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THE INVOLVEMENT OF THE STEROL RESPONSE ELEMENT BINDING PROTEINS IN LIPOGENESIS IN THE SEB-1 SEBACEOUS MODEL SYSTEM

A Thesis in

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by

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ABSTRACT

Excessive lipid production is an integral factor in the pathogenesis of acne. As such, an understanding of the molecular signaling involved in sebaceous gland lipid production is needed to identify therapeutic targets to improve acne. The sterol response element binding proteins (SREBPs) are a class of transcription factors known to regulate lipid production, particularly in response to insulin in the liver. We hypothesize that the SREBPs are important in sebaceous gland lipid metabolism, and thus represent a potential drug target for the treatment of acne.

The correlation between severity of acne and insulin-like growth factor-1 (IGF-1) levels in women led us to investigate the effects of IGF-1 on sebaceous lipid production and SREBP-1 levels. The work described in this thesis demonstrates that IGF-1 increases both lipogenesis and SREBP protein levels in SEB-1 sebocytes.

Furthermore, a variety of growth factors have been shown to increase SREBP mRNA and protein in many different model systems. However, the signaling pathway(s) that are important to transducing the growth factor signal seems to vary by cell type. We have found that IGF-1 stimulation activates both the PI3-K and the MAPK/ERK pathways in SEB-1 cells. As both of these pathways have been found to be responsible for growth factor stimulated increases of SREBP, we sought to determine if one or both pathways were important for the IGF-1 induced lipogenesis and increase in SREBP that we have observed in the SEB-1 cells. We report that treatment with the PI3-K inhibitor LY294002 completely blocks IGF-1 induced lipogenesis and SREBP transcription, translation, and processing in SEB-1 cells whereas the MAPK/ERK pathway inhibitor

PD98059 has no effect on lipogenesis or SREBP protein induction. These data indicate the PI3-K is the primary signaling pathway involved in lipogenesis in SEB-1 sebocytes.

In summary, this work provides evidence that SREBP-1 is a potential drug target for the reduction of lipid production in the treatment of acne. Furthermore, these data provide a rationale for the investigation of specific inhibitors to the individual members of the PI3-K family for treatment of acne.

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List of Abbreviations

°C	degrees Celsius
µ	micron
µCi	microcurie
µM	micromolar
15-Lox 2	15-lipoxygenase form 2
ACC	acetyl-coenzyme A carboxylase
ALS	acid-labile subunit
ACP	acyl carrier protein
ANOVA	analysis of variance
ATP	adenosine triphosphate
bHLH-ZIP	basic-helix-loop-helix leucine zipper domain
BP	blocking peptide
BSA	bovine serum albumin
C	cholesterol
CBP	CREB binding protein
CFU	colony forming unit
cm	centimeter
CO	cholesterol oleate
CoA	coenzyme A
COPII	coatomer protein II
COX	cyclooxygenase

cpm	counts per minute
CRSP	cofactor required for Sp1 activation
CTP	cytidine triphosphate
Da	dalton
DMEM	Dulbecco's modified eagle medium
DN	dominant-negative
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ED_{50}	effective dose 50
EGF	epidermal growth factor
EGTA	ethyleneglycol-O, O'-bis (2-aminoethyl)-N, N, N', N'-tetraacetic acid
ELISA	enzyme-linked immunosorent assay
ERK	extracellular signal-regulated kinase
ETYA	eicosatetraynoic acid
FAR	fatty acyl-CoA reductase
FAS	fatty acid synthase
FOH	fatty alcohol
FOX	forkhead
FOXO	forkhead class O
GDP	guanosine diphosphate
GFP	green fluorescent protein
GPAT	glycerol-3-phosphate acyltransferase
GSK3	glycogen synthase kinase 3

GTP	guanosine triphosphate
HETE	hydroxyeicosatetraenoic acid
HMG CoA	3-hydroxy-3-methylglutaryl coenzymeA
HNF-4	hepatic nuclear factor-4
HPETE	hydroperoxyeicosatetraenoic acid
HPLC	high performance liquid chromatography
IGF	insulin-like growth factor
IGF-1R	insulin-like growth factor type 1 receptor
IGF-2R	insulin-like growth factor type 2 receptor
IGFBP	insulin-like growth factor binding protein
IL-1α	interleukin-1α
IR-A	insulin receptor-A
IR-B	insulin receptor-B
IRS	insulin receptor substrate
JNK	c-Jun-N terminal kinase
kDa	kilodalton
KGF	keratinocyte growth factor
LDL	low-density lipoprotein
Lox	lipoxygenase
LXRE	liver X receptor response element
M	molar
MAPK	mitogen-activated protein kinase
MBC	methyl-β-cyclodextrin

MDI	methylisobutylxanthine, dexamethasone, and insulin
min.	minute
mL	milliliter
mm	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid
mTor	mammalian target of rapamycin
N	normal
nCi	nanocurie
NDGA	nordihydroguaiaretic acid
NF-Y	nuclear factor-Y
ng	nanogram
nM	nanomolar
no.	number
OA	oleic acid
P. acnes	propionibacterium acnes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE3b	Phosphodiesterase 3b
PDK	phosphoinositide-dependent kinases
PDGF	platelet derived growth factor
PI3-K	phosphoinositide 3-kinase
PIK fyve	FYVE domain-containing phosphotidylinositol 3 phosphate 5-kinase

PKB	protein kinase B (Akt)
PMSF	phenylmethylsulfonyl fluoride
PPARγ	peroxisome proliferators-activated receptorγ
PVDF	polyvinylidene fluoride
PX-1	pancreatic duodenal homeobox-1
QPCR	quantitative polymerase chain reaction
RAR	retinoic acid receptor
REST-XL	relative expression statistical tool
RNA	ribonucleic acid
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
S1P	site-1 protease
SCAP	SREBP-cleavage activating protein
SE	standard error
SH2	Src homology 2
siRNA	small interfering RNA
SP-1	stimulating protein-1
SQ	squalene
SRE	sterol response element
SREBP	sterol response element binding protein
SV40	simian virus 40
TAG	triglyceride (triacylglycerol)
TF	transcription factor

TLC	thin layer chromatography
TNFα	tumor necrosis factor-α
US FDA	United State Food and Drug Administration
UV	ultraviolet
wk	week

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Chapter 1

Literature review

1.1 Introduction

Acne vulgaris is a skin condition that has been described by scientists as early as Aristotle (Grant 1951). Characterized by seborrhea, comedones, and inflammatory papules, acne is most common during adolescence, though it can persist longer (Jansen 1998). Acne is specific to the sebaceous gland, which are specialized structures that secrete lipid. Sebaceous glands are located in all anatomical locations of human skin with the exception of the soles of the feet and palms of the hands. The largest glands are found on the face, chest, and upper back which, incidentally, are the areas most prone to acne lesions (Ebling 1992). While not a life-threatening affliction, the low self-esteem as well as the mental and physical scarring caused by acne warrants research that seeks to cure this disease.

The exact causes of acne are yet to be elucidated, but there is a known hormonal component. Recently, both insulin and insulin-like growth factor-1 (IGF-1), along with androgens have been shown to increase sebaceous activity, a known factor in acne pathogenesis (Deplewski and Rosenfield 2000).

A potential mediator of this increased sebaceous activity is a class of transcription factors called the sterol response element binding proteins (SREBPs) that regulates transcription of several lipogenic genes. In light of these findings, we hypothesized that

insulin or IGF-1 can act through the SREBP transcription factors to regulate lipid production in the sebaceous gland.

In the first section of the literature review, I will detail the epidemiological data that has been gathered on acne, the causes and most current treatments of acne, the anatomy of the sebaceous gland, and finally, models used to study the sebaceous gland. In the second section I will describe the insulin and IGF systems, and how they affect lipogenesis and acne. Finally, in the third section I will give an overview of the research on SREBPs, and describe how these transcription factors could be involved in the manifestation of acne.

1.2 Acne

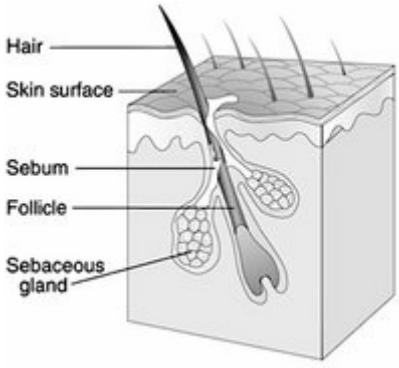
1.2.1 Epidemiological data

Acne affects approximately 50 million Americans (White 1998). Most people with acne are in their teenage years, and 79-95% of the adolescent population will be affected by acne (Cordain, Lindeberg et al. 2002). It has also been reported that some degree of facial acne is found in 54% of women and 40% of men older than 25 years of age, of which 12% of the women and 3% of the men will have this condition persist into the middle age range (Goulden, Stables and Cunliffe 1999).

1.2.2 Causes of Acne

Currently, acne is attributed to four major causes which include:

1. Increased sebum (skin lipid) production
2. Abnormal keratinization leading to partial obstruction of the follicle (comedo formation)
3. Proliferation of the anaerobic bacteria *Propionibacterium acnes* within the follicle
4. Inflammation



(taken from http://en.wikipedia.org/w/index.php?title=Sebaceous gland&oldid=73072526)

Figure 1: **Cartoon drawing of the sebaceous gland.** Sebceous glands are associated with hair follicles. As the sebocytes differentiate, cells fill with lipid until they rupture and the lipid content of the cells is extruded to the skin surface.

These factors are interrelated, and all contribute to the severity of the lesion. The increased sebum production coupled with the partial obstruction of the follicle provides both nourishment and an anaerobic environment in which the *P. acnes* bacteria can thrive. Human sebum is comprised primarily of triglycerides (41%), wax esters (25%), free fatty acids (16%), and squalene (12%) (Cheng and Russell 2004). The *P. acnes* bacteria cleaves triglycerides in the sebum for nourishment, releasing free fatty acids which stimulate the release of keratinocyte cytokines including IL-1α, TNFα, and

EGF/TGFα. It is these factors that mediate the inflammatory response (Downie, Sanders and Kealey 2002). Eventually, the lesion remits, but the mechanism by which this occurs remains a mystery, though de-differentiation and shrinkage of the sebaceous gland is known to be associated with remittance (Downie, Sanders et al. 2002).

Though the etiology of acne lesions is understood, there remains confusion among the general public and discord in the scientific community regarding the triggers of acne. A recent study surveyed new patients who were receiving medical attention for acne. This study revealed that hormones were the most common response as to what the patients perceived as the cause of their acne. These data are presented to show what the general public believes about the triggers of acne, and it is interesting to note that diet is the third most popular response (see Table **1**-Tan, Vasey and Fung 2001).

In the 1950's, dermatologists also believed that diet was the primary cause of acne (Wolf, Matz and Orion 2004). While the connection between diet and acne had fallen out of favor, primarily due to the lack of solid scientific studies supporting it, it has recently again received some attention.

Table **1**: **Factors believed by patients to cause acne.** Taken from (Tan, Vasey et al. 2001)

Factor	No. of responses	% Responses/patients
Hormones	50	64%
Genetics	30	38%
Diet	25	32%
Poor skin hygiene	23	29%
Infection	14	18%
Other	1	1%

Cordain *et al.* performed an interesting study in which they concluded that diet can be an important trigger of acne. This group studied two populations of humans who were found to be devoid of acne. The Kitavan islanders occupied an island near New Guinea and existed primarily as horticulturalists and fishermen. Their island did not have electricity, telephones, or motor vehicles at the time of the study in 1990 (Cordain, Lindeberg et al. 2002).

The second group that was found to be without acne is the Ache hunter-gatherers (Cordain, Lindeberg et al. 2002). This group resides in eastern Paraguay. Unlike the Kitavan islanders, the Aches made contact with western civilization in the mid 1970's. Their diet was estimated to be 8% western foods, with the remainder being cultivated goods and wild game.

From the data gathered on the Kitavians and Aches, Cordain *et al* put forth the idea that a diet consisting of low glycemic load foods will "cause a decrease in serum testosterone and fasting glucose levels while improving insulin metabolism and increasing concentrations of sex hormone binding globulin" (Cordain, Lindeberg et al. 2002). Interestingly, both insulin and IGF-1 have been shown to increase lipogenesis in the rat preputial sebaceous model (Deplewski and Rosenfield 1999).

It has also been hypothesized that consumption of milk may cause acne (Danby 2005). Danby argues that the collection of growth factors in bovine milk, including IGF-1, can stimulate sebaceous gland production of lipid. Another study implicates dairy intake with acne, but they also mention that dairy products can induce endogenous IGF-1 (Adebamowo, Spiegelman et al. 2005). It is difficult to critically evaluate some of these

data because of its dependence on the recall bias of a group of women aged 34-51 years, and their ability to remember how much milk they drank in their teenage years.

In response to groups which favor an important role of diet in acne development, it has been pointed out that 3 decades ago-before the explosion of soda and candy in schools-acne was a common problem for American teenagers (Bershad 2003). This indicates that it is not the high levels of refined sugar in the current Western diet that is to blame for the prevalence of acne. Additionally, the isolated groups of people studied by Cordain *et al* share genetic backgrounds and a similar environment, both of which cannot be removed from the equation (Thiboutot and Strauss 2002). There has never been a study that establishes a causal link between acne and diet, while it has been shown that diets consisting of foods believed to cause break outs by patients, failed to do so in a non-double blinded experiment.

In summary, diet has never been shown to directly cause acne. However, it is reasonable to suggest that as food intake affects hormone levels, this may have an impact on sebaceous activity, but there are certainly other factors involved.

Several interesting studies have been performed in humans testing the hypothesis that there is a genetic component to acne. A retrospective study performed by Goulden *et al.* recruited 204 acne cases and 144 non-acne control volunteers to study this issue. A detailed pedigree including all first-degree relatives was made totaling 1203 and 856 relatives (respectively). The individuals with acne cases, and non-acne control volunteers, answered questions about themselves and their own acne in addition to providing information about their relatives and acne. Two hundred and three relatives of individuals with acne also had acne, compared with only 42 first-degree relatives for the

control group (Goulden, McGeown and Cunliffe 1999). This data shows that the risk of adult acne occurring in a relative of a patient with adult acne is significantly greater than the risk for the control volunteer (odds ratio is 3.93; $p<0.001$).

Another large retrospective twin study based on 458 pairs of monozygotic twins and 1099 pairs of dizygotic twins (all women) found that 14% of the twins reported a history of acne. Further, using genetic modeling, it was shown that 81% of the variance of acne was attributable to additive genetic effects, while the remaining 19% was attributed to unshared environmental factors (Bataille, Snieder et al. 2002). These data are confirmed by the recent findings of Evans *et al* who attribute 31-97% of phenotypic acne to additive genetic effects in a prospective study of twins (Evans, Kirk et al. 2005).

1.2.3 Treatments for Acne

There are currently several treatments for acne, many of which can be used in combination with one another to increase their efficacy. Americans spend over $100 million per year on over-the-counter acne products and hundreds of millions more on doctor visits and prescriptions (Fielding and Ulene 2002). In this section, I will give the rationale for each class of drug along with the side effects for the current treatments of acne.

1.2.3.1 Topical retinoids

Topical retinoids have been used for more than 30 years and have been shown to effectively treat acne. The reference standard for this class is all-*trans* retinoic acid, though adapalene, and tazarotene are now available (Shalita 1998). These compounds reduce the hyperkeratinization that leads to comedone formation (Guzzo 1996). Retinoids act by binding to the retinoid receptors. Tretinoin is a photolabile compound and is also a photoirritant; patients undergoing topical retinoid treatment are urged to avoid sun exposure (Shalita and Strauss 1998). Skin irritation is also observed in patients undergoing topical retinoid treatments. Patients using topical retinoids alone typically experience a 40-70% reduction in comedones and inflammatory lesions. For this reason, topical retinoids are often paired with a topical or systemic antibiotic for severe forms of acne (Haider and Shaw 2004). A summary of clinical trial data with topical retinoids is shown in Table 2.

Table 2: **Summary of clinical trials using topical retinoids.**

Source	No. of Patients	Study Type	Length of treatment (wk)	Type of Acne	Treatment	% Reduction inflam. lesion	% Reduction noninflam. lesion	% Reduction total lesion
Cunliffe et al, 1988*	900	Meta-analysis	12	Mild to moderate facial acne	Adapalene 0.1% gel	52	58	57
					Tretinoin 0.025% gel	51	52	53
Salita et al, 1999*	449	Randomized, double-blind, placebo controlled multicenter	12	Mild to moderate facial acne	Tazarotene 0.1% gel	42	55	52
					Tazarotene 0.05% gel	39	45	44
					Vehicle	30	35	33
Leyden et al, 2002*	169	Randomized, double-blind, multicenter	12	Mild to moderate facial acne	Tazarotene 0.1% gel	56	60	…
					Tretinoin 0.1% gel	46	38	...
Webster et al, 2001*	143	Randomized, double-blind, multicenter	12	Mild to moderate facial acne	Tazarotene 0.1% gel	54	55	…
					Tretinoin 0.025% gel	44	42	...
Lucky et al, 2001*	237	Randomized, double-blind, multicenter	12	Mild to moderate facial acne	Adapalene 0.1% cream	36	38	38
					Vehicle	19	20	20
Webster et al, 2002*	145	Randomized, double-blind, multicenter	12	Mild to moderate facial acne	Tazarotene 0.1% gel	70	71	…
					Adapalene 0.1% gel	55	48	...
Leyden et al, 2001*	164	Randomized, double-blind, multicenter	15	Mild to moderate facial acne	Tazarotene 0.1% gel	54	58	…
					Adapalene 0.1% gel	57	55	...

…this measure was not assessed in the study Adapted from Haider and Shaw 2004.

1.2.3.2 Topical antimicrobials

Topical therapy for acne began in the 1960s with benzoyl peroxide formulations, and expanded significantly with utilization of topical antibiotics in the 1980s. Topical antimicrobials work primarily by bacteriostatic actions which reduce the number of *P. acnes* bacteria. Currently clindamycin, erythromycin, tetracline, and benzoyl peroxide are available as topical antimicrobials. *P. acnes* levels as high as 10^5 per follicle have been observed in acne patients, compared to 10^4 per cm^2 of skin in non-acne patients

(Leyden 1998). A secondary mechanism by which antimicrobial therapy can work is by a decrease in inflammatory mediators which can occur without death of the *P. acnes* bacteria (Webster, Leyden et al. 1982). It has been established that *P. acnes* is highly susceptible to a range of antibiotics including macrolides and tetracyclines (Leyden 1998), although recent antibiotic-resistant strains of *P. acnes* have emerged. For this reason, it is recommended that topical antibiotic therapy be combined with benzoyl peroxide, a topical antimicrobial therapy for which resistant *P. acnes* has not been seen (Gollnick, Cunliffe et al. 2003). In fact, a recent study comparing the efficacy of clindamycin and erythromycin in the 1970's versus recent publications concludes that there is diminished efficacy which the authors attribute to antibiotic resistance (Simonart and Dramaix 2005). Common side effects from this class include erythema, peeling, dryness, and burning. A summary of recent clinical trials for topical antibiotics is shown in Table **3**.

Table 3: **Summary of clinical trials with topical antimcrobials.**

Source	No. of Patients	Length of treatment (wk)	Type of Acne	Treatment	% Reduction inflam. lesion	% Reduction noninflam. lesion	% Reduction total lesion
Becker et al, 1981	358	8	Mild to moderate acne	Clindamycin phosphate	66	…	…
				Clindamycin hydrochloride	63	...	…
				Vehicle	42	...	...
Dobson and Belknap, 1980	253	12	Mild to moderate acne	Erythromycin 1.5% solution	70	26	40
				Vehicle	5	55	30
Lesher et al, 1985	225	12	Mild to moderate acne	Erythromycin 2%	46	…	…
				Vehicle	19	...	...
Jones and Crumley, 1981	156	12	Moderate to severe facial acne	Erythromycin 2%	51	…	…
				Vehicle	33	...	...
Habbema et al, 1989	122	12	Moderate to severe facial acne	Erythromycin-4% zinc solution	85	68	…
				Erythromycin-2% lotion	46	49	...
Lookingill et al, 1997	334	11	Mild to moderate facial acne	Clindamycin-1%/BP 5% gel	61	36	…
				Clindamycin-1% gel	35	9	…
				BP5% gel	39	30	…
				Vehicle	5	0	...
Cunliffe et al, 2002	79	16	Mild to moderate facial acne	Clindamycin-1% plus/BP 5% gel	…	…	53
				Clindamycin-1%	...	...	28

…this measure was not used in the study Adapted from Haider and Shaw, 2004

1.2.3.3 Systemic antibiotics

The most common oral antibiotics for acne treatment are tetracycline, doxycycline, minocycline, and erythromycin. Used for more severe acne cases, oral antibiotics kill *P. acnes* in addition to inhibiting bacterial lipase activity (Haider and Shaw 2004). Due to the risk of antibiotic resistance of *P. acnes*, systemic therapy is almost always used in conjunction with another acne treatment, though antibiotic-resistant strains of other bacteria is still a risk with this therapy.

1.2.3.4 Hormonal treatment

Hormonal treatment for acne is only for women, as these treatments decrease the effects of androgens. Elevated serum androgen levels have been found in women with acne (Thiboutot, Gilliland et al. 1999). Anti-androgenic compounds include oral contraceptives and androgen-receptor blockers such as flutamide, spironolactone, and cyproterone acetate. The United States Food and Drug Administration has not approved any androgen-receptor blockers for use in treating acne. Oral contraceptives suppress ovarian androgen production, lowering the serum androgen levels (Haider and Shaw 2004). As hormonal therapy targets the excessive sebum production associated with acne, it is often used in combination with a topical retinoid or antimicrobial therapy (Thiboutot and Lookingbill 1998).

1.2.3.5 Oral Isotretinoin

Oral isotretinoin, or 13-*cis* retinoic acid, was first used in 1982 and has had a profound impact on severe acne patients. Isotretinoin prevents differentiation of the basal cells in the sebaceous gland. Due to the lack of differentiation, the cells do not fill with lipid and rupture, causing as much as a 90% decrease in sebum production (Shalita and Strauss 1998). Additionally, there is also evidence that isotretinoin has a beneficial impact upon follicular epithelial differentiation and has anti-inflammatory properties (Shalita and Strauss 1998). The typical course of treatment is 4-6 months, and the effects can last as long as three years.

Despite the tremendous benefits of isotretinoin, there are substantial drawbacks. First, the mechanism by which isotretinoin works is not yet understood. It is believed that isotretinoin may be converted to tretinoin which then binds various nuclear receptors, however, recent work has shown that isotretionoin causes cell cycle arrest and apoptosis in a retinoid acid receptor (RAR) independent manner in SEB-1 sebocytes, and that this cannot be duplicated by 9-cis or all-trans retinoic acid (Nelson, Gilliland et al. 2006). Second, there are significant health and regulatory issues for patients being treated with isotretinoin. Typical side effects include dry lips, dry skin, dry eyes, decreased night vision, headache, epistaxis, and backache. Additionally, mild to moderate elevation in liver enzymes and in serum lipid indices may be present. For this reason, patients are recommended to have cholesterol, fasting triglyceride, and liver functions tests performed at the beginning of therapy, and then four and eight weeks into the course of treatment (Haider and Shaw 2004). Finally, isotretinoin is a known teratogen. Female patients

must have a negative pregnancy test, use two forms of contraception, and submit to a pregnancy test monthly as long as they are being treated with isotretinoin. Additionally, the prescribing physician must identify on each prescription that the patient has met qualifications and signed a consent form. Isotretinoin is the second drug (after Thalidomide) whose use is now restricted within a registry system as mandated by the FDA in 2006.

1.2.4 Sebaceous gland model systems

A model system is needed because sebaceous glands differentiate and rupture in primary culture, making it difficult to have enough viable cells for an experiment. In an ideal model 1) the entire gland would be used, 2) the gland should be morphologically similar to that of man with a sebaceous follicle, infundibulum, lobules, and a piliary unit, 3) the model should be androgen sensitive, and 4) the model should be economical of both material and time (Plewig and Luderschmidt 1977). The following sections describe the progress that has been made in developing various sebaceous models, and briefly provide the benefits and disadvantages of each.

1.2.4.1 Rat/mouse preputial model

The preputial gland in rodents is used for territorial marking. These glands are holocrine glands that mature in a manner similar to sebaceous glands and have been shown to be androgen responsive (Potter, Prutkin and Wheatley 1979). Preputial glands

have been used as an androgen responsive model since the 1950's. The primary limitation of this gland as a sebaceous model is that the composition of lipid produced by the preputial gland differs significantly from the lipid composition of human sebum (Nikkari 1974). Additionally, the preputial glands do not contain a piliary unit as sebaceous glands do, which may or may not be an important factor (Plewig and Luderschmidt 1977). One other disadvantage of this model is that it is not possible to test compounds by topical administration to the preputial glands. Only systemic compounds can be tested.

In 1979 it was shown that cells isolated from the mouse preputial gland tumor could be grown in monolayer (Potter, Prutkin et al. 1979) and this work was extended in 1989 when Rosenfield *et al* showed that rat preputial glands can be disbursed into a single cell suspension and grown on a layer of 3T3 fibroblasts (Rosenfield 1989). These cells grow in monolayer and express K4, a keratin found in sebaceous cells (Laurent, Mednieks and Rosenfield 1992). However, cells grown in monolayer do not decrease in number when treated with retinoic acid (Laurent, Mednieks et al. 1992).

Overall, the preputial gland/cells grown in monolayer are viable models, with limitations that must be considered when interpreting data. Most importantly, the lipid composition and differentiation process is different than that for sebocytes.

1.2.4.2 Hamster ear/flank model

Like the rat and mouse preputial gland, the flank organ (costovertebral gland) of the hamster is also used by the animal for territorial marking. These glands are similar to

human sebaceous glands in that they have an infundibulum, a sebaceous duct, multiple lobules, and a piliary unit which enters from below the gland (Plewig and Luderschmidt 1977). The flank organs are visible paired structures located on the back of the hamster. A benefit of this model is that hair can be shaved from the hamster and topical application of a drug can be made to one flank organ, while the other flank can serve as a control. This eliminates animal to animal variation. The flank organ is approximately 6-8mm in diameter, much larger than the human sebaceous gland. Like the human sebaceous gland, the flank organ is responsive to androgens (Chen, Puy et al. 1995), though numerous compounds that have shrunk the flank organ have failed to be effective in humans (Franz, Lehman et al. 1989).

Another model used is the hamster ear model. The skin on the inside of the hamster ear contains a dense layer of sebaceous glands. These glands have similar morphology to the human sebaceous gland, similar turnover time, and are also androgen responsive (Matias and Orentreich 1983). The glands are larger in males, and also vary in size depending on location on the ear. Each ear contains 8 to 15 glands that can be seen using a microscope under a low objective (Plewig and Luderschmidt 1977). Like the flank organ model, the hamster ear sebocytes can be used for topical application of drugs, and further, they may be a better model than the flank organ because their size is similar to human sebaceous glands--0.1750mm^2 compared to 0.2175mm^2 for humans in sagittal sections (Plewig and Luderschmidt 1977). The hamster ear sebocytes decrease in size when treated with isotretinoin (Geiger 1995), although for other treatments the hamster ear sebaceous glands tend to respond to treatments in the same way the flank organs do, limiting their usefulness as a model.

To date, no animal model has been found predictive in assessing the effects of anti-acne drugs in humans (Geiger 1995). Because acne is an exclusively human disease and the fact that sebaceous gland activity and differentiation is species specific, many have attempted to create a model using human sebocytes (Nikkari 1974). It should be pointed out that there is "feline acne" and "canine acne", but the use of the term acne in this case is a misnomer, as for a species to have acne, there must be blackheads, whiteheads, and inflammatory papules. There has been no other species whose "acne" lesions meet these criteria.

1.2.4.3 Growth of human sebocytes in monolayer

There have been several reports describing various ways to grow primary sebocytes. Most are variations on one of two techniques (Xia, Zouboulis et al. 1989; Doran, Baff et al. 1991). This work preceded the advent of growing sebaceous glands in organ culture. Most importantly, cells in primary culture exhibit an incomplete differentiation (Zouboulis, Xia et al. 1998).

To circumvent the difficulties in collecting sufficient human skin, two cell lines have been created by SV40 immortalization of primary sebocytes. The SEB-1 cell line (Thiboutot, Jabara et al. 2003) and SZ95 cell line (Zouboulis, Seltmann et al. 1999). Both cell lines 1) have been passaged for several years, 2) are androgen responsive, 3) produce lipid including triglycerides, squalene, and wax esters, 4) possess markers characteristic of sebocytes, and 5) have proliferation inhibited by 13-*cis* retinoic acid. Though much more convenient and practical for large scale studies, immortalized

sebocytes do not fully differentiate as evidenced by the decreased amount of squalene and wax esters produced compared to sebum.

1.2.4.4 Growth of human sebaceous cells/glands in primary culture

It has been demonstrated that excised human sebaceous glands can be grown for up to 7 days in organ culture (Guy, Ridden and Kealey 1996). Human chest skin from cardiac surgery is sheared and maintained on polycarbonate filters. In this environment, sebocytes differentiate as they would *in vivo*. Importantly, sebaceous glands maintained in organ culture respond to steroids and 13-*cis* retinoic acid as do sebaceous glands *in vivo*. The primary drawbacks for this model include: difficulty in obtaining skin, difficulty in preparing the glands for culture, limitations of the size of experiments that can be performed, and experiments are limited to treatments of up to 7 days from excision.

Clearly there are benefits and drawbacks to each model system. It is important to be aware of the shortcomings of each model when interpreting data, and more work is needed to provide a sebaceous model that more accurately reflects the intact human sebaceous gland.

1.3 IGF and Insulin

1.3.1 Insulin

Insulin was first isolated in 1921 by Banting and Best who administered pancreatic islet cell extracts to a diabetic young boy (Banting 1922). The amino acid sequence was determined in 1960 by Sanger and complete synthesis of the protein was accomplished in 1963. Insulin is formed from a single gene in most species, though both the mouse and rat have two genes which yield two insulin molecules that differ in two amino acids (Davis 1996).

An essential metabolic hormone, insulin is initially formed by the beta cells of the pancreas as a 110 amino acid peptide called preproinsulin. After crossing the rough endoplasmic reticulum membrane, the 24 amino acids on the amino terminus are cleaved and 3 disulfide bonds are formed on the resulting proinsulin molecule. In the Golgi complex, four basic amino acids and a connecting peptide are cleaved by proteolysis yielding two peptide chains (the A and B chain) which contain two intersubunit disulfide bonds and an additional disulfide bond in the A chain (Davis 1996). The A chain is usually composed of 21 amino acids, and the B chain is 30 amino acids; the molecular weight of an insulin molecule is 5800 Da.

Insulin stimulates glucose uptake, protein metabolism, lipid metabolism, as well as RNA and DNA synthesis (Kahn and White 1988). Fasting concentrations of insulin in portal blood are 2-4 ng/mL, while the peripheral circulation has a fasting concentration of 0.5ng/mL (0.1nM) (Davis 1996). After feeding, an immediate increase in insulin concentration in portal blood is observed, followed shortly thereafter by a lesser increase

in peripheral insulin concentration. The half-life of insulin *in vivo* is 6-7 minutes, and 50% of insulin that reaches the portal vein is destroyed before reaching the general circulation. Most degradation of insulin occurs in the liver, kidney, and muscle (Duckworth 1988). Insulin has high affinity for its receptor, by which its effects are mediated. Interestingly, the binding properties of the insulin receptor are more highly conserved throughout nature than the insulin molecule itself (Muggeo, Ginsberg et al. 1979).

1.3.2 Insulin-like growth factor-1 and 2 (IGF-1 and 2)

The IGF system consists of two peptide hormones, IGF-1 and IGF-2, along with myriad circulating binding proteins and cell surface receptors. There are six binding proteins identified to date, in addition to the IGF-1 and IGF-2 cell surface receptors (Denley, Cosgrove et al. 2005). IGF-1 is the most studied member of the group, and was discovered in 1957 when researchers observed that rat serum stimulated the incorporation of $^{35}SO_4$ into chondroitin sulfate in cartilage, while serum from hypophysectomized rats did not stimulate ^{35}S incorporation. Chondroitin sulfate is responsible for the elasticity property of cartilage. It should be noted that addition of growth hormone to the media of the incubated cartilage did not increase ^{35}S incorporation, but they found that addition of growth hormone, now known to cause the release of IGF-1, to hypophysectomized rats, increased ^{35}S incorporation. This led researchers to conclude that growth hormone itself does not stimulate growth processes; rather it induces formation of mediators that carry out this action (Salmon and Daughaday 1957). These factors were initially called

sulfation factors, later they were called somatomedins, and now insulin-like growth factors (Daughaday, Hall et al. 1972).

1.3.2.1 IGF structure

IGF-1 is 7649 daltons and is comprised of 70 amino acids, while IGF-2 is 7471 daltons and is 67 amino acids. Both molecules have three intra-chain disulfide bridges. Compared to one another, there is a 70% amino acid identity (Rosenfeld, Lamson et al. 1990). Both molecules also have a high sequence homology to proinsulin, though they each are composed of a single polypeptide chain (Rinderknecht and Humbel 1978).

1.3.2.2 IGF function

Both IGF-1 and IGF-2 exert their actions by binding to the IGF-1 receptor. As shown in Table 4, IGF-1 binds the IGF-1 receptor with a higher affinity than IGF-2, and also is 3 to 5 times more potent in stimulating DNA synthesis (Froesch, Schmid et al. 1985).

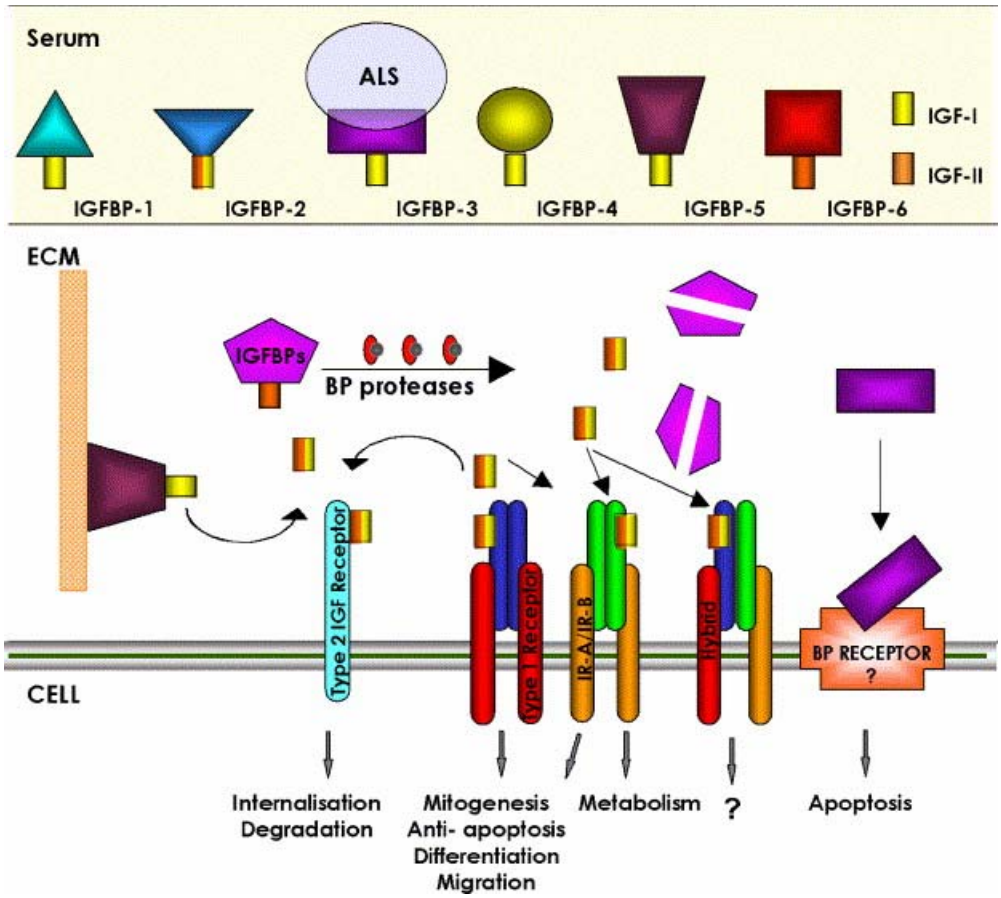
Administration of IGF-1 to hypophysectomized rats restores growth, enhances sulfate inorporation into proteoglycans, increases the synthesis of protein, RNA, and DNA, and promotes transport of amino acids and glucose into muscle. In addition, IGF-1 also increases lipogenesis in adipose tissue, increases renal plasma flow and increases glomerular filtration rate (Froesch, Schmid et al. 1985). Of more pertinence to this work, IGF-1, insulin, or growth hormone stimulates differentiation in the rat preputial

sebaceous model. Growth hormone stimulates differentiation the most, implying a role for growth hormone alone, while IGF-1 stimulates DNA synthesis more than any other treatment (Deplewski and Rosenfield 1999).

1.3.2.3 IGF regulation

IGFs are hormones that act both in an endocrine as well as a paracrine/autocrine manner. Ninety percent of IGF is produced by the liver where synthesis is stimulated by growth hormone released from the pituitary gland. Very little IGF is stored in tissue; rather it is rapidly secreted (Froesch, Schmid et al. 1985), though there is a large amount of inactive IGF bound to proteins in serum. IGF circulates with 99% bound to the insulin-like growth factor binding proteins (IGFBPs) and acts in an endocrine manner. Additionally, all cells examined have been shown to have the capacity to produce IGF which would act in a paracrine/autocrine manner. IGF levels in humans remain quite constant, largely due to the actions of IGFBPs and the IGF-2 receptor, both of which will be discussed in the next section.

IGF-1 can be present in serum at up to 1µg/mL without causing hypoglycemia, though only a few nanograms are needed to have a significant impact on cell growth. This regulation is accomplished by the IGFBPs. There are six IGFBPs which bind IGF with greater affinity than the IGF receptor (Duan and Xu 2005). In addition, the IGFBPs stabilize IGF, increasing its half life from 10 minutes to 15 hours. IGFBPs also serve to transport IGF from the liver to the peripheral circulation (Figure 2-Denley, Cosgrove et al 2005).



Taken from Denley *et al*, 2005

Figure 2: **There is a complex interplay between the members of the IGF system.** The IGF system consists of four receptors (IGF-2R, IGF-1R, two IR isoforms, and IGF-1R:IR hybrids). Most IGF circulates bound to IGFBP3 and the protein called acid-labile subunit (ALS). Upon cleavage by a protease or binding to the extracelluar matrix (ECM), free IGF is released and binds to IGF-1 receptors, insulin receptors, and hybrid receptors. It is also possible that IGFBPs may bind to a receptor to mediate their own biological actions (proposed BP receptor).

IGFBPs are secreted proteins produced by the liver in a growth hormone independent manner, though IGF-1 has been found to control IGFBP-3 expression (Froesch, Schmid et al. 1985). The six IGFBPs share a highly conserved N-terminal domain, a conserved C-terminal domain, and a linker that varies. IGFBP-3 and 5 also

bind the acid labile domain through their C-domains. Despite their high degree of conservation, each IGFBP has different structural and biochemical features such as glycosylation, molecular weight, cell surface binding, heparin binding sequence, nuclear localization signal, and serine phosphorylation sites (Duan and Xu 2005).

IGFBP-1 is found exclusively in the liver, while all other forms are present in both the liver and peripheral tissue. Interestingly, IGFBP-2, 3, and 5 have been shown to both inhibit or potentiate the effects of IGF. It has been proposed that IGFBPs can bind proteoglycans which provides a reservoir of IGF in close proximity to the IGF-1 receptor (Bach, Headey and Norton 2005). There is also evidence that IGFBPs possess biological activities that are ligand independent, whereby they bind to unknown receptors or proteins to mediate a biological response (Duan and Xu 2005). For instance, IGFBPs bind to integrins to cause cell migration.

As IGFBPs work to control the amount of active IGF released from the serum, the IGF-2 receptor appears to clear excess IGF. This receptor has some signaling activity as it is linked to a G-protein, and receptor stimulation results in the PKCα mediated release of acetylcholine from the rat hippocampal formation, though this is the only report of important IGF-2 signalling (Hawkes, Jhamandas et al. 2006). The IGF-2 receptor consists of a long extracellular domain containing 15 domains consisting of 124-192 amino acids, a 23 amino acid transmembrane domain, and a small 164 amino acid cytoplasmic domain (Hassan 2003). There is no homology between IGF-2R and either IGF-1 or the insulin receptor (IR) and no ATP binding site. The type 2 receptor has been identified as the cation-independent mannose 6-phosphate receptor which is involved in cellular transport of lysosomal enzymes (Rosenfeld, Lamson et al. 1990). Lending further credence to the

hypothesis that the primary function of the IGF-2 receptor is to clear IGF from the serum is the fact that the IGF-1 receptor has been shown to be downregulated in response to insulin or IGF-1, while the number of IGF-2 receptors increases in response to insulin or IGF-1 (Rechler and Nissley 1985).

1.3.3 Insulin receptor, IGF-1 receptor, hybrid receptors

Both the insulin receptor and the IGF-1 receptor are transmembrane glycoproteins, where two α subunits and two β subunits comprise a single receptor. In both receptors, the α subunit is extracellular, while the β subunit is a transmembrane protein that has tyrosine protein kinase activity. Additionally, the α subunit of the insulin receptor is alternatively spliced, where one variant excludes exon 11 (A form) and the other includes exon 11 (B form) (Kahn and White 1988). This alternative splicing has been shown to affect the affinity of both insulin and IGF-1 binding to the insulin receptor. The IR/IGF-1R has been divided into six extracellular structural domains, and three intracellular structural domains (Figure 3). There is a high degree of amino acid sequence identity between the IGF-1 and insulin receptor (Siddle, Urso et al. 2001).

Taken from Siddle *et al.* 2001

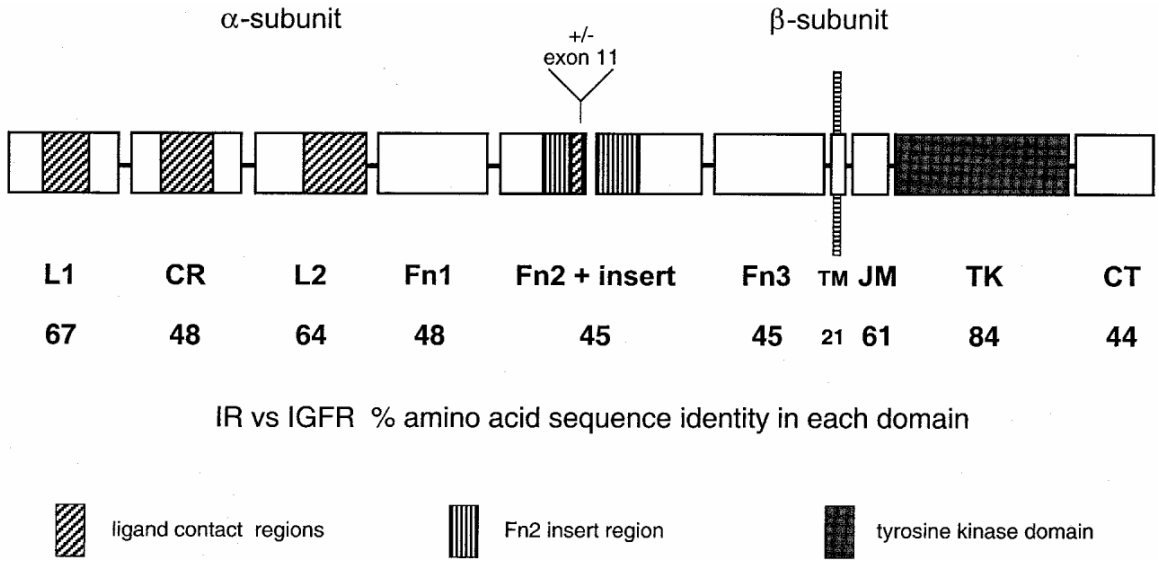


Figure 3: **The insulin receptor and IGF-1 receptor have several conserved domains.** The IR and IGF-1R contain the following structural domains: L1and L2 (β helices), CR (cystein-rich), Fn1, Fn2, Fn3, (fibronect type III; seven-stranded β-sandwich), TM (transmembrane), JM (juxtamembrane), TK (tyrosine kinase), and CT (C-terminal). Numbers represent the percentage of amino acid sequence identity within each domain.

In addition to binding the ligand, the α subunits also prevent activation of the β subunit, as elimination or modification of the α subunit has been shown to result in constitutive activation of the β subunit (Kahn 1994). Another form of receptor has been observed but not well characterized is the hybrid receptor. This receptor is also a tetramer; however the hybrid receptor contains one insulin receptor α and β subunit paired with one IGF-1 receptor α and β subunit. Some functional implications of the hybrid receptor will be described later.

The high degree of homology between the IGF-1R and the IR in the alpha chain presents challenges in dissecting the signaling pathways of each receptor. The insulin receptor can bind IGF-1 with an affinity 100-fold less than it binds its native ligand.

Likewise, the IGF-1 receptor has an affinity 100-1000 times weaker for insulin than for its natural ligand (Jones and Clemmons 1995). Hybrid receptors bind insulin with lower affinity than native insulin receptors, and to act more like IGF-1 receptors (Siddle, Urso et al. 2001) further complicating the process of dissecting individual signaling cascades from these receptors. A summary of the binding properties of the various insulin and IGF receptors to each ligand is presented (Table 4).

Table 4: **Affinities of insulin, IGF-1, and IGF-2 for various types of receptors in the IGF system.**

	IGF-1 (ED_{50} nM)	IGF-2 (ED_{50} nM)	Insulin (ED_{50} nM)
IGF-1R	0.2	0.6	>100
IR-A	120	0.9	0.2
IR-B	366	11	0.3
Hybrid-A	0.3	0.6	3.7
Hybrid-B	2.5	15	>100
IGF-2R	0.4×10^{-3}	0.2	No binding

Based on Dentin, Girard and Postic 2005

The insulin receptor and the IGF-1 receptor are members of the tyrosine kinase family, more specifically, the class-II tyrosine receptor family that features cysteine-rich sequences and heterotetramers linked by disulfide linkages.

By definition, members of the receptor tyrosine kinase family have the following four characteristics:

1) an extracellular ligand binding domain

2) an intracellular regulatory domain

3) an intracellular tyrosine kinase domain

4) a transmembrane domain

The insulin receptor is found on every cell in the body. Receptor number per cell ranges from 40 on erythrocytes to 200,000 receptors per cell on adipocytes and hepatocytes (Kahn and White 1988). The IGF-1 receptor, on the other hand has been found on all cell types with the exception of mature B cells (Valentinis and Baserga 2001).

The binding of insulin or IGF-1 to its receptor is a quick, reversible process (Perz and Torlinska 2001). Upon binding of the ligand to the extracellular α domain, the receptor has a rapid intramolecular phosphorylation of several tyrosines in the β subunit. In this process, ATP serves as the phosphate donor (Kahn and White 1988). This phosphorylation is then transferred to additional proteins that interact with the the activated β subunit, most notably the insulin receptor substrate proteins (IRS 1,2,3, and 4) and the Src homology proteins (Shc). Phosphorylation of these proteins triggers two major signaling pathways: the mitogen actiaved protein kinase (MAPK) and the phosphotidylinositol 3-kinase (PI3-K) pathway which will be discussed in more detail in the following sections (White, Maron and Kahn 1985; Siddle, Urso et al. 2001; Grimberg 2003). Additionally, upon binding, both insulin (Smith, Cobb et al. 1985) and IGF-1 (Chow, Condorelli and Smith 1998; Grimberg 2003) receptors aggregate on the plasma membrane where they are internalized in clathrin-coated pits. Once inside the cell, they are either degraded or recycled back to the cell surface (Davis 1996). It seems that internalization is important for some aspects of insulin and IGF-1 signaling, though details of this mechanism are unknown (Goldfine 1987). The half life of an insulin receptor is 8-12 hours (Kahn and White 1988).

The β chains which are responsible for transmitting the signal upon ligand binding exhibit a very high degree of sequence identity between the IGF-1 receptor and the IR (Figure **3**). Additionally, the effects of the IGF-1 receptor and the IR are exerted primarily by phosphorylating IRS proteins. Recent work performed by several groups has described methods of signal transduction by the IGF-1 receptor and the IR that occurs apart from the phosphorylation cascade described above. Siddle and co-workers described a role for proteins to interact directly with either the insulin or IGF-1 receptor in a manner not related to the phosphorylated tyrosines in the β subunit (Siddle, Urso et al. 2001). Dalle and colleagues showed that the IGF-1 receptor can signal through a heterotrimeric G-protein containing $G_{\alpha i}$, which the insulin receptor does not require. Additionally, the insulin receptor uses $G_{\alpha q/11}$, whereas the IGF-1 receptor does not (Dalle, Ricketts et al. 2001). Furthermore, Povsic and coworkers showed that β-arrestin, a cytosolic protein that plays a central role in G-protein coupled receptor signaling, mediates IGF-1 activation of PI-3 kinase, while it has no effect on insulin signaling (Povsic, Kohout and Lefkowitz 2003). Finally, β-arrestin has also been shown to attenuate insulin-induced degradation of IRS-1, leading to increased insulin signaling via IRS-1 (Usui, Imamura et al. 2004). This “non-classical insulin/IGF-1 signaling” hypothesis is in its early stages, but could explain how the IGF-1 and IR have such similar connections to the traditional signaling pathways, yet different effects on cellular processes (Kim and Accili 2002).

1.3.3.1 PI3-K signaling pathway

The means by which IGF-1 and insulin exert their biological effects are not fully understood. The two signal transduction pathways activated by insulin and IGF-1 that are most studied are the PI3-K and MAPK cascades. Undoubtedly, there are other pathways activated by insulin and IGF-1, but for the purposes of this review, only the two most common will be covered (Scrimgeour, Blakesley et al. 1997).

Upon ligand binding to the IGF-1 or IR, autophosphorylation occurs on the receptor. IRS proteins interact with the phoshorylated receptor and are themselves phosphorylated. Phosphorylated IRS proteins in turn intereact with the regulatory 85 kDa subunit of the PI3-K, and this results in phosphorylation in both SH2 domains. The 110 kDa subunit of the activated PI3-K protein then converts phosphoinositide 3,4-bisphosphate into phosphoinositide 3,4,5 trisphosphate. This, in turn, recruits phosphoinositide-dependent kinases (PDKs) which have the ability to interact with pleckstrin-homology domains (Shepherd 2005). Because of this characteristic, PDKs can activate other protein kinases including Akt (protein kinase B) by phosphorylation (Siddle, Urso et al. 2001). Activated Akt phosphorylates many substrates to control biological signaling, including glucose transport, protein synthesis, glycogen synthesis, proliferation, and cell survival (White 2002-Figure 4).

Taken from Shepherd, 2005.

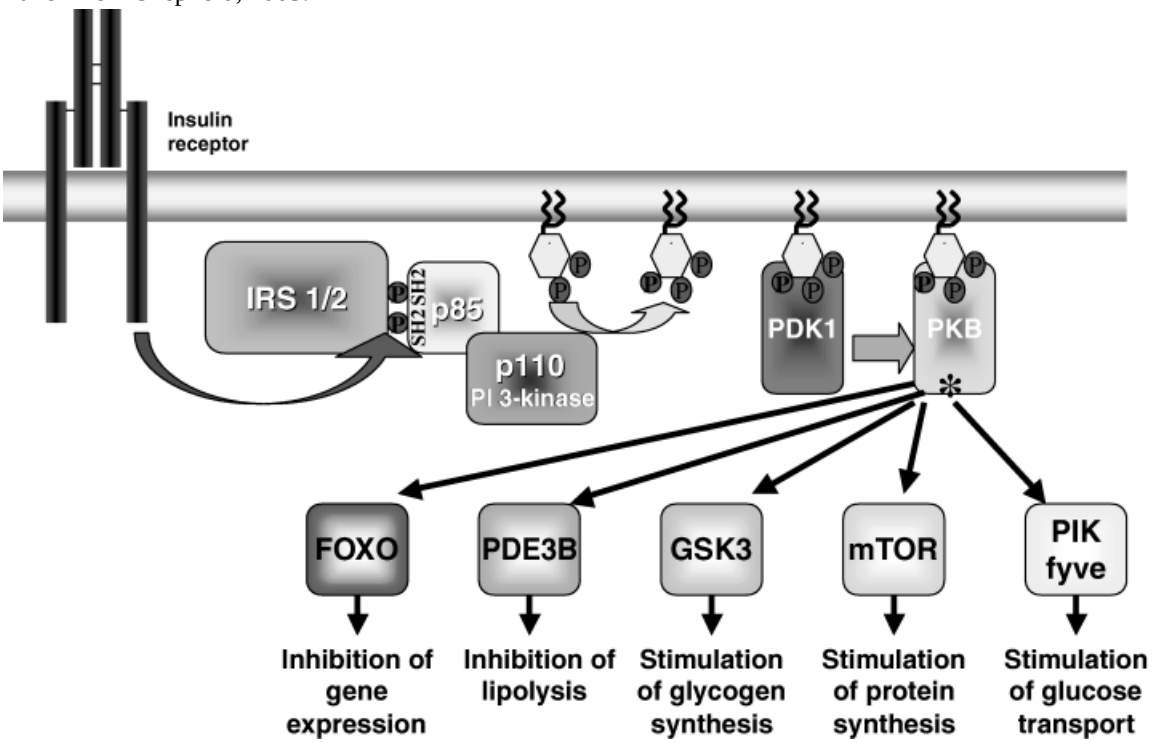


Figure 4: **Activation of the PI3-K pathway via the insulin or IGF-1 receptor.** In this diagram, PKB is used in place of Akt. Abbreviations used in this figure: IRS (insulin-responsive substrate), PDK1 (phosphoinositied-dependent kinase 1), PKB (protein-kinase B), FOXO (forkhead boxO), PDE3B (phosphodiesterase 3b), GSK3 (glycogen synthase kinase 3), mTOR (mammalian target of rapamycin), PIK fyve (FYVE domain-containing phosphotidylinositol 3 phosphate 5-kinase).

1.3.3.2 MAPK

In addition to the IRS proteins, the phosphorylated insulin/IGF-1 receptor interacts with src-homology 2 (SH2) containing adaptor proteins such as growth factor receptor-bound protein-2 (Grb-2) (Siddle, Urso et al. 2001). Grb-2 then binds to a nucleotide-exchange protein called SOS which recruits Ras and loads GTP onto the Ras molecule (Werner and Le Roith 1997). Ras then recruits and phosphorylates Raf (Avruch, Zhang and Kyriakis 1994). Phosphorylated Raf begins the classical MAPK

cascade, which includes subsequent phosphorylations of MEK and ERK. A graphical depiction of how the insulin signal is transduced though the MAPK pathway is shown (Figure 5). Acitvated ERK regulates growth factor responsive targets in both the nucleus and cytosol to regulate gene transcription.

Taken from Anderson, 2006.

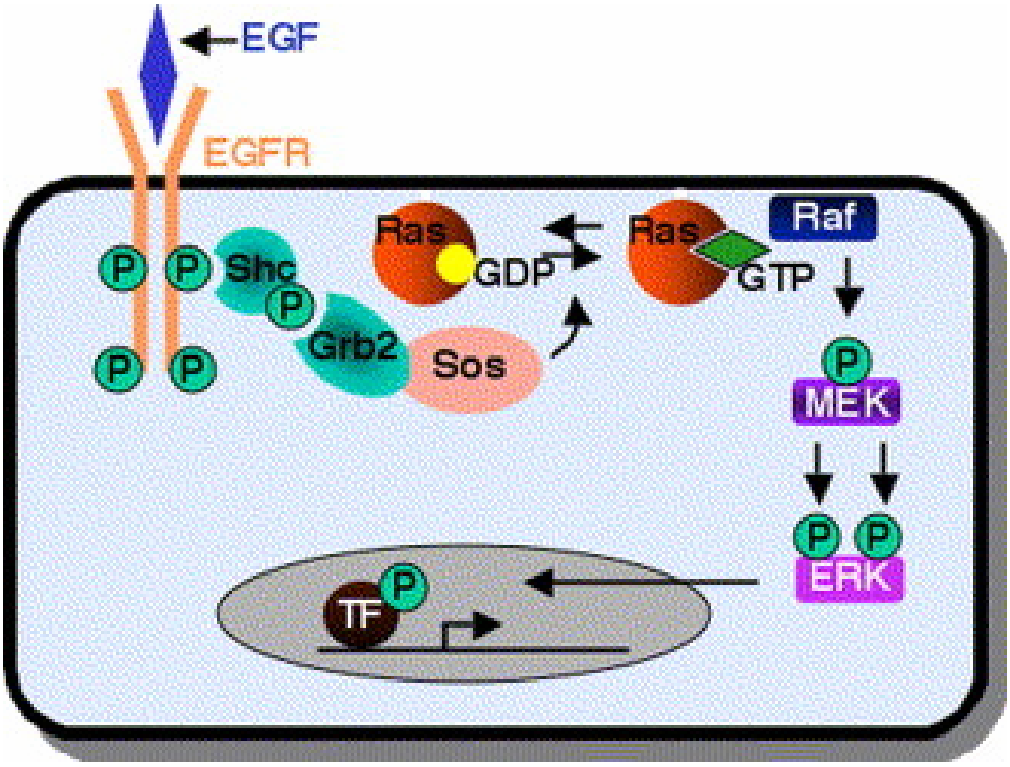


Figure 5: **The classic MAPK (Ras/Raf/MEK/ERK) pathway.** Activation of an RTK by PDGF, EGF, insulin, or IGF-1 results in activation of the MAPK pathway. Abbreviations used: (Grb2) growth factor receptor-bound protein-2, TF (transcription factor), GDP (guanosine diphosphate), GTP(guanosine triphosphate), ERK (extracellular signal-regulated kinases).

It is important to note that the MAPK and PI3-K pathways are not independent of one another. It is possible that IRS proteins also modulate the MAPK pathway; likewise, SHC can also be involved in both the MAPK and PI3-K pathways (LeRoith and Roberts 2003). Additional evidence for crosstalk between the PI3-K and MAPK pathways is

found in the fact that in some cells where IGF-1 simulates AKT, AKT can phosphorylate Raf of the MAPK pathway on an inhibitory serine residue resulting in a net decrease of MAPK activity (O'Connor 2003).

1.3.4 The effects of insulin and IGF-1 on lipogenesis

Much of the information garnered to date about the effects of insulin and IGF-1 on lipogenesis has been gained using the 3T3-L1 adipoctye model created by Green and Kehinde (Green and Kehinde 1975). The 3T3-L1 preadipocytes were cloned from 3T3 mouse embryo fibroblasts. Though not adipocytes, when treated with a cocktail of methylisobutylxanthine, dexamethasone, and insulin (MDI cocktail), the cells differentiate in monolayer culture with morphological and biochemical characterstics of adipocytes. Most notably, the activity of the enzyme fatty acid synthase (FAS) is increased by 19.5 fold in differentiated 3T3-L1 adipocytes compared to the activity FAS activity in 3T3-L1 preadipocytes (Student, Hsu and Lane 1980). FAS catalyzes the seven steps in the conversion of acetyl-CoA and malonyl-CoA to palmitate and has been shown to be regulated at the transcriptional level by insulin in 3T3-L1 adipocytes, and further, this is mediated by the PI3-K pathway (Wang and Sul 1998). Additional adipocyte and adipocyte-like cell lines have been made (Negrel, Grimaldi and Ailhaud 1978; Chapman, Knight et al. 1984) and data from these lines largely corroborates the findings from 3T3-L1 cells (Rosen, Walkey et al. 2000).

3T3-L1 preadipocytes can grow normally in medium containing serum. The entire MDI cocktail is not necessary to achieve differentiation; rather, differentiation can

be induced by treatment with 2 μM insulin or 10-20 nM IGF-1. The high dose of insulin is likely acting through the IGF-1 receptor, which incidentally outnumbers the insulin receptor in preadipocytes by a ratio of 2 to 1 (Smith, Wise et al. 1988). Insulin is the only component of the MDI cocktail capable of activating the PI3-K pathway, which is essential to differentiation. Using RNA interference to downregulate Akt, differentiation was blocked with MDI treatment in 3T3-L1 preadipocytes, indicating that IGF-1 receptor activation causes differentiation of 3T3-L1 preadipocytes via the Akt pathway, though a contribution from the insulin receptor cannot be ruled out in this study (Xu and Liao 2004).

There is evidence for insulin and IGF-1 inducing lipogenesis in a variety of other systems. First, it has been shown that FAS mRNA is increased in a time-dependent manner with insulin or IGF-1 treatment in fetal rat brown adipocytes. Further, lipid content is increased in these cells with these treatments over a 24 hour period in culture (Teruel, Valverde et al. 1996). Second, both insulin and IGF-1 have also been shown to increase lipogenesis in the rat preputial sebaceous model (Deplewski and Rosenfield 1999). Finally, IGF-1 administration to human sebocytes grown in organ culture exhibit a robust increase in lipogenesis (Downie, Sanders et al. 2002).

1.4 Lipid Biosynthesis and Control by Sterol Response Element Binding Proteins

1.4.1 Lipid Biosynthesis

In mammals, there are two primary pathways responsible for lipogenesis: the fatty acid biosynthesis pathway and the cholesterol biosysnthesis pathway. Both begin with the same substrate-the two carbon acetate molecule-but that is the end of their biochemical similarities. The primary product of the fatty acid pathway is palmitate, while the primary product of the cholesterol biosynthesis pathway is cholesterol. The palmitate created by the fatty acid pathway can be modified by various enzymes to make different fatty acids, triglycerides, and wax esters, while different molecules can be the result of the cholesterol biosynthesis pathway by intermediates leaving the pathway before the final cholesterol product is produced. This happens in sebaceous glands with squalene production.

In humans, cholesterol production is tightly regulated as humans lack the cellular machinery to recovery energy stored in cholesterol, rendering it a metabolic dead end. Fatty acids, on the other hand, are made by cells to store excess energy and there are efficient means by which this energy can be recovered. The following sections give more detail on the mechanism of each pathway and the involvement of sterol response element binding proteins (SREBPs) in each.

1.4.1.1 Cholesterol Biosynthesis

All 20 enzymes in the animal cell cholesterol synthesis pathway have been shown to be regulated by SREBP-1 or 2. The major steps in cholesterol biosynthesis have been defined (Figure 6-Goldstein, DeBose-Boyd and Brown 2006). The 27 carbon molecules in cholesterol are all derived from acetate fragments. The intial step in cholesterol biosynthesis is the conversion of acetyl-CoA into isoprene units by HMG-CoA synthase and HMG-CoA reducatse. Interstingly, HMG-CoA reductase is the target of the statin class of cholesterol-lowering drugs and has also been shown to be a transcriptional target of SREBP (see Table 5 for lipogenic genes known to be regulated by SREBPs).

Taken from Goldstein *et al*, 2006.

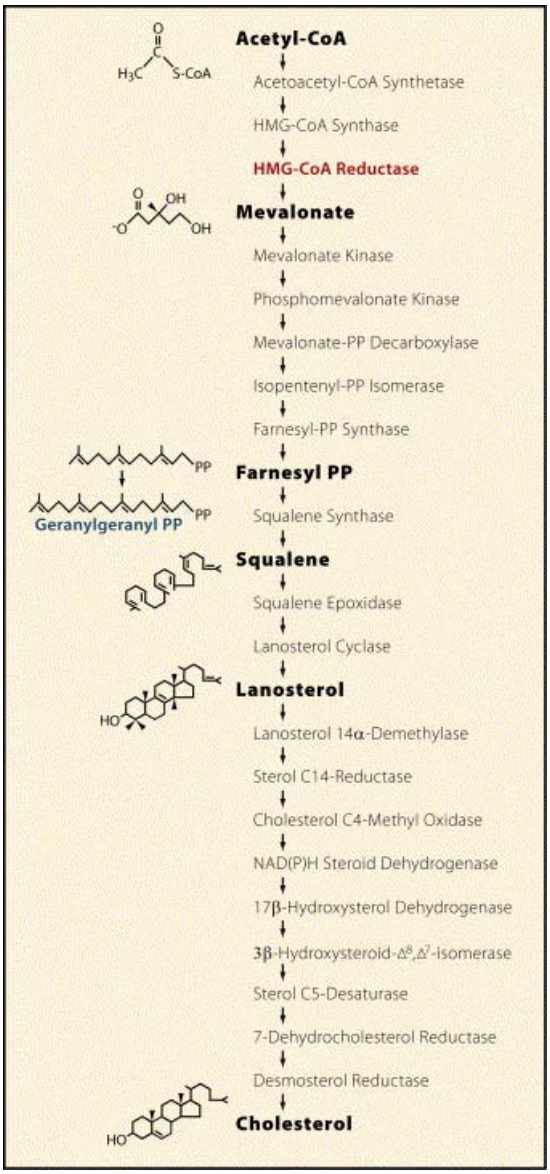


Figure **6**: **The cholesterol biosynthesis pathway in humans.** Mevalonate, farnesyl pyrophosphate, squalene, and lanosterol are the key intermediates. All enzymes in this pathway have been shown to be regulated by SREBPs.

Squalene is of particular interest for this review because it is an intermediate in the cholesterol biosynthesis pathway, but is produced as an end-product in the sebaceous gland. In all other tissues, squalene is quickly converted in lanosterol and subsequently cholesterol. For unknown reasons, sebaceous glands produce squalene as an end product.

It has been suggested that sebaceous glands lack the ability to efficiently cyclize squalene to form other sterols, though they possess the enzymes necessary for cyclization (Thiboutot and Strauss 2003). Nonetheless, squalene is comprised of six isoprene units and is formed by the reactions catalyzed by squalene synthase. Though squalene is a significant product of sebaceous glands *in vivo*, immortalized sebaceous cells produce minimal amounts of squalene. Three possible reasons for this are: 1) The high demand for membrane cholesterol in rapidly dividing immortalized sebocytes requires the cells to convert squalene into cholesterol, 2) The enzymes responsible for conversion of squalene to lanosterol, and ultimately cholesterol, may not function well in the oxygen deficient environment of the sebaceous gland, 3) The expression of many metabolic enzymes are lost in cell culture, for instance cytochrome P450s in hepatocytes.

1.4.1.2 Fatty Acid Synthesis

Fatty acid synthesis occurs in the cytosol and can best be described in two parts. The first part is the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA. This reaction represents the commited step in fatty acid biosynthesis and serves as a rate controlling step. Acetyl-CoA carboxylase (ACC) is the enzyme that catalyzes this reaction; ACC has been shown to be regulated by SREBP-1 at the transcriptional level (Rawson 2003).

The second group of fatty acid biosynthesis reactions is controlled by fatty acid synthase (FAS). FAS is the key enzyme in fatty acid biosynthesis and is also regulated by SREBP-1 (Rawson 2003). FAS is initially loaded with one acetate molecule and one

malonyl-CoA. First the malonyl group of malonyl-CoA is decarboxylated. This two-carbon fragment is then reduced, dehydrated, and further reduced, resulting in the initial acetyl group being extended by two carbon molecules. These reactions are repeated 6 more times with malonyl-CoA serving as the carbon source to form palmitoyl-ACP. Palmitoyl-ACP is hydrolyzed by palmitoyl thioesterase to yield palmitate (16:0) (Figure 7).

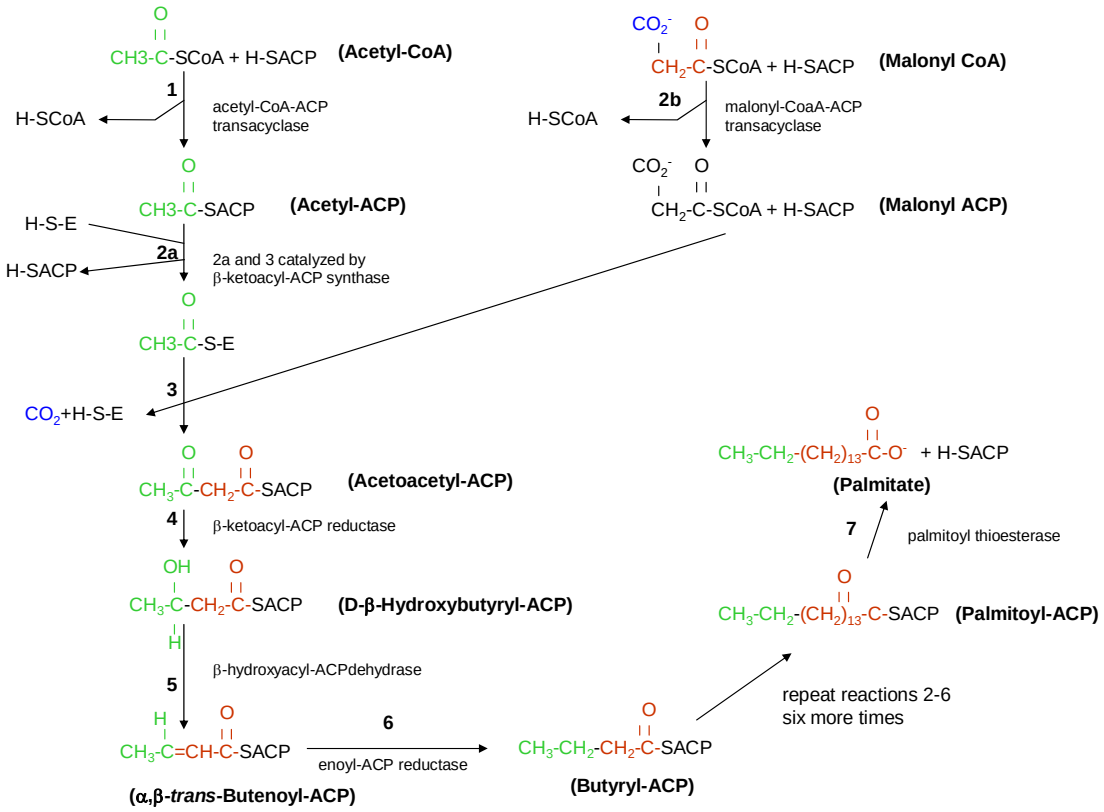


Figure 7: **The primary product of the fatty acid pathway in mammals is palmitate.** One acetyl-ACP molecule is combined with malonyl-ACP in a reaction catalyzed by FAS. Steps 2b-6 are repeated seven times to yield one pamitate molecule. Abbreviations used: ACP (acyl-carrier protein), S (serine), E (condensing enzyme)

The 16:0 palmitate is the most common product of fatty acid synthesis and serves as the precursor of longer chain saturated and unsaturated fatty acids. This occurs through the actions of various elongases and desaturases. Elongases located in the endoplasmic reticulum and mitochondria add two carbons at a time to the acyl-CoA produced by fatty acid synthase. Mammals have four different terminal desaturases that add double bonds to the acyl-CoA at the Δ^9–, Δ^6–, Δ^5–, and Δ^4– positions. Triglycerides and wax esters are the two major components of sebum that are synthesized from the acyl-CoA molecules modified by elongases and desaturases. The synthesis of these two classes of molecules will be discussed in the following sections.

1.4.1.2.1 Triglyceride synthesis

Triacylglycerols (TAG), also called triglycerides, are produced by the combination of fatty acyl-CoA esters produced by FAS with glycerol-3-phosphate. In liver tissue under normal physiological conditions, 93% of TAG synthesis occurs in the mitochondria and endoplasmic reticulum in a reaction catalyzed by glycerol-3-phosphate acyltransferase (GPAT) (Coleman and Lee 2004). Like many other enzymes controlling fatty acid synthesis, GPAT has also been shown to be controlled by SREBP-1 (Ericsson, Jackson et al. 1997). The reaction catalyzed by GPAT represents both a committment step and the rate limiting step in triglyceride synthesis.

Alternatively, TAG can also be produced by dihydroxyacetone phosphate acyltransferase. This occurs in the endoplasmic reticulum and peroxisomes, however

this accounts for only a small amount of total TAG production under normal physiological conditions and converges with the GPAT pathway at the lysophosphatidic acid step (Figure **8**).

Taken from Voet and Voet *Biochemistry* 2nd edition, page 689, 1995.

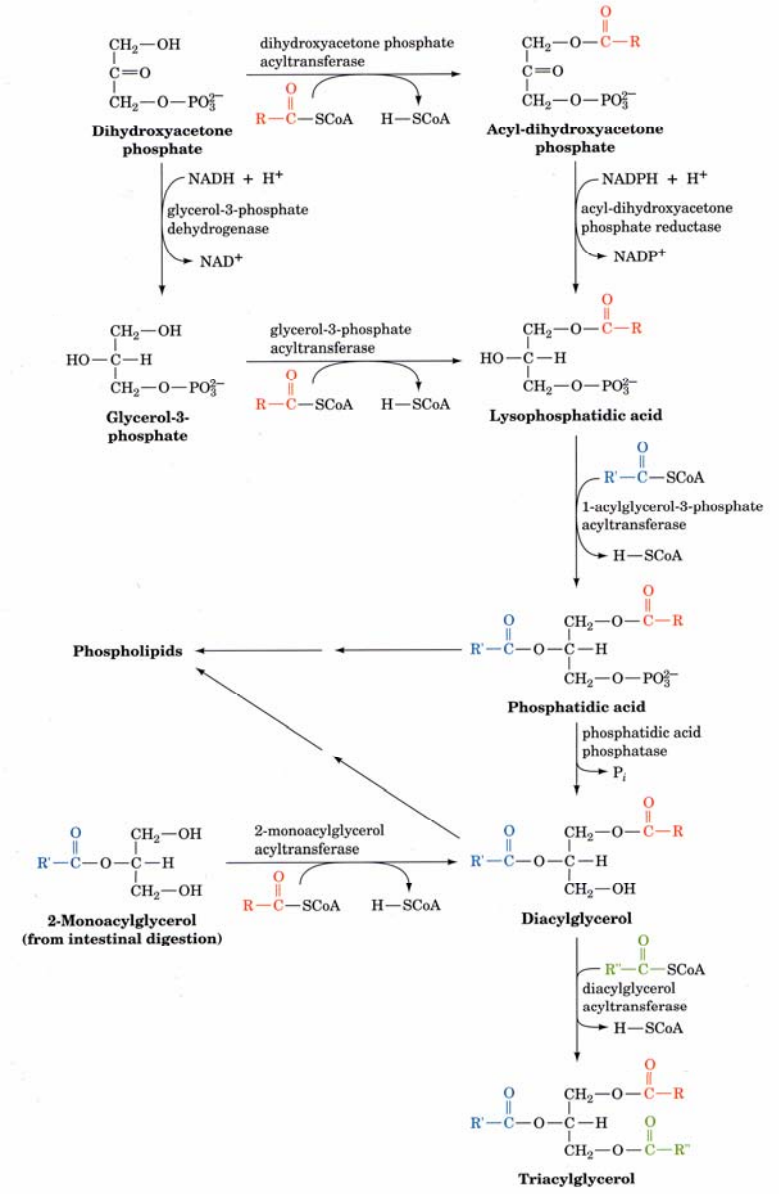


Figure **8**: **Triglyceride synthesis in mammals occurs by two pathways that converge at lysophosphatidic acid.**

1.4.1.2.2 Wax ester biosynthesis

Recently, tremendous progress has been made in elucidating the molecular pathway by which wax esters are made in mammals. It is currently believed that a two-step biochemical process is responsible for wax production (Figure 9). The first step involves the formation of a fatty alcohol from fatty acyl-CoA (produced by fatty acid synthase). This reaction is catalyzed by the recently cloned fatty acyl-CoA reducatase (FAR) (Cheng and Russell 2004). There are two isozymes of FAR, which are both present in mouse eyelid and rat preputial glands. Fatty alcohols have two metabolic fates; they are converted to ether lipids by a pathway involving at least 7 enzymes, or they are converted to wax monoesters by wax synthase (Cheng and Russell 2004).

Taken from Cheng and Russell, 2004a.

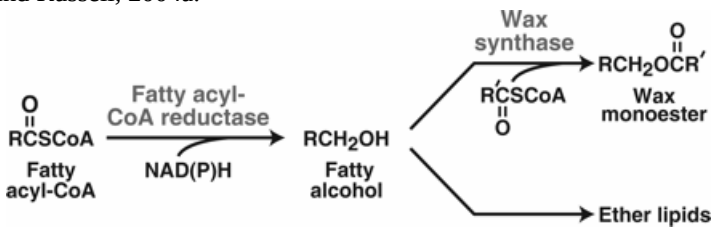


Figure 9: **The biochemical pathway by which wax esters are produced**.

Wax synthase conjugates a long chain fatty alcohol to a fatty acyl-CoA forming a wax monoester (Cheng and Russell 2004). Like fatty acyl-CoA reductase, higher mRNA levels of wax synthase are found in rat preputial glands and mouse eyelid than in other tissues. Validation of this model was recently shown when co-expression of the cDNA for wax synthase along with fatty acyl-CoA reductase led to the formation of wax esters (Cheng and Russell 2004). A potential role for SREBP regulation of the enzymes controlling wax ester biosynthesis has not been examined.

Table 5: **Genes involved in fatty acid biosynthesis and cholesterol metabolism controlled by SREBPs.**

Fatty Acids	Sterols
Acetyl CoA synthetase	ATP citrate lyase
Acetyl CoA carboxylase	Acetoacetyl CoA thiolase
Fatty acid synthase	HMG CoA synthase
Long-chain fatty acyl elongase	HMG CoA reductase
Stearoyl CoA desaturase	Mevalonate kinase
Glycerol-3-phosphate acyl-transferase	Mevalonate pyrophosphate decarboxylase
Pyruvate kinase	Geranylgeranyl pyrophosphate synthase
CTP:phosphocholine cytidylyltransferase	Isopentenyl pyrophosphate isomerase
	Farnesyl pyrophosphate synthase
	Squalene synthase
	Squalene epoxidase
	Lanosterol synthase
	Lanosterol 14α-demethylase
	Lathosterol oxidase
	7-dehydrocholesterol reductase
	LDL receptor

Taken from Rawson, 2003 and Coleman and Lee, 2004.

1.4.2 Sterol Response Element Binding Proteins

There are three known members of the SREBP family. SREBP-1a and 1c are encoded by the same gene, located on chromosome 17p.11.2 in humans. Two different transcriptional start sequences (and two different promoters) give rise to the transcripts which differ only in the first exon. The amino terminus of the 1c protein contains 6 negatively charged amino acids, whereas the amino terminus of the 1a protein is longer and contains 12 negatively charged amino acids. This difference accounts for the more potent activation of transcriptional targets that has been observed by SREBP-1a protein when compared to the SREBP-1c protein (Shimano, Horton et al. 1997). The other

member of the family, SREBP-2 is located on human chromosome 22q.13 and has 47% identity to the 1a and 1c transcripts (Hua, Wu et al. 1995).

All three members of the SREBP family share the following structures: an NH_2-terminal transcription factor domain of approximately 480 amino acids, a middle hydrophobic region of approximately 80 amino acids containing two hydrophobic trans-membrane segments, and a COOH-terminal regulatory domain of approximately 590 amino acids (Figure **10**-Brown and Goldstein 1997).

Taken from Brown and Goldstein, 1997.

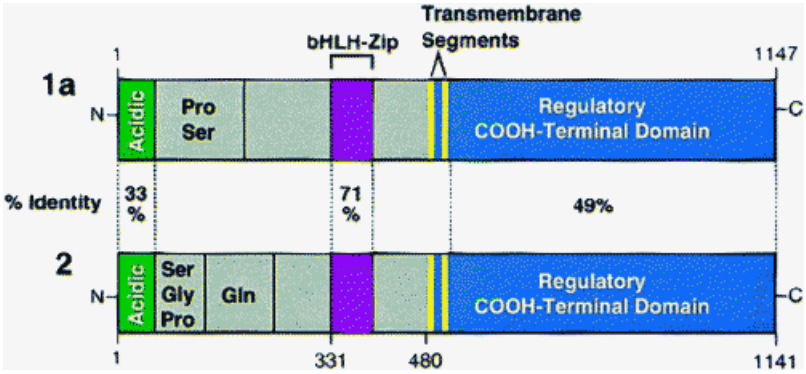


Figure **10: Domain structures of human SREBP-1a and SREBP-2 proteins.** The sequence of SREBP-1c (not shown) is identical to SREBP-1a except for a shortened NH_2 acidic domain.

As suggested by the name, SREBPs bind sterol response elements (SREs). An SRE is a decamer containing the nucleotide sequence ATCACCCCAC. SREBPs fit into a larger protein class called basic-helix-loop-helix-leucine zipper (bHLH-ZIP) proteins. Normally, bHLH-ZIP proteins target the DNA sequence CANNT; the SREBPs bind this sequence in addition to the SRE which contains the direct repeat of CAC. The SREBP proteins also differ from other members of the bHLH-ZIP in that SREBPs are larger proteins (Yokoyama, Wang et al. 1993).

1.4.2.1 Transcriptional control/activators of SREBPs

The fact that the SREBP-1a and 1c transcripts are driven by different promoters allows for differential transcriptional regulation, and the ratios between the transcripts differ in different tissue types and also in cell lines. The human SREBP-1c promoter contains an SRE, an SP-1 site (stimulating protein-1), an NF-Y site (nuclear factor-Y), an LXRE (liver X receptor response element), an HNF-4 site (hepatic nuclear factor-4), and a PX-1 site (pancreatic duodenal homeobox-1) (Tarling, Salter and Bennett 2004). In contrast, the SREBP-1a promoter contains only two Sp1 sites which are weakly activated by insulin (Zhang, Shin and Osborne 2005).

SREBP-1c predominates in most tissues including liver, white adipose tissue, skeletal muscle, adrenal gland, and brain, while the SREBP-1a protein is the dominant form in cells with a high capacity for proliferation such as the spleen, intestines, and cell lines (Shimomura, Shimano et al. 1997). This presents a challenge in the interpretation of data from cells grown *in vitro*. To take it one step further, in a recent article from the laboratory of Michael Brown and Joseph Goldstein, pioneers in SREBP research, they wrote, “we were unable to find a cultured hepatocyte cell line that retains the ability to respond to insulin by upregulating SREBP-1c mRNA” (Chen, Liang et al. 2004). It seems in many cases, cell lines have so much SREBP-1a which lacks the promoter elements to have a robust response to insulin and so little SREBP-1c, it is difficult to model SREBPs using culture systems.

The pieces of the puzzle that determine exactly how transcription of SREBP mRNA is activated are still falling into place. The best characterized organ in the study

of SREBPs is the liver. It has been demonstrated that insulin increases SREBPs, which in turn, increase lipogenesis in rodent liver. This effect is mediated by IRS-1, as a dominant-negative form of IRS-1 abrogates the insulin induced SREBP-1c mRNA in primary hepatocytes (Matsumoto, Ogawa et al. 2002). Additional cell types have been shown to have an increase in SREBP mRNA in response to insulin treatment, including primary multinucleated myotubules (Guillet-Deniau, Mieulet et al. 2002), cultured rat hepatocytes (Azzout-Marniche, Becard et al. 2000), myocytes, and cultured adipocytes (Nadeau, Leitner et al. 2004). The mechanism by which SREBP transcription is activated will be discussed later.

In addition to insulin, there have been several other molecules that induce SREBP transcription. Foretz and co-workers contend that glucose is the main activator of lipogenic genes in response to carbohydrate feeding in rodent livers, and their work shows that the effects are mediated by SREBP-1 (Foretz, Pacot et al. 1999). Other work has shown that the glucose induction of SREBP-1 is not induced by fructose, xylose, or galactose, nor by the glucose analogs 2-deoxy glucose or 3-o-methyl glucopyranose (Hasty, Shimano et al. 2000). Additionally, platelet-derived growth factor (PDGF) (Kotzka, Muller-Wieland et al. 1998), keratinocyte growth factor (KGF) (Chang, Wang et al. 2005), and androgens (Heemers, Maes et al. 2003) have all been shown to increase SREBP-1 mRNA.

As many different molecules can activate SREBP transcription, there is little wonder that SREBPs were dubbed, “master regulators of lipid homestasis” in a recent review (Eberle, Hegarty et al. 2004). Amidst the myriad activators, a consensus molecular pathway by which SREBP transcription is activated is yet to be found. The

leading candidates are the PI3-kinase/Akt pathway and the MAPK/Erk pathway. Akt induces SREBP-1 which mediates the lipogenic effect seen in several lung cancer cell lines (Chang, Wang et al. 2005; Porstmann, Griffiths et al. 2005). Additionally, Fleischmann *et al.* used an inducible Akt construct to determine that in primary liver cells, induction of Akt increases SREBP-1 mRNA, while MAPK inhibitors have no effect (Fleischmann and Iynedjian 2000). Insulin has also been shown to activate SREBPs via the PI3-kinase pathway in cultured rat hepatocytes (Azzout-Marniche, Becard et al. 2000). Conversely, it has been reported that SREBPs are regulated by the MAPK pathway and unaffected by PI3-Kinase inhibitors in HepG2 cells deficient in SREBP-1 (Kotzka, Muller-Wieland et al. 2000). Additionally, in myocytes, MAPK inhibitor PD98059 blocked the insulin induced increase of SREBP-1, while PI3-Kinase inhibitors had no effect. In the same study, the authors also reported that the converse is true in 3T3-L1 preadipocytes, validating the argument that the mechanism by which various growth factors activate SREBP transcription is cell type specific (Nadeau, Leitner et al. 2004).

In summary, the SREBP field is young and there is still substantial confusion and conflict surrounding the molecular pathways that activate the SREBPs. Adding to the confusion is the fact that early studies simply reported an increase in SREBP-1 and did not differentiate between the SREBP-1a and 1c transcript.

1.4.2.2 SREBP activation by proteolytic cleavage

The SREBP proteins are embedded in the membrane of the endoplasmic reticulum. They cross the membrane twice in a hairpin fashion with both ends of the protein in the cytosol. Only a small portion of the protein is within the lumen of the endoplasmic reticulum. To form the active transcription factor, the amino terminus of the protein is cleaved, which translocates to the nucleus and binds to SREs in the promoters of various lipogenic genes. The cleavage of SREBPs is regulated in an elegant manner which is described below.

The primary regulators of SREBP processing are the proteins SREBP-cleavage-activating protein (Scap) and Insig (insulin-induced gene). Scap is an integral membrane protein that contains a sterol-sensing domain and multiple tryptophan-aspartate repeats (WD repeats) which mediate protein-protein interactions. To date, two Insig proteins have been identified: Insig-1 and Insig-2. Insig-1 was the first of the group discovered and was named "Insig" as the mRNA transcript increased in response to insulin (Mohn, Laz et al. 1991). It was later discovered that Insig-1 transcription was activated by insulin as a result of insulin's effect on SREBP-1c. Insig-2 is expressed at a low, but constitutive level which does not seem to be affected by insulin. Both Insigs are extremely hydrophobic, with only short sequences at both the amino and carboxy terminus projecting into the cytoplasm (Goldstein, DeBose-Boyd et al. 2006).

Scap exists in the cells bound to SREBPs through its WD domain. When there are sufficient levels of sterols in the cell, Scap undergoes a conformational change in its sterol-sensing domain, which then binds to Insig-1 or 2. When bound to Insig, the

Scap/SREBP complex remains in the endoplasmic reticulum, as this binding prevents the formation of COPII vesicles. As expected, an overexpression of Insig inhibits SREBP processing and reduces insulin-induced lipogenesis in transgenic mouse liver (Engelking, Kuriyama et al. 2004). When sterols are low, a conformational change in Scap prevents Insig from binding, which then allows COPII vesicles to form. The COPII vesicle transports the SREBP/Scap complex from the endoplasmic reticulum to the Golgi (Figure **11**). Under low sterol conditions, Insig is ubiquitinated and degraded in proteasomes (Gong, Lee et al. 2006). Because the Insig promoter contains an SRE, activation of SREBP increases the amount of Insig protein, which is degraded unless the cellular sterol levels are high enough that no more activated SREBP is needed.

Taken from Goldstein *et al*, 2006.

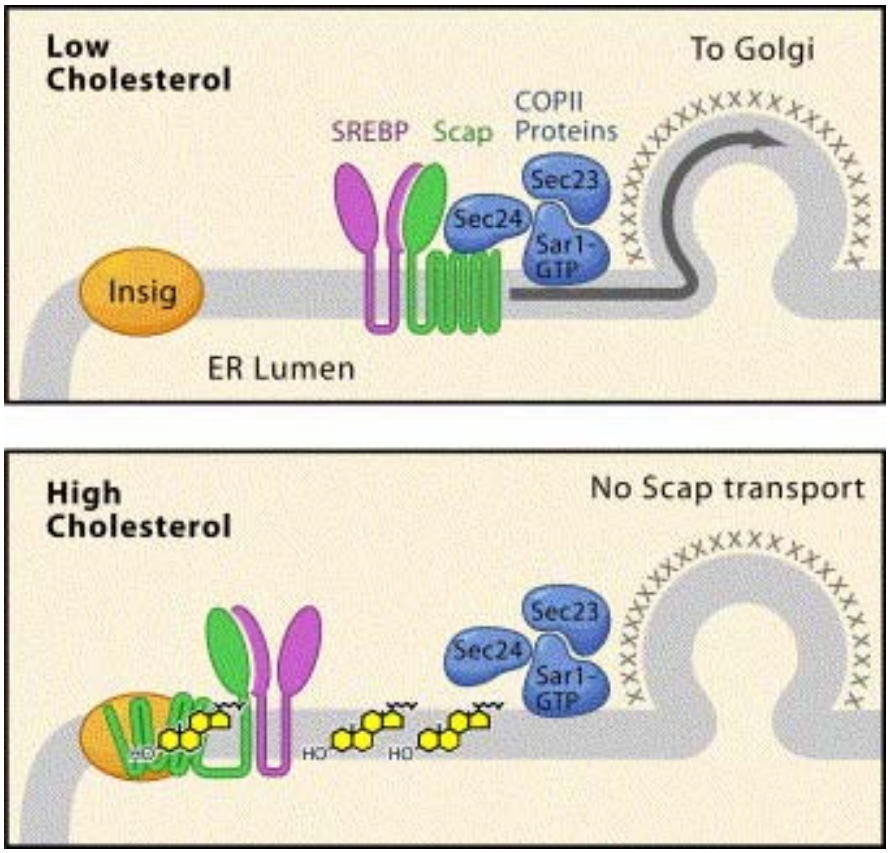


Figure 11: **Insig and SCAP regulate SREBP processing.** In low cholesterol conditions, a conformational change in Scap causes Insig to release, allowing a COPII vesicle to form, transporting the SREBP/Scap complex to the Golgi.

In the Golgi, two sequential cleavages release the amino portion of the SREBP protein, which acts as the active transcription factor in the nucleus. The first cleavage is performed by Site-1 protease (S1P) of the subtilisin family which cleaves the SREBP on the luminal loop. Cleavage by S1P is in turn regulated by sterols (Wang, Sato et al. 1994). Site-2 protease (S2P) is a hydrophobic protease and contains an HEXXH zinc-binding motif which is typical of the family of metalloproteinases. S2P is different enough from other metalloproteinases that is included in its own family, M50, which has at least 68 other members in bacteria, archae, plants, and animals. S2P cleaves the

SREBP in a membrane spanning point; cells without S2P cannot release the active form of SREBP to enter the nucleus (Figure 12-Rawson 2003). The result is that the amino terminus of the SREBP is cleaved from the rest of the protein, and goes into the nucleus to activate transcription of genes containing SREs.

Taken from Rawson, 2003.

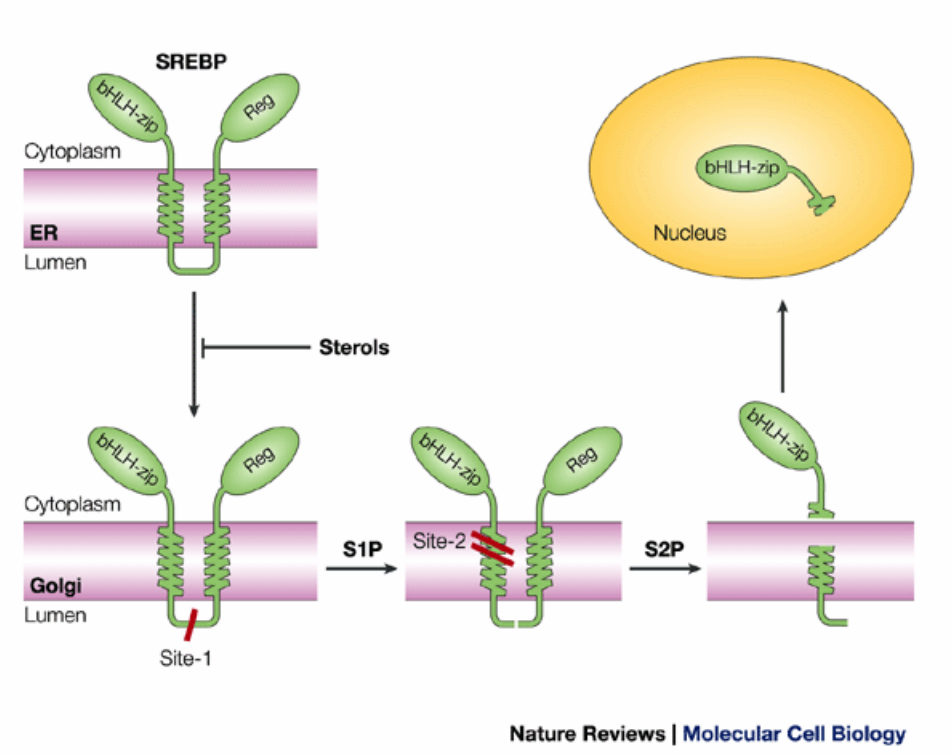


Figure 12: **The activation of the SREBP precursor occurs by two sequential cleavages.** Site-1 protease and Site-2 protease cleave the SREBP, releasing the mature basic-helix-loop-helix transcription factor from the regulatory domain of the protein.

1.4.2.2.1 Post-translation modifications and protein:protein interactions of SREBPs

Recently, there have been reports of post-translational modifications of the mature SREBP protein. All reports deal with the protein after it has been cleaved and has entered the nucleus. To date, phosphorylation, acetylation, ubiquitination, and interations with other proteins have been demonstrated, though the effects of all the modifications are not clear.

Several phosphorylation sites have been found on the mature SREBP proteins. The first report of SREBP phosphorylation showed that serine 117 of the mature SREBP-1a protein is a target of Erk phosphorylation in insulin or PDGF stimulated cells. Further, mutation of this serine resulted in the abrogation of the increased LDL receptor transcription that is induced by insulin treatment (Roth, Kotzka et al. 2000). While the debate continues as to whether or not SREBP-1c is phosphorylated by Erk, it has also been reported that PKB can phosphorylate purified SREBP-1c *in vitro* (Eberle, Hegarty et al. 2004).

The significance of phosphorylation is still unknown for the SREBP protein. Additional phosphorylation sites in the C-terminus of the mature SREBP-1a protein have been found. Further, these sites are hyperphosphorylated when the cell is in the mitotic phase of the cell cycle (Bengoechea-Alonso, Punga and Ericsson 2005). It is postulated that phosphorylation could affect the conformation of the protein, the binding of co-activators, or stability. Evidence for the phosphorylation affecting the stability has been found recently, as phosphorylation of the mature SREBP protein has been shown to

attract proteasome machinery via its ubiquitination (Sundqvist, Bengoechea-Alonso et al. 2005).

The attachment of a polyubiquitin chain to a lysine reside marks a protein for rapid degradation by the 26 S proteasome. The mature SREBP has been shown to be ubiquitinated and degraded, but there is no evidence that the precursor protein is ubiquitinated (Hirano, Yoshida et al. 2001). It is also interesting to note that in order for SREBP to be ubiquitinated, the protein must be transcriptionally active. Mutation of the DNA binding domain stabilizes the SREBP protein, showing that ubiquitination is dependent upon the DNA binding ability of the protein (Sundqvist and Ericsson 2003).

It seems that everything from activation of transcription to ubiquitination of SREBPs involves interaction with other proteins. Among the most important proteins for SREBP interaction is the p300/CBP complex and cofactor required for Sp1 activation (CRSP). CBP and p300 are co-activators that bridge transcriptional machinery and DNA specific transcriptional activators. Both CBP and p300 have acetyltransferase activity and are involved in acetylation of histones and altering chromatin structure (Ericsson and Edwards 1998). Interaction with p300 results in acetylation of SREBP which enhances its stability, as the acetylation occurs at sites where ubiquitin would be attached, blocking ubiquitination and stabilizing the transcript (Eberle, Hegarty et al. 2004). Interestingly, disruption of the interaction between SREBP and endogenous p300/CBP results in inhibition of SREBP dependent transcription and also stabilizes the protein in the nucleus (see Figure **13**-Sundqvist and Ericsson 2003). Additionally, CRSP has been shown to bind the C-terminal domain of RNA polymerase II which may help attract RNA

polymerase II to the promoter (Naar, Taatjes et al. 2002). The significance of the interaction with SREBP and CRSP has yet to be elucidated.

Figure taken form Sundqvist and Ericsson, 2003.

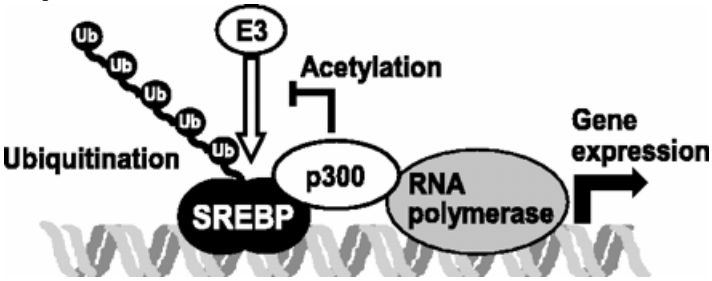


Figure 13: **SREBP proteins are ubiquitinated and degraded in a transcription dependent manner.** Interaction with p300/CBP is required for SREBP transcription, and SREBP is acetylated by p300, protecting it from ubiquitination. E3 is an enzyme that catalyzes the addition of ubiquitin molecules to various proteins.

Finally, twist2, also known as dermo-1, interacts with SREBP-1c in a yeast two-hybrid system. Twist2 is expressed in adipose and liver tissue, and overexpression of twist2 blocked the transcriptional activity of SREBP-1c by preventing it from binding target promoters. Further, histone deacetylase inhibitors blocked this inhibition, implying that this inhibition is partly dependent on chromatin modifications (Lee, Lee et al.).

Chapter 2

IGF-1 Induces Lipid Production in SEB-1 Cells Via SREBP-1

2.1 Introduction

Sebum production is pivotal in the pathogenesis of acne. An understanding of the factors that regulate this process is important in designing strategies to improve acne. The peak incidence of acne occurs during adolescence when serum IGF-1 (insulin-like growth factor-1) levels reach their peak during the growth spurt (Deplewski and Rosenfield 1999), and IGF-1 levels correlate positively with severity of acne in women with clinical acne (Cappel, Mauger and Thiboutot 2005). The IGF-1 receptor is expressed on sebocytes and can be activated by either IGF-1 or high doses of insulin (Rudman, Philpott et al. 1997).

Insulin, in combination with the hormones methylisobutylxanthine and dexamethasone, is commonly used to induce the differentiation of 3T3-L1 preadipocytes into adipocytes in culture, a process which is characterized by a marked increase in lipogenesis (Student, Hsu et al. 1980). A role for IGF-1 and insulin in stimulating sebaceous gland lipogenesis was first demonstrated in rat preputial sebocytes (Deplewski and Rosenfield 1999).

The sterol response element-binding proteins (SREBPs) are nuclear transcription factors that regulate the synthesis of cholesterol and fatty acids. As suggested by their name, SREBPs bind sterol response elements in the promoters of genes involved in

lipogenesis, including fatty acid synthase, long-chain fatty acyl elongase, stearoyl CoA desaturase, HMG CoA synthase, HMG CoA reducatase, and squalene synthase (Horton 2002). SREBPs are produced as precursors embedded in the endoplasmic reticulum. In sterol-depleted cells, two sequential cleavages release the amino terminal fragment of the protein, which in turn enters the nucleus and binds to promoters of genes containing sterol response elements, thus activating transcription (Hua, Sakai et al. 1996; Sakai, Duncan et al. 1996). There are three known SREBPs that regulate synthesis of cholesterol and fatty acids. SREBP-1a and c are derived from a single gene with alternate transcription start sites, yielding a different first exon (Shimomura, Shimano et al. 1997). SREBP-2 is the third member of the family and is believed to be more active in cholesterol homeostasis, though there is some functional overlap between the two forms (Sakai, Duncan et al. 1996).

The goal of this study was to examine the mechanism(s) by which adipogenic hormones increase lipogenesis in human SEB-1 sebocytes and to test the hypothesis that SREBPs are key regulators of this process. Here we report that: (1) the increased lipogenesis observed in SEB-1 cells treated with methylisobutylxanthine, dexamethasone, and a high dose of insulin (MDI) is attributable to insulin; (2) physiologically relevant doses of IGF-1 increase lipogenesis in SEB-1 sebocytes; and (3) increased expression of SREBP-1 mRNA and protein mediates the increase in lipid production by IGF-1 and augments the increase induced by 1 μM insulin or 20 ng/mL IGF-1 in SEB-1 sebocytes. From this we conclude that insulin and IGF-1 can potentially activate lipogenesis by different mechanisms. These data also suggest that the IGF-1 receptor is instrumental in

activating the SREBP pathway with consequent downstream increases in lipogenesis in SEB-1 cells.

2.2 Materials and Methods

2.2.1 Cell culture

SEB-1 (passage 22-24) SV40 immortalized human sebocytes were grown to confluence in all experiments unless stated otherwise, and were cultured in standard medium consisting of DMEM (Invitrogen, Carlsbad, CA), 5.5 mM glucose/Ham's F-12 3:1 (Invitrogen), fetal bovine serum 2.5% (HyClone, Logan, UT), adenine 1.8 x 10^{-4} M (Sigma, St. Louis, MO), hydrocortisone 0.4 µg/mL (Sigma), insulin 10 ng/mL (Sigma), epidermal growth factor 3ng/mL (Austral Biologicals, San Ramon, CA), and cholera toxin 1.2 x 10^{-10} M (Sigma) (Thiboutot, Jabara et al. 2003).

2.2.2 Cell treatments

To determine if adipogenic hormones induce differentiation of SEB-1 cells, cells were grown to 80% confluence in standard medium and treated with a combination of (MDI) methylisobutylxanthine (0.5 mM) (Sigma), dexamethasone (0.3 µM) (Sigma), and insulin (1.74 µM) (Sigma) in medium containing DMEM 5.5 mM glucose, 10% fetal bovine serum, and antibiotics for 72 hours (Student, Hsu et al. 1980). Additional plates were assayed using the appropriate drug vehicle controls in the same medium.

For all treatments with insulin or IGF-1, confluent cells were washed twice with phosphate-buffered saline and treated with the appropriate hormone or vehicle in DMEM with no additives or serum for 24 hours. Thus, the control media in experiments with insulin or IGF-1 consisted of DMEM without additives plus the appropriate vehicle. IGF-1 (Invitrogen) was diluted 1:1,000 in 0.1 M acetic acid with 0.1% BSA. The final concentration was 20 ng/mL. Insulin was also added 1:1,000 in 0.005 N HCl.

2.2.3 Lipogenesis assay

The incorporation of ^{14}C-acetate into lipids was used as a measure of lipogenesis. SEB-1 cells were cultured in standard medium until confluent. Cells were washed twice with PBS, and then treated in different experiments with a combination of adipogenic hormones or each hormone individually as above for 72 hours. In experiments examining the effects of IGF-1 on lipogenesis, cells were treated with IGF-1 or 1:1000 of the vehicle (0.1% BSA in 0.1 M acetic acid) in DMEM without additives for 24 hours. In all experiments cells were then removed with trypsin and an aliquot was taken for a cell count which was used to normalize the data. The remaining cells were suspended in a solution containing 1 µCi of ^{14}C-acetate (New England Nuclear, Boston, MA) in DMEM. Ambient air in the tubes was replaced with argon gas to mimic the low oxygen tension in sebaceous glands found *in vivo* (Evans, Schrlau et al. 2006) and samples were incubated for 2 hours at 37° with agitation. After the two-hour incubation, samples were extracted twice with ethyl ether and non-radioactive carrier lipids (purchased individually from Sigma) in petroleum ether were added to aid in visualization following thin layer

chromatography. These included cholesterol, stearyl alcohol (fatty alcohol), oleic acid (fatty acid) triolein (triglyceride), cetyl palmitate (wax ester), cholesterol oleate (cholesterol ester) and squalene. Following evaporation of organic solvent, samples were dissolved in a small volume of ethyl acetate and spotted on 20 cm silica gel thin layer chromatography plates (Macherey-Nagel, Easton, PA) which were then run until the solvent front reached 19.5 cm in hexane, followed by 19.5 cm in benzene, and finally to 11 cm in hexane:ethyl ether:glacial acetic acid (70:30:1). Plates were dried 5 minutes after each solvent. Lipid spots were visualized using iodine and bromothymol blue/sodium hydroxide blue spray dye. All lipid-containing zones were circled, excised, and radioactivity counted in a liquid scintillation counter. Negative controls for each experiment consisted of plates of untreated cells where radioactivity was added and cells were incubated on ice for 2 hours (lipids were extracted and analyzed in parallel). All experiments were repeated three times with each sample in triplicate within each experiment. Data were analyzed using the Student's t-test and considered significant if $p<0.05$ compared to the control.

2.2.4 Western blot

Cytoplasmic and nuclear lysates were obtained using the Ne-Per kit (Pierce, Rockford, IL) following manufacturer's instructions. A protease inhibitor cocktail (Sigma) containing 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin was added. Both the mature and precursor forms of the SREBP-1 proteins were found in the nuclear fraction in SEB-1 cells. Western blot for the

endoplasmic reticulum protein GRP-78 revealed that this marker was present in both the nuclear and cytoplasmic fraction in a variety of cell lines (Figure **17**). Thus, all Western blots probed using the SREBP-1 (K-10) antibody are the nuclear fraction. Total protein content of each sample was determined using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Twenty-five micrograms of protein was run on a 4-12% Bis-Tris NuPage polyacrylamide gel (Invitrogen). Protein was then transferred to a polyvinylidene fluoride (PVDF) membrane and probed using standard methods. The SREBP-1 (K-10) antibody was obtained from Santa Cruz and used at a dilution of 1:2000. Preliminary experiments showed that this antibody reacts non-specifically with a protein at 80 kDa. This was confirmed to be nonspecific using the SREBP-1 (K-10) blocking peptide (Santa Cruz). The actin antibody (as a loading control) and the goat anti-rabbit secondary antibody (7074) were obtained form Cell Signaling Technologies (Beverly, MA) and were used at 1:5000 and 1:2000 dilutions respectively. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to film. Films of blots were analyzed and quantified by densitometry with QuantityOne Software (Bio-Rad) after background subtraction. Western blots were repeated a minimum of three tiems. Data were analyzed using Student's t-test and results were considered significant if $P<0.05$.

2.2.5 Quantitative RT-PCR

Quantitative RT-PCR (QPCR) was performed to compare the ratios of SREBP-1a and 1c mRNA transcripts in SEB-1 sebocytes and human sebaceous glands. For QPCR

with SEB-1 cells, cells were grown in standard medium for 6 days. On day 7, cells were washed twice with PBS and treated with insulin, IGF-1 or the appropriate vehicle in serum-free DMEM for 14 hours. RNA was isolated and complementary DNA was generated from 4.2 µg RNA/reaction primed with oligo-dT using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). QPCR was performed using the Brilliant SYBR Green QPCR Core Reagent Kit in an Mx4000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA). TATA-binding protein was used as a reference gene. The following primer sequences were used: TATA-binding protein upstream (GenBank number NM_003194) 5'c acg gca ctg att ttc agt tct, TATA-binding protein downstream primer 5'ttc ttg ctg cca gtc tgg act, SREBP-1a upstream primer (GenBank number NM_004176) 5'gct gct gac cga cat cga a, SREBP-1c upstream primer (GenBank number NM_001005291) 5'gga gcc atg gat tgc act tt, and SREBP-1a, c downstream primer 5'tca aat agg cca ggg aag tca. Data were analyzed using the REST-XL© program (Pfaffl, Horgan and Dempfle 2002). A p-value <0.05 was considered significant.

Facial skin samples were obtained under a protocol approved by the Institutional Review Board of the Pennsylvania State University College of Medicine; informed consent was obtained form the subjects and the study was conducted according to the principles outlined in the Declaration of Helsinki Principles. Sebaceous glands were microdissected as previously described (Thiboutot, Harris, Iles, Cimis, Gilliland and Hagari 1995). Owing to a limited number of sebaceous samples, cycle threshold values (Ct) were transformed using the formula $(2^{-Ct})x\ 10^7$ as described (Spangler, Goddard, Avena, Hoebel and Leibowitz 2003) and normalized to TATA-binding protein. The relative difference between SREBP-1c and 1a is a ratio of normalized 1c to 1a.

The ratio of insulin receptor A-type (exon 11-) to insulin receptor B-types (exon 11+) was deteremined by relative QPCR as described previously (Trivedi, Gilliland, Zhao, Liu and Thiboutot 2006). SEB-1 sebocytes were grown to 50% confluence and the RNA was extracted. Both primer/probe sets were purchased from ABI. For the insulin receptor A-type (GenBank number AB208861) the primer/probe set Hs00965956_m1 was used. For the B-type (GenBank number NM_00208) the primer/probe set Hs00169631_m1 was used.

2.3 Results

2.3.1 Insulin is the component in MDI media that increases lipogenesis in SEB-1 cells.

Statistically significant increases in the incorporation of ^{14}C acetate into sebaceous lipids were noted when SEB-1 cells were treated with methylisobutylxanthine (0.5 mM), dexamethasone (0.3 µM), and insulin (1.74 µM) together with 10% fetal bovine serum (Figure **14**). Specifically, cholesterol was increased 4.4-fold, fatty alcohol was increased 3.6-fold, triglycerides were increased 4.8 fold, cholesterol oleate was increased 9.5-fold, and squalene was increased 14.2 fold. However, when SEB-1 cells were treated with each of the three components of MDI medium individually, only insulin significantly increased lipogenesis (Figure **15**). There were no significant changes in lipogenesis when cells were treated with either methylisobutylxanthine or dexamethasone alone (data not shown).

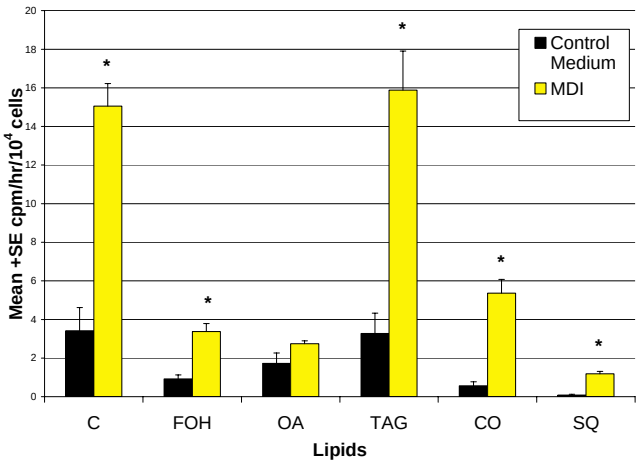


Figure **14: Treatment of SEB-1 sebocytes with the adipogenic cocktail MDI increases ^{14}C acetate incorporation into non-polar lipids.** SEB-1 sebocytes were treated with methylisobutylxanthine (0.5 mM), dexamethasone (0.3 µM), and insulin (1.74 µM) for 72 hours. Cells were then incubated with ^{14}C acetate for two hours. Lipids were extracted and separated by thin layer chromatography. Units are expressed as mean ±SE cpm/hr/10^4 cells. Significance was determined by Student's t-test with a P-value <0.05 considered significant and denoted by an *. Abbreviations used are as follows: C=cholesterol, FOH=fatty alcohol, OA=oleic acid, TAG=triglycerides, CO=cholesterol oleate, and SQ=squalene.

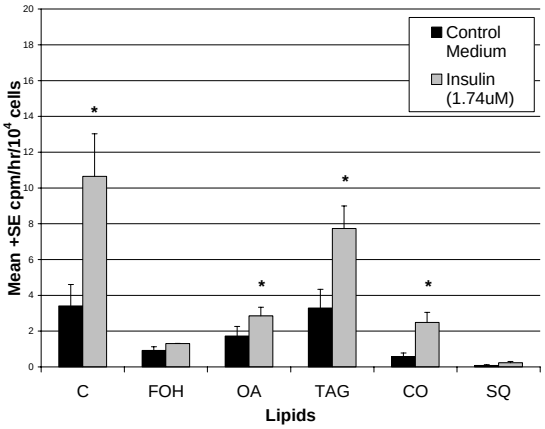


Figure 15: **Treatment with insulin alone can duplicate the increase in lipogenesis observed in MDI treated SEB-1 sebocytes.** SEB-1 sebocytes were treated with insulin (1.74 µM) for 24 hours. Cells were then incubated with ^{14}C acetate for two hours. Lipids were extracted and separated by thin layer chromatography. Abbreviations are the same as Figure 14.

2.3.2 Lipogenesis is increased by 100 nM insulin, 1µM insulin, and 20 ng/mL IGF-1 in SEB-1 sebocytes.

Because insulin at high concentrations is capable of acting through the IGF-1 receptor (Prisco, Romano et al. 1999), we tested whether a physiologically relevant dose of IGF-1 (20 ng/mL) could mimic the lipogenic effect observed with 1.74 µM insulin. We also wanted to determine if a lower dose of insulin would cause the same increase in lipogenesis as the high dose of insulin which was likely acting through both the insulin and the IGF-1 receptors.

Statistically significant increases in the production of cholesterol, cholesterol oleate, fatty alcohol, and triglycerides were noted when SEB-1 cells were treated with 100 nM insulin. Treatment with either 20 ng/mL IGF-1 or 1 µM insulin significantly

increased all eight lipid species assayed when compared to the vehicle control (Figure 16). Further, the increases induced by 1 µM insulin were significantly greater than those induced by 100 nM insulin in all eight lipid classes assayed; and significantly greater incorporation of acetate into cholesterol, oleic acid, triglycerides, and was esters was achieved with IGF-1 when compared to 100 nM insulin.

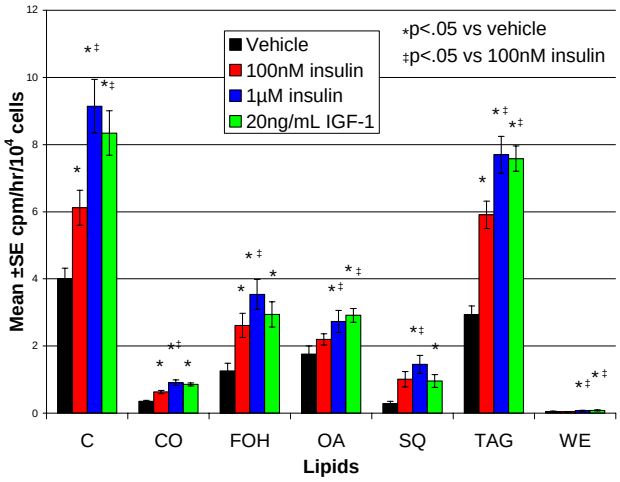


Figure 16: **Treatment of SEB-1 sebocytes with 100 nM insulin increases lipogenesis, though to a lesser extent than 1 µM insulin or 20 ng/mL IGF-1.** SEB-1 sebocytes were treated for 24 hours with 100 nM insulin, 1 µM insulin, or 20 ng/mL IGF-1. Cells were then incubated with ^{14}C acetate for 2 hours. Lipids were extracted and separated by thin layer chromatography. Units are expressed as mean ±SE cpm/hr/10^4 cells. Analysis of variance was used to determine statistical significance. Date by treatment interaction was observed for some data points. A P-value <0.05 in comparison to the vehicle was considered statistically significant and denoted with an *, while a P-value of <0.05 compared to 100 nM insulin is denoted by an ‡. Abbreviations used are as follows: C=cholesterol, FOH=fatty alcohol, OA=oleic acid, TAG=triglycerides, CO=cholesterol oleate, and SQ=squalene.

2.3.3 The precursor SREBP-1 is found in both the nuclear and cytoplasmic fractions when SEB-1 cells are fractionated using the Ne-Per kit.

Western blots probing for SREBP-1 using protein lysates separated into nuclear and cytoplasmic fractions by the Ne-Per© kit from Pierce indicated that there was actually more the precursor SREBP-1 protein in the nuclear fraction than there was in the cytoplasmic fraction (approximately 70% of precursor SREBP protein was in the nuclear fraction). As the endoplasmic reticulum is connected to the nucleus, we reasoned that the proprietary detergents used in the kit may not completely solubilize the endoplasmic reticulum, which would explain the presence of the precursor SREBP-1 in the nuclear fraction. To this end, we performed a Western blot probing for the endoplasmic reticulum specific heat-shock protein GRP-78, using both the nuclear and cytoplasmic fractions from the Ne-Per kit. This blot, showed that enoplasmic proteins are found in both the nuclear and cytoplasmic fractions in a variety of cell lines (Figure **17**).

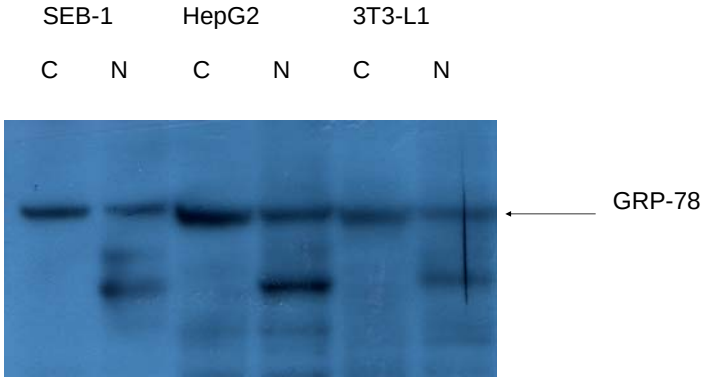


Figure 17: **Endoplasmic reticulum proteins are found in both the nuclear and cytoplasmic fractions when protein is isolated using the NePer kit (Pierce).** A Western blot probing for the endoplasmic reticulum protein GRP-78 was performed. Cytoplasmic lysates (denoted by a "C") and nuclear lysates (denoted by an "N") were isolated from various cell lines to determine if the Ne-Per kit solubilized the endoplasmic reticulum.

2.3.4 Non specific band using the SREBP-1 (K-10) antibody

The SREBP-1 (K-10) polyclonal antibody is targeted to an epitope in the extreme amino terminus of the SREBP protein and therefore recognizes the 120 kDa precursor SREB-1 protein, the 55 kDa mature SREBP-1 protein, and a band slightly larger than the mature protein, at about 80 kDa. In many blots where the cells were stimulated with IGF-1 or insulin, in addition to the increased expression of the two SREBP-1 protein forms, the intensity of the band at 80 kDa would also increase. Though unlikely, we wanted to determine if this band could be the cleaved fragment that results from the processing of the precursor protein into the mature protein. Western blot analysis was

performed where the primary SREBP-1 antibody was incubated with its blocking peptide; in this blot, both the precursor and mature SREBP bands were removed, while the band at 80 kDA remained indicating that this band is indeed non-specific for SREBP-1 (Figure 18).

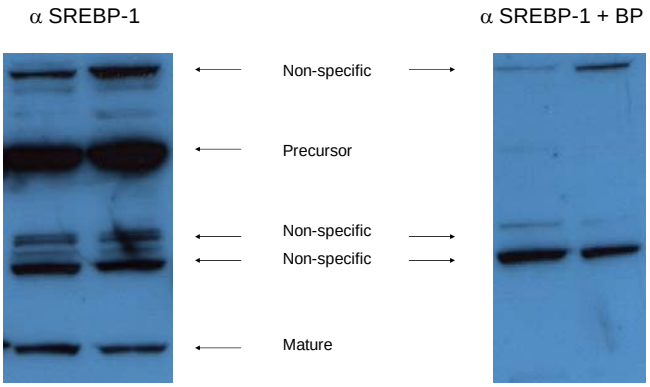


Figure 18: **The SREBP-1 (K-10) polyclonal antibody has several non-specific bands.** This antibody was used in every blot throughout this report that probes for SREBP-1. Note the position of the non-specific bands, as these bands are present in the blot on the left and the blot on the right where the antibody was pre-incubated with the blocking peptide (BP). In some blots, the intensity of these bands changes with IGF-1 stimulation.

2.3.5 SREBP-1 protein is induced in SEB-1 cells by IGF-1 and 1 µM insulin, but not by 100 nM insulin

Since SREBPs regulate numerous genes involved in lipid metabolism, we tested whether the effects of IGF-1 on lipogenesis were mediated by the SREBPs. Western blot demonstrated that both the precursor and mature forms of the SREBP-1 protein were increased by 6- and 3-fold, respectively, in response to IGF-1 (Figure 19). Additionally, the precursor was increased by 2.7-fold and the mature protein was increased by 1.7-fold

in response to 1 μM insulin, a dose known to activate the IGF-1 receptor. Interestingly, although 100 nM insulin is capable of increasing lipogenesis, it did not increase SREBP protein expression. These results suggest that the moderate increase in lipogenesis in SEB-1 cells induced by 100 nM insulin does not involve upregulation of SREBP-1, while the greater increase seen with 1 μM insulin or 20 ng/mL IGF-1 are at least, in part, mediated by the SREBP pathway.

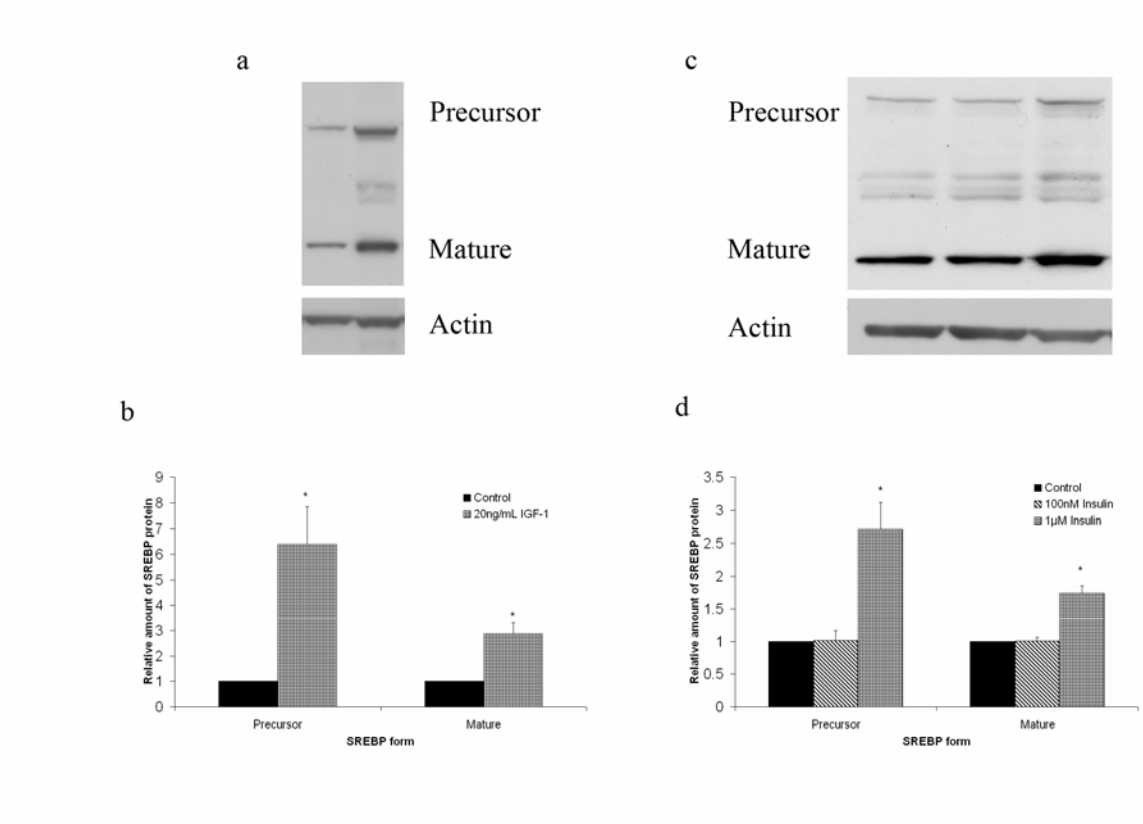


Figure 19: **Both forms of the SREBP-1 protein are increased in SEB-1 sebocytes treated with 20 ng/mL IGF-1 or 1 μM insulin, but not with 100 nM insulin.** SEB-1 sebocytes were treated for 24 hours with insulin or IGF-1. Protein was isolated and a Western blot was performed probing with antibodies to SREBP-1 and actin. Panel a is a representative blot probed for SREBP-1 following 20 ng/mL IGF-1 treatment. The first lane is SEB-1 cells treated with vehicle, while the second lane is SEB-1 cells stimulated with IGF-1. This blot was normalized to actin and quantified by densitometry as shown in panel b. Panel c is a representative blot probed for SREBP-1 and normalized to actin following insulin treatment: 100 nM (lane 2) and 1 μM (lane 3). Panel d is the densitometry analysis of the Western blots of cells treated with insulin. All experiments were repeated a minimum of 5 times. Densitometer data were compared to vehicle treated blots and Student's t-test was performed. A p-value of >0.05 was determined to be statistically significant.

2.3.6 Both the SREBP-1a and SREBP-1c mRNA transcripts are increased in SEB-1 cells treated with IGF-1 and insulin.

Quantitative reverse-transcription PCR (QPCR) was performed to determine if the SREBP-1a or 1c transcripts were increased in response to IGF-1 or insulin treatment. Treatment of SEB-1 sebocytes with 20 ng/mL IGF-1 (Figure **20**) produced a statistically significant increase in mRNA for both SREBP-1a (3.3 fold increase) and SREBP-1c (4.7 fold increase). Treatment with either 100 nM insulin or 1 µM insulin also increased SREBP-1a and SREBP-1c mRNA (also shown in Figure **20**). The increase in response to 100 nM insulin was surprising, since no change in SREBP-1 protein was noted at this dose (Figure **19**). The increase in both SREBP-1a and 1c transcripts in response to 100 nM insulin, combined with the lack of increase in SREBP-1 protein at this dose, implies a level of post-trancriptional regulation that has not been previously described for SREBP-1.

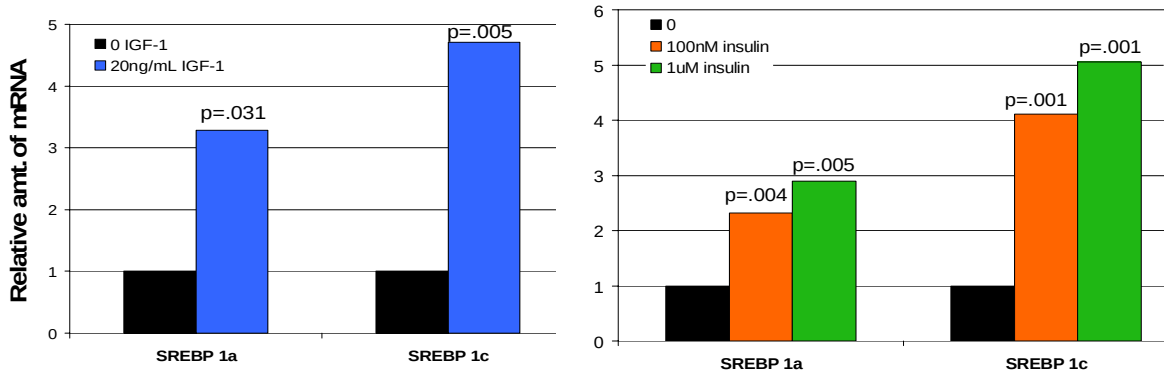


Figure 20: **Both SREBP-1a and SREBP-1c mRNA transcripts are increased in SEB-1 sebocytes treated with 20 ng/mL IGF-1, 100 nM insulin, or 1 µM insulin.** SEB-1 sebocytes were treated with IGF-1 (left panel) or insulin (right panel) for 14 hours. Following treatment, RNA was isolated and subjected to QPCR to determine relative abundance of the SREBP-1a and SREBP-1c transcript in comparison to vehicle-treated cells. Data were analyzed using the REST program and Student's t-test was performed. A p-value >0.05 was considered statistically significant. RNA was extracted from five different plates and analyzed by QPCR.

2.3.7 The ratios of SREBP-1c:SREBP-1a differ between human sebaceous glands and SEB-1 sebocytes.

Several reports have indicated that the SREBP-1c:1a ratio is markedly different in cell lines compared to tissue (Shimomura, Shimano et al. 1997). It is typical for the SREBP-1c form to predominate in tissue, while immortalized cell lines have much more SREPP-1a than 1c. We performed QPCR to compare the respective SREBP-1c:1a ratios and found this to be true for SEB-1 cells as well. Sebaceous glands had a 1c:1a ratio of nearly 10:1, whereas, the SEB-1 cell line had a SREBP-1c:1a ratio of about 0.6:1 (Figure 21). As the 1c transcript abundance is increased to a greater extent than the 1a transcript with IGF-1 treatment, a theoretical calculation of SREBP-1c:1a mRNA with

IGF-1 stimulation predicts that the ratio is 1:1 with IGF-1 stimulation in SEB-1 sebocytes.

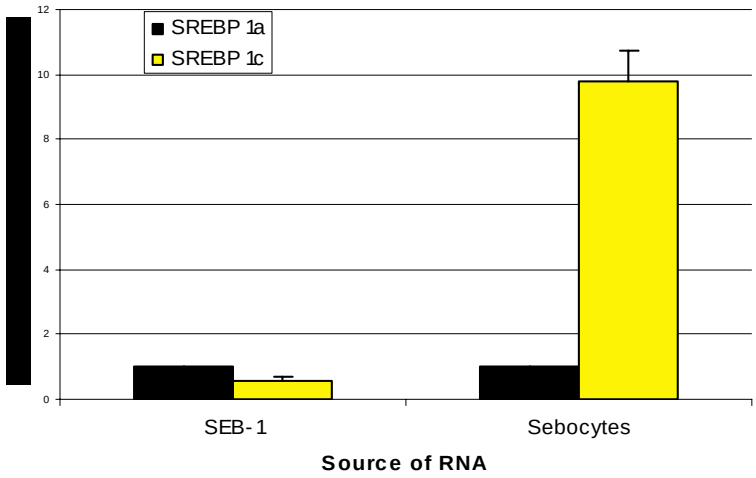


Figure 21: **The ratio of SREBP-1c:1a mRNA in SEB-1 sebocytes is 0.59:1, while the ratio is 9.8:1 in the sebaceous gland.** Human sebaceous glands were microdissected. RNA was extracted from the glands and subjected to QPCR to determine the relative ratio of SREBP-1c:1a. RNA was also extracted from SEB-1 cells to determine the SREBP-1c:1a ratio by QPCR. Data represent the mean ratio of five determinations ± SE.

2.3.8 The ratio of insulin receptor isoform A:isoform B is 5.1:1 in SEB-1 sebocytes.

The isoform of insulin receptor present in cells can have a profound impact on the insulin signaling. There have been two isoforms of the insulin receptor described: insulin receptor isoform A (IR-A) lacks exon 11, while insulin receptor isoform B (IR-B) retains exon 11. IR-A has been shown to have a higher affinity for insulin and a higher internalization rate than IR-B (Yamaguchi, Flier, Yokota, Benecke, Backer and Moller 1991), though IR-B has greater phosphorylation activity (Kellerer, Lammers, Ermel, Tippmer, Vogt, Obermaier-Kusser, Ullrich and Haring 1992). It has also been shown

that IR-A and IR-B signal through different PI3-K isoforms (Leibiger, Leibiger et al. 2001). Finally, IR-B sends differentiation signals and is generally found in differentiated tissues; while IR-A preferentially sends mitogenic and antiapoptotic signals (Sciacca, Prisco et al. 2003). We performed QPCR to determine which isoform was prevalent in the SEB-1 sebocytes. We found that IR-A (exon 11-) was more prevalent than IR-B (exon 11+) by a ratio of about 5.1:1 (Figure 22).

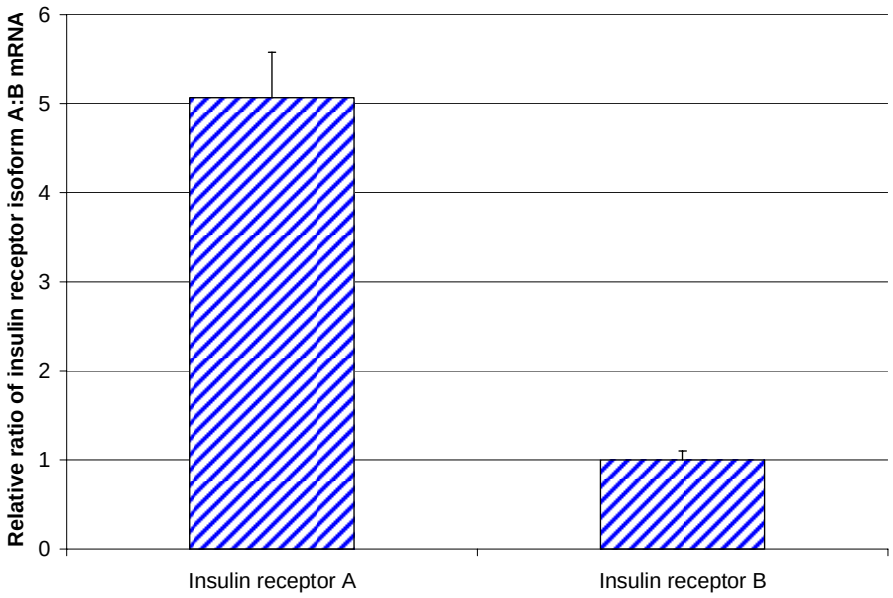


Figure 22: **The ratio of insulin receptor isoform A:isoform B mRNA is 5.1:1 in SEB-1 sebocytes.** RNA was extracted from SEB-1 sebocytes and QPCR was performed to determine the ratio of insulin receptor isoform A to insulin receptor isoform B. Data represent the mean ratios of five determinations ± SE.

2.4 Discussion

Acne is the most common skin condition observed by clinicians, and at times it has been considered "physiologic" in the adolescent population (Kligman 1974). Sebum

production by the sebaceous gland is fundamental in the pathophysiology of acne and, apart from isotretinoin or hormonal therapy; there are no effective means to reduce sebum production. An understanding of the factors that regulate sebum production is crucial to making advances in acne therapy (Harper and Thiboutot 2003).

While sebocytes are epithelial cells and adipocytes are mesenchymal cells, similarities exist in that each of these cell types make lipid as part of their process of differentiation, as first noted by Rosenfield and co-workers (Rosenfield, Kentsis et al. 1999). The adipogenic hormones methylisobutylxanthine, dexamethasone, and insulin induce the differentiation of preadipocytes into adipocytes, a process which is accompanied by a change in morphology, increased expression of the peroxisome proliferators-activated receptor γ (PPARγ), and the accumulation of intracellular lipid (Rosen, Walkey et al. 2000). In this chapter, we show that adipogenic hormones increase lipid production in SEB-1 sebocytes, similar to their actions in adipocytes, and that this effect can be induced by insulin. Additionally, this effect can be reproduced by IGF-1.

Both insulin and IGF-1 are members of the receptor tyrosine kinase subfamily. It is crucial to note that although each molecule has its own receptor, insulin can bind to the IGF-1 receptor with an affinity 100-fold less than to its native receptor. Likewise, IGF-1 will bind to the insulin receptor with 100-1,000 times less affinity than it has for its own receptor (Jones and Clemmons 1995). This matter is further complicated by alternative splicing that has been observed for the insulin receptor, which alters both the signaling and affinity for insulin, IGF-1, and IGF-II (Denley, Wallace et al. 2003). As 1 μM insulin can activate the IGF-1 receptor in addition to the insulin receptor (Prisco, Romano et al. 1999), we also treated the SEB-1 cells with a lower dose of insulin (100 nM) or

with IGF-1 (20 ng/mL). The lower dose of insulin was able to induce lipogenesis to a lesser extent than higher concentrations, but IGF-1 induced lipogenesis to a significantly greater extent than 100 nM insulin, and to approximately the same levels as 1 µM insulin (Figure **16**). From this we conclude that IGF-1 receptor activation is a key driver of lipogenesis in SEB-1 sebocytes. This hypothesis is supported by the fact that 3T3-L1 preadipocytes can be differentiated by IGF-1 or 2 µM insulin, from which it was suggested that IGF-1 is the essential regulator of differentiation in the 3T3-L1 line (Smith, Wise et al. 1988).

Our data support those of Deplewski and Rosenfield (1999) who found increased lipid content in rat preputial cells following treatment with insulin and IGF-1. IGF-1 at 1 nM (7.7 ng/mL) stimulates differentiation to the same degree as 1 µM insulin in the rat preputial sebocyte model. In another study, isolated human sebaceous glands from chest skin that were grown in the presence of 50 ng/mL IGF-1 for 3 days showed an increase in ^{14}C acetate incorporation into non-polar lipids that was comparable to that achieved by 1.8 µM insulin (Downie, Sanders et al. 2002; Downie 2004). Our data demonstrate that IGF-1 at a physiological dose (20 ng/mL) increases lipogenesis to the same extent as insulin at 1 µM. Taken together, these data indicate that the lipogenic enzymes in sebaceous glands and sebocytes are maximally stimulated by physiologically relevant doses of IGF-1.

A possible mechanism underlying this increase in rate of lipogenesis in response to insulin and IGF-1 treatment involves the SREBP pathway. The SREBPs are key transcriptional regulators of lipogenic enzymes (Horton, Goldstein and Brown 2002). Their expression is upregulated in response to insulin in tissues such as liver, fat, and

skeletal muscle (Kim, Sarraf et al. 2002). We hypothesized that the effects of insulin and IGF-1 on lipogenesis in SEB-1 sebocytes may be mediated via SREBPs.

When SEB-1 cells were treated with 20 ng/mL IGF-1 or 1 μM insulin, we found that both the precursor and cleaved SREBP-1 protein levels were increased, while 100 nM insulin had no effect on SREBP-1 protein. The modest increase in lipogenesis in cells treated with 100 nM insulin (this dose is still well above physiological insulin levels) combined with the lack of induction of SREBP-1 protein in this group provides evidence for an SREBP-1 independent mechanism by which sebaceous gland lipogenesis can be increased. However, activation of the IGF-1 receptor, and ultimately, SREBP-1 is required for maximal stimulation.

The two transcripts of the SREBP-1 protein are formed from a single gene with alternate transcription start sites which yields two mRNA species that have different first exons, but are identical from the second exon onward (Shimomura, Shimano et al. 1997). In this first exon, the 1a variant has a much longer activation domain, making a much more potent activator of sterol response elements than the 1c variant. As such, SREBP-1c has been reported to be regulated more at the transcriptional level, particularly by insulin, while SREBP-1a appears to be regulated at the protein processing stage (Eberle, Hegarty et al. 2004). Here, we show that in SEB-1 sebocytes, both the SREBP-1a and SREBP-1c mRNA transcripts are increased in response to IGF-1 treatment and two different doses of insulin. The increase in SREBP-1c mRNA was expected as this response to insulin has been well characterized (see Eberle, Hegarty et al. 2004). However, the increase in SREBP-1a transcript was not as robust as the increase observed for SREPB-1c. This may be attributed to the fact that the 1a promoter contains only two

Sp1 elements which are relatively weak activators of transcription (Zhang, Shin et al. 2005). Sp1 elements have been shown to activate transcription in response to insulin (Samson and Wong 2002), which is most likely responsible for the increase in response to IGF-1 as well. In agreement with our findings, increases in SREBP-1a mRNA have been reported in 3T3-L1 adipocytes when differentiated by adipogenic hormones (Shimomura, Shimano et al. 1997). SEB-1 cells, like HepG2 cells (1c:1a ratio <1:2), or 3T3-L1 adipocytes (no SREBP-1c detected-only 1a) have more SREBP-1a compared to SREBP-1c, which is reversed compared to the tissues from which these cell lines were derived (Shimomura, Shimano et al. 1997).

Whereas, numerous studies link the action of insulin to SREBPs, there is a very limited body of work connecting the IGF-1 and SREBP pathways, none of which has been investigated in the sebaceous gland. IGF-1 increases low-density lipoprotein receptor expression in HepG2 cells. Significantly less activation of a low-density lipoprotein receptor reporter construct is noted however, in a HepG2-derived cell line that exhibits decreased expression of SREBP-1 (Streicher, Kotzka et al. 1996). Although artificial and indirect, these data indicate that the effect of IGF-1 on low-density lipoprotein receptor expression may be mediated by SREBP-1 which would support the relationship between IGF-1 and SREBP-1 we report here. In addition, a functional IGF receptor is necessary for insulin to induce SREBP protein and its downstream effects in an immortalized brown fat cell line (Mur, Arribas et al. 2003). In this model, IGF-1 can stimulate fatty acid synthase RNA (Arribas, Valverde and Benito 2003). Fatty acid synthase may correlate directly with SREBP activity (Kim, Sarraf et al. 1998), though there are exceptions (Palmer, Rutter and Tavare 2002; Louveau and Gondret 2004).

Taken together, these studies generally support our finding that IGF-1 activates SREBP-1, which in turn increases *de novo* lipid production. To our knowledge, we are the first to directly show an increase in SREBP-1 protein in response to IGF-1.

Our data suggest that different molecular mechanisms might underlie the increase in lipogenesis induced by low doses of insulin (acting via the insulin receptor) compared to IGF-1 and 1 μM insulin (acting at least predominantly via the IGF-1 receptor). Each of these treatments increased mRNA for SREBP-1a and SREBP-1c, yet 100 nM insulin failed to increase SREBP-1 protein. The reasons for this discrepancy are not apparent but could involve differences in transcript or protein stability. Additional studies are needed to test these alternatives.

Androgens are major regulators of sebaceous gland function. Interestingly, the SREBP pathway has been implicated in the actions of androgens in stimulating lipogenesis in sebaceous glands. Rosignoli *et al*. showed that the increased lipogenesis observed in the hamster ear model in response to androgen treatment is mediated by the SREBP pathway (Rosignoli, Nicolas et al. 2003), a finding supported by the data presented here.

Our work confirms that SREBP proteins are indeed important mediators of sebaceous lipid metabolism. An understanding of the mechanisms by which insulin and IGF-1 increase SREBP-1 expression will move us one step closer to understanding the underlying molecular mechanisms that govern skin lipid production. Finally, this work demonstrates that SREBP proteins, considered to be, “Master regulators of lipid homeostasis” (Eberle, Hegarty et al. 2004) are important in the sebaceous gland and are significantly regulated by IGF-1. The SEB-1 sebocyte model could be a useful tool for

dissecting the differences between insulin and IGF-1 signaling which may provide further insight into the factors regulating human sebum production and the development of acne.

Chapter 3

Activation of PI3-K is essential for IGF-1 induced lipogenesis in SEB-1 sebocytes

3.1 Introduction

Sebum production is critical in the pathogenesis of acne. In order to design rational therapeutics for acne, it is essential to understand the molecular signaling pathways that drive the increased sebogenesis observed in many acne patients. Insulin-like growth factor (IGF-1) levels reach their peak in adolescents during the growth spurt, and then decline, which coincides with the incidence and severity of acne in many individuals (Deplewski and Rosenfield 1999). Additionally, our lab has demonstrated that IGF-1 levels correlate positively with severity of acne in women with clinical acne (Cappel, Mauger et al. 2005) and that IGF-1 activates the sterol response element binding protein (SREBP) pathway and increases lipogenesis in cultured SEB-1 sebocytes (Smith, Cong et al. 2006).

As implied by their name, SREBPs bind sterol response elements (SREs), which are nucleotide sequences found in the promoters of several lipogenic genes in the cholesterol and fatty acid biosynthesis pathways. There are three members in the SREBP family: SREBP-1a, SREBP-1c, and SREBP-2. SREBP-1a and 1c are transcribed by alternative start sequences, which are spliced to form an identical protein from the second exon onward. The longer first exon found in SREBP-1a makes it the more potent activator of transcription.

SREBP proteins exist embedded in the endoplasmic reticulum, bound to the SREBP-cleavage-activating protein (SCAP). SCAP tethers the SREBPs to the membrane by association with Insig proteins. When cellular sterols are low, SCAP undergoes a conformational change which causes it to dissociate from Insig, resulting in the SREBP/SCAP being transported to the Golgi by way of COPII vesicles. In the Golgi, two sequential cleavages release the amino terminus of the SREBP protein (approximately 490 amino acids) which then translocates to the nucleus. The end result is an increased amount of the active SREBP transcription factor in the nucleus which activates transcription of several lipogenic genes (Goldstein, DeBose-Boyd et al. 2006).

Transcription of the SREBPs has been shown to be activated by insulin, platelet derived growth factor (PDGF), epidermal growth factor (EGF), IGF-1, and keratinocyte growth factor (KGF). It is possible that the molecular pathway by which each of these stimuli activates transcription and/or increase SREBP-1 protein abundance may not be the same. Additionally, it is also becoming apparent that the specific molecular signaling pathway that transduces the signal upstream of SREBP translation may be cell/tissue type specific (Table **6**). For instance, it has been shown that insulin increases SREBP expression in L-6 skeletal muscle cells via the MAPK/ERK pathway, and antagonism of the PI3-K pathway has no effect on this; yet in 3T3-L1 adipocytes, insulin increases SREBP expression via the PI3-K pathway and antagonism of the MAPK/ERK pathway has no effect (Nadeau, Leitner et al. 2004).

Table 6: **A brief summary of experiments in the literature that have shown SREBPs to be stimulated.** A variety of growth factors and three different molecular pathways are implicated in the induction of SREBP mRNA or protein.

Stimuli	Pathway utilized	Cell type/line	SREBP 1a, 1c, or 2?	mRNA or protein?	Reference
IGF-1	PI3-K	Chinese Hamster Ovary	SREBP-2	Mature protein only	(Du, Kristiana et al. 2006)
Insulin	MAPK/ERK	Myocytes (L-6)	SREBP-1	mRNA and protein	(Nadeau, Leitner et al. 2004)
Insulin	PI3-K	3T3-L1 adipocytes	SREBP-1	mRNA and protein	(Nadeau, Leitner et al. 2004)
Insulin	PI3-K (didn't examine others)	Primary rat hepatocytes	SREBP-1c	mRNA	(Matsumoto, Ogawa et al. 2002)
Insulin	PI3-K (didn't examine others)	HepG2	SREBP-1a and 1c	Protein	(Borradaile, de Dreu and Huff 2003)
Insulin	PI3-K, found MAPK/ERK not important	Primary hepatocytes	SREBP-1c	mRNA	(Fleischmann and Iynedjian 2000)
Insulin	PI3-K (didn't examine others)	Cultured rat hepatocytes	SREBP-1c	mRNA and protein	(Azzout-Marniche, Becard et al. 2000)
KGF	PI3-K and MAPK/JNK. MAPK/ERK not important	H292 pulmonary epithelial cells	SREBP-1c	mRNA and protein	(Chang, Wang et al. 2005)
PDGF	PI3-K (didn't examine others)	Human fibroblasts	SREBP-1c and 2	mRNA and protein	(Demoulin, Ericsson et al. 2004)
VEGF	PI3-K (didn't examine others)	Primary human vasucular endothelial cells	SREBP-1c and 2	mRNA and protein	(Zhou, Yao et al. 2004)
Inducible Akt	Akt	A variety of cell lines	SREBP-1	mRNA and protein	(Fleischmann and Iynedjian 2000)

In this paper, we sought to determine the molecular signaling pathways by which IGF-1 stimulation of SEB-1 sebocytes increases SREBP-1 mRNA and protein. It has been shown that both the MAPK/JNK and MAPK/ERK cascades, in addition to the PI3-K pathway can be activated by IGF stimulation. Further, all three of these pathways have been implicated in activation of SREBP in at least one model system (Table **6**). In this paper we report IGF-1 activates the PI3-K and MAPK/ERK pathways in SEB-1 sebocytes, while the MAPK/JNK and MAPK/p38 pathways are not stimulated. Moreover, pharmacological antagonism of the MAPK/ERK pathway has no effect upon SREBP-1 mRNA, protein, or on IGF-1 induced lipogenesis. Most importantly, IGF-1 activates the PI3-K pathway and this activation mediates the increase in SREBP-1 mRNA and protein in SEB-1 sebocytes. Finally, inhibition of the PI3-K pathway with inhibitor LY294002 completely blocks the increase in lipogenesis in SEB-1 cells in response to IGF-1.

3.2 Materials and methods

3.2.1 Cell culture

SEB-1 (passage 22-24) SV40 immortalized human sebocytes were grown to confluence in all experiments unless stated otherwise, and were cultured in standard medium consisting of DMEM (Invitrogen, Carlsbad, CA), 5.5 mM glucose/Ham's F-12 3:1 (Invitrogen), fetal bovine serum 2.5% (HyClone, Logan, UT), adenine 1.8 x 10^{-4} M (Sigma, St. Louis, MO), hydrocortisone 0.4 µg/mL (Sigma), insulin 10 ng/mL (Sigma),

epidermal growth factor 3ng/mL (Austral Biologicals, San Ramon, CA), and cholera toxin 1.2 x 10^{-10} M (Sigma) (Thiboutot, Jabara et al. 2003).

3.2.2 Cell treatments

For treatments with IGF-1, SEB-1 cells were plated at 7.6 x 10^5 in a 100mm dish, or 1.3 x 10^5 in a 35mm dish and grown for 6 days. On the sixth day, media was removed, cells were washed twice with PBS, and cells were given DMEM containing 5.5mM glucose. IGF-1 was added 1:1000 in 0.1M acetic acid plus 0.1% BSA for 24 hours. MEK inhibitor PD98059 was used as a pharmacological inhibitor of the MAPK/ERK pathway, and cells were pretreated with the compound 30 minutes prior to IGF-1 stimulation. Likewise, the cell permeable PI3-K inhibitor LY294002 was also administered 30 minutes prior to IGF-1 treatment. Both compounds were suspended in DMSO and the final concentration of DMSO in the media was 0.1%.

3.2.3 Lipogenesis Assay

The incorporation of ^{14}C-acetate into lipids was used as a measure of lipogenesis. SEB-1 cells were plated at 1.3 x 10^5 cells per 35mm dish and cultured 6 days in growth medium. On day six, cells were treated with IGF-1 and appropriate inhibitors/vehicles for 24 hours. Cells were then removed with trypsin and an aliquot was taken for a cell count which was used to normalize the data. The remaining cells were suspended in a solution containing 1µCi of ^{14}C-acetate (New England Nuclear, Boston, MA) in DMEM.

Ambient air in the tubes was replaced with argon or nitrogen gas to mimic the low oxygen tension in sebaceous glands found *in vivo* (Evans, Schrlau et al. 2006) and samples were incubated for 2 hours at 37° C with agitation. After the two-hour incubation, samples were extracted twice with ethyl ether and non-radioactive carrier lipids (purchased individually from Sigma) in petroleum ether were added for visualization following thin layer chromatography. These included cholesterol, stearyl alcohol (fatty alcohol), oleic acid (fatty acid) triolein (triglyceride), cetyl palmitate (wax ester), cholesterol oleate (cholesterol ester) and squalene. Following evaporation of organic solvent, samples were dissolved in a small volume of ethyl acetate and spotted on 20 cm silica gel thin layer chromatography plates (Macherey-Nagel, Easton, PA) which were then run until the solvent front reached 19.5 cm in hexane, followed by 19.5 cm in benzene, and finally to 11 cm in hexane:ethyl ether:glacial acetic acid (70:30:1). Plates were dried 5 minutes after each solvent. Lipid spots were visualized using iodine and bromothymol blue/sodium hydroxide spray dye. All lipid-containing zones were circled, excised, and radioactivity counted in a liquid scintillation counter. Negative controls for each experiment consisted of plates of untreated cells where radioactivity was added and cells were incubate on ice for 2 hours; lipids were extracted and analyzed in parallel. All experiments were repeated three times with each sample in triplicate within each experiment. Data were analyzed by ANOVA and considered significant if a p-value of <0.05 was observed compared to control.

3.2.4 Western blot

Cytoplasmic and nuclear lysates were obtained using the Ne-Per kit (Pierce, Rockford, IL) following manufacturer's instructions. A mammalian protease inhibitor cocktail containing 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin was added (Sigma). Both the mature and precursor forms of the SREBP-1 proteins were found in the nuclear fraction in SEB-1 cells. Total protein for each sample was determined using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Twenty-five micrograms of protein was run on a 4-12% Bis-Tris NuPage polyacrylamide gel (Invitrogen). Protein was then transferred to a polyvinylidene fluoride membrane (PVDF) and probed using standard methods. The SREBP-1 (K-10) antibody was obtained from Santa Cruz and used at a dilution of 1:2000. The phospho-ERK, phospho-Akt, phospho-p38, phospho-JNK, actin (as a loading control) and the goat anti-rabbit secondary antibody were obtained form Cell Signaling Technologies (Beverly, MA). Blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to film. Western blots were repeated a minimum of three times.

As an alternative to actin as a loading control, some membranes were stained with SimplyBlue SafeStain (Invitrogen) for 20 minutes and destained in water for 10 minutes as specified in the manufacturer's instructions.

3.2.5 Quantitative RT-PCR

For QPCR, cells were grown in standard medium for 6 days. On the sixth day, cells were washed twice with phosphate-buffered saline and treated with 20 ng/mL IGF-1

and the appropriate inhibitor/vehicle in serum-free DMEM for 14 hours. RNA was isolated and complementary DNA was generated from 4.2 µg RNA/reaction primed with oligo-dT using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). QPCR was performed using the Brilliant SYBR Green QPCR Core Reagent Kit in an Mx4000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA). TATA-binding protein was used as a reference gene. The following primer sequences were used: TATA-binding protein upstream (GenBank number NM_003194) 5'c acg gca ctg att ttc agt tct, TATA-binding protein downstream 5'ttc ttg ctg cca gtc tgg act, SREBP-1a upstream (GenBank number NM_004176) 5'gct gct gac cga cat cga a, SREBP-1c upstream (GenBank number NM_001005291 5'gga gcc atg gat tgc act tt, and SREBP-1a, c downstream 5'tca aat agg cca ggg aag tca. Primer pairs for both FAS (product number Hs00188012_m1) and LDLR (product number Hs00181192_m1) were purchased from ABI. Data were analyzed using the REST-XL© program (Pfaffl, Horgan et al. 2002). A p-value <0.05 was considered significant.

3.2.6 Nucleofection of SEB-1 cells with siRNA

SEB-1 cells were transfected with various concentrations of siRNA using the Nucleofection Kit T (Amaxa). In previous experiments, nucleofection conditions were optimized for the SEB-1 cell line using a plasmid that codes for the green fluorescent protein (GFP). Transfection efficiency as determined by counting GFP positive cells was found to be 60-70% using the Amaxa Nucleofection kit. Briefly, $1.4*10^6$ SEB-1 cells were resuspended in nucleofection reagent "T" with appropriate siRNA, and shocked on

program T-20 using the proprietary Nucleofector Device (Amaxa). Following the shock, 500 µL RPMI 1640 medium was added to the cells and the cells were plated in normal SEB-1 growth medium. Cells were assayed for SREBP-1 protein expression 48 and 72 hours post-nucleofection by Western blot. Three different siRNAs sequences were used: Qiagen product Hs_SREBF1_1_HP siRNA (Qiagen 1) designed to target CCC AGT GGT CTG GCT GCT CAA employed the following siRNA sequences: sense-CAG UGG UCU GGC UGC UCA A, antisense-UUG AGC AGC CAG ACC ACU G. Qiagen product Hs_SREBF1_3_HP siRNA (Qiagen 3) targeted TGC GGA GAA GCT GCC TAT CAA and used the sense-CGG AGA AGC UGC CUA UCA A, and the antisense-UUG AUA GGC AGC UUC UCC G. A standard siRNA that has no mammalian target gene was also obtained form Qiagen and used as a negative control nucleofection; the sense sequence was-UUC UCC GAA CGU GUC ACG U, and the antisense sequence was-ACG UGA CAC GUU CGG AGA A. Concentrations of siRNA used were 100, 200, 500, or 750 picomoles. Additionally, the siRNA sequence used by (Porstmann, Griffiths et al. 2005) was employed in the SEB-1 line. The sequence sense-GGA AGA GUC AGU GCC ACU Gtt and antisense-CAG UGG CAC UGA CUC UUC Ctt was synthesized by Ambion (Austin, TX). A scrambled siRNA was also nucleofected alongside the siRNA targeting SREBP-1.

3.2.7 Complexing cholesterol with methyl-β-cyclodextrin and cell treatments

To facilitate uptake by the cells, cholesterol and 25-OH cholesterol were complexed with Methyl-β-cyclodextrin (MBC) as carriers in aqueous medium as

described by (Brown, Sun, Feramisco, Brown and Goldstein 2002). The final concentration of each lipid was 2.5 mM in water. The molar ratio of sterol:MBC was 1:10.

3.2.8 Retroviral infection of SEB-1 cells

To create a stable population of clones containing a dominant-negative SREBP-1 construct, a retroviral strategy was employed. To produce the virus, glycerol shocked 293T cells were transiently co-transfected with the pBABE-SREBP-1DN with puromycin resistance designated "pBABE-ADD1-DN", kindly provided by (Kim and Spiegelman 1996) or the pBABE-puro empty vector along with the amphotropic packaging vector 4070 using Lipofectamine 2000 (Invitrogen). In this construct, transcription in mammalian cells is driven by the LTR promoter. Supernatant was collected 24-36 hours later and the supernatant was titered.

SEB-1 cells (passage 22) were plated $18*10^4$ in a T-75 flask 24 hours prior to infection. Viral titers were as follows: pBABE empty 100 cfu/ml *3.5mL, pBABE ADD-1 (1-403) 40 cfu/mL*3.8mL, and pBabe ADD-1 DN 10 cfu/mL*2.4mL. All of the viral supernatant for each construct was applied to SEB-1 cells and 10µg/mL polybrene was added to enhance viral uptake. The cells were incubated for 6 hours to allow for infection, at which point medium was replaced with SEB-1 medium containing 0.25µg/mL puromycin to select for stable clones.

3.3 Results

3.3.1 Neither MAPK/p38 nor MAPK/JNK is phosphorylated in SEB-1 cells in response to IGF-1 treatment at 24 hours.

To determine if the MAPK/p38 pathway may tranduce the signal by IGF to increase lipogenesis, a Western blot was performed to determine the phosphorylation status of p38 in SEB-1 cells stimulated with IGF-1. We did not observe activation of p38 in reponse to IGF-1 in SEB-1 cells (Figure 23).

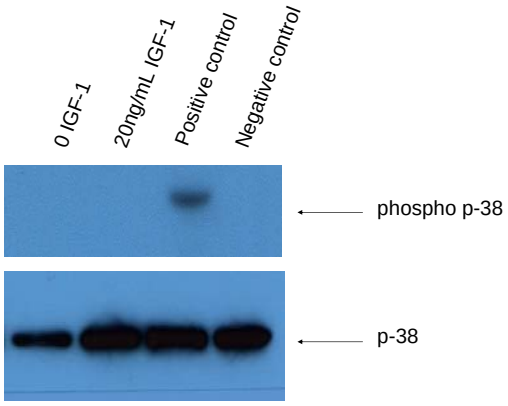


Figure 23: **p38 is not phosphorylated by SEB-1 cells in response to IGF-1 stimulation.** A Western blot probing for phosphorylated MAPK/p38 reveals that SEB-1 sebocytes do not phosphorylate p38 in response to IGF-1. C-6 glioma cells treated with anisomycin serve as a positive control for phosphorylated p38, while unstimulated C-6 glioma cells are the negative control.

Additionally, a Western blot was also performed under the same experimental conditions probing for activation of the MAPK/JNK pathway which showed that the JNK pathway was not stimulated by IGF-1 treatment (Figure 24).

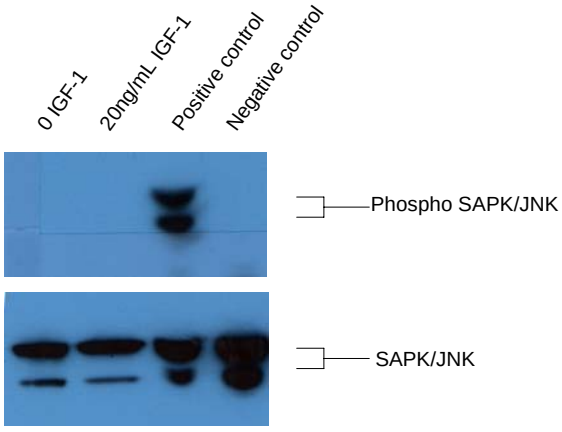


Figure 24: **SAPK/JNK is not activated by IGF-1 stimulation of SEB-1 cells.** A Western blot probing for phosphorylated JNK reveals that SEB-1 sebocytes do not phosphorylate JNK in response to IGF-1. Total cell extracts from 293 cells treated with UV light serve as a positive control for phosphorylated JNK, while normal 293 extracts serve as the negative control.

3.3.2 The MAPK/ERK pathway is activated by IGF-1 treatment in SEB-1 cells

Stimulation of the MAPK/ERK pathway is indicated by phosphorylation of p44/p42 (ERK 1 and 2). IGF-1 caused a robust increase in the amount of phosphorylated p44/p42 in SEB-1 sebocytes after 24 hours of stimulation (Figure 25). Furthermore, pretreatment of the SEB-1 cells for 30 minutes prior to the addition of IGF-1 with 50 µM of the MEK inhibitor PD98059 prevented the activation of this pathway (Figure 25). No toxicity was observed at 50 µM PD98059.

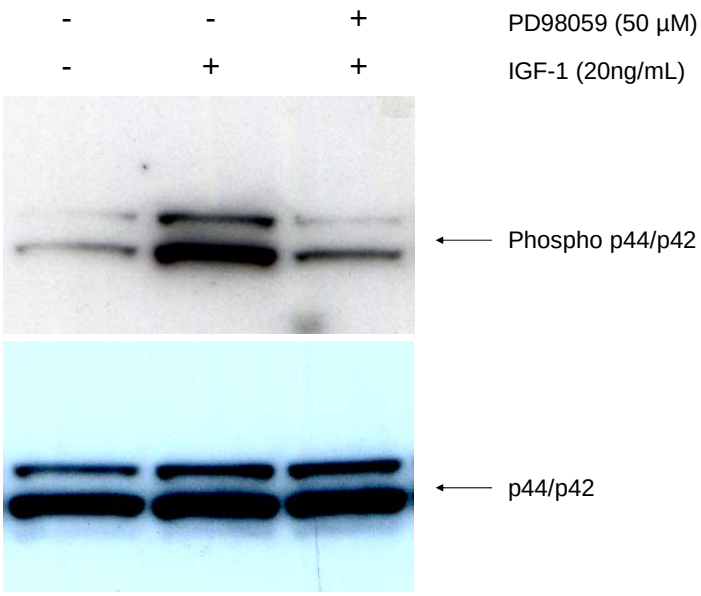


Figure 25: **IGF-1 stimulation of SEB-1 cells increases phosphorylation of p44/p42. This activation is blocked by 50 µM PD98059 treatment**. Western blot reveals that p44/p42 is phosphorylated by IGF-1 in SEB-1 cells and that this stimulation can be blocked by 50 µM PD98059 treatment. The phospho p44/p42 blot was stripped and re-probed with an antibody that recognizes total p44/p42 regardless of phosphorylation status to serve as a loading control.

3.3.3 The PI3-K pathway is activated by IGF-1 treatment in SEB-1 cells

Phosphorylation of Akt is used to indicate activation of the PI3-K pathway. SEB-1 sebocytes have very low amounts of phosphorylated Akt when maintained in medium without serum. However, the addition of IGF-1 to this same medium caused a robust increase in phosphorylated Akt (Figure 26), accumulating to a high level after 24 hours. Importantly, we also demonstrate that this induction could be blocked quite potently by the addition of 50 µM of the PI3-K inhibitor LY294002 30 minutes prior to IGF-1

stimulation (Figure 26). Additionally, we also showed (Figure 26) that 1 µM insulin activates the Akt pathway. Interstingly, 100 nM insulin does not lead to an increase in phosphorylated Akt protein. No toxicity was observed with 50 µM LY294002 treatment.

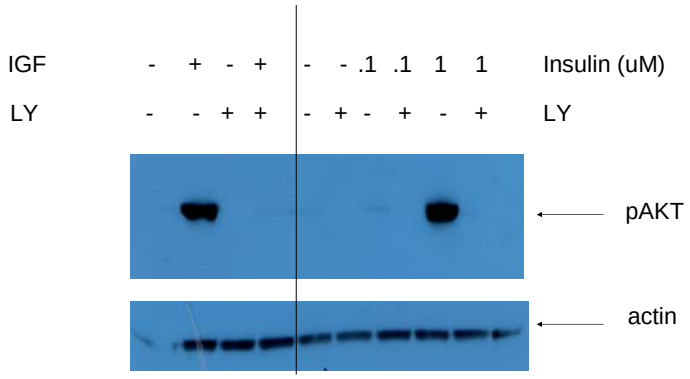


Figure 26: **Western blot reveals that Akt is phosphorylated by IGF-1 at 24 hours (left panel). Further, this induction is blocked by the addition of PI3-K inhibitor LY294002.** A pre-treatment for 30 minutes with 50 µM LY294002 blocks the phosphorylation of Akt which is induced by IGF-1 at 24 hours. On the right side of the figure, cells treated with 100 nM insulin show a miniscule increase in phosphorylated Akt, while 1 µM insulin, which can act through the IGF-1 receptor also increases phosphorylated Akt. This activation is also blocked by 50 µM LY294002.

3.3.4 MAPK inhibition with PD98059 has no effect on IGF-1 induction of SREBP-1 mRNA or protein.

We demonstrated (Figure 25) that the MAPK inhibitor PD98059 can block the activation of the p44/p42 pathway by IGF-1. We have also shown that the addition of IGF-1 causes an increase in SREBP-1 mRNA and protein (see Figures 19 and 20). To determine if the MAPK pathway plays a role in the IGF-1 induction of SREBP-1, we

treated SEB-1 cells with IGF-1 and/or PD98059. Addition of the MAPK inhibitor does not block the IGF-1 induced increase in SREBP-1 mRNA or protein (Figure 27).

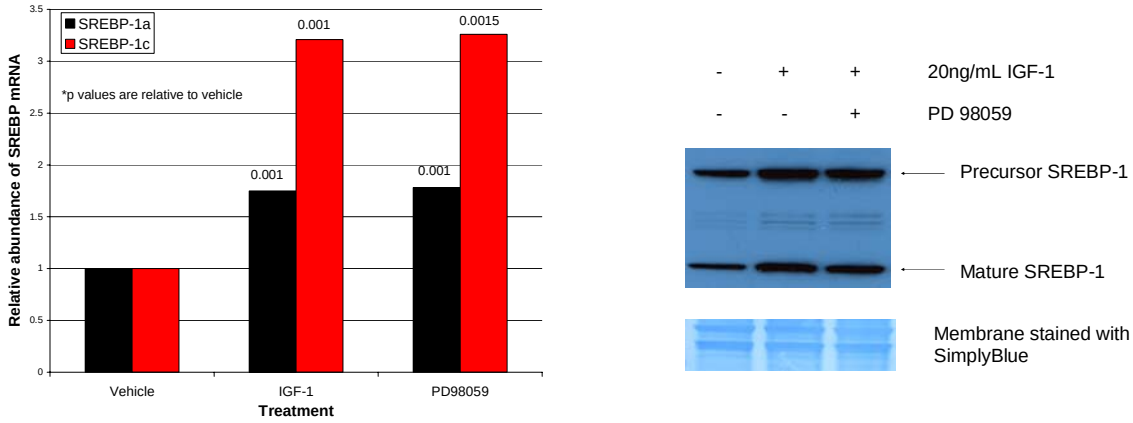


Figure 27: **Inhibition of the MAPK kinase pathway does not alter IGF-1 induced increases in SREBP-1 mRNA or protein in SEB-1 cells.** SEB-1 cells were treated with IGF-1 and/or MEK inhibitor PD98059. In the panel on the left, QPCR was used to show that the SREBP-1 mRNA transcript is still induced, despite the inhibition of the MAPK pathway. On the right, a Western blot confirms these findings, showing that SREBP-1 protein is also induced by IGF-1 when the MAPK pathway is antagonized. After being probed for SREBP-1, the membrane was then stained with Simply Blue to ensure even loading.

3.3.5 PI3-K inhibition with LY294002 blocks IGF-1 induced increases in SREBP-1 protein and mRNA.

Having shown that 50 µM LY294002 effectively blocks Akt activation in response to IGF-1 (Figure 26), we treated SEB-1 cells with LY294002 and/or IGF to determine if the activation of Akt was essential for the previously observed IGF-1 induced increase of SREBP-1 mRNA and protein. We found (Figure 28) that inhibition of the PI3-K/Akt pathway by LY294002 prevented the IGF-1 induced increase of SREBP-1 mRNA and protein.

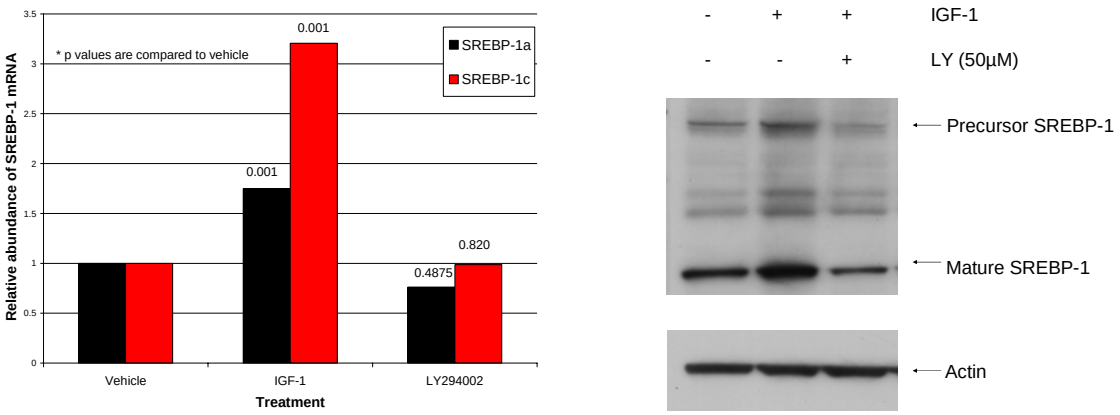


Figure **28**: **Inhibition of the PI3-K pathway prevents IGF-1 induced increases in SREBP-1 mRNA and protein in SEB-1 cells.** SEB-1 cells were treated with 50 μM LY294002 and/or 20 ng/mL IGF-1 for 24 hours. The panel on the left shows that the SREBP-1a and 1c mRNA is not induced by IGF-1, as determined by QPCR, when LY294002 is added to the media. The panel on the right is a Western blot that confirms the QPCR data that the SREBP-1 protein is not induced by IGF-1 when the PI3-K pathway is inhibited.

3.3.6 Activation of the PI3-K pathway is essential for IGF-1 induced lipogenesis in SEB-1 cells.

Since both the PI3-K and MAPK pathways are activated in SEB-1 cells by IGF-1, we next wanted to dissect the role of each of these signaling pathways in the increased lipogenesis in response to IGF-1 treatment. We know that PD98059 has no effect on SREBP activation by IGF-1, and keeping in line with our central hypothesis that SREBPs are important mediators of lipid metabolism in SEB-1 cells, we hypothesized that treatment of SEB-1 cells with a MAPK inhibitor will reduce lipogenesis to a much lesser degree than treatment with a PI3-K inhibitor would. The ^{14}C acetate incorporation assay was performed on SEB-1 cells treated with IGF-1 alone, or with the addition of PD98059 or LY294002. IGF-1 increased lipogenesis as we have shown previously (Figure **29**).

Additionally, treatment with MAPK inhibitor PD98059 had no effect on this induction of lipogenesis. However, when cells were treated with LY294002, IGF-1 did not increase lipogenesis, as cells in this treatment group produce a lipid profile nearly identical to the group that received no IGF.

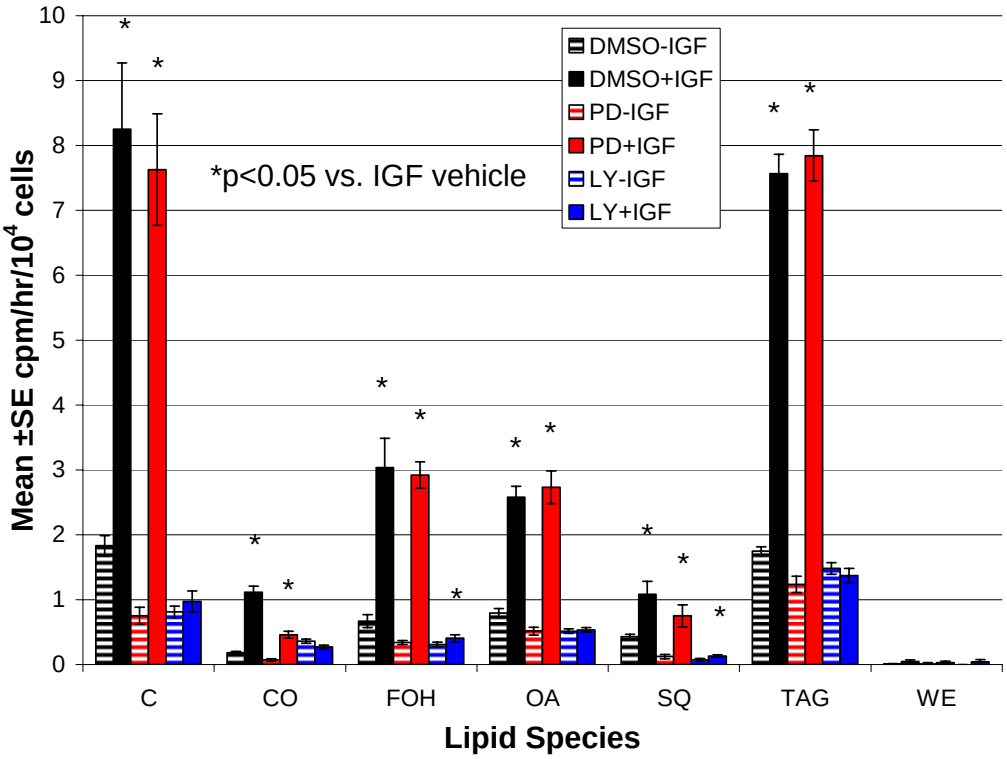


Figure 29: **PI3-K inhibitor LY294002 completely blocks IGF-1 induced lipogenesis, while MAPK inhibitor PD98059 has no effect.** The ^{14}C incorporation assay was performed on SEB-1 cells treated with IGF-1 and/or a pharmacological inhibitor of PI3-K or MAPK. Here we show that IGF-1, again, induces a robust increase in lipogenesis (black striped bars vs solid black bars). Interestingly, addition of 50 µM PD98059 to cells treated with IGF-1 has no effect on the IGF-1 induced lipogenesis (striped red bars vs solid red bars). Most importantly, the addition of 50 µM PI3-K inhibitor LY294002 blocks any induction of lipogenesis when cells are stimulated with IGF-1 (blue striped bars vs blue solid bars).

3.3.7 Attempts to inhibit SREBP-1 function in SEB-1 cells

3.3.7.1 siRNA

We employed an siRNA approach in an effort to knockdown SREBP-1 mRNA, and ultimately, the protein. We first used proprietary algorithms from Qiagen to select optimal target sequences in the SREBP-1 transcript for these molecules. A non-specific siRNA that targets a sequence that is not present in mammalian cells was used as a negative control for these experiments. These molecules, called Qiagen 1 and Qiagen 3, proved to be unsuccessful in altering the SREBP-1 protein content of SEB-1 cells (Figure 30).

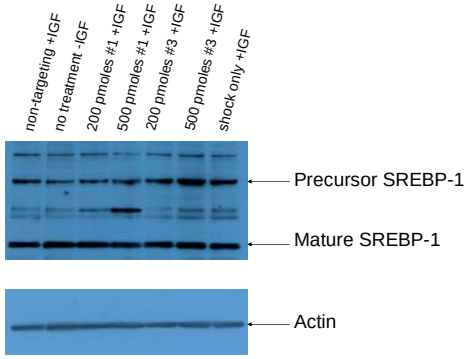


Figure 30: **The Qiagen 1 and Qiagen 3 siRNA sequences do not effectively reduce SREBP-1 protein content in SEB-1 cells.** Twenty four hours post-nucleofection, cells were stimulated with IGF-1 or vehicle for an additional 24 hours. At this point, a Western blot was performed to determine the amount of SREBP-1 protein.

After some time, an siRNA sequence was published where the authors convincingly demonstrated effective knockdown of SREBP-1 expression (Porstmann, Griffiths et al. 2005). The sequence, which is detailed in the methods section and called

"Porstmann siRNA", was ordered from Ambion. A challenge of working with the SEB-1 cell line is that the cells are difficult to transfect using traditional cationic means. We have found that the Amaxa Nucleofection can achieve a transfection efficiency of approximately 60% as assessed using GFP expression constructs (Figure **31**). We nucleofected the SREBP siRNA in concentrations of 150, 350, or 500 picomoles of siRNA per 100 mm plate. Additionally, control plates were nucleofected with various concentrations of a scrambled siRNA control. Cells were stimulated with 20 ng/mL IGF-1 48 hours post-nucleofection, then protein was harvested 24 hours later. We found that the siRNA used by Porstmann and coworkers did not decrease SREBP-1 protein when cells are stimulated with IGF-1 (Figure **32**). The half life of SREBP-1 nuclear protein has been reported to be 3 hours in COS cells (Hirano, Yoshida et al. 2001) and 4.5 hours in primary hepatocytes which is increased to 8 hours in the presence of insulin (Yellaturu, Deng et al. 2005).

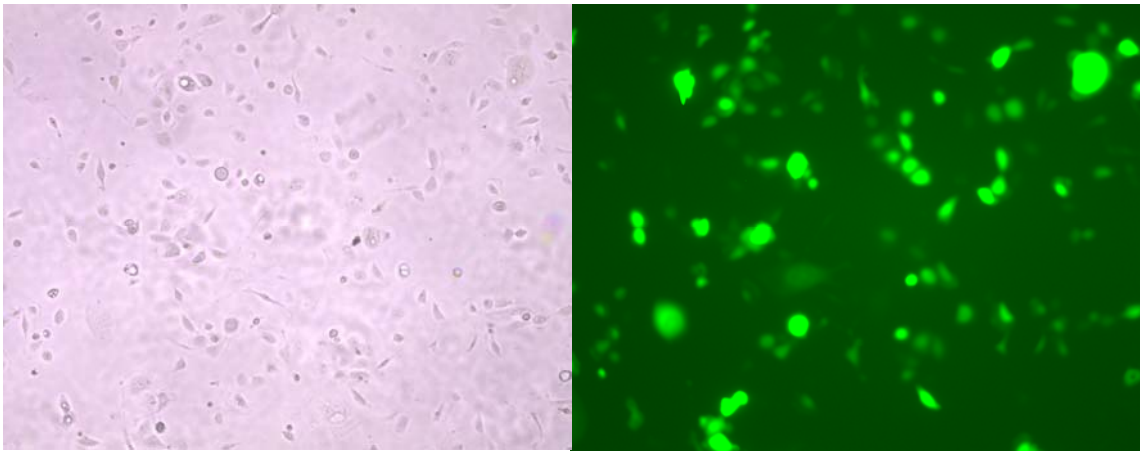


Figure 31: **The transfection efficiency of SEB-1 cells using Amaxa Nucleofection technology is approximately 60%.** SEB-1 cells were transfectected with a GFP construct using the Amaxa Nucleofection kit. The percentage of cells expressing GFP was assessed 24 hours post-transfection. The image on the left is the bright field photograph of the same field which is shown under fluorescence in the photograph on the right.

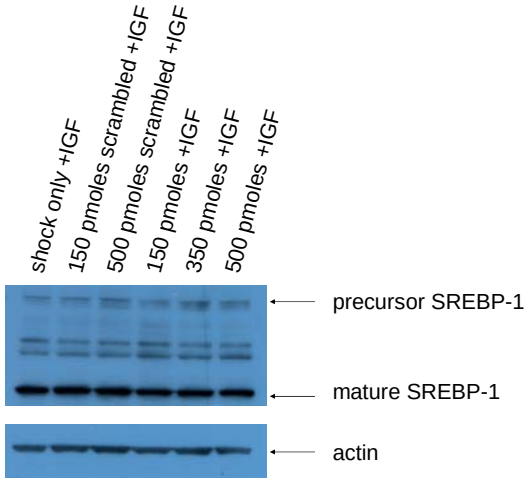


Figure 32: **The siRNA sequence published by Porstmann *et al* for SREBP-1 has no effect on SEB-1 SREBP-1 protein levels.** A western blot was performed on SEB-1 cells nucleofected with an SREBP-1 siRNA. Cells were stimulated with IGF-1 for 24 hours. This blot reveals no change in mature SREBP-1 protein between the scrambled siRNA control and the treatment groups.

3.3.7.2 Inhibition of SREBP by feedback inhibition

There have been reports that treatment of cultured cells with 25-OH cholesterol or cholesterol could decrease SREBP-1 protein expression, albeit through different mechanisms (Brown, Sun et al. 2002). It is believed that cholesterol causes a conformational change in SCAP, while 25-OH cholesterol may inhibit SREBP processing by inducing translocation of cholesterol from the plasma membrane to the ER, as 25-OH cholesterol does not trigger the sterol sensing domain in SCAP. Both cholesterol and 25-OH cholesterol were complexed with methyl-β-cyclodextrin so they can be taken up by the cell in the aqueous medium. Pre-treament of SEB-1 cells with either of the MCB complexed sterols for 30 minutes prior to addition of IGF-1 for 24 hours resulted in little, if any, decrease in SREBP-1 protein (Figure 33).

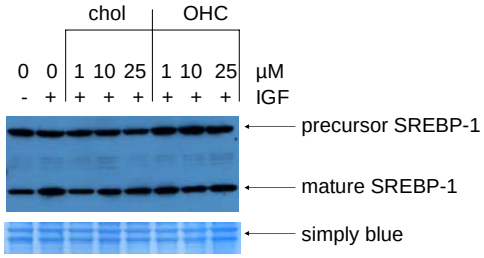


Figure 33: **Treatment with 25-OH cholesterol or cholesterol has little effect on IGF-1 induced increases in SREBP protein in SEB-1 cells.** SEB-1 cells were stimulated with IGF-1 and treated with either cholesterol or 25-OH cholesterol. Protein lysates were collected and a Western blot probing for SREBP-1 was performed.

3.3.7.3 Creation of a SEB-1 cell line that expresses a dominant-negative SREBP-1

SREBPs dimerize when they bind DNA. A dominant-negative SREBP has been created that contains a substitution in the DNA binding domain such that the molecule can still dimerize with the endogenous SREBP, but this dimer is unable to activate transcription because it can't bind DNA. Thus the dominant-negative protein will dimerize with the endogenous protein resulting in an ablation of SREBP DNA binding (Kim and Spiegelman 1996). As clonal variation in lipogenesis can be significant, a retroviral approach was used where many clones could be pooled into a population. SEB-1 cells were stably infected with the dominant-negative SREBP (Figure **34**), however, the resulting population, SEB-1-DNSREBP, had been passaged numerous times and showed significant contact inhibition in its growth. Additionally, lipogenesis performed on this cell line with IGF-1 stimulation did not show a difference between this line and the SEB-1 cells stably infected with the vector only (data not shown). Western blot of the SEB-1-DNSREBP cell line showed that this line possessed only a fraction of the dominant-negative protein compared to the endogenous protein. It has been shown that a ratio of 2:1 in favor of the dominant-negative protein is required to block SREBP activity (Kim and Spiegelman 1996). This level of DN protein observed is not sufficient to block SREBP activity.

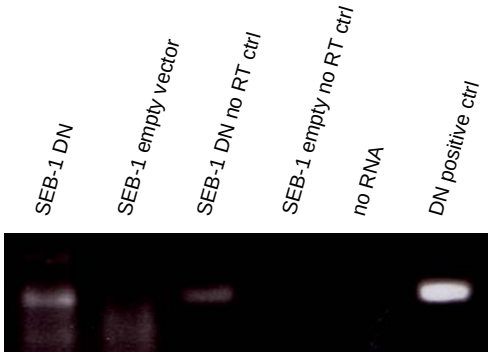


Figure 34: **RT-PCR of the SEB-1-DNSREBP RNA shows that the dominant-negative SEB-1 cells contain some RNA for the DN protein.** The second and fourth lanes show that SEB-1 cells transfected with the empty vector is negative for the dominant-negative protein. The presence of a band in lane 3 indicates that this transcript is integrated into the DNA, and that there is some DNA contamination in the RNA prep.

3.3.8 Analysis of downstream targets of SREBP

We next sought to determine if the increase in nuclear SREBP protein was accompanied by an increase in the mRNA levels of SREBP transcriptional targets. Following 14 hours of IGF-1 treatment, SEB-1 RNA was isolated and QPCR was performed to determine the relative amounts of fatty acid synthase (FAS) and low density lipoprotein receptor (LDLR), the transcription of both has been shown to be regulated by SREBPs (Streicher, Kotzka et al. 1996; Shimano, Horton et al. 1997; Kim, Sarraf et al. 1998). Here we show that IGF-1 increases both FAS and LDLR mRNA in SEB-1 sebocytes, however it is important to note that the addition of LY294002 blunts the induction of FAS, but is incapable of inhibiting the increase in LDLR mRNA (Figure 35).

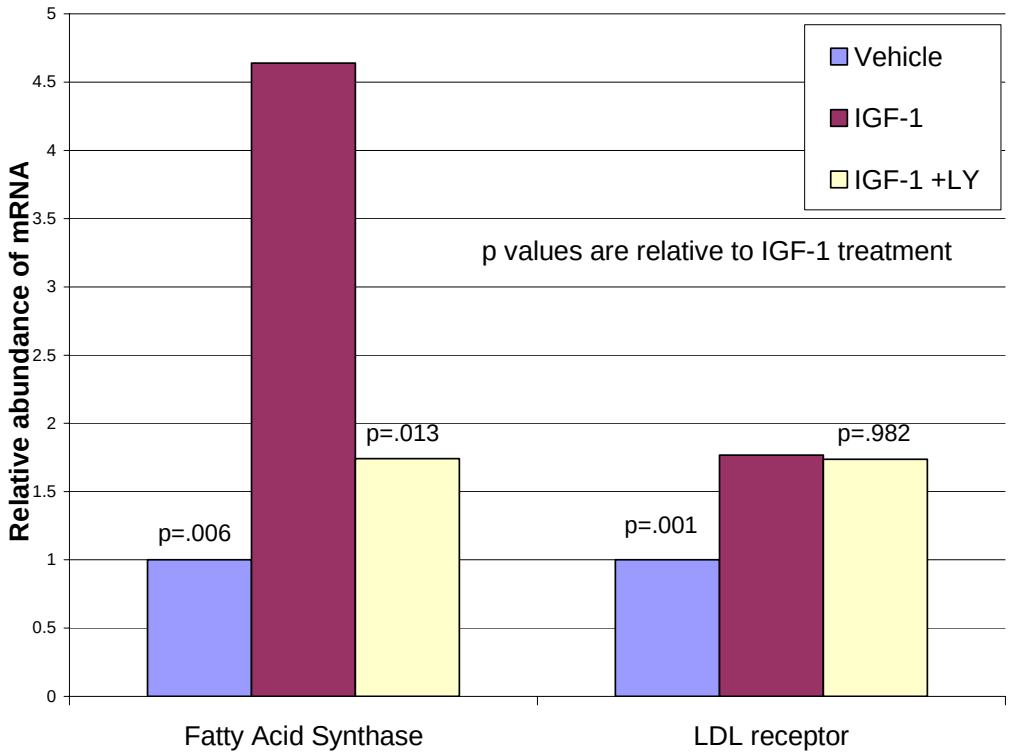


Figure 35: **Both FAS and LDLR mRNA are increased in response to IGF-1. This effect on LDLR is unaffected by LY294002.** IGF-1 induces a robust increase in FAS mRNA. Addition of LY294002 (which blocks SREBP induction) reduces this increase. On the other hand, LDLR mRNA is also increased in response to IGF-1; however, this is not affected by PI3-K inhibition.

3.4 Discussion

It is well established in the literature that SREBP mRNA and protein is increased in a variety of cell lines in response to a litany of growth factors. Previous work in our lab has shown that in the SEB-1 sebaceous model system, a physiological dose of insulin does not increase SREBP-1 protein, while IGF-1 does (Smith, Cong et al. 2006). There are conflicting reports as to which molecular pathway(s) are involved in carrying the

growth factor signal that eventually leads to induction of SREBPs. In this study, we sought to determine which molecular pathway(s) are involved in the induction of SREBP-1 protein by IGF-1 treatment in SEB-1 sebocytes, and futher, to determine which pathway(s) are important for the increase in lipogenesis observed when SEB-1 cells are stimulated with IGF-1. The motivation behind these studies is to gain a better understanding of the molecular pathways involved in lipogenesis, to allow subsequent development of drugs targeting key members of the lipogenic pathway to decrease sebum production. For the sake of completeness, all three arms of the MAPK pathway were examined (p38, SAPK/JNK, and p44/p42) in addition to the PI3-K pathway.

First, we found that neither the p38 nor the SAPK/JNK pathways are activated in SEB-1 cells in response to IGF-1 stimulation (Figures **23** and **24**). The fact that these pathways are not activated is not surprising, as they are both typically associated with cell stress, and it has been shown that IGF-1R activation can actually down-regulate both pathways (Galvan, Logvinova et al. 2003). Study of these pathways was undertaken because keratinocyte growth factor (KGF) activates SREBP protein and lipogenesis in the H292 pulmonary epithelial cancer cell line via the SAPK/JNK pathway, providing further evidence that SREBP activation is largely cell-type specific (Chang, Wang et al. 2005).

As expected, SEB-1 sebocytes stimulated with IGF-1 display a strong activation of the p44/p42 (ERK 1 and 2) MAPK pathway (Figure **25**) and the PI3-K pathway (Figure **26**). This MAPK and PI3-K activation is blocked by the traditional inhibitors PD98059 and LY294002 respectively. As activation of the SREBP proteins has been shown to increase transcription of several lipogenic genes (Rawson 2003), we

hypothesized that one or both pathways are responsible for the increase in SREBP and ultimately, the increase in lipogenesis we have observed previously with IGF treatment (Smith, Cong et al. 2006).

As far as SREBP activation is concerned, we found that inhibition of the p44/p42 MAPK pathway with PD98059 had no effect on SREBP-1a or SREBP-1c mRNA, the translation of SREBP-1 protein, or the processing of the protein into the mature form (Figure **27**). Numerous reports, particularly by groups using myocytes, had previously found the MAPK pathway to be linked to the SREBPs. Here we establish that the MAPK pathway is not tied to the SREBP pathway in SEB-1 sebocytes.

Interestingly, inhibition of the PI3-K pathway with LY294002 blocked transcription of SREBP-1a and 1c mRNA, translation of SREBP-1 protein, and also decreased the amount of the mature SREBP-1 protein found in the nucleus of IGF-1 stimulated SEB-1 cells. This clearly implicates the PI3-K/Akt pathway as the pathway by which IGF-1 mediates the increase in SREBP-1 activity in SEB-1 sebocytes. As an interesting aside, previous work done in our lab has shown that insulin can only increase lipogenesis to a limited extent in comparison to IGF-1. Here we show that 100 nM insulin does not cause phosphorylation of Akt (Figure **26**), further illustrating that many factors work in concert to increase lipogenesis.

The fact that the amount of mature SREBP-1 protein increases with IGF treatment indicates that, in addition to possible transcriptional/translation control, Akt activation also affects SREBP processing. Data presented in this paper supports the recent findings that Akt is involved in ER-to-Golgi transport of the SCAP/SREBP complex (Du, Kristiana et al. 2006). Though the authors were dealing with SREBP-2, they report that

they also found an increase in fatty acid synthase which is largely regulated by SREBP-1; therefore, these findings likely extend to SREBP-1.

To determine if an increase in nuclear SREBP-1 protein has an effect of on transcription of genes with SREs in their promoter in SEB-1 sebocytes, we performed QPCR on cells stimulated with IGF-1 assaying for FAS and LDLR mRNA. We found that FAS mRNA, the most important enzyme in fatty acid biosynthesis, is increased 4.5 fold when SEB-1 cells are treated with IGF-1, and this effect is significantly attenuated when cells are treated with IGF-1 and LY294002 simultaneously (Figure **35**). This expression pattern parallels that of SREBP protein expression we have described previously (Figure **28**). Additionally, we assayed for LDLR expression under the same treatment conditions, as the LDLR has been shown to be regulated by SREBPs and is important for cholesterol homeostasis (Streicher, Kotzka, Muller-Wieland, Siemeister, Munck, Avci and Krone 1996). We report a slight, yet statistically significant increase in LDLR mRNA in SEB-1 cells treated with IGF-1 (Figure **35**). Surprisingly, this increase was not attenuated with the addition of LY294002, from which we can conclude that SREBP-1 protein does not impact LDLR expression in SEB-1 sebocytes.

We put forth two hypotheses as to why LDLR expression is not regulated by the SREBP-1 in SEB-1 sebocytes. First, most of the work on SREBPs has been done in the liver, a tissue in which a significant portion of the cellular demand for cholesterol is satisfied by absorption through the LDLR. As such, the LDLR is crucial for proper liver function. We propose that, physiologically speaking, the LDLR is not as important in sebocytes as it is in hepatocytes. It has been shown that human sebocytes express the LDL receptor, though the degree of expression and the importance of exogenous

cholesterol in the sebocyte remain unknown (Smythe, Greenall and Kealey 1998). Furthermore, the extent to which SEB-1 sebocytes express the LDLR has not been studied.

Second, SREBP-2 is more involved in cholesterol metabolism than SREBP-1 (Rawson 2003). This was recently confirmed when Ringseis and coworkers showed that conjugated linoleic acids increase LDLR expression through SREBP-2, and that SREBP-1 was not an important mediator of this effect (Ringseis, Konig et al. 2006). In light of this, it stands to reason that the increase in SREBP-1 may not have a profound effect on cholesterol biosynthesis in SEB-1 cells, and further, SREBP-2 is most likely not induced by IGF-1 in SEB-1 sebocytes.

To fully validate the SREBP proteins as targets to reduce lipogenesis, we have attempted to inhibit SREBP processing with sterols, knock-down SREBP expression with siRNA, and block SREBP function with a dominant negative SREBP construct. All three methods were not successful, for unclear reasons.

Cholesterol and 25-OH cholesterol are potent regulators of SREBP activity, working presumably on SCAP to prevent release of SREBP from the ER. We treated SEB-1 cells with either cholesterol of 25-OH cholesterol and found only minor differences (if any), in the amount of mature SREBP-1 protein between cells treated with a sterol and IGF compared to cells treated with the sterol vehicle and IGF (Figure **33**). Given the small decrease in SREBP-1 protein with this treatment, and the fact that we are looking for production of cholesterol and other lipids; the possibility for non-specific feedback inhibition on lipogenesis using sterol treatments is too great to pursue further experiments with these particular inhibitors.

The use of siRNA is widely utilized to knock down specific genes. At the time of our first attempt to knock down SREBP-1, there were no published siRNA sequences that target SREBP-1 available. Qiagen has an algorithm to identify a few sequences to which siRNA could be targeted in the SREBP-1 gene, though there are other, most likely better ways to identify accessible sites (Pan and Clawson 2006). These siRNA molecules, (Figure **30**) had no effect on SREBP-1 protein. We later tried a siRNA sequence that was previously reported to successfully reduce SREBP-1 (Porstmann, Griffiths et al. 2005), but again no downregulation was observed (Figure **32**). For both sequences, it is possible that there was actually a decrease in mRNA transcript, since we only assayed for protein expression. However, for our purposes, if IGF-1 has effects at either/both the translational and at the protein processing stages, simple destruction of the mRNA transcript would not be a useful tool for our application.

Finally, we utilized a dominant-negative SREBP-1 construct generously provided to us by Bruce Spiegelman's lab at Harvard. This construct is the 403 amino acids at the N-terminus of the SREBP-1 protein (Kim and Spiegelman 1996). The endogenous mature SREBP-1 protein is 497 amino acids. It was reported (Kim, Spotts et al. 1995) that the 403 amino acids at the N-terminus form a "super potent" activator of transcription of genes with SREs, and it was further discovered that changing a single amino acid in the DNA binding domain will still allow the protein to dimerize with the endogenous SREBP protein, but will render the dimer unable to bind DNA to activate transcription, creating a dominant-negative.

We therefore used a retroviral construct to infected SEB-1 cells. The viral titer expressing the DN-SREBP sequence was low (approximately 30 cfu/mL compared to the

10^5-10^6 cfu/mL produced in the original paper). This can be attributed to the fact that the authors were infecting a cell line of mouse origin, and thus used the Bosc23 cell line which efficiently packages the virus for infection of mouse cells. In our case, the DN-SREBP had to be packaged in an amphotropic virus to infect human cells which may have had an impact on the viral titer. Nonetheless, we got about 50 colonies which were pooled into a population to measure lipogenesis. However, the low titer caused two problems: 1) each cell that was infected would only be infected with one copy of the virus, and 2) many cell divisions were required before the stable population could provide enough cells for characterization and downstream assays.

The end result was that the dominant-negative SREBP had no effect on IGF-1 induced lipogenesis in SEB-1 cells. Western blot confirms that there was insufficient translation of the DN protein to bind a significant amount of the endogenous SREBP, particularly when it was induced by IGF-1. Additionally, the dominant-negative cells were barely growing and exhibiting contact inhibition, whereas they normally grow until tightly packed on the plate. The cells infected with the empty vector were still growing normally, but we believe that the number of cell divisions and the high passage number of the dominant-negative cell line were the reasons for this altered growth. In the original paper describing the dominant-negative construct, the authors note that there was no altered growth (Kim and Spiegelman 1996). To repeat this experiment, the dominant-negative would need to be put into a vector that yields a significantly greater titer.

The most important endpoint for treatment of acne, and what differentiates these studies from other SREBP studies, is our focus on the endpoint of lipogenesis. Sebum, the lipid product of sebocytes, consists primarily of cholesterol, triglycerides, and wax

esters. Here we show that treatment with LY294002 blocks the robust induction of lipogenesis in response to IGF-1 treatment for all lipids assayed, while treatment with PD98059 has no effect on the IGF-1 induced lipogenesis in SEB-1 cells (Figure **29**). These data provide a rationale for investigation of members of the PI3-K pathway as drug targets for topical applications to decrease sebum production; the broad inhibition of PI3-K by LY294002 as an oral agent renders it undesirable as a systemic therapy for humans.

The PI3-K enzyme consists of a p110 and a p85 subunit, each of which has several isoforms. The p110α isoform is activated in response to insulin (Knight, Gonzalez et al. 2006). Research is currently underway to develop small molecule drugs to several p110 isoforms. As it has been recently learned that p110α is frequently mutated in human tumors, this class may come to the forefront and be useful as a topical application to reduce sebum production (Samuels, Wang et al. 2004).

In summary, we have shown that the PI3-K pathway is important for the IGF-1 induced increase of SREBP-1 protein, and more importantly, lipogenesis. Inhibition of this pathway completely blocks IGF-1 induced lipogenesis in SEB-1 sebocytes. As the p110α subunit of the PI3-K molecule has recently been shown to mediate the insulin signal, it is plausible that the small molecules designed to inhibit this form of the protein may be useful in reducing lipogenesis.

Chapter 4

15-Lipoxygenase 2 is not endogenously produced in SEB-1 cells

4.1 Introduction

Lipoxygenases are non-heme iron containing enzymes that catalyze the reaction that adds an oxygen molecule to a free fatty acid substrate creating a hydroxyl fatty acid. Humans have 5-lipoxygenases, 12-lipoxygenases, and 15-lipoxygenases. Of particular interest to dermatologists is the 15-lipoxygenase 2 (15-Lox 2), as this particular lipoxygenase has very limited tissue distribution and is found in the skin, particularly the sebaceous gland. The products of lipoxygenase molecules often have significant implications in signal transduction, as will be detailed later in this section (Brash 1999). 15-Lox 2 was cloned from the hair follicle and is differentiated from 15-Lox 1 by its tissue distribution and its preferred substrate. 15-Lox 1 is almost ubiquitously expressed, while 15-Lox 2 is expressed only in lung, prostate, cornea, and the skin—all epithelia or tissues having a major epithelial component. It is not expressed in heart, brain, placenta, liver, skeletal muscle, pancreas, kidney, spleen, thymus, ovary, small intestine, colon, retina, or iris (Brash, Boeglin and Chang 1997). As for substrate specificity, 15-Lox 1 prefers linoleic acid as its substrate, while 15-Lox 2 uses arachidonic acid as a substrate almost exclusively.

Arachidonic acid is a 20-carbon fatty acid that contains four double bonds beginning at the Ω-6 position. This molecule can also be called 5, 8, 11, 14-

eicosatetraenoic acid, and is often denoted as: 20:4-6. Arachidonic acid is released from the cell membrane by phospholipase A2. Following release from the membranes, arachidonic acid is oxygenased by one of four routes: the cyclooxygenase pathway (COX), the lipoxygenase pathway (LOX), p450 epoxygenase, and the isoprostane pathways (Chang, Schneider et al. 2005).

15-Lox 2 converts arachidonic acid into 15-hydroperoxyeicosatetraenoic acid (15-HPETE), which rapidly converts to the hydroxyl derivative 15-HETE (Figure **36**). The actions of 15-HETE are not completely understood, but it is known, for example, that 15-HETE can act as a peroxisome proliferators-activated receptor γ (PPARγ) agonist (Flores, Li, McHugh and Aneskievich 2005).

Taken from Voet and Voet 1995, *Biochemistry* 2nd edition, page 707, 1995.

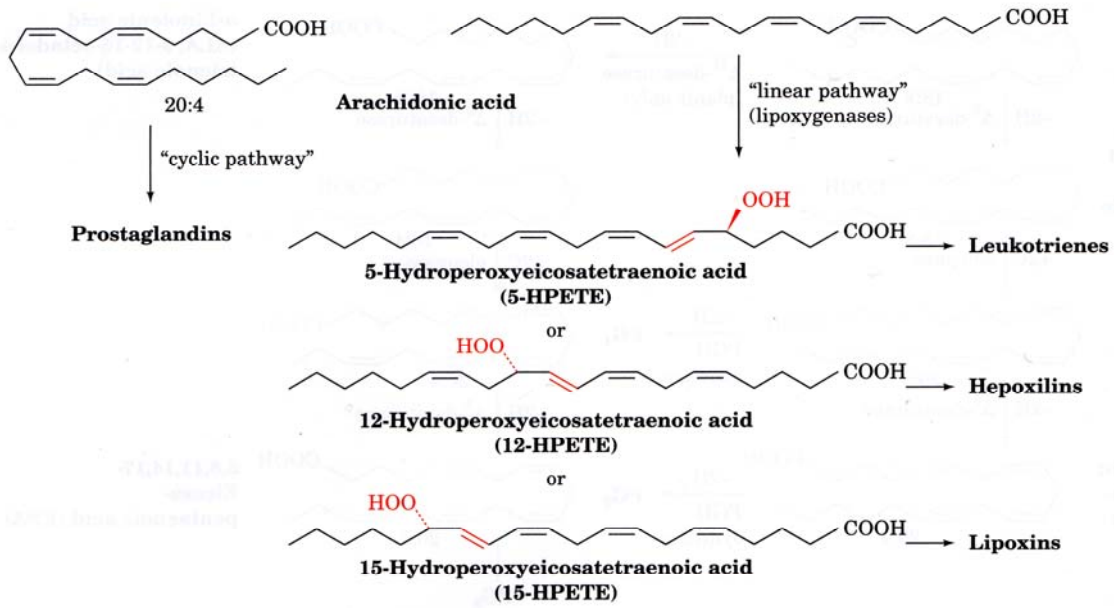


FIGURE 25-66 The cyclic and linear pathways of arachidonic acid metabolism.

Figure 36: **Arachidonic acid is metabolized by both the cyclic and non-cyclic pathways.** The cyclic pathway is catalyzed by cycloxygenase enzymes to yield prostaglandins, while lipoxygenases form various HPETE molecules in the linear pathway.

4.2 Rationale and hypothesis

The goal of this project was to determine if downregulation of 15-Lox 2 by an antisense oligonucleotide or ribozyme will result in a decrease in lipid production in sebocytes. In 1967, Strauss *et al* found that the non-specific lipoxygenase/cycloxygenase inhibitor 5:8:11:14-tetraynoic acid (ETYA) was able to suppress sebum production in all subjects and to improve acne in a small number of cases when administered orally to humans (Strauss, Pochi and Whitman 1967). Additionally, the fact that 15-HETE can activate PPARγ, a known regulator of sebaceous gland lipid production led us to formulate the

following hypothesis: The observed decreased lipogenesis in humans in response to topical ETYA application is caused by antagonism of 15-Lox 2, resulting in a decrease of 15-HETE. 15-HETE is a pro-lipogenic lipid, perhaps acting via activation of PPARγ. Additionally, the fact that 15-Lox 2 has been shown to have limited tissue distribution also made this enzyme a more attractive target for our studies.

4.3 Materials and methods

4.3.1 Western blot

Cells were lysed and protein was collected using a lysis buffer comprised of 50 mM Tris pH 7.4, 30 mM sodium deoxycholate, 150 mM sodium chloride, 1 mM EGTA, and 1% IGEPAL. The protease inhibitors PMSF (1 mM), aprotinin (1µg/mL), leupeptin (1µg/mL), and pepstatin (1µg/mL) were also added. Protein lysate was run on a polyacrylamide gel, transferred to a nylon membrane, and probed for 15-Lox 2 using a primary antibody from Oxford Biomedical.

4.3.2 RT-PCR

RNA was isolated from SEB-1 cells using Trizol Reagent (Invitrogen) following the manufacturer's protocol. RNA was diluted to 200 ng/µL. Reverse transcription was performed using the Sensiscript RT kit (Qiagen) using the reverse primer 5'ttcaatgccgatgcctgtgt (Claw 2262). PCR was then performed on the RT product using

standard molecular protocols and the following primers: 5'actacctcccaagaacttcccc (forward-Claw 2263) and Claw 2262 as the reverse primer. The PCR product was then run on a 1% agarose gel.

4.3.3 Lipogenesis assay

Lipogenesis was performed as described (Smith, Cong et al. 2006) In some cases, plates were exposed to the phosphorimager rather than being cut into pieces and quantified by the scintillation counter. A 48 hour exposure to the phosphorimager was required to detect a signal for all lipids with the exception of wax esters of which an insufficient amount is produced by SEB-1 cells for detection by this method. Autoradiographic [^{14}C] micro-scales were purchased from Amersham, each strip containing 2 nCi of radioactivity. This strip is ^{14}C incorporated into rat brain tissue that is fixed in paraffin. The strip was cut into 9 pieces and mounted asymmetrically on a microscope slide. As each TLC plate was exposed to the phosphorimager, this reference slide was also included to provide a means to normalize data between experiments. The R^2 value for this normalization was found to be 0.9949 when comparing data collected from the same plates with different exposure times to the phosphorimager, multiplied by a correction factor as determined by the signal strength from the normalization slide.

4.3.4 Transient transfection/cell sorting

SEB-1 cells were transfected using Effectene (Qiagen) following manufacturer's instructions. Transient transfection efficiency was determined to be approximately 20%.

SEB-1 cells were transfected with pIRES-hrGFP-Lox2 or the empty vector. GFP positive cells were sorted using a MoFlo Cell Sorter (Cytomation) in the Molecular Core Facility at the Penn State College of Medicine.

4.3.5 Quantification of 15-HETE by HPLC and ELISA

Two methods were used to quantify 15-HETE production. Samples were extracted with methanol:dichloromethane (2:1). The organic phase was dried under N2 gas, and the solid was resuspended in 50 µL methanol. 950 µL water was added and the sample was purified with a reverse-phase sep-pak C18 column (Waters) according to manufacturer's instructions. Lipids were eluted in 1 mL methanol and dried down. Reverse phase HPLC analysis was carried out on a Beckman System Gold with a Luna 5µm C18 column using a solvent system of methanol:water:acetic acid (80:20:0.01) at a flow rate of 1.1 ml/min. The retention time for 15-HETE is 14 minutes.

The Correlate-EIA 15(S)-HETE Enzyme Immunoassay kit was used for an absolute quantification of 15-HETE. Lipids were extracted as described (Kempen, Yang et al. 2001). Briefly, cells were trypsinzed and lipids extracted using 1:1 hexane:ethyl acetate in the presence of citric acid and butylated-hydroxytoluene to prevent free radical peroxidation. Samples were vortexed, centrifuged, and the upper organic layer was removed. The lower aqueous layer was extracted twice more, and the extracted organic

layers were pooled and dried under a stream of N_2 gas. The dried sample was then resupsended in assay buffer from the Correlate-EIA kit, and applied to the microplate according to the manufacturer's instructions.

4.4 Results and discussion

This project began in 2001, four years after the discovery and cloning of 15-Lox 2. The SEB-1 sebaceous cell model was to be utilized to study the effects of 15-Lox 2 inhibition on lipid production. The summer before I joined the lab, approximately 2/3 of 15-Lox 2 was cloned from the SEB-1 cells, though it was, "extremely difficult and had to be done in many pieces" (personal communication with M. Aros).

To validate the SEB-1 model as appropriate for studying 15-Lox 2 inhibition, Western blot was performed to ensure that the cells expressed the 15-Lox 2 protein. The only commercially available antibody lacked a positive control, a blocking peptide, and had what would later be discovered to be a non-specific band at 70 kDa, very close to the 76kDA 15-Lox 2 protein. Western blot analyses using SEB-1 lysate led to the erroneous assumption that the SEB-1 cells did, in fact, produce 15-Lox 2 protein (Figure 37).

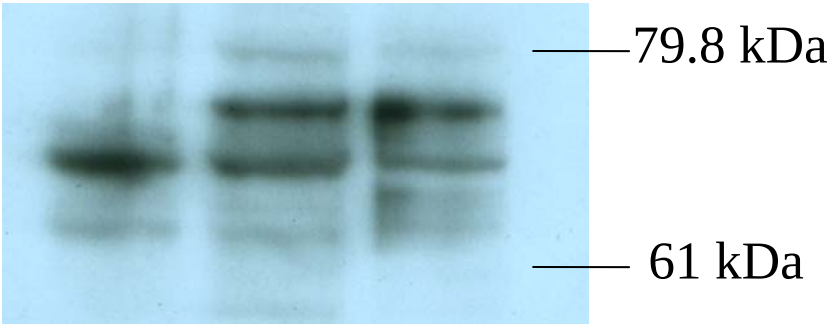


Figure 37: **Western blot of SEB-1 lysate probing for 15-Lox 2.** The lane on the far left is HaCat (immortalized human keratinocytes) lysate, the middle lane is SEB-1 lysate, and the far right lane is SEB-2 lysate. It was later learned that both of these bands are non-specific and that the neither the SEB-1, the SEB-2, nor the HaCat cells possess 15-Lox 2 protein.

Under this assumption, we next wanted to determine if a non-specific pharmacological lipoxygenase inhibitor could decrease lipogenesis in SEB-1 cells. Nordihydroguaiaretic acid (NDGA) and morin are both lipoxygenase inhibitors that have an effect on all known lipoxygenases. SEB-1 cells were treated with morin or NDGA and a ^{14}C acetate incorporation assay was performed as a measure of lipogenesis. NDGA significantly reduced the rate of lipogenesis of cholesterol, cholesterol oleate, and squalene, while treatment with morin has no effect (Figure 38). This reduction in lipogenesis could not be attributed wholly to 15-Lox 2, as NDGA also inhibits 15-Lox 1, 5-Lox, and 12-Lox.

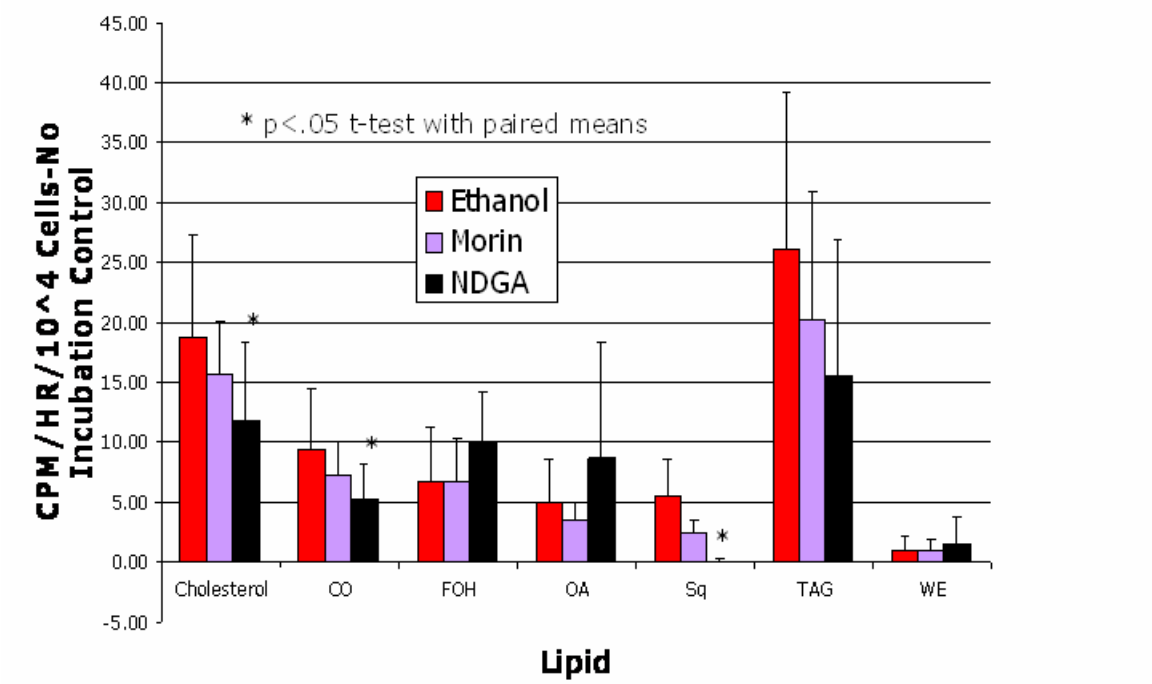


Figure **38**: **NDGA reduced the rate of ^{14}C acetate incorporation into cholesterol, cholesterol oleate, and squalene in SEB-1 cells, while morin did not affect rate of lipogenesis for any lipid species assayed.**

We next sought to determine how much 15-HETE was being produced by the SEB-1 cells, and if NDGA treatment was able to decrease the 15-HETE production. Reverse-phase high performance liquid chromatography (HPLC) was utilized to determine how much 15-HETE was produced. It was from this study that we learned that there was no detectable 15-HETE being produced by the SEB-1 cells, even when the cells were pre-treated with arachidonic acid (data not shown).

At this point, a paper was published using a GFP-15-Lox 2 construct (Tang, Bhatia et al. 2002). Dr. Tang at the MD Anderson Cancer Center was kind enough to send us this construct. The construct was transiently transfected into SEB-1 cells and

used as a positive control for a western blot. This experiment allowed us to conclude that the SEB-1 sebocytes clearly do not produce endogenous 15-Lox 2 protein (Figure 39).

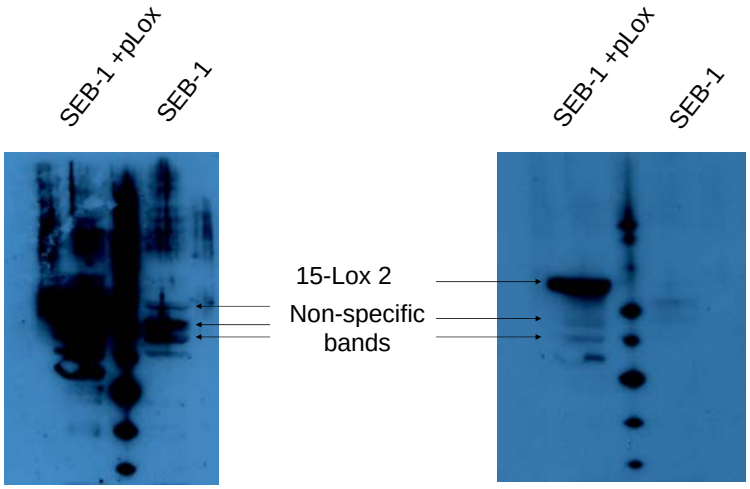


Figure 39: **SEB-1 cells do not express endogenous 15-Lox 2 protein at detectable levels.** Western blot shows that SEB-1 cells transiently transfected with pLox produce a protein slightly larger than the non-specific bands previously thought to be 15-Lox 2. The two blots shown in this figure are of the same blot. The longer exposure on the left is shown to demonstrate that there was, in fact protein loaded in the SEB-1 lane. The blot on the right shows a strong band in the lane containing SEB-1 cells transfected with pLox that represents 15-Lox 2. This band is absent in the SEB-1 lane.

To study the effects of 15-Lox 2 in the SEB-1 sebocytes, we transiently transfected the GFP-15-Lox 2 vector into SEB-1 cells. After testing a panel of lipid based transfection methods, it was determined that Effectene (Qiagen) had a transfection efficiency of 20% which, although poor, was the highest by a large margin among the panel tested. We transiently transfected SEB-1 cells with GFP-15-Lox 2 or the empty vector and sorted them for GFP positive cells. The cells were then incubated with ^{14}C acetate and the lipogenesis assay was performed. This method proved to be extremely time consuming and did not give us enough cells to perform the assay, so it was

determined that a cell line stably expressing 15-Lox 2 would have to be created. Additionally, we had transiently transfected SEB-1 cells with 15-Lox 2 and assayed for 15-HETE production. With pre-treatment of arachidonic acid, we found using a reverse ELISA from Assay Designs that SEB-1 cells with 15-Lox 2 transiently transfect produced 1.49 ng of 15-HETE/5x10^6 cells, while the SEB-1 cells transiently transfected with the empty vector produced 0.284 ng of 15-HETE/ 5x10^6 cells. Comparatively, untransfected SEB-1 cells produced 0.396 ng of 15-HETE/5x10^6 cells as shown in (Table 7).

Table 7: **Summary of 15-HETE production in SEB-1 cells transiently transfected with 15-Lox 2.**

Cells assayed	**ng 15-HETE/5x10^6 cells**
SEB-1 transiently transfected with 15-Lox 2	1.49
SEB-1 transiently transfected with empty vector	0.28
Untransfected SEB-1 cells	0.40

The 15-Lox 2 was subcloned from the GFP construct into a vector without the GFP tag (which may interfere with functionality) and renamed pLox. SEB-1 cells were transfected with pLox using Effectene and then selected in G418 for a number of weeks. Individual colonies were trypsinized using cloning rings and put into individual plates. In all over 100 colonies were picked and plated. Amazingly, by the time these clones had grown enough to be tested, all were negative for 15-Lox 2 when assayed by Western blot or RT-PCR.

At this point, we found an article where the authors state that, “all prostate cancer cells examined lost expression of 15-Lox 2, even SV-40 immortalized but non-

transformed prostate epithelial cells lose 15-Lox 2 expression" (Tang et al 2002). Additionally, another paper by the same group reported that they had successfully transfected 15-Lox 2 into LNCap prostate cancer cells, but expression of 15-Lox 2 was lost by passage 3 (Bhatia, Maldonado et al. 2003).

In light of the papers that were published describing growth arrest in cells stably transfected with 15-Lox 2, our own experiences confirming this in the SEB-1 cells, and the realization that there were no immortalized cell lines that produce 15-Lox 2 protein, we decided not to pursue this project any further.

Since then, it has been shown that 15-Lox 2 mRNA and protein levels increase in serially passaged normal human prostate cells, which precedes replicative senescence. As mentioned earlier, stable transfection of 15-Lox 2 into primary prostate cancer cells results in a passage-related senescence phenotype, while infection of normal human prostate cells with a retrovirus containing 15-Lox 2 induces partial cell cycle arrest and senescence (Bhatia, Tang et al. 2005). In light of these findings, the authors have labeled 15-Lox 2 as a tumor suppressor gene.

At this point in time, we do not have a suitable model system to study 15-Lox 2 and the effects of 15-HETE on sebaceous lipid metabolism. 15-Lox 2 may still be a viable target for acne, but lacking the appropriate model, this project cannot move forward. Because primary sebocytes are nearly impossible to grow in culture, a determination of the prospects of 15-Lox 2 as a target for acne drugs cannot be pursued until an appropriate model becomes available.

Chapter 5

Global Discussion

5.1 Introduction

As increased sebum production (lipogenesis) is a primary cause of acne, it can be reasoned that if lipogenesis could be decreased by administration of pharmacological compounds, a viable treatment for acne would be born. Isotretinoin, currently the most effective drug for treatment of acne, significantly decreases sebaceous gland activity (Trivedi, Cong et al. 2006). Unfortunately, this drug has a very undesirable side-effect profile, and a search for alternate drugs is warranted.

In this thesis, we have shown that 1) IGF-1 stimulates lipogenesis in SEB-1 sebocytes, 2) The increased lipogenesis in response to IGF-1 occurs concomitant with an increase in SREBP-1 protein, and 3) The PI3-K molecular signaling pathway transduces the IGF-1 signal resulting in an increase in lipogenesis and, further, the PI3-K pathway is essential for IGF-1 induced lipogenesis.

The molecular pathways delineated in this thesis highlight potential drug targets that can be exploited by specific compounds to decrease lipogenesis in the sebaceous gland. While the pathogenesis of many diseases may be attributable to PI3-K, acne may be easier to treat with small molecular inhibitors due to the accessibility of the sebaceous gland by topical application. Additionally, because the sebaceous gland has no known function, inhibition of sebaceous gland differentiation should have few (if any) side

effects. In this final chapter, I will evaluate each of the potentially druggable targets that have arisen from these studies and discuss the potential of each.

5.2 Druggable targets in the PI3-K pathway

There are 15 kinases in the PI3-K family, with distinct expression patterns, regulation, and substrate specificity (Katso, Okkenhaug et al. 2001). As the PI3-K pathway is involved in cell metabolism, adhesion, growth, and proliferation (Cantley 2002), it is unreasonable to disrupt the pathway with a broad inhibitor. The PI3-K inhibitor used in these studies, LY294002, was intended to be a research tool rather than a therapeutic molecule (Vlahos, Matter et al. 1994), and, as such, is specific for PI3-K, but acts very broadly within the PI3-K class (Knight and Shokat 2005).

The fact that the PI3-K pathway is one of the most frequently mutated pathways in cancer (Engelman, Luo and Cantley 2006) has led to a significant amount of effort devoted to both understanding the role of each isoform and developing isoform specific small molecule inhibitors. Of the 15 members of the PI3-K pathway, the three that are of interest to us for insulin signaling are classified as class Ia PI3-Kinases. The class Ia PI3-Ks are heterodimers that consist of a regulatory p85 subunit, and the catalytic p110 subunit (Engelman, Luo et al. 2006). This family contains those that are activated by tyrosine kinases, and includes p110α, p110β, and p110δ (Engelman, Luo et al. 2006).

Due to their importance in many deadly (and lucrative) diseases, there have been several reports of specific inhibitors for individual isoforms of the class Ia PI3-Ks (Sadhu, Masinovsky et al. 2005; Jackson, Schoenwaelder et al. 2005). Perhaps most

relevant for our study, Knight and co-workers used a panel of small molecule PI3-K inhibitors that are in preclinical development which exhibit a specificity for each isotype of PI3-K to determine which isotype(s) were important for insulin signaling (Knight, Gonzalez et al. 2006). They found that the compounds targeting p110α block the effects of insulin *in vivo*, while p110β inhibitors have no effect. Thus, PI3-Kα may be a viable target for a topical application to treat acne.

The studies described in this thesis illustrate the importance of the PI3-K pathway for sebaceous gland lipid production and put the acne research field in a position to benefit from the multiple, potent PI3-K compounds being created to inhibit individual PI3-K isoforms to treat other ailments.

5.3 Druggable targets in the SREBP pathway

A positive correlation between lipogenesis and SREBP protein has been shown in sebocytes in this thesis; this relationship has also been established in other models (Chang, Wang et al. 2005; Porstmann, Griffiths et al. 2005). In the following sections, I will discuss the potential targets to control SREBP expression at three different sites: release from the endoplasmic reticulum, cleavage in the golgi, and finally targeted disruption of the SREBP mRNA.

5.3.1 SCAP

Another attractive target to disrupt the lipogenesis pathway is the SREBP-cleavage-activating protein (SCAP). SREBPs exist in the endoplasmic reticulum bound to SCAP, and SCAP is bound to Insig (Goldstein, DeBose-Boyd et al. 2006). When cellular sterols are low, SCAP undergoes a conformational change, causing it to dissociate from Insig. SCAP and the SREBP proteins are then transported to the golgi via COP II vesicles for processing (Figure **11**).

Until recently, both 25-OH cholesterol and cholesterol were believed to be inhibitors of SCAP, with a proposed mechanism of action that each bound to the sterol sensing domain of SCAP, thus retaining SREBPs in the endoplasmic reticulum. However, Adams and co-workers showed that 25-OH cholesterol does not, in fact, interact with SCAP (Adams, Reitz et al. 2004). It has since been proposed that there is a 25-OH cholesterol sensor in the cell that is tied to the SREBPs by unknown mechanisms. It has been confirmed that cholesterol physically interacts with and inhibits SCAP as previously believed (Adams, Reitz et al. 2004). This information is a setback for a potential topical acne treatment, as 25-OH cholesterol can passively enter cells and, as such, would be easier to administer than cholesterol which needs to be complexed with a carrier molecule to cross the plasma membrane (Brown, Sun et al. 2002).

5.3.2 S1P/S2P

Perhaps the most promising targets in this pathway are the two enzymes located in the golgi that cleave the SREBP to its active transcription factor, site-1 protease (S1P),

and site-2 protease (S2P). Once transported to the golgi, SREBPs are cleaved by S1P and S2P to form the active transcription factor which then translocates to the nucleus (Figure **12**-Goldstein, DeBose-Boyd et al. 2006).

S1P, also called subtilisin kexin isozyme-1, is a serine protease (Basak, Stewart et al. 2004). This enzyme is fairly well understood and will be discussed in detail here. On the other hand, little is known about S2P aside from the fact that it is an extraordinarily hydrophobic zinc metalloprotease that cleaves SREBPs (Ye, Dave et al. 2000). Because of this, S1P will be the focus of this section, though future studies on S2P may yield data that validates it as a target.

Evidence of the importance of SREBP processing by S1P was first shown by Debose-Boyd and coworkers who found that when S1P is artificially relocated from the golgi to the endoplasmic reticulum, SREBP transport was no longer necessary for processing (DeBose-Boyd, Brown et al. 1999). The non-specific serine protease inhibitor, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) has been shown to be a potent inhibitor of S1P with an IC_{50} in the 200-800nM range (Basak, Stewart et al. 2004). More importantly, AEBSF successfully inhibits nuclear accumulation of SREBP protein (Okada, Haze et al. 2003). Modifications have been made to AEBSF by Pullikotil *et al.* to make a panel of molecules more specific for S1P which can serve as a prototype for a specific S1P inhibitor (Pullikotil, Vincent et al. 2004).

The biggest obstacle to using S1P inhibitors to reduce lipid production is the potency at which this compound would need to act. Using an S1P inducible promoter, it has been demonstrated that inhibition of S1P results in a decrease in lipogenesis, though nearly a 100% reduction in S1P is necessary to obtain these results (Yang, Goldstein et

al. 2001). Even if a small molecule with the potency to effectively eliminate S1P could be produced, there would be potential safety issues, as homozygous germ-line disruptions of S1P are embryonic lethal in mice (Yang, Goldstein et al. 2001).

5.3.3 Targeted disruption of the SREBPs

There is also the possibility of using siRNA/antisense oligonucleotide/ribozyme targeted to SREBPs to ultimately decrease lipid production. There has been no clinicial or pre-clinical work published on this, largely due to the delicate nature of cholesterol homeostasis in the body.

However, as the sebaceous glands have no known function, it may be possible to topically apply one of these therapies to decrease skin lipid production. The fact that cells need cholesterol in their membranes could be a potential problem with this study. Another obstacle associated with this type of experiment is that the dermatology field would not be able to follow the lead of the cholesterol-lowering drug discovery field, as those interested in lowering serum cholesterol would not try to directly target the SREBPs.

5.4 The Forkhead BoxO transcription factors

An additional target that has emerged recently, which is closely related to this work, is the family of forkhead transcription factors (FOX). Specifically, FOX class O (FOXO) has been shown to be phosphoryalted in response to activation of the insulin

receptor. FOXO exists in the nucleus until it is phosphorylated, then it is rapidly shuttled into the cytoplasm (Brunet, Bonni et al. 1999). FOXO proteins have been shown to work by sequestering other transcription factors, inducing the expression of repressor proteins, and by recruiting co-repressors to various promoters (Zhang, Patil et al. 2006). It has recently been shown that expression of a mutant FOXO that cannot be excluded from the nucleus results in a significant decrease in SREBP-1 expression and lipogenesis in both a transgenic mouse liver using a liver-specific promoter and in hepatocytes grown in culture (Zhang, Patil et al. 2006). Additionally, FOXO has been shown to antagonize PPARγ (Dowell, Otto et al. 2003), and constitutive activation of FOXO prevents differentiation of preadipocytes (Nakae, Kitamura et al. 2003) and myoblasts (Hribal, Nakae et al. 2003). It is quite possible that FOXO is also involved in sebocyte differentiation. There are no FOXO inhibitors, though a FOX class M1 inhibitor has been recently described (Radhakrishnan, Bhat et al. 2006). FOXO represents another potential target for the treatment of acne, though a definitive link and a better understanding of the interactions between FOXO and SREBP-1 is needed.

5.21 Conclusion

In conclusion, the SREBPs are important regulators of sebocyte differentiation. There are likely factors other than IGF-1 that regulate their expression in the sebaceous gland, but the work described here certainly warrants further investigation in the SREBPs and their processing enzymes as druggable targets to decrease skin lipid production. Our

working model of the importance and interaction of SREBPs in sebaceous gland lipid metabolism is show below (Figure **40**).

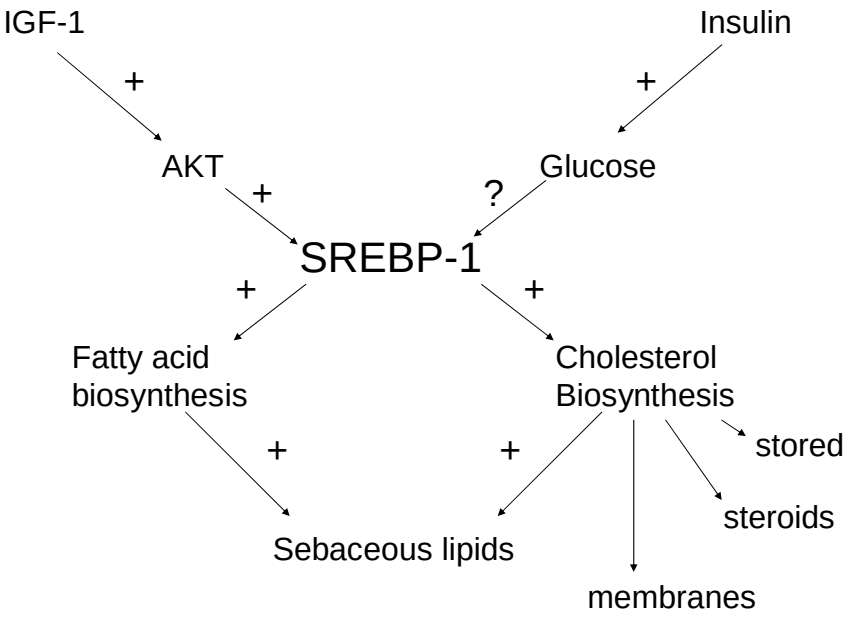


Figure **40**: **Model by which SREBPs are primary regulators of sebaceous gland lipid production.** We have shown that IGF-1 increases the phosphorylation of AKT. Additionally, phosphorylation of AKT is required for the both the IGF-1 induced increase in lipogenesis and also for the increase in nuclear SREBP-1 protein. SREBPs have been shown to regulate transcription of several enzymes in both the fatty acid biosynthesis and cholesterol biosynthesis pathways. We hypothesize that inhibition of SREBP activation will result in decreased sebaceous gland lipogenesis. Insulin does not increase SREBPs directly, though it does increase cellular glucose uptake. Glucose has also been reported to regulate SREBPs in liver tissue (Foufelle and Ferre 2002), but has yet to be investigated in the sebaceous gland.

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ABSTRACTS AND PUBLICATIONS

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