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Regulation of 7-Dehydrocholesterol Reductase by Vitamin D3

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REGULATION OF 7-DEHYDROCHOLESTEROL REDUCTASE BY VITAMIN D_3

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
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By
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ABSTRACT OF DISSERTATION

Regulation of 7-Dehydrocholesterol Reductase by Vitamin D_3

7-Dehydrocholesterol (7-DHC) is the substrate of 7-dehydrocholesterol reductase (DHCR7) in the cholesterol synthesis pathway. Keratinocytes in human skin possess the enzymes necessary for cholesterol synthesis but are also responsible for vitamin D_3 synthesis from 7-DHC by exposure to UVB irradiation. It has been well established that DHCR7 is regulated by the SREBP pathway in the regulation of cholesterol synthesis, but little is known about the regulation of DHCR7 by the vitamin D pathway. In this study, the regulation of DHCR7 activity by vitamin D was explored. Treatment of adult human epidermal keratinocyte (HEKa) cells with vitamin D_3 resulted in a rapid decrease in DHCR7 activity which was not due to changes in the amount of enzyme present. This suppression of activity was observed only in HEKa cells, a primary cell line cultured from normal human skin, and not in an immortalized skin cell line (HaCaT cells) nor in two liver-derived hepatoma cell lines. Because vitamin D_3 treatment of HEKa cells did not change the content of lanosterol nor 7-DHC, these results suggest that vitamin D_3 rapidly down-regulates the entire cholesterolgenesis pathway, presumably at a very early step in the pathway. 25-Hydroxyvitamin D_3, the first metabolite and circulating form of vitamin D_3, had a lesser effect on DHCR7 activity, while 1,25-dihydroxyvitamin D_3, the activated form of the vitamin, had no effect on DHCR7, indicating that the vitamin D receptor is not involved. The decrease in DHCR7 activity was due neither to the dephosphorylation of the enzyme, an established mechanism of inactivation, nor to direct inhibition by vitamin D_3. Vitamin D_3 markedly inhibited proliferation and induced differentiation of HEKa cells, suggesting a possible role for hedgehog signaling in the decrease in DHCR7 activity.

KEYWORDS: Cholesterol, 7-Dehydrocholesterol reductase, Vitamin D_3, HEKa, Hedgehog signaling

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To Wenlong and Lucy

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1.1.1 Cholesterol function

Cholesterol is an essential lipid in many eukaryotic organisms (Burger, Gimpl et al. 2000). Mammalian cells require cholesterol for membrane biogenesis, cell growth, and synthesis of steroid hormones and bile acids (Liscum and Munn 1999).

Cholesterol is a key component of biological membranes. It is nonrandomly distributed between cellular organelles, subdomains of membranes, and leaflets of the membrane bilayer. In eukaryotic cells, about 65%-90% of cellular free cholesterol may reside in the plasma membrane where its average concentration is about 200-300 μg/mg protein. Cholesterol influences many of the biophysical properties of membranes such as fluidity and permeability. It can modulate membrane receptor function by either binding directly to the protein thereby altering, for example, the conformation of the protein, or influencing the receptor indirectly by changing the biophysical properties of the membrane lipid bilayer (Burger, Gimpl et al. 2000).

As cholesterol is present in the lipoprotein membranes of the nucleus, mitochondria, and microsomes, as well as the plasma membrane, it is not surprising that a supply of cholesterol would be required for the membrane proliferation that must accompany cell growth(Siperstein 1984). This was first supported by the observation that 25-hydroxy- or 20-hydroxycholesterol, which

inhibits HMG-CoA reductase, the rate limiting enzyme in cholesterol synthesis, blocked the growth and proliferation of mouse L cells (Chen, Kandutsch et al. 1974). Further exploration suggested that cholesterol is required for cell growth during the early G1 phase of the cell cycle (Quesney-Huneeus, Galick et al. 1983) and loss of cholesterol feedback control is observed in premalignant and malignant cells (Siperstein 1984).

Cholesterol is the precursor molecule in the synthetic pathways of steroid hormones and bile acids. The rate-limiting step of steroid hormone biosynthesis occurs in mitochondria where free cholesterol (from the hydrolysis of cholesterol esters) is converted to pregnenolone by cholesterol side-chain cleavage enzyme (P450scc). Further metabolism of pregnenolone leads to the steroidogenesis of progestogens, mineralocorticoids, glucocorticoids, androgens, and estrogens (Hanukoglu 1992). In the liver, cholesterol is converted to bile acids via cytochrome P450-mediated oxidation. In the human and rat, the primary bile acids are cholic acid and chenodeoxycholic acid (Andersson, Davis et al. 1989) which are secreted into the lumen of the intestine to form deoxycholic acid and lithocholic acid, respectively, functioning as emulsifiers to facilitate the absorption of dietary fat and fat-soluble vitamins.

1.1.2 Cholesterol homeostasis

Hypercholesterolaemia is one of the major causes of atherosclerosis (Bhatnagar, Soran et al. 2008). To maintain cholesterol homeostasis in mammals, three separate metabolic pathways have developed and are coordinately regulated.

Two pathways function in the supply of cholesterol to cells. The first one is the endogenous biosynthetic pathway in which cholesterol is synthesized from acetate precursors. The second one is the exogenous pathway in which dietary cholesterol is absorbed by the intestine. The liver responds to the dietary cholesterol by decreasing hepatic cholesterol synthesis (Lu, Lee et al. 2001). A third pathway functions in the catabolism of cholesterol and involves the conversion of the molecule into bile acids (Andersson, Davis et al. 1989).

1.1.2.1 Cholesterol biosynthesis, uptake, storage and metabolism

The endoplasmic reticulum (ER) is the site of cholesterol biosynthesis (Maxfield and Wustner 2002). Synthesis (Fig 1.1) starts with one molecule of acetyl CoA and one molecule of acetoacetyl CoA, which are hydrated by 3-hydroxy-3-methyglutaryl CoA (HMG-CoA) synthase to form HMG-CoA. This molecule is then reduced to mevalonate by HMG-CoA reductase, the rate-limiting enzyme in the cholesterologenic pathway (Goldstein and Brown 1990). Mevalonate is then converted into isopentenyl pyrophosphate. Squalene, which is composed of six isopentenyl pyrophosphate units, is cyclized into lanosterol by the action of squalene monooxygenase. Finally, lanosterol is converted into cholesterol through a 19-step process (Vance and Van den Bosch 2000). Notably, 7-dehydrocholesterol reductase catalyzes the last step in cholesterol synthesis in mammals and ergosterol synthesis in fission yeast (Espenshade and Hughes 2007).

Dietary sources of cholesterol from egg yolks and cheese, etc. are available for use by cells through the low-density lipoprotein (LDL) pathway (Goldstein and Brown 1977). Free cholesterol in the diet is solubilized into micelles formed from bile acids and then absorbed into enterocytes. Within enterocytes, cholesterol is esterified and packaged into chylomicrons which are subsequently secreted into the circulation. The cholesterol-carrying lipoprotein particles in the blood are then delivered to the liver by LDL receptor-mediated internalization (Lee, Gordon et al. 2001). It consists of an ordered sequence of events in which LDL is first bound to a high-affinity receptor (LDL receptor) on the cell surface, and is then internalized by endocytosis and delivered to lysosomes where the cholesteryl esters are hydrolyzed to free cholesterol for cell use.

Adipose tissue is the major site of cholesterol storage. Most of the cholesterol in fat tissue is localized in the central oil droplet of the fat-storing adipocyte (Angel and Farkas 1974).

Four enzymes are critical to bile acid biosynthesis: CYP7A1, CYP7B1, CYP27 and CYP8B. The conversion of cholesterol to the primary bile acids, cholic acid and chenodeoxycholic acid (CDCA) occurs via either the classic or mitochondrial pathway. In the classic pathway, the first and rate-limiting step is the addition of a hydroxyl group on position 7 of the steroid nucleus by the enzyme cholesterol 7α-hydroxylase (CYP7A1 gene product). This reaction is followed by over a dozen reactions to form the primary bile acids. In the mitochondrial pathway, the side-chain of cholesterol is firstly hydroxylated by the mitochondrial CYP27 gene product to generate substrate for the oxysterol 7α-hydroxylase (CYP7B1 gene

product). This pathway preferentially produces CDCA, whereas the classic pathway produces both cholic acid and CDCA (Repa and Mangelsdorf 2000).

1.1.2.2 Regulation of cholesterol homeostasis

Cholesterol homeostasis is predominantly achieved through repression of transcription of genes that govern the synthesis of cholesterol and its receptor-mediated uptake from plasma lipoproteins (Goldstein and Brown 1990). Mammalian cells lack the capacity to directly degrade the four-membered ring structure of cholesterol; thus, the bile acid pathway represents the major mechanism for disposal of cholesterol from the body (Andersson, Davis et al. 1989).

The regulation of the cholesterol biosynthetic pathway is of particular importance because cholesterol must be supplied for many cellular functions. Failure in control of the pathway leads to solid crystal formation within cells or deposition of cholesterol in arteries, initiating atherosclerosis (Brown and Goldstein 1997). A finely tuned end-product feedback suppression mechanism is developed to govern cholesterol biosynthesis. This is established by the finding that diets rich in cholesterol decreased cholesterol de novo synthesis. Several enzymes are involved in this regulation including HMG-CoA synthase, HMG-CoA reductase, farnesyl diphosphate synthase, and squalene synthase (Brown and Goldstein 1997). HMG-CoA reductase is the most extensively studied since it controls the rate-limiting step in cholesterol biosynthesis. HMG-CoA reductase is among the most highly regulated enzyme in nature (Goldstein and Brown 1990). Addition of

HMG-CoA reductase inhibitor, such as compactin and lovastatin, to cultured cells yields a 200-fold induction in HMG-CoA reductase protein within a few hours (Nakanishi, Goldstein et al. 1988). This is the result of the multiplicative effect of smaller changes occurring at three levels: induction of transcription produces an eightfold increase in mRNA levels, each mRNA is translated at a fivefold higher rate, and the enzyme molecules are degraded fivefold more slowly (Goldstein and Brown 1990). Further studies revealed that a family of membrane-bound transcription factors called sterol regulatory element binding protein (SREBP) and the sterol regulatory element-1 (SRE-1) in the promoter of the HMG-CoA reductase gene are responsible for the cholesterol-mediated inhibition of transcription (Osborne, Goldstein et al. 1985). In addition, the NH_2-terminal membrane-attachment domain of HMG-CoA reductase has a sterol-sensing function in which the regulated degradation of this enzyme is dependent on when sterols accumulate (Brown and Goldstein 1997). Another level of regulation on HMG-CoA reductase is the rapid inhibition of the enzyme by phosphorylation of serine871 through the action of AMP-activated protein kinase (AMPK) (Omkumar, Darnay et al. 1994). Statins inhibit HMG-CoA reductase to lower serum cholesterol and thus reduce the risk for cardiovascular disease. Green and black tea extracts also inhibit HMG-CoA reductase by directly inhibiting the enzyme and activating AMPK and the concomitant phosphorylation of HMG-CoA reductase (Singh, Banerjee et al. 2009).

The other major source of cholesterol is derived from LDL receptor-mediated endocytosis. Patients who suffered from familial hypercholesterolaemia have

genetic defects involving specific steps in the LDL pathway. As a result, the cells fail to bind and take up the lipoprotein with high affinity. Because these cells are unable to utilize LDL-cholesterol, they must satisfy their cholesterol requirement by synthesizing large amounts of cholesterol de novo (Goldstein and Brown 1977). In vitro, in the absence of LDL, animal cells maintain high activities of the HMG-CoA synthase and HMG-CoA reductase, thereby synthesizing an excessive amount of cholesterol. When LDL is present, HMG-CoA synthase and reductase activities decline by more than 90%. When cellular sterols rise or when cell growth ceases and cholesterol demand declines, the LDL receptor gene is repressed (Goldstein and Brown 1990).

To balance the pool of cholesterol, cells develop an intricate series of reactions to modify cholesterol to form bile acids: small, soluble, and detergent-like molecules so that excess cholesterol can be readily eliminated. The catabolism of cholesterol to bile acids is transcriptionally regulated by both feed-forward and feedback signaling pathways mediated by cholesterol-derived oxysterols and bile acids, respectively (Repa and Mangelsdorf 2000). LXRα is activated by oxysterols to increase *Cyp7α* transcription (Janowski, Willy et al. 1996). When fed high cholesterol diets, rats and mice respond in part by synthesizing more bile acids through the classic pathway, whereas LXRα$^{-/-}$ mice fail to induce *Cyp7α* transcription and accumulate enormous amount of cholesterol in the liver. The most active ligands for LXRα are the 24-hydroxycholesterol and 24,25-epoxycholesterol (Russell 1999). Oxysterols also block the cleavage of SREBPs and the subsequent migration of the amino-terminal fragments to the nucleus to

activate the expression of many genes in the cholesterol supply and fatty acid synthesis pathways (Lund, Kerr et al. 1998). FXR, a bile acid receptor, is a global regulator of bile acid metabolism, modulating both synthetic output in the liver and recycling in the intestine. When the bile acid pool size in an animal is increased by dietary bile acids, transcription from *Cyp7α* is decreased and that from ileal bile acid binding protein (IBABP), a transporter on the apical surface of the hepatocyte, is increased (Russell 1999).

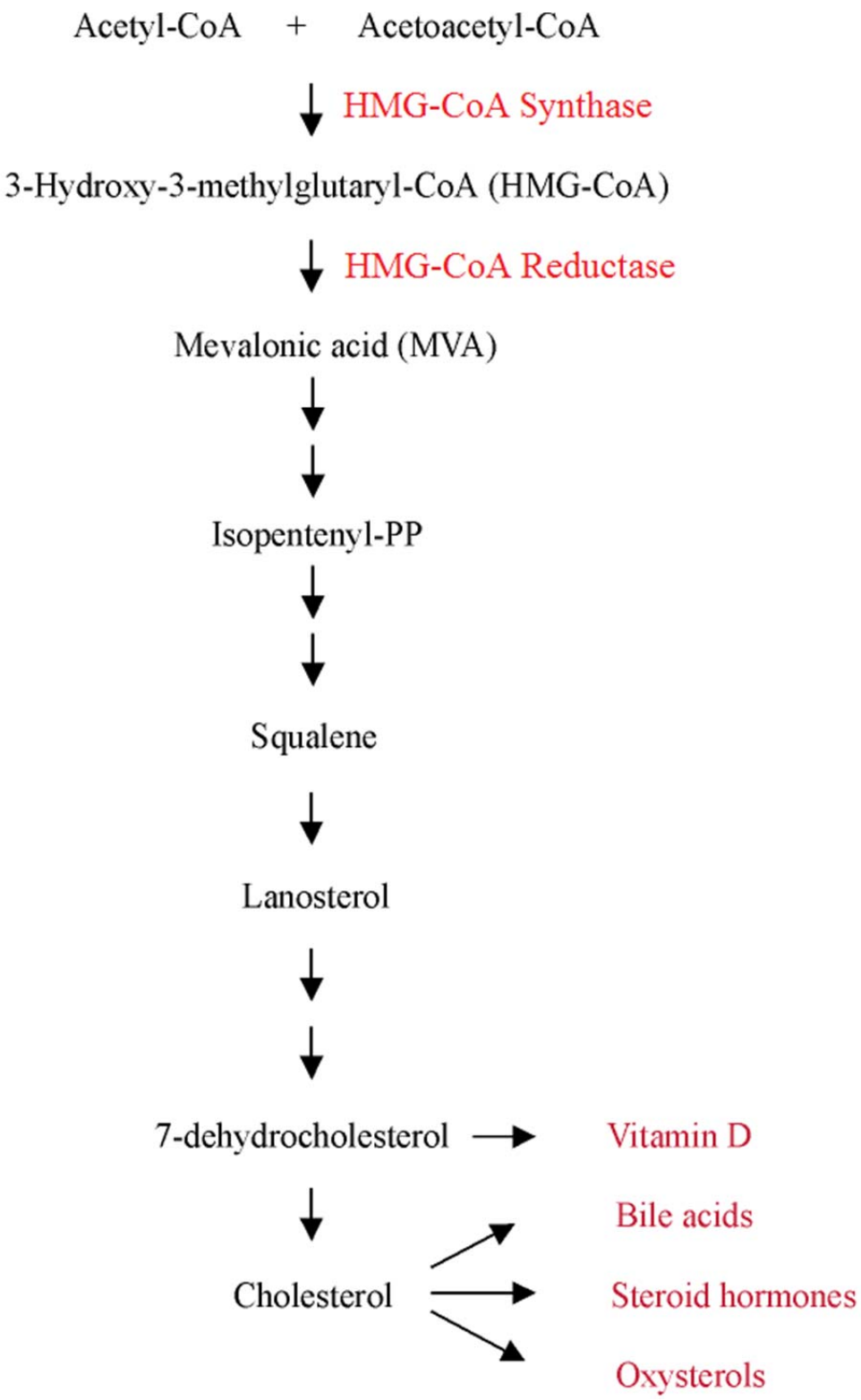


Figure 1.1 Overview of the cholesterol biosynthetic pathway [modified from (Herman 2003)].

1.2 Structure and function of mammalian skin

1.2.1 Function of mammalian skin

Skin is the frontier of the mammalian body which shields the host from a variety of insults including mechanical cuts and abrasions, UV-irradiation, chemicals, pathogenic microorganisms, etc. To survive these insults, the skin performs a variety of specialized functions such as thermoregulation, sensation, immunological protection, and water and electrolyte preservation (Ziboh and Chapkin 1988, Feingold 2007, Proksch, Brandner et al. 2008).

Another function of the skin is that it is the site where vitamin D photosynthesis occurs. Indeed, the epidermis is the major source of vitamin D for the body (Bikle 2011).

1.2.2 Structure of mammalian skin

The mammalian skin is composed of two primary layers, the epidermis and the dermis. Epidermis is the first line of defense and protection (Alonso and Fuchs 2003).

The epidermis is composed of multiple layers (Fig 1.2). From the inner to the outermost are the stratum basale, the stratum spinosum, the stratum granulosum (SG) and the stratum corneum (SC). Within the epidermis, the major cell type is the keratinocyte which undergoes a continuous process of self-renewal as they progressively differentiate towards the outermost layer of the tissue (Swanson 2004).

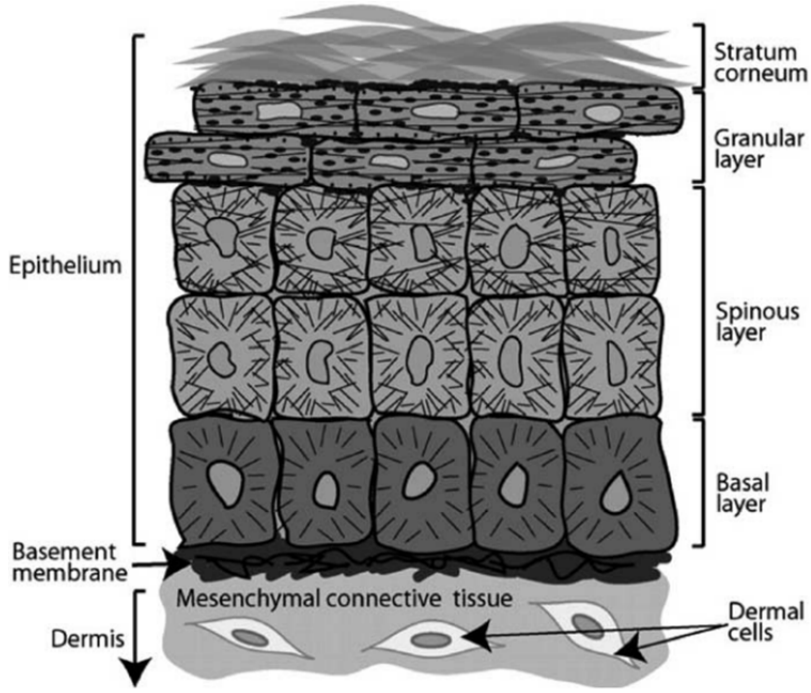


Figure 1.2 A diagram of human skin layers (Swanson 2004).

Keratinocytes of the basal layer are relatively undifferentiated (Alonso and Fuchs 2003). The primary function of the basal cell layer is that of mitotic activity in which the epidermal stem cells and the transiently amplifying cells pass through a series of physiological and biochemical events to proliferate. As the population of basal cells expands, some cells detach from the basement membrane and begin to move outward toward the skin surface (Nicoll and Cortese 1972, Alonso and Fuchs 2003). High resolution radioautography analysis of the kinetics on human epidermal cell populations showed that epidermal cell renewal time was between 13 to 18 days. Interestingly, these cells migrate at different speeds and are not rigidly bound to each other (Nicoll and Cortese 1972). As epidermal cells move away from the basal cell layer they enter the synthetic stage of differentiation, during which they are no longer dividing but are concentrating on

the formation of three major cellular constituents: the filamentous bundles, the membrane coating granules, and the keratohyalin granules. The second major phase of differentiation, the transformation phase, is the conversion of the granular cell into a cornified cell (Nicoll and Cortese 1972). At the molecular level, markers are indicators of the progress of differentiation. As the basal epidermal cells commit to differentiation, keratins K5 and K14 are downregulated while K1 and K10 are upregulated. Profilaggrin is produced when the spinous cells reach the granular layer and filaggrin is synthesized in the transition zone between the granular and cornified layer. Involucrin is a marker of differentiated keratinocytes of the cornified envelope (Swanson 2004).

The stratum corneum consists of corneocytes surrounded by a neutral lipid-enriched extracellular matrix. The mechanical strength of the skin is provided by the corneocytes, which are encased by a cornified envelope consisting of extensively cross-linked proteins. The hydrophobic extracellular lipid matrix provides the barrier to the movement of water and electrolytes (Feingold 2007).

1.2.3 Regulation of epidermal lipogenesis

Mammalian epidermis is an active site of de novo lipid biosynthesis (Monger, Williams et al. 1988). As keratinocytes differentiate, there is a shift in intercellular lipid composition from a mixture of polar lipids and neutral lipids to sphingolipids and neutral lipids which have an important effect on both barrier function and desquamation (Elias and Feingold 1988).

It is generally accepted that the barrier function of the skin is mainly dependent on the stratum corneum (Nicoll and Cortese 1972) and the lipids are important for the cutaneous primary function (Grubauer, Feingold et al. 1987). Unlike the lipids that constitute most biological membranes, the lipids in the extracellular matrix of the stratum corneum are unique. The lipid mixture consists of roughly equimolar concentrations of ceramides, free fatty acids, and cholesterol while little phospholipid is present (Feingold 2007). Ceramides are particularly critical for barrier function because they are long-chain and highly saturated (Elias and Feingold 1988). A more recent study proposed that the lipids are organized as stacked bilayers of fully extended ceramides with cholesterol molecules associated with the ceramide sphingoid moiety and free fatty acid at the ceramide fatty acid end (Iwai, Han et al. 2012).

Both in vivo and in organ culture, the rate of epidermal lipogenesis is higher in the stratum granulosum of neonatal mouse epidermis than in the subjacent basal/spinous layers (Elias and Feingold 1988). The lipid biosynthetic activity in vitro also was two- to fivefold higher in the stratum granulosum than in basal/spinous layers while other forms of metabolic activity such as protein and DNA synthesis are diminishing. The surprising observation that the highest rates of de novo synthesis occurred in differentiating, outer epidermal cell layers rather than in the proliferating compartment suggests that the epidermal lipid biosynthesis may be largely directed towards provision of the cutaneous permeability barrier, rather than in supplying lipids for membrane synthesis in response to requirements for cellular growth and replication (Monger, Williams et

al. 1988). This is further supported by the evidence that epidermal cholesterol synthesis is increased during permeability barrier repair (Proksch, Elias et al. 1990) and topical application of statins which inhibit HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis, disrupts barrier function (Proksch, Feingold et al. 1992).

Cutaneous cholesterol synthesis is virtually autonomous. Although basal cells are capable of taking up cholesterol from the circulation, cholesterol synthesis in the upper layers is influenced neither by dietary cholesterol nor by circulating cholesterol levels (Proksch, Elias et al. 1990). The autonomy is due to the absence of LDL receptors on the plasma membranes of keratinocytes committed to differentiation whereas undifferentiated keratinocytes on the basal layer have LDL receptors (Williams, Mommaas-Kienhuis et al. 1987, te Pas, Lombardi et al. 1991).

1.3 Vitamin D production, metabolism and function in mammalian skin

1.3.1 Role of vitamin D in calcium homeostasis

Vitamin D has been widely known to maintain calcium homeostasis. It either can be synthesized in the skin from 7-dehydrocholesterol (7-DHC) upon exposure to UV irradiation, or be taken in the diet. Vitamin D in the circulation is bound to vitamin D binding protein and is transported to the liver where it is hydroxylated at position 25 to form 25-hydroxyvitamin D [$25(OH)D_3$]. $25(OH)D_3$ is further hydroxylated in the kidney by the enzyme CYP27B1 to form 1,25-dihydroxyvitamin D [$1,25(OH)_2D_3$], the most active form of vitamin D and the ligand for vitamin D receptor (VDR). $1,25(OH)_2D_3$ acts at the intestine, kidney, and bone to regulate blood calcium levels. When there is a need to increase blood calcium levels, $1,25(OH)_2D_3$ increases intestinal calcium absorption. If this is insufficient, $1,25(OH)_2D_3$ works in concert with parathyroid hormone (PTH) in the kidney to promote calcium reabsorption from the distal tubule, and in the skeletal system to release calcium from bones. $1,25(OH)_2D_3$ acts through the VDR in nucleus of cells in target tissues. The VDR heterodimerizes with the retinoid X receptor (RXR), and the VDR/RXR complex binds to VDR-responsive elements found in or around target genes, resulting in the transcription of these genes (Christakos, Hewison et al. 2013).

More recently, however, the discovery of the VDR in tissues that have no involvement in calcium homeostasis and bone health (e.g., skin, placenta, pancreas, breast, prostate and colon cancer cells, and activated T cells) has led

to exploration of its broader biological significance (Christakos, Hewison et al. 2013).

1.3.2 Regulation of vitamin D production and metabolism in mammalian skin

1.3.2.1 Regulation of vitamin D production in mammalian skin

The immediate product of 7-DHC ring-opening after ultraviolet irradiation is previtamin D_3 (pre-D_3) (Holick, Richtand et al. 1979). The formation of pre-D_3 is rapid and reaches plateau within hours. Subsequently it undergoes a temperature-catalyzed rearrangement of the triene structure at 37˚C to form vitamin D_3, which is also known as cholecalciferol (Bikle 2011). Further irradiation increased only the biologically inactive photoisomers, lumisterol and tachysterol which are photochemically generated from pre-D_3. The generation of these preD_3 metabolites appears to limit preD_3 accumulation and vitamin D_3 intoxication in human skin during excessive exposure to the sun (Holick, MacLaughlin et al. 1981).

The spectral character of natural sunlight has a profound effect on the photochemistry of 7-DHC in human skin, with the optimum wavelengths for the production of preD_3 between 295 and 300 nm (MacLaughlin, Anderson et al. 1982).

The photoproduction of pre-D_3 is not only governed by UV light, but more importantly, by the availability of 7-DHC content because skin 7-DHC content is entirely derived from biosynthesis in the skin, and the highest skin concentrations of 7-DHC are found in the epidermis (Esvelt, DeLuca et al. 1980). After exposure

to sunlight, the synthesis of pre-D_3 reaches a plateau at about 10 to 15 percent of the original 7-DHC content in vivo (Holick, MacLaughlin et al. 1981) and 70 percent in vitro (Nemanic, Whitney et al. 1983). 7-DHC levels can be modulated by calcium, hydrocortisone, EGF, and 1,25$(OH)_2D_3$ in the circulation (Esvelt, DeLuca et al. 1980, Nemanic, Whitney et al. 1983). Interestingly, the production of 7-DHC is 5-8 times greater than the production of desmosterol, the final intermediate in the alternative ∆24 reductase pathway of cholesterol synthesis (Herman 2003). As human keratinocytes show a preferential utilization of the Δ^7-reductase vs. Δ^{24} pathway, the Δ^7-reductase may be the rate-limiting enzyme in post-lanosterol cholesterol biosynthesis in the epidermis. Thus, the Δ^7-reductase may serve as a control point for both cholesterologenesis and vitamin D_3 generation in the skin (Nemanic, Whitney et al. 1983).

1.3.2.2 Metabolism of vitamin D_3 to its biologically active products

The epidermis is not merely the site of vitamin D_3 photoproduction. Epidermal keratinocytes are the only cells in the body which possess the complete vitamin D signaling pathway, including the VDR, the 25-hydroxylase (CYP27A1) and the 1α-hydroxylase (CYP27B1), allowing these cells to generate the active VDR ligand, 1,25$(OH)_2D_3$. The production of 1,25$(OH)_2D_3$ in the skin could serve not only a local need in regulating the unique epidermal functions of vitamin D production and keratin synthesis, but also a systemic need in times of reduced production of 1,25$(OH)_2D_3$ by the kidney (Bikle, Nemanic et al. 1986, Vantieghem, Kissmeyer et al. 2006, Bikle 2011).

$1,25(OH)_2D_3$ negatively regulates its own levels within the keratinocyte. This feedback inhibition is due to induction of 25(OH)D 24-hydroxylase (CYP24A) that converts $25(OH)D_3$ and $1,25(OH)_2D_3$ to $24,25(OH)_2D_3$ and $1,24,25(OH)_3D_3$, respectively (Bikle 2011). The increased production of $24,25(OH)_2D_3$ as $1,25(OH)_2D_3$ production declined is correlated with the early events of differentiation such as expression of transglutaminase activity and the levels of a precursor protein for the cornified envelopes, involucrin (Pillai, Bikle et al. 1988).

The expression and activity of CYP27B1 also changes with differentiation, with the highest expression in the stratum basale in vivo and the greatest enzymatic activity in the undifferentiated cells in vitro (Bikle 2011).

1.3.3 The role of vitamin D in skin cancer

Since the first observation of the positive correlation between latitude of residence and incidence of colon cancer, numerous studies have evaluated the potential role of 1,25-dihydroxyvitamin D_3 and vitamin D receptor (VDR) for anticancer activity (Tang, Xiao et al. 2011, Bikle, Elalieh et al. 2013). Three mechanisms have been established to underlie the protective role of vitamin D in tumor formation: (1) inhibition of proliferation and promotion of differentiation; (2) induction of DNA damage repair resulting from UV radiation; (3) inhibition of hedgehog signaling. For the purpose of this dissertation, mechanisms one and three will be addressed.

1.3.3.1 Vitamin D inhibits proliferation and promotes differentiation of keratinocytes

The unexpected finding of the VDR in the epidermis (Stumpf, Sar et al. 1979) led to studies of the role of $1,25(OH)_2D_3$ in the skin. Hosomi *et al.* made the first observation with mouse epidermal cells that $1,25(OH)_2D_3$ induces keratinocyte differentiation dose-dependently at concentrations of 0.12 nM or more. The size and density of the cells became larger and lighter, respectively, during the course of differentiation. $1,25(OH)_2D_3$ also stimulated formation of a cornified envelope. (Hosomi, Hosoi et al. 1983). Similar observations of morphologic and biochemical differentiation were seen in cultured human epidermal keratinocytes grown under defined, serum-free conditions (Smith, Walworth et al. 1986). $1,25(OH)_2D_3$ also increases the expression of differentiation markers such as involucrin, transglutaminase, loricrin, and filaggrin while inhibiting proliferation. Mice lacking the VDR or the enzyme (CYP27B1) that produces its ligand, $1,25(OH)_2D_3$, show defective epidermal differentiation manifesting as reduced levels of involucrin and loricrin and loss of keratohyalin granules (Bikle 2011).

$1,25(OH)_2D_3$ at subnanomolar concentrations either promotes or inhibits keratinocyte proliferation. Concentrations above 10^{-9} M are generally found to have only an antiproliferative effect which is accompanied by an increase in TGF-β and a reduction in c-myc and the EGF receptor mRNA levels. The mechanisms underlying the proproliferative actions are not known (Bikle, Ng et al. 2001).

Interestingly, expression of the VDR is highest in the basal layers (Stumpf, Sar et al. 1979) and both the VDR and the production of $1,25(OH)_2D_3$ are reduced in the later stages of differentiation indicating a feedback regulation (Bikle, Ng et al. 2001).

1.3.3.2 Vitamin D inhibits hedgehog signaling pathway in keratinocytes

1.3.3.2.1 Hedgehog signaling pathway

Hedgehog (Hh) proteins comprise a family of secreted signaling molecules essential for developmental patterning of structures in animal embryogenesis. The 45 kDa hedgehog protein precursor undergoes autocatalytic cleavage mediated by its carboxyl-terminal domain to yield a 20 kDa amino-terminal fragment (HH-N) responsible for all known Hh signaling activity (Porter, Young et al. 1996). The HH-N possesses a covalently linked cholesterol moiety on the carboxyl end and is palmitoylated at the amino-terminus. This dual lipid modification of the Hh has important effects on its properties, both enhancing its membrane association and potentiating its secretion and range of action (Gallet, Ruel et al. 2006, Ingham, Nakano et al. 2011). A decreased level of cholesterol leads to impaired Hh autoprocessing and diminished responsiveness to the Hh signal (Cooper, Wassif et al. 2003). Originally discovered in *Drosophila melanogaster*, three vertebrate homologs of the *Hh* gene have been identified including *sonic hedgehog* (*SHH*), *desert hedgehog* (*DHH*) and *Indian hedgehog* (*IHH*) (Bale and Yu 2001).

The hedgehog signal is received and transduced at the membrane via a receptor complex consisting of patched and smoothened (Bale and Yu 2001). In the absence of hedgehog, the function of smoothened is inhibited by patched. When hedgehog binds to patched, this inhibition of smoothened is lost resulting in the activation of the zinc finger-containing GLI protein family which function as transcriptional activators of a variety of genes in the nucleus (Ingham, Nakano et al. 2011).

1.3.3.2.2 Hedgehog signaling in skin cancer

Hh signaling has a multitude of functions in embryonic and adult tissues, mediating fundamental processes such as cell fate specification, proliferation and patterning, tissue morphogenesis and homeostasis (Ingham, Nakano et al. 2011). On the other hand, it is also identified as a potentially important stimulator of carcinogenesis when dysregulated. This can occur via mutations in the genes encoding components of the pathway or by excess production of Hh ligand by the tumor or stromal cells (Tang, Xiao et al. 2011). For example, germline mutations of *patched* cause Gorlin syndrome, an autosomal dominant disorder characterized by multiple skin cancers, other tumors and congenital anomalies of the brain, bones and teeth (Bale and Yu 2001).

Overexpression of Hh in mice can induce the development of basal cell carcinoma (BCC), a tumor originating from undifferentiated pluripotent cells in the skin (Zhou, Jia et al. 2006). BCC is the most common of all human cancers, representing at least one-third of all cancers diagnosed in the US each year

(Bale and Yu 2001) and affecting approximately 1 million Americans per year. The pivotal molecular abnormality in BCC is constitutive activation of the Hh signaling pathway in 10% to 20% of tumors by mutational activation of smoothened and in the great majority of the others is associated with mutational inactivation of patched1 (Tang, Xiao et al. 2011). The Ptch1+/- mouse developed as the first practical model of murine BCC, is quite susceptible to the development of BCC following UV radiation (Bikle, Elalieh et al. 2013).

1.3.3.2.3 Potential roles of vitamin D_3 and 7-dehydrocholesterol reductase (DHCR7) in hedgehog signaling

Mice lacking the VDR have constitutively active hedgehog signaling, whereas 1,25-dihydroxyvitamin D_3 can suppress this pathway in normal skin. So loss of 1,25-dihydroxyvitamin D_3 and/or VDR regulation of Hh signaling is one of the causes of the increased susceptibility of the epidermis to malignant transformation (Bikle, Elalieh et al. 2013).

The suppression of the Hh pathway occurs not only via the genomic actions of 1,25-dihydroxyvitamin D_3 acting through its receptor, but through the direct binding of vitamin D_3 to smoothened. Thus, vitamin D_3 may potentially serve as a chemopreventive and/or chemotherapeutic agent for BCCs (Bijlsma, Spek et al. 2006, Tang, Xiao et al. 2011, Bikle, Elalieh et al. 2013).

Interestingly, a defect in (or the absence of) DHCR7 that leads to the accumulation of its substrate, 7-DHC, inhibits smoothened activity. AY-9944, an inhibitor of DHCR7, also results in the repression of smoothened (Bijlsma,

Peppelenbosch et al. 2006). Although not as potent as vitamin D_3, 7-DHC directly binds to and inhibits the action of smoothened (Bikle, Elalieh et al. 2013). These results underline the important role of DHCR7 in keratinocytes to maintain a balance between cholesterol and vitamin D_3 production.

1.4 The potential role of 7-dehydrocholesterol reductase in vitamin D deficiency

7-DHC, the substrate of DHCR7, is also the precursor of vitamin D_3. Thus it may be of importance to characterize biochemical and genetic aspects of DHCR7 to understand and ultimately prevent and treat vitamin D deficiency.

1.4.1 The biochemical characterizations of human 7-dehydrocholesterol reductase

The human 7-dehydrocholesterol reductase (DHCR7) gene is on chromosome 11q13 (Fitzky, Witsch-Baumgartner et al. 1998) and the mRNA is ubiquitously transcribed but most abundant in adrenal gland, liver, testis, and brain. Human DHCR7 enzyme is a membrane-bound protein with a predicted molecular mass of 55 kDa and six to nine putative transmembrane segments with the N terminus oriented toward the cytosol (Fitzky, Witsch-Baumgartner et al. 1998, Moebius, Fitzky et al. 1998).

7-Dehydrocholesterol reductase (DHCR7) catalyzes the final step in cholesterol synthesis on the smooth endoplasmic reticulum, reducing the C (7-8) double bond in 7-dehydrocholesterol (7DHC) by using NADPH as the electron donor. Substrate specificity assays indicate that the reduction of the double bond at C-7,8 requires binding of the enzyme protein with the B-ring of the sterol substrate that contains a double bond at C-5,6 and that the reaction is hindered by substituents located on the apolar side-chain and epimerization of the hydroxyl group in ring A to a 3α-configuration. The enzymatic activity is increased by

phosphorylation and inhibited by AY9944 (IC_{50} 0.013 μM), BM15766 (IC_{50} 1.2 μM) and triparanol (IC_{50} 14 μM) (Moebius, Fitzky et al. 1998, Shefer, Salen et al. 1998). The expression of the DHCR7 gene is directly activated by the transcription factor SREBP-2, dedicated to the synthesis of cholesterol (Horton, Goldstein et al. 2002). Some antipsychotic drugs such as clozapine, chlorpromazine, and haloperidol and antidepressants such as imipramine are able to induce the transcription of DHCR7 gene by activating the SREBPs (Lauth, Rohnalter et al. 2010).

A distinguishing characteristic of DHCR7 is the presence of the sterol-sensing domain (SSD) attributed to its role in cholesterol homeostasis and cholesterol-linked signaling. It is increasingly appreciated that cholesterol has the potential to affect organismal development, thus deficiency of DHCR7 has been shown to be responsible for causing developmental malformations (Kuwabara and Labouesse 2002). Smith-Lemli-Optiz Syndrome (SLOS), which is an autosomal recessive disease, is caused by a defect of the enzyme DHCR7 and consequently low serum cholesterol levels. The clinical spectrum of SLOS includes different morphogenic abnormalities such as limb/skeletal and urogenital anomalies, growth and mental retardation, as well as failure to thrive and behavioral problems (Jira, Waterham et al. 2003).

1.4.2 The genetic variations of 7-dehydrocholesterol reductase in vitamin D deficiency

25-Hydroxyvitamin D, the primary circulating form of vitamin D, is used to determine a person's vitamin D status and a level less than 20 ng per milliliter is defined as vitamin D deficiency (Holick 2007). Genome-wide association study (GWAS) of 25-hydroxyvitamin D suggested that single-nucleotide polymorphisms (SNPs) in or near genes involved in vitamin D synthesis or activation are associated with low 25-hydroxyvitamin D level and elevated risk of vitamin D deficiency. In both European and Chinese populations, the variants of genes involved in vitamin D transport (GC), hydroxylation (CYP2R1, CYP24A1), and interestingly, cholesterol synthesis (DHCR7) are suggestive to influence plasma 25-hydroxyvitamin D status (Ahn, Yu et al. 2010, Wang, Zhang et al. 2010, Lu, Sheng et al. 2012).

Chapter 2: Experimental rationale

Both cholesterol and vitamin D_3 are indispensable for the proper function of skin. DHCR7 activity likely influences skin 7-DHC content available for both cholesterologenesis and vitamin D_3 photosynthesis. It was suggested that the photoproduction of vitamin D_3 is not only governed by UV light, but more importantly, by the availability of 7-DHC, which is entirely derived from biosynthesis in the skin (Esvelt, DeLuca et al. 1980). I hypothesized that as vitamin D_3 accumulated it might promote the activation of DHCR7 to limit further vitamin D_3 production by increasing the conversion of 7-DHC to cholesterol, thereby depleting 7-DHC levels in the keratinocyte. Alternatively, vitamin D_3 might enhance its own production by suppressing DHCR7 activity, thereby making more 7-DHC available for photoconversion to vitamin D_3. It has been established that DHCR7 could be activated by phosphorylation and inactivated by dephosphorylation (Shefer, Salen et al. 1998), and so I wished to determine if vitamin D_3 regulated DHCR7 activity by these mechanisms. Direct inhibition by vitamin D or allosteric modulation of enzyme activity was also considered. More long-term regulation, through changes in enzyme levels mediated by increased or decreased gene expression and protein stability were also evaluated. The involvement of the vitamin D receptor in the regulation of DHCR7 by vitamin D_3 was to be determined since it regulates the transcription of numerous genes in skin and other tissues (Bikle 2011). The effect of UVB irradiation on DHCR7 activity in keratinocyte cells was examined based on the supposition that 7-DHC

in keratinocytes would be converted into vitamin D_3. The effect of vitamin D_3 on cholesterolgenesis was also investigated.

Specific Aims

Specific Aim 1: Determine if vitamin D_3, 25-hydroxyvitamin D_3, 1,25-dihydroxyvitamin D_3, and UV light modulate the activity and expression of DHCR7 in whole cells. This aim had two parts: 1) to determine if different forms of vitamin D regulate DHCR7 activity; 2) to determine if the response of DHCR7 to vitamin D is specific to keratinocytes. Rat hepatoma (McA-RH7777), human hepatoma (HepG2), human immortal keratinocyte (HaCaT), and adult human epidermal keratinocyte (HEKa) cells would be cultured and treated with different forms of vitamin D. Only HEKa cells would be exposed to UV light, which provides an alternative way to generate vitamin D_3, to test the effect of UVB on DHCR7 activity in keratinocytes.

Specific Aim 2: Determine the mechanism by which vitamin D regulates DHCR7 activity. Possible mechanisms include: 1) phosphorylation/dephosphorylation; 2) transcriptional regulation by the vitamin D receptor (VDR); 3) direct binding of vitamin D_3 to DHCR7, either as an inhibitor or as an allosteric modulator; and 4) signaling through the hedgehog pathway to modulate DHCR7 activity. It has been shown that addition of ATP to rat microsomes increases DHCR7 activity and phosphatase treatment prevents this activation and decreases DHCR7 activity. The effect of ATP on DHCR7 activity would be determined in mouse microsomes, McA-RH7777 cells, HepG2 cells, HaCaT cells and HEKa cells. If confirmed in HEKa cells, I would attempt to identify the protein kinase involved. The response of DHCR7 to 1,25-dihydroxyvitamin D_3 would be used to determine if the vitamin D receptor is involved in the regulation of DHCR7. The ability of

vitamin D_3 to modulate DHCR7 activity when added directly to cell lysates would also be determined. To evaluate the role of the hedgehog signaling pathway in vitamin D regulation of cell differentiation and DHCR7 activity, I would determine if vitamin D_3 and cyclopamine, an inhibitor of hedgehog signaling, inhibit proliferation, induce differentiation of HEKa cells, and affect DHCR7 activity.

Specific Aim 3: Determine if vitamin D_3 affects other enzymes in the cholesterologenic pathway. Changes in the activity of the pre-sterol synthesis steps would be evaluated by treating the cells with a lanosterol demethylase inhibitor (ketoconazole) and then measuring lanosterol accumulation in vitamin D_3-treated and untreated cells. The effect of vitamin D_3 treatment on the activity of lathosterol 5-desaturase, the enzyme that immediately precedes DHCR7 in the cholesterolgenic pathway, would be measured directly.

Chapter 3: Materials and methods

List of Chemicals, Buffers, and Reagents

Chemicals

The following chemicals were used in these studies and were purchased from standard suppliers: Dipotassium phosphate (K_2HPO_4), dithiothreitol (DTT), nicotinamide, ethylenediaminetetraacetic acid (EDTA), nicotinamide adenine dinucleotide phosphate ($NADP^+$), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, sodium hydroxide, adenosine-5'-triphosphate (ATP), magnesium chloride ($MgCl_2$), methyl-β-cyclodextrin (MβCD),

ergosterol (2.5 mM stock in 13% MβCD), brassicasterol, stigmasterol (dissolved in ethanol), cholecalciferol (dissolved in ethanol), 7-dehydrocholesterol (in 13% MβCD), cholesterol, lanosterol, lathosterol (dissolved in 13% MβCD), 25-hydroxycholecalciferol (dissolved in ethanol), 1,25-dihydroxyvitamin D (dissolved in ethanol),

ethanol, isobutanol, and hexanes.

Buffers and solutions

Ethanolic NaOH solution: 1 M NaOH, 70% ethanol

Lower Tris Buffer: 1.5 M Tris base, 0.4% w/v SDS, pH 8.8

Upper Tris Buffer: 0.5 M Tris base, 0.4% w/v SDS, pH 6.8

5X Running Buffer: 125 mM Tris base, 1 M Glycine, 0.5% w/v SDS

1X Electroblot Transfer (ET) Buffer: 25 mM Tris base, 200 mM Glycine, 0.1% w/v SDS, 20% methanol

10X TBS: 200 mM Tris base, 1.37 M NaCl, pH 7.6

TBST (Wash Buffer): 1X TBS, 0.2% Tween 20

Blocking Buffer: 5% w/v Bovine Serum Albumin

9% resolving gel for 3 SDS-PAGE gels: 7.5 ml of lower Tris, 8.8 ml of 30% Acyl-bis, 12.3 ml of ddH_2O, 450 µl of 10% APS, 15 µl of TEMED

3.3% stacking gel for 3 SDS-PAGE gels: 2.5 ml of upper Tris, 1.1 ml of 30% Acyl-bis, 6.2 ml of ddH_2O, 150 µl of 10% APS, 20 µl of TEMED

4X SDS-PAGE sample loading buffer (10 ml): 2.4 ml of 1 M Tris-Hcl (pH 6.8), 0.8g of SDS, 4.0 ml of 100% glycerol, 0.5 ml of β-mercaptoethanol, 4.0 mg of bromophenol blue, 3.1 ml of H_2O.

Hepatoma or HaCaT cell culture (1 L): 13.4 g of Dulbecco's Modified Eagle's Medium (DMEM) powder, 3.7 g of sodium bicarbonate, ddH_2O, pH 7.3. Filter to sterilize. Supplement with 10% fetal bovine serum and 1% penicillin-streptomycin.

HEKa cell culture: EpiLife medium, Human Keratinocyte Growth Supplement.

Trypsin/EDTA, Defined trypsin neutralizer

Dulbecco's phosphate-buffered saline (DPBS)

Reagents

Halt™ protease inhibitor cocktail, PhosSTOP phosphatase inhibitor cocktail tablets,

M-PER® mammalian protein extraction reagent (Thermo Scientific),

Pierce™ BCA protein assay kit,

TRIzol® reagent.

Table 3.1: Primary antibodies used in this dissertation:

Host	Primary Antibodies	Dilution	Supplier
Rabbit	DHCR7	WB 1:500	Sigma, Cat. No: D7695
Mouse	Involucrin	WB 1:1000	Sigma, Cat. No: I9018
Mouse	β-actin	WB 1:5000	Sigma, Cat. No: A1978

Table 3.2: Secondary antibodies used in this dissertation:

Secondary Antibodies	Dilution	Supplier
ECL Plex goat-α-rabbit IgG-Cy3	1:2500	GE Healthcare, Cat. No: 28901106
ECL Plex goat-α-mouse IgG-Cy5	1:2500	GE Healthcare, Cat. No: PA45009

Cell Culture

HEKa cells between passages 1 to 5 were cultured in EpiLife medium (GIBCO) supplemented with EpiLife Defined Growth Supplement (EDGS) in 100 mm cell culture plate at 37°C under a humidified atmosphere of 5% CO_2. The culture medium was changed after 36 hours and every other day thereafter, until the cultures were approximately 50% confluent, after which the medium was changed every day until the culture was approximately 80% confluent. Chemicals were added according to experimental design.

McA-RH7777 rat hepatoma cells, HepG2 human hepatoma cells, and HaCaT human keratinocyte cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen) in 100 mm cell culture plates at 37°C under a humidified atmosphere of 5% CO_2. The culture medium was changed every 2 days. Chemicals were added according to experimental design.

Preparation of lysates: Cells were washed twice with ice cold Dulbecco's phosphate-buffered saline (GIBCO) and scraped from the plates, pelleted by centrifugation (200 x g for 10 minutes) and lysed with M-PER mammalian protein extraction reagent (Thermo Sientific) supplemented with 1x protease and phosphatase inhibitors on ice for 30 minutes. The lysates were cleared by centrifugation (18,300 x g, 4°C for 10 minutes) and the supernatant was used immediately.

Activity Assays

Microsomal/Cell Lysate 7-Dehydrocholesterol Reductase Activity Assay

Microsomal or lysate protein was incubated in a final volume of 0.5 ml (pH 7.3) containing 100 µg microsomal/cell lysate protein, 60 µM ergosterol, 100 mM K_2HPO_4, 1 mM DTT, 0.1 mM EDTA, 30 mM nicotinamide, and an NADPH-generating system (3.4 mM $NADP^+$, 30 mM glucose-6-phosphate, 0.3 IU glucose-6-phosphate dehydrogenase); in some experiments, 50 mM ATP and 50 mM $MgCl_2$ were included. After incubation at 37˚C for various time-periods, the reaction was stopped by the addition of 1 ml of 1 M ethanolic NaOH and the mixture was allowed to stand for 1 h at 37˚C. After adding 0.5 ml water, the products were extracted twice with 2 ml of hexanes which subsequently were evaporated under nitrogen. Extracts were derivatized, identified, and quantified by GC/MS at the UK Mass Spectrometry facility, focusing on mass ions 363 (ergosterol), 380 (brassicasterol), and 394 (stigmasterol, internal standard). DHCR7 activity was interpreted as the ratio of brassicasterol/stigmasterol.

Intact Cell 7-Dehydrocholesterol Reductase Activity Assay

Ergosterol (60 µM) dissolved in methyl-β-cyclodextrin was added to the cell culture medium and the cells were incubated in a 37°C, 5% CO_2/95% air, humidified cell culture incubator for designated times according to experimental design. Stigmasterol (1 µg) was added to the cell culture medium before harvesting the cells with 2 ml of ethanolic NaOH. The cell culture medium and harvested cells were extracted with hexanes. After centrifugation, the

supernatant was evaporated and analyzed by GC/MS at the UK Mass Spectrometry facility, focusing on mass ions 363 (ergosterol), 380 (brassicasterol), and 394 (stigmasterol, internal standard). DHCR7 activity was interpreted as the ratio of brassicasterol/stigmasterol.

Lathosterol-5-Desaturase Activity Assay

Reaction mixtures were prepared by combining 100 mM K_2HPO_4, 1 mM DTT, 100 µM EDTA, 100 µM lathosterol, 100 µg lysate protein, and 2.5 mM NADH. Samples were assayed as described in the cell lysate 7-dehydrocholesterol reductase activity assay.

GC/MS Sample Analysis by Wsearch32

Compounds were identified by specific mass-to-charge ratio (*m/z*) and quantified as the integrated area of the peak of *m/z* at the specific retention time. 7-Dehydrocholesterol reductase activity was calculated as the Peak Area of Brassicasterol/Peak Area of Stigmasterol. Lathosterol-5-desaturase activity was measured as the Peak Area of 7-Dehydrocholesterol/Peak Area of Stigmasterol. Lanosterol accumulation was measured as the Peak Area of Lanosterol/Peak Area of Stigmasterol.

Table 3.3: Compound mass-to-charge ratio (*m/z*) and retention time

Compound	*m/z*	Retention Time (min)*
7-Dehydrocholesterol	351	24.20
Cholesterol	368	23.60
Cholecalciferol	351	21.63
25-Hydroxycholecalciferol	341	24.13
1,25-Dihydroxyvitamin D_3	343	25.90
Lanosterol	393	25.78
Lathosterol	353	24.30
Brassicasterol	380	24.16
Ergosterol	363	24.68
Stigmasterol	394	25.28

*Retention time may vary based on the adjustment of the GC/MS machine.

UVB Irradiation

HEKa cells were exposed to UVB irradiation after reaching 80% confluence. Cells were washed with DPBS before exposure. The cell culture plate was placed in the Lucite chamber with the cover on the plate. Exposure to UVB was

for sufficient time to receive desired dosing (100 J/m^2 and 200 J/m^2). Cell culture medium was added back to cells immediately after the irradiation and incubation continued in a 37°C, 5% CO_2/95% air, humidified cell culture incubator for 3 hours or 24 hours according to experimental design.

Gel electrophoresis and immunoblotting

Fifty micrograms of cell lysate protein was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 8% gels and electroblotted to nitrocellulose sheets. The membrane was blocked with Blocking Buffer for 30 minutes at room temperature and then incubated overnight at 4°C in buffer mixed with Wash Buffer and Blocking Buffer (4:1) containing primary antibody to DHCR7, involucrin, or β-actin. The immunoblot was developed with Cy3- or Cy5-conjugated secondary antibody at room temperature for 1.5 hours while being protected from light. The fluorescent blot was then scanned on the Typhoon FLA 9000 Scanner and quantitatively analyzed by the ImageQuant software.

RT-PCR

RNA isolation

Total RNA was isolated from cells with the use of TRIzol reagent. The concentration of total RNA was determined with the use of the Nanodrop instrument.

Reverse Transcriptase reaction

In a nuclease-free tube, 200 ng of random primers was added along with 1 µl of dNTP mix (10 mM each nucleotide), 200 ng total RNA in a total volume of 12 µl in diethylpyrocarbonate-treated water. The mixture was heated to 65°C for 5 minutes and quickly chilled on ice. The mixture was collected by brief centrifugation and 4 µl of 5x First-Strand Buffer, 1 µl of SuperScript II Reverse Transcriptase, 2 µl of 0.1 M DTT, and 1 µl of RNase OUT was added. The reaction was incubated at 25°C for 10 minutes, followed by 50 minutes at 42°C and inactivated by incubation at 70°C for 15 minutes. The synthesized cDNA was aliquoted and stored at -20°C.

Real-time PCR

Gene-specific primers for real-time PCR were designed using Primer 3 software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The sequences of the primers are shown in Table 3.4. The concentrated cDNA template was diluted 6-fold in a 1:5 serial dilution to achieve the final concentration used in the PCR reaction. Each 20 µl of PCR reaction mixture contained 5 µl of diluted cDNA template, 10 µl of 2x SYBR Green PCR Master Mix, 0.4 µl of Forward Primer (100 nM final), 0.4 µl of Reverse Primer (100 nM final), and 4.2 µl of nuclease-free water. The plate was sealed thoroughly to prevent evaporation. PCR was carried out in the 7900HT thermal cycler as follows: Cycling conditions were 2 minutes at 50°C to activate, initial denaturation for 10 minutes at 95°C, followed by 40 cycles of denaturation for 15 seconds at 95°C and 1 minute of annealing/extension at 60°C.

The detection and quantitation of target gene expression levels was done by using the comparative C_T method.

Table 3.4: Primer sequence for gene expression

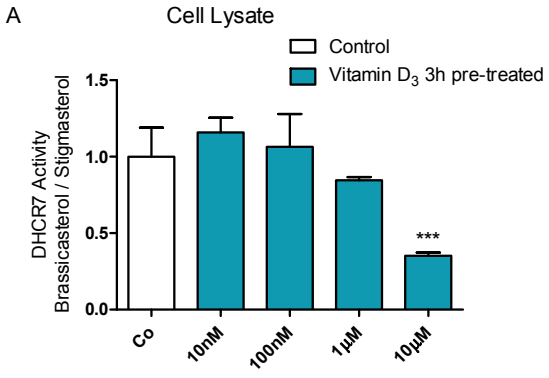
Abbreviation	Gene Name	Accession Number	Forward/Reverse primers
DHCR7	7-Dehydrocholesterol reductase	NM_001163817.1	Fw GCAACCCAACATTCCCAAAG
			Rv TCGCCAGTGAAAACCAGTC
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046.4	Fw ACATCGCTCAGACACCATG
			Rv TGTAGTTGAGGTCAATCAAGGG

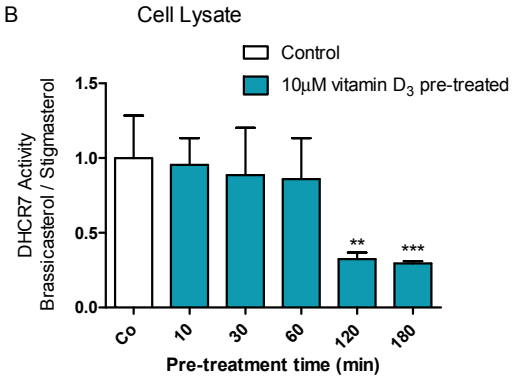
CHAPTER FOUR: Results

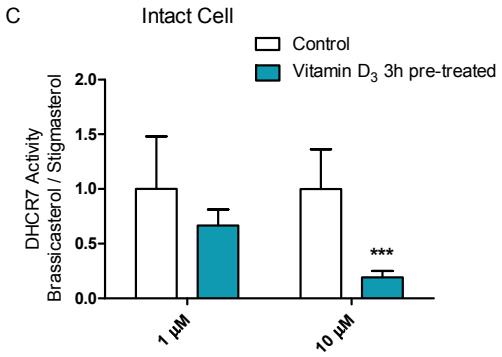
Vitamin D_3 decreases 7-dehydrocholesterol reductase activity in adult human epidermal keratinocytes

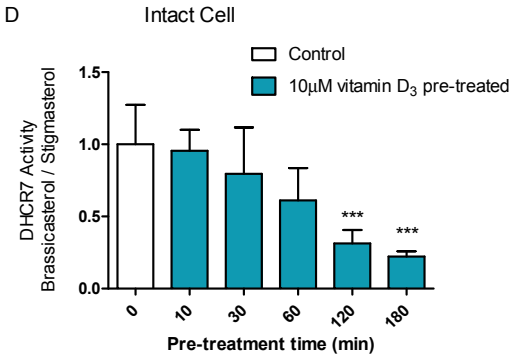
7-Dehydrocholesterol (7-DHC) is the substrate of 7-dehydrocholesterol reductase (DHCR7) for cholesterol synthesis. Keratinocytes in human skin possess the machinery for cholesterol synthesis and are also responsible for vitamin D_3 synthesis from 7-DHC by exposure to UVB irradiation. Therefore, it is important for keratinocytes to maintain a balance between cholesterol synthesis, which forms an important component of the skin barrier function, and vitamin D_3 production. A recent genome-wide association study (GWAS) suggested that SNPs in DHCR7 are associated with vitamin D deficiency (Ahn, Yu et al. 2010). This provides us with an insight into understanding the regulation of vitamin D_3 synthesis in skin and a potential target for drug therapy. In order to pursue this goal, we first asked whether DHCR7 responds to vitamin D_3 treatment. Hypothetically, vitamin D_3 could increase DHCR7 activity, favoring cholesterol synthesis, through a feedback mechanism so as to decrease 7-DHC content in keratinocytes against further vitamin D_3 production. To test this hypothesis, adult human epidermal keratinocytes (HEKa) were used to measure the DHCR7 response to vitamin D_3 treatment in HEKa cell lysates and in intact cells. Unexpectedly, vitamin D_3 decreased DHCR7 activity to 85% and 35% compared to control after 3 hour treatment at 1 μM and 10 μM concentrations, respectively (Fig 4.1A). The decrease in activity was evident as early as 2 hours after vitamin D_3 treatment of whole cells when measured with keratinocyte lysates (Fig 4.1B).

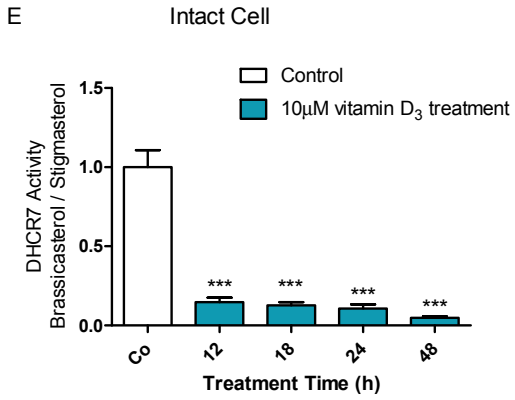
Interestingly, DHCR7 is more sensitive to vitamin D_3 in intact cells as the activity decreased more than that in cell lysates (67% and 19% at 1μM and 10μM concentrations, respectively) (Fig 4.1C) and continuously decreased from 96% (after 10 minute treatment) to 22% (after 180 minute treatment) compared to control (Fig 4.1D). This rapid down-regulation of DHCR7 activity by vitamin D_3 is at the post-translational level as I saw no change in the amount of DHCR7 protein over the course of the treatment (Fig 4.1G). In comparison to this immediate effect after short-term (3 h) treatment, long-term (up to 48 h) treatment of HEKa cells with 10 μM of vitamin D_3 suppressed DHCR7 activity nearly completely (Fig 4.1E). Recovery from even a short treatment with vitamin D_3 was slow, with DHCR7 activity returning to about 60% of its original levels at 24 h (Fig 4.1F).

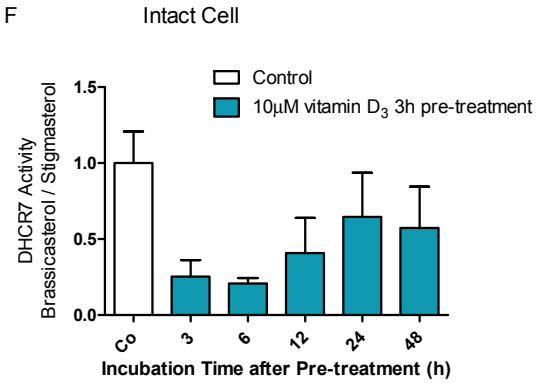

A
Cell Lysate
Control
Vitamin D3 3h pre-treated
DHCR7 Activity
Brassicasterol / Stigmasterol
1.5
1.0
0.5
0.0
***
Co
10nM
100nM
1μM
10μM


B
Cell Lysate
Control
10μM vitamin D3 pre-treated
DHCR7 Activity
Brassicasterol / Stigmasterol
1.5
1.0
0.5
0.0
**
***
Co
10
30
60
120
180
Pre-treatment time (min)


C
Intact Cell
Control
Vitamin D3 3h pre-treated
DHCR7 Activity
Brassicasterol / Stigmasterol
2.0
1.5
1.0
0.5
0.0
***
1 μM
10 μM


D
Intact Cell
Control
10μM vitamin D3 pre-treated
DHCR7 Activity
Brassicasterol / Stigmasterol
1.5
1.0
0.5
0.0
***
***
0
10
30
60
120
180
Pre-treatment time (min)


E
Intact Cell
Control
10μM vitamin D3 treatment
DHCR7 Activity
Brassicasterol / Stigmasterol
1.5
1.0
0.5
0.0
***
***
***
***
Co
12
18
24
48
Treatment Time (h)


F
Intact Cell
Control
10μM vitamin D3 3h pre-treatment
DHCR7 Activity
Brassicasterol / Stigmasterol
1.5
1.0
0.5
0.0
Co
3
6
12
24
48
Incubation Time after Pre-treatment (h)

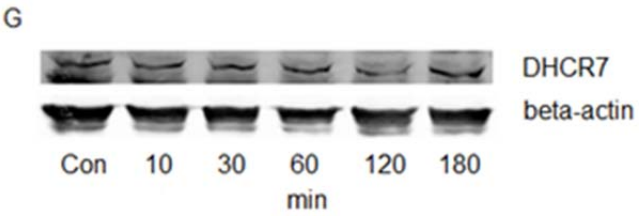
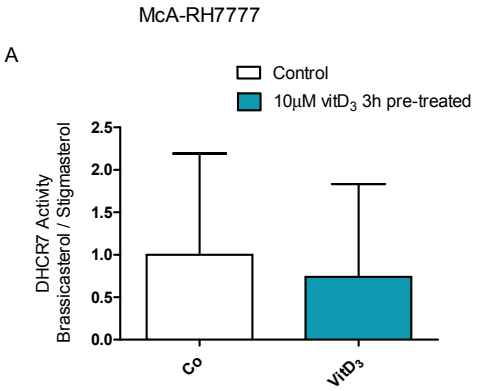
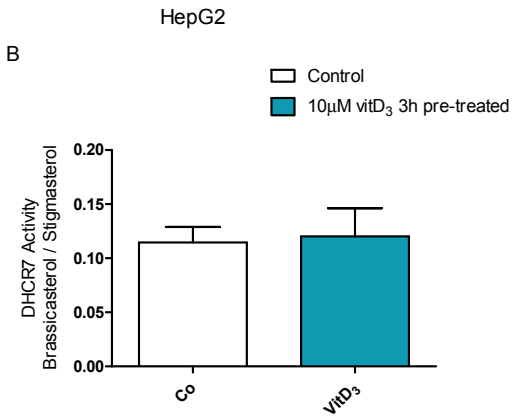

G
DHCR7
beta-actin
Con
10
30
60
120
180
min

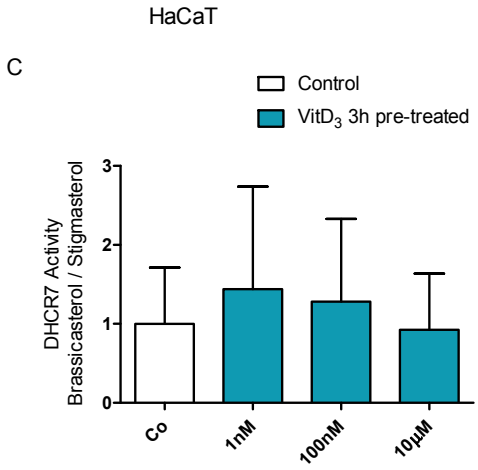
Figure 4.1: Vitamin D_3 decreases 7-dehydrocholesterol reductase activity in HEKa cells. Measurement of DHCR7 activity in cell lysates (A and B) and in intact cells (C and D) after vitamin D_3 treatment (3 h) at various concentrations (A and C) and after 10 μM of vitamin D_3 treatment at various time points (B and D). E, Measurement of DHCR7 activity in intact cells after continuous treatment with 10 μM of vitamin D_3 and the restoration of DHCR7 activity after discontinuation of treatment (F). G, protein expression was measured at various time points after treatment of cells with 10 μM of vitamin D_3 by immunoblot analysis. Values represent the mean and S.D. of 3 or more experiments; values that are statistically different from the control are indicated by asterisks, as determined by t-test or one-way ANOVA analysis of variance with Dunnett's multiple comparisons post hoc test, $p < 0.05$.

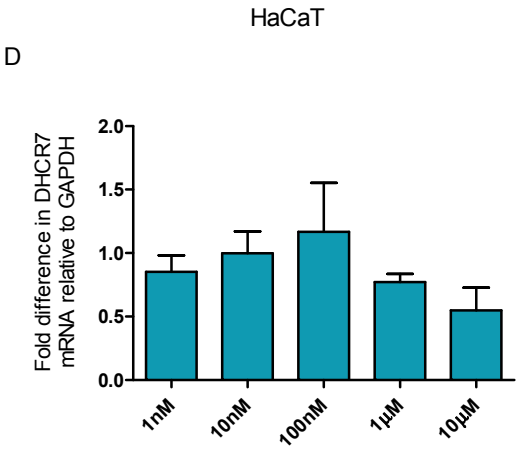
Vitamin D_3 has no effect on DHCR7 activities in McA-RH7777, HepG2, and HaCaT cells

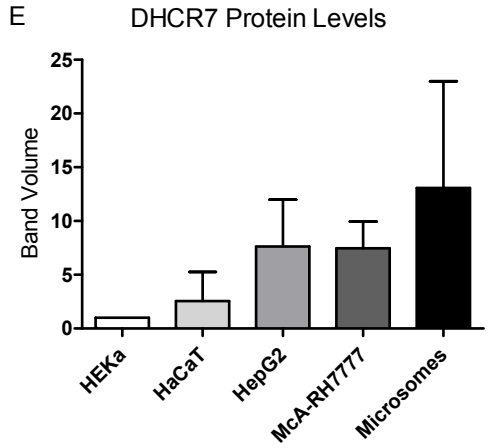
Because skin is the only site of vitamin D_3 production, it is not necessary for other organs, especially the liver, to maintain a balance between cholesterol and vitamin D_3 production. This leads to the rationale that vitamin D_3 should only regulate DHCR7 activity in the skin. Therefore, rat hepatoma (McA-RH7777) and human hepatoma (HepG2) were used to test this hypothesis. As expected, neither of these cell lines responded to a 3-h, 10 μM vitamin D_3 treatment (Fig 4.2A and B). For comparison, immortalized human keratinocytes (HaCaT cells) were also tested for their ability to regulate DHCR7 activity in response to vitamin D_3. Surprisingly, vitamin D_3 had no effect on DHCR7 activity in this immortalized skin cell line (Fig 4.2C), nor did it change DHCR7 mRNA levels (Fig 4.2D). These observations indicate that the regulation of DHCR7 activity by vitamin D_3 is restricted to normal keratinocytes. During these studies I noted that DHCR7 protein levels in HepG2 cells were 7-fold greater than that in HEKa cells (Fig 4.2E) while its activity was just 50% of that in HEKa cells (Fig 4.2F). This suggests that DHCR7 activity may be regulated post-translationally in this liver cell line by means other than vitamin D_3 levels.


A
McA-RH7777
Control
10μM vitD3 3h pre-treated
DHCR7 Activity
Brassicasterol / Stigmasterol
2.5
2.0
1.5
1.0
0.5
0.0
Co
VitD3


B
HepG2
Control
10μM vitD3 3h pre-treated
DHCR7 Activity
Brassicasterol / Stigmasterol
0.20
0.15
0.10
0.05
0.00
Co
VitD3


C
HaCaT
Control
VitD3 3h pre-treated
DHCR7 Activity
Brassicasterol / Stigmasterol
3
2
1
0
Co
1nM
100nM
10μM


D
HaCaT
Fold difference in DHCR7
mRNA relative to GAPDH
2.0
1.5
1.0
0.5
0.0
1nM
10nM
100nM
1μM
10μM


E
DHCR7 Protein Levels
Band Volume
25
20
15
10
5
0
HEKa
HaCaT
HepG2
McA-RH7777
Microsomes

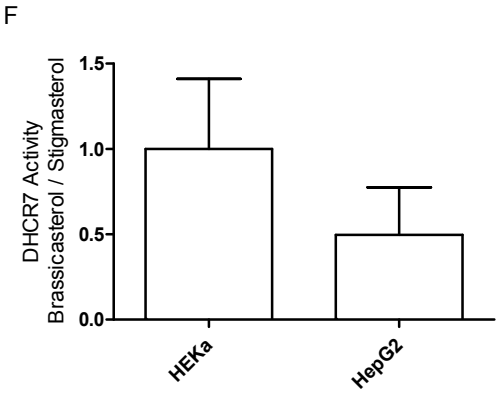
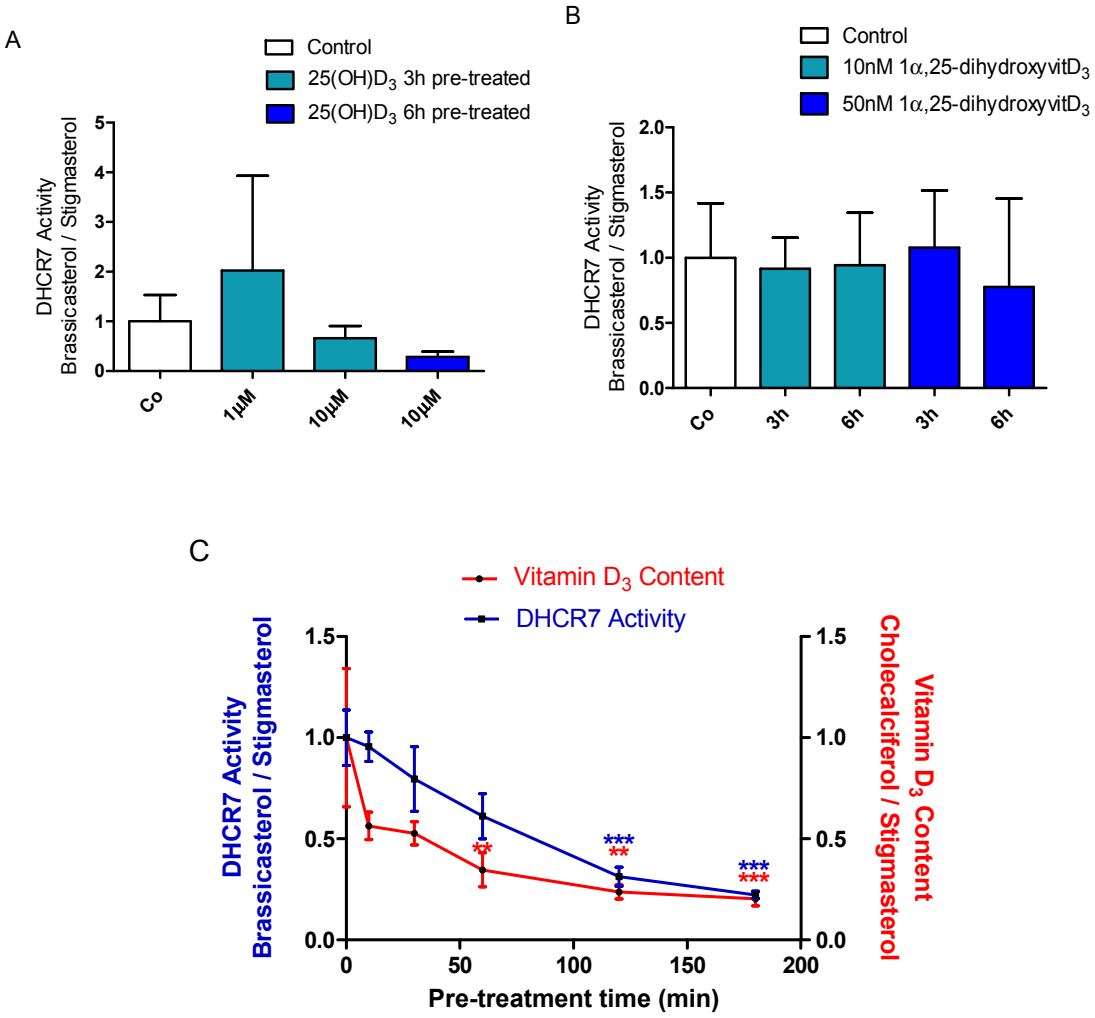

F
DHCR7 Activity
Brassicasterol / Stigmasterol
1.5
1.0
0.5
0.0
HEKa
HepG2

Figure 4.2 Vitamin D_3 has no effect on DHCR7 activities in McA-RH7777, HepG2, and HaCaT cells. Activity was measured in cell lysates after a 3-h treatment with vitamin D_3 (A, B, and C). DHCR7 mRNA levels were measured in HaCaT cells after a 24-h treatment with vitamin D_3 at various concentrations (D). DHCR7 protein expression was measured in four cell lines and in mouse microsomes (E). The DHCR7 activities in HEKa and HepG2 were measured and compared to each other (F). Values represent the mean and S.D. of 3 experiments; values that are statistically different from the control are indicated by asterisks, as determined by t-test or one-way ANOVA analysis of variance with Dunnett's multiple comparisons post hoc test, $p < 0.05$.

Effects of 25-hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3 on DHCR7 activity in HEKa and HaCaT cells

After being formed in keratinocytes, vitamin D_3 binds to vitamin D binding protein in the blood and is subsequently hydroxylated to form 25-hydroxyvitamin D_3 and then 1α,25-dihydroxyvitamin D_3 in the liver and the kidney, respectively. Keratinocytes also possess the enzymes necessary to synthesize 25-hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3. More importantly, the receptor for 1α,25-dihydroxyvitamin D_3, the vitamin D receptor (VDR), is expressed in keratinocytes and has been suggested to be involved in cancer prevention (Bikle, Elalieh et al. 2013). The 25-hydroxylated form of vitamin D_3 is less inhibitory to DHCR7 than is vitamin D_3 in HEKa cells, decreasing activity to approximately 30% of control levels after 6 h treatment (Fig 4.3A). 1α,25-Dihydroxyvitamin D_3 had no effect on DHCR7 activity in HEKa and HaCaT cells (Fig 4.3B&D).This is further supported by the observation that neither 25-hydroxyvitamin D_3 nor a metabolite of 25-hydroxyvitamin D_3 was synthesized in vitamin D_3-treated HEKa cells (Fig 4.3E). On the other hand, vitamin D_3 levels decreased by half 10 minutes after addition to HEKa cell culture (Fig 4.3C). This significant decrease in vitamin D_3 content was more rapid than that of DHCR7 activity, suggesting the involvement of metabolite(s) of vitamin D_3, other than 25-hydroxyvitamin D_3 or 1α,25-dihydroxyvitamin D_3, in the regulation of DHCR7 activity.


A
Control
25(OH)D3 3h pre-treated
25(OH)D3 6h pre-treated
DHCR7 Activity
Brassicasterol / Stigmasterol
0
1
2
3
4
5
Co
1μM
10μM
10μM
B
Control
10nM 1α,25-dihydroxyvitD3
50nM 1α,25-dihydroxyvitD3
DHCR7 Activity
Brassicasterol / Stigmasterol
0.0
0.5
1.0
1.5
2.0
Co
3h
6h
3h
6h
C
Vitamin D3 Content
DHCR7 Activity
DHCR7 Activity
Brassicasterol / Stigmasterol
Vitamin D3 Content
Cholecalciferol / Stigmasterol
0.0
0.5
1.0
1.5
0
50
100
150
200
Pre-treatment time (min)
***
**
**
***
***
D
HaCaT
Control
1,25(OH)2D3 3h pre-treated
1,25(OH)2D3 24h pre-treated
DHCR7 Activity
Brassicasterol / Stigmasterol
0.0
0.5
1.0
1.5
2.0
Co
1nM
10nM
100nM
1nM
10nM
100nM

E

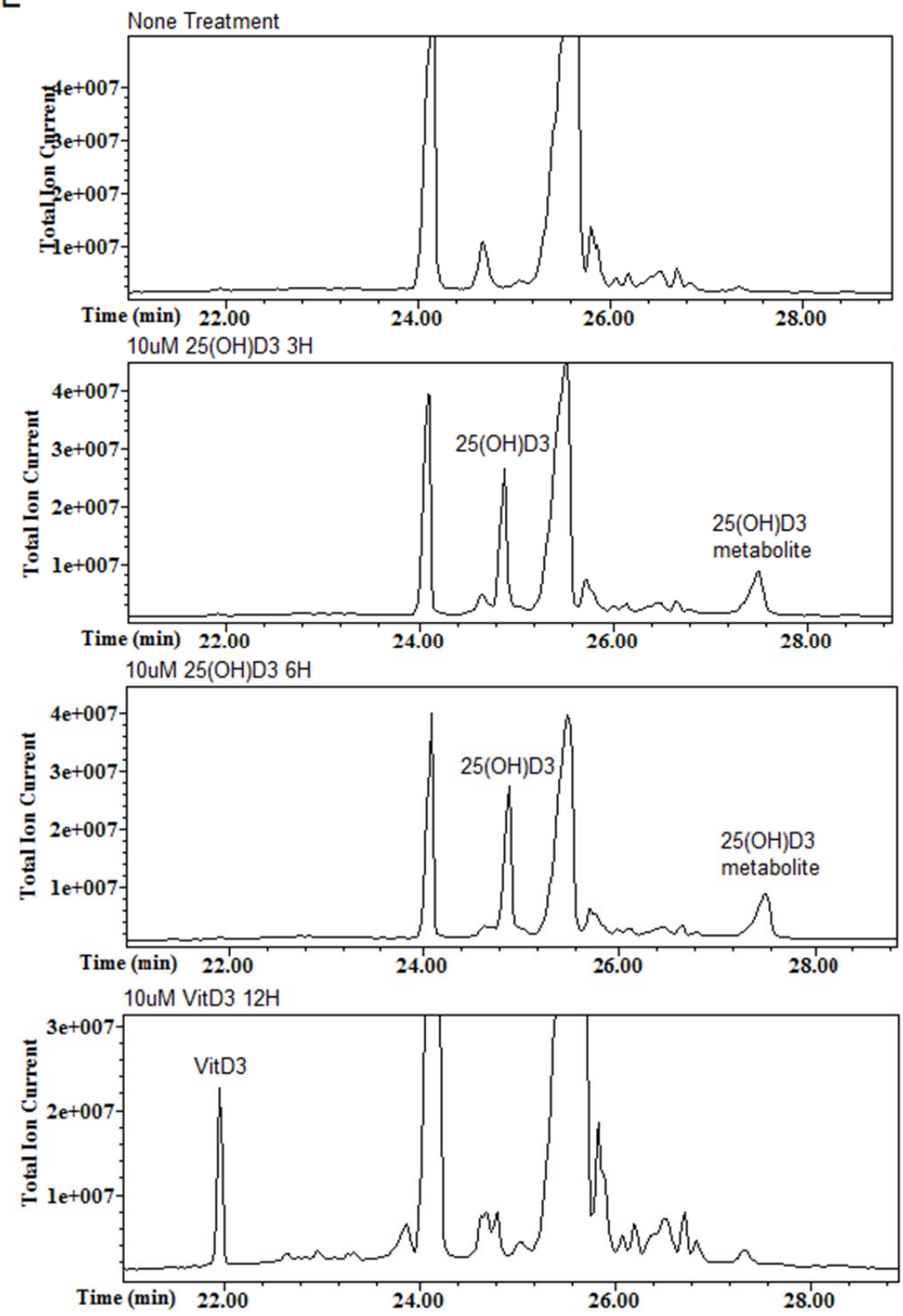

None Treatment
Total Ion Current
4e+007
3e+007
2e+007
1e+007
Time (min)
22.00
24.00
26.00
28.00
10uM 25(OH)D3 3H
25(OH)D3
25(OH)D3
metabolite
10uM 25(OH)D3 6H
25(OH)D3
25(OH)D3
metabolite
10uM VitD3 12H
VitD3

Figure 4.3 Effect of 25-Hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3 on DHCR7 activity in HEKa and HaCaT cells. DHCR7 activity was measured in intact HEKa cells after treatment with 25-hydroxyvitamin D_3 (A) or 1α,25-dihydroxyvitamin D_3 (B) at various concentrations and time points. The rate of decrease in vitamin D_3 content is compared to that of DHCR7 activity after vitamin D_3 treatment (C). Gas chromatographic profiles of HEKa untreated and cells treated with vitamin D_3 or 25-hydroxyvitamin D_3 is shown (E). Values represent the mean and S.D. of 3 or more experiments; values that are statistically different from the control are indicated by asterisks, as determined by one-way ANOVA analysis of variance with Dunnett's multiple comparisons post hoc test, $p < 0.05$.

Vitamin D_3 does not change lanosterol and 7-dehydrocholesterol content in HEKa cells

7-Dehydrocholesterol (7DHC) is the precursor for both cholesterol and vitamin D_3 synthesis. Given that vitamin D_3 decreased DHCR7 activity, I anticipated that 7DHC would accumulate in keratinocytes treated with vitamin D_3. Unexpectedly, 7DHC content did not change after vitamin D_3 pre-treatment (Fig 4.4D). There was also no change in the activity of lathosterol 5-desaturase which catalyzes the conversion of lathosterol to 7DHC, the step that immediately precedes DHCR7 (Fig 4.4C). Lanosterol, the first intermediate containing the core steroid structure, is formed further upstream in the cholesterol synthesis pathway. The inhibition of lanosterol 14-demethylase by ketoconazole provided a way to examine changes in the early steps in the cholesterol synthesis pathway after vitamin D_3 treatment. The content of lanosterol in HEKa cells was not changed significantly after short-term (3 hour) or long-term (24h) treatment with vitamin D_3, although there was a trend toward a decrease in the content of this vitamin D and cholesterol precursor (Fig 4.4A&B). This indicates that vitamin D_3 may suppress the early steps in the cholesterol/vitamin D_3 synthesis pathway.

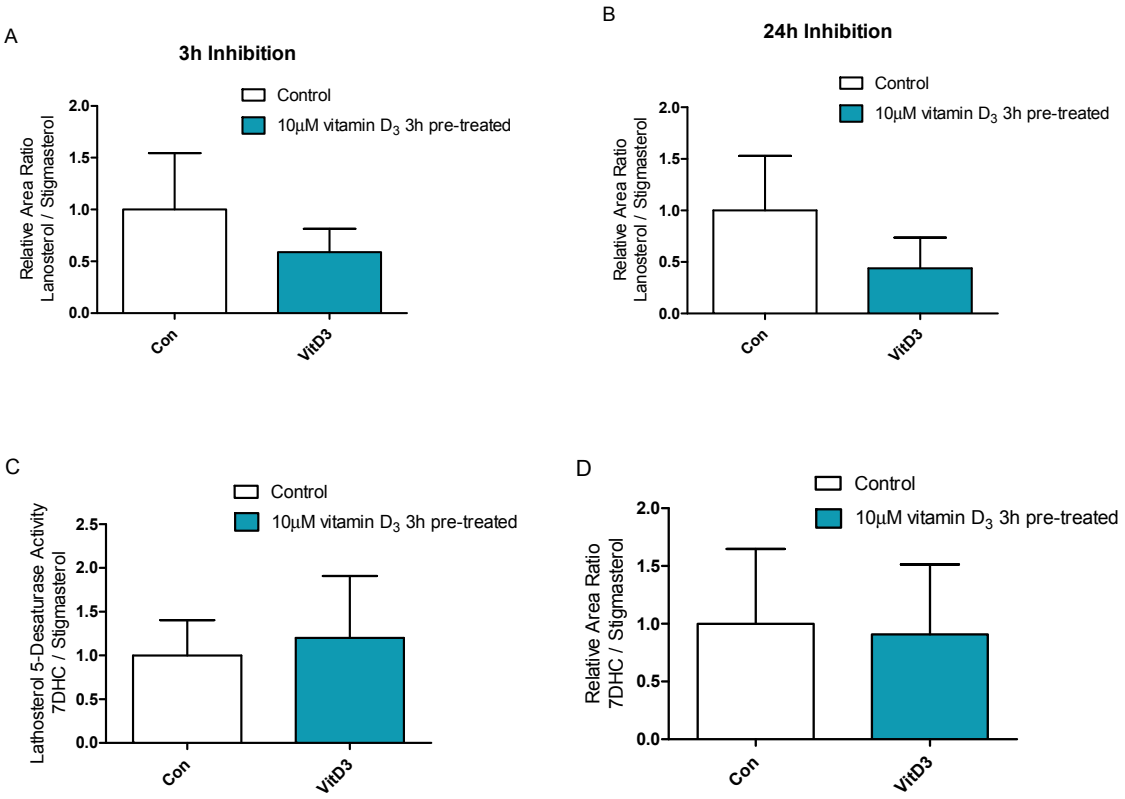


Figure 4.4 Vitamin D_3 does not change lanosterol and 7-dehydrocholesterol content in HEKa cells. Cells were treated with 10 μM of vitamin D_3 for 3 hours and then incubated with 10 μM of ketoconazole for 3 hours (A) or 24 hours (B) and the accumulated lanosterol was measured. Lathosterol activity was measured as the conversion of lathosterol to 7DHC (C). The content of 7DHC was measured in HEKa cells untreated or treated with vitamin D_3 (D). Values represent the mean and S.D. of 3-5 experiments; values that are statistically different from the control are indicated by asterisks, as determined by t test analysis, $p < 0.05$.

UVB irradiation does not affect DHCR7 activity

When keratinocytes are exposed to UVB irradiation 7DHC in these cells is converted into vitamin D_3. Therefore, it is possible that DHCR7 may be regulated by UVB irradiation through the product vitamin D_3. However, changes in DHCR7 activity following UVB irradiation were not observed (Fig 4.5A&B).

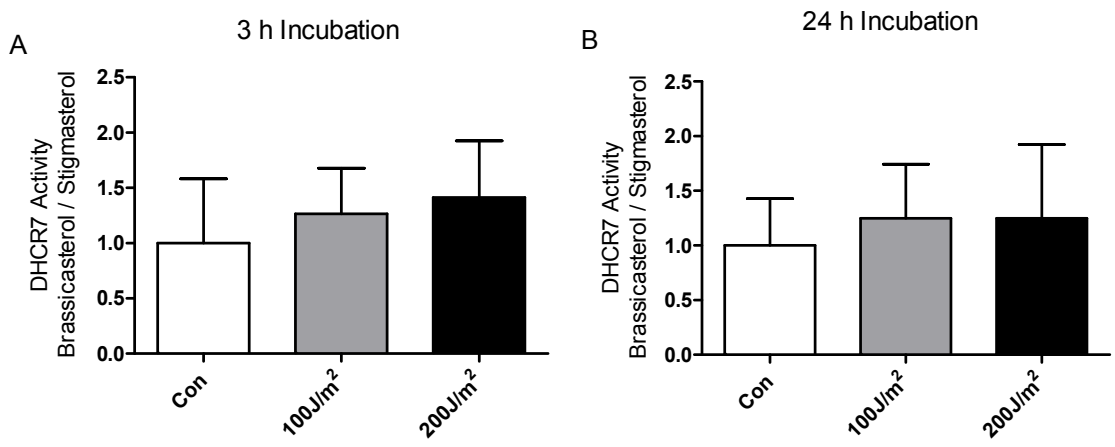


Figure 4.5 UVB does not regulate DHCR7 activity. HEKa cells were exposed to UVB with two dosings: 100 J/m^2 and 200 J/m^2. After exposure, cells were cultured for another 3 hours (A) or 24 hours (B) and DHCR7 activity was measured. Values represent the mean and S.D. of 3-4 experiments; values that are statistically different from the control are indicated by asterisks, as determined by one-way ANOVA analysis of variance with Dunnett's multiple comparisons post hoc test, $p < 0.05$.

Regulation of DHCR7 activity by vitamin D_3 is not due to phosphorylation/dephosphorylation

Shefer et al. (Shefer, Salen et al. 1998) demonstrated that DHCR7 in rat liver can be activated by phosphorylation and inactivated by dephosphorylation. I was able to confirm the activation of DHCR7 by ATP in mouse microsomes (Fig 4.6A) and rat hepatoma cell lysates (Fig 4.6B), but not in human hepatoma cell lysates (Fig 4.6C) or in human HaCaT cell lysates (Fig 4.6D). Moreover, neither ATP nor protein phosphatase 1 changed DHCR7 activity in cell lysates from both non-treated and vitamin D_3-treated human HEKa cells (Fig 4.6E), suggesting that the reduction of DHCR7 activity by vitamin D_3 is not due to dephosphorylation. These results also suggest that the response of DHCR7 to ATP may be species-dependent.

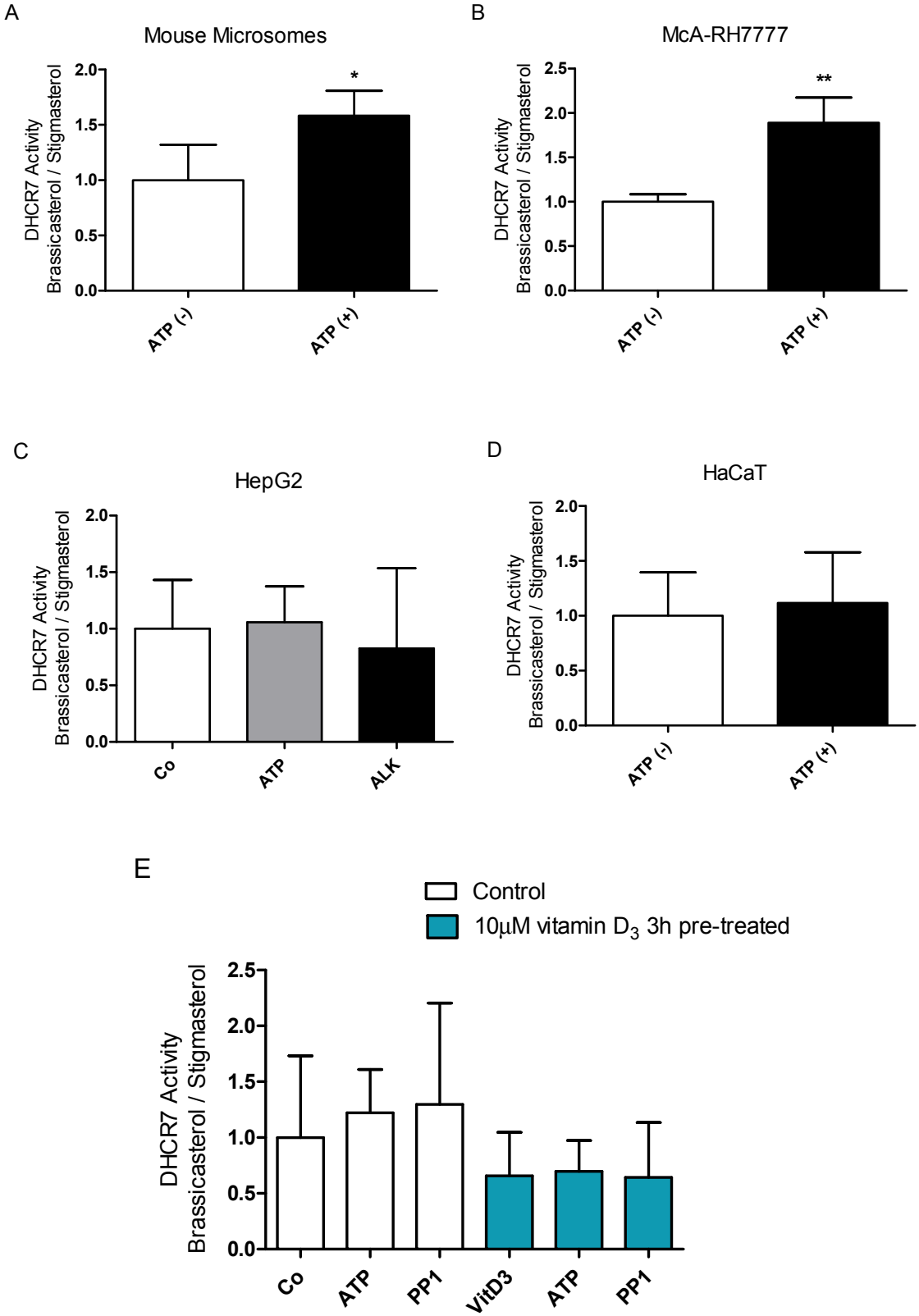

A
Mouse Microsomes
DHCR7 Activity
Brassicasterol / Stigmasterol
2.0
1.5
1.0
0.5
0.0
*
ATP (-)
ATP (+)
B
McA-RH7777
DHCR7 Activity
Brassicasterol / Stigmasterol
2.5
2.0
1.5
1.0
0.5
0.0
**
ATP (-)
ATP (+)
C
HepG2
DHCR7 Activity
Brassicasterol / Stigmasterol
2.0
1.5
1.0
0.5
0.0
Co
ATP
ALK
D
HaCaT
DHCR7 Activity
Brassicasterol / Stigmasterol
2.0
1.5
1.0
0.5
0.0
ATP (-)
ATP (+)
E
Control
10µM vitamin D3 3h pre-treated
DHCR7 Activity
Brassicasterol / Stigmasterol
2.5
2.0
1.5
1.0
0.5
0.0
Co
ATP
PP1
VitD3
ATP
PP1

Figure 4.6 Modulation of DHCR7 activity by vitamin D_3 is not due to changes in the phosphorylation state of the enzyme. Measurement of DHCR7 activity in mouse microsomes (A), rat hepatoma (B), human hepatoma (C), human HaCaT (D), and human HEKa (E) cell lysates. ATP or phosphatases were added to cell lysates (C & E). Values represent the mean and S.D. of 3 or more experiments; values that are statistically different from the control are indicated by asterisks, as determined by t-test or one-way ANOVA analysis of variance with Dunnett's multiple comparisons post hoc test, $p < 0.05$.

Regulation of DHCR7 activity by vitamin D_3 is not due to direct binding of vitamin D_3 to DHCR7 in HEKa cells

Instead of treating HEKa cells in culture with vitamin D_3, vitamin D_3 was added to cell lysates. By this means I could determine if vitamin D_3 directly inactivates DHCR7 and thereby provide a possible mechanism by which vitamin D_3 down-regulates DHCR7 activity. The reaction was initiated either immediately after vitamin D_3 addition to the cell lysate or after a 3-hour incubation of the lysate with vitamin D_3. The activity of DHCR7 did not change in the presence of low and high concentrations of vitamin D_3 or after pre-incubation with a low concentration of vitamin D_3 (Fig 4.7). The pre-incubation with 100 μM of vitamin D_3 decreased DHCR7 activity significantly. However, this concentration is far beyond the physiologic concentration, so even though vitamin D_3 decreased DHCR7 activity, this is not the likely mechanism by which vitamin D_3 regulates DHCR7 activity in HEKa cells.

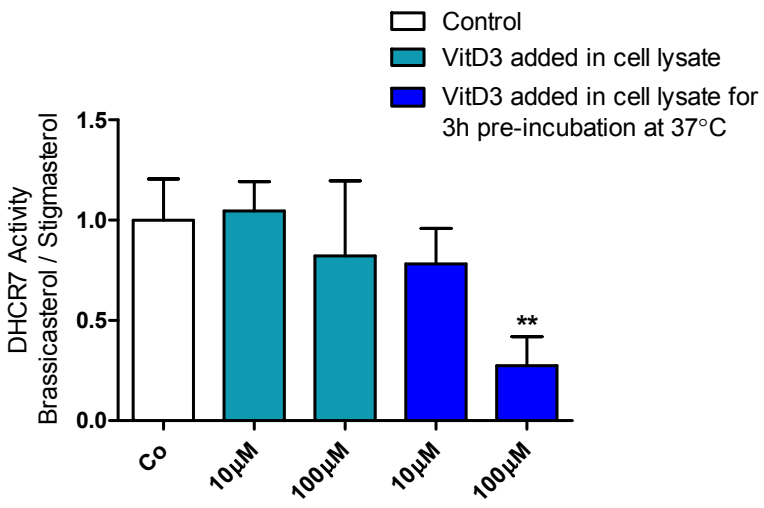


Figure 4.7 Vitamin D_3 did not inhibit DHCR7 in HEKa cell lysates. DHCR7 activity was measured immediately after the addition of vitamin D_3 to HEKa cell lysates or after a 3 hour pre-incubation with vitamin D3 at 37°C. Values represent the mean and S.D. of 3 or more experiments; values that are statistically different from the control are indicated by asterisks, as determined by one-way ANOVA analysis of variance with Dunnett's multiple comparisons post hoc test, $p < 0.05$.

Vitamin D_3 inhibits proliferation and induces differentiation of HEKa cells

During the long-term treatment of HEKa cells with vitamin D_3, the cells gradually became elongated and flat, and grew slowly (Fig 4.8B). It has been reported that vitamin D induces cell differentiation (Bikle, Ng et al. 2001) and inhibits proliferation in basal cell carcinomas (Tang, Xiao et al. 2011). The anti-proliferative and pro-differentiating effects of vitamin D_3 were determined with HEKa cells. After 48 hour treatment, 10 μM vitamin D_3 (Fig 4.8B), 10 μM 25-hydroxyvitamin D_3 (Fig 4.8D), and 100 nM 1,25-dihydroxyvitamin D_3 (Fig 4.8F) significantly inhibited the growth of HEKa cells. In contrast, 10 μM 7-DHC (Fig 4.8C), 10 nM 1,25-dihydroxyvitamin D_3 (Fig 4.8E) and 10 μM cholesterol (Fig 4.8G) did not have antiproliferative effects on HEKa cells. Vitamin D_3 induced expression of involucrin, a marker of cell differentiation (Fig 4.9A). Interestingly, 25-hydroxyvitamin D_3 and a high concentration (100 nM) of 1,25-dihydroxyvitamin D_3 also induced HEKa cell differentiation (Fig 4.9C&E). 7-DHC (Fig 4.9B) and 10 nM of 1,25-dihydroxyvitamin D_3 (Fig 4.9D) did not induce HEKa cell differentiation.

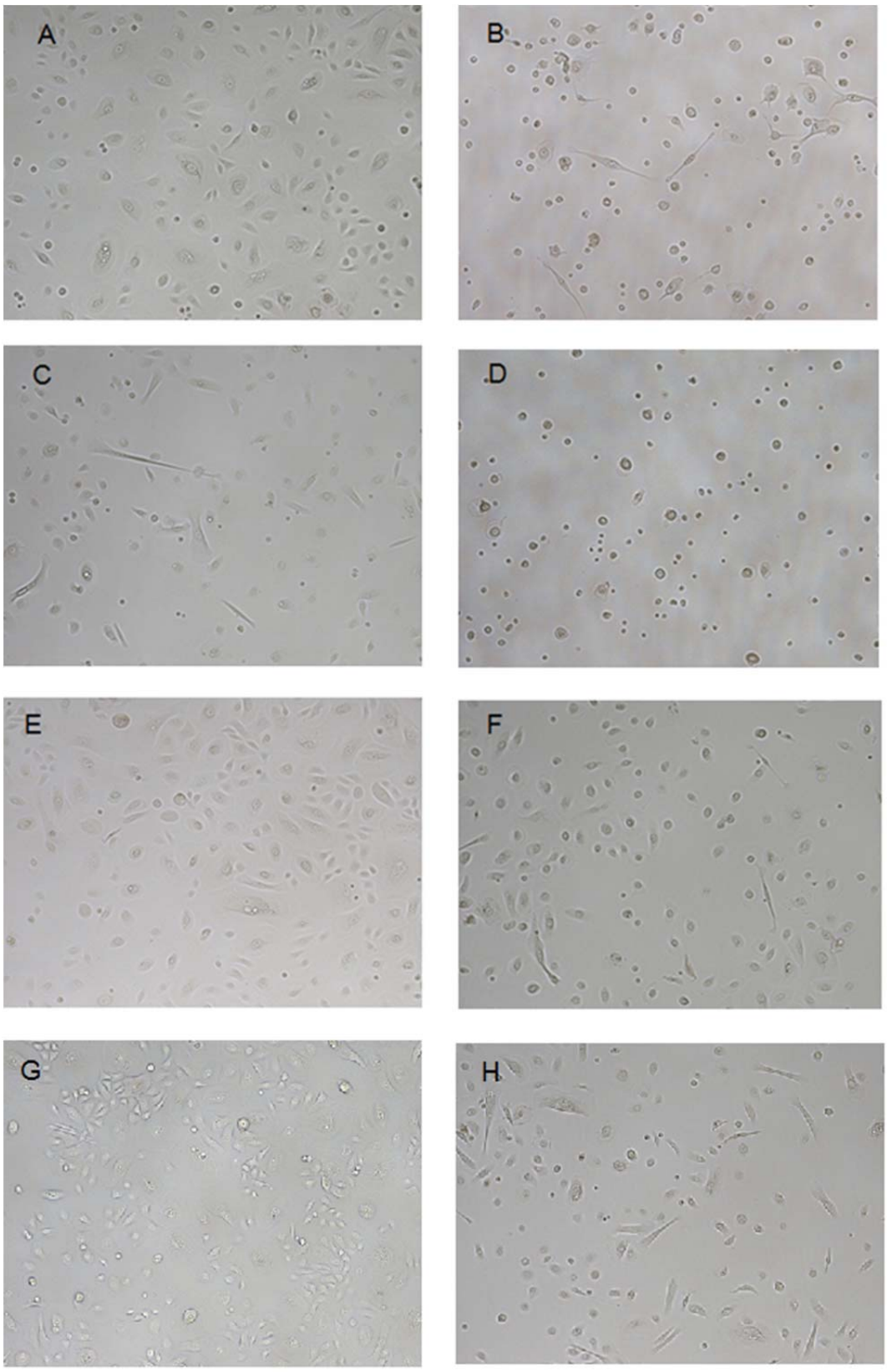

A
B
C
D
E
F
G
H

Figure 4.8 Proliferation of HEKa cells. Untreated cells (A) and cells treated with 10 μM of vitamin D_3 (B), 10 μM 7DHC (C), 10 μM 25(OH)D_3 (D), 10 nM 1,25$(OH)_2D_3$ (E), 100 nM 1,25$(OH)_2D_3$ (F), 10 μM cholesterol (G), or 10 μM cyclopamine (H) for 48 hours.

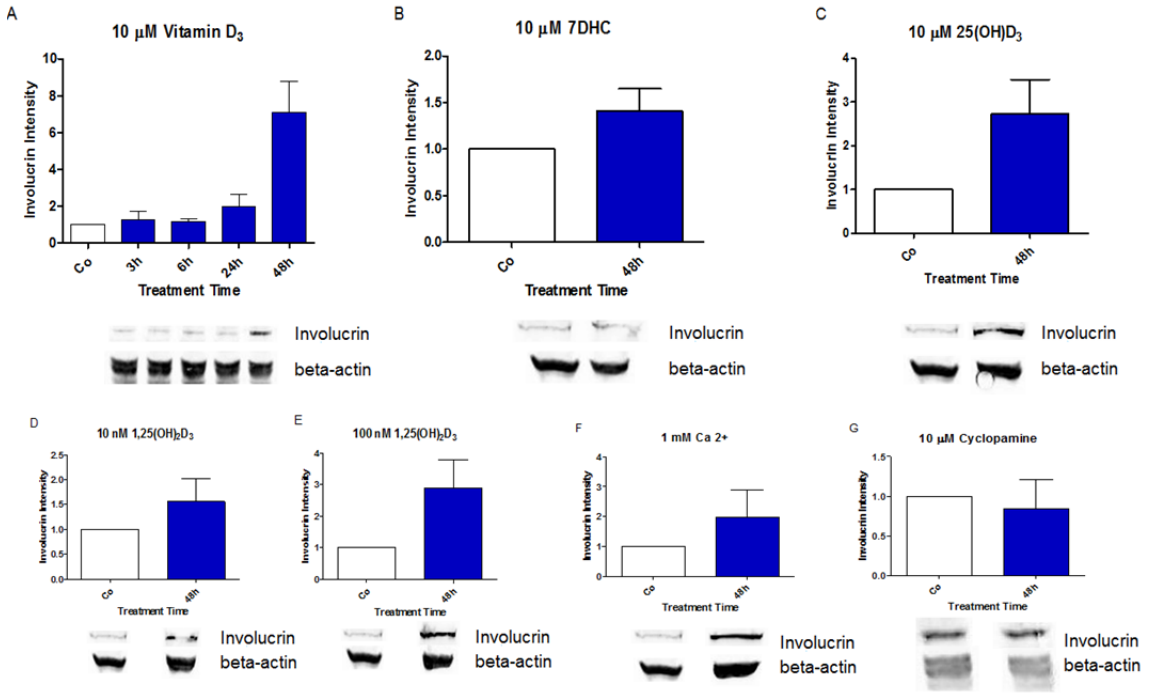


Figure 4.9 Differentiation of HEKa cells after treatment. Measurement of the expression of involucrin in HEKa cells after a 48-hour treatment with 10 µM vitamin D_3 (A), 10 µM 7DHC (B), 10 µM 25(OH)D_3 (C), 10 nM 1,25$(OH)_2D_3$ (D), 100 nM 1,25$(OH)_2D_3$ (E), 10 µM $CaCl_2$ (F), or 10 µM cyclopamine (G).

Effect of cyclopamine on DHCR7 activity

According to published results (Bijlsma, Spek et al. 2006, Tang, Xiao et al. 2011), vitamin D_3 may inhibit HEKa cell proliferation by inhibiting the hedgehog signaling pathway. In my studies cyclopamine, an inhibitor of hedgehog signaling, inhibited HEKa cell proliferation (Fig 4.8H), although not as potently as vitamin D_3. Unlike vitamin D_3, cyclopamine did not induce differentiation of HEKa (Fig 4.9G). This led me to investigate the effect of cyclopamine on DHCR7 activity and the possibility that hedgehog signaling is involved in the regulation of DHCR7 activity by vitamin D_3. Interestingly, a 3h pre-treatment with 10 μM of cyclopamine decreased DHCR7 activity by 50% ($P=0.12$), and no further inhibition after 6h pre-treatment (Fig 4.10).

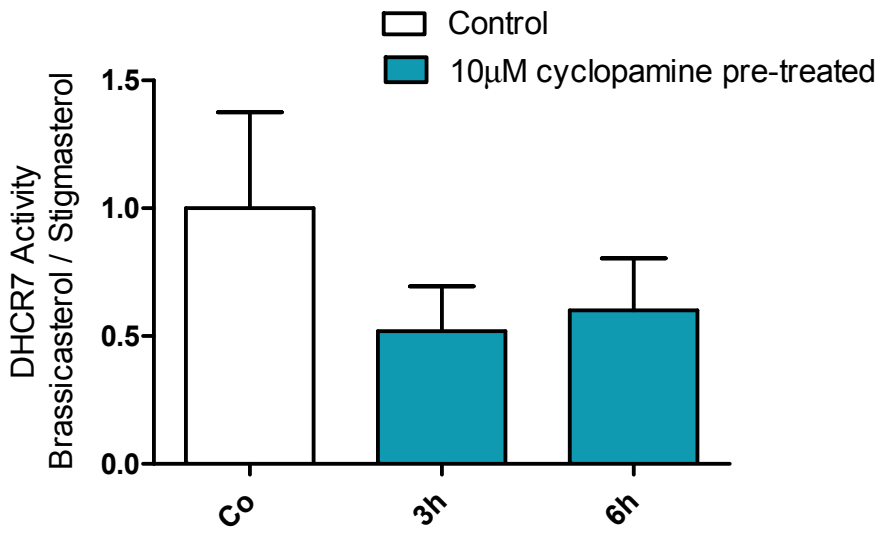


Figure 4.10 Effect of cyclopamine on DHCR7 activity. Measurement of DHCR7 activity after 3-h and 6-h pre-treatment with cyclopamine. Values represent the mean and S.D. of 3-4 experiments; values that are statistically different from the control are indicated by asterisks, as determined by one-way ANOVA analysis of variance with Dunnett's multiple comparisons post hoc test, $p < 0.05$.

Chapter 5: Summary of results

DHCR7 occupies the pivot-point between cholesterol and vitamin D_3 synthesis in skin. It is regulated by the SREBP pathway to generate the proper amount of cholesterol, but little is known about the regulation of DHCR7 by the vitamin D pathway. This is important because DHCR7 activity in skin cells likely affects the amount of 7-DHC available for vitamin D_3 photosynthesis. It was suggested that the photoproduction of vitamin D_3 is not only governed by UV light, but more importantly, by the availability of 7-DHC, which is entirely derived from biosynthesis in the skin (Esvelt, DeLuca et al. 1980). I hypothesized that as vitamin D accumulated it might promote the activation of DHCR7 to limit further vitamin D production by increasing the conversion of 7-DHC to cholesterol and depleting 7-DHC levels in the keratinocyte cell. An alternative hypothesis was that vitamin D might enhance its own production by suppressing DHCR7 activity, thereby making more 7-DHC available for photoconversion to vitamin D.

The first aim of my work was to determine if DHCR7 is regulated by vitamin D_3. In order to pursue this goal, adult human epidermal keratinocytes (HEKa cells, obtained from a commercial supplier) were chosen, as they are primary cells harvested from human skin, and are the major cell type responsible for vitamin D production. Cultured cells were treated with vitamin D_3 at various concentrations and for various lengths of time and DHCR7 activity was measured in two ways: in cell lysates and in intact cells.

Indeed, DHCR7 activity did respond to vitamin D_3 treatment. Treatment with vitamin D_3 resulted in a rapid decrease in DHCR7 activity in as little as 2 hours. This rapid down-regulation of DHCR7 activity by vitamin D_3 was not due to changes in the amount of DHCR7 protein in the cells over the course of the experiment. Long-term treatment of HEKa cells with vitamin D_3 suppressed DHCR7 activity to an even greater degree, and activity did not return fully to pre-treatment levels even 48 h after removal of vitamin D from the media. I also exposed HEKa cells to UVB irradiation on the supposition that 7-DHC in keratinocytes would be converted into vitamin D_3 and thereby decrease DHCR7 activity. However, DHCR7 activity did not change in response to UVB irradiation.

A follow-up study was designed to determine if the hydroxylated forms of vitamin D_3, 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3, are also able to regulate DHCR7 activity in HEKa cells. Compared to the rapid and strong inhibitory effect of vitamin D_3, the effect of 25-hydroxyvitamin D_3 was modest, while 1,25-dihydroxyvitamin D_3, the activated form of the vitamin that binds the vitamin D receptor, had no effect on DHCR7 activity. Vitamin D_3 rapidly disappeared from HEKa cells after addition to the media but I did not see evidence that 25-hydroxyvitamin D_3, or the 1,25-dihydroxy form of the vitamin were being formed. Thus, other unidentified metabolites may be responsible for the decrease in DHCR7 activity.

Three other cell lines were also used to evaluate the ability of DHCR7 to respond to vitamin D_3 treatment: rat hepatoma (McA-RH7777), human hepatoma (HepG2), and immortalized human keratinocyte (HaCaT) cells. The rationale was that skin

is the only organ exposed to sunlight and where both cholesterol and vitamin D_3 are synthesized. The results were consistent with this rationale in that DHCR7 from both rat and human hepatoma cells did not respond to vitamin D_3 treatment. Unexpectedly, vitamin D_3 did not affect DHCR7 activity in HaCaT cells. I did note that the DHCR7 protein level in HEKa cells was 7-fold lower than that in HepG2 cells while the activity in HEKa cells was 2-fold greater.

After treatment of HEKa cells with vitamin D_3 for 3 hours, DHCR7 activity decreased to 19% of the original levels, but 7-DHC content was unchanged. There was also no change in the activity of lathosterol 5-desaturase which catalyzes the conversion of lathosterol to 7DHC, the step that immediately precedes DHCR7. The content of lanosterol, an intermediate formed further upstream in the cholesterol synthesis pathway, was not changed significantly after short-term (3 hour) or long-term (24h) treatment with vitamin D_3, although there was a trend toward a decrease in the content of this vitamin D and cholesterol precursor. These results suggest that vitamin D_3 rapidly down-regulates cholesterolgenesis presumably at a very early step in the pathway, as neither lanosterol nor 7DHC accumulate in vitamin D_3-treated cells.

Shefer et al. (Shefer, Salen et al. 1998) demonstrated that DHCR7 in rat liver can be activated by phosphorylation and inactivated by dephosphorylation. This is the only rapid regulation of DHCR7 reported to date. I was able to replicate the activation of DHCR7 by ATP in mouse microsomes and in rat hepatoma cells, but not in human hepatoma cells nor in human keratinocytes, HaCaT and HEKa.

In addition, phosphatases such as alkaline phosphatase and protein phosphatase 1 did not decrease DHCR7 activity in either keratinocyte line.

The vitamin D receptor might be expected to be involved in the regulation of DHCR7, given that 1,25-dihydroxyvitamin D_3 modulates the transcription of numerous genes in skin and other tissues. However, DHCR7 activity was not affected by 1,25-dihydroxyvitamin D_3 treatment, even at a pharmacologic concentrations, in either keratinocyte cell line. Therefore, it does not appear that the VDR is involved in the rapid regulation of DHCR7 by vitamin D_3.

Other means of suppressing DHCR7 activity, including direct inhibition by vitamin D or allosteric modulation of activity were also considered, as AY-9944 and BM 15.766 are established non-competitive inhibitors of DHCR7, binding either to the free enzyme or to the enzyme-substrate complex (Shefer, Salen et al. 1998). I did not find evidence for a direct inhibition of DHCR7 by vitamin D_3 in my studies. Addition of vitamin D_3 to HEKa cell lysates did not change DHCR7 activity and only extended incubation of the cell lysate with a high concentration of vitamin D_3 produced a decrease in DHCR7 activity.

An unexpected observation was that vitamin D_3 inhibited proliferation and induced differentiation of HEKa cells. In healthy adult human skin, keratinocytes undergo a continuous process of self-renewal as they progressively differentiate and move to the outermost layer of the tissue (Swanson 2004). Little is known about the effect of vitamin D_3 on this physiologic process even though it has been suggested that vitamin D_3 inhibits proliferation of basal cell carcinoma

through the hedgehog pathway (Tang, Xiao et al. 2011). My studies show that vitamin D_3, 25-hydroxyvitamin D_3, and 1,25-dihydroxyvitamin D_3 (100 nM) inhibit the growth of HEKa cells. Vitamin D_3 also induced differentiation, increasing involucrin expression after 48 hours of treatment. 25-Hydroxyvitamin D_3 also induced involucrin expression, but not as potently as vitamin D_3. 1,25-Dihydroxyvitamin D_3 only induced involucrin expression at a high concentration (100 nM). 7-DHC and 1,25-dihydroxyvitamin D_3 (10 nM) had no effect on HEKa cell growth or induction of involucrin expression.

It has been suggested that vitamin D_3 is a potent inhibitor of hedgehog signaling (Hh), binding to *Smo* at the same site as cyclopamine, an inhibitor of Hh and a potential agent for cancer therapy (Bijlsma, Spek et al. 2006). Therefore, the possible involvement of Hh in vitamin D3-mediated regulation of DHCR7 activity was determined by testing the effect of cyclopamine on DHCR7 activity. Although not as potent as vitamin D_3, treatment of HEKa cells with cyclopamine for 3 hours inhibited DHCR7 activity by 50%. This interesting result suggests a possible role of Hh in the regulation of DHCR7 by vitamin D_3.

Chapter 6: Discussion

Skin is the only organ where both cholesterol and vitamin D_3 are synthesized. In this dissertation I have attempted to test the hypothesis that the activity of DHCR7 governs the availability of 7-DHC for vitamin D_3 photoproduction by controlling the conversion of 7-DHC to cholesterol. It has been reported that 7-DHC is entirely derived from biosynthesis in the skin (Esvelt, DeLuca et al. 1980). Therefore it is important to understand the regulation of DHCR7 by both end-products in keratinocytes, the major cell type responsible for vitamin D_3 production in skin. It is well established that DHCR7 is regulated by the SREBP pathway to generate proper amounts of cholesterol, but little is known about the regulation of DHCR7 by the vitamin D pathway.

In the present study, I investigated the regulation of DHCR7 by vitamin D_3 in keratinocytes. By treating cultured HEKa cells with vitamin D_3 at various concentrations and for various length of time, I determined that vitamin D_3 is capable of DHCR7 repression. The inhibitory effect is rapid at a pharmacological concentration (10 μM), decreasing DHCR7 activity by 70% after treatment for as little as 2 hours without changing DHCR7 protein levels. This result suggests that vitamin D_3 inhibits the conversion of 7-DHC to cholesterol and regulates the synthesis of itself in a positive feedback manner. Similar observations were published by other groups that chronic dosing of vitamin D-deficient rats with 1,25-dihydroxyvitamin D_3 caused a 4-fold increase of 7-DHC content in skin (Esvelt, DeLuca et al. 1980) and long-term (4 weeks) exposure of rats to ultraviolet irradiation, which should result in vitamin D_3 generation, increased 7-

DHC content by 60% in skin (Takada 1983). However, unlike these two *in vivo* studies, in my studies 7-DHC content in cultured HEKa cells was not changed after a 3-h treatment with vitamin D_3. This may be due to high basal levels of 7-DHC in keratinocytes and/or the short length of treatment. It was shown that the 7-DHC content of skin was much greater than that found in liver (250:1, μg/g) (Takada, Ito et al. 1981). Therefore, although vitamin D_3 markedly inhibited DHCR7 activity, 7-DHC content was not likely to increase to a significant degree in 3 hours. In addition, my studies showed that the lanosterol content and the activity of the 7-DHC generating enzyme, lathosterol-5-desaturase, were also unchanged, indicating that vitamin D_3 rapidly down-regulates cholesterologenesis, presumably at a very early step in the pathway.

In my studies, the regulation of DHCR7 activity by vitamin D_3 was found only in HEKa cells, and was not evident in rat hepatoma cells, human hepatoma cells, or in immortalized human keratinocyte cells. I also observed that DHCR7 protein levels in HEKa cells were 7-fold lower than the levels measured in HepG2 cells, but the activity was 2-fold higher. Clearly, DHCR7 in keratinocytes exhibits some unique characteristics with regard to cholesterologenesis and vitamin D_3 production.

In 1983, Nemanic et al. suggested that keratinocytes can adjust DHCR7 activity over a wide range to control both cholesterologenesis and vitamin D_3 generation in skin (Nemanic, Whitney et al. 1983). Very few studies had focused on the regulation of DHCR7 until 1998 when Shefer et al. suggested that DHCR7 exists in two forms: an active phosphorylated form and an inactive dephosphorylated

form (Shefer, Salen et al. 1998). In my studies, therefore, I explored possible mechanisms by which DHCR7 is regulated, focusing on the rapid inhibitory effect of vitamin D_3.

First of all, I tested the possibility that vitamin D_3 promotes the dephosphorylation of DHCR7. I replicated the activation of DHCR7 by ATP in mouse microsomes and rat hepatoma cells, but ATP had no effect on DHCR7 activity in HepG2 cells, HaCaT cells, or in HEKa cells, suggesting that the different responses of DHCR7 to ATP are species- and cell-type dependent. Addition of phosphatases such as alkaline phosphatase and protein phosphatase 1 did not decrease DHCR7 activity in HepG2 or HEKa cells, indicating that dephosphorylation of DHCR7 was not likely to be the mechanism by which vitamin D_3 inactivates this enzyme in HEKa cells.

Direct inhibition or allosteric modulation of enzyme activity by vitamin D_3 was also considered as a possible mechanism by which vitamin D_3 inactivates DHCR7. Incubation of cell lysates with vitamin D_3 did not change DHCR7 activity except upon incubation in the presence of a very high concentration (100 μM) at 37°C for 3 h. This concentration is 10-fold higher than that used to treat cells. This lack of a direct effect of vitamin D_3 on DHCR7 raised the possibility that vitamin D_3 binds to a membrane receptor on whole cells, initiating a signaling cascade to decrease DHCR7 activity. It was reported recently that vitamin D_3 can bind with high affinity to the membrane protein “smoothened” (Smo), to inhibit hedgehog signaling (Bijlsma, Spek et al. 2006). Hedgehog (Hh) plays a role in development and stimulates carcinogenesis when dysregulated (Tang, Xiao et al. 2011).

Interestingly, vitamin D_3 inhibited proliferation and induced differentiation of HEKa cells after a 48 h treatment. In support of the report that vitamin D_3 serves as an inhibitor of Hh signaling by binding to Smo, cyclopamine, a known inhibitor of hedgehog signaling, also decreased DHCR7 activity in HEKa cells after a 3 h treatment. Testing the possible involvement of hedgehog signaling in the regulation of DHCR7 by vitamin D_3 certainly warrants further investigation; one interesting experiment would be to determine the effect of vitamin D_3 on DHCR7 activity in Smo $^{-/-}$ animal models.

Esvelt et al. suggested that the vitamin D receptor was involved in the increase of 7-DHC content in rat skin induced by 1,25-dihydroxyvitamin D_3 (Esvelt, DeLuca et al. 1980). This is possible because in their study rats were given 1,25-dihydroxyvitamin D_3 subcutaneously for 7 days, long enough for increases in the transcription and translation of vitamin D receptor target genes, resulting in the accumulation of 7-DHC. On the other hand, it has also been suggested that 1,25-dihydroxyvitamin D_3 is able to exert a direct and rapid action on its target cells by binding to membrane receptors other than the traditional vitamin D receptor (Nemere, Schwartz et al. 1998). In my study, however, DHCR7 in HEKa cells did not respond to low nor high concentrations of 1,25-dihydroxyvitamin D_3 after a 3 h treatment.

In my study, vitamin D_3 rapidly disappeared from HEKa cells after addition to the media but I did not see evidence that 25-hydroxyvitamin D_3, nor the 1,25-dihydroxy form of the vitamin were being formed. Thus, other unidentified

metabolites may be ultimately responsible for the more prolonged decrease in DHCR7 activity.

Takada suggested that the accumulation of 7-DHC after ultraviolet irradiation might be due to the ability of photosynthesized vitamin D_3 to prevent the conversion of 7-DHC to cholesterol (Takada 1983). In my study I showed that vitamin D_3 treatment decreased DHCR7 activity in HEKa cells. However, DHCR7 activity in HEKa cells was not affected by UVB irradiation. This discrepancy may be due to different systems (*in vivo* vs. *in vitro*). Although HEKa cells are primary cells harvested from human skin, keratinocytes in cultured media may be different from those in epidermis in terms of 7-DHC content, DHCR7 protein level, and enzymatic activities. The response of DHCR7 to UVB irradiation may also require an epidermal microenvironment which is absent in monolayer culture. In addition, the exposure of HEKa cells to UVB irradiation *in vitro* might not generate enough vitamin D_3 to decrease DHCR7 activity. Nemanic et al. (Nemanic, Whitney et al. 1983) were first able to show the production of vitamin D_3 in monolayer human keratinocytes by exposing the cells to UVB with total dose of 5000 J/m^2. These cells were also pre-treated with AY-9944, an inhibitor of DHCR7, resulting in the accumulation of a large quantity of 7-DHC to ensure the production of vitamin D_3. In my study the cells received a much lower dose of UVB (100 J/m^2 or 200 J/m^2) and no DHCR7 inhibitor was present. This 50-fold lower level of UVB exposure is commonly used *in vitro* for DNA repair and immunosuppression studies (Enk, Sredni et al. 1995) but may have been insufficient to generate enough vitamin D_3 to regulate DHCR7. In order to

determine the response of DHCR7 to UVB irradiation, a better *in vitro* system needs to be developed.

In summary, vitamin D_3 inhibited DHCR7 activity at a pharmacological concentration only in HEKa cells. Whether vitamin D_3 regulates DHCR7 activity at lower, and perhaps more physiological concentrations is unclear. However, the decrease in DHCR7 activity, the overall decrease in cholesterologenesis, and the inhibition of proliferation and promotion of differentiation may all be mediated through the inhibition of hedgehog signaling by vitamin D_3.

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Academic Degrees

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Honors and Awards

- Kentucky Opportunity Fellowship 2012 – 2013
- Best Abstract Competition 3rd place, Experimental Biology 2012, Integrative Systems, Translational and Clinical Pharmacology Division, American Society for Pharmacology and Experimental Therapeutics, April, 2012.
- ISTCP Young Investigators Award Platform 3rd place, Experimental Biology 2012, Integrative Systems, Translational and Clinical Pharmacology Division, American Society for Pharmacology and Experimental Therapeutics, April, 2012.

Publications

- Porter TD, Banerjee S, Stolarczyk EI, **Zou L** (2011) Suppression of cytochrome P450 reductase expression in hepatoma cells replicates the hepatic lipidosis observed in hepatic POR-null mice. *Drug Metab. Dispos.* 39, 966-973.

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Oral Presentations

- Regulation of 7-Dehydrocholesterol Reductase by Vitamin D, Drug Discovery Division, Department of Pharmaceutical Sciences, May, 2011.
- Cholecalciferol Increases 7-Dehydrocholesterol Reductase Activity in Adult Human Epidermal Keratinocytes, Drug Discovery Division, Department of Pharmaceutical Sciences, February, 2012.
- Cholecalciferol increases 7-dehydrocholesterol reductase activity in adult human epidermal keratinocytes, ISTCP Young Investigators Award Platform Session, Experimental Biology 2012, Integrative Systems, Translational and Clinical Pharmacology Division, American Society for Pharmacology and Experimental Therapeutics, April, 2012.

Conference Abstracts

- **Zou, L.**, Li, L., and Porter, T.D. (2009) Cytochrome P450 reductase is not necessary for 7-dehydrocholesterol reductase activity. Gill Heart Institute Cardiovascular Research Day, Oct 15, Lexington, KY.
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- **Zou, L**. and Porter, T.D. (2012) Cholecalciferol increases 7-dehydrocholesterol reductase activity in adult human epidermal keratinocytes. Experimental Biology, Apr 21-25, San Diego, CA.