IGF-1* in the epidermis (HK1.IGF-1 and BK5.IGF-1) demonstrated a direct correlation between enhanced IGF-1R signaling and susceptibility to two-stage skin carcinogenesis (125, 160). Further studies using these transgenic mouse models identified two potential mechanisms that could account for the enhancing effect of *IGF-1* overexpression on epithelial carcinogenesis: i) direct effects on signaling downstream to PI3K/Akt/mTOR; ii) and indirect effects on EGFR downstream signaling to PI3K/Akt/mTOR due to IGF-1 mediated EGFR activation (93, 125, 160, 182). Overexpression of *Akt* or a constitutively active form of Akt (BK5.Akt wt, BK5.Aktmyr) further confirmed a critical role for Akt and mTOR signaling in the modulation of two-stage skin carcinogenesis. These studies demonstrated that heightened Akt activation

enhanced cellular proliferation, through effects on cell cycle progression related proteins, and inhibited cell survival due to inhibition of apoptosis related proteins (98). While no further analysis of the effect of *IGF-1* overexpression on EGFR activation was conducted, several mechanisms for IGF-1 mediated IGF-1R/EGFR crosstalk have been suggested and include: IGF-1 induced ectodomain shedding of EGFR ligands, IGF-1 induced transcription of EGFR and EGFR ligands, and IGF-1R/EGFR heterodimerization (86-90).

In the present study, we utilized diets of varying caloric density to determine the impact of both positive and negative energy balance on multistage carcinogenesis in mouse skin. We then conducted short-term experiments using lean (30% CR), normal (15% CR), overweight (10 Kcal%) and obese (DIO diet, 60 Kcal%) mice to determine potential mechanisms whereby dietary energy balance modulates epithelial carcinogenesis. Specifically, we evaluated diet-induced changes in epidermal hyperproliferation and examined the impact of dietary manipulation on growth factor signaling and levels of cell cycle regulatory proteins following TPA treatment. Finally, using both *in vitro* and *in vivo* approaches, we determined the impact of IGF-1 on IGF-1R and EGFR crosstalk. Collectively, our findings suggest that dietary energy balance affects susceptibility to epithelial carcinogenesis, due at least in part to diet-induced changes in IGF-1R and EGFR signaling, which then alters levels of cell cycle related proteins, thus regulating epidermal proliferation. These diet-induced

changes in IGF-1R and EGFR activation are thought to be mediated, at least in part, by levels of circulating IGF-1 which then modulates IGF-1R/EGFR crosstalk.

Materials and Methods

Chemicals. TPA was purchased from Alexis Biochemicals (San Diego, CA). Bromodeoxyuridine (BrdU) was purchased from Sigma Chemicals Co. (St. Louis, MO), and 7,12-dimethylbenz[*a*]anthracene (DMBA) was obtained from Eastman Kodak Co. (Rochester, NY). Recombinant human IGF-1 (rhIGF-1) was purchased from Sigma Aldrich (St. Louis, MO) and epidermal growth factor (EGF) was purchased from BD Biosciences (San Jose, CA).

Animals. ICR female mice (3-4 weeks of age) were purchased from Harlan Teklad (Indianapolis, IN). Mice were group housed for all experiments; however, mice maintained on a 30% CR regimen were separated into dividers for a period of one hour for feeding purposes. Mice were weighed before randomization into experimental groups and then weighed bimonthly for the duration of both the long- and short-term experiments.

Diets. All diets were purchased in pellet form from Research Diets, Inc. (New Brunswick, NJ). Four diets ranging in caloric density were used for both the long- and short-term studies: 30% CR diet (D03020702), 15% CR diet (D03020703),10 Kcal% fat (control, D12450B, *ad libitum*) and 60 Kcal% fat (DIO, D12492, *ad libitum*). These diets have been previously described (9). CR was achieved by administering a daily aliquot equivalent to either 70% or 85% of the daily amount of total energy consumed by the control diet group. Both CR diets were

isonutrient relative to the control group. For short-term experiments, mice were placed on the control diet for a one-week equilibration period, after which they were maintained on the four experimental diets for 15 weeks. Mice were sacrificed by either CO_2 asphyxiation (serum collection) or cervical dislocation and tissues and blood were collected, processed and stored as described below.

Serum analysis and body fat composition. Blood was collected immediately following CO_2 asphyxiation. Samples were spun for 7 minutes at 7500 RPM. The supernatant (serum) was collected and flash frozen in liquid nitrogen for later analysis. Serum IGF-1 levels were determined using a radioimmunoassay (RIA) kit (Diagnostic Systems Laboratories, Webster, TX). Serum insulin, leptin and adiponectin were analyzed using a Lincoplex (Linco Research, Inc., St. Charles, MO) Luminex bead assay kit on a BioRad Bio-Plex Workstation (Hercules, CA). Percent body fat was determined at the termination of the experiments by DXA scanning using a GE Lunar Piximus II densitometer, as previously described (183).

Two-stage skin carcinogenesis experiment. ICR female mice were placed on the 10 Kcal% fat control diet at 7 weeks of age and initiated with 25 nmol of 7,12-dimethylbenz[*a*]anthracene (DMBA) (Eastman Kodak Co., Rochester, NY). Four weeks following initiation, mice were randomized into groups and placed on the four dietary treatment regimens (n=30/diet), on which they were maintained for

the duration of the experiment. Four weeks following diet commencement, mice were promoted twice weekly with 3.4 nmol of 12-O-tetradecanoylphorbol-13-acetate (TPA) (Alexis Biochemicals, San Diego, CA) for 50 weeks. Tumor incidence (percent mice with papillomas) and tumor multiplicity (average number of tumors per mouse) were determined weekly until multiplicity reached a plateau (29 weeks). Carcinoma incidence and the average number of carcinomas per mouse were determined weekly from the time of initial detection until 50 weeks of tumor promotion. Suspect squamous cell carcinomas (SCCs) were collected for histopathological confirmation and carcinoma counts were adjusted to reflect pathological evaluation.

Histological analysis of epidermal hyperproliferation. ICR mice were maintained on the four diets, as described above, for 15 weeks, after which they were treated twice weekly for two weeks with either acetone vehicle or 3.4 nmol of TPA (n=3/diet/treatment). Mice were injected with bromodeoxyuridine (BrdU) (Sigma Aldrich, St. Louis, MO) (100ug/g body weight) thirty minutes prior to sacrifice and whole skin sections were excised, processed and evaluated for both epidermal thickness and labeling index as previously described (49).

Preparation of epidermal protein lysates. For short-term in vivo experiments, ICR mice were maintained on the diets for 15 weeks, after which they were treated with a single application of either acetone vehicle or 3.4 nmol of TPA.

Mice were sacrificed 6H (acetone, TPA) or 18H (TPA) following treatment, epidermis was scraped and protein lysates were prepared as previously described (49). Epidermal scrapings from six mice were pooled for each treatment/timepoint.

Cell culture. C50 cells (a gift from Dr. Susan Fischer, MD Anderson – Science Park Research Division, Smithville, TX) were cultured as previously described (184), however they were plated directly in KGM-2 media (Lonza, Basel, Switzerland) without calcium. When cells reached ~80% confluency, they were serum and growth factor starved for 24 hours. Plates were then stimulated with either 25 ng/mL recombinant human IGF-1 (rhIGF-1) (Sigma Aldrich, St. Louis, MO) or 10 ng/mL epidermal growth factor (EGF) (BD Biosciences, San Jose, CA) and harvested at multiple timepoints for either protein or RNA isolation.

Immunoprecipitation and Western blot analysis. Immunoprecipitation and Western blot analyses were performed using lysates prepared from either epidermis of ICR mice maintained on diets of varying caloric density or cultured mouse keratinocytes stimulated with IGF-1 or EGF. For co-immunoprecipitation experiments, lysates were precipitated with either IGF-1R (Cell Signaling, Danvers, MA) or EGFR (Millipore, Billerica, MA) antibodies using the Dynabead Protein G IP kit (Invitrogen, Carlsbad, CA). Western blots analyses were performed as previously described (49). Blots were probed with the following

antibodies against: phospho EGFR (Y1086), phospho ErbB2 (Y1248) (AbCam, Cambridge, MA); phospho IGF1-R/insulin receptor (IR; Y1135/1136), IGF-1Rβ total, phospho Akt (S473), Akt total, phospho mTOR (S2448), mTOR total, phospho S6 ribosomal (S235/236), phospho GSK3β (S9), GSK3β total, phospho Foxo3a (S253), phospho Stat3 (Y705), total Stat3, cyclin D1, cyclin E, c-myc, p27 (Cell Signaling Technologies, Danvers, MA); EGFR (Millipore, Billerica, MA); p21 (Thermo Fisher Scientific, Waltham, MA); beta actin (Sigma Aldrich, St. Louis, MO); cyclin A, phospho IRS-1 (Y989), phospho IRS-1 (Y632), phospho IRS-1 (Y465), and phospho IRS-1 (Y941) (Santa Cruz Biotechnology, Santa Cruz, CA).

Real time quantitative reverse transcriptase polymerase chain reaction (qPCR). For short-term in vivo experiments, ICR mice were maintained on the diets for 15 weeks, after which they were treated with a single application of either acetone vehicle or 3.4 nmol of TPA. Mice were sacrificed 6H (acetone, TPA) or 18H (TPA) following treatment and epidermis was scraped for RNA isolation (n=3/group). For *in vitro* experiments, C50 cells were stimulated with either IGF-1 or EGF and harvested for RNA. RNA for both *in vivo* and *in vitro* experiments was isolated using the Qiagen RNeasy protect mini kit (Qiagen, Valencia, CA). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). 1 ug of total RNA was then used to synthesize cDNA using the High Capacity cDNA Archive Kit (Applied

Biosystems, Foster City, CA). qPCR was then performed using the ABI 7900HT Fast Real Time PCR System (ABI) with assays on demand specific to *Cdkn1a*, *Cdkn1b*, *TGF-α*, *HB-EGF*, *amphiregulin* and *EGFR*. RNA was normalized to *GAPDH* using the ABI Mouse *GAPDH* Endogenous Control kit. Sequence Detection System software from ABI was then used for data analysis. The experimental Ct was normalized against the *GAPDH* control product. The ΔΔCt method was used to determine the amount of product relative to that expressed by 10 Kcal% fat acetone derived RNA (1-fold, 100%). All measurements were duplicated.

Immunohistochemical staining for p27 and p21. Mice were maintained on the four dietary regimens for 15 weeks, after which they were treated with a single application of acetone vehicle or 3.4 nmol of TPA. Mice were sacrificed 6 and 18 hours following treatment and whole skin sections were excised and processed for histological analysis. Skin sections were deparaffinized and rehydrated in xylene substitute, followed by 100% and 95% ethanol in water. Sections were blocked with 3% H_2O_2 in water for 10 minutes. Skin sections were boiled in 10 mM citrate buffer (pH 6.0) for 10 minutes, and then allowed to cool for 20 minutes. Non-specific antibody binding was blocked using Biocare Blocking Reagent for 10 minutes. Skin sections were incubated with p21 (Santa Cruz Biotechnology, Santa Cruz, CA) or p27 (BD Transduction Laboratories, San Jose, CA) antibody at a 1:10,000 dilution overnight at 4°C, and then incubated

with biotinylated rabbit-anti-mouse antibody at a 1:250 dilution for 15 minutes at room temperature. Skin sections were then incubated with streptavidin-horseradish peroxidase conjugate for 30 minutes at room temperature, after which they were developed with diaminobenzidine.

Results

Dietary energy balance and two-stage skin carcinogenesis. To evaluate the effect of dietary energy balance on epithelial carcinogenesis, we conducted a two-stage skin carcinogenesis experiment using ICR mice maintained on diets of varying caloric density. For this experiment, ICR female mice were initiated with 25 nmol of DMBA. Four weeks following initiation, mice were randomized to four experimental diet groups (30% CR, 15% CR, 10 Kcal%, 60 Kcal%) and maintained on these diets for the duration of the study, thus generating lean (30% CR), normal (15% CR), overweight (10Kcal% fat) and obese (60 Kcal% fat) body weight phenotypes. Four weeks following diet commencement, mice were promoted twice weekly with 3.4 nmol of TPA for 50 weeks. To determine the impact of dietary energy balance on tumor promotion, tumor incidence (incidence of papillomas) and tumor multiplicity (average number of papillomas per mouse) were determined weekly for 29 weeks, at which point tumor multiplicity plateaued (Fig. 4.1). Both 30% and 15% significantly inhibited tumor multiplicity (48% inhibition and 25% inhibition, respectively, when compared to the overweight control) ($p < 0.05$; Mann-Whitney U test), however no significant differences were observed between the overweight control and DIO groups (Fig. 4.1A, Table 4.1). Dietary manipulation had no significant effect on the incidence of papillomas (Fig. 4.1B).

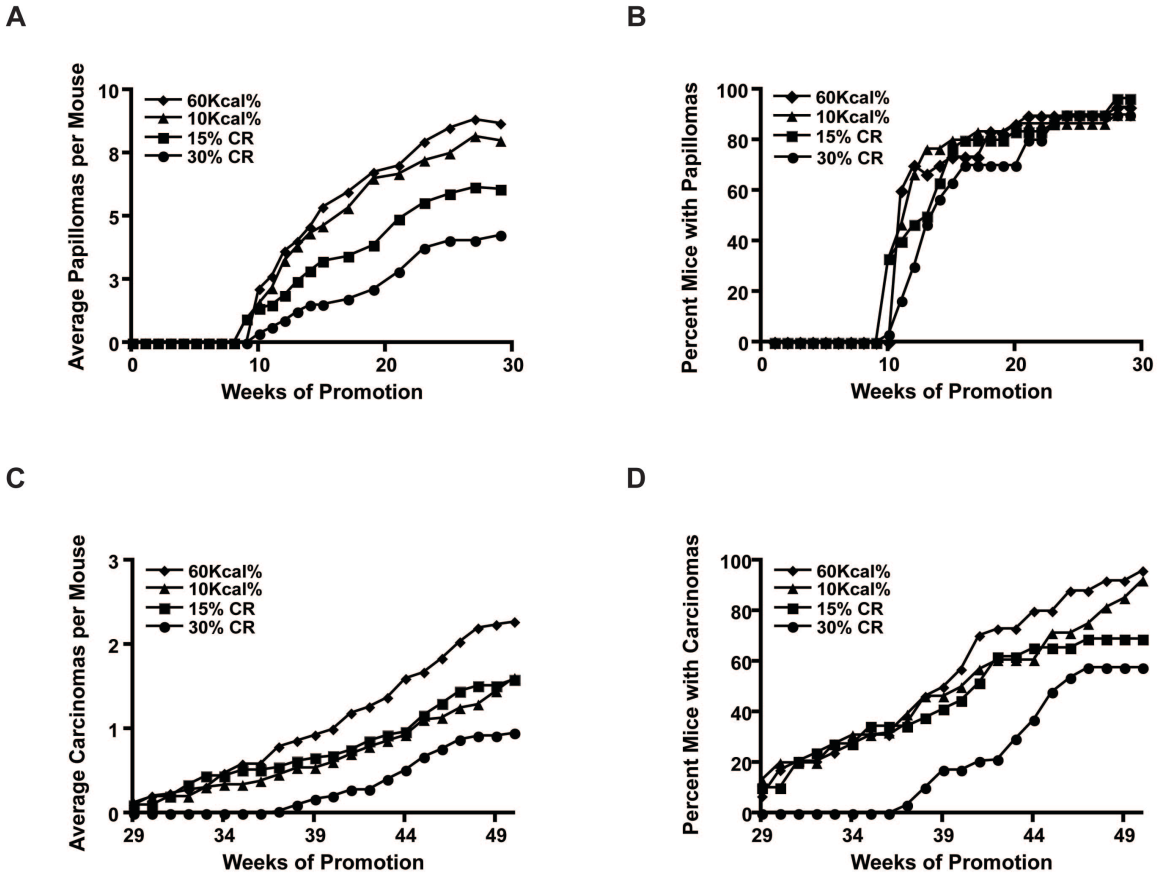

Figure 4.1. Effect of dietary energy balance on two-stage skin carcinogenesis in ICR female mice. ICR female mice were initiated with 25 nmol of DMBA. Four weeks following initiation, mice were placed on four experimental diets and maintained on these diets for the duration of the study (n=30/diet), thus generating lean (30% CR), normal (15% CR), overweight (10 Kcal% fat) and obese (60 Kcal% fat) phenotypes. Following a four week diet equilibration period, mice were promoted twice weekly with 3.4 nmol of TPA for the duration of the study. A, Tumor multipliciaty; B, Tumor incidence; C, Average number of carcinomas per mouse; D, Carcinoma incidence. CR (30%, 15%) significantly reduced tumor multiplicity; 30% CR significantly reduced, while DIO (60 Kcal%) significantly increased the average number of carcinomas per mouse ($p < 0.05$; Mann-Whitney U test). CR (30%, 15%) significantly reduced carcinoma incidence ($p < 0.05$; χ^2 test).

To determine the impact of dietary energy balance on tumor progression, promotion with 3.4 nmol of TPA continued for an additional 21 weeks, during which carcinoma incidence and the average number of carcinomas per mouse were determined weekly. As confirmed by histopathological analysis, 30% CR significantly reduced both carcinoma incidence ($p < 0.05$, χ^2 test) and the average number of carcinomas per mouse ($p < 0.05$, Mann-Whitney U test), while DIO significantly increased the average number of carcinomas per mouse ($p < 0.05$, Mann-Whitney U test) (Figure 4.1 and Table 4.1). However, there were no significant differences in the rate of malignant conversion among the four dietary treatment groups (Table 4.1). An additional two-stage skin carcinogenesis experiment (same dietary manipulation, but using 6.8 nmol of TPA for the promotion stage) yielded similar findings (data not shown). Taken together, these data suggest that negative energy balance inhibits, while positive energy balance enhances tumor promotion, but not the conversion of papillomas to carcinomas in this model for epithelial carcinogenesis.

Effect of dietary energy balance on weight distribution, body fat and serum protein levels following 50 weeks of tumor promotion in ICR mice. To further characterize the effect of long-term dietary energy balance manipulation on ICR female mice, body mass, percent body fat and circulatory protein levels (i.e., IGF-1, insulin, leptin, adiponectin) were evaluated at the conclusion of the 50-week two-stage skin carcinogenesis study (Table 4.2). Body mass and percent body

Table 4.1. Effect of dietary energy balance on tumor promotion and tumor progression using the two-stage skin carcinogenesis protocol.

Experimental Diet	*Number of Mice*	*Papillomas per Mouse*[1]	*Carcinoma Incidence (%)*	*Carcinomas per Mouse*	*Conversion Ratio*[2]
30% CR	**26**	**4.27**[a]	**57.7**[b]	**0.96**[a]	**0.23**
15% CR	**29**	**6.17**[a]	**69.0**[b]	**1.59**	**0.26**
10 Kcal% fat	**27**	**8.2**	**92.3**	**1.58**	**0.20**
60 Kcal% fat	**25**	**8.86**	**96**	**2.28**[a]	**0.26**

[1]Data taken at 29 weeks of promotion with 3.4 nmol of TPA after which the papilloma response had reached a plateau. [2]Ratio of the average number of SCCs at week 50 to average number of papillomas at week 29. [a]Significantly different from all groups ($p < 0.05$, Mann-Whitney U test). [b]Significantly different from 10 Kcal% fat and 60 Kcal% fat groups ($p < 0.05$, $\chi 2$ test).

fat were significantly different among all groups ($p < 0.05$, Mann-Whitney U test). CR (both 30% and 15%) significantly reduced levels of circulating IGF-1, insulin and leptin, and increased levels of adiponectin (30% CR), as compared to both the overweight control and DIO groups ($p < 0.05$, Mann-Whitney U test). Despite significant differences in both body mass and percent body fat, no significant differences in the levels of circulatory proteins were observed between the overweight control and DIO groups. The similarity in levels of circulatory proteins in the DIO and overweight control groups, may explain, in part, the inability of DIO to further potentiate the effects of tumor promotion during two-stage skin carcinogenesis.

Effect of dietary energy balance manipulation on weight distribution, body fat and levels of circulatory proteins in ICR mice. To evaluate potential mechanisms whereby dietary energy balance modulates two-stage skin carcinogenesis, additional diet studies were conducted to generate lean, normal, overweight and obese mice. For these studies, mice were maintained on the four previously described diets for 15 weeks. Body mass, percent body fat and levels of circulatory proteins were then determined at study end (Table 4.3). As shown in Table 4.3, body mass and percent body fat correlated directly with caloric consumption, yielding significant differences in both parameters among all groups ($p < 0.05$, Mann-Whitney U test). Similarly, levels of circulatory proteins were significantly different across the spectrum of negative to positive energy

Table 4.2. Effect of dietary energy balance on body mass and serum protein levels following 50 weeks of tumor promotion.

	30% CR	*15% CR*	*10 Kcal% fat*	*60 Kcal%*
Body Mass (g) Study Start	31.3 ± 0.6	29.5 ± 1.5	31.1 ± 1.1	31.9 ± 0.9
Body Mass (g) Study End [c]	26.7 ± 0.3	35.0 ± 0.4	41.4 ± 0.9	50.0 ± 1.8
Percent Body Fat [c]	13.7 ± 1.1	27.1 ± 2.0	33.2 ± 2.9	49.2 ± 5.4
IGF-1 (ng/mL)	61.4 ± 13.6[a,b]	186 ± 23.9[a,b]	330.5 ± 47.5[a]	339.5 ± 27.1[b]
Insulin (pg/mL)	187.1 ± 25.1[a,b]	404 ± 140.4[a,b]	872.8 ± 190.8 [a]	904.0 ± 199.0[b]
Leptin (pg/mL)	386.5 ± 134.3[a,b]	2322 ± 724.1[a,b]	12887 ± 1223 [a]	17540 ± 3872[b]
Adiponectin (ng/mL)	22221.7 ± 1773.1[a,b]	19751.4 ± 1960.9	16165.3 ± 1730.1[a]	14979.7 ± 1904.2[b]

ICR female mice were initiated with 25 nmol of DMBA. Four weeks following initiation, mice were placed on four experimental diets and maintained on these diets for the duration of the study (n=30/diet), thus generating lean, normal, overweight and obese phenotypes. Mice were then promoted twice weekly with 3.4 nmol of TPA for 50 weeks. After a two-week washout period, mice were sacrificed and serum was collected from a subset of mice (n≥4). Data represents mean ± SEM. [a,b]Significantly different from values with the same lettering; [c]significantly different values amongst all groups ($p < 0.05$, Mann-Whitney U test).

Table 4.3. Effect of dietary energy balance on body mass and serum protein levels following 15 weeks of diet consumption.

	30% CR	*15% CR*	*10 Kcal% fat*	*60 Kcal%*
Body Mass (g) Study Start	30.8 ± 0.7	30.9 ± 1.1	30.8 ± 0.5	31.2 ± 0.6
Body Mass (g) Study End [a]	23.3 ± 0.5	34.9 ± 0.9	40.3 ± 1.2	55.9 ± 1.3
Percent Body Fat [a]	19.0 ± 1.0	33.0 ± 1.3	38.5 ± 1.8	49.7 ± 1.8
IGF-1 (ng/mL) [a]	57.6 ± 4.4	101.9 ± 11.3	193.3 ± 13.9	241.3 ± 14.9
Insulin (pg/mL) [a]	178.2 ± 43.7	418.7 ± 45.0	738.9 ± 86.8	1300 ± 173.6
Leptin (pg/mL) [a]	439.8 ± 126.1	2464 ± 298.8	4484 ± 514.2	7663 ± 869.6
Adiponectin (ng/mL)	20924.1 ± 1639[b,c]	19902.8 ± 1048.3[d,e]	16611.6 ± 732.9[b,d]	17299.5 ± 816.6[c,e]

ICR female mice (n≥22) were maintained on four experimental diets for 15 weeks, thus generating lean, normal, overweight and obese phenotypes. Serum and epidermis were collected following sacrifice for analysis. Data represents mean ± SEM. [a]Significantly different value amongst all groups; [b,c,d,e]significantly different from values with the same lettering ($p < 0.05$, Mann-Whitney U test).

balance, with the greatest differences occurring between the caloric extremes ($p < 0.05$, Mann-Whitney U test). Specifically, CR reduced, while DIO increased levels of circulating IGF-1, insulin and leptin, findings which are consistent with previous short-term diet studies conducted in our laboratory (110). In contrast, levels of adiponectin inversely correlated with caloric consumption, with significant differences occurring between each CR group and the overweight control and DIO groups ($p < 0.05$, Mann-Whitney U test). The mice characterized in Table 4.3 were then used in subsequent experiments to further evaluate potential mechanisms of inhibition or enhancement associated with negative and positive energy balance.

Dietary energy balance and TPA-induced epidermal hyperproliferation. Given the effect of dietary energy balance on the tumor promotion phase of two-stage skin carcinogenesis, we next evaluated diet-induced changes in the epidermal proliferative response following treatment with TPA. For these studies, ICR female mice were maintained on the four diets for 15 weeks, as described above, and then treated twice weekly for two weeks with either acetone vehicle or 3.4 nmol of TPA. Forty-eight hours following the final treatment, mice were sacrificed; whole skin sections were then processed and examined for epidermal hyperplasia (epidermal thickness) and labeling index (BrdU incorporation). Figure 4.2A shows representative sections stained with BrdU, which correspond to both acetone vehicle and TPA treated skins excised from lean, normal, overweight

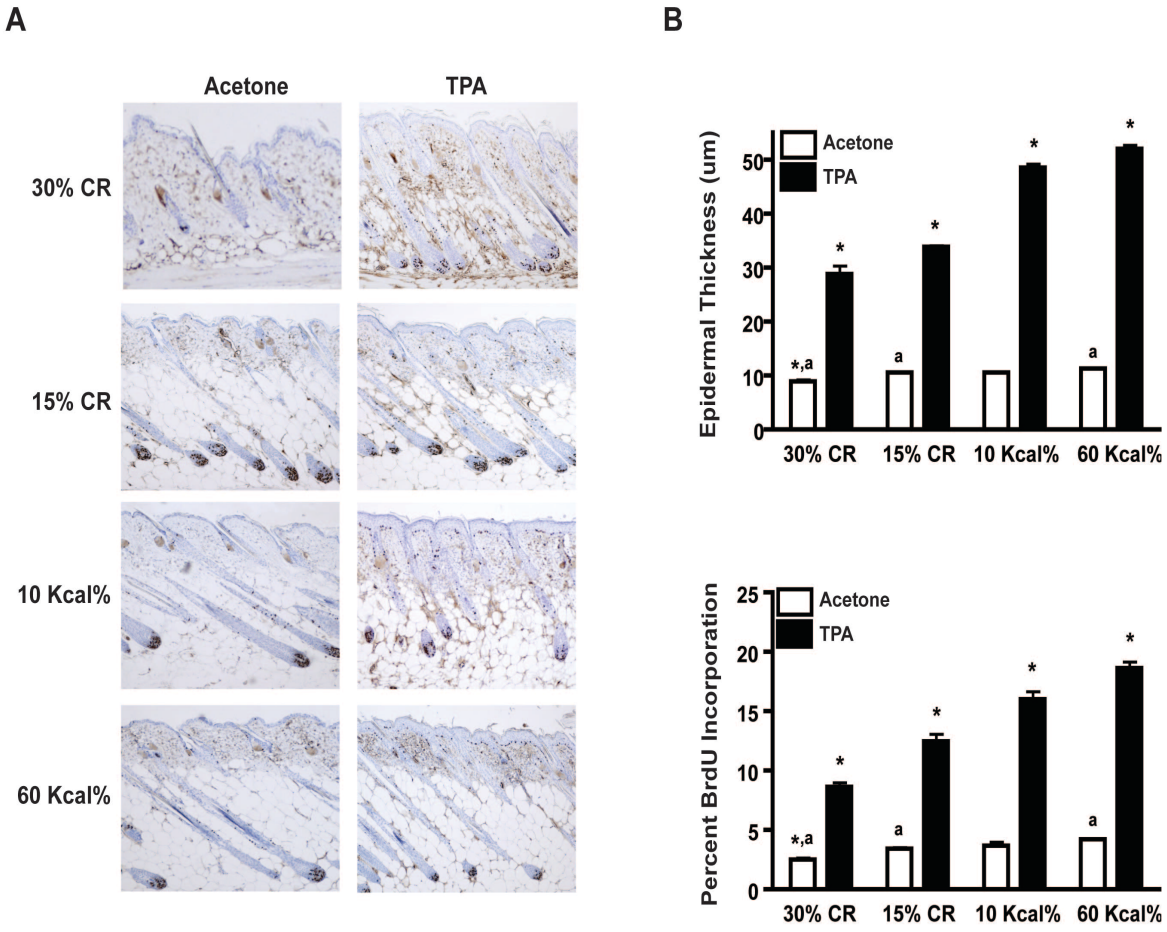

Figure 4.2. Effect of dietary energy balance on epidermal proliferative response. ICR female mice were maintained on the four experimental diets for 15 weeks; after which they were treated with either acetone vehicle (white bars) or 3.4 nmol of TPA (black bars) (n=3/group), twice weekly for two weeks. A, Representative paraffin embedded skin sections stained with BrdU; B, Epidermal thickness (upper panel) and percent BrdU incorporation (lower panel). *Indicates statistically different from all similarly treated experimental groups; [a] significantly different from values with the same lettering ($p < 0.05$; Mann-Whitney U test).

and obese mice. Quantitative analysis of diet-induced changes in epidermal hyperproliferation are shown in Figure 4.2B. In the absence of TPA treatment, moderate CR (30%) significantly reduced both epidermal thickness and labeling index, as compared to all other dietary groups, but mild CR (15%) only significantly reduced both parameters when compared to the DIO group ($P < 0.05$, Mann-Whitney U test). No differences in either epidermal thickness or percent BrdU incorporation were observed between the overweight control and DIO groups in the absence of TPA treatment. In contrast, both epidermal thickness and percent BrdU incorporation were significantly different among all dietary groups following TPA treatment, with the greatest differences occurring between the 30% CR and DIO groups ($p < 0.05$, Mann-Whitney U test). Thus, negative energy balance reduced and positive energy balance increased epidermal hyperproliferation in the presence and absence of TPA treatment.

Effect of dietary energy balance on growth factor signaling. To identify the underlying mechanisms that link dietary energy balance and epithelial carcinogenesis, we performed Western blot analyses to evaluate diet-induced changes in growth factor signaling. For these studies, ICR mice were maintained on the four diets, as described previously, and were treated with a single application of either acetone or 3.4 nmol of TPA. Mice were sacrificed 6H following treatment and epidermal lysates were prepared for Western blot analyses. As shown in Figure 4.3, phosphorylation of the EGFR, IGF-1R and

EGFR/IGF-1R downstream targets (i.e., Akt, mTOR, S6 ribosomal, GSK-3β, Foxo3a, Stat3) correlated directly with caloric density in the absence of TPA treatment, corroborating previous findings which suggested that dietary energy balance modulated steady-state growth factor signaling (110). These diet-induced changes in EGFR/IGF-1R signaling were further enhanced following TPA treatment, with the greatest differences in phosphorylation occurring between the 30% CR and DIO groups (Figure 4.3A, 4.3B). Specifically, 30% CR significantly reduced activation and/or phosphorylation of the EGFR, the IGF-1R, Akt, mTOR, S6 ribosomal, GSK-3β, Foxo3a and Stat3, as compared to the DIO group following TPA treatment ($p < 0.05$, Student's t-test; Figure 4.3B).

Dietary energy balance and levels of cell cycle regulatory proteins. In light of the observed effects of dietary manipulation on tumor promotion, epidermal hyperproliferation and growth factor signaling, we evaluated diet-induced changes in cell cycle regulatory proteins. Lean, normal, overweight and obese mice, described above, were treated with a single application of acetone vehicle or 3.4 nmol TPA. Mice were sacrificed 6 and 18 hours following treatment, epidermis was collected for both Western blot and qPCR analysis, and whole skin sections were excised for immunohistochemical evaluation. Initial experiments were performed to evaluate changes in positive cell cycle regulatory protein levels. In the absence of TPA treatment, CR reduced cyclin D1, cyclin E,

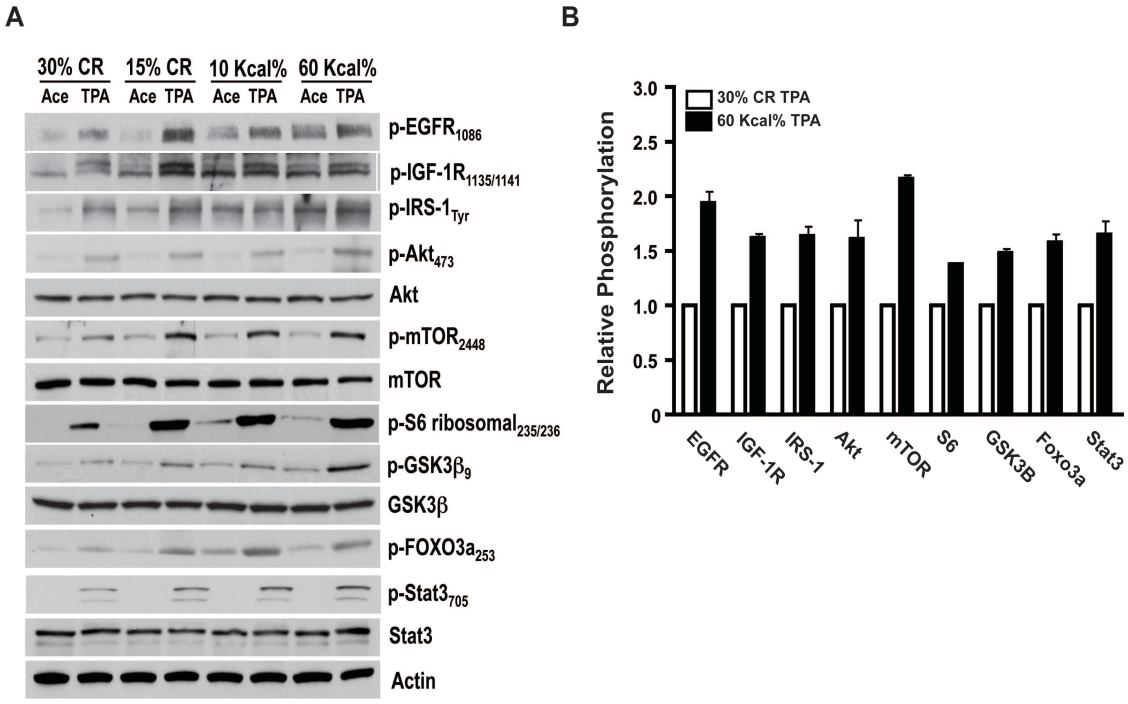

Figure 4.3. Effect of dietary energy balance on growth factor signaling. ICR female mice were maintained on four experimental diets for 15 weeks, thus generating lean, normal, overweight and obese phenotypes. Mice were treated with a single application of acetone vehicle (white bars) or 3.4 nmol of TPA (black bars) and sacrificed 6 hours following treatment (n=6/group). Epidermal lysates were pooled and prepared for Western blot analysis. A, Western blot analyses of dietary energy balance effects on EGFR and IGF-1R signaling; B, Densotometric graph that represents the mean ± SEM of three independent experiments in which Western blot data was normalized to both actin and total protein. Differences between 30% CR and 60 Kcal% fat TPA treated samples are all significant ($p < 0.05$; Student's t-test).

cyclin A and c-myc levels, as compared to DIO, however these differences were not significantly different (see Figure 4.4A). Consistent with previously published data from our laboratory, TPA treatment led to increased levels of cyclin D1 (18H), cyclin E (6H, 18H), cyclin A (6H, 18H) and c-myc (6H, 18H) (99 and see Figure 4.4A). The TPA-mediated increases in cyclin D1, cyclin E, cyclin A and c-myc were significantly reduced by 30% CR, as compared to DIO, at the 18H timepoint for all four proteins ($p < 0.05$, Student's t-test). Diet-induced changes in c-myc levels following TPA treatment were also significant at the 6H timepoint ($p < 0.05$, Student's t-test).

Additional Western blot analyses were performed to determine the impact of dietary energy balance on levels of negative cell cycle regulatory proteins. In the absence of TPA treatment, levels of both p27 and p21 were significantly higher in 30% CR mice, as compared to DIO mice (see Figure 4.4B). Following TPA treatment levels of p27 were reduced and levels of p21 were increased, regardless of timepoint. In contrast to the observed diet-induced changes in positive cell cycle regulatory proteins, levels of both p27 and p21 inversely correlated with caloric consumption. Specifically, both p27 and p21 levels were significantly higher in 30% CR mice, as compared to DIO mice, 6H following treatment with TPA ($p < 0.05$, Student's t-test). In addition, a similar trend was observed at the 18H timepoint, however, these differences were not significantly different.

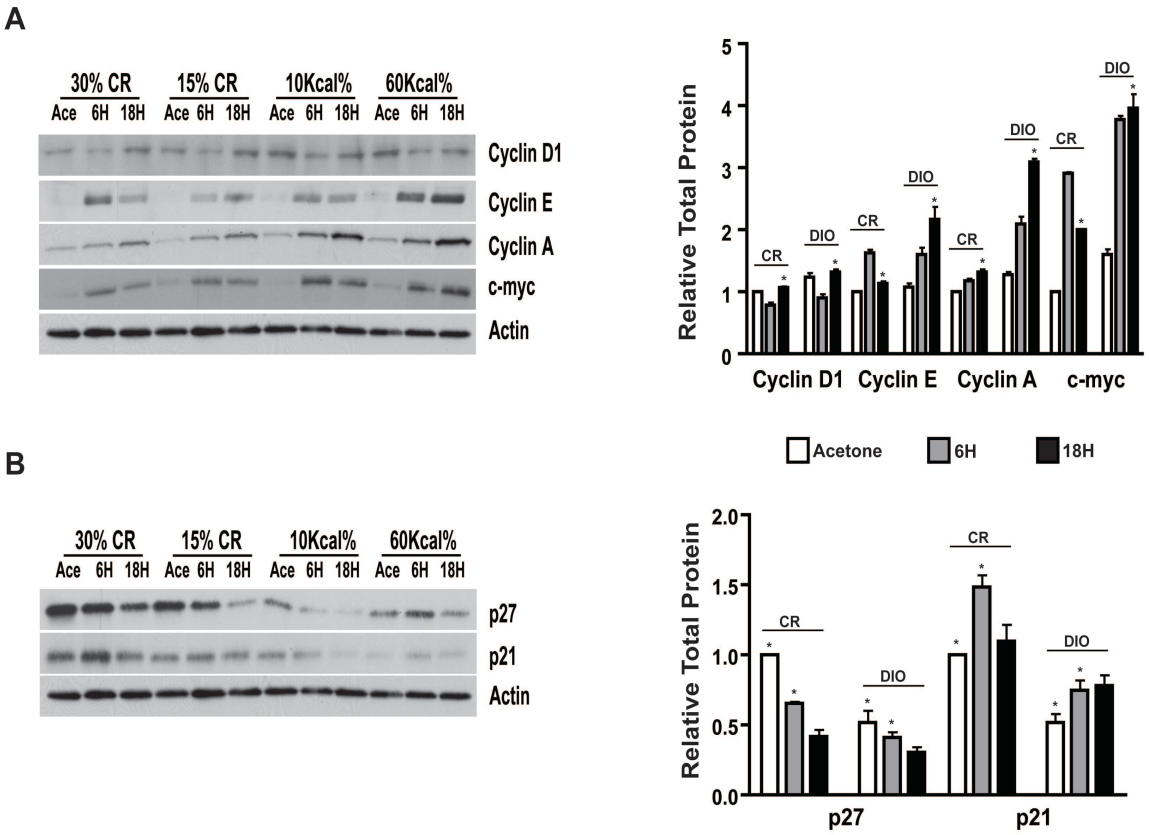

Figure 4.4. Effect of dietary energy balance on cell cycle regulatory proteins. ICR female mice were maintained on four experimental diets for 15 weeks, thus generating lean, normal, overweight and obese phenotypes. Mice were treated with a single application of acetone vehicle (white bars) or 3.4 nmol of TPA and sacrificed at 6 (gray bars) or 18 (black bars) hours following treatment (n=6/group). Epidermal lysates were pooled and prepared for Western blot analysis. A, Western blot analyses and quantitation of dietary energy balance effects on positive cell cycle regulatory proteins; B, Western blot analyses and quantitation of dietary energy balance effects on negative cell cycle regulatory proteins. Densotometric graphs represent the mean ± SEM of three independent experiments in which Western blot data was normalized to actin. *Denotes significantly different value from the corresponding timepoint ($p < 0.05$; Student's t-test).

Additional experiments were conducted to evaluate potential mechanisms whereby dietary energy balance modulates p21 and p27 (for review of regulatory mechanisms refer to 185, 186). Epidermal RNA isolated from the lean, normal, overweight and obese mice was analyzed using qPCR to evaluate diet-induced changes in p27 (*Cdkn1b*) and p21 (*Cdkn1a*) mRNA expression both in the presence and absence of TPA treatment. CR (both 30% and 15% CR), significantly increased expression of p27 mRNA in the absence of TPA treatment ($p < 0.05$, Mann-Whitney U test), however no differences were observed following TPA treatment (Fig 4.5A). In contrast, no diet-induced changes in p21 mRNA expression were observed in the absence of TPA, however, 30% CR significantly increased p21 mRNA expression 6H following TPA treatment ($p < 0.05$, Mann-Whitney U test; Fig. 4.5B). Additionally, immunohistochemical evaluation of whole skin sections stained for both p27 and p21 suggested an effect of caloric consumption on nuclear localization of these negative cell cycle regulatory proteins. Specifically, 30% CR increased the intensity of nuclear staining for p27 and p21 both in the presence and absence of TPA treatment, independent of the effects of TPA on total protein levels (Fig. 4.6). Collectively, these data suggest that dietary energy balance, at least in part, modulates tumor promotion and epidermal proliferation through both direct and indirect effects on cell cycle regulatory proteins.

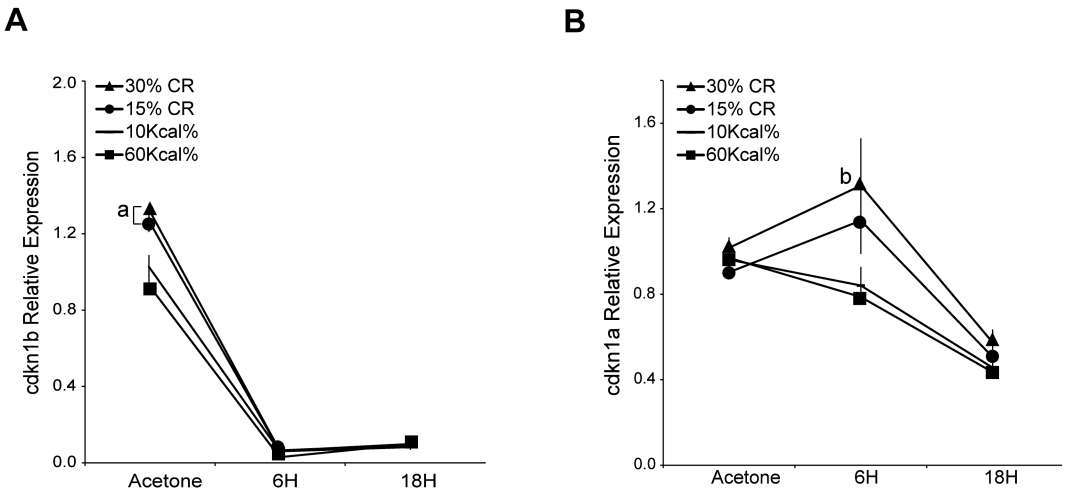

Figure 4.5. Effect of dietary energy balance on mRNA expression of negative cell cycle regulatory proteins. ICR female mice were maintained on four experimental diets as described in Figure 4.3. Mice were treated with a single application of acetone vehicle or 3.4 nmol of TPA and sacrificed 6 and 18 hours following treatment (n=3/group). RNA was prepared from epidermis and analyzed using qPCR. A, Effect of dietary energy balance on *cdkn1b* mRNA expression; B, Effect of dietary energy balance on *cdkn1a* mRNA expression. [a] Significantly different from 10 Kcal% and 60 Kcal% fat groups, [b] significantly different from all groups ($p < 0.05$, Mann-Whitney U test).

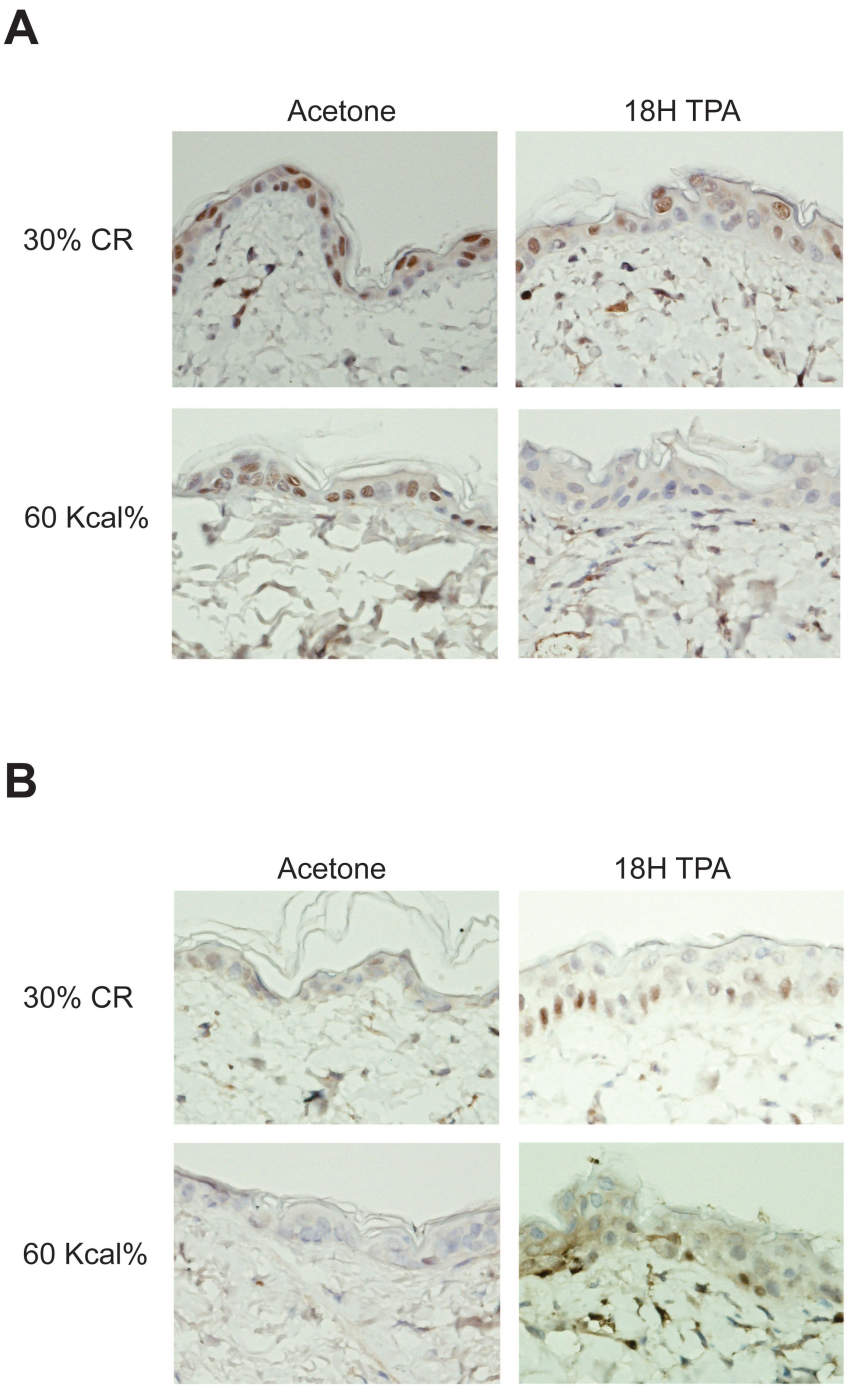

Figure 4.6. Effect of dietary energy balance on p27 and p21 localization. ICR female mice were maintained on four experimental diets as described in Figure 4.3. Mice were treated with a single application of acetone vehicle or 3.4 nmol of TPA and sacrificed 6 and 18 hours following treatment (n=3/group). Whole skin sections were excised for immunohistochemical analysis of p27 and p21 localization. A, p27; B, p21.

Activation of the IGF-1R stimulates IGF-1R/EGFR crosstalk in cultured mouse keratinocytes. Both current and previous data suggest a role for IGF-1 in modulating the effect of dietary energy balance on epithelial carcinogenesis. A reduction in circulating IGF-1, either due to genetic (LID mouse model) or dietary manipulation (CR) attenuates both IGF-1R and EGFR growth factor signaling, suggesting a potential for crosstalk between these two receptors (49, 110). To determine the role of IGF-1 in modulating signaling through both the IGF-1R and the EGFR, we conducted a series of experiments using C50 cells (cultured mouse keratinocytes), cultured as previously described (187). Initial experiments were performed to determine if IGF-1 stimulation had an effect on activation of not only the IGF-1R, but also the EGFR and ErbB2. For these experiments, C50 cells were stimulated with 25 ng/mL of IGF-1, harvested at multiple timepoints (0, 5, 10, 15, 30, 60, 90, 120 minutes) and analyzed for IGF-1R (as measured by IRS-1), EGFR and ErbB2 activation (i.e., phosphorylation) using Western blot analysis. As shown in Figure 4.7, IGF-1 induced activation of all three cell surface receptors. Phosphorylation of the EGFR was rapid, with the greatest activation occurring 5 minutes following IGF-1 stimulation, and despite a steadily decreasing trend in EGFR activation at later timepoints, phosphorylation was maintained above the basal level, even at 120 minutes. EGFR phosphorylation

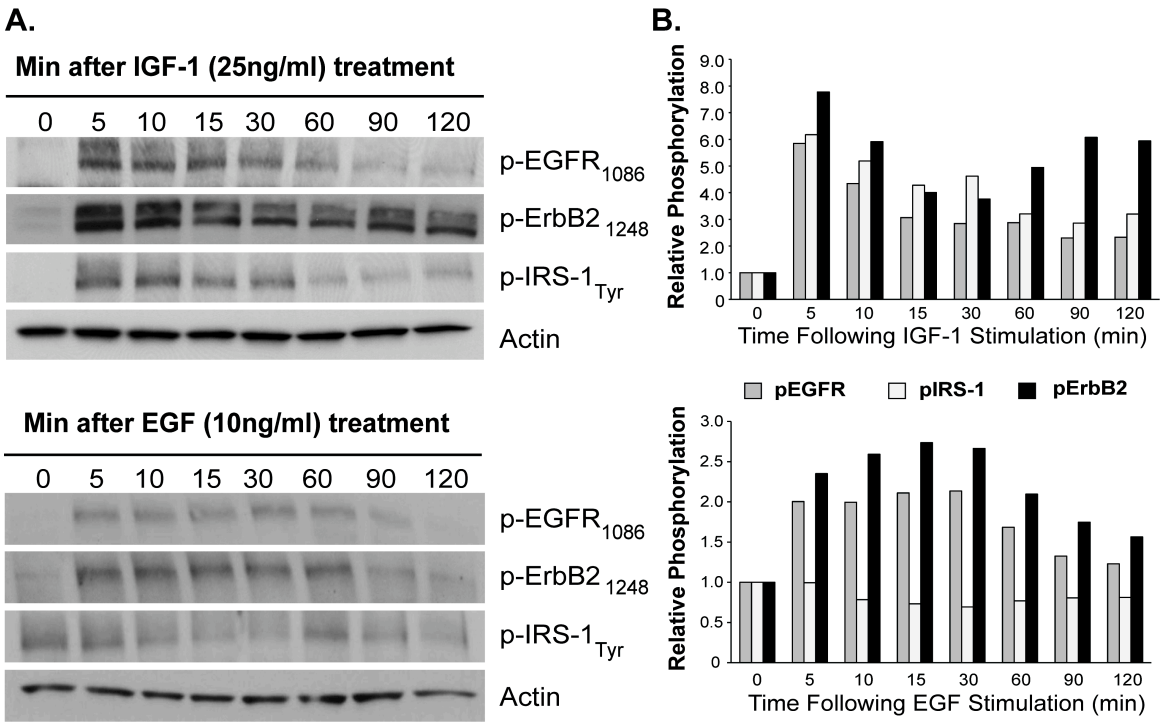

Figure 4.7. Effect of IGF-1 and EGF stimulation on growth factor receptor activation. C50 cells were cultured to ~80% confluency, serum and growth factor starved for 24 hours and stimulated with either IGF-1 (25 ng/mL) or EGF (10 ng/mL). Cells were then harvested at multiple timepoints (0, 5, 10, 15, 30, 60, 90, 120) and lysate was prepared for Western blot analysis. A, Western blot analyses targeting EGFR, ErbB2 and IGF-1R activation status. Upper panel corresponds to IGF-1 stimulation, while the lower panel corresponds to EGF stimulation; B, Quantitation of corresponding Western blots. Data shown represents an average of at least two independent experiments.

correlated directly with IGF-1 induced activation of the IGF-1R (again see Fig. 4.7). Early ErbB2 phosphorylation also paralleled IGF-1R and EGFR activation; however, ErbB2 activation was maintained to a greater extent at later timepoints. An additional experiment was performed in which C50 cells were stimulated with 10 ng/mL of EGF and harvested at the same timepoints described above to determine if direct activation of the EGFR could induce IGF-1R phosphorylation. EGF stimulation activated both the EGFR and ErbB2, with the greatest activation occurring between 15 and 30 minutes; however, there was no effect of EGF stimulation on IGF-1R activation (again see Fig. 4.7).

Additional experiments were performed to evaluate mechanisms whereby IGF-1 induced activation of the EGFR. Previous studies have identified several potential mechanisms for IGF-1R and EGFR crosstalk (86-90). We began by evaluating the ability of IGF-1 or EGF to induce heterodimerization between the IGF-1R and the EGFR. Co-immunoprecipitation experiments were performed using cell lysates prepared from similarly treated and harvested C50 cells. As shown in Figure 4.8, IGF-1 stimulation induced an increase in IGF-1R/EGFR association, but EGF had no effect. Additional experiments were performed to determine the impact of IGF-1 on EGFR and EGFR ligand mRNA expression. We performed qPCR analysis using RNA extracted from C50 cells (stimulated with IGF-1 or EGF and harvested at the previously described timepoints) and evaluated the relative expression of *EGFR*, *TGF-α*, *HB-EGF* and *amphiregulin*.

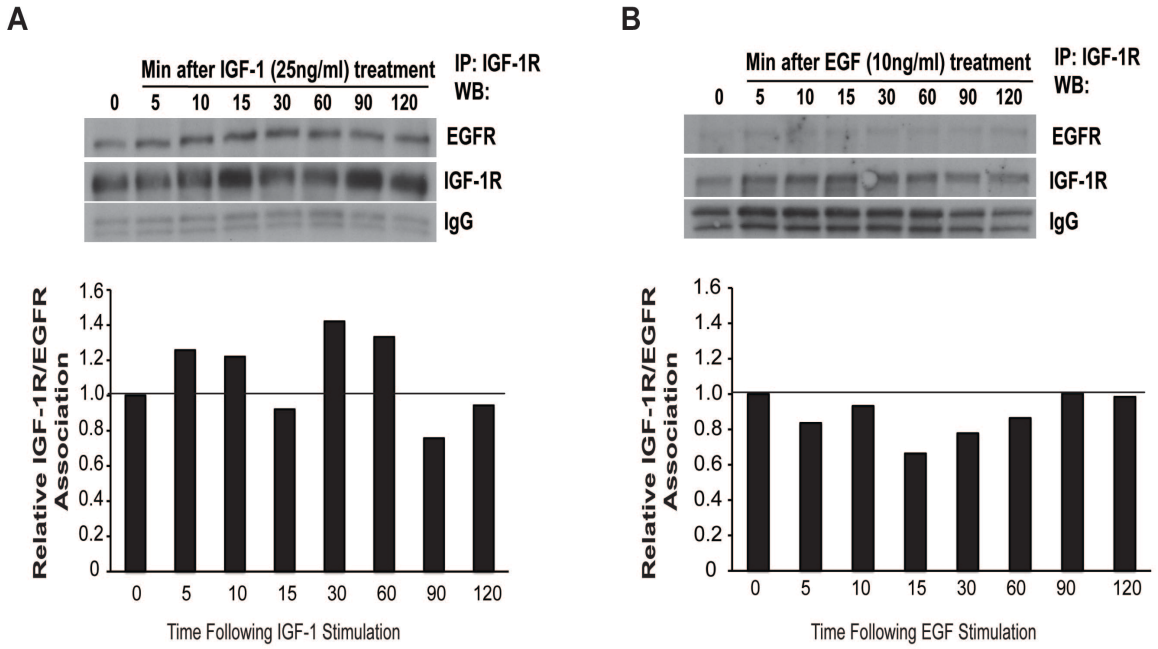

Figure 4.8. Effect of IGF-1 and EGF stimulation on IGF-1R/EGFR association. C50 cells were harvested and cultured as described in Figure 4.7 and lysate was prepared for co-immunoprecipitation experiments. A, Effect of IGF-1 stimulation on IGF-1R/EGFR association. Upper panel corresponds to co-immunoprecipitation with IGF-1R and subsequent Western blot analyses for EGFR and IGF-1R. Lower panel shows the corresponding quantitation. EGFR and IGF-1R were first normalized to IgG and then normalized to eachother; B, Effect of EGF stimulation on IGF-1R/EGFR association. Upper and lower panels correspond to the same parameters presented in A. Data shown represents a single experiment.

Both IGF-1 and EGF stimulation induced heightened expression of *HB-EGF* and *amphiregulin*, with a greater induction occurring with EGF treatment (Fig. 4.9B, C). These relative increases in mRNA expression, however, were observed only at later timepoints (≥ 60 minutes). We observed no increase in *TGF-α* and *EGFR* mRNA expression following stimulation of C50 cells with either IGF-1 or EGF (Fig. 4.9A, D). These findings suggest that IGF-1 modulates both IGF-1R and EGFR activation due to receptor crosstalk. IGF-1 induced IGF-1R and EGFR heterodimerization may partially mediate early phosphorylation of the EGFR, while IGF-1 mediated changes in EGFR ligand mRNA expression may account for sustained activation of the EGFR. Ectodomain shedding of EGFR ligands following IGF-1 stimulation may act as an additional mechanism of early EGFR phosphorylation, however, this mechanism was not evaluated in the current study. In contrast to the effects of IGF-1 on EGFR activation, EGF stimulation had no effect on IGF-1R phosphorylation or induction of an IGF-1R/EGFR association, findings that imply that reciprocal crosstalk is absent.

Dietary energy balance and IGF-1R/EGFR crosstalk in vivo. After having identified mechanisms whereby IGF-1 modules IGF-1R/EGFR crosstalk in cultured mouse keratinocytes, we next evaluated the impact of dietary energy balance on IGF-1R/EGFR crosstalk in vivo. For these experiments, ICR female mice were maintained on the 30% CR and DIO diets previously described for 15 weeks. The lean and obese mice were then treated with a single application of

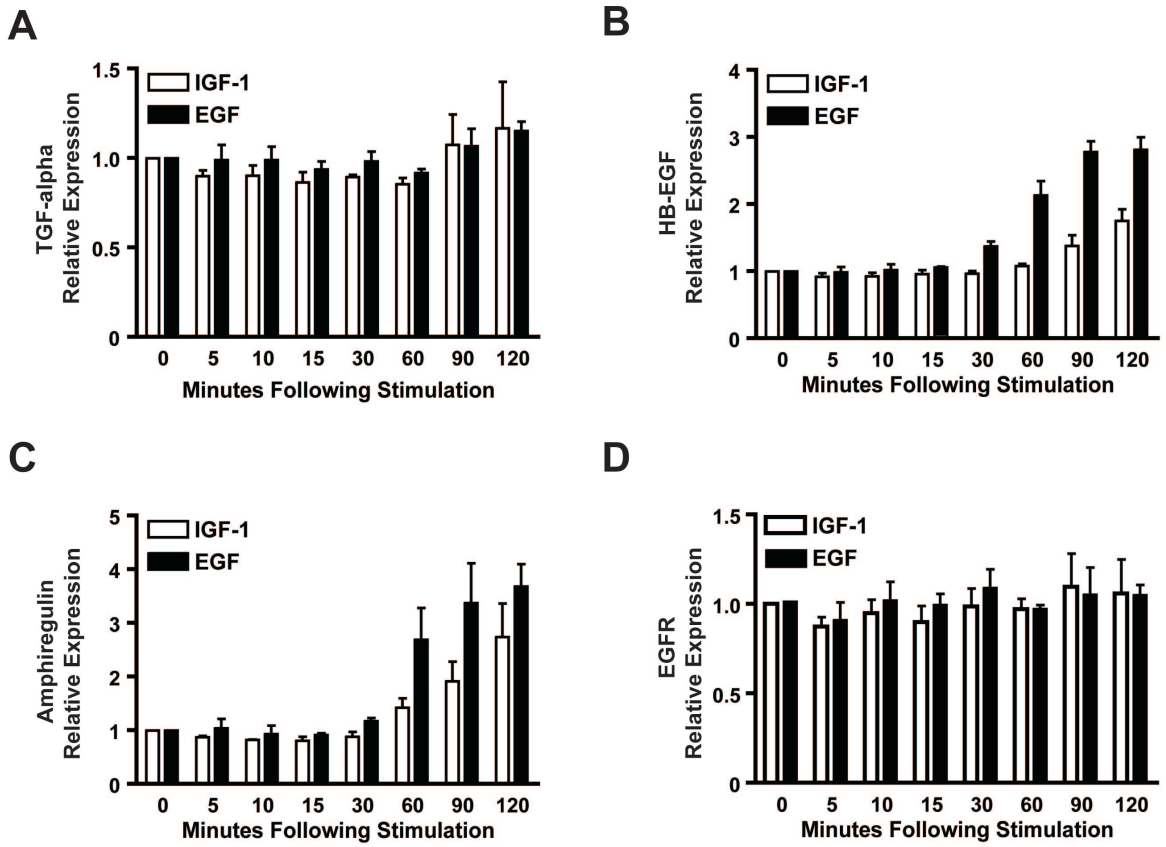

Figure 4.9. Effect of IGF-1 and EGF stimulation on EGFR ligand expression. C50 cells were cultured and harvested as described in Figure 4.7. RNA was isolated and mRNA expression was analyzed using qPCR. A, *TGF-α*; B, *HB-EGF*; C, *Amphiregulin*; D, *EGFR*. Data shown represents the average ± SEM of three independent experiments.

acetone vehicle or 3.4 nmol of TPA and sacrificed 6 or 18 hours later. Epidermal lysates and epidermal RNA were prepared for Western blot and qPCR analysis. Co-immunoprecipitation experiments were performed to evaluate diet-induced changes in IGF-1R/EGFR heterodimerization. As shown in Figure 4.10, DIO increased the relative association between the IGF-1R and the EGFR 6 hours following TPA treatment, while CR reduced this association. We next performed qPCR analysis to determine the impact of dietary energy balance on *EGFR* and EGFR ligand mRNA expression (i.e., *TGF-α*, *HB-EGF*, *amphiregulin*, *EGFR*). DIO significantly increased levels of mRNA for EGFR ligands, as compared to 30% CR, both in the presence and absence of TPA treatment (Fig. 4.11). Specifically, DIO significantly increased mRNA expression of *TGF-α* (18H), *HB-EGF* (acetone, 6H) and *amphiregulin* (6H), while there were no significant differences in *EGFR* mRNA expression. These findings suggest that dietary energy balance modulates IGF-1R/EGFR crosstalk *in vivo* at least in part due to diet-induced changes in both IGF-1R and EGFR heterodimerization and EGFR ligand mRNA expression.

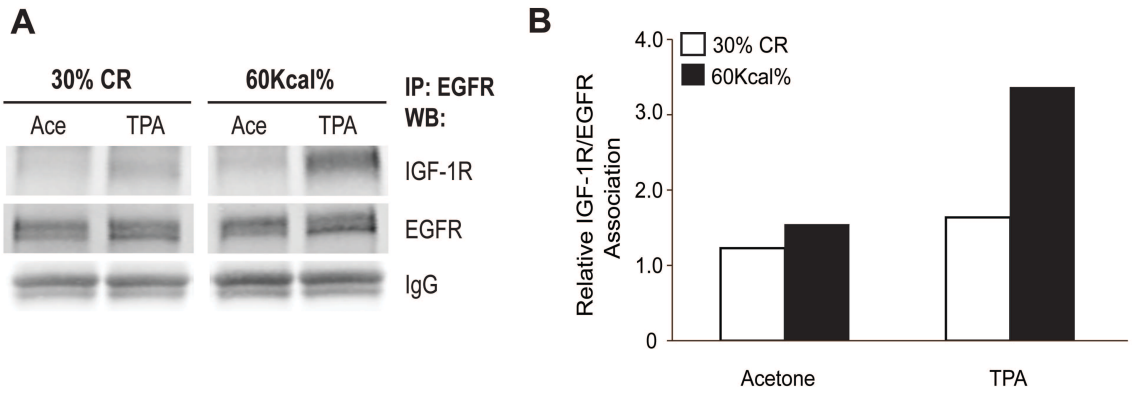

Figure 4.10. Effect of dietary energy balance on IGF-1R and EGFR heterodimerization. ICR female mice were maintained on a 30% CR and 60 Kcal% fat diet and treated with TPA, as described in Figure 4.3 (n=6/group). Epidermal lysates were pooled and prepared for co-immunoprecipitation experiments. A, Co-immunoprecipitation with EGFR and subsequent Western blot analyses for EGFR and IGF-1R; B, Desotometric graph representing the Western blots shown in A. EGFR and IGF-1R were first normalized to IgG and then normalized to eachother. Data shown represents a single experiment.

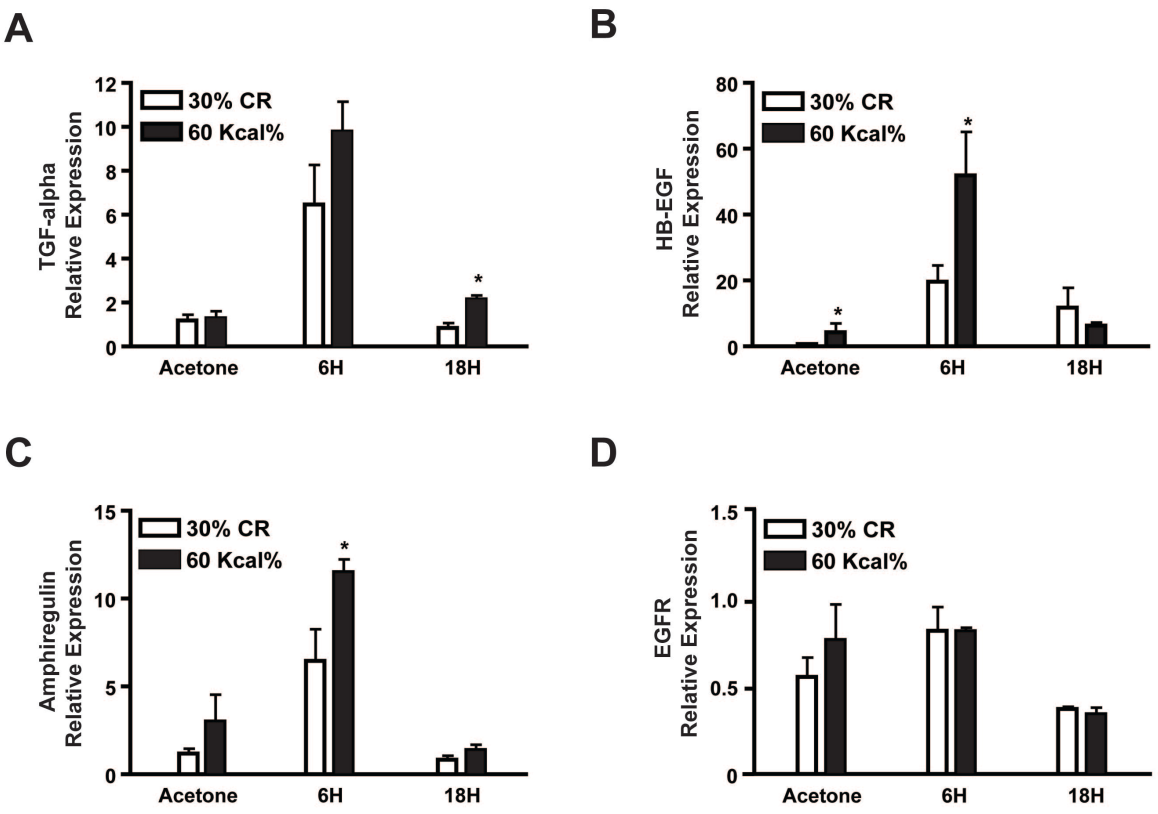

Figure 4.11. Effect of dietary energy balance on EGFR ligand mRNA expression. ICR female mice were maintained on the 30% CR and 60 Kcal% fat diets described in Figure 4.3 for 15 weeks. Mice were treated with a single application of acetone vehicle or 3.4 nmol of TPA and sacrificed 6 or 18 hours following treatment (n=3/group). Epidermal RNA was isolated for qPCR analysis and relative mRNA expression was determined. A, *TGF-α*; B, *HB-EGF*; C, *Amphiregulin*; D, *EGFR*. Data represents the average ± SEM. * Denotes significantly different value from 30% CR group at the corresponding timepoint ($P < 0.05$, Mann-Whitney U test).

Discussion

The current study was conducted to determine: i) the impact of dietary energy balance on epithelial carcinogenesis; ii) potential mechanisms whereby caloric consumption modulates two-stage skin carcinogenesis; iii) the role of IGF-1 in mediating IGF-1R/EGFR crosstalk in mouse keratinocytes. We began by determining the impact of caloric consumption on epithelial carcinogenesis using the two-stage skin carcinogenesis protocol. Consistent with previously published data, CR (30% and 15%) significantly inhibited tumor promotion, as compared to both the overweight control and DIO groups, but had no inhibitory effect on tumor progression (measured by rate of malignant conversion) (6, 100, 101). With regard to DIO, we observed no significant effect on either tumor promotion or progression when compared to the overweight control, despite a significant increase in both body mass and percent body fat, findings similar to studies conducted by Birt et. al. (105). Interestingly, levels of IGF-1, insulin and leptin did not significantly differ between the overweight control and DIO groups, suggesting that a threshold may have been reached in which further caloric consumption had no impact on levels of circulatory proteins and/or tumor response. This notion of a threshold for the effects of fat consumption on tumorigenesis has been previously published, although not using the two-stage skin carcinogenesis model. Several mammary carcinogenesis studies conducted in both rats and mice have reported that despite significant differences in body mass, no significant increase in tumor incidence occurred when dietary fat

consumption exceeded 20 to 30 Kcal% (188-190). Although our study employed a lower percent fat diet (10 Kcal%), this threshold concept could explain the lack of a further effect of DIO on tumor promotion, due to differences in dietary composition, diet consumption and mode of tumor induction between the current and previous studies. In summary, the current study evaluated the effect of both moderate (30%) and mild (15%) CR, as well as the effect of DIO, on epithelial carcinogenesis; thus providing a more thorough evaluation of the effects of dietary energy balance on two-stage skin carcinogenesis, as compared to earlier reports. Collectively, our data suggests that negative energy balance (30% and 15% CR) inhibited, while positive energy balance (overweight control, DIO) enhanced tumor promotion, with no effect on the conversion of papillomas to squamous cell carcinomas.

Given the effect of dietary energy balance on two-stage skin carcinogenesis, we next conducted a series of experiments to determine potential mechanisms whereby negative energy balance inhibits and positive energy balance enhances epithelial carcinogenesis. To begin, we evaluated diet-induced changes in epidermal hyperproliferation both in the presence and absence of treatment with TPA. In the absence of TPA treatment, moderate (30%) and mild (15%) CR significantly reduced both epidermal thickness and labeling index, as compared to DIO. In contrast, no significant differences in epidermal hyperproliferation were observed between the overweight control and

DIO groups, findings that are consistent with data published by Lautenbach et. al., which reported no difference in BrdU incorporation in normal mammary glands excised from either normal weight control or obese mice (191). When evaluating diet-induced changes in the epidermal proliferative response to TPA treatment, we observed a significant effect of energy consumption across the spectrum of negative to positive energy balance on both epidermal thickness and labeling index, with the greatest differences occurring between the 30% CR and DIO groups. Several studies have demonstrated similar effects of energy balance manipulation (DIO and/or varying levels of CR) on cellular proliferation in both normal and tumorigenic tissue (e.g., mammary, colon, liver, epidermis, bladder) (191-196). These findings suggest that dietary energy balance may modulate epithelial carcinogenesis through diet-induced changes in epidermal proliferation.

Previously published data from our laboratory suggests a role for both IGF-1R and EGFR activation in the modulation of TPA-induced epidermal hyperproliferation and multistage carcinogenesis in mouse skin. Specifically, inhibition of TPA-induced EGFR activation *in vivo*, using a tyrosine kinase inhibitor (RG13022), significantly reduced epidermal hyperplasia and proliferation in SENCAR mice (168). In contrast, overexpression of *IGF-1*, using either HK1.IGF-1 or BK5.IGF-1 transgenic mice, significantly enhanced the epidermal proliferative response to TPA treatment, as well as tumor multiplicity during two-stage skin carcinogenesis (125, 160). Given the association between IGF-

1R/EGFR activation and epidermal proliferation, as well as findings from recently published data which demonstrated an effect of caloric consumption on steady-state activation of both the IGF-1R and the EGFR (110), we performed Western blot analyses to evaluate diet-induced changes in IGF-1R and EGFR activation following TPA treatment. CR significantly attenuated activation of both the IGF-1R and the EGFR, as compared to DIO. Additional analyses were performed to evaluate the effect of dietary energy balance on signaling downstream of the IGF-1R and the EGFR, specifically to Akt, mTOR and their downstream substrates. CR consistently inhibited, while DIO consistently enhanced phosphorylation of Akt, mTOR, S6 ribosomal, GSK3β and Foxo3a. Xie et. al. reported a similar reduction in TPA-mediated Akt activation following dietary calorie restriction (104). Taken together, these data suggest that negative energy balance may inhibit, while positive energy balance may enhance epidermal hyperproliferation and multistage carcinogenesis in mouse skin due to differential activation of the IGF-1R and the EGFR, resulting in altered downstream signaling to Akt and mTOR.

Heightened activation of Akt (BK5.IGF-1, BK5.Aktwt transgenic mice) has been shown to specifically increase levels of positive cell cycle regulatory proteins (cyclins D, A, E, c-myc), thus providing a mechanism for the up-regulation of cellular proliferation in response to tumor promotion (93, 98). Given the effect of dietary energy balance on Akt activation, we evaluated diet-induced

changes in cell cycle regulatory proteins. In the current study, levels of positive cell cycle regulatory proteins correlated directly with caloric consumption (significant with TPA treatment – 18H timepoint), although significant differences between the 30% CR and DIO groups were not observed in the absence of TPA treatment. In contrast, levels of negative cell cycle regulatory proteins inversely correlated with caloric consumption, with significant differences occurring between the caloric extremes both in the presence (6H) and absence of TPA treatment. Several studies by Thompson and colleagues have reported similar modulation of cell cycle regulatory proteins (40-42). Forty percent CR consistently reduced levels of cyclins D1, E and A and increased levels of p27 and p21 in mammary carcinomas (40, 41). The more severe CR (40%) and the malignant nature of the tissue examined (mammary carcinomas) may have exacerbated the effects of CR on cell cycle regulation, as compared to the current study; however the findings remain relevant. Additional findings by Jiang et. al., suggest that energy restriction not only modulates levels of cell cycle regulatory proteins, but also regulates activity of cell cycle inhibitors and activators (40). Specifically, 40% CR enhanced cyclin dependent kinase (cdk) complex formation with both p27 and p21 and inhibited complexing of cdks with cyclin D1 and cyclin E, thus inhibiting cell cycle progression (40). These findings may explain, in part, the ability of CR to inhibit epidermal proliferation, in the absence of TPA treatment, regardless of the non-significant reduction in levels of cell cycle progression related proteins. Additional studies evaluated diet-induced

changes in the regulation (e.g., mRNA expression, protein localization) of cell cycle inhibitory proteins, thus providing further evidence for the role of dietary energy balance in the modulation of epithelial carcinogenesis. The current study demonstrates that both CR and DIO modulate cell cycle progression through diet-induced changes in levels or activity of cell cycle regulatory proteins. These changes in cell cycle regulation result in altered epidermal proliferation, thus providing a plausible mechanistic explanation for the effects of dietary energy balance on two-stage skin carcinogenesis.

As noted in the Introduction and shown in Tables 4.2 and 4.3, levels of circulating IGF-1 are differentially regulated by dietary energy balance manipulation, and changes in this globally active circulatory protein are hypothesized to regulate many of the effects of caloric consumption on tumorigenesis. Recently published data from our laboratory, using the liver IGF-1 deficient (LID) mouse model, demonstrated a direct correlation between genetically reduced levels of circulating IGF-1 and inhibition of two-stage skin carcinogenesis, similar to the effects of CR (Figs. 3.1 and 4.1) (49). Evaluation of IGF-1 mediated changes in epidermal growth factor signaling, using both the LID mouse model and dietary energy balance manipulation, demonstrated alterations in activation of both the IGF-1R and the EGFR (CR, LID reduced; DIO increased) following treatment with TPA (Figure 4.3) (49). Additional data from our laboratory demonstrated that overexpression of *IGF-1* (HK1.IGF-1 transgenic

mouse model) increased epidermal activation of the EGFR following TPA treatment, thus providing further evidence for the role of IGF-1 in mediating signaling through both the IGF-1R and the EGFR (125). Taken together, these findings suggest that IGF-1 may modulate the effects of dietary energy balance on epithelial carcinogenesis due at least in part to receptor crosstalk between the IGF-1R and the EGFR.

In the current study, we established that IGF-1 specifically induced activation of not only the IGF-1R, but also the EGFR and ErbB2 using C50 cells (cultured mouse keratinocytes), findings that confirm previously published data showing IGF-1 mediated activation of the EGFR (55, 89, 90, 197). These earlier reports, however, do not evaluate the effect of IGF-1 stimulation on ErbB2 activation. We hypothesize that several mechanisms are responsible for the observed effect of IGF-1 on ErbB2 phosphorylation, including: i) EGFR/ErbB2 heterodimerization (early timepoints) (for review see 198); ii) MMP-mediated generation of a truncated, kinase active form of ErbB2 (early timepoints) (199); iii) and IGF-1R/ErbB2 heterodimerization (either full length or truncated form of ErbB2 – later timepoints) (200). Further evaluation of IGF-1 effects on ErbB2 will be conducted in future experiments. Additional studies were performed to evaluate the effect of EGF on both IGF-1R and EGFR activation. Similar to studies conducted by Roudabush et. al. and El-Shewy et. al., we observed no effect of EGF stimulation on IGF-1R activation, suggesting that IGF-1R/EGFR

crosstalk may be unidirectional (89, 90). Further mechanistic studies determined that IGF-1 modulates activation of the EGFR in cultured keratinocytes through multiple mechanisms, including induction of IGF-1R/EGFR heterodimerization and heightened EGFR ligand mRNA expression. These IGF-1 induced changes may account for sustained activation of the EGFR, observed at later timepoints, however, they do not fully explain the rapid induction of EGFR phosphorylation observed following IGF-1 stimulation. Previous studies identified IGF-1 mediated ectodomain shedding of EGFR ligands as an additional mechanism whereby IGF-1 induces EGFR phosphorylation (55, 89, 90, 197), findings which could account for this early induction of EGFR activation by IGF-1. The effects of IGF-1 on EGFR ligand ectodomain shedding are currently being analyzed. Finally, we conducted in vivo experiments to determine if dietary energy balance modulates IGF-1R/EGFR crosstalk. CR not only reduced IGF-1R/EGFR heterdimerization following TPA treatment, but also significantly reduced mRNA expression of EGFR ligands, as compared to DIO. These findings suggest that CR reduced, while DIO increased activation of the IGF-1R and the EGFR, in part due to diet-induced changes in IGF-1R and EGFR crosstalk.

In conclusion, we have shown that CR reduced, while DIO increased epidermal growth factor signaling through the IGF-1R and the EGFR following TPA treatment. *In vitro* experiments using IGF-1 stimulation in cultured mouse keratinocytes, as well as earlier *in vivo* studies using the LID mouse model

suggest that levels of circulating IGF-1, which are modulated by dietary energy balance, regulate activation of both the IGF-1R and the EGFR due to receptor crosstalk. These diet-induced changes in IGF-1R and EGFR activation then altered downstream signaling to Akt and mTOR, subsequently modulating cell cycle regulation. Specifically, CR inhibited, while DIO enhanced cell cycle progression, findings that correlated directly with diet-induced changes in epidermal proliferation. Taken together, these effects of dietary energy balance on IGF-1R/EGFR signaling and crosstalk, cell cycle regulation and epidermal proliferation provide a plausible mechanism for the inhibitory effects of negative energy balance and the enhancing effects of positive energy balance on susceptibility to two-stage skin carcinogenesis.

Chapter V

Final conclusions and future directions.

The prevalence of obesity in the United States continues to rise among both adults and children, creating a virtual epidemic that is associated with multiple health implications. Amongst these obesity-related diseases, a direct association between obesity and increased risk of developing or mortality from multiple cancers has been clearly established (1, 10). While the obesity-cancer link has been well defined, the molecular mechanisms underlying the enhancing effects of obesity remain unclear. In contrast, CR has been shown to consistently inhibit tumorigenesis in animal models, regardless of tumor type or mode of induction (4). This method of cancer prevention, however, presents translational limitations, given the degree of CR that is required to achieve maximal inhibition. Similar to the association between obesity and cancer, the mechanisms underlying the inhibitory effects of CR are not well established. Given this link between energy balance and cancer and the dramatic increase in the incidence of obesity, there is an urgent need for mechanistic studies that identify the potential molecular targets that account for the enhancing or inhibitory effects of obesity and CR on tumorigenesis. Identification of these molecular mechanisms could provide the necessary information for intervention strategies that could facilitate a reduction in obesity related cancers, as well as identify molecular targets for CR mimetics that could function in a chemopreventive fashion.

The overall aim of the project presented herein was to determine the effect of both positive and negative energy balance on epithelial carcinogenesis and to identify potential mechanisms underlying the energy balance-cancer link. From these studies, we were able to demonstrate the following: i) positive energy balance enhanced, while negative energy balance inhibited multistage carcinogenesis in mouse skin (Chapter IV); ii) the epidermal proliferative response correlated directly with caloric consumption (Chapter IV); iii) CR reduced, while DIO increased both steady-state and TPA-induced epidermal growth factor signaling through the IGF-1R and the EGFR, as well as signaling downstream to Akt and mTOR (Chapter II, IV); iv) CR and DIO similarly modulated steady-state growth factor signaling in both the liver and DL prostate in multiple mouse strains (Chapter II); v) levels of positive cell cycle regulatory proteins (i.e., cyclin D1, E, A and c-myc) correlated directly with caloric consumption, while levels of negative cell cycle regulatory proteins (p27 and p21) inversely correlated with caloric consumption in mouse epidermis following TPA treatment (Chapter IV); vi) reduced levels of circulating IGF-1, in the absence of dietary manipulation (LID), reduced susceptibility to two-stage skin carcinogenesis, inhibited epidermal hyperproliferation and attenuated IGF-1R and EGFR growth factor signaling during tumor promotion, in a manner similar to CR (Chapter III); vii) IGF-1 modulated activation of both the IGF-1R and the EGFR in cultured mouse keratinocytes (Chapter IV); viii) IGF-1 induced IGF-1R/EGFR heterodimerization and EGFR ligand mRNA expression in cultured

mouse keratinocytes (Chapter IV); and finally ix) CR reduced, while DIO increased the association between the IGF-1R and the EGFR, as well as EGFR ligand mRNA expression following TPA treatment (Chapter IV). Taken together, these findings suggest that dietary energy balance affects susceptibility to epithelial carcinogenesis due to alterations in epidermal growth factor signaling. Specifically, levels of circulating IGF-1, which directly correlate with caloric consumption, modulate signaling through both the IGF-1R and the EGFR due to the presence of IGF-1R/EGFR crosstalk (see Figure 5.1 for summary of crosstalk mechanisms observed *in vitro* and *in vivo*). These diet-induced changes in IGF-1R and EGFR activation (CR reduces, DIO increases) subsequently alter downstream signaling to Akt and mTOR, which then modulates levels of cell cycle regulatory proteins. This effect of dietary manipulation on cell cycle progression then alters epidermal hyperproliferation, thus inhibiting (CR) or enhancing (DIO) susceptibility to epithelial carcinogenesis (see Figure 5.2 for summary of diet-induced changes in cellular signaling and cellular response).

While the data presented fulfills the project aims, further *in vitro* and *in vivo* studies could be performed to provide a more comprehensive evaluation of the effects of IGF-1 on IGF-1R and EGFR crosstalk. Additional studies could be conducted to determine which of the three mechanisms (i.e., mRNA expression, IGF-1R/EGFR heterodimerization, ectodomain shedding of EGFR ligands) is most critical for the effects of IGF-1 on EGFR activation. For these studies,

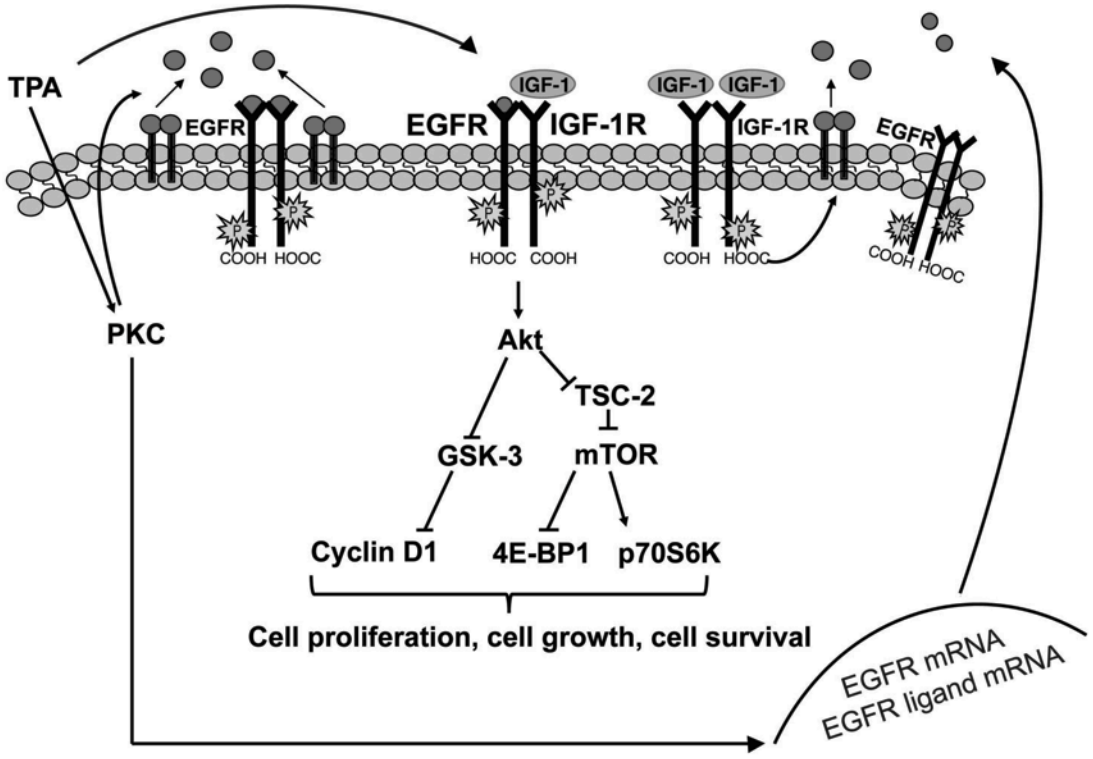

Figure 5.1. Proposed mechanisms of crosstalk between the IGF-1R and the EGFR in keratinocytes. Activation of the IGF-1R by IGF-1 leads to activation of the EGFR. In vitro, IGF-1 rapidly induces EGFR phosphorylation, in part due to an IGF-1R/EGFR association. We hypothesize that an additional mechanism may be occurring in which IGF-1 induces EGFR ligand ectodomain shedding. Our data suggests that sustained EGFR activation may be mediated by IGF-1 induced changes in EGFR ligand expression. In vivo, TPA treatment leads to an increase in IGF-1R/EGFR association, which is further enhanced with DIO. Furthermore, CR reduces, while DIO increases EGFR ligand mRNA expression both in the presence and absence of TPA treatment. Differential regulation of ectodomain shedding may contribute to the effects of energy consumption on IGF-1R/EGFR crosstalk and downstream signaling.

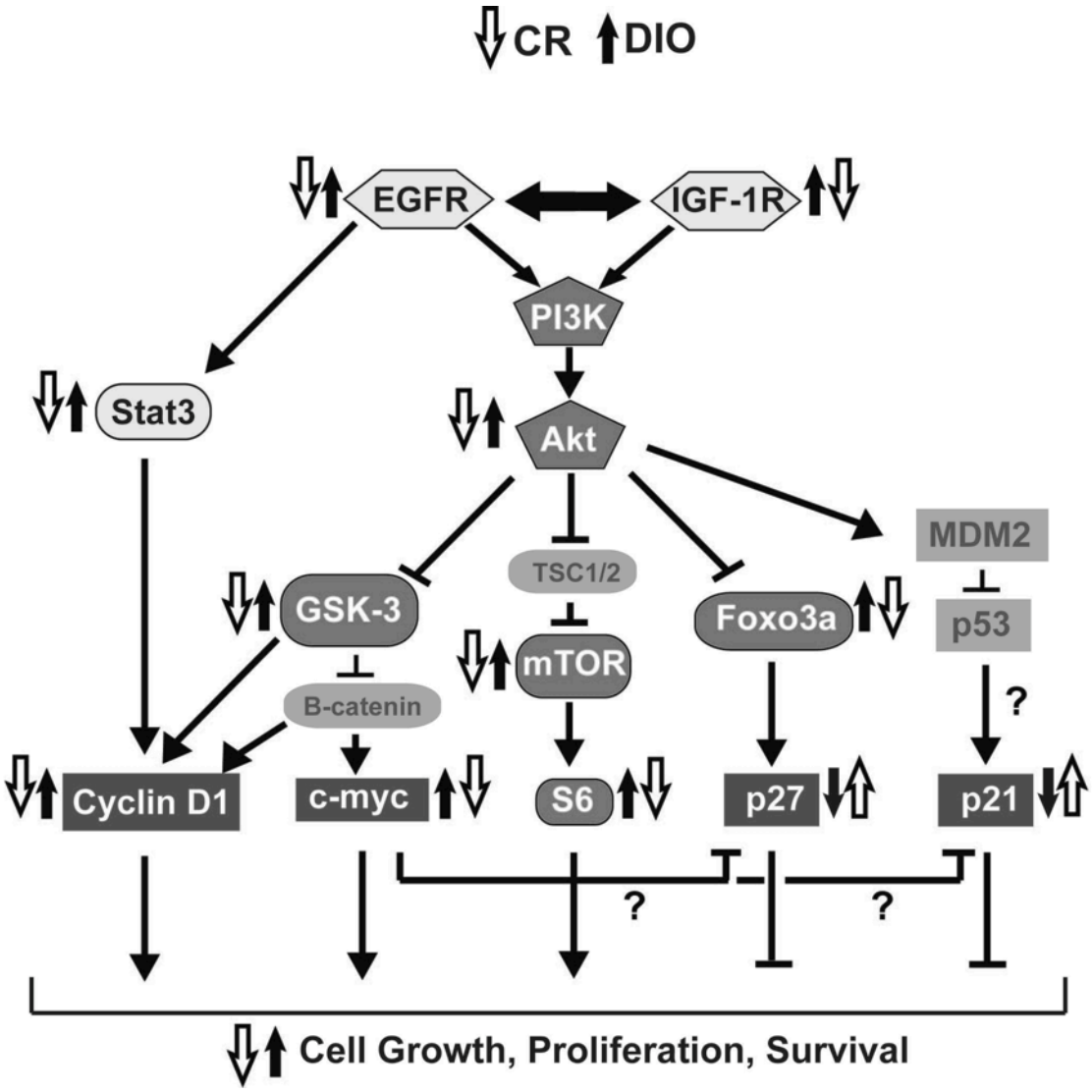

Figure 5.2. Proposed mechanism whereby dietary energy balance modulates epithelial carcinogenesis. DIO (black arrows) increases signaling through the IGF-1R and the EGFR, potentially due to IGF-1R/EGFR crosstalk, thus leading to heightened activation of Akt and mTOR. Subsequently, DIO increases protein levels of positive cell cycle regulators such as c-myc and cyclins A, E and D1 while decreasing the levels of negative cell cycle regulatory proteins (p21 and p27). Overall, this leads to increased cell growth, proliferation, and survival during tumor promotion. In contrast, CR (white arrows) leads to the opposite effects on these signaling pathways and cell cycle regulators, leading to a reduction in cell growth, proliferation, and survival during tumor promotion. Collectively, these changes in cellular signaling modulate susceptibility to epithelial carcinogenesis in mouse skin.

both pharmacologic (inhibit ectodomain shedding, bind available cleaved EGFR ligands) and genetic approaches (siRNA against EGFR ligands) could be utilized either alone or in combination, to evaluate the ability of IGF-1 to induce EGFR activation, as well as determine the mechanism that exerts the greatest effects on IGF-1 mediated cellular proliferation and cellular survival. Additional experiments, in which the EGFR is inhibited (siRNA, shRNA or a pharmacologic approach), could provide further mechanistic data. These studies would specifically determine the contribution of EGFR activation in the modulation of IGF-1 induced phosphorylation of Akt and mTOR, as well as the overall biological outcome. Together, these studies would more clearly establish the importance of IGF-1R and EGFR crosstalk, as well as determine the critical underlying mechanisms that contribute to the effects of IGF-1 on cancer-related cellular signaling pathways in mouse keratinocytes.

In vivo studies suggest that dietary energy balance modulates IGF-1R and EGFR crosstalk, however, it is unclear if these dietary effects are directly mediated by changes in levels of circulating IGF-1. Several experiments could be conducted to determine if IGF-1 is in fact modulating receptor crosstalk *in vivo*. First, we could evaluate the impact of *IGF-1* overexpression (BK5.IGF-1 transgenic mouse model), in the absence of dietary manipulation, on IGF-1R and EGFR crosstalk following treatment with TPA. Specifically, using BK5.IGF-1 transgenic mice, we could determine if *IGF-1* overexpression induces IGF-1R

and EGFR heterodimerization, as well as increases mRNA expression of EGFR ligands. These same experiments could be performed using the LID mouse model to determine if genetically reduced levels of circulating IGF-1 can function to inhibit crosstalk between the IGF-1R and the EGFR in a manner similar to CR. Finally, we could conduct a study in which CR mice are infused with recombinant human IGF-1, thus raising the level of circulating IGF-1 to those observed in DIO mice, and then evaluate IGF-1R/EGFR heterodimerization and EGFR ligand mRNA expression.

In addition to the proposed *in vitro* and *in vivo* studies that examine IGF-1R and EGFR crosstalk, further studies could be conducted to evaluate the impact and underlying mechanisms of IGF-1 on activation of other ErbB family members. As mentioned in Chapter IV, IGF-1 stimulated rapid phosphorylation of not only the EGFR, but also ErbB2, and this phosphorylation was sustained at later timepoints. Several mechanisms could account for these effects of IGF-1 on ErbB2 activation, including: i) EGFR/ErbB2 heterodimerization (early timepoints) (for review see 198); ii) MMP-mediated generation of a truncated, kinase active form of ErbB2 (early timepoints) (199); iii) and IGF-1R/ErbB2 heterodimerization (either full length or truncated form of ErbB2 – later timepoints) (200). Using approaches similar to those already proposed, we could determine the primary mechanism whereby IGF-1 modulates ErbB2 activation and the role that ErbB2 plays in IGF-1 mediated effects on cellular proliferation and survival. These

findings could then be evaluated *in vivo* using the animal models previously mentioned.

Finally, the data presented in the current project suggests that disruption of IGF-1R/EGFR crosstalk and downstream signaling to Akt and mTOR would function to inhibit, at least in part, the enhancing effect of positive energy balance on epithelial carcinogenesis. Findings from the proposed studies would identify the primary mechanism whereby IGF-1R activation modulates EGFR phosphorylation, thus providing a potential target for pharmacologic intervention. This would enable inhibition of IGF-1R and EGFR crosstalk without the use of broad-spectrum inhibitors of either receptor tyrosine kinase. Studies could then be conducted to evaluate the ability of this inhibitor of IGF-1R and EGFR crosstalk, either alone or in combination with mTOR and/or Akt specific inhibitors, to attenuate the effects of positive energy balance on TPA-induced cellular signaling, epidermal proliferation and two-stage skin carcinogenesis. Should these inhibitors be ineffective, additional studies could be conducted to evaluate the feasibility of using an IGF-1R or an EGFR specific inhibitor, either alone or in combination, as a chemopreventive strategy. Further studies could be conducted to determine if these inhibitors could mimic the anticancer effects of moderate CR (30% CR), in the absence of DIO, either alone or in combination with a milder CR regimen. Findings from these experiments could provide further insight that could then be used in translational chemoprevention studies.

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