The Significance of Enzyme 3β-hydroxysterol-Δ24 reductase in Cholesterol Biosynthesis and Steroidogenesis: An *in vitro* Model to study Desmosterolosis

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ABSTRACT

Desmosterolosis is an autosomal recessive condition in which affected individuals lack expression of the final enzyme in the cholesterol biosynthetic pathway, 3β-hydroxyΔ^{24}cholesterol reductase (DHCR24). The enzyme is responsible for converting desmosterol to cholesterol. It is characterized by several congenital abnormalities, as well as high circulating levels of desmosterol accompanied by hypocholesterolemia.

This study was intended to examine the molecular disruptions involved in desmosterolosis and determine the potential impact on steroidogenesis using an *in vitro* approach. Due to the importance of cholesterol in cell-signaling and cell membrane integrity, it was found that desmosterol was a poor substitute for cholesterol in cell membranes. Cells deprived of cholesterol for 45 minutes rapidly underwent morphological changes and presumably became apoptotic. This effect was potentiated in cells with DHCR24 knock-down. Additionally, cells responded by increasing levels of cholesterol biosynthetic factors such as, SREBP1, SREBP2, SCAP, S1P, as well as the enzyme HMG Co A reductase all of which were measured using real-time PCR. The increase in these transcripts indicates that desmosterol cannot be sensed by the sterol sensing domain (SSD). Following cholesterol repletion, cells were able to restore a certain level normalcy in terms of morphology and the production of the transcripts listed above.

Cholesterol is required for steroidogenesis in the adrenal cortex. We examined whether or not desmosterol could be used as a steroidogenic substrate. We measured levels of steroidogenic acute regulatory protein (StAR) as well as measured cortisol production with DHCR24 knock-down following cholesterol depletion and repletion with and without trophic stimulation. We found that in cells stimulated with vasoactive intestinal polypeptide, devoid of cholesterol and with endogenous synthesis impaired at the level of DHCR24, there was a marked increase in cholesterol transcription components indicating that desmosterol was a poor steroidogenic substrate. Interestingly, cortisol production was similar in desmosterol-producing and in normal cells, indicating that desmosterol may have been substituting for cholesterol.

RESUME

La desmostérolose est une maladie autosomale récessive dans laquelle les individus atteints ont un déficit de l'expression de l'enzyme terminale du processus de biosynthèse du cholestérol, connue sous le nom de 3β-hydroxyΔ ²⁴cholesterol réductase (DHCR24). Cette enzyme est chargée de convertir le desmostérol en cholestérol. Cette maladie est caractérisée par plusieurs anomalies congénitales, ainsi que par des taux élevés de desmostérol dans le sang accompagnés d'hypocholestérolemie.

L'objectif de cette étude a été d'examiner les anomalies provoquées par la desmostérolose au niveau moléculaire ainsi que son impact sur la stéroïdogenèse en utilisant une approche *in vitro*. Nous avons, pour cela, utilisé la technique d'interférence par des ARN pour diminuer l'expression de l'enzyme DHCR24 reproduisant ainsi les conditions de la desmostérolose dans notre modèle cellulaire.

Le cholestérol participe à de nombreux processus de signalisation cellulaire et joue un rôle important dans la stabilité des membranes constituant les cellules. Au cours de notre étude nous avons constaté que le desmostérol du fait de sa structure n'est pas capable de se substituer au cholestérol pour remplir ces fonctions. En effet, les cellules dépourvues de cholestérol pendant 45 minutes ont systématiquement subi un changement de morphologie et ont par la suite présenté les caractéristiques de cellules en apoptose. Ce phénomène était encore plus prononcé dans les cellules où l'expression de DHCR24 avait été diminuée expérimentalement. Nous avons également examiné si le desmostérol pouvait être reconnu par la protéine dite 'domaine de reconnaissance des stérols' (SSD) qui *in vivo* stimule la synthèse endogène de cholestérol quand ses niveaux dans la circulation sanguine sont trop bas. Pour cela, nous avons mesuré les taux d'expression des protéines suivantes : 'sterol regulatory element binding protein' (SREBP), 'SREBP cleavage activating protein' (SCAP), HMG CoA reductase (HMGCoA) impliquée dans la synthèse du cholestérol. Suite à la déplétion en cholestérol, nous avons observé une augmentation de l'expression de ces facteurs dans les cellules. Ceci indique que le desmostérol ne peut pas être reconnu par la SSD. Nous l'avons confirmé en réintroduisant du cholestérol ce qui se traduisit par un retour à des niveaux d'expression normaux de ces facteurs.

Le cholestérol est également nécessaire pour la stéroïdogenèse du cortex surrénal. Nous avons donc examiné les effets d'un remplacement du cholestérol par le desmostérol sur la fonction stéroïdogène de cellules surrénaliennes. Pour ce faire, nous avons déterminé les niveaux d'expression de la protéine 'Steroidogenic Acute Regulatory Protein' (StAR) ainsi que des protéines SREBP, SCAP, HMGCoA et nous avons mesuré la production de cortisol à la suite d'un knock-down de DHCR24. Nos résultats indiquent qu'il y a une augmentation des facteurs impliqués dans la synthèse du cholestérol dans les cellules stimulées par le 'vasoactive intestinal polypeptide' (VIP) pour induire une sécrétion de cortisol et dépourvues en cholestérol. Ceci tend à prouver une nouvelle fois que le desmostérol ne peut se substituer à toutes les fonctions du cholestérol. Cependant, il est intéressant de souligner que la production de cortisol et l'expression de la StAR sont similaires dans les cellules normales et celles produisant principalement du desmostérol. A ce niveau, le desmostérol semble avoir remplacé le cholestérol.

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LIST OF ABBREVIATIONS

ACC.....Adrenocortico Carcinoma
ACTH.....Adrenocorticotropic hormone
ACTHR.....Adrenocorticotropic hormone receptor
ATCC.....American type culture collection
CHILD.....Congenital hemidysplasia with ichtyosiform erythroderma and limb defects
CLCD.....Cholesterol-loaded cyclodextrin
DHCR7.....Dehydrocholesterol reductase7
DHCR24.....3β-hydroxysterol-24-reductase (dehydrocholesterol reductase24)
Disp.....Dispatched
EIA.....Enzyme ImmunoAssay
HMG CoA...Hydroxyl methyl glutaryl coenzyme A reductase
Hh.....hedgehog protein
MCD.....methyl-β-cyclodextrin
Ptc.....Patched
PACAP.....Pituitary adenylate cyclase activating peptide
StAR.....Steroidogenic acute regulatory protein
SRE.....Sterol Regulatory Element
SREBP.....Sterol Regulatory Element Binding Protein
SCAP.....Sterol regulatory element binding protein Cleavage Activating Protein
SSD.....Sterol sensing domain
Smo.....Smoothened
TF.....Transcription Factor
VIP.....Vasoactive Intestinal Peptide

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CHAPTER I. INTRODUCTION

A number of inherited disorders of cholesterol biosynthesis have been identified in the last 15 years. The cholesterol/isoprenoid biosynthetic pathway is characterized by a number of different intermediates all of which require enzymes for catalysis. The absence of these enzymes has emphasized the important role of cholesterol in maintaining cell membrane integrity, as well as its critical role in cell signalling cascades and the formation of bile acids, steroid hormones, vitamin D, oxysterols, and meiosis activating sterols (MAS). It is also proven to be an essential component for the post-translational modification of hedgehog signalling molecules, a family of morphogen proteins required for embryonic patterning and segment polarity. Cholesterol can be obtained either from endogenous *de novo* synthesis or exogenously. Low levels of cellular cholesterol are detected by the SREBP/SCAP proteins which can act to regulate the transcription of genes necessary to make cholesterol biosynthetic enzymes.

Attributable to the large amount of enzymes involved in the synthesis of cholesterol, it is not surprising that there have been several genetic defects resulting from dysfunctional enzymes within the pathway. Cholesterol biosynthesis can be divided into pre- and post-squalene sections which consist of the proximal (from acetate to lanosterol) and distal (lanosterol to cholesterol) sections respectively. The only known disorder of the pre-squalene section of the pathway involves the loss of function of the mevalonate kinase enzyme resulting in mevalonic aciduria (Hoffmann et al., 1986). However several heritable diseases associated with mutations in the post-squalene pathway have since

been discovered including Smith-Lemli-Opitz syndrome (SLOS), Desmosterolosis, Latherosterolosis, Congenital hemidysplasia with ichtyosiform erythroderma and limb defects (CHILD), hydrops-ectopic calcification-moth-eaten skeletal dysplasia, X-linked dominant chondroplasia and Antley-Bixler syndrome (Ngozi et al., 2001).

The focus of this thesis is on Desmosterolosis. Desmosterolosis is an autosomal recessive disorder caused by a mutation in the 3β-hydroxysterol-Δ-24-reductase gene which is responsible for the conversion of desmosterol to cholesterol. The disease was first reported in 1998 by Fitzpatrick and colleagues and is characterized by a number of congenital defects which result from a defective 3β-hydroxysterol-Δ24-reductase (DHCR24) leading to the accumulation of desmosterol and hypocholesterolemia. The importance of cholesterol is emphasized within this disorder since the result of DHCR24 absence has proven to be lethal at least in humans causing peri-natal death. Due to the requirement of cholesterol as the precursor for steroidogenesis, it is suspected that cholesterol-related diseases have altered levels of steroid production. (Andersson et al., 2002)

All steroid hormones utilize cholesterol as the normal precursor. Cholesterol trafficking inside the cell plays a vital role in the regulation of hormone synthesis in steroid-producing cells. Upon stimulation, cholesterol within the cytoplasm is rapidly transported across the inner mitochondrial membrane by the Steroidogenic Acute Regulatory (StAR) protein where CYP11A1 (cytochrome P450 side-chain cleavage) enzyme is located and is responsible for the conversion of cholesterol to pregnenolone, the precursor for all steroids. As a by-product of side-chain cleavage, 4-methylpentanal is

also released. Since the latter is cytotoxic, it is reduced to isocaproic acid and isocapryl alcohol by the aldose-ketose reductase superfamily (Lefrançois-Martinez et al., 1999). The effect of substituting desmosterol for cholesterol may modify the bioactivity of these reductases due to the presence of a double bond at C3 in the released side-chain which in turn may affect steroidogenesis and/or cytotoxicity. It is suspected, given the consequences of substituting desmosterol for cholesterol in cell membranes, desmosterol may change the way StAR binds to membranes which would result in an alteration of the rate at which sterols are translocated across mitochondrial membranes. The end result of this would be a decrease in steroidogenesis and possibly the accumulation of lipids in steroidogenic cells (Zadworny et al. 2003)

Accordingly, the effects of desmosterol were assessed on the human cell-line H295R, which was derived from an adrenocortical carcinoma of the zona fasciculata. This cell line produced cortisol under appropriate induction conditions. In order to mimic desmosterolosis, DHCR24 was knocked-down using RNA interference.

CHAPTER II. REVIEW OF LITERATURE

2.1.1 Cholesterol structure

Cholesterol, (5α-cholesta-5-en-3β-ol) is a 27 carbon monounsaturated sterol containing a 4 fused ring structure of perhydrocyclopentano-phenathrene with molecular formula $C_{27}H_{46}O$ (Shieh et al., 1980). The molecule consists of four 6-carbon rings which forms a structure with a chair-like appearance. Cholesterol has many optical isomers forming both cis- and trans- structures.

2.1.2 Desmosterol Structure

Desmosterol, (5α cholesta 5, 24-dien-3β-ol or 24 dehydrocholesterol) is the immediate precursor of cholesterol. The only structural difference lies in the double bond located at carbon 24. The enzyme 24 dehydocholesterol reductase is responsible for the conversion of desmosterol to cholesterol (Frantz and Schroeper, 1967, Bae and Paik 1997, Waterham and Wanders, 2000).

2.2 Cholesterol Functions

Cholesterol is found ubiquitously in animals and is a primary constituent of eukaryotic cell membranes. It controls membrane fluidity and hence integrity via the presence of acyl chains embedded in the membrane. In erythrocyte plasma membranes, it affects the function of hexose transporters (Conolly, 1985). It is also found throughout both the central and peripheral nervous systems in the plasma membranes intertwined with phospholipids and myelin (Clayton 2005). Cholesterol is also a major constituent of

both lipid rafts and caveolae which function in cell signalling and signal transduction (Papanikalou, 2005). It is also known that cholesterol and possibly its intermediates can be used as precursors for steroid hormones, vitamin D, Ubiquinone, Heme A, Dolichol, and Prenylated proteins (King, 1996). Additionally, cholesterol has been deemed critical in the post-translational modification of hedgehog proteins, which are responsible for segment polarity during embryonic development (Porter, 1996).

2.2.1 Cholesterol in lipid rafts

Most cholesterol within cells is localized in the membrane. The amount of lipids within a membrane depends on the cell type and function (Liscum, 1995). Lipids within the bilayer act predominantly as a solvent for the proteins throughout the membrane (Singer and Nicolson, 1972). The majority of membrane cholesterol is located either within lipid rafts or caveolae. Lipid rafts are microenvironments located within cell membranes which are important for regulating cellular signal transduction. They are typically composed of sphingolipids (SPH), free cholesterol (FC) molecules, and varying levels of proteins (Simons, and Toomre, 2000). The lipids can occur heterogeneously in lipid microdomains within cell membranes (Fielding and Fielding, 2002). These lipid microdomains are characterized by the presence of a number of proteins called glycosylphosphatidylinositol (GPI)-anchored proteins but are deficient in caveolins making them distinct from caveolae. Cholesterol generally acts as a spacer among sphingolipids (Simons and Ikonen, 1997). Sphingolipid-cholesterol rafts are involved in transporting proteins in the endocytic pathway. (Simons and Ikonen, 1997). The depletion of cellular cholesterol significantly affects the properties of GPI-anchored proteins

leading to their random dispersion on the cell surface (Rothberg et al., 1990) thus affecting cell signalling properties (Stulnig et al., 1997).

Lipid rafts are considered to be in a liquid-ordered state, characterized by the rigid packing of lipids but allowing for their high lateral mobility and are often referred to as detergent-resistant membranes (DRMs) (Brown, D.A., and London, E., 1997, Brown, and Rose, 1992). The importance of cholesterol in the cell membrane can be greatly emphasized by the impact of cell viability in the absence of cholesterol. When cholesterol is removed from the membrane, signal transduction and therefore cell-survival is compromised. As a result, cholesterol-depleted cells typically undergo apoptosis. The incorporation of unsaturated phospholipids into the membrane causes loose-packing of raft components resulting in a liquid-disordered membrane that is solubilised upon the addition of mild detergents (Vainio et al., 2006). It has previously been shown that lathosterol incorporates into DRMs at least as efficiently as cholesterol. (Lusa et al., 2003). Additionally, lathosterol formed rafts that were at least as detergent-resistant as cholesterol-containing rafts (Wang et al., 2004) It was also previously discovered that 7-dehydrocholesterol was stronger at domain-promoting than cholesterol (Xu et al., 2001). When cholesterol is replaced by desmosterol within the cell membrane, however, there is an alteration of membrane fluidity. Desmosterol has a very similar structure to cholesterol, however the desmosterol side-chain contains an unsaturated hydrocarbon bond between C24 and C25 thereby altering the flexibility of the side-chain (Vainio et al., 2006). The side-chain penetrates the membrane and is thought to prevent close-packing of membrane components. As a result, when substituted within the membrane, desmosterol increases the membrane fluidity as the side-chain creates gaps within the

membrane. Indeed, Vainio et al., 2006 discovered that the order within membranes is significantly decreased when desmosterol is substituted for cholesterol in the membrane, however, desmosterol maintains a more rigid (and thus viable) membrane than does the complete absence of sterols.

2.2.2 Cholesterol in caveolae

Cells receive extracellular information in the form of signals through the use of cell surface (protein) receptors. Although the signal transduction relies on the binding of ligand to its cell membrane receptor, the composition of lipids within the cell membrane have a great influence on transduction (Fielding and Fielding, 2002). Caveolae are protein-, cholesterol- and sphingolipid-rich flask-shaped invaginations found within plasma membranes of mammals, especially in endothelial cells and adipocytes. Caveolae rely on cholesterol for survival (Rothberg et al., 1990). They typically contain more cholesterol than sphingolipids when compared to lipid rafts and are primarily used in the transport of molecules between cells and are capable of both endocytosis and exocytosis of a variety of molecules (Anderson, 1998). Caveolae are devoid of GPI proteins but contain proteins called caveolins in which 3 isoforms are known to exist: caveolin1, 2, and 3. Caveolin-1 directly binds with cholesterol (Murata et al., 1995). This occurs likely in order to maintain the critical concentration of cholesterol required for invagination (Lu et al., 2002). Caveolae are found to co-purify with receptor kinases, including the platelet-derived growth factor receptor (PDGF-R), the insulin receptor (IR), protein kinase A (PKA), protein kinase C (PKC). Caveolae are rapidly lost upon administration of cyclodextrin (a cholesterol-sequestering oligosaccharide) possibly due to the loss of caveolins from the membrane which results from cholesterol absence. The importance of

membrane cholesterol in caveolae function was illustrated by Lu et al. who showed that when cholesterol is removed from the cell membrane and endogenous production is impaired, cells rapidly underwent apoptosis due to inadequate Akt-bad signalling. Previous scientists have shown that the insulin receptor is present within caveolae (Gustavsson et al., 2003;Kimura et al., 2002, Nystrom et al., 1999, Balbis et al., 2004 as stated in Lu et. al., 2006). Lu et al. 2006 demonstrated that inhibition of cholesterol biosynthesis at the level of DHRC24 impairs the insulin-signalling cascade.

2.3 Functions of Desmosterol:

Desmosterol is normally present in the sperm head along with other sterols and the efflux of desmosterol is highly critical for the maturation and capacitation of spermatozoa (Nimmo and Cross, 2003). Desmosterol can also structurally and functionally replace cholesterol in the retina (Fliesler, 2000). Additionally, knock-out cholesterol-free mice were generated where cholesterol was substituted for desmosterol and were found to be viable (Wechsler et al., 2003). Desmosterol is also found in high abundance in the developing central nervous system and is found to accumulate just prior to the onset of myelination (Hinse and Shah, 1971). It is still unclear as to whether this accumulation is required for the normal CNS myelination or whether it reflects the limited synthesis of DHCR24 in this tissue (Porter 2002)

2.4 Cholesterol Biosynthesis

Isoprenoid synthesis is found in all living organisms. Cholesterol is an isoprenoid and is synthesized from the acetyl CoA from fatty acid degradation through a series of

approximately 40 enzymatic steps (Zadworny et al., 2003). The required enzymes are distributed among the cytoplasm, the ER and peroxisomes within cells (Liscum, 2002). The pathway is divided into 2 sections which are separated by the intermediate squalene (Figure 1). The pre-squalene pathway comprises the synthesis of lanosterol (30 Carbon: the first sterol produced resulting from the condensation of squalene) from acetyl coA (2 carbon) while the post-squalene pathway begins with lanosterol and results in cholesterol. The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, which catalyses the reaction forming mevalonate from HMG CoA, represents the rate-limiting step of cholesterol biosynthesis.(Goldstein and Brown, 1990). Mevalonate can then be converted to isopentenyl pyrophosphate and further to cholesterol and other isoprenoids (Goldstein and Brown, 1990). HMGCoA reductase resides in the ER. The activity of HMG CoA reductase and therefore the synthesis of mevalonate is controlled by a number of regulatory mechanisms which is in part regulated by the level of circulating LDL (Brown et al., 1973, Brown and Goldstein, 1980). These include the efficiency of gene transcription followed by mRNA translation and finally rate of protein break down and, regulation of enzymatic activity (Goldstein and Brown, 1990). The synthesis and degradation of this enzyme is tightly regulated via the presence of cholesterol and non sterol mevalonate-derived products. This enzyme is rapidly degraded when cholesterol is present in sufficient amounts within the cell (Navdar et al. 2003).

The formation of squalene, catalyzed by the enzyme squalene synthetase is the first enzymatic step which is unique to the biosynthesis of cholesterol (Nwokora et al., 2001).

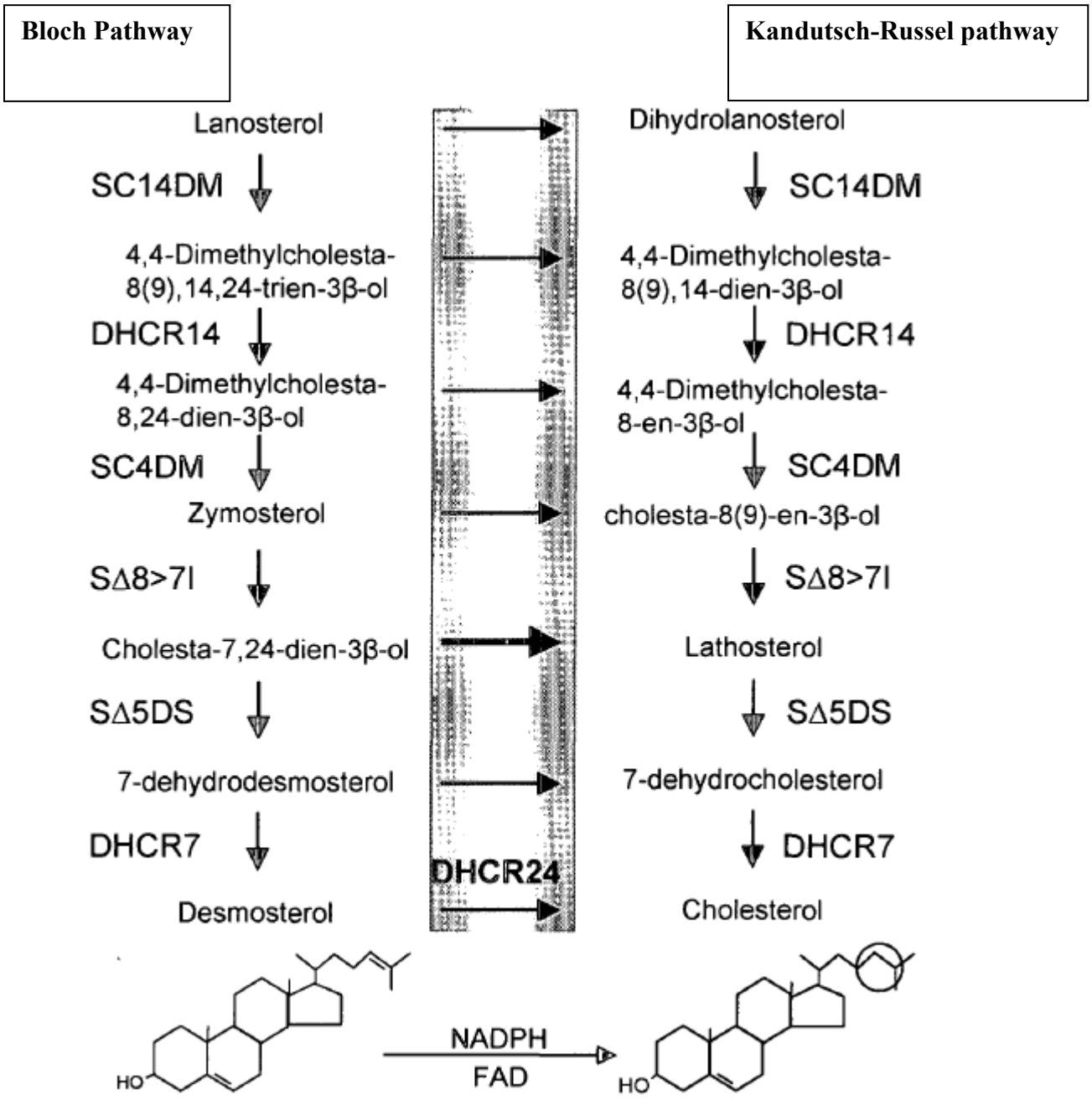

Figure 1. Post-squalene cholesterol biosynthesis. Zadworny et al., 2002

The synthesis of cholesterol from lanosterol can then occur from one of 2 pathways. The Bloch pathway begins with the conversion of lanosterol to 4,4-dimethylcholesta8(9), 14,24trien-3β-ol by the enzyme 3β-hydroxysterol C14 demethylase (CYP51) (Figure 1). This comprises the rate-limiting step in the post-squalene portion of the pathway. The

demethylation involves 3 consecutive oxidation steps (Waterman and Lepesheva, 2005). The result of this enzymatic conversion which is then converted to 4,4,-dimethylcholesta-8,24-dien-3β-ol by the enzyme 3β-hydroxysterol Δ 14 reductase (cytochrome P450, 51 A1/CYP51 A1), which is converted to zymosterol by the enzyme 3β-hydroxysterolC4 demethylase complex. Zymosterol is then converted to cholesta-7,24-dien-3 β-ol by the enzyme 3β-hydroxysterol Δ 8 Δ7 isomerase, which is converted to 7-dehydrodesmosterol by 3β-hydroxysterol Δ 5 desaturase, which is converted to desmosterol by 3 β-hydroxysterol Δ 7 reductase, (7DHCR) and finally converted to cholesterol by the action of 3 β-hydroxysterol Δ 24 reductase. On the other hand, the Kandutsch-Russell pathway is similar but implies that DHCR24 acts early in the pathway (immediately after lanosterol formation), therefore making slightly different intermediates. In this pathway, the catalytic activity of 7DHCR encompasses the final conversion to cholesterol. It seems that DHCR24 will use a number of substrates, depending on which are available (Zadworny et al., 2004). In 1997, Bae and Paik established that among the 3 substrates cholesta-7,24-dienol, zymosterol and desmosterol, DHCR24 preferred cholesta-7,24-dienol as a substrate. They established that C-24 reduction of sterol intermediates takes place immediately after isomerisation of 3β-hydroxysterol Δ 8 Δ7 in the 19-step conversion of lanosterol into cholesterol. Conversely, under normal conditions, the first reaction in the 19-step lanosterol transformation into cholesterol should be catalysed by the 14α-demethylase, and this has been considered to be the rate-limiting step (Gaylor, 1981 as stated in Bae and Paik, 1997)

2.5 Regulation of Cholesterol Biosynthesis

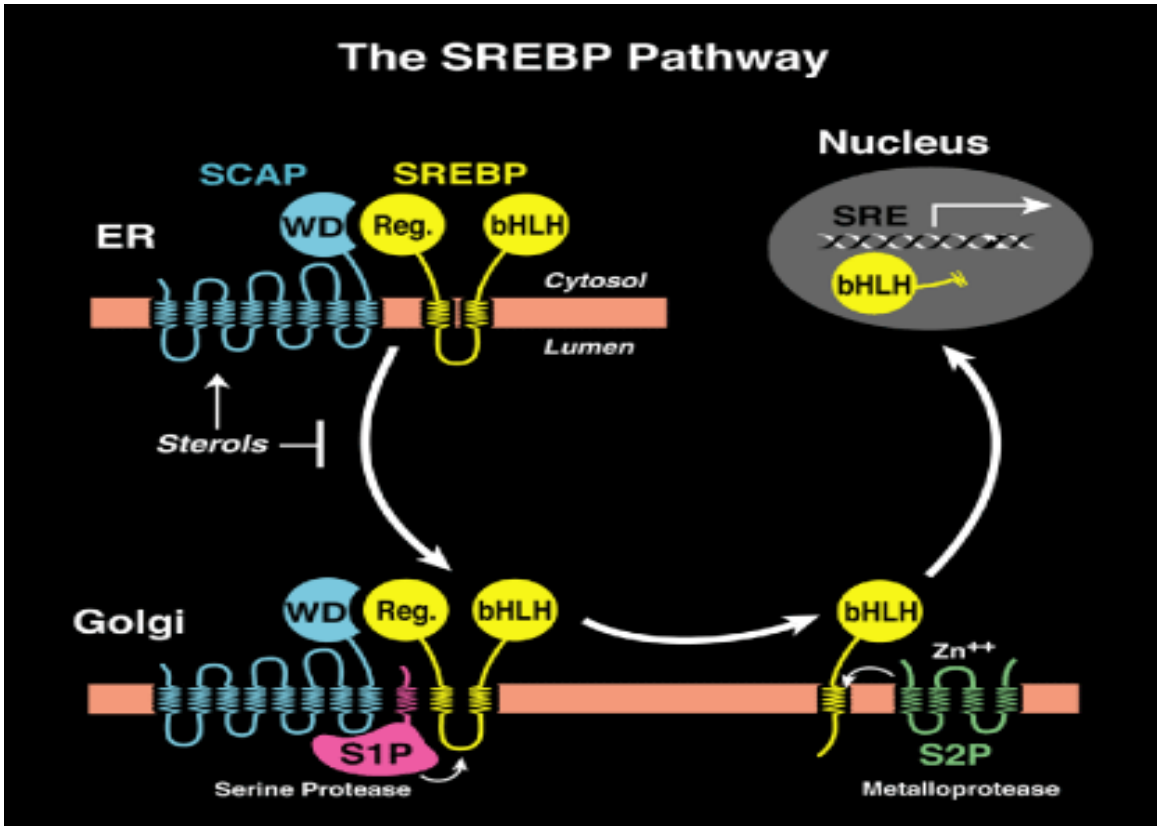

Figure 2. Regulation of Cholesterol Biosynthesis Brown and Goldstein, 2008

Animal cells must regulate cholesterol production in order to avoid excessive levels. The regulation of endogenous cholesterol synthesis is tightly regulated by the sterol regulatory element binding protein (SREBP) pathway, which is also responsible for the regulation of fatty acid synthesis (Figure 2). SREBP is a transcription factor located within the ER. When low sterol levels are detected, SREBP is escorted with the help of the Sterol regulatory element binding protein Cleavage Activating Protein (SCAP) at which time it can translocate to the nucleus, binding to the Sterol Regulatory Element and turn on the transcription of genes whose proteins (enzymes) are required for cholesterol

synthesis. These enzymes include HMG CoA Reductase, HMG CoA Synthase, farnesyl diphosphate synthase, and squalene synthase (Goldstein and Brown, 1990; Osborne, 1995, Guan et al.1995, Ericsson et. al ,1996).

2.5.1 Sterol Regulatory Element Binding Protein/SREBP Cleavage Activating Protein

Three SREBP proteins have been identified in mammals. SREBP 1a and 1c are located within the same gene on chromosome 17p11.2. They are identical with the exception of their first exon. The third, SREBP2 is located on chromosome 22q13 (Brown and Goldstein, 1997). Recently synthesized SREBPs are found in a hairpin structure in the ER, where they remain until they are required by the cell to stimulate synthesis of cholesterol biosynthetic enzymes (Goldstein et al., 2002) The SREBP contains 3 regions. The first faces the cytosol and consists of approximately 480 amino acids. This region functions as a basic helix-loop-helix leucine zipper transcription factor. The second region contains about 90 amino acids and acts as a membrane-anchoring domain consisting of two membrane-spanning domains separated by a 30 amino acid hydrophilic loop projecting into the lumen of the ER. The third region consists of approximately 590 amino acids and contains a regulatory region that extends into the cytosol. The sterol levels within a cell are responsible for the activity of all 3 SREBP isoforms. The SCAP protein contains a sterol sensing domain and acts as an escort of SREBP (Goldstein et al., 2002) Depleted cellular sterol levels are therefore detected by SCAP, which then acts to transport SREBP from the ER to the Golgi complex. In the golgi, the SREBP undergoes 2 cleavages. The first cleavage occurs by the action of a

serine protease, site 1 protease (S1P), cleaving the SREBP in the luminal loop between its two membrane-spanning sequences thus creating two halves and creating a cleavage site for another enzyme Site-2 Protease (S2P). S2P, a metalloprotease, cleaves the amino-terminal within a transmembrane domain of SREBP. Following the second cleavage, the amino-terminal bHLH-Zip domain exits the golgi complex and, carrying 3 hydrophobic residues at its carboxyl-terminal, enters the nucleus where it can participate in transcription of target genes. SCAP is a membrane-bound protein containing 2 distinct domains. The amino-terminal with 730 amino acids containing alternating hydrophobic and hydrophilic sequences. It is thought that these residues form up to 8 membrane-spanning domains. The hydrophilic carboxyl-terminal of SCAP consists of 546 amino acid residues organized into four repeats. (Brown and Goldstein, 1997). The importance of SCAP in regulating cholesterol biosynthesis is apparent in cells devoid of the protein. More specifically, cells lacking SCAP do not have a mechanism to detect low sterol levels nor a SREBP escort. They must therefore rely on exogenous sources of cholesterol for survival. Most importantly, SCAP is found to have an amino-terminal sequence with high resemblance to the sequence of HMG CoA. HMG Co A contains the same 8 transmembrane domains. The two proteins are also similar in that both amino-terminals contain the sterol regulation. In sterol-depleted cells, HMG CoA can remain in the cell for a prolonged period of time in order to carry out cholesterol synthesis (Goldstein et al., 2002).

2.5.2 Sterol-Sensing Domains (SSD)

Insig-1 is short for insulin-induced gene 1 and is located on chromosome 7q36 and is a transmembrane protein located in the ER and expressed in most tissues. With high cellular sterol levels, Insig-1 binds to the SSD of SCAP and acts to retain SCAP within the ER. This in turn prevents the further production of sterols. Due to the presence of a sterol-sensing domain, when sterol levels are high, the insig-1 protein is responsible for maintaining the SCAP protein in the ER therefore preventing transfer and processing of SREBP thus preventing transcription of HMG CoA. (Yang et al., 2002; Yabe et al., 2002). Additionally, this enzyme is rapidly degraded after binding to insig-1 within the ER (Navdar et al., 2003). At this point, it is uncertain as to whether or not SCAP is capable of detecting all sterols to prevent endogenous synthesis. In fact lanosterol does not bind the SSD of SCAP (Song et al., 2005)

2.6 DHCR24

DHCR24 was found to be a gene expanding 9 exons and 8 introns containing 46kb of DNA located on chromosome 1p31.1-p33 as described by Waterham et al. in 2001. The mRNA sequence contains an open reading frame of 1548 base pairs. The protein contains 516 amino acid residues. DHCR24 was found to be expressed in a number of different tissues, mostly (but not exclusively) in those exerting high levels of steroidogenesis. Some of these include the brain and spinal cord, liver, lung, testis, prostate but highest level was found to be in the adrenal gland (Greeve et al., 2000). Waterham and colleagues in 2001 also determined the inherent need of FAD and NADPH as reducing agents for maximal enzymatic activity. Within the cholesterol

pathway, it is the only enzyme acting on the cholesterol side-chain. All other enzymes involved in the pathway catalyze reactions within the four rings of the cholesterol molecule.

2.6.1 DHCR24 and Alzheimer`s Disease

Greeve and colleagues in 2000 discovered that DHCR24 is down-regulated in the necrotic tissue in the brains of Alzheimer`s disease patients. They concluded that oxidative stress in neural tissue induced by the presence of desmosterol within the cell membranes was causing elevated levels of apoptosis. The suspected mechanism of increased apoptosis was through the alteration of caspase 3 activity.

2.6.2 DHCR24 as protection against oxidative stress

Kuehnle et al. in 2008 have confirmed the importance of DHCR24 in protection of cells against oxidative stress. They established that up-regulation of DHCR24 upon oxidative stress is simply due to increased cholesterol production, possibly through the prosurvival cellular protein Akt, while upon chronic exposure to oxidative stress, DHRC24 levels decline below baseline. They suspect that the down-regulation seems to be another prosurvival mechanism of DHCR24 interaction with tumour suppressor p53. It is not surprising then that DHCR24 is preferentially up-regulated in a number of tumours, including adrenocortical carcinomas, prostate, and ovarian tumours. Additionally, Khuenle et al., 2008 found that in three independent cultures of S/P and SM2 cell-lines, there was a strong correlation between DHCR24, HMG CoA reductase, and SREBP1. Di Stasis et al. (2005) indicated that DHCR24 is expressed at higher levels in metastatic

melanoma compared to primary melanoma. They further discovered that expression of DHCR24 by melanoma cells is associated with resistance to apoptosis induced by oxidative stress.

2.7 Cholesterol-Related Disorders

There have been 8 disorders of cholesterol biosynthesis identified. Cholesterol-related disorders are typically characterized by several congenital deformities and serious developmental delay. The only disorder identified in the pre-squalene pathway involves a deficiency in the enzyme mevalonate kinase (Figure 3). There are two conditions that arise from defects in this enzyme: mevalonic aciduria (MKD) and hyperimmunoglobulinemia D syndrome (HIDS) (Van der meer et al, 1984). The conditions are autosomal recessive and characterized by skeletal abnormalities (including facial), microcephaly, cataracts, anemia, hepatosplenomegaly, many psychomotor impairments, hypotonia, myopathy, and cerebellar ataxia, depending on the levels of elevated mevalonic acid. Affected individuals also suffer from recurrent inflammatory spells. Hyperimmunoglobulinemia D syndrome (HIDS) is a milder form of mevalonic aciduria typically have 10- to 100-fold increases in urinary mevalonic acid as opposed to 5000- to 50000-fold elevations in MKD.

2.7.1 Post-Squalene Cholesterol-Related Disorders

The remaining 6 diseases result from enzyme deficiencies within the post-squalene pathway. The four autosomal recessive conditions of the post-squalene pathway include Smith-Lemli-Opitz syndrome (SLOS) due to a defective 3β-hydroxysterol delta 7

reductase, Lathosterolosis, due to a defective 3 β-hydroxysterol Δ5-desaturase, Desmosterolosis, caused by a defective 3 β –hydroxysterol-Δ24 reductase and Greenberg skeletal dysplasia, caused by a defective 3 β-hydroxysterol Δ-14-reductase. The 2 remaining conditions are X-linked dominant and consist of Conradi-Hunermann-Happle syndrome (CDPX2) and CHILD which are caused by defective sterol-Δ8-Δ7 isomerase and sterol C-4 demethylase respectively.

2.7.1.a. Smith Lemli-Opitz

The first post-squalene disorder to be identified was Smith-Lemli-Opitz syndrome (SLOS). This condition is a result of a deficiency in the 7 dehydrocholesterol reductase gene (DHCR7) and is characterized by high levels of the metabolite 7-dehydrocholesterol, hypocholesterolemia, dismorphic facial features, mental retardation, as well as a number of other abnormalities. Patients were found to have a 100-fold increase in 7-dehydrocholesterol the immediate cholesterol precursor in the Kandutsch-Russell pathway (Kandutsch and Russell, 1960). Incidence of SLOS is about 1 in 40,000. Steroid hormone production had also been found to be altered in SLOS patients. One of the earliest studies by Chasalow et al. 1985 found abnormally high levels of dehydroepiandrostenone sulphate (DHEA-S) but low levels of testosterone in two newborns with SLOS while an older infant (30 months) and a child (5 years) were found to have abnormally low levels of DHEA-S.

Human Genetic Defects of Cholesterol Biosynthesis

	Mevalonic aciduria and HIDS	RSH/SLOS	Desmosterolosis	HEM dysplasia	CDPX2	CHILD
Inheritance	AR	AR	AR	AR	XLD	XLD
Enzyme defect	Mevalonate kinase	3β-Hydroxysteroid Δ^7-reductase	3β-Hydroxysteroid Δ^{24}-reductase	3β-Hydroxysteroid Δ^{14}-reductase[a]	3β-Hydroxysteroid Δ^8,Δ^7-sterol isomerase	3β-Hydroxysteroid dehydrogenase
Accumulating intermediates	Mevalonic acid	7-DHC and 8-DHC	Desmosterol	Cholesta-8,14-dien-3β-ol and cholesta-8,14,24-trien-3β-ol	8-DHC and cholest-8(9)-en-3β-ol	Lathosterol and 4-methyl-sterols
Chromosomal location	12q24	11q12–13	NR[b]	NR	Xp11.22–11.23	Xq28
Gene symbol	*MVK*	*DHCR7*	*DHCR24*	—	*EBP*	*NSDHL*

Figure 3. Human Genetic Defects of Cholesterol Biosynthesis Nwokoro et al., 2001

2.7.2. Desmosterolosis

Desmosterolosis was first discovered in 1998 by Fitzpatrick *et. Al.* and has not been studied as extensively. It was found to be an autosomal recessive condition characterized by a number of congenital abnormalities as well as hypocholesterolemia (Waterham *et al.*, 2001). It is a genetic defect in which affected individuals have deficiencies in the 3β-hydroxysterol-Δ24 reductase or dehydrocholesterol reductase 24 (DHCR24) enzyme. The phenotype displayed by the first-identified individual paralleled that of SLOS patients which is what led scientists to believe that it was caused by an inborn error in cholesterol biosynthesis. DHCR24 is responsible for the conversion of desmosterol to cholesterol which is the last step in the Bloch pathway of cholesterol biosynthesis. More specifically, it is required to saturate/reduce the double bond located between C24 and C25. There have been only 2 reported incidences of this condition. The first patient (XX) was of European descent. She was delivered at 34 weeks of age by emergency caesarean section due to fetal distress. She presented with a number of deformities and died shortly after

birth due to respiratory complications. Her deformities included: macrocephaly, frontal bossing, hypoplastic nose, posteriorly-rotated low-set ears, cleft palate, micrognathia, ambiguous external genetalia, short limbs, and generalized osteosclerosis. Upon mass spectroscopy gas chromatography analysis, the patient was found to have low levels of cholesterol and high levels of the precursor desmosterol. Upon examination, her parents also presented with mildly elevated levels of desmosterol confirming the autosomal recessive nature of the disease. The second patient (XY) was also of European descent as reported by Andersson *et al.* in 2002. He presented with many of the same deformities, however his condition was less severe. His phenotype includes microcephaly, dismorphic facial features, limb anomalies, agenesis of the corpus callosum, severe developmental delay, as well as altered steroid hormone levels. In fact, Andersson *et al.* were measuring ion levels at the age of two weeks, and discovered abnormal levels of sodium and potassium (133 and 5.6 mEq/L) indicating a potential deficiency in mineralocorticoids. Additional examinations were performed when the patient reached 3 years of age and indicated that aldosterone:renin (22.8, normal range 28–920) levels were low, furthermore, the patient had normal renin (1.7; normal range 1.7–11.2 ng/ml/hr) levels but abnormally low levels of aldosterone. (39; normal range 83–970 pmol/L). (Andersson et al., 2002). With the information regarding steroid levels obtained from the patient at 3-years of age remain non-conclusive with regards to steroid levels in desmosterolosis patients. The reason is that at that age, cholesterol required for steroidogenesis can be obtained entirely from dietary supplementation. The less severe phenotype of XY patient is due to the functional capacity of the enzyme. The XX patient possessed 3 missense mutations in the DHCR24 gene resulting in an enzyme with 1% functional activity while

XY patient had a 20% functional allele (Waterham *et al.*, 2001). The boy was found to have normal cholesterol levels but a 100-fold increase in desmosterol. In all inborn cholesterol errors, there is a wide variety of phenotype, which depends on the level of enzymatic activity retained within the mutated allele (Mirza et al.,2006).

2.8 Mouse Models of Desmosterolosis

A mouse model for Desmosterolosis has been established by gene knock-out of DHCR24 (Wechsler et al., 2003) .Surprisingly, the null mice closely resembled the wild-type counterparts, however they showed slower growth patterns initially, and males exhibited severely degenerated testis. In contrast, Mirza et al., 2006 noted that null mice born to dhcr24+/- parents died shortly after birth and there was approximately 32-52% of pre-natal death of knock-outs (vs 10-17%: Wechsler *et. al.*,2003). The mice exhibited phenotypes similar to that seen in desmosterolosis, namely that they showed signs of poor mental development (inability to suckle), slower growth, and wrinkleless skin. The reason for the discrepancy in results is unknown, however the DHCR24 knock-out was bred into mice with a different genetic background (Mirza *et al*., 2006).

2.9 Cholesterol and Hedgehog processing

Many of the phenotypic characteristics of cholesterol-related diseases can be partially explained by disruptions of hedgehog signalling during embryogenensis. Hedgehog is a family of proteins which act as morphogens to induce distinct cell fates based on a concentration gradient (Ingham and McMahon, 2001). This family of proteins was isolated in the 1990s in *Drosophila melanogaster* and in humans was found to

consist of 3 members; sonic hedgehog (Shh), desert hedgehog (Dhh), and Indian hedgehog (Ihh) all of which contribute to patterning during development. Shh is the most widely studied and is responsible mostly for the development of the CNS and limb-bud formation, Ihh is responsible primarily for cartilage and bone deposition, as well as activation of hematopoeisis (Ingham and McMahon, 2001). Hedgehog is synthesized as a 45kD precursor protein which undergoes catalytic cleavage to form a 20kD protein originating from the amino terminal and comprising the active form of the molecule and a 25kD fragment from the carboxy terminal of the original protein which mediates the catalytic. The mechanism of this cleavage involves the formation of a thioester intermediate that undergoes nucleophilic substitution. Cholesterol was found to contribute to the catalytic cleavage since in its absence, the molecule remains in its precursor form and the dispersion of the molecule from the site of synthesis to the site of action is altered (Porter *et al.*, 1996). It was originally thought that the absence of hedgehog autoprocessing due to low sterol levels was primarily responsible for the deformities in cholesterol-deficiency diseases (Guy, 2000). More recently, however, it was discovered that the hedgehog signal response is more sensitive to inhibition by mutational or pharmacological sterol depletion than is hedgehog autoprocessing (Cooper et al.,2003).

Following the cleavage of the protein, the active hedgehog protein undergoes 2 post-translational modifications including the covalent attachment of a palmitate and cholesterol residues to the amino and carboxyl terminals respectively to form the fully active hedgehog molecule. The attachment of lipid moieties is important in the regulation of the exit of the molecule from its site of synthesis. Dispatched, a transmembrane protein

containing a sterol sensing domain (SSD) seems to be at least in part responsible for the regulated release of functional hedgehog from the site of synthesis. Once released from the cell, hedgehog can act at a distance to induce cellular fates. Signal transduction is effected by hedgehog binding to its receptor Patched (Ptc) (Ingham et al., 1991). Patched also contains a SSD. The binding of hedgehog to Patched derepresses inhibition of patched on smoothened (Smo). Smoothened is a transmembrane protein (Alcedo et al., 1996, van den Heuvel and Ingham, 1996) and upon hedgehog binding, its inhibition by patched is removed allowing it to activate or repress the GLI family of transcription factors. The GLI family of transcription factors contains both transcriptional activators and transcriptional repressors. In the absence of hedgehog, when Patched is binding smoothened thus inhibiting its activity, the levels of GLI repressor activity overrides the GLI activator. This in turn suppresses hedgehog target gene expression. In the absence of the cholesterol moiety, the delivery of signalling activity is disrupted allowing the random and more rapid spread of hedgehog molecules. This in turn alters the threshold levels required to induce distinct cell fates and therefore disrupts normal development (Ingham, 2001) and causes more constant concentrations regardless of the distance the molecule must travel.

Additionally, when cholesterol is removed from cell cultures through the use of a lipid-sequestration agent (such as cyclodextrin), hedgehog auto-processing is affected. Indeed, it was discovered that, when cells were cultured in lipid-deprived serum and in the absence of enzymes DHCR24, DHCR7, or Sc5d, upon administration of cyclodextrin, hedgehog signalling was impaired to varying levels depending on the amount administered (Cooper et al., 2003)

2.10 Steroidogenesis

All steroids are synthesized from the same precursor, cholesterol which can be derived from endogenous stores, serum derived lipoprotein or from *de novo* cholesterol synthesis. The differential expression of these enzymes throughout the different zones of the adrenal cortex allows for the variety of hormones to be secreted. The main regulators of mineralocorticoid secretion are angiotensin II and potassium. On the other hand ACTH is the principle regulator of glucocorticoids and androgens.

Steroidogenesis is the process whereby cholesterol is converted to steroid hormones through a series of enzymatic reactions. Within the adrenal cortex, steroid synthesis involves the coordinated actions of 5 forms of cytochrome P450 (CYP) and 3β-hydroxysteroid dehydrogenase (3β-HSD) all of which are distributed throughout the mitochondria and ER (Rainey et al. 2004). CYP11A1 and CYP11B (representing the first and last enzymes of steroid synthesis respectively) are both located on the matrix side of the inner mitochondrial membrane, while HSD3B, CYP21, and CYP17 (intermediate enzymes) are located in membrane of the smooth ER (Ishimura et al., 1997). This indicates that the initial and final steps in the synthesis deoxycorticosterone and deoxycortisol occur in the inner mitochondrial membrane and that the intermediate steps in steroidogenesis of take place in the smooth ER (Guo et al., 2003). Several organs, including the ovary, testis, adrenal gland, brain, placenta, and adipose tissue, have the ability to synthesize biologically active steroids. Only the ovary, testis and adrenal are capable of *de novo* steroid production (Sanderson, 2006).

2.10.1 StAR

Steroid hormone production is in part limited to the amount of available substrate, cholesterol as well as the production of steroidogenic enzymes. In response to tropic hormones, cholesterol is initially transported across the inner mitochondrial membrane membrane by the Steroidogenic Acute Regulatory (StAR) protein. This represents the rate-limiting step of steroidogenesis. It appears following stimulation of steroidogenic cells with tropic hormones. (Strauss *et al.*, 2003). StAR contains 285 amino acids and contains a StAR-related lipid transfer (START) domain is required for the binding of sterols (Strauss et al., 2003). StAR was discovered to be synthesized as a 37kD precursor protein which is modified to its active form of 30kD within the mitochondria. When the cholesterol-loaded StAR protein reaches the mitochondrial membrane, the carboxyl terminal of the protein associates with the sterols in the outer membrane, causing cholesterols on the outer membrane to translocate to the inner membrane where sterol levels are lower. Continuous StAR synthesis is required to sustain steroidogenesis. Likewise, the protein must be phosphorylated in order to be active and carry out cholesterol transport. The most well-known condition associated with StAR malfunction is congenital adrenal lipoid hyperplasia (CAH) first described by Prader and Gurtner, in 1955. Individuals with this condition have impaired steroid biosynthesis and during the early post-natal period, are found to have an enlarged adrenal cortex with high lipid (cholesterol) content since there is an absence of steroid production and thus lack of negative feedback to the hypothalamus.

It has yet to be demonstrated whether StAR will use other sterols as steroidogenic substrates. DeWitt et al., 1962 were administering triparanol to human subjects and recording the effects on bile acid and steroid hormone production. Using radiolabeled desmosterol, they concluded that in fact, desmosterol can be directly converted into both bile acids and steroid hormones. Given the number of years which have passed since this study took place, some of the conclusions that were drawn are questionable. First, there were many uncertainties as to whether or not what they were observing was actually derived from sterol precursors, as well, whether or not the steroids found in urine were representative of the steroids that were being produced.

2.10.2 Steroidogenic Enzymes

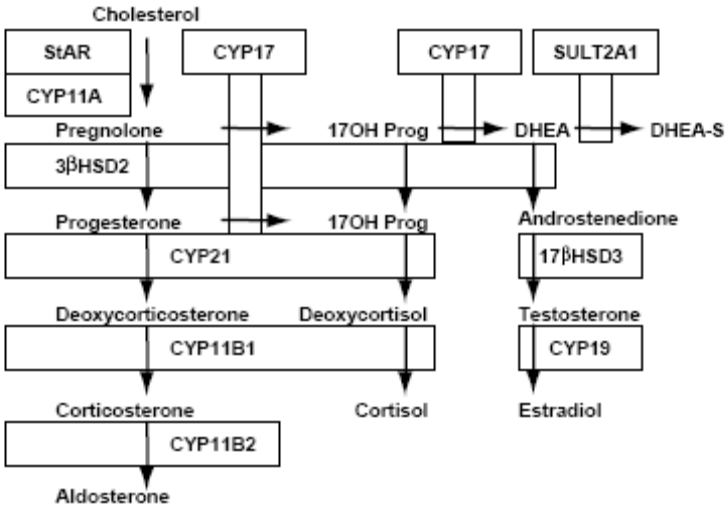

Figure 4. Steroidogenic pathway

Within the adrenal cortex, steroid synthesis involves the coordinated actions of 5 forms of cytochrome P450 and 3B-HSD, all of which are distributed throughout the mitochondria and the ER. Within the inner mitochondrial membrane, the cholesterol side

chain is cleaved via the CYP 11A1 (or P450 side chain cleavage) enzyme. The result of this cholesterol side chain cleavage is the intermediate pregnenolone. CYP11A1 is substrate specific, with cholesterol being the only known substrate (Lambeth, 1986). The biochemical features of the structure of cholesterol are important in the binding of CYP11A1. These features include the 3B-hydroxyl group, planarity of the molecule, and the side-chain organization in the C20-C22 region of the side-chain (Lambeth, 1986). 7-dehydrocholesterol and vitamin D have previously been shown to be acceptable substrates for CYP11A1 (Guryey et al., 2003). Pregnenolone can then be used as a precursor molecule for a number of steroid hormones. For example, pregnenlone can be converted to progesterone, through the action of 3β-hydroxysteroid dehydrogenase (3β-HSD). The same enzyme is capable of converting 17α OH-Pregnenolone to 17α OH-Progesterone and for the conversion of dehydroepiandrostenone to androstendione. The CYP17 enzyme has two different enzymatic activities. The first activity is a hydroxylase activity and is responsible for converting pregnenolone to 17 α pregnenolone as well as converting progesterone to 17 α-progesterone. The second enzymatic activity of CYP17 is a 17,20 lyase and is responsible for the conversion of 17 α OH pregnenolone to dehydoepiandrostenone (DHEA). DHEA can then be converted to a sulphate counterpart through the actions of enzyme DHEA sulfotransferase (SULT2A1). The CYP21 enzyme is responsible for the conversion of progesterone and 17 α progesterone to deoxycorticosterone and deoxycortisol respectively. CYP11B1 enzyme is then responsible for converting deoxycorticosterone and deoxycortisol to corticosterone and cortisol respectively. Further conversion of corticosterone by CYP11B2 results in aldosterone.

2.10.3 Steroidogenesis and the adrenal

A number of peptide hormones are released from the anterior pituitary gland and travel through circulation to target sites which causes the release of hormones leading to a negative feedback on the further release of hormones from the pituitary (Brokken *et al*., 2004). ACTH is a 39 amino acid hormone derived from proopiomelanocortin (POMC) that regulates adrenal glucocorticoid and androgen production by the zona fasciculata and zona reticularis respectively (Slawick *et al.* 2004). The adrenal gland is the most important steroidogenic tissue in the human body and, unlike the gonad steroids, essential for survival (Addison, 1855 as stated in Sanderson, 2006). Multiple pathways of steroidogenesis are present in the normal adrenal cortex. The enzymes present are those involved in the formation of glucocorticoids, mineralocorticoids, and weak androgens. There are also small amounts of estrogens and some progesterone produced as intermediates (Hall, 1984, Nussdorfer, 1986, Gazdar et al., 1990). Steroid production from the adrenal cortex is under tight regulation by the Hypothalamic-Pituitary-Adrenal (HPA) axis. Corticotropin Releasing Hormone (CRH) is secreted from the hypothalamus in response to low levels of steroid hormone which in turn stimulates the release of adrenocorticotropic hormone (ACTH). ACTH is secreted and can bind to G-coupled receptors located on the adrenal cortex to stimulate the production of steroid hormones, mainly glucocorticoid and androgen production via the protein kinase A pathway. Binding of ACTH results in both an acute and a chronic effects (Guo and Chung, 1999). The acute effect occurs within minutes to hours and increases the availability of cholesterol activating cholesterol esterase as well as the StAR protein (Stocco, 2001, Guo et al., 2003) and steroidogenic factor 1 (SF1/Ad4BP), a transcription factor involved in

the synthesis of steroidogenic genes (CYP/CytP450 enzymes). SF/Ad4BP is activated following its phosphorylation, mediated by cAMP (Morohashi and Omura,1996, Morohashi, 1992). Following its phosophorylation, it acts to turn on the transcription of steroidogenic enzyme genes which represents the chronic effect of ACTH binding and takes place after several hours (Guo and Chung, 1999)

2.10.4 Cortisol

Glucocorticoid production is essential for glucose homeostasis as well as the stress response of mammals and is important in the development of the fetal lung immune modulation, and the development and normal maintenance of a variety of tissues. Cortisol is the predominant corticosteroid and can be used as an indicator of adrenal function (de Wreeth et al., 2003)

2.10.5 Steroidogenesis and the Fetal Adrenal

Steroid secretion is important for development and maintenance of pregnancy. At about mid-gestation, the fetal adrenal is of its largest proportional size and consequently responsible for high levels of steroid synthesis. The primary steroid being produced is dehydroepiandrostenone sulphate (DHEA-S). The fetal adrenal produces more adrenal androgen per day (Rainey et al, 2004) and expresses higher levels of the transcripts encoding enzymes involved in cholesterol production than do adult adrenals (Rainey et al., 2001) including a 7.6 fold greater level of HMG Co A reductase and 8.3 fold cytochrome P450 lanosterol 14α demethylase (CYP51). Additionally, it has been observed that DHRC24 is also highly expressed in the fetal adrenal (4-fold increase)

(Rainey et al., 2001, 2002, 2004). The high levels of DHEA-S are required by the placenta to produce estrogens such as Estriol and Estetrol, the primary circulating estrogen during pregnancy. It is suspected that estriol plays a critical role in angiogenesis and thus maintenance of pregnancy and development **(**Buster, 1983**).** Although the incidence of desmosterolosis is quite low, there is a possibility that it is responsible for a number of pre-natal losses due to a deficiency in steroidogenesis. Deficient steroidogenesis could also, at least, in part explain the seriously altered phenotype seen in affected individuals.

2.11 H295R

H295R is an adrenocorticocarcinoma (ACC) derived cell-line which is commonly used to study steroid hormone synthesis due to its ability to produce high levels of adrenal steroids (Gazdar et al., 1990). The tumour sample was obtained from a 48-year-old black woman who was subsequently was found to have elevated levels of cortisol, aldosterone, and 17-ketosteroids H295R is a modified substrain of the original cell-line H295 and was established by selecting only cells that adhere to plastic culture dishes (unattached cells were discarded). (Rainey et al., 2004). The cells are responsive to a number of trophic factors and are capable of steroid synthesis in serum-free and in cholesterol-free medium (Gazdar et al. 1990). Cells were also noted to have high numbers of mitochondria as well as a prominent golgi apparatus, both of which are conducive to high levels of steroid production and secretion (Nussdorfer., 1986). Furthermore, the cells are capable of synthesizing all adrenal steroids.

2.12 RNAi

The discovery of RNA interference and the use of post-transcriptional gene silencing has been a useful tool when studying gene function. It was first discovered in *Caenohabditis elegans* (Fire et al., 1998) and can occur from one of two methods. The first method is through short interfering RNA (siRNA), while the second is performed using micro RNAs. Gene silencing through siRNA occurs as a result of the introduction of double-stranded RNA (dsRNA) into cells. The strength and duration of the silencing depends on the efficiency of the transfection, the amount of dsRNA and on the ability of an siRNA to silence its target (Hannon and Rossi, 2004). dsRNA must first be stably expressed in a transfection vector. Following transfection, it can then be introduced into target cells. Once it enters the cell, the dsRNA is incorporated into a tetramer of proteins called the RNA-induced Silencing Complex (RISC). This complex binds to the target sequence and with the catalytic activity of the DICER (a ribonuclease-III-like enzyme) cleaves the targeted mRNA and degrades it. The targeted mRNA will therefore not be translated to protein. RNAi is a much more efficient technique than the use of knock-outs.

The objective of the current study was to use H295R cells as a model of adrenal steroidogenesis in desmosterolosis patients. Steroidogenic and sterol parameters were assessed in the cell line following trophic stimulation in the presence and absence of DHCR24 suppression via RNA interference.

CHAPTER III. MATERIALS AND METHODS

3.1 Cell Culture

Human adrenocortical carcinoma cell-line NCI-H295R was obtained from American Type Culture Collection (ATCC). Cell's were cultured in monolayer is a 75cm² Corning cell culture flask containing Dulbecco's modified DMEM and Ham's F12 (Sigma Aldrich) in a 1:1 ratio supplemented with 2% Insulin Transferrin Selenite (ITS) + premix and 7.5% NuSerum (BD BioSciences) and containing antibiotics Streptomycin (50 µg/ml) and ampicillin (100IU/ml). Medium was changed of 2-3 times weekly. The cells were trypsinized for sub-culture approximately every 6-7 days. The cells were then counted using a hemacytometer and diluted to attain a ratio of 1:4 for subculturing in 75 cm² culture flasks. For all cell culturing experiments, cells at 80% confluency were harvested and were grown in 8.5x13cm Corning Costar 12-Well-Cell Culture Clusters (Fisher) with a plating density of $5x10^5$ cells/well. All treatments were conducted in triplicates and independently replicated a minimum of two times.

3.2 RNA interference

The DHCR24 gene was suppressed using RNA interference; more specifically, the gene was knocked down using short interfering (si) RNA. Pre-designed siRNA (Table 2) and siPORT NeoFX transfection agent were purchased from Ambion Inc Texas, USA and were assayed for effective gene silencing. Optimization experiments were conducted in order to determine the effectiveness of the 3 siRNAs for all knock-down experiments. All siRNAs were used at a final concentration of 5 nM. siRNA 2 was most effective at gene

silencing and was therefore used for all subsequent knock-down experiments. For all experiments, cells were plated at a density of $5x10^5$cells /ml. Cells were first trypsinized from a 75cm^2 culture flask, counted with a hemacytometer and diluted to obtain a final concentration of $5x10^5$cells/ml. 2 µl of siPORT NeoFX transfection agent per well was mixed with 48 µl of OPTIMEM (Sigma) medium and incubated at room temperature for 10 minutes. 2 µl siRNA (5nM final concentration) was then added to 48 µl of OptiMEM medium. These two solutions were then mixed and incubated at room temperature for 10 minutes. 100 µl of this transfection mixture was transferred to a 12-well plate. 900 µl of the cells and medium were layered on top of the transfection complex and incubated at 37°C with 5% CO_2 for 24 hours. Control cells were subjected to the same treatment without inclusion of siRNA.

3.3 ACTH, Forskolin, and VIP stimulation

The response of cells to trophic stimulation by ACTH, VIP, and Forskolin were tested in preliminary experiments. Cells were stimulated with graded doses of ACTH (0.25nM, 0.5nM, 1nM, 2nM, 4nM). After having determined the optimum dose of 1nM, ACTH was used at time intervals of 2, 24, and 34 hours. Minimal increases in DHCR24 activity were detected. It was therefore decided to detect for the presence of ACTH receptors (ACTHR) since some H295R are found to have baseline levels of ACTHR. The following primers were used:S: ACTGTCCTCGTGTGGTTTTG and AS:AGAGATGAAGACCCCGAGCAG and minimal levels of ACTHR were detected by conventional PCR. Therefore, Forskolin was used to activate the second messenger pathway and hence steroidogenesis. Forskolin was used at a concentration of $1x10^{-4}$ M to

$1x10^{-7}$ M and the optimal forskolin concentration was found to be $1x10^{-5}$ M. Next, a combination of Forskolin and VIP was used in attempt to determine if there were advantages of using both a cAMP analogue and an adenylate cyclase activator. Cells were stimulated with graded doses of Forskolin as well as a combination of Forskolin and VIP followed by graded doses of VIP alone. All cells were seeded onto 12-well plates at a density of $5x10^{5}$ cells/well and grown for 24 hours. The medium was replaced for medium containing $1x10^{-5}$ M forskolin (Sigma Aldrich) which remained for 24 hours. Following 24-hour forskolin stimulation the medium was replaced with serum-free medium containing 5nM VIP for 8 hours

3.4 VIP treatment

Following preliminary studies using forskolin, forskolin and VIP, and VIP alone, it was decided that VIP would be the most effective trophic stimulator due to its ability to specifically stimulate the specific PKA pathway to stimulate steroidogenesis. Cells were treated with $5.0x10^{-8}$M, $1.0x10^{-7}$M, $2.0x10^{-7}$ M VIP for 4 hours to determine a dose response curve. $1.0x10^{-7}$M treatment gave the greatest response. A time course of optimum time that cells should be incubated with VIP was conducted. Cells were therefore treated with $1x10^{-7}$M VIP and incubated for (0.5, 1, 2,4,8 hours). The optimum result of stimulation took place over 2 hours. For all further VIP treatments, cells were stimulated and incubated for 2 hours.

3.5 Cyclodextrin treatment

Cholesterol was removed from cells using methyl-β-cyclodextrin. DHCR24 suppressed cells were obtained from the incubator 24 hours post-transfection. The culture medium was removed and was replaced with methyl-β-cyclodextrin (2%) containing media and cells were incubated for 45 minutes. The solution was prepared according to Liu et al., 1986.

3.6 Cholesterol-loaded cyclodextrin treatment

Following cholesterol depletion, some cells were repleted with cholesterol using cholesterol-loaded cyclodextrin, prepared as follows: 200 mg cholesterol (Sigma-Aldrich) was dissolved in 1 ml chloroform while 1 g cyclodextrin powder was dissolved in 2 ml methanol. 0.45 ml of the cholesterol solution was then added to the cyclodextrin solution (90 mg cholesterol, 1 g CD). This was mixed and placed under a stream of nitrogen gas for 1 hour to evaporate the solvents. The remaining powder (cholesterol-loaded cyclodextrin) was then used at 0.5 mg CLCD per 1ml media. Cells were treated for 2 hours.

3.7 RNA Isolation:

Total RNA was extracted from cells using TRIzol. Following aspiration of the medium, 1ml of TRIzol reagent was added to each well and the cells were lysed by pipetting up and down. The solution was transferred to a 1.5 ml eppendorf tube and 20% chloroform was added. The solution was then mixed by shaking and centrifuged at 9000 rpm for 10 minutes. The supernatant was then removed and transferred to a fresh tube where 500 µl

isopropyl alcohol was added and mixed. The tubes were then centrifuged for 10 min at 9000 rpm. The alcohol was then discarded and the RNA pellet was briefly dried. The pellet was then washed with 75% alcohol and again centrifuged at 9000rpm for 10 minutes. The alcohol was again discarded and the pellet was air-dried for approximately 5 minutes and finally, re-suspended in 10 µl of DEPC-treated water. The RNA was then treated with DNase I (Invitrogen) to remove traces of contaminating genomic DNA. In brief, 1µg of RNA was treated with 1µl DNase I and incubated at room temperature for 10 minutes, at which time, RNA was re-extracted using TRIzol RNA was then stored at -80°C for further use. RNA concentration and quality was assessed using a Spectrophotometer (Nanodrop). Good Quality RNA was defined by having a 260/280 value of 1.8-2.0.

3.8 Reverse Transcription

RNA volumes were adjusted to contain 500 ng of RNA to reverse transcribe single stranded cDNA. In a 0.6 ml PCR reaction tube, 1 µl of dNTP (10 µM), 1 µl of random hexamers (Amersham Biosciences), 500 ng of RNA and DEPC-treated water of variable amounts depending on RNA volume (to obtain a final volume of 11 µl), were mixed and heated to 65°C for 5 minutes followed by a 3 minute quick chill on ice. To this reaction, 1 µl DTT, 1 µl RNase inhibitor and 4 µl 5X first-strand buffer were added and heated to 42°C for 2 minutes. 1 µl (200 units) of Superscript III (invitrogen) was then added to each tube and the reaction was carried out at 42°C for 50 minutes followed by 70°C for 15 minutes. The cDNA was then stored at -20°C for further use.

3.9 Real-Time Quantitative PCR

Quantitative PCR was carried out in an ABI Prism 7500 Sequence Detection System. The 20 µl reaction mix contained 10 µl PCR SYBR green master mix (Applied Biosystems), 0.4 µl of 5 µM forward and reverse primers, 2 µl cDNA, 7.6 µl DEPC-treated water. The conditions for DHCR24 and StAR were 95°C for 10 minutes followed by 39 cycles of 95°C for 15 seconds and 60°C for 1 minute. The conditions for HMG CoA, S1P, SCAP, SREBP1 and 2 were identical except 40 cycles were used. The primers used were designed to span introns; sequences and expected sizes of amplicons are listed in table 1. Amplifications were conducted in triplicate. For absolute quantification, a standard curve was generated using serial dilutions of known quantities of the same gene of interest. For all measurements of gene expression, the threshold cycle (Ct) value was taken. The absolute values of gene expression were extrapolated from the curve. The ratio of 18S RNA (Ambion) to gene of interest was used to normalize and correct the gene expression. Specificity of the amplifications was determined by melting curve analysis (63ºC for 15s to 95ºC in 0.1ºC/s increments).

3.10 Enzyme Immunoassay for Cortisol

For estimation of cortisol production, the cortisol EIA kit (Cayman chemical, MI) was used. Briefly, 50 µl of the cell medium was added to the strip plates coated with mouse monoclonal antibody against cortisol. 50 µl of cortisol tracer and 50 µl of cortisol antibody were then added to the wells. The plate was then incubated overnight at 4°C. The plate was developed with Ellman's reagent and read in a plate reader. Cortisol

standards were used to create the standard curve from which the actual values were extrapolated.

3.11 Sterol Analysis

For sterol analysis, cell pellets were homogenized and saponified for 1 h with 4% KOH in ethanol at 60°C. Five micrograms of coprostanol were added as an internal standard. The samples were then extracted in an equal volume of ethyl acetate, dried under nitrogen and derivitized with BSFTA plus 1% TMCS (Pierce). Samples were analyzed by both gas chromatography/flame ionization detection (Agilent 6890) and gas chromatography/mass spectrometry (Trace Thermo Finnigan) using a Phenomenex ZB-1701 column (30mm x0.32mm x 0.25 mm).

3.12 Statistical Analysis

Statistical significance was calculated using a 95% confidence interval with a t-test. Significance is indicated on figures by a star.

Table 1. Primers used for Q-PCR

Name of Gene	Sense Primer	Antisense Primer	Expected Size (bp)
DHCR24	GAAGCGAGGTCCTGCATGAG	TTGTGGTACAAGGAGCCATCAA	131
StAR	CCACTTGCATGGTGCTTCAC	TGGGACAGGACCTGGTTGAT	121
SREBP1	CGACATCGAAGACATGCTTCAG	GGAAGGCTTCAAGAGAGGAGC	178
SREBP2	CAAGATGCACAAGTCTGGCG	GCTTCAGCACCATGTTCTCCTG	98
SCAP	GCCATCCAGGAGTTCTGTCTC	CATCCGGCGAATGTCAATG	105
HMG CoA	CTGTCATTCCAGCCAAGGTTG	GTCCACAGGCAATGTAGATGG	167
S1P	CGTGTTAAACCTCAGCATCGG	CCATAAAGAGGTCCGTCATTGC	126

Table 2. siRNA used for RNA interference

Name of Gene	Sense siRNA	Antisense siRNA
DHCR24	CCAAGAAACAGAUUGUCCGTT	CGGACAAUCUGUUUCUUGGTG
DHCR24	CGCUAUCUGUGCCAAGUUCTT	GAACUUGGCACAGAUAGCCTC
DHCR24	GCUGUAGUUAAUUUCAGUGTT	CACUGAAAUUAACUACAGCTG

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CHAPTER IV. RESULTS

4.1 Response of DHCR24 to siRNA

Following preliminary experiments to establish optimal conditions, cells were transfected at a density of $5x10^5$ cells/well using 3 different siRNAs for DHCR24 (Ambion) (Table 2.). All were used at a concentration of 5 nM. Transfection was done using siPORT lipofection (Ambion) for a final siRNA concentration of 5nM. Control cells were subjected to the same transfection conditions, however did not receive siRNA. All siRNAs significantly knocked down DHCR24 (Figure 5), however siRNA 2 was most effective with knock-down of 90%. Sterol analysis (Figure 6) confirmed that RNA interference resulted in a significant accumulation of desmosterol in these cells. Control cells had approximately a 1.5-fold increase in cholesterol content than did DHCR24 suppressed cells with VIP stimulation. This difference was found to be significant ($p<0.05$). A 1.4-fold higher content of cholesterol was also observed in control cells in the absence of VIP stimulation

4.2 Effect of ACTH treatment on DHCR24 and StAR levels of expression

H295R cells were seeded onto 12-well plates at a density of $5x10^5$ cells/well and grown for 2 days in usual growth medium and under standard conditions. The medium was then replaced with medium containing 1nM ACTH and cells were kept under standard conditions for 24 hours. The medium was again replaced with medium containing 10 nM ACTH for 8 hours. Total RNA was isolated, reverse transcribed and the resulting cDNA was used to detect expression of StAR and DHCR24 using conventional PCR and

analysis of average density of bands on agarose gel was detected. As indicated in figure 7, pre-treatment with 1nM ACTH for 24 hours did not show any significant differences in DHCR24 levels of expression, similar results were obtained for StAR level of expression. Likewise, further treatment with 10nM ACTH for 8 hours showed no significant difference in DHCR24 and StAR levels of expression. There have been contradictory reports about the presence of ACTH receptors in H295R cells. It was then decided to detect for the presence of ACTH receptors within the cell-line. Analysis by PCR and Q-PCR suggested that levels of ACTHR were low and were not affected by any of the treatments including ACTH, VIP or Forskolin in various combinations. This explained the absence of responsiveness to ACTH stimulation and suggests that ACTH receptors are down-regulated in H295R.

4.3 Time-dependent effect of Forskolin on DHCR24 and StAR

H295R cells were seeded onto 12-well plates at a density of $5x10^5$ cells/well and grown for 24 hours in usual growth medium and under standard conditions. The medium was then changed with medium containing 10^{-5} M forskolin and allowed to grow for 24 hours. Cells were grown in forskolin-containing medium for 24 hours and harvested at 1,2,4,6,12, and 24 hours (Figure 8). For this experiment, a time-dependent effect of Forskolin on DHCR24 expression was measured (Figure 8). Total RNA was extracted, reverse transcribed and expression of DHCR24 and StAR were analyzed using real-time PCR. The values were corrected to expression of 18S RNA and expressed in picograms. A similar time-course was observed in expression of StAR. In the time course study, a 1.4-fold DHCR24 increase was observed after 2 hours of culture with forskolin. The level

of DHCR24 expression increased with time until 6 hours where expression level peaked with a greater-than 2.5-fold increase. After 6 hours, levels of DHCR24 decreased from peak values but at 12 and 24 hours, expression levels were still 2-fold greater than in the control. Forskolin is a cAMP analogue and therefore is a potent adenylate cyclase activator and stimulates both DHCR24 and StAR expression via the cAMP second messenger pathway. Stimulation with forskolin results in steroidogenesis. Vasoactive intestinal peptide is also a potent activator of steroidogenesis and thus cholesterol biosynthesis acting through the cAMP second messenger pathway.

4.4 Effect of Forskolin and VIP on DHCR24 and StAR

H295R cells were seeded onto 12-well plates at a density of $5x10^5$ cells/well and grown for 24 hours in usual growth medium and under standard conditions. The medium was then changed with medium containing 10^{-5} M forskolin and allowed to grow for 24 hours. Medium was then changed again for medium containing 10^{-5} M forskolin and 10 nM VIP and grown for an additional 8 hours. As observed in Figure 9, Forskolin treatment for 24 hours caused a 1.4-fold increase in DHCR24 expression and the response was potentiated by treatment with VIP. Pretreatment with forskolin and subsequent treatment with VIP increased the expression of DHCR24 by 1.7-fold. The effect of forskolin and VIP on DHCR24 was similar to that observed in StAR expression (Figure 10). Forskolin increased the level of StAR expression by 2.4-fold and the response was potentiated to 3.6-fold with a combination of forskolin and VIP treatment. VIP alone is a potent activator of StAR and DHCR24 expression, having caused a 1.3-fold increase in

DHCR24 and a 2.5-fold increase in StAR expression. Both Forskolin and VIP are potent adenylate cyclase activators.

4.5 Effect of Forskolin and VIP on DHCR24 suppressed cells

H295R cells were transfected with DHCR24 siRNA and seeded onto 12-well plates at a density of $5x10^5$ cells/ and grown for 24 hours in usual growth medium and under standard conditions. Cells were then pretreated with forskolin for 24 hours and treated with VIP for 8 hours. Cells were then harvested and RNA was isolated, reverse transcribed, and cDNA was amplified using quantitative PCR. Treatment with 10^{-7}M Forskolin resulted in a 1.8-fold increase in expression of DHCR24 (Figure 11). Further treatment with 10nM VIP resulted in a 2-fold increase in expression of DHCR24. Stimulation with Forskolin and VIP in the absence of DHCR24 suppression resulted in a 1.5-fold increase in level of DHCR24 expression compared to stimulated cells with DHCR24 knock-down. Although the expression of DHCR24 increased following trophic stimulation, the increase was only about 50% of that observed in non-transfected cells (Figure 11).

Similarly, treatment with 10^{-7}M forskolin caused a 2.7-fold increase in StAR expression with and a 3.7-fold increase with 10nM VIP treatment in DHCR24-suppressed cells (Figure 12). Forskolin and VIP stimulated the expression of StAR similarly in non-suppressed cells.

4.6 Effects of VIP on DHCR24, HMG CoA reductase, SREBP1, SREBP2, SCAP, S1P, and StAR in control and DHCR24-suppressed cells

H295R cells were seeded at $5x10^5$ cells/well and transfected with siRNA for DHCR24 and cultured under standard conditions in normal growth medium for 24 hours. Medium was then changed for serum-free medium containing $1.0x10^{-7}$M. Cells were harvested following a 2-hour incubation with VIP. RNA was isolated, reverse-transcribed and cDNA was amplified using real-time PCR. DHCR24 levels were significantly higher ($p<0.05$) in control cells both with and without VIP stimulation (Figure 13A.). HMG CoA reductase levels were highest in DHCR24-supressed cells with VIP stimulation (Figure 14A.). Similarly, highest levels of SREBP1 (Figure 15A.), SCAP (Figure 16A.), and SREBP2 (Figure 17A.) expression was noted in the same treatment group although the difference was only found to be significant in SREBP1 level of expression. S1P level of expression was found to be highest in DHCR24-suppressed cells with no VIP stimulation (Figure 18A.), although similar levels of expression were seen in DHCR24-suppressed cells with VIP stimulation. Similar levels of StAR expression were noted in DHCR24-suppressed and control cells with VIP stimulation as well as in DHCR24-suppressed and control cells without VIP stimulation (Figure 19A.).

4.7 Effect of cholesterol depletion and repletion on cellular sterol levels as well as on levels of expression of HMG CoA Reductase, SREBP1, SREBP2, SCAP, S1P , and StAR

H295R cells were seeded and transfected with siRNA for DHCR24 and cultured under standard conditions in normal growth medium for 24 hours. Medium was then changed for serum-free medium containing 2% methyl-β-cyclodextrin. Treatment took place over a 45 minute time period at which time medium was changed again for serum-free media containing cholesterol-loaded cyclodextrin for 2 hours. VIP was then used to stimulate steroidogenesis and cells were cultured for another 2 hours at which time cells were harvested. Total RNA was extracted after each treatment and gene expression was detected using quantitative PCR. DHCR24, SREBP1 and 2, S1P, HMG CoA reductase, SCAP, and StAR gene expression were all quantified using real-time PCR.

Sterol profiles were confirmed using gas-chromatography-mass spectroscopy analysis. (Figure 20). It was illustrated that there was negligible desmosterol levels in control cells therefore DHCR24-suppressed cells which were cholesterol-depleted and repleted had significantly higher levels ($p<0.05$) of desmosterol prior to and following delipidation. All cells in which cholesterol was depleted using methyl-β-cyclodextrin showed lower sterol levels following trophic stimulation by VIP. Sterol levels increased following repletion with cholesterol-loaded methyl-β-cyclodextrin.

DHCR24 level of expression was found to be significantly higher ($p<0.05$) in control cells treated with MCD and VIP than in DHCR24-supressed cells (Figure 13B.).

Significantly higher levels of DHCR24 were also observed in control cells following cholesterol repletion and stimulation with VIP than in DHCR24-suppressed cells.

Conversely, the level of expression of HMG CoA reductase, SREBP1, SCAP, and S1P were found to be significantly higher ($p<0.05$) in DHCR24-suppressed cells treated with MCD and VIP than in control cells (Figure 14B, 15B, 16B and18B. respectively). This difference was also observed in DHCR24-suppressed cells following cholesterol repletion with CLCD and VIP stimulation.

SREBP2 level of expression was found to be significantly higher ($p<0.05$) in DHCR24-suppressed cells treated with CLCD and VIP than in control cells (Figure 17B.).

There was no significant difference observed in StAR level of expression between DHCR24-suppressed and control cells with the cyclodextrin treatments (Figure 19B.). With the exception of DHCR24 and SREBP1 (figures 13 and 15 respectively), levels of HMG CoA reductase, SCAP, SREBP2, S1P, and StAR increased following delipidation (Figures 14, 16-19, respectively, A. vs. B.)

4.7.1 Effect of cholesterol depletion and repletion on cell morphology in DHCR24-suppressed and control cells

H295R cells were seeded and transfected with siRNA for DHCR24 and cultured under standard conditions in normal growth medium for 24 hours. Medium was then changed for serum-free medium containing 2% methyl-β-cyclodextrin. Treatment took place over

a 45 minute time period at which time medium was changed again for serum-free media containing cholesterol-loaded cyclodextrin for 2 hours. VIP was then used to stimulate steroidogenesis and cells were cultured for another 2 hours. Digital images were taken throughout the various treatments. Figure 21.1.a shows the morphology of H295R cells in the absence of treatment prior to transfection with normal growth medium. Following cholesterol depletion with MCD, cells undergo a change in morphology causing them to recede slightly (Figure 21.1.b). Receding was observed to a greater extent in DHCR24-suppressed cells with cholesterol depletion (Figure 21.1.c)

Control cells which were then repleted with cholesterol began re-attaching to the culture flask (Figure 21.2.d). This was similarly observed in DHCR24-suppressed cells, although to a lesser extent (Figure 21.2.e). An even greater extent of re-attachment was observed was observed in following cholesterol repletion and VIP stimulation, in control cells (Figure 21.2.f) although cell integrity appears to be slightly decreased when compared to control H295R cells. Re-attachment with impaired cell integrity is seen to a greater extent in DHCR24-suppressed cells subject to the same treatments (Figure 21.2.g)

4.7.2 Effect of cholesterol depletion and biosynthesis inhibition at the level of DHCR24 on level of cortisol production

Higher levels of cortisol production were observed in control cells treated with MCD and VIP than in cells with DHCR24-suppression (Figure 22B.). A similar trend was observed in cells treated with CLCD and VIP, however, no significant difference was observed in either case.

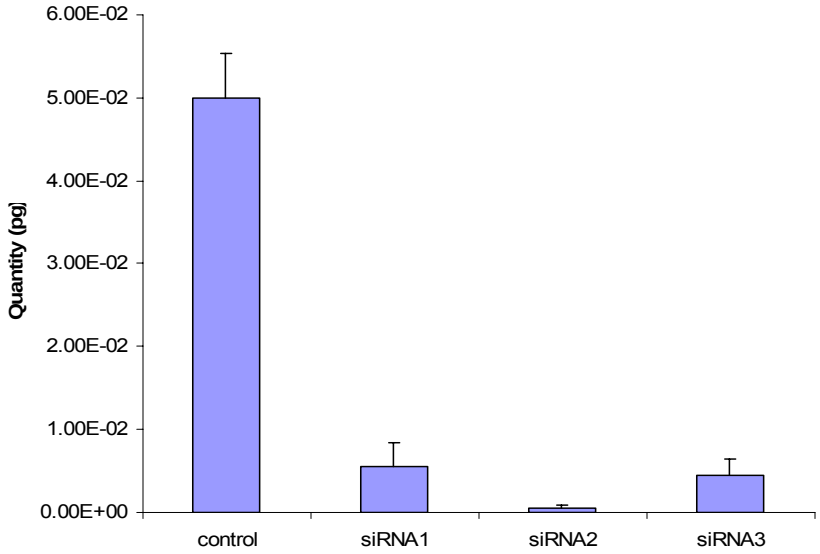


Figure 5. Responsiveness of DHCR24 to 3 different siRNAs. H295R cells were transfected at 5nM with one of three siRNAs and incubated under standard conditions for 24 hours. Control cells are those which were not treated with siRNA but were subjected to the same conditions, siRNA1,2, and 3 represent the 3 different siRNAs listed in table 2.

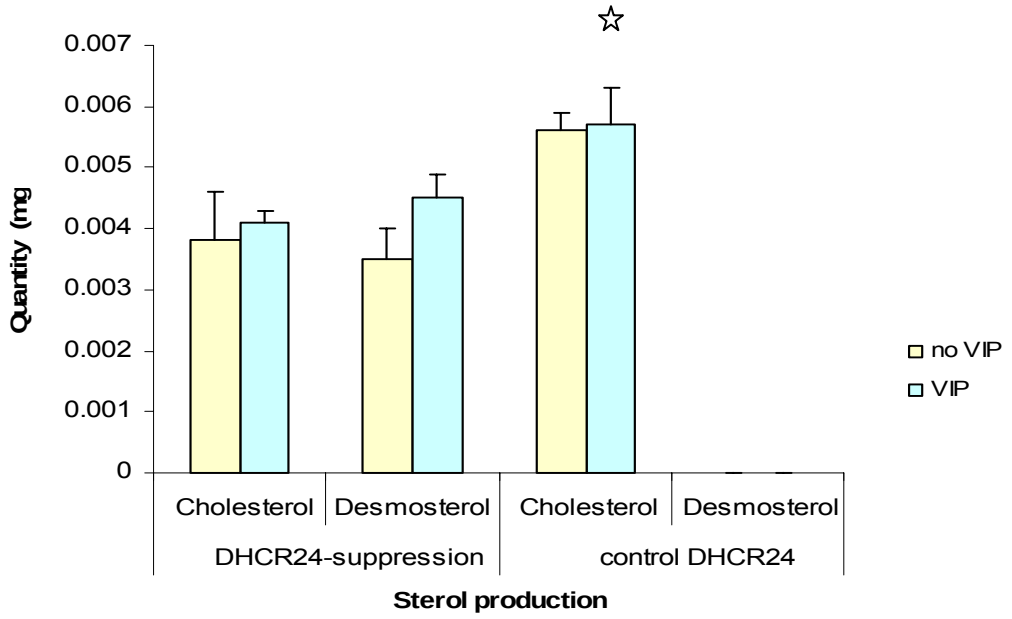


Figure 6. Gas chromatography-mass spectroscopy analysis of cholesterol and desmosterol contents in control and DHCR24-suppressed cells. The values represent cholesterol and desmosterol content as measured by gas chromatography-mass spectroscopy in cells subjected to DHCR24 suppression and control cells, some of which were exposed to VIP stimulation.

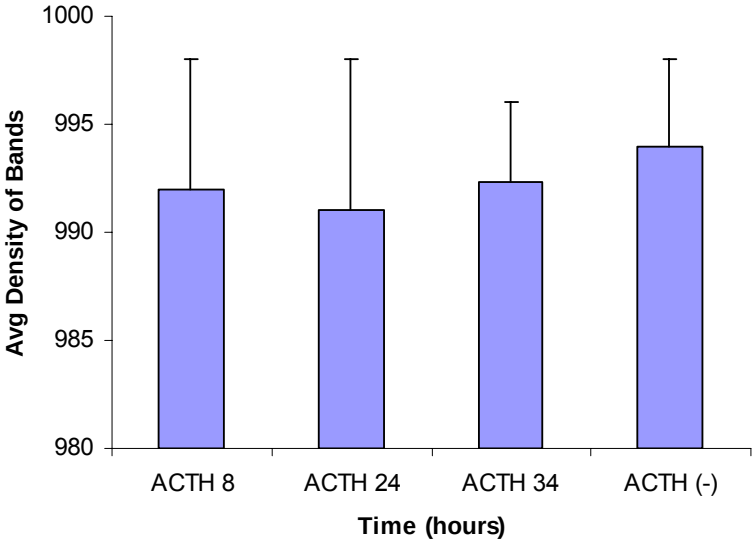


Figure 7. Effect of ACTH on DHCR24. ACTH 8 represents pre-treatment with ACTH for 8 hours, ACTH 24 represents pre-treatment for 24 hours. ACTH 34 represents pre-treatment for 34 hours and ACTH (-) represents no ACTH treatment. Results are based on RT PCR.

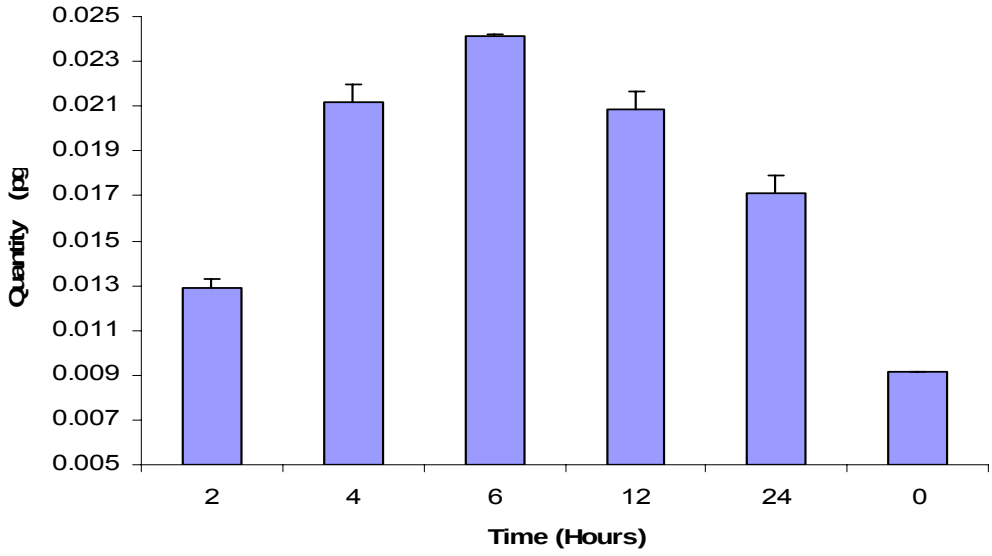


Figure 8. Time-dependent effect of forskolin on level of expression of DHCR24. Forskolin was used at a concentration of 10^{-5}M. The time course is represented in hours and the mRNA expression in picograms, done in Real-Time PCR and corrected to expression of 18S.

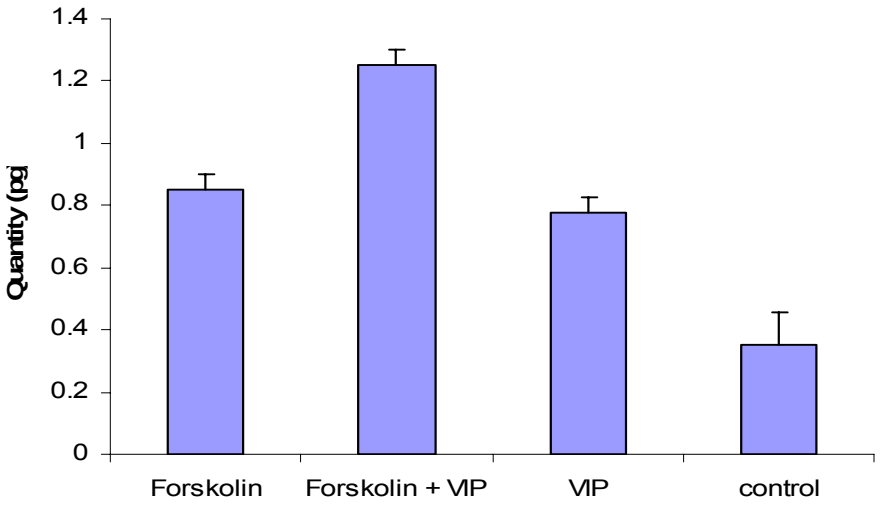


Figure 9. Effect of Forskolin and VIP on level of expression of DHCR24. The values represent gene expression corrected to 18S in real-time PCR. Forskolin (10^{-5}M) corresponds to treatment at for 24 hours, Forskolin (10^{-5}M) + VIP (10nM) represents treatment with Forskolin for 24 hours and treatment with VIP (10nM) for 8 hours. Control cells have not been treated

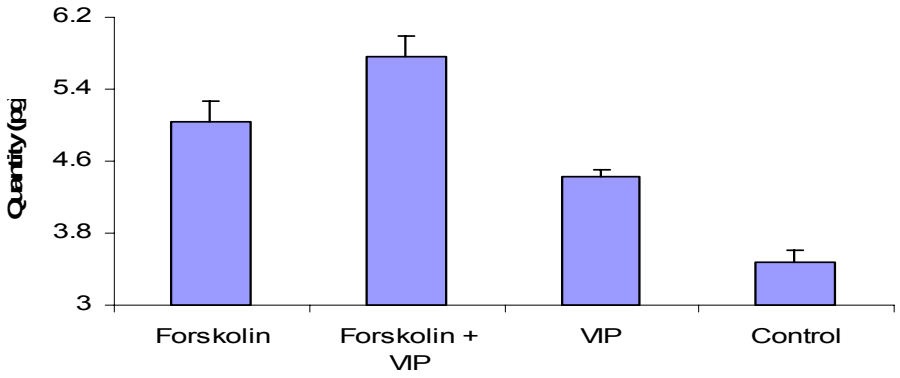


Figure 10. Effect of Forskolin and VIP on level of expression of StAR. The values represent gene expression corrected to 18S in real-time PCR. Forskolin (10^{-5}M) corresponds to treatment for 24 hours and Forskolin (10^{-5}M) +VIP (10nM) represents treatment of Forskolin treatment for 24 hours followed by VIP (10nM) treatment for 8 hours. Control cells have not been treated

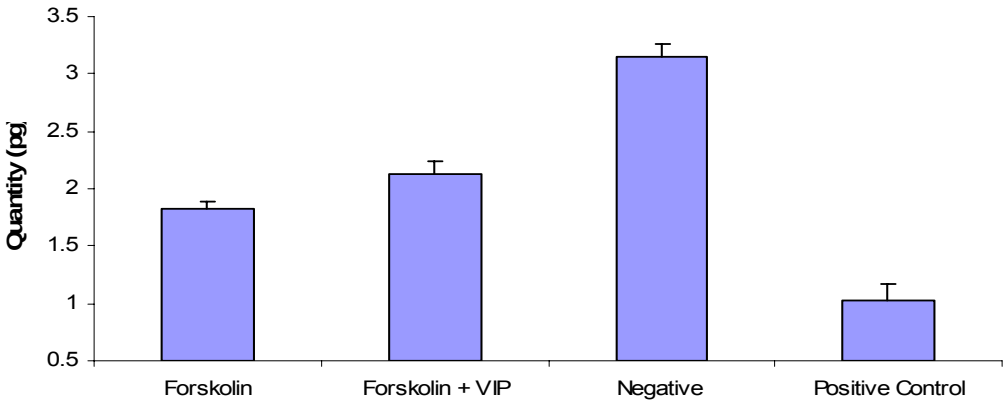


Figure 11. Effect of Forskolin and VIP on level of expression of DHCR24 in DHCR24 suppressed cells. The values represent gene expression corrected to 18S in real-time PCR. Forskolin (10^{-5}M) corresponds to treatment for 24 hours and Forskolin (10^{-5}M) +VIP (10nM) represents treatment of Forskolin treatment for 24 hours followed by VIP (10nM) treatment for 8 hours. Negative and positive control cells were stimulated using Forskolin and VIP with the conditions listed above and positive control cells have been subjected to DHCR24-suppression

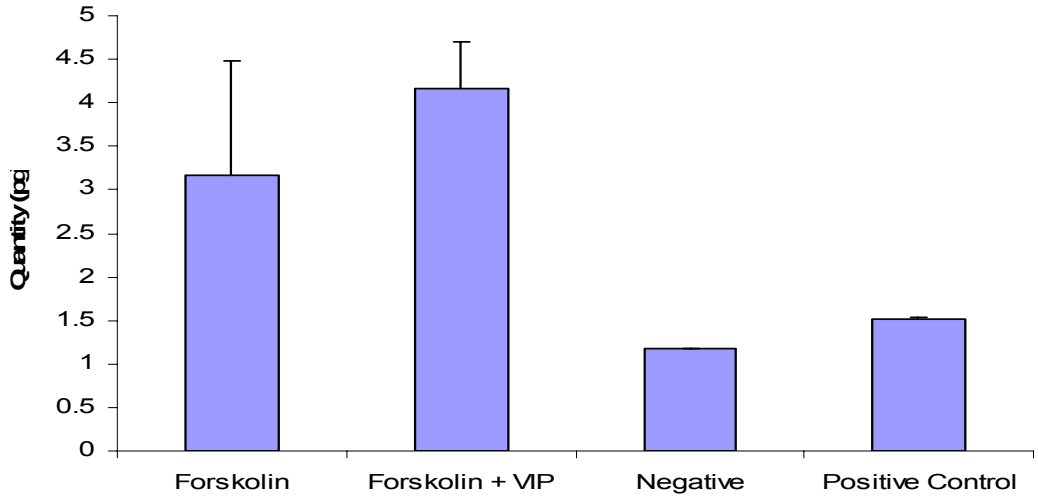


Figure 12. Effect of Forskolin and VIP on level of expression of StAR in DHCR24 suppressed cells. The values represent gene expression corrected to 18S in real-time PCR. Forskolin (10^{-5}M) corresponds to treatment for 24 hours and Forskolin (10^{-5}M) +VIP (10nM) represents treatment of Forskolin treatment for 24 hours followed by VIP (10nM) treatment for 8 hours. Negative and positive control cells were stimulated using Forskolin and VIP with the conditions listed above and positive control cells have been subjected to DHCR24-suppression.

Cyclodextrin Treatment

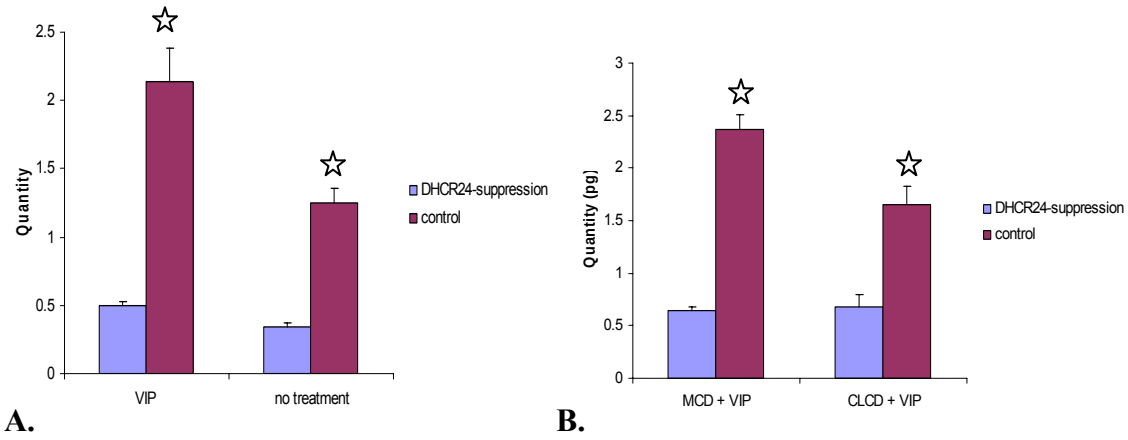


Figure 13. DHCR24 level of expression in control and DHCR24-suppressed cells. Values represent gene expression corrected to 18S values in real-time PCR. VIP represents VIP treatment for 2 hours and no treatment represents no treatment following transfection. MCD +VIP involves the treatment of cells with 2% MCD for 45 minutes followed by 2 hour of culture with serum-free medium following 2 hours of stimulation with VIP. CLCD + VIP represents treatment of cells with 2% MCD for 45 minutes, CLCD treatment for 2 hours and VIP treatment for 2 hours.

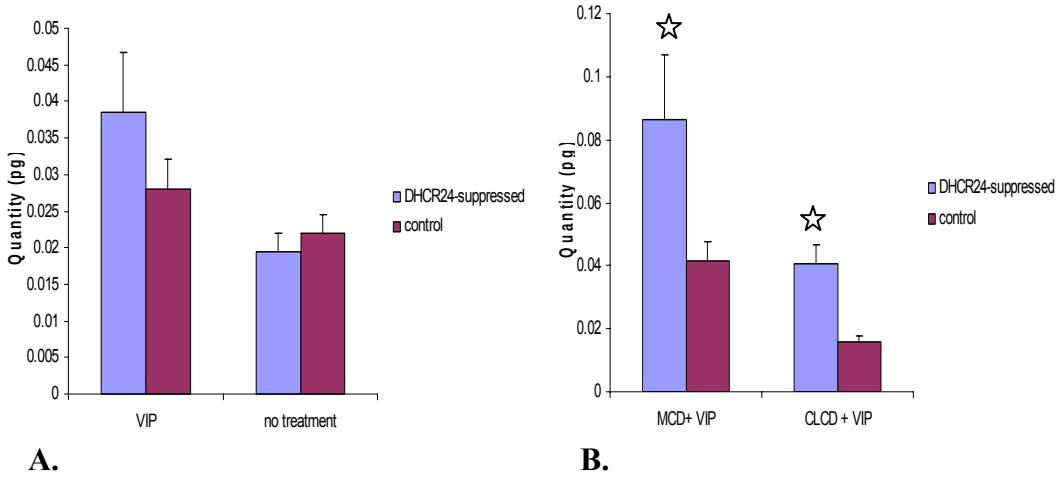


Figure 14. HMG CoA reductase level of expression in control and DHCR24-suppressed cells. Values represent gene expression corrected to 18S values in real-time PCR. VIP represents VIP treatment for 2 hours and no treatment represents no treatment following transfection. MCD +VIP involves the treatment of cells with 2% MCD for 45 minutes followed by 2 hour of culture with serum-free medium following 2 hours of stimulation with VIP. CLCD + VIP represents treatment of cells with 2% MCD for 45 minutes, CLCD treatment for 2 hours and VIP treatment for 2 hours.

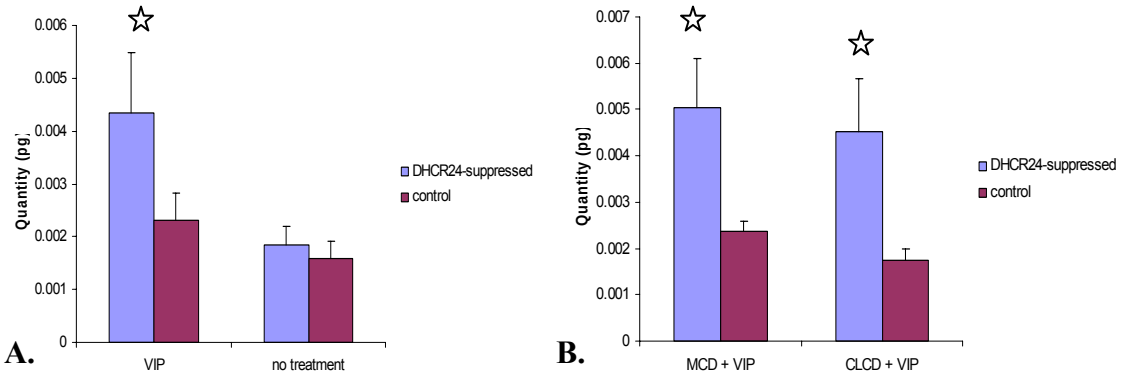


Figure 15. SREBP1 level of expression in control and DHCR24-suppressed cells. Values represent gene expression corrected to 18S values in real-time PCR. VIP represents VIP treatment for 2 hours and no treatment represents no treatment following transfection. MCD +VIP involves the treatment of cells with 2% MCD for 45 minutes followed by 2 hour of culture with serum-free medium following 2 hours of stimulation with VIP. CLCD + VIP represents treatment of cells with 2% MCD for 45 minutes, CLCD treatment for 2 hours and VIP treatment for 2 hours.

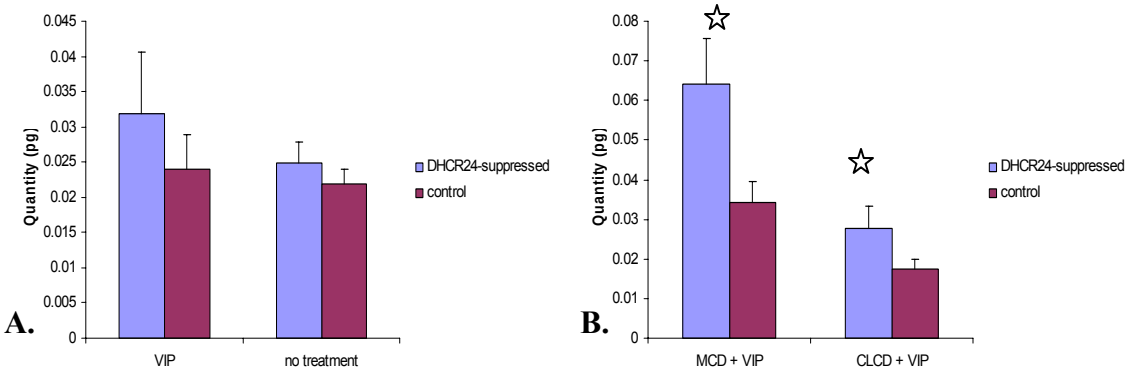


Figure 16. SCAP level of expression in control and DHCR24-suppressed cells. Values represent gene expression corrected to 18S values in real-time PCR. VIP represents VIP treatment for 2 hours and no treatment represents no treatment following transfection. MCD +VIP involves the treatment of cells with 2% MCD for 45 minutes followed by 2 hour of culture with serum-free medium following 2 hours of stimulation with VIP. CLCD + VIP represents treatment of cells with 2% MCD for 45 minutes, CLCD treatment for 2 hours and VIP treatment for 2 hours.

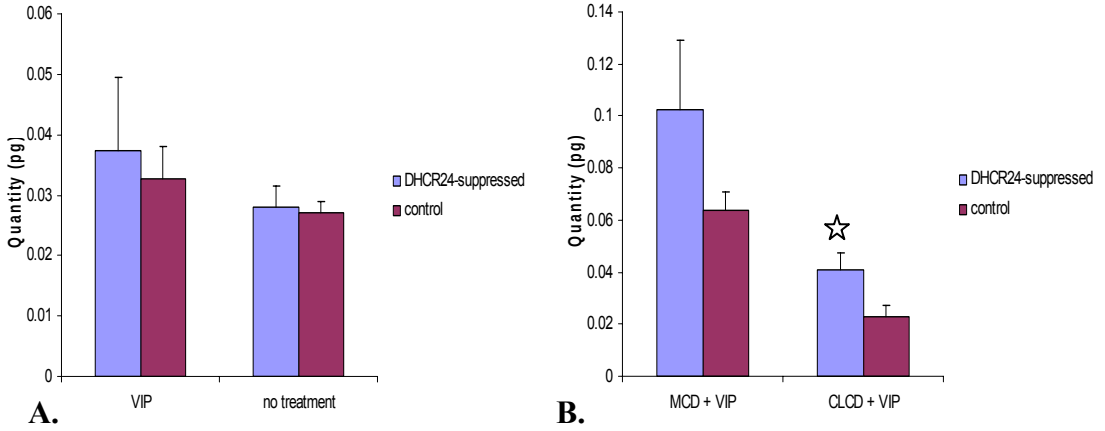


Figure 17. SREBP2 level of expression in control and DHCR24-suppressed cells. Values represent gene expression corrected to 18S values in real-time PCR. VIP represents VIP treatment for 2 hours and no treatment represents no treatment following transfection. MCD +VIP involves the treatment of cells with 2% MCD for 45 minutes followed by 2 hour of culture with serum-free medium following 2 hours of stimulation with VIP. CLCD + VIP represents treatment of cells with 2% MCD for 45 minutes, CLCD treatment for 2 hours and VIP treatment for 2 hours.

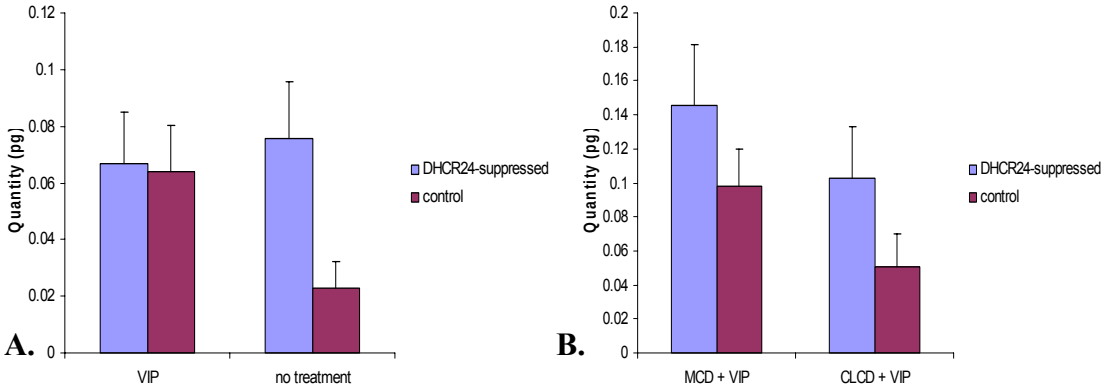


Figure 18. S1P level of expression in control and DHCR24-suppressed cells. Values represent gene expression corrected to 18S values in real-time PCR. VIP represents VIP treatment for 2 hours and no treatment represents no treatment following transfection. MCD +VIP involves the treatment of cells with 2% MCD for 45 minutes followed by 2 hour of culture with serum-free medium following 2 hours of stimulation with VIP. CLCD + VIP represents treatment of cells with 2% MCD for 45 minutes, CLCD treatment for 2 hours and VIP treatment for 2 hours.

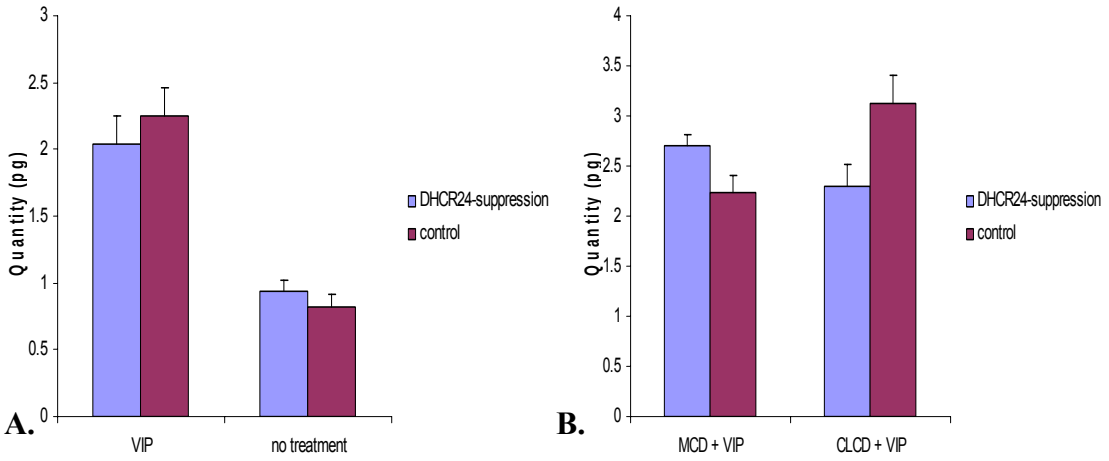


Figure 19. StAR level of expression in control and DHCR24-suppressed cells. Values represent gene expression corrected to 18S values in real-time PCR. VIP represents VIP treatment for 2 hours and no treatment represents no treatment following transfection. MCD +VIP involves the treatment of cells with 2% MCD for 45 minutes followed by 2 hour of culture with serum-free medium following 2 hours of stimulation with VIP. CLCD + VIP represents treatment of cells with 2% MCD for 45 minutes, CLCD treatment for 2 hours and VIP treatment for 2 hours.

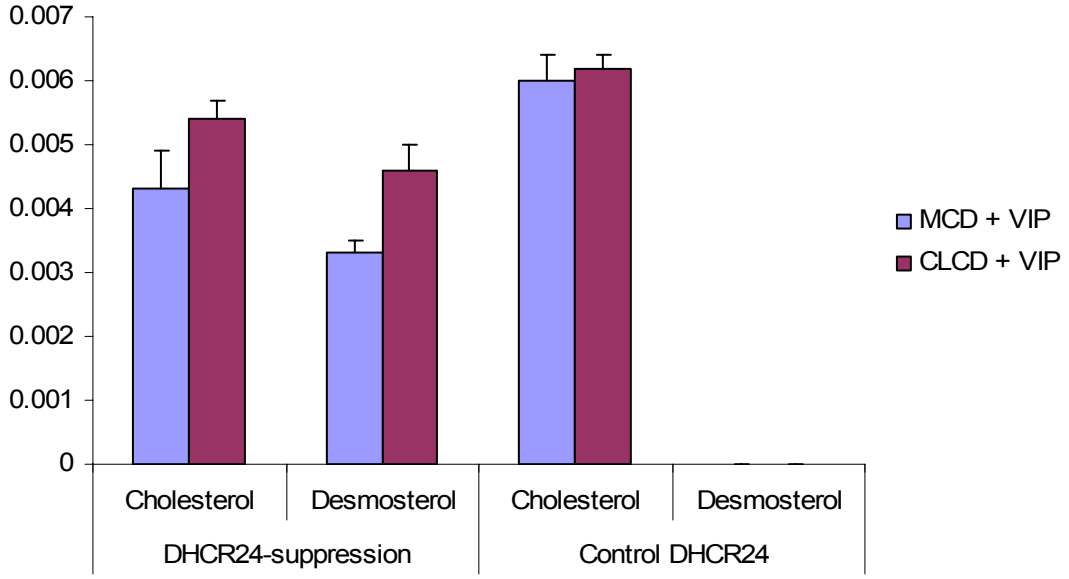
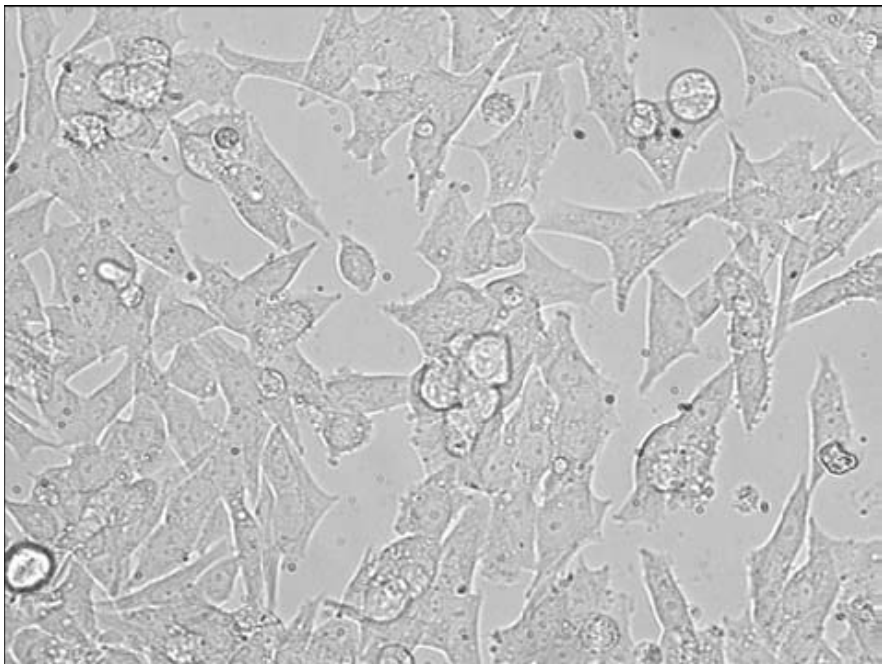
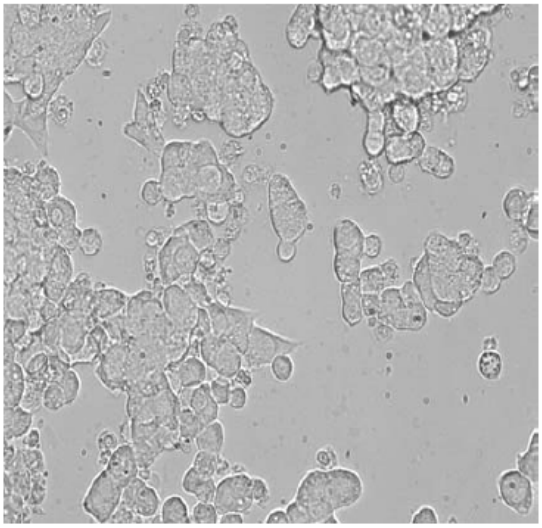


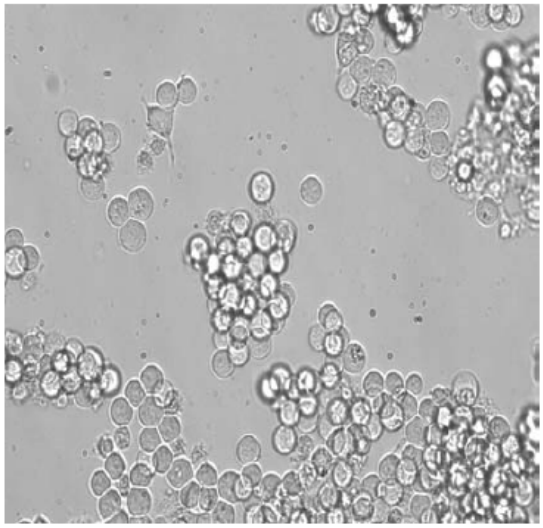
Figure 20. **Gas chromatography-mass spectroscopy analysis of cholesterol and desmosterol contents in control and DHCR24-suppressed cells subjected to cholesterol depletion and repletion with cyclodextrin.** The values represent cholesterol and desmosterol content as measured by gas chromatography-mass spectroscopy in cells subjected to DHCR24 suppression and control cells, some of which were exposed to VIP stimulation



a) Control H295R cells

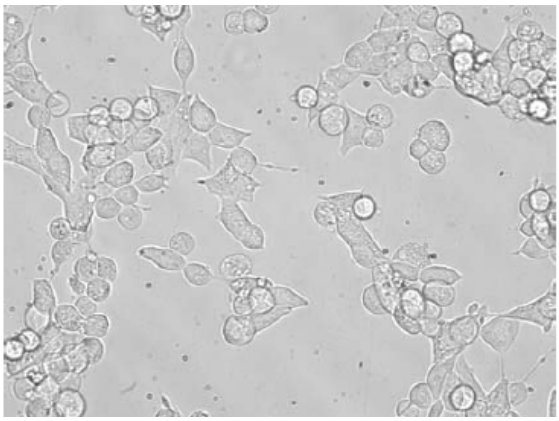


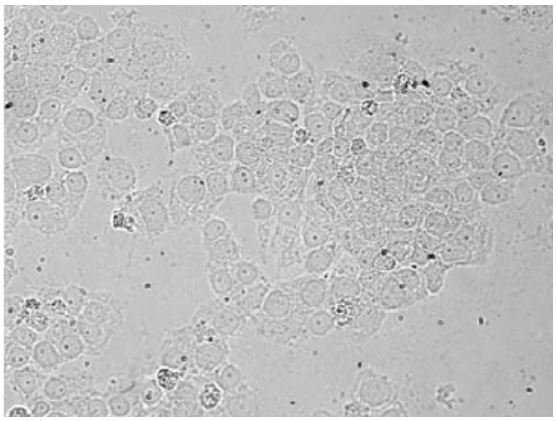
b) Control cells with MCD



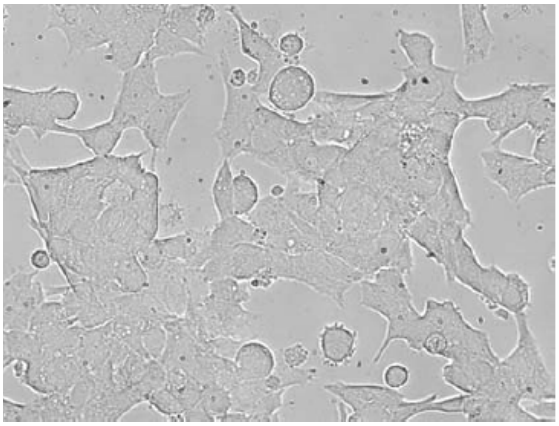
c) Suppressed cells with MCD

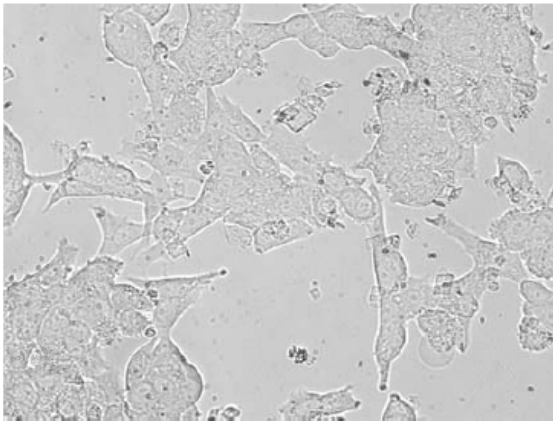
Figure 21.1. Cell Morphology (Cholesterol-depleted) . a) Control H295R cells grown for 24 hours b) Control cells treated with 2% MCD for 45 minutes c) DHCR24-suppressed cells treated with 2% MCD for 45 minutes





d) Control cells with MCD + CLCD **e) Suppressed cells with MCD + CLCD**





f) Control cells with MCD+CLCD+VIP **g) Suppressed cells with MCD+CLCD+VIP**

Figure 21.2. Cell Morphology (Cholesterol repletion and VIP treatment).Control H295R cells treated with 2% MCD for 45 minutes and supplemented with CLCD for 2 hours e) DHCR24 suppressed cells treated with 2% MCD and supplemented with CLCD for 2 hours f) normal cells treated with MCD, CLCD and stimulated with VIP for 8 hours g) DHCR24 suppressed cells treated with MCD, CLCD and stimulated with VIP for 8 hours.

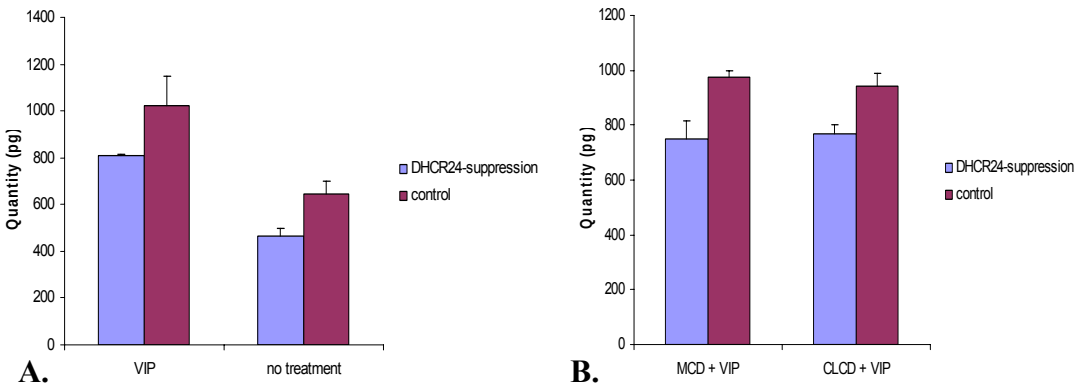


Figure 22. Enzyme Immuno Assay for cortisol in control and DHCR24-suppressed cells. Values represent gene expression corrected to 18S values in real-time PCR. VIP represents VIP treatment for 2 hours and no treatment represents no treatment following transfection. MCD +VIP involves the treatment of cells with 2% MCD for 45 minutes followed by 2 hour of culture with serum-free medium following 2 hours of stimulation with VIP. CLCD + VIP represents treatment of cells with 2% MCD for 45 minutes, CLCD treatment for 2 hours and VIP treatment for 2 hours.

CHAPTER V: DISCUSSION

In the current study we successfully reduced DHCR24 activity by 70-90% (depending on the trial) using RNA interference. Since DHCR24 is the final enzyme in the Bloch cholesterol biosynthetic pathway, lower levels of DHCR24 are associated with lower cellular levels of cholesterol and elevated levels of desmosterol. During the trial, in addition to biosynthesis inhibition, cells were depleted of cholesterol using methyl-β-cyclodextrin. The objective was to mimic sterol conditions representative of an individual with desmosterolosis and evaluate potential effects on steroidogenesis.

5.1 Trophic Stimulation

H295R cell-line has previously demonstrated to be well correlated to the expression in normal human adrenal (Oskarsson *et al*., 2006). ACTH is responsible for glucocorticoid and androgen production in the adrenal glands, using cholesterol as a substrate. Upon stimulation by ACTH, the adrenal gland cells begin producing cholesterol to be used for steroidogenesis, or use exogenous cholesterol. Following a series of treatments with ACTH, we discovered that ACTH is virtually ineffective at increasing steroidogenesis which was indicated by DHCR24 levels. Upon further investigation, we discovered that the reason for this was the absence of ACTH receptor in H295R. This was in accordance with observations by Rainey et al., 2004 who stated that some H295R cell-lines are either partially or completely non-responsive to ACTH. We therefore decided to detect levels of ACTHR expression and discovered them to be down-regulated. For this reason, it was decided to use another trophic stimulator. Forskolin was selected for this purpose. Forskolin, a cAMP analogue, is a potent stimulator of steroidogenesis stimulating a

number of second messenger pathways. When used in combination with VIP, the trophic response was potentiated. Our studies indicated that Forskolin caused a 2.4-fold increase in StAR expression, Forskolin in combination with VIP caused a 3.6-fold increase in StAR expression and VIP alone caused a 2.5-fold increase in StAR expression. In our experiments, we saw that VIP was a potent stimulator of DHCR24 and StAR as it increased expression of both genes by approximately 2.5-fold.

VIP receptors are found throughout many areas of the human body, including both the cortex and medulla of the adrenal gland (Conconi et al., 2006). VIP has been previously shown to independently stimulate stimulated cAMP synthesis in C6 rat glioma cells derived from rat glioblastoma multiforme (Sokolowska and Nowk, 2006). VIP potently and directly stimulates secretion of cortisol from these adrenocortical cells of human origin via an adenylate cyclase-coupled VIP receptor (Cobb et al., 1997). VIP specifically stimulates the PKA pathway, stimulating several steroidogenic enzymes as well as StAR transcription and therefore cortisol production (Rainey et al., 1993). VIP was therefore assayed independently of Forskolin additionally. We discovered that VIP used at a slightly higher concentration than when used in combination with Forskolin could sufficiently stimulate steroidogenesis.

Steroidogenesis was also measured directly via enzyme immunoassay for cortisol. Both control and suppressed cells produced increased cortisol levels in response to VIP. However the increase in cortisol production was moderate in suppressed cells when compared to normal cells. This may be explained by a compensatory mechanism in suppressed cells. There seems to be sufficient evidence that desmosterol does not

adequately replace cholesterol in membranes, but that it may be an adequate substrate for steroidogenesis. There are findings that support the idea that desmolase, the enzyme responsible for converting cholesterol to pregnenolone can tolerate side chain variations. This study indicates that there is at least a partial tolerance for steroidogenesis. The exact mechanism for this sterol substitution is still unknown and more research must be conducted. Since StAR expression levels were unaltered by DHCR24 suppression and forskolin and VIP adequately stimulated StAR expression, it is possible that desmosterol was an adequate steroidogenic substrate.

5.2 Effect of Cholesterol depletion and synthesis inhibition

Cholesterol has previously proven to be of critical importance for many aspects of development including its crucial role in the post-translational modification of hedgehog proteins as well as in prenatal steroid hormone formation. It is also of paramount importance for many physiological functions. First off, all evidence supports the idea of the need for cholesterol to maintain cell membrane integrity, as well as intracellular signaling cascades due to its role in both caveolae (Razani et al., 2002) as well as in lipid raft formation (Kenworthy, 2002). Cholesterol is also important for the synthesis of some fat-soluble vitamins, mitosis-activating sterols, bile acids and steroid hormones.

5.3 Effect on DHCR24

In this study, following DHCR24 knock-down, we expected lower levels of DHCR24 in suppressed cells. Indeed, control cells with and without VIP treatment were found to produce significantly higher levels of DHCR24 than those with DHCR24 suppression. To

confirm the hypothesized sterol profile this gas chromatography-mass spectroscopy was performed (Figure 6.). DHCR24-suppressed cells were found to be producing lower levels of cholesterol and began producing desmosterol as an end product instead. The response was potentiated by the removal of cholesterol with cholesterol-loaded cyclodextrin (Figure 20).

It has previously been demonstrated that the removal of cholesterol from cells using methyl-β-cyclodextrin treatment disrupts caveolae function and thus impairs insulin signaling (Gustavsson et al., 1999). The current study has confirmed the crucial importance of cholesterol and hence DHCR24 for the maintenance of cell membrane integrity and cell survival. Previous research has shown that, in fact desmosterol may be able to substitute for cholesterol in the cell membrane although there is an alteration of membrane fluidity and hence overall cell integrity (Huster et al., 2005). It was found that efflux of biosynthetic desmosterol from cells is 3 times more efficient than that of cholesterol (Phillips et al., 1998). This was partially supported by the discovery that certain strains of mice could in fact be phenotypically normal in the complete absence of DHCR24 (Wechsler et al., 2001). Although prenatal losses occurred, the living mice were relatively normal showing some growth retardation as well as testis generation in males. Similarly, Heverin et al. 2007 discovered that DHCR24 knock-out mice were viable, exhibiting no substantial morphological defects, however males also exhibited testis degeneration and females had compromised oocyte maturation which is possibly due to inadequate pre- and post-natal steroid synthesis. Both sexes therefore have compromised fertility. Indeed, Vainio et al., 2006, established that desmosterol does not replace cholesterol in membranes. As a result, cells which have impaired cholesterol synthesis at

the level of DHCR24 are more susceptible to apoptosis, which is known to be in part due to a disturbance in insulin Akt-Bad signaling which results ultimately in apoptosis (Lu et al., 2006). Our study was in accordance with these findings. Following cyclodextrin administration, cell morphology was greatly altered. Cells began to detach from the surface of the culture dish and upon cholesterol repletion with cholesterol-loaded cyclodextrin many cells were able to regain their integrity and re-attach to the surface. It is noteworthy that the length of time in which cells are treated with cyclodextrin is crucial to avoid high numbers of irreversibly damaged cells. Indeed, comparisons of tissues with high levels of DHCR24 with tissues which are relatively DHCR24 deficient reveal an increase in cell death suggesting that DHCR24 serves as a cell-death inhibiting protein. Similar anti-apoptotic function was suggested in the brain, in which DHCR24 expression is reduced in the regions affected by Alzheimer's disease (Greeve et al.2000).

5.4 Effects on cell morphology

Following a series of treatments including DHCR24 knock-down, delipidation, and trophic stimulation we observed that both suppressed and normal cells physically shrunk in size and rounded up indicating the cells were possible undergoing apoptosis. However, suppressed cell showed more pronounced physical changes than normal cells indicating severe deficiency of cholesterol. This indicates an increased susceptibility towards cholesterol depletion in DHCR24 suppressed cells.

5.5 Effect on HMG CoA reductase, SREBP1, SREBP2, S1P, SCAP

The suppressed cells are deficient in DHCR24 meaning that they are unable to produce adequate levels of cholesterol. They are instead producing desmosterol and are

cholesterol deficient. Since the cells are grown in serum-free medium, there is no exogenous source of cholesterol, the cells must therefore rely on endogenous synthesis to meet cellular requirements, particularly when steroidogenesis is stimulated. The low levels of cholesterol are detected by the sterol sensing domains (SSD) of proteins such as SCAP, and HMG CoA reductase. This detection of sub-normal cholesterol levels results in the transcription of cholesterol biosynthetic factors such as SREBP1, SREBP2, S1P, SCAP. Together these four components act in attempt to increase levels of cholesterol by increasing transcription of cholesterol biosynthetic enzymes. This explains the rise in these four factors as well as in HMG CoA in DHCR24-suppressed cells, an effect which is potentiated with the removal of cholesterol with MCD.

In measuring factors that are responsible for cholesterol biosynthesis, we were in fact measuring the need for cholesterol in the cell. We measured the response of the cell which was depleted of cholesterol with DHCR24 inhibition as well as those which were depleted and shortly thereafter repleted with cholesterol following VIP stimulation.

The most significant findings were seen in DHCR24 knock-down cells which were cholesterol-depleted and stimulated. This resulted in a significant rise in SREBP1 and 2, SCAP, and HMG CoA Reductase transcripts. S1P levels were also elevated but not significantly. The cellular SSD are detecting low cellular sterol in DHCR24 impaired cells and react by inducing cholesterol biosynthesis. Desmosterol can be sensed by the SCAP SSD and can induce a conformational change similar to that induced by cholesterol (Brown et al., 2002) Heverin et al. 2007 noticed elevated levels of hepatic SREBP1c, SREBP2, HMG CoA Reductase levels were all increased in mice which were

DHCR24 deficient which was consistent with our findings. Both SREBP1c and 2 may be upregulated as a consequence of sterol depletion. It is known that SREBP-2 preferentially activates genes of cholesterol metabolism whereas SREBP-1c preferentially activates genes of fatty acid and triglyceride metabolism (Horton et al., 2002).

Contrary to our findings with SREBP, Rodriguez-Acebes et al., 2009 have shown that desmosterol is able to regulate the SREBP pathway similarly to cholesterol. More specifically, desmosterol inhibited SREBP1 and SREBP2 as observed in both J774 and L cells. The difference could lie within the difference in cell-lines. Accordingly, there was no increase in SREBP target genes in the presence of desmosterol within the 2 cell-lines.

Additionally, expression of HMG CoA, levels for suppressed cells treated with CLCD +VIP were similar to those of suppressed cells with only VIP (0.40 pg vs 0.39 pg respectively). This was found to be true for SCAP (0.030 pg vs 0.032 pg respectively), SREBP1 (0.005 pg vs 0.004 pg) and SREBP 2 (0.041 pg vs 0.037 pg). This indicates that cholesterol was effectively restored via CLCD.

5.6 Effect on StAR

Our initial speculations regarding levels of StAR expression were that, in an environment devoid of cholesterol, levels of expression would be reduced, however, there were no significant differences found in any of the treatments. This could suggest that desmosterol or another precursor sterol is an adequate substrate for steroidogenesis.

5.7 Potential roles of DHCR24

DHCR24 has, in this study as well as in many others, proven to be of great importance and has several biological roles. It has been shown to be crucial in the development of the lungs (Mirza et al., 2008 a) as well as in the epidermis (Mirza et al., 2006, Mirza et al., 2008 b). Additionally, DHCR24 has antiapoptotic effects; its free-radical scavenging abilities protect cells from oxidative stress (Lu et al., 2008). Elevated levels of DHCR24 have been associated with a number of cancers including melanomas (Di Stasi et al., 2005), adrenal cortex tumours (Luciani et al., 2004), pituitary adenomas (Luciani et al., 2005), and prostate tumours (Soloman et al., 2004).

Additionally, cells with reduced levels of DHCR24 have reduced sterol levels (Heverin et al., 2007) and hence increased need for cholesterol production. This phenomenon explains the rise in SREBP1 and 2, SCAP, S1P, HMG CoA Reductase observed in the current study. The cellular sterol sensing domains (SSD) are detecting low levels of cholesterol in cells with DHCR24 impaired function and react by inducing cholesterol biosynthesis. This effect is amplified in cells which have been depleted of cholesterol by MCD which have not had cholesterol replenishment with CLCD.

It was also found in mouse trials that although mice may be phenotypically normal, they may have alterations of vital organs. For example, Mirza et al., 2008a discovered that DHCR24 knock-out mice are characterized by severe lung hypoplasia, indicating the potential importance of DHCR24 in lung development. Another study discovered severe dermopathy in DHCR24-/- mice which in turn was lethal. The study therefore concluded the need for cholesterol in normal skin development. All DHCR24 deficient mice died

within a few hours after birth (Mirza et al., 2006). They further discovered that knockout mice had increased expression of aquaporin-3 in the epidermis (Mirza et al., 2008b)

It was previously demonstrated that desmosterol can act as a substitute for cholesterol in the synthesis of normal bile acids (Heverin, et al. 2007) although it is still uncertain as to whether is can act as a substrate for steroid hormones.

5.8 Significance to Desmosterolosis

In humans, the low levels of expression of DHCR24 results in the condition termed desmosterolosis. Extremely low DHCR24 expression levels have proven to be fatal. The first-identified desmosterolosis patient died shortly after birth of respiratory distress. She was characterized by dysmorphic facial features and other congenital abnormalities. Following genotyping, she was found to have a 1% functional DHCR24 allele (Fitzpatrick et al., 1998). The only known living patient contains a 20% functional allele. He is severely compromised mentally and suffers from several congenital abnormalities that paralleled those seen in the XX patient (Waterham et al., 2001). XY was found to have altered steroid levels which is what led us to explore the possibility that steroidogenesis is hindered by low cholesterol/high desmosterol levels. Although his levels were not significantly altered, the steroid measurements were made at the age of 3 when cholesterol could be obtained almost entirely from endogenous sources (i.e. from a cholesterol-rich diet) (Zadworny et al.,2003). It is known that adrenal insufficiency may be fatal as demonstrated by the anesthetic etomidate (Ullerus et al., 2008). Indeed, administration to critically ill patients resulted in adrenal insufficiency followed by death (Ledingham and Watt, 1983). The proper function of the adrenal cortex and resulting

secretion of cortisol and aldosterone are of a vital function to the organism ((Harvey and Everett, 2003;Harvey *et al*., 2007, Ullerus et al., 2008).

This study has confirmed the importance of cholesterol as an essential biological molecule in mammals. Indeed, desmosterolosis is a tragic condition which, along with other similar conditions, further emphasizes the requirement for cholesterol. Although the occurrence of desmosterolosis is extremely low, there is suspicion that it occurs more frequently but results in prenatal loss and therefore remains undetected. It is yet to be determined whether the phenotype is due to low cholesterol production or the abundance of precursor sterols is responsible for the phenotypic disturbances of affected individuals, or a combination of both characteristics. The variation in phenotypes among the cholesterol-related disorders suggests that it could be due to the toxic effects of the abundance of substrate rather than inadequate cholesterol.

CHAPTER VI. REFERENCES

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