Vitamin D and non-melanoma skin cancer

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DECLARATION

Doctor of Philosophy Declaration

"I, Natalie Nemazannikova, declare that the PhD thesis entitled [title of thesis] is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

Signature  Date 15.09.16

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ABBREVIATIONS

calcitriol – dihydroxycholecalciferol

calcidiol – hydroxycholecalciferol

7-DHC – 7-dehydrocholesterol

AK – actinic keratosis

AP-1 – activating protein-1

Apaf – apoptotic protease activating factor 1

BCC – basal cell carcinoma

bFGF – basic fibroblast growth factor

BSA – bovine serum albumin

C-24 – carbon 24

C-25 – carbon 25

CYP24A1 –vitamin D3 24-hydroxylase gene

CYP27A1 – vitamin D 25-hydroxylase gene

CYP27B1 – calcidiol 1-monooxygenase gene

CYP450 – cytochrome P450

DAB – diaminobenzidine

DRIP – vitamin D receptor interacting protein

EGF – endothelial growth factor

EGFR – EGF receptor

ERK1/2 – extracellular-signal-regulated kinases 1/2

FBS – fetal bovine serum

FGF3 – fibroblast growth factor 3

FGFR3 – FGF receptor 3

GLI – zinc finger protein GLI

HR – hairless protein

HRP – horseradish peroxidase

HSP70 – 70 kDa heat shock protein

kDa – kiloDalton

LSAB – labelled streptavidin biotin

MAPK – mitogen-activated protein kinase

MEK – mitogen-activated protein kinase kinase

MMP-1 – matrix metalloproteinase-1

MF – malignant fibroblasts

MK – malignant keratinocytes

NF-κB – nuclear factor kappa B

NMSC – non-melanoma skin cancer

PCNA - proliferating cell nuclear antigen

PBS – phosphate-buffered saline

PF – primary fibroblasts

PK – primary keratinocytes

PTCH – tumour suppressor protein patched

PTH – parathyroid hormone

PVDF – polyvinylidene difluoride

ROS – reactive oxygen species

RXR – retinoid X receptor

SCC – squamous cell carcinoma

SCCIS – squamous cell carcinoma in situ

SDS-PAGE – sodium dodecyl sulphate–polyacrylamide gel electrophoresis

SHH – sonic hedgehog

SMO – smoothened

SOD – superoxide dismutase

SRC – steroid receptor activator

TBS – Tris-buffered saline

TBST – Tris-buffered saline/Tween-20

TGF – transforming growth factor

TKI – tyrosine kinase inhibitor

UV – ultraviolet

VDBP – vitamin D binding protein

VDR – vitamin D receptor

VDRE – vitamin D response element

VEGF – vascular endothelial growth factor

VEGFR – vascular endothelial growth factor receptor

PUBLICATIONS

Alcantara MB, **Nemazannikova N**, Elahy M, Dass CR. Pigment epithelium-derived factor upregulates collagen I and downregulates matrix metalloproteinase 2 in osteosarcoma cells, and colocalises to collagen I and heat shock protein 47 in fetal and adult bone. J Pharm Pharmacol. 2014 Aug 31. doi: 10.1111/jphp.12289 [Epub ahead of print].

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Nemazannikova N, Antonas K, Dass CR. Role of vitamin D metabolism in cutaneous tumour formation and progression. J Pharm Pharmacol. 2013 Jan; 65(1): 2–10. doi: 10.1111/j.2042-7158.2012.01527.x.

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ABSTRACT

Limited information is available on the direct role of vitamin D metabolites (calcidiol and calcitriol) in skin carcinogenesis. For most individuals, skin cancer can be readily managed with surgery; however, some patients may face life-threatening neoplasia. Sun exposure, specifically ultraviolet radiation, is a causative agent for development of skin cancer, though, somewhat ironically, sunlight through the endogenous production of vitamin D may have a protective effect against some skin cancers.

To gain insights into the role of vitamin D metabolites in progression of non-melanoma skin cancers (NMSC), the effects of calcidiol and calcitriol on malignant human keratinocyte (SCC4 cells) proliferation and migration was assesed. It was shown that calcidiol (100,000 nM) and calcitriol (100 nM) exerted partial inhibition of SCC-4 cell proliferation, whilst lower doses had no obvious inhibitory effects. Interestingly, calcidiol at lower concetrations (250 nM) inhibited cell migration; in contrast to calcitriol (10 nM), which appeared to have a stimulatory effect on migration of metastatic SCC-4 cells.

To determine whether vitamin D metabolites have an influence on the expression of vitamin D hydroxylases (CYP27A1, CYP27B1 and CYP24A1) and tumour promoting markers (EGFR and FGFR3) in NMSC, the expression of these proteins were assessed in response to various doses of calcidiol (and calcitriol) in cultured malignant SCC-4 keratinocytes.

In addition, the status of vitamin D enzymes and receptor were studied in human NMSC and normal skin tissues. Immunohistochemical analysis of human NMSC tissues [actinic keratosis (AK), squamous cell carcinoma (SCC), basal cell carcinoma (BCC) and squamous cell carcinoma in situ (SCCIS)] revealed diminished expression of the anabolic vitamin D metabolic enzyme, CYP27B1, compared to normal skin. The lack of CYP27B1 expression has been associated with neoplastic skin lesion progression. Futhermore, the status of intratumoural expression of CYP24A1 and CYP27A1 and vitamin D receptor (VDR) showed that in neoplastic skin the expression of hydroxylases is altered compared to normal skin and pre-cancerous AK lesions. In fact, expression of VDR was similar in AK, BCC, SCCIS, but not in SCC (where poor to moderate expression was noted). CYP24A1 was found to be highly elevated in all tumours, contrastingly to normal skin. Interestingly, CYP27B1 immunoreactivity was noted to have prominent staining in normal skin. In addition, intratumoural expression of EGF, FGF, VEGF, PCNA, FGFR3 and EGFR was demonstrated within the same study for the first time.

It was demonstrated that SCC-4 cells and normal keratinocytes have differential response in expression of CYP27A1, CYP27B1 and CYP24A1 to antiproliferative doses of calcidiol and

calcitriol, which appears not to be VDR-dependent. Furthermore, SCC-4 cells appear to be dose dependent, where FGFR3 inhibition was noted only in response to calcidiol at higher doses (> 100,000 nM).

Cumulative results of immunohistochemical and immunoblot studies suggest that vitamin D enzymes, CYP27B1 and CYP24A1 may be involved in the regulation of neoplastic growth. In conclusion, it was demonstrated that calcidiol has an inhibitory role in neoplastic keratinocytes proliferation and migration of metastatic SCC-4 keratinocytes. Hence, calcidiol appears to have an inhibitory effect on NMSC and has an influencial effect on FGFR3.

IUBMB Enzyme Nomenclature

EC 1.14.13.13

Accepted name: calcidiol 1-monooxygenase

Reaction: calcidiol + NADPH + H^+ + O_2 = calcitriol + $NADP^+$ + H_2O

Glossary:
calcidiol = 25-hydroxyvitamin D3 = (3S,5Z,7E)-9,10-seco-5,7,10(19)-cholestatriene-3,25-diol

calcitriol = 1α,25-dihydroxyvitamin D3 = (1S,3R,5Z,7E)-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol

Other name(s): 25-hydroxycholecalciferol 1-hydroxylase; 25-hydroxycholecalciferol 1-monooxygenase; 1-hydroxylase-25-hydroxyvitamin D3; 25-hydroxy D3-1α-hydroxylase; 25-hydroxycholecalciferol 1α-hydroxylase; 25-hydroxyvitamin D3 1α-hydroxylase

Systematic name: calcidiol, NADPH: oxygen oxidoreductase (1-hydroxylating)

Links to other databases: BRENDA, EXPASY, KEGG, Metacyc, CAS registry number: 9081-36-1

References:

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EC 1.14.13.159

Accepted name: vitamin D 25-hydroxylase

Reaction: calciol + O_2 + NADPH + H^+ = calcidiol + $NADP^+$ + H_2O

Glossary: calciol = cholecalciferol = (3S,5Z,7E)-9,10-seco-5,7,10(19)-cholestatriene-3-ol = vitamin D3

calcidiol = 25-hydroxyvitamin D3 = (3S,5Z,7E)-9,10-seco-5,7,10(19)-cholestatriene-3,25-diol

Other name(s): vitamin D2 25-hydroxylase; vitamin D3 25-hydroxylase; CYP2R1

Systematic name: calciol,NADPH:oxygen oxidoreductase (25-hydroxylating)

Comments: A microsomal enzyme isolated from human and mouse liver which bioactivates vitamin D3.

While multiple isoforms (CYP27A1, CYP2J2/3, CYP3A4, CYP2D25 and CYP2C11) are able to catalyse the reaction in vitro, only CYP2R1 is thought to catalyse the reaction in humans in vivo [4].

Links to other databases: BRENDA, EXPASY, KEGG, Metacyc, PDB, CAS registry number:

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EC 1.14.13.126

Accepted name: vitamin D3 24-hydroxylase

Reaction: (1) calcitriol + NADPH + H^+ + O_2 = calcitetrol + $NADP^+$ + H_2O

(2) calcidiol + NADPH + H^+ + O_2 = secalciferol + $NADP^+$ + H_2O

Glossary: calcidiol = 25-hydroxyvitamin D3

calcitriol = 1α,25-dihydroxyvitamin D3

calcitetrol = 1α,24R,25-trihydroxyvitamin D3

secalciferol = (24R)-24,25-dihydroxycalciol = 24R,25-dihydroxyvitamin D3

Other name(s): CYP24A1

Systematic name: calcitriol,NADPH:oxygen oxidoreductase (24-hydroxylating)

Comments: A heme-thiolate enzyme (P-450). The second donor, NADPH, donates electrons through EC 1.18.1.2, ferredoxinÑNADP+ reductase and a [2Fe-2S] ferredoxin. The enzyme can perform up to 6 rounds of hydroxylation of the substrate calcitriol leading to calcitroic acid. The human enzyme also shows 23-hydroxylating activity leading to 1,25 dihydroxyvitamin D3-26,23-lactone as end product while the mouse and rat enzymes do not.

Links to other databases: BRENDA, EXPASY, KEGG, Metacyc, PDB, CAS registry number:

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CHAPTER 1.

LITERATURE REVIEW, HYPOTHESIS, AIMS AND OBJECTIVES

1.1 Non-melanoma skin cancers: an increasing cause of morbidity and mortality in Australia

Australia has the highest incidence of skin cancer in the world and the mortality is steadily increasing (Statistics, ABo 2012, Australian Bureau of Statistics, ABS; <http://www.abs.gov.au/Ausstats/abs>, viewed 12 December 2015).

There are different types of skin cancers originating in the epidermis and/or the dermis of the skin, which can be classified as melanoma or as non-melanoma skin cancers (NMSCs). While there were more than 800,000 NMSCs treated in Australia in 2011, 543 Australians died from NMSCs that year (Sinclair, 2013). Cancer Council Victoria's national household surveys have estimated NMSC to be three times more prevalent than all other forms of cancers diagnosed in Australia. Indeed, 118,000 Australians were treated for squamous cell carcinoma (SCC) in 2002, with a further 256,000 treated for basal cell carcinoma (BCC) (http://www.skincancer.gov.au/internet/skincancer), (viewed 10 April 2015).

Ultraviolet (UV) radiation is the most significant contributing factor for these cancers. Numerous studies have shown a direct association between solar radiation and an increased risk of skin cancers (Bachelor & Bowen, 2004; Oberyszyn, 2008; Godar et.al., 2009). This association is of major importance, as the incidence of NMSC in Australia is amongst the highest in the world. In 2002, almost 2 % of the Australian population were treated for NMSC. These numbers are expected to rise in the future (Staples et.al., 2006). Intensive UVB exposure in childhood and adolescence is causative for the initiation of basal cell carcinoma (BCC), whereas the major cause of squamous cell carcinoma (SCC) development is chronic UVA exposure (Bachelor & Bowden, 2004; Situm et.al., 2008). Epidemiological studies convincingly suggest that the majority of all melanoma cases are caused by excessive exposure to sunlight. In contrast to SCC, melanoma risk seems not to be associated with cumulative exposure, but instead with intermittent exposure to sunlight.

Undeniably, the exposure to solar UVB radiation may lead to a number of harmful effects, including sunburn, genetic mutations, melanoma, NMSC, cataracts and photoaging of the skin (Afaq et.al., 2005). The avoidance of sun overexposure, frequent use of sun-screens and protective clothing should be made compulsory. Indeed, the development of skin cancer can be avoided by adequate skin protection from solar irradiance. However, the minimal solar exposure (approximately 12 minutes full body exposure per day) is essential for allowing adequate vitamin

D synthesis in the skin, which is vital for human health and prevention of diseases, including cancer (Garland et.al., 2007; Garland et.al., 2011). UVB is known as the main etiological cause for BCC and SCC initiation and the major factor for endogenous vitamin D synthesis. It is hard to resolve the vitamin D dilemma and firmly conclude that UVB exposure leads to detrimental effects, such as carcinogenesis. Epidemiological studies are conflicting on the beneficial role of vitamin D in skin cancer. In fact, in an earlier published study conducted in 73,366 women with 4 years follow up, the oral supplementation of vitamin D intake had no association between vitamin D and NMSC risk (such as BCC) (Hunter et.al., 1992). Another study investigated calcidiol and calcitriol serum levels in 88 Brazilian breast cancer patients (with 35 healthy controls) submitted for mammoplasties or resection of benign lesions. No difference was noted between plasma calcidiol levels in breast cancer patients and control groups, however, low calcitriol serum concentrations were associated with increased risk of breast cancer (Lindelöf et.al., 2012).
In contrast, the antiproliferative activity of calcitriol is shown in human androgen-sensitive non-metastatic prostate cancer cells (LNCaP). Inhibition of cellular growth and proliferation by calcitriol has synergistic effects to 20(S)-protopanaxadiol (aPPD) treatment. Moreover, the effect combined with calcitriol therapy has amplified antiprolifertive effect, hence demonstrating calcitriol as a promising therapeutic. In skin carcinogenesis the influence of vitamin D was shown in 1,608 randomly selected elderly males, where a correlation between higher calcidiol levels (above 30 ng/mL) was associated with decreased NMSC (Tang et.al., 2010).

The anticancer properties of vitamin D have been well acknowledged (World Health Organisation International Agency for Research on Cancer, 2008). Oral supplementation of cholecalciferol – vitamin D precursor, (compound that converts to active vitamin D hormone), may benefit patients with prostate cancer, by lowering prostate specific antigen and stabilising overall plasma parathyroid hormone (PTH) level (Marshall et.al., 2012). The supplementation with 4000 IU/d over the period of one year resulted in elevated circulating calcidiol (25(OH)D) (Marshall et.al., 2012) to a range recently recommended by The Endocrine Society practice guidelines (Holick et.al., 2011). These guidelines, are in agreement with the Food and Nutritional Board (IOM, The Institute of Medicine) on vitamin D a recommended intake, with tolerable upper intake level of 10000 IU/d. However these recommendations of the IOM are not always supported by others authors. For example, an inverse correlation between the expression of Ki67 (proliferation marker) and calcitriol levels in prostate tissue has been reported without adverse toxic effects even at doses as high as 40000 IU daily (Wagner et.al., 2013). Serum calcidiol and calcitriol levels are also increased with high oral supplementation, whilst plasma and urinary

calcium levels are unaffected (Wagner et.al., 2013). Interestingly, the anticancer effects of calcidiol on prostate cells suggests, that the biological effects of calcidiol are via binding directly to vitamin D receptor (VDR) (Munetsuna et.al., 2014). It is not unreasonable to suspect similar mechanism of calcidiol actions in the epidermal keratinocytes. Furthermore, vitamin D analog inhibits breast cancer cell proliferation. In particular, calcitriol and its anologue (with 21-methyl group replacement with a 5,5,5-trifluoro-4-hydroxy-4-(trifluoromethyl)-2-pentynyl group), BXL0124, exerts anti-proliferative and suppressive actions on the expression of putative stem cell markers (including PCNA, proliferative cancer antigen) in mammary gland in situ ductal carcinoma cell line (Wahler et.al., 2015). A relationship between serum calcidiol and risk of breast cancer is evident, where 50 % reduction in risk of breast cancer occurs when circulatory levels of vitamin D (calcidiol) is above 50 ng/ml compared to patients with calcidiol serum levels < 13 ng/ml (Garland et.al., 2007).

Whilst there is evidence suggesting the clinical benefits of vitamin D supplementation in some cancers, clinical justification is required for skin cancers. The effects of calcidiol and calcitriol in the development and progression of non melanoma skin cancer is not well understood. Interestingly, epidemiological studies in a subtropical Australian community indicated no association between vitamin D status and skin cancer incidence. The findings suggest that skin carcinogenesis as a result of high sun exposure cannot be counteracted by high vitamin D status (van der Pols et.al., 2013). Thus, it is logical to suggest that high sun exposure should be avoided in order to achieve optimal vitamin D status.

Numerous publicity campaigns are in place to reduce sun exposure, with the goal of reducing the incidence of skin cancers, and so affecting overall health and predisposition to oncogenic conditions in the future. It would be interesting to see the outcomes of the comprehensive Australian SunSmart program – which involves covering exposed body parts with sunscreen, wearing hats and sunglasses, and minimising sun exposure, in the prevention of skin cancer incidence in the next few decades.

1.2 Vitamin D

Vitamin D relates to fat-soluble secosteroids, responsible for enhancing intestinal absorption of calcium and phosphate. Vitamin D is involved in ionic homeostasis essential for bone maintanance and plays a crucial role in skeletal and muscular health (Leyssens et.al., 2014; Mostafa & Hegazy, 2014). Most commonly referred vitamin D metabolites in this thesis are calcidiol and calcitriol. Although of interest, vitamin D_2, or ergocalciferol, synthesised by fungi and yeasts, will not be

further discussed in this thesis. The structural characteristics of these compounds are shown in Table 1.1 and described in section 1.4.

Vitamin D is a fat-soluble vitamin responsible for ionic homeostasis and intestinal absorption of calcium, iron, magnesium, phosphate and zinc. As the nomenclature of vitamin D compounds can be confusing, it was decide to use the terminoloty of International Union of Pure and Applied Chemistry (IUPAC) accepted names, such as calcidiol and calcitriol (http://www.chem.qmul.ac.uk/iupac/misc/D.html>, viewed 20 August 2014). In this thesis the names of vitamin D compounds are used in accordance to IUPAC nomenclature recommendations. Even though the trivial names calcidiol (for 25-hydroxyvitamin D, 25OHD) and calcitriol (1,25-dihydroxyvitamin D, 1,25(OH)2D), have not come into widespread use, it was decided to refer to both compounds using their IUPAC recommended nomenclature.

Vitamin D synthesis is initiated in the skin by sunlight; however, overexposure to sunlight is the main cause of skin cancer. Calcitriol promotes calcium absorption in the gut, and maintains and regulates serum calcium and phosphate balance for bone mineralisation (Lips, 2006). Calcitriol is required for bone growth and remodelling via osteoblasts and osteoclasts (Haussler et.al., 2010). Its deficiency leads to thin and brittle bones, causing significant health impact. Vitamin D is known to prevent rickets in children and osteomalacia in adults (Stamp, 1980; Holick, 2006a; Pettifor and Prentice, 2011).

The active form of vitamin D is categorised as one of the most important biomolecules maintaining calcium levels (Armbrecht et.al., 1996). Calcitriol indirectly stimulates ionic absorption in the small intestine. The major regulator of this process is parathyroid hormone (PTH), which is part of a negative feedback loop to maintain calcium levels in the extracellular fluid. PTH secretion is stimulated by hypocalcaemia, and it works to simulate mechanisms to increase calcium ion levels. PTH stimulates the release of calcium ions from bone, in part by stimulating bone resorption (Carpenter, 1989). Calcitriol renal synthesis is also stimulated by PTH.

It is understood that circulating calcidiol insufficiency is associated with numerous diseases involving the skeleton. For instance, in rickets development in children, a primary outcome is the failure to maintain normal calcium homeostasis, which leads to no mineralisation of the growth plate of growing bones (Pettifor and Prentice, 2011). Interestingly, maternal vitamin D deficiency, which is usually measured by circulatory calcidiol, is a causative factor for vitamin D status in infants, and their risk of developing infantile nutritional rickets (Thandrayen & Pettifor, 2010). The age-associated decrease in mass and quality of bone (osteoporosis) and muscle (sarcopenia) leads to an exponential increase in risk for osteoporosis-related fracture, which are associated with

lower vitamin D levels observed with advancing age (Binkley, 2012). When combined with calcium supplementation, vitamin D has preventative functions against osteoporosis (Haussler et.al., 2011). In addition, low vitamin D levels have been linked to an increased risk of diabetes, some cancers, cardiovascular disease, multiple sclerosis, mental health problems and altered immunity (Ma et.al., 2004; Pérez-López et.al., 2009; Krishnan et.al., 2010; Grant, 2010; Gröschel et.al., 2012).

1.3 The role of vitamin D in skin cancer development

The skin is composed of epidermal, dermal and hypodermal layers, which assist in mechanical and biochemical protection (Garland et.al., 2009). The epidermis comprises layers of keratinocytes, which undergo a process of continual self-replacement that declines with age. During differentiation, keratinocytes undergo desquamation as they move towards the epidermal surface (Staiano-Coico & Higgins, 1992). The basal layer (stratum basale) forms the basal lamina at the border of the epidermis with the dermis (Figure 1.1). Keratinocytes of the basal layer can proliferate readily when required, furnishing newly formed cells for the upper differentiating layers (Clausen & Potten, 1990). Renewal of epidermal cells via cell division is tightly controlled by two sub-populations of basal epidermal cells: stem cells and transit amplifying cells (Luderer & Demay, 2010). Transit keratinocytes have limited proliferative capacity, whereas epidermal stem cells have an unlimited capacity for self-renewal (Clausen & Potten, 1990). Neoplastic keratinocytes can undergo self-renewal and produce malignant stem cells and progeny keratinocytes, which can result in tumour formation. It has been shown that the absence of a functional vitamin D receptor (VDR) leads to impaired function of keratinocyte stem cells by causing a defect in keratinocyte differentiation, both in vitro and in vivo (Luderer & Demay, 2010).

UV rays (both, UVA and UVB) penetrate the skin epidermis, causing photolesions and cellular damage (Sarkany, 2011). The exposure of the skin to solar radiation is a stressful event and triggers cutaneous defence mechanisms, such as alterations in proteins responsible for differentiation, as well as growth factors, and protection from DNA damage, to counter this threat (Norris et.al., 1997; Wong et.al., 2004). The protective response of epidermal keratinocytes against photocarcinogenesis is activation of DNA repair or pro-apoptotic mechanisms (Mason et.al., 2010).

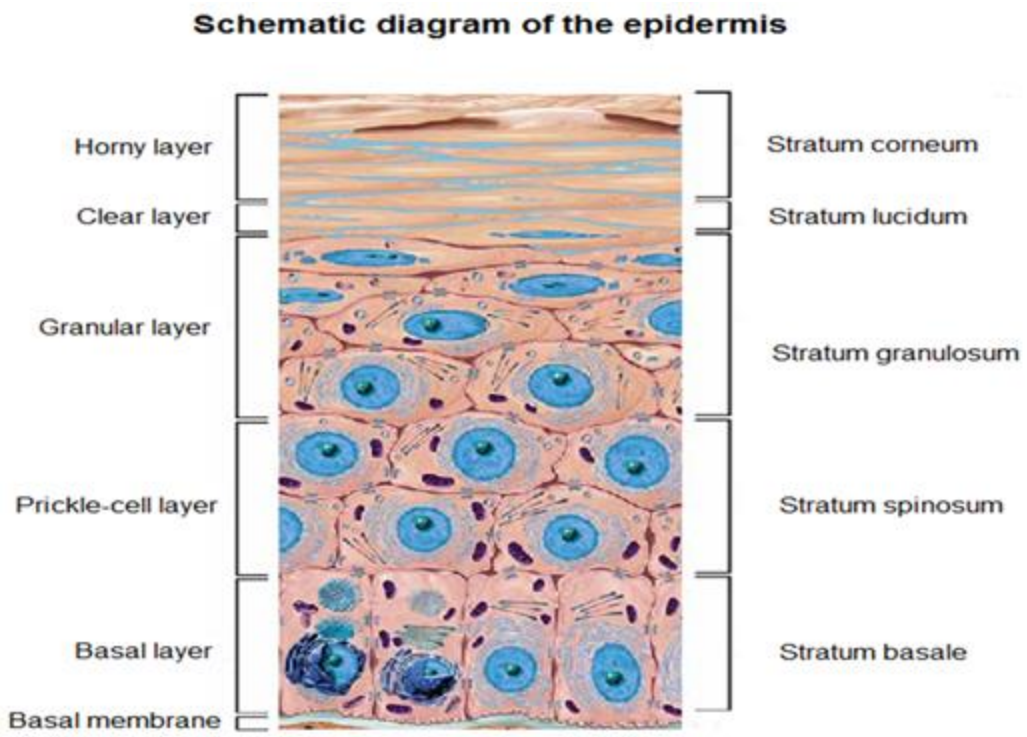


Figure 1.1. Epidermal architecture.

Schematic representation of epidermal keratinocytes. The basal cells change, through differentiation, into flat horny skin cells that are without nuclei. During the differentiation process, keratinocytes permanently withdraw from the cell cycle, initiate expression of epidermal differentiation markers, and move suprabasally to become part of the stratum spinosum (prickle-cell layer), then the stratum granulosum (granular layer), and eventually become corneocytes in the stratum corneum (horny layer), which are shed. Human keratinocyte turnover, from epidermal stem cells of basal layer to desquamation and shedding, occurs every 40–56 days. Keratinocytes that have completed their differentiation program and have lost their nucleus and cytoplasmic organelles will eventually be shed through desquamation, as new cells keep proliferating and progressing towards the outer epidermal layer.
Adapted from (Halprin, 1972; Koster, 2009);
<http://www.intranet.tdmu.edu.ua/data/kafedra/internal/lik_tex/classes_stud/en/pharm/prov_pharm/ptn/Technology%20of%20cosmetic%20products/5/practical%201.htm>, viewed 5 May 2014.

Epidermal keratinocytes are also known to express various antioxidant enzymes such as superoxide dismutase (SOD), which protect cells against oxidative insult (Haruna et.al., 2008). The intracrine (within a cell) synthesis of vitamin D in human epidermal keratinocytes activates multiple signal transduction pathways that have protective effects against DNA damage caused by solar exposure (De Haes et.al., 2005). Solar irradiance is a major source of DNA damage and free radical production in keratinocytes (De Haes et.al., 2005). Interestingly, it was reported that UV-exposed cutaneous nerve cells are capable of expressing neuroglobin, a protein that scavenges free

radicals (Gruber & Holtz, 2009). The fundamental living cells of the skin exhibit synergistic responses towards the detrimental events of solar exposure (Figure 1.2). It is not well understood how various cells within cutaneous layers communicate following damage.

Keratinocytes are known to produce mitochondrial antioxidant enzymes, such as SOD, glutathione peroxidase, glutathione reductase, copper- and zinc-dependent superoxide dismutase (CuZnSOD), catalase and manganese-dependent superoxide dismutase (MnSOD), when exposed to UV radiation. The optimal dose of solar exposure is yet to be determined, but there is no doubt that there is an essential requirement for endogenous calcidiol and calcitriol synthesis in this process. In addition, calcitriol inhibits UVB-induced damage of human keratinocytes, and possibly reduces accumulation of reactive nitrogen species and subsequently reduces oxidative and nitrosative damage (De Haes et.al., 2005; Song et.al., 2013a). Furthermore, calcidiol regulates the expression of antimicrobial and pro-inflammatory peptides such as cathelicidin (Schrumpf et.al., 2012). This regulation requires local conversion of calcidiol into the active hormone calcitriol by renal enzyme, CYP27B1 (Schrumpf et.al., 2012). Moreover, pretreatment of ex vivo cultured human skin cells with high physiological concentrations of calcitriol (1 nM), prior to irradiation, results in decreased thymine dimer accumulation, hence reduction in DNA damage (Song et.al., 2013a). While some reports suggest photoprotective actions of calcitriol, further studies are required to determine the efficacy of vitamin D metabolites against solar irradiance.

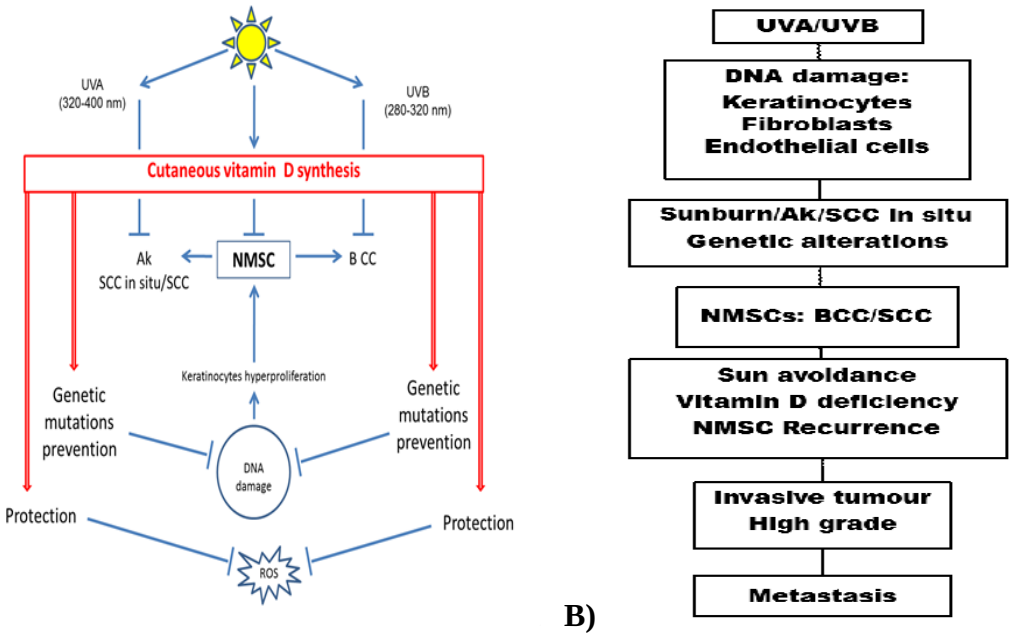


Figure 1.2. Vitamin D protective effects against NMSC.

A) Vitamin D modulation of cutaneous carcinogenesis. Activation of cutaneous vitamin D synthesis via UVB; subsequent protection from DNA mutations and damage from free radicals.

B) Vitamin D deficiency and NMSC recurrence. Vitamin D deficiency (calcidiol and calcitriol) can be associated with NMSC, as DNA damage by solar irradiation triggers formation of premalignant lesions such as AK and SCC in situ; most NMSC patients prevent further damage to the skin by avoiding sun exposure. The result of this avoidance is vitamin D deficiency and recurrence of NMSC.

AK, actinic keratosis; BCC, basal cell carcinoma; NMSC, non-melanoma skin cancer; SCC; squamous cell carcinoma; SCC in situ, squamous cell carcinoma in situ; UVA, ultraviolet A; UVB, ultraviolet B.

Accumulation of genetic alterations over a lifetime of excessive sun exposure, viral infections and immune-compromised conditions are the major risk factors in the development of NMSC (Afaq et.al., 2005; Arora & Attwood, 2009; Bachelor & Bowden, 2004; Holick, 2006b; Moan et.al., 2007; Schauber & Gallo, 2008). Sun exposure has been linked to pathological conditions of the skin, including sunburn, skin aging, melanomas and NMSC (Reichrath, 2006). Studies have shown that UVA (320-400 nm) exposure can cause oxidative stress (Basu-Modak et.al., 2003; Song et.al., 2013a). Skin acts as a first line of defence against oxidative stress (i.e. damage via free radicals; Gruber & Holtz, 2009).

The endogenous synthesis of vitamin D and its role in the prevention of cutaneous carcinogenesis is still poorly understood. It is suggested that vitamin D synthesis in the epidermis has several protective qualities. Low physiological dose (10^{-8} M) of calcitriol (active vitamin D) inhibits keratinocyte proliferation and differentiation (Smith et.al., 1986). It was also demonstrated that calcitriol at concentrations of 10^{-7} M inhibits proliferation of human keratinocyte cell line (HaCaT) (Reichrath et.al., 2010).

The effect of calcitriol is versatile and depends on the cell type origin (as well as whether it is a primary, cancerous or immortalised cell lines), applied dose and duration of the exposure. Studies show inhibitory effects of calcitriol action on primary epidermal keratinocytes, melanocytes, facial sebocytes and some types of squamous cell carcinoma cell lines, at concentrations much greater than physiological (range of 10^{-6} – 10^{-9} M) (Koizumi et.al., 1997; Reichrath et.al., 2004; Krämer et.al., 2009; Chiang et.al., 2013; Reichrath et.al., 2010). However, very low physiological concentrations (10^{-11} M) calcitriol stimulates proliferation of keratinocytes (Lehmann, 2005). Interestingly, the stimulatory effect of calcitriol has been shown at quite high physiological doses (10^{-5} – 10^{-7} M) in normal human fibroblasts (Gruber & Anuszewska, 2002).

It has been suggested that some of the genetic alterations that are induced by chronic sun exposure in individuals with pre-malignant skin lesions can be reverted by well-balanced and maintained vitamin D supplementation (or its endogenous accumulation) (Baeke et.al., 2008; Nürnberg & Reichrath, 2009).

Interestingly, newly diagnosed patients with melanocytic skin cancers (MSCs) are less prone to subsequently developing malignant melanoma if they have sufficient levels of calcidiol (Baeke et.al., 2008; Nürnberg & Reichrath, 2009). An inverse relationship exists between 25(OH)D levels and risk of malignant melanoma (Field & Newton-Bishop, 2011). Similarly in NMSC patients, higher serum levels of 25(OH)D are associated with a decreased risk of having a history of NMSC. Further, the beneficial role of calcidiol against various cancers is apparent in numerous studies

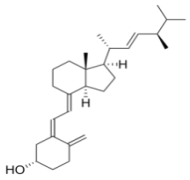
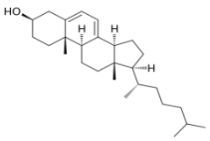
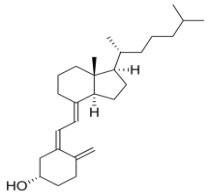
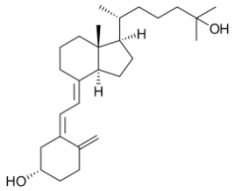
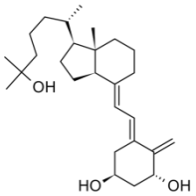
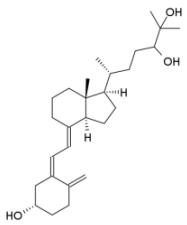
(Banerjee & Chatterjee, 2003; Giovannucci et.al., 2005; Bouillonet et.al., 2006; Holick, 2006b; Deeb et.al., 2007; Dixon et.al., 2007; Moan et.al., 2007; Lehmann, 1997; Krishnan et.al., 2010; Mason et.al., 2010; Vinceti et.al., 2011). Higher serum 25(OH)D levels are associated with better survival and reduced risk of metastasis (Garland et.al., 2009; Grant & Mohr, 2009; Ng et.al., 2009). Moreover, an inverse relationship between VDR and tumour growth has been noted in animal models, where VDR deficient mice are predisposed to benign and malignant neoplasm formation (Teichert et.al., 2011).

Whilst these reports agree on an inverse association between vitamin D and risk of skin cancer, some epidemiological studies state otherwise. Increased levels of circulatory 25(OH)D are associated with increased risk of non-melanoma and melanoma skin cancers (Liang et.al., 2012; Afzal et.al., 2013). It was reported in over 28 year follow-up study plasma 25(OH)D levels were measured in 10,060 individuals. Of these individuals, 590 individuals developed non-melanoma skin cancer while 78 individuals developed melanoma skin cancer (Afzal et.al., 2013). It was apparent that there was a correlation between high 25(OH)D levels with the incidence of non-melanoma skin cancers. However, it is acceptable that association can not prove causation. Hence, it is imperative to understand the complex relationship between vitamin D and NMSC, and whether vitamin D contributes to skin cancer or acts as a preventative agent.

1.4 Vitamin D metabolism

Vitamin D can be obtained from dietary sources, including fish, dairy, egg yolk and mushrooms. However, the major source of vitamin D is exposure to sunlight. 7-dehydrocholesterol (7-DHC) absorbs solar UVB radiation and results in the production of pre-vitamin D3 which is thermally isomerised to vitamin D3 (Tian & Holick, 1995). This isomerisation is a reversible process, and able to interconvert pre-vitamin D back to cholecalciferol.

Table 1.1. Nomenclature of vitamin D compounds.

IUPAC recommended name	Compound structure	Alternative names
Ergocalciferol		Vitamin D_2, D_2
7-dehydrocholecalciferol		Provitamin D, 7-DHC
Cholecalciferol		Calciol; an inactive, unhydroxylated form of vitamin D, previtamin D, (6Z)-Tacalciol
Calcidiol		Hydroxyvitamin D, , 25-hydroxycholecalciferol, 25-hydroxyvitamin D
Calcitriol		Calfediol, dihydroxyvitamin D, 1,25-dihydroxycholecalciferol, 1,25-dihydroxyvitamin D; active vitamin D_3 hormone
(24R)-Hydroxycalcidiol		24,25-Dihydroxycholecalciferol, 24,25-dihydroxyvitamin D, inactive vitamin D

Adapted from (Norman et.al., 2008); <http://www.chem.qmul.ac.uk/iupac/misc/D.html>, viewed 24 July 2014.

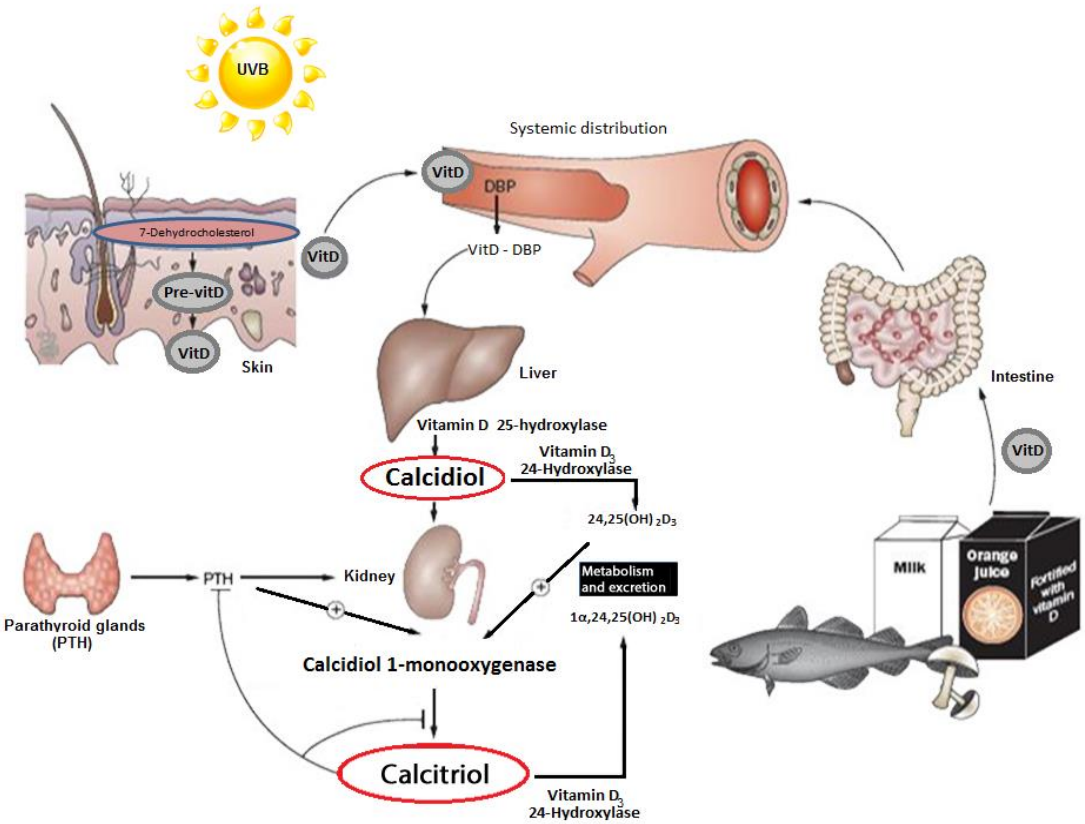


Figure 1.3. Physiology of vitamin D metabolism

Vitamin D synthesis is initiated in the skin in response to UVB exposure, or it is absorbed from the diet. The pre-vitamin D is preferentially transported by vitamin D binding protein (DBP) to the liver, and finally to the kidneys, where it undergoes sequential hydroxylation step to yield the hormonally active form, calcitriol (by calcidiol–monooxygenase, or CYP27B1). Calcidiol and calcitriol may induce the expression of vitamin D3 24-hydroxylase (CYP24A1), which is involved further in its metabolism and excretion. Adapted from <http://arayofhealth.wordpress.com/2011/01/09/vitamin-d-metabolism/>, viewed 1 July 2015

Vitamin D is not strictly classified as a vitamin, as it is not an essential dietary compound, but rather an endogenous pre-hormone produced by sequential metabolic steps. The molecular structure of vitamin D compounds consists of a cyclopentanoperhydrophenanthrene ring, which is closely related to that of classic steroid hormones such as oestradiol, cortisol and aldosterone (Norman, 2008). In this respect, cacitriol has the same root as all other steroid hormones; however, it is often known as a secosteroid because one of the rings of its cyclopentanoperhydrophenanthrene structure has a broken carbon–carbon bond, as shown in Table 1.1.

It has been shown in the past that the classic vitamin D endocrine function occurs via regulation of the circulating levels of calcitriol, whose synthesis is dependent on ionic homeostasis and other endocrine requirements (Banerjee & Chatterjee, 2003). Decreases in endogenous levels of calcitriol are known to regulate its own production via activation of PTH, which stimulates renal production of calcitriol and controls serum concentrations of calcium and phosphate. When the circulating concentration of calcitriol reaches a threshold level, renal synthesis of calcitriol declines rapidly (Norman, 2008). Interestingly, current research has shown that in some people with the clinical symptoms of a mild hypercalcemia, with circulatory 25(OH)D is in the range 51-100 ng/mL, had no association with low PTH levels, which would expected to be suppressed, and lack of other clinical symptoms (Dudenkov et.al., 2015)

1.4.1 25-hydroxylation of vitamin D

Calcidiol synthesis is regulated by the hepatic microsomal and mitochondrial cytochrome P450 monooxygenase, vitamin D 25-hydroxylases, encoded by the microsomal (*CYP2R1, CYP2J2/3, CYP3A4, CYP2D25* and *CYP2C11* and mitochondrial (*CYP27A1*) genes (Vantieghem, 2006; Zhu & DeLuca, 2012). It seems as CYP27A1 is no longer considered to be a principal hydroxylase resulting in calcidiol formation in knockout mice, where deletion of CYP2R1 led to 50 % reduction in calcidiol serum levels, compared to wild type (Zhu & DeLuca, 2012). Interestingly, in CYP27A1 knockout mice, the loss of CYP27A1 does not cause rickets or bone abnormalities associated with vitamin D deficiency, instead CYP271 knockout mice exhibit very high serum calcidiol levels and a significant reduction in bile acid production, with large impact on lipid metabolism (Rosen et.al., 1998). Hence, CYP27A1 has minimal contribution in calcidiol production in mice. However, later findings suggest that only mitochondrial CYP27A1 is involved in hydroxylation of vitamin D precursor, dehydrocholesterol (DHC), resulting in the formation of 25-Hydroxy-7-DHC (Endo-Umeda et.al., 2014). Interestingly, 25-Hydroxy-7-DHC activates VDR, whilst 7-DHC and 26/27-hydroxy-7-DHC does not. Moreover, the only product of

CYP27A1 hydroxylation (but not CYP2R1), 25-Hydroxy-7-DHC, activates VDR and modulates transactivation of liver X receptor (LXR) (Endo-Umeda et.al., 2014).

Hence, CYP27A1 is the key enzyme in the conversion of cholecalciferol to calcidiol in the process of mitochondrial (CYP27A1) and microsomal (CYP2R1) hydroxylation at carbon-25 of vitamin D (Nürnberg & Reichrath, 2009). An inherited mutation in the CYP2R1 gene can lead to low circulating levels of calcidiol and classic symptoms of vitamin D deficiency, as the substitution of a proline for a leucine at amino acid 99 eliminates the enzymatic activity of CYP27A1 (Cheng et.al., 2004).

The plasma level of calcidiol is commonly used as an indicator of endogenous vitamin D status (Schuster, 2011). The complex mechanism of VDR activation and numerous contributing factors in endogenous synthesis of vitamin D metabolites warrants further studies.

1.4.2 Renal and extra-renal calcidiol 1-monooxygenase (CYP27B1)

Calcidiol 1-monooxygenase, (from now on referred to CYP27B1), encoded by the gene *CYP27B1*, is a member of the mitochondrial cytochrome P450 enzyme family (Schuster, 2011). CYP27B1 (often referred as 25-hydroxycholecalciferol 1-hydroxylase; 25-hydroxycholecalciferol 1-monooxygenase; 1-hydroxylase-25-hydroxyvitamin D_3; 25-hydroxy D_3-1α-hydroxylase; 25-hydroxycholecalciferol 1α-hydroxylase; and 25-hydroxyvitamin D_3 1α-hydroxylase) produces hormonally active vitamin D – (calcitriol) – from the precursor hormone calcidiol in the kidney (Lips 2006). The expression of this enzyme has also been detected in a number of tissues, including the skin (Krishnan et.al., 2010; Bachelor & Bowden, 2004; Holick, 2006a). CYP27B1 is a critical enzyme in calcium homeostasis; however, its role in other human tissues is not completely clear (Zehdner et.al., 2000).

Numerous studies describe CYP27B1 role in cancer cells, including those from colon, prostate, mammary gland and skin (Tangpricha et.al., 2001; Hewison et.al., 2004; Deeb et.al., 2007). These studies indicate the significance of CYP27B1 in neoplastic formation and progression (Brożyna et.al., 2013; Diesel et.al., 2004). Polymorphisms observed in genes encoding proteins involved in calcitriol homeostasis are associated with tumour growth and cancer progression. It was shown in colon cancer cells that CYP27B1 proteins containing single-nucleotide polymorphisms (SNPs) had reduced enzymatic activity compared to the wild-type protein (Jacobs et.al., 2013). Deregulation of CYP27B1 during cancer development contributes to abrogation of tumour suppressive effects triggered by calcitriol (Jacobs et.al., 2013).

It was believed that calcitriol would carry out the functions of an endocrine hormone only (Lips, 2006). However, calcitriol performs an array of functions including maintenance of serum

calcium and phosphorus levels, it is required for skeletal mineralisation, has roles in the cell cycle and differentiation, and acts as a tumour regulatory factor (Bikle et.al., 2011b; Oda et.al., 2004; Garland et.al., 2011; Deeb et.al., 2007; Wu et.al., 1996; Ebert et.al., 2006).

The antitumourigenic functions of calcidiol have received significant attention in recent years (Lou et.al., 2004; Geng et.al., 2011; Krishnan et. al., 2013). The malfunction of vitamin D metabolism observed in malignant cells is an indicator for an inverse correlation between serum calcidiol status and cancer development (Friedrich et.al., 2005). 25-hydroxylation plays an important role in maintaining normal tissue homeostasis, and dysregulation of this mechanism may contribute to tumour development and aggressiveness (Diesel et.al., 2004; Cross et.al., 2011).

Vitamin D enzymatic activity plays a significant role in the pathogenesis and progression of melanoma skin cancers (Brożyna et.al., 2013; Reichrath, 2006). The expression of CYP27B1 in melanocytic skin cancers is directly correlated with tumour behaviour and overall survival (Brożyna et.al., 2013). Although most patients with NMSC can be considered to be at lower risk of morbidity or mortality compared to melanoma, each year an increasing number of patients are diagnosed with advanced NMSC, including SCC and BCC (Australian Bureau of Statistics, ABS; <http://www.abs.gov.au/Ausstats/abs>, viewed 12 April 2016).

The cellular loss of CYP27B1 activity has been shown in human melanocytic skin cancers to correlate with tumour phenotype and behaviour, and its lack affects the survival of melanoma patients, suggesting a role in the pathogenesis and progression of cancer (Egan, 2009; Field & Newton-Bishop, 2011; Vinceti et.al., 2011; Brożyna et.al., 2013). However, it is less clear, whether CYP27B1 has similar effects in the pathogenesis of NMSC.

1.4.3 Vitamin D catabolism and vitamin D3 24-hydroxylase (CYP24A1)

Mitochondrial vitamin D3 24-hydroxylase (encoded by the gene *CYP24A1*), (from now on referred as CYP24A1) is responsible for the hydroxylation at carbon-24 of calcitriol and calcidiol (Luo et.al., 2012). This hydroxylation contributes to calcitriol degradation, resulting in its transformation into short-lived water-soluble products, such as calcitroic acid (Figure 1.3), which have reduced biological activity and are excreted in bile (Jones, 1999; Luo et.al., 2012). 24-hydroxylation is an important enzymatic process that contributes to terminating excess calcidiol and calcitriol (Annalora et.al., 2010). Vitamin D signaling and antitumour activity appears to be dependent on the expression of CYP24A1 as predominantly catabolic inactivator of the active vitamin D hormone, calcitriol, in the vast majority of normal tissues (Luo et.al., 2012). The catalysis by CYP24A1 results in several reactions with the final production of inactive, water-soluble vitamin D by-product in bile. In addition, CYP24A1 expression becomes deregulated in

numerous human tumours (Chen et.al., 2011; Luo et.al., 2012). Although elevated CYP24A1 expression has been reported in some skin cancers, the evidence that it plays a causative role in neoplasia is still not clear (Brożyna et.al., 2014).

CYP24A1 enzymatic expression is broadly distributed in the target tissues, including kidney, intestine, bone, and skin (Bouillon et.al., 2006). In target cells, calcitriol triggers its own degradation by up-regulation of CYP24A1 expression, to attenuate the action of calcitriol. CYP24A1 overexpression is noted in many cancers, such as colorectal and lung, and has been associated with tumorigenesis and metastasis (Chen et.al., 2011; Luo et.al., 2012; Peehl et.al., 2004).

1.4.4 Vitamin D binding protein

The primary carrier of vitamin D in the circulation is vitamin D binding protein (VDBP), which regulates the bioavailability of calcitriol (White & Cooke, 2000). Calcidiol can enter renal cells (and possibly keratinocytes, which expresses megalin receptor (Adly et.al., 2010)) via the VDBP transport system, where it is converted to the active hormone, which has modulatory functions that influence gene expression. Conversely, calcitriol has low affinity for the transport protein VDBP and high affinity for VDR, principal receptor regulating the transcription of several target genes in vitamin D target cells (Alshahrani & Aljohani, 2013).

The relationship between alterations in VDBP and the risk of vitamin D-associated diseases (such as malfunction in transportation of vitamin D metabolites and immune disorders) has been reported in numerous studies (Christakos et.al., 2003; Noya, 2012; White & Cooke, 2000). Circulatory VDBP, also known as GC-globulin, is well known for its role in the transport of vitamin metabolites. VDBP is an abundant protein in plasma. It is the primary transporter of calcidiol, and therefore has a role in regulating its circulatory levels (Christiansen et.al., 2007; Speeckaert et.al., 2006). It regulates free (unbound) calcidiol, and has tissue and cell type specific utilisation (White & Cooke, 2000). Other roles include actin scavenging and fatty acid binding. In addition, VDBP has a role in diabetes mellitus, early-stage breast cancer, oral squamous cell carcinoma, aggressive periodontitis, idiopathic temporal lobe epilepsy, Alzheimer's and Parkinson's diseases, and myopia (Malik et.al., 2013). It has been shown to be downregulated in patients with sepsis, neuromyelitis optica, cutaneous malignant melanoma, hepatocellular carcinoma and breast cancer (Malik et.al., 2013). Further studies are required to determine the relationship of VDBP, calcidiol status and skin cancers.

1.5 Vitamin D receptor

Vitamin D receptor (VDR) belongs to the large superfamily of transcriptional regulatory factors, which includes the steroid and thyroid hormone receptors, the retinoid X receptors and retinoic acid receptors. VDR is encoded by a relatively large gene located on chromosome 12q13. It consists of the noncoding 5′ promoter region that is encoded by exons 1A, 1B and 1C, exons 2 and 3, which encode the DNA binding domain, and exons 6 to 7, which encode the ligand-binding region (Pike et.al., 2011). Different domains of VDR are involved in different functions, including DNA binding, receptor dimerisation, gene transactivation and cofactor activation (Mizwicki & Norman, 2011).

VDR has a similar domain structure as other nuclear receptors. It comprises 427 amino acids encompassing a short amino-terminal activation function 1 (AF1) domain, a DNA binding domain (DBD) containing two zinc fingers, a flexible hinge region that includes nuclear localisation signals, a ligand binding domain (E1) and a carboxyl-terminal transcriptional activation domain (AF2; Figure 1.4) (Pike & Meyer, 2012; Ondková et.al., 2006). Upon calcitriol binding to the ligand binding domain, VDR is stabilised by phosphorylation in the DNA binding domain. VDR associates with the retinoid X receptor (RXR) through dimerisation domains in E/F (Carlberg & Campbell, 2013).

VDR is known to mediate the activity of numerous antiproliferative genes and plays a role in the regulation of the cell cycle in many cancers (van Leeuwen & Pols, 2005; Bouillon et.al., 2006; Spina et.al., 2007; Guan et.al., 2013; Welsh, 2011). The binding of calcitriol to the nuclear VDR is vital for VDR-mediated regulation of various transcriptional factors (Guan et.al., 2013; Li et.al., 2007; Spina et.al., 2007; Srinivasan et.al., 2011). Altered expression of VDR can affect the expression of cytochrome P450 hydroxylases (Brown et.al., 1999; Banerjee & Chatterjee, 2003). Indeed, VDR knockout mice exhibit extremely high rates of skin cancer development (Bikle et.al., 2013).

In addition, vitamin D deficient (diet induced) VDR knockout mice lack vitamin D calcemic effects, and have extremely high serum levels of calcitriol (Nakagawa et.al., 2004). The overexpression of *CYP27B1* gene that is responsible for calcitriol synthesis inhibits metastatic growth of lewis lung carcinoma cells (Nakagawa et.al., 2004). These findings establish a critical role for calcitriol, in lung metastasis (Nakagawa et.al., 2004). Furthermore, studies indicate that VDR ablation contributes to diminished production of calcidiol and calcitriol in skin cancer in both animal models and in humans (Banerjee & Chatterjee, 2003; Campbell et.al., 2009 ; Jiang et.al., 2013; Correa et.al., 2002). VDR acts as an important determinant of cellular sensitivity to

the actions of calcitriol and calcidiol. It remains unclear, however, whether all types of skin cancers have alterations in VDR and if these alterations contribute to cancer progression. These are important points, as vitamin D pathways are able to modulate carcinogenesis only if cells express and maintain functional VDR (Chung et.al., 2009; Keisala et.al., 2009; Whitfield et.al., 2001; Savkur et.al., 2005).

The anti-proliferative actions of calcitriol have been shown to be mediated by VDR in mice (Chung et.al., 2009). Treatment with calcitriol results in tumour growth inhibition in wild type mice expressing VDR compared to VDR knockout mice. This study demonstrated that tumours from VDR knockout mice were relatively resistant, suggesting that calcitriol-mediated growth inhibition is VDR-dependent and loss of VDR can lead to abnormal tumour growth (Chung et.al., 2009). Moreover, VDR mediated mechanism of protection against skin cancer formation was shown in keratinocytes lacking VDR (Jiang et.al., 2014). VDR deficient keratinocytes have increased expression of oncogenes and decreased expression of tumor suppressor genes, an ideal predisposition to skin cancer formation in mice (Jiang et.al., 2013). In addition, VDR knockout mice have poorer survival, early alopecia, thickened skin, enlarged sebaceous glands and development of epidermal cysts and premature ageing (Keisala et.al., 2009). Futhermore, it was established that the common polymorphisms in human VDR have an impact on receptor function and its transactivational potency (Whitfield et.al., 2001).

Calcitriol is produced within keratinocytes and other tissues, which also express VDR. Most of the biological actions of calcitriol are mediated through VDR, which is a member of the nuclear receptor superfamily and acts as a ligand-dependent transcription factor with co-activators (Haussler et.al., 2011). After renal hydroxylation, calcitriol is released into the circulatory system, where it binds to its carrier protein, VDBP. Delivered to target cells, calcitriol dissociates from VDBP, presumably enters the cell by diffusion, and binds to nuclear VDR to regulate gene transcription (Lips 2006). VDR is expressed in most tissues; it is known to regulate over 900 genes and has a broad range of biological actions, including ionic homeostasis, modulation of cellular proliferation, differentiation, migration and inhibition of angiogenesis (Schuster, 2011).

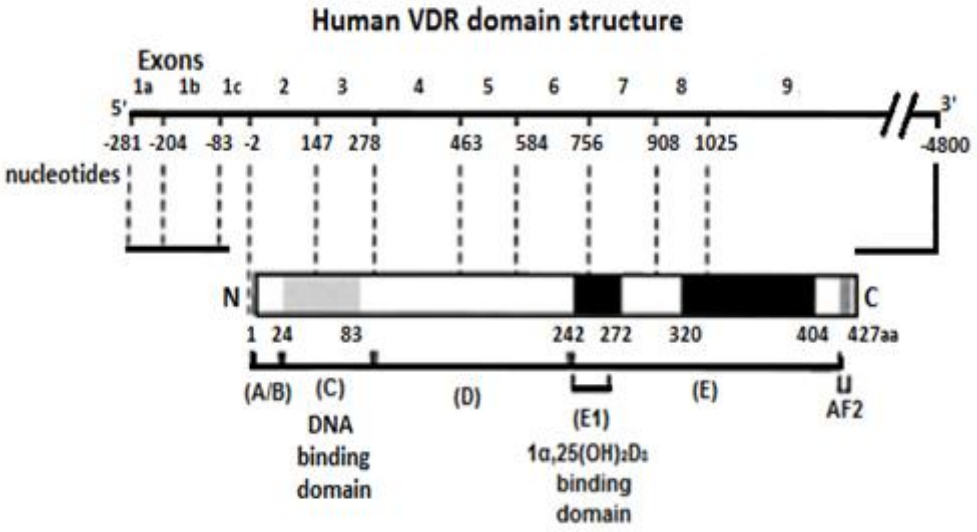


Figure 1.4. Schematic representation of the human *VDR* gene domain structure.

VDR encompasses an AF1 domain (A/B), a DNA binding domain containing (C), a flexible hinge region (D) containing nuclear localisation signals, a ligand binding domain (E1) and an AF2 domain.

Adapted from (Carlberg & Campbell, 2013).

VDR requires heterodimerisation with co-regulatory proteins for effective DNA interaction (Mizwicki & Norman, 2011). Following binding of calcitriol to VDR and heterodimerisation with RXR, the calcitriol–VDR–RXR complex binds to vitamin D response elements (VDREs) in the promoter regions of specific genes, thus initiating target gene transactivation (Oda et.al., 2004). Several co-regulatory (or cofactor) proteins have been identified: RXR, steroid receptor activator complex (SRC), as well as numerous co-activator and co-suppressor protein complexes.

1.5.1 VDR genomic and non-genomic responses

VDR mediates the regulation of gene expression controlling tumour cell growth and development (Palmer et.al., 2008). The wide expression of VDR among various tissues (including epithelial lining, muscular-skeletal system, endocrine glands, blood cells and neurons) indicates that its actions are not restricted to classical vitamin D target tissues and suggest that ligand mediated VDR genomic and nongenomic regulation occurs by endocrine, paracrine, and autocrine mechanisms (Scolletta et.al., 2013). Following binding to its nuclear receptor, calcitriol heterodimerises with one of the RXRs: RXR-α, RXR-β or RXR-γ (Yang et.al., 2012). The genomic pathway is illustrated in Figure 1.5. Numerous proteins form complexes with the activated VDR–RXR heterodimers to promote (or inhibit) transcriptional activity (Lefebvre et.al., 2010; Marshall et.al., 2012).

The complex network of VDR-regulated genes encompasses cellular events such as cell cycle and apoptosis regulation, differentiation, oncogenesis, and immune and metabolic responses.

The rapid responses (Figure 1.5) mediated by calcitriol bound to its receptor occurs within minutes (1–2 min to 15–45 min) (Haussler et.al., 2011; Norman et.al., 2002; Norman et.al., 2004). This is in contrast with genomic responses, which generally take several hours to days to be fully apparent, and which can be blocked by inhibitors of transcription and translation (Norman et.al., 2004). Rapid responses include transcaltachia, insulin secretion, endothelial cell and smooth muscle cell migration, and osteoblast Cl^- and Ca^{2+} regulation (via endocytosis and exocytosis) (Huhtakangas et.al., 2004).

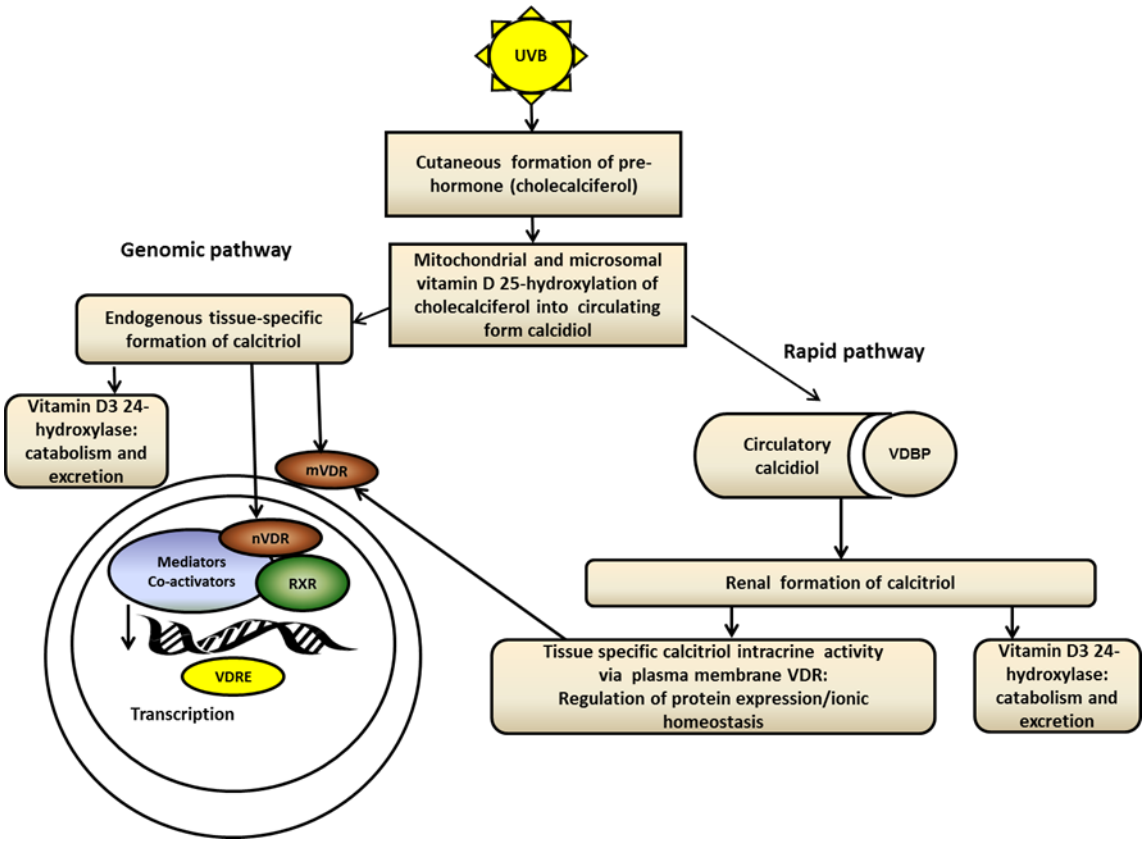


Figure 1.5. Schematic representation of vitamin D pathways.

Cutaneous cholecalciferol undergoes 25-hydroxylation, converting it to calcidiol. Rapid response pathway: serum calcidiol binds to VDBP (DBP) to reach the kidneys, for hydroxylation to calcitriol by CYP27B1. Genomic pathway: renal and non-renal (tissue specific) 1α-hydroxylation by calcidiol 1-monooxygenase (CYP27B1) and formation of calcitriol. Liganded VDR influences genetic mediators of transcriptional activity with involvement of VDRE.

Calcitriol; calcidiol; RXR, 9-cis retinoid X receptor; DBP, vitamin D binding protein; VDR, vitamin D receptor; mVDR, membrane VDR; nVDR, nuclear VDR; VDRE, vitamin D response element.

1.5.2 VDR transcriptional activation by calcitriol

Two major co-activator complexes have been identified: the steroid receptor activator complex (SRC), consisting of the p160 family of SRC1, SRC2 and SRC3 co-activators; and the vitamin D receptor interacting protein (DRIP) mediator complex (Sebag et.al., 1992). To form a VDR-mediating protein complex (the major VDR binding complex in proliferating keratinocytes), VDR is required to bind to calcitriol to enable formation of DRIP and SRC complex. SRC coordinates VDR-mediated regulation of keratinocytes differentiation, whereas DRIP facilitates the ability of VDR to regulate cellular proliferation (Bikle et.al., 2010). Calcitriol regulates keratinocyte proliferation and differentiation via the DRIP mediator complex (Oda et.al., 2007). In addition, vascular endothelial growth factor (VEGF), a vascular permeability factor that is involved in the regulation of angiogenic activity, was reported to be VDR dependant. Alterations in VEGF expression have effects in cancer development and metastasis. Calcitriol regulates VEGF production via the VEGF–VDR–RXR promoter complex (Cardus et.al., 2009).

VDR regulation of transcription is directed by an orchestra of transcription factors, receptors, bound ligands and DNA response elements, which work together towards inhibition or stimulation of gene expression (Marshall et.al., 2012). VDR expression has been identified in most tissues in humans, although its expression is altered in cancer tissues. The underlining mechanism resulting in these changes in VDR expression is not clear.

1.5.3 VDR transcriptional repression by calcitriol

VDR-dependent suppression of gene transcription by VDR–RXR regulates multiple pathways. One of the VDR-inhibitory pathways involve nuclear receptor co-repressor(s), which alters the architecture of chromatin in the vicinity of the target gene to heterochromatin (Carlberg & Campbell, 2013). As a repression regulator, liganded VDR binds to co-repressor rather than co-activator (Bikle et.al., 2010).

Studies have identified a protein that acts as a suppressor of VDR in the skin, known as hairless (HR) (Thompson & Beaudoin, 2006). The structure and function of this protein were studied in unpigmented and immunocompetent hairless mice. These mice are a good animal model, as they allow for manipulation of the skin by application of topical agents and easy visualisation of the cutaneous response to UV rays, acute photobiological responses and skin carcinogenesis (Benavides et.al., 2009). Loss of, or mutations in, HR in a cycling hair follicle are responsible for the phenotypic development of hairless mice, and also result in baldness in humans. Interestingly, the *HR* gene was found to inhibit calcitriol-mediated transcription, by binding to and repressing VDR action, as described in keratinocytes (Bikle et.al., 2007). The

region of VDR interaction with HR overlaps the DNA sites responsible for interaction with other steroid hormone receptors, although the precise amino acids involved in the interaction of HR with VDR have not been determined (Hsieh et.al., 2003). HR is also known to suppress VDR activity both in the absence and presence of vitamin D (Thompson & Beaudoin, 2006). There is mounting evidence that HR is a co-suppressor of VDR in animals and humans (Thompson et.al., 2006; Benavides et.al., 2009; Hsieh et.al., 2003). Similar to co-activators, the co-suppressors can be very specific for different genes in different tissues.

1.5.4 Hypercalcaemia and vitamin D toxicity

Excessive supplementation of vitamin D can cause toxicity (Alshahrani & Aljohani, 2013). Importantly, calcitriol has low affinity for the transport of protein VDBP and high affinity for VDR (Alshahrani & Aljohani, 2013). Conversely, calcidiol can enter the cell via the VDBP transport system, where it is converted to active hormone resulting in modulatory functions that influence signal transduction.

Vitamin D supplementation has a positive association, in particular in asthma, diabetes, stroke, multiple sclerosis, cognitive decline, dermatopathlogical conditions and influenza infection (Grant et.al., 2009; Kesby, 2011). Vitamin D deficiency is a very common condition amongst patients with infectious diseases (both bacterial and viral, especially respiratory infections), falls and fractures, cardiovascular disease, autoimmune diseases and cancers (Grant et.al., 2009, Grant, 2012). However, in some conditions, such as cardiovascular diseases and infectious diseases, the role of vitamin D requires further investigation. While some documented literature suggests intoxication as a result of high doses of vitamin D (156,000 to 2,604,000 IU of vitamin D daily), the Endocrine Society recommendation is 4000 IU daily uptake for everyone over 8 years old (Holick et. al., 2011). Furthermore, in Crohn's disease low levels of vitamin D is associated with the disease (Suibhne et.al., 2012). However, it was reported in 2 Crohn's disease patients that excessive serum $1\alpha,25(OH)_2D$ levels, corresponded to tissue-specific enhanced expression of CYP27B1, whilst 25(OH)D levels remained unchanged, suggesting that calcidiol has nothing to do with calcitriol toxicity, known to be a direct precursor of calcitriol (Bosch, 1998).

Numerous clinical studies are currently in progress: VITAL, 2000 IU (International Units (IU) of vitamin D) daily (USA) and FIND, 1600-3200IU daily (Finland) to determine the effects of vitamin D in cancer and cardiovascular disease; ViDA, 100,000-200,000 IU monthly (NZ) and its effects in cardiovascular disease, respiratory disease and fractures; VIDAL, 60,000 IU monthly (UK) to assess it role in longevity (Kupferschmidt, 2012). The aim of these studies is to evaluate the efficacy of vitamin D therapy in oncological conditions, cardiovascular disease, diabetes,

cognitive function and longevity. The outcome of these trials will be available after 2017 (Kupferschmidt, 2012). At present, there is great amount of controversy regarding the appropriate concentration of vitamin D to be used, the effects of vitamin D deficiency and its potential toxic effects.

Meanwhile, it was suggested, that calcidiol toxicity may occur with circulatory concentrations beyond 500 nM (Vieth, 2006). It was also suggested that vitamin D daily prolonged intake of 250 μg (10,000 IU) likely poses no risk of adverse effects in almost all individuals in the general population (Vieth, 2007). On average, vitamin D intake of 5,000 IU/day achieves serum calcidiol (or 25(OH)D) concentrations between 100–150 nM (NIH, Department of Health and Human services, FDA: <http://ods.od.nih.gov/factsheets/VitaminD-HealthProfessional//>, July 2015). Most recent population-based study, involving over 20,000 people, measured 25(OH)D. This study found the levels of circulatory calcidiol in some people 1714 (8.4%), 123 (0.6%), and 37 (0.2%) over 50, 80, and 100 ng/mL, respectively. Where serum 25(OH)D values were not significantly related with serum calcium values or with the risk of hypercalcemia. This study reports that the incidence of circulatory 25(OH)D values over 50 ng/mL increased significantly between 2002 and 2011, without a corresponding increase in acute clinical toxicity (Dudenkov et.al., 2015).

With no hypercalcaemic effects observed for calcidiol at the highest dose of 40,000 IU/day (Garland et.al. 2011), it may provide health beneficial effects (Finamor et.al., 2013). In addition, the risks of vitamin D toxicity shown in a longitudal study (1985-1991), were of over-fortified vitamin D in milk that was supplied to 11,000 households. According to hospital discharge records and laboratory data, there were identified cases of clinical hypervitaminosis D (ranging 56-696 ng/ml) in the affected communities during that period (Blank et.al., 1995). High circulatory vitamin D levels are associated with anorexia, weight loss, weakness, fatigue, disorientation, vomiting, dehydration, polyuria, constipation and in severe cases, death. However, it was suggested that this could be the cause of a pre-existing susceptibility for the development of hypervitaminosis D following exposure to overfortified milk (Blank et.al., 1995). It is well accepted that vitamin D toxicity to be associated with varying degrees of hypercalcaemia, hypercalciuria and some additional complications (Alshahrani & Aljohani, 2013). However, circulatory 25(OH)D as high as 125 nM in some individuals was noted without clinical signs of intotoxicication (Dudenkov et.al., 2015). Solar exposure itself does not lead to toxicicity as the excessively generated vitamin D metabolites are very sensitive to sunlight and undergo photolysis upon excessive exposure. The excess formed vitamin D undergoes photodegradation to a variety

of photoproducts, such as 5,6-transvitamin D, suprasterol I, and suprasterol II (Webb, et.al., 1989). It does not come as a surprise, that excessive and unprotected sun exposure can result in sunburn and/or various types of skin cancers. Due to limited clinical studies it is difficult to establish firm conclusions on vitamin D toxic dose, optimal daily dose required for health maintenance, and its cancer preventative or therapeutic dose. While the optimal circulatory vitamin D range is unclear, it is difficult to accurately identify its toxic levels. Interestingly, epidemiological studies, involving 3,667 participants, demonstrated a dose–response relationship between oral intake of vitamin D (as cholecalciferol) and corresponding endogenous concentrations of calcidiol (Garland et.al., 2011). The increases in circulatory calcidiol levels were found were associated with decreases in its absorbtion, hence, it was suggested that doses the amount of 40000 IU were not toxic (Garland et.al., 2011). Moreover, safe serum calcidiol concentrations within the range of 75–220 nM have been reported and with suggestions that plasma calcidiol concentration must rise above 750 nM to produce toxic effects (Vieth, 1999; Jones, 2008).

In 1977 a study of 39 patients, diagnosed with metabolic bone disease or hypoparathyroidism, were treated with high cholecalciferol doses for 12 weeks. Interestingly, hypercalcaemia due to vitamin D intoxication was associated with levels greater than 1000-1250 nM (Gertner & Domenech, 1977). Patients with plasma calcidiol levels below 250 nM had treatment failure (Gertner & Domenech, 1977). Moreover, in human cultured immortalised keratinocytes (oral epithelial cells line OKF6-TERT2) minor cytotoxicity of calcidiol is noted, while higher calcidiol doses (10000 and 20000 nM) inhibited cellular viability (Wang et.al., 2013). Indeed, high doses of calcidiol inhibit cellular proliferation in vitro. Moreover, significant reduction in cellular proliferation (70 and 50 % respectively) is noted in cultured human immortalised prostate cells with varying concentrations of calcitriol (0.01–10 nM) and calcidiol (1–100 nM) (Munetsuna, 2014). It was determined that concentrations of the intracellularly accumulated calcitriol are approximately 0.01–0.1 nM following treatment with 10–100 nM of calcidiol (Munetsuna, 2014). Interestingly, it was shown that cell derived calcitriol has much lower inhibitory effects on cellular growth and *CYP24A1* mRNA expression than calcidiol supplementation. Conversely, vitamin D therapy could represent “danger”, as noted in a large scope study, in patients of a large integrated healthcare organisation without prior diagnosis of atrial fibrillation. It was noted that 260 of 130,000 evaluated patients had calcidiol levels in the amount greater than 100 ng/dL and was associated with an increase in atrial fibrillation (Smith et.al., 2011). However, there was no greater risk of incidence of atrial fibrillation at vitamin D concentrations between 21-40 ng/dL; althought there were associations with high prevalence of

comorbidities (i.e., hypertension, heart failure, diabetes, and renal failure). Perhaps, vitamin D at high concentrations have health beneficial effects to counteract the pre-existing comorbidities (Smith et.al., 2011). Likewise, latest sudy conducted over 12 years at Rotterdam, Netherlands (with recruitment of 3,395 people) have found no connection between cuirculatory 25(OH)D levels and atrial fibrillation. This study considered D deficiency at circulatory 25(OH)D levels below 50nM, insufficiency between 50nM and 75nM, while serum 25(OH)D concentrations equal to and above 75nM were considered as adequate average (Vitezova et.al., 2015).

Interestingly, the relationship between vitamin D and cardiovascular health, indicates that vitamin D reduces inflammation (specifically, C–reactive protein, CRP) (Amer and Qayyum, 2012). In the study of asymptomatic adults the relation between serum calcidiol and C-reactive protein (CRP) was examined. It was demonstrated independently amongst traditional cardiovascular risk factors, an inverse relation between calcidiol circulatory levels (<21 ng/ml) and CRP. It was also shown that calcidiol at levels >=21 ng/ml was associated with increased serum CRP, suggesting the possible role of vitamin D supplementation to reduce inflammation is beneficial only among those with lower serum calcidiol (Amer and Qayyum, 2012). This is indicative that antiinflamatory vitamin D effects may occur only among patients with a lower serum calcidiol. Further investigations are required to support these observations. Indeed, the existing suggested guidelines are not very clear on what is considered deficient, optimal, therapeutic and excessive vitamin D dose. Accepted recommendations are summarised in Table 1.2.

Table 1.2 Circulatory vitamin D levels in health and disease conditions

Circulatory vitamin D levels			
Deficiency dose	Optimal dose	To promote health effects	Toxic
<30 nM	50-75nM	75-100 nM	> 220 nM

Adapted from Balvers et.al., 2015.

In order to apply the optimal therapeutic dose against skin carcinogenesis, it is important to establish that vitamin D deficiency has an impact on the development of skin cancers leading to decreased risk of development and progresion of disease. More defined understanding on vitamin D metabolism warrants further investigations to accurately evaluate vitamin D deficiency, toxicity and optimal dose that is required for sustainability of human health.

1.6 Antitumour activity of calcitriol and calcidiol

Results of clinical and epidemiological studies suggest that calcidiol deficiency is associated with increased risk of various cancers (Gröschel, 2016; Shannan et.al., 2007; Leyssens et.al., 2013; Garland et.al., 2009). Increased serum levels of circulating calcidiol are correlated with decreased incidence and aggressiveness of metastasis in prostate, breast, colon and other cancers (Shannan et.al., 2007; Leyssens et.al., 2013). Calcidiol and calcitriol have well-recognised properties that allow it to be used for the prevention and management of various cancers. It has been shown that mice lacking VDR are predisposed to developing skin tumours after chronic ultraviolet (UV) exposure. Such studies suggest that vitamin D production, and subsequent signalling through VDR in the skin, may have evolved in part as a protective mechanism against epidermal cancer formation (Bikle et.al., 2013).

It is clear that vitamin D metabolism is connected to the molecular pathways involved in the development and prevention of skin cancer. Mice with metabolic alterations (in particular, deficiency in VDR) display hyperproliferation in the hair follicle and epidermis, and decreased epidermal differentiation.

Cutaneous vitamin D pathways have been extensively described (Bikle et.al., 2011a; Bikle et.al., 2013; De Haes et.al., 2005; Olds et.al., 2008; Reichrath, 2006; Reichrath, 2010). In UV-exposed epidermis, 7-DHC may impact the amount of cholecalciferol, that is produced (Olds, 2008). It was shown that 7-DHC undergoes transformation to pre-vitamin D under the influence of solar irradiation. The first step of this process requires UVB (290-315 nm) induced formation of pre-vitamin D, via opening the electrocyclic ring between carbon 9 and 10. Once pre-vitamin D is formed, it begins to thermally isomerise to cholecalciferol (Tian & Holick, 1995). The thermal formation of cholecalciferol from pre-vitamin D is an intramolecular reversible process, and due to this reason cholecalciferol and its precursor, pre-vitamin D, always interconvert. This contrasts markedly with all other known steroids (Tian & Holick, 1995). The formation of active hormone calcitriol is required transformation of pre-vitamin D to cholecalciferol, following by formation of calcidiol and via renal CYP27B hydroxylation synthesis of calcitriol, that is known to have an influence on many genes, hence influence cellular growth and development.

Decreases in serum calcitriol concentration is reported to have an association with carcinogenesis, in particular breast cancer progression (de Lyra et.al., 2006). There is evidence that calcitriol plays a protective role against solar exposure and provides genetic stability to exposed epidermal cells (Reichrath, 2006; Trémezaygues et.al., 2010; Tang et.al., 2012; Bikle et.al., 2013). Calcidiol and calcitriol signalling pathways contribute to prevention of skin cancer

formation by regulating keratinocyte proliferation and differentiation, and promoting DNA recovery and repair following solar exposure-mediated damage (Chatterjee, 2001; Bikle, 2004; Dowd et.al., 2005; Bikle et.al., 2013; Carlberg & Campbell, 2013). While calcitriol and calcidiol trigger various effects in normal and neoplastic cell signalling, it is clearly involved in the regulation of cellular fate. Table 1.3 shows the effects of vitamin D in normal and neoplastic cutaneous cells.

Table 1.3. Effect of vitamin D metabolites on cutaneous cell proliferation, migration and growth.

Compound	Applied dose	Human cell type	Effects	References
calcidiol	10^{-5}–10^{-7} M	Normal fibroblasts	Stimulates cell growth	(Gruber & Anuszewska, 2002)
calcitriol	10^{-5}–10^{-7} M	Normal fibroblasts	Stimulates cell growth	(Gruber & Anuszewska, 2002)
calcitriol	10^{-6}–10^{-10} M	Normal fibroblasts	Inhibits cell growth in dose dependent manner	(Clemens et.al., 1982)
calcidiol	10^{-7} M	Immortalised facial sebocytes	Moderate suppression of cell proliferation	(Krämer et.al., 2009)
calcitriol	10^{-7}–10^{-9} M	Immortalised human facial sebocytes	Dose-dependent and significant stimulation of cell proliferation	(Krämer et.al., 2009)
calcitriol	10^{-6}–10^{-9} M	Tongue cancer cell line (SCC-25)	Inhibits cell growth	(Chiang et.al., 2013)
calcitriol	10^{-7} M	Cutaneous squamous cell carcinoma cells (SCL-1)	Inhibits cell growth and proliferation	(Reichrath et.al., 2010)
calcitriol	10^{-7} M	Spontaneously immortalised (‘normal’) human keratinocytes (HaCaT)	Mild suppression of cell proliferation	(Reichrath et.al., 2010)
calcitriol	10^{-8}- 10^{-7} M	Spontaneously immortalised (‘normal’) human keratinocytes (HaCat)	Stimulation of cell growth was noted at dose 10^{-8} M, while inhibition were required 10^{-7} M	(Hill et.al., 2015)
calcitriol	10^{-7} M	Human melanoma cell lines	Inhibits cell proliferation	(Reichrath et.al., 2004)
calcitriol	10^{-6}–10^{-8} M	Primary epidermal keratinocytes	Inhibits cell proliferation	(Koizumi et.al., 1997)

calcitriol	10^{-8}–10^{-10} M	Primary epidermal keratinocytes	Inhibits cell density and influences keratinocytes differentiation	(Smith et.al., 1986)

Both vitamin D metbolites have been shown to affect to some degree epidermal and dermal cells. Interestingly, most studies used a high dose of these compounds to achieve the observed effects on cutaneous cells.

1.6.1 Vitamin D effects on tumour microenvironment, angiogenesis and metastasis

Keratinocytes are the predominant cell type in the epidermis. They play a central and unique role in cutaneous vitamin D metabolism. Epidermal health largely relies on cutaneous vitamin D status (intracellulat production of both, calcidiol and calcitriol) and regulatory growth factors that control cellular proliferation, differentiation and death. The molecular interactions that occur between epithelial and stromal cells to drive tumour growth and 'invasive' behaviour illustrate the roles of vitamin D metabolites (calcidiol and calcitriol) as anticancer agents (Fleet, 2008; Geng et.al., 2011).

Calcidiol plays a regulatory role in prostatic tumour stromal development (Lou et.al., 2003). This occurs via regulation of cellular migration, proliferation and extracellular matrix (ECM) degradation (You et.al., 2003; Lippens et.al., 2004; Pampalakis & Sotiropoulou, 2007; Puccinelli et.al., 2010). Calcitriol inhibits proteolytic degradation of the surrounding stroma, inhibiting the mobility of mutated cells and their adhesion to the ECM (Bachelor & Bowden, 2004). Tumour stroma formation is also driven by the differentiation of fibroblasts into endothelial cells (Kon & Fujiwara, 1994). Fibroblasts play a role to play in angiogenesis (the formation of new blood vessels from pre-existing vessels) by being able to act as endothelial cell precursors in newly formed blood vessels (Kon & Fujiwara, 1994). The new blood vessels deliver vital nutrients for tumour growth and development and create pathways from local to systemic circulations.

The progression of mutated lesions into BCC or SCC largely relies on structural and vascular support for tumour growth that leads to overall tissue rearrangement. Some NMSCs, such as BCCs, rarely metastasise due to limitations in neovascularisation that are imposed by the local stroma (Reichrath et.al., 1999; Nguyen, 2004; Sidoroff & Thaler, 2010). The angiogenic mechanism is tightly regulated by cellular signals that can be inhibited by calcitriol (Ben-Shoshan et.al., 2007). The angiogenic switch is crucial for cutaneous tumour metastasis.

Studies have shown that calcitriol inhibits tumour angiogenesis and prevents metastasis. Calcitriol acts on freshly sprouting and immature blood vessels by inhibiting the production of angiogenic factors and disrupting the ECM (Fratzl-Zelman et.al., 2003; Boyan et.al., 2007;

Pampalakis & Sotiropoulou, 2007). Calcitriol has the ability to interrupt stromal signals in the skin tumour microenvironment, and hence can influence the development of cutaneous cancers. The regulatory activity of calcitriol may have an inhibitory effect on the tumour microenvironment and certain growth factors (Smola et.al., 1993; Gabison et.al., 2009).

VEGF receptor (VEGFR) and epidermal growth factor receptor (EGFR) both have a synergistic effect on neoplastic cells (Smola et.al., 1993; Ben-Shoshan et.al., 2007; Lichtenberger et.al., 2009; Mackenzie et.al., 2011). Calcitriol has been shown to inhibit EGFR expression through VDR in vitro using human ovarian cancer cells (Shen et.al., 2011). It was also demonstrated that calcitriol treatment triggers growth arrest of hyperplastic thyroid cells by suppressing EGFR growth-promoting signals (Dusso et.al., 2004). Crosstalk may occur between these pathways, where VDREs in VEGFR and EGFR promoters inhibit the expression of these genes, thereby suppressing tumour growth and development (Lichtenberger et.al., 2009).

1.6.2 Vitamin D effects on ErbB receptor tyrosine kinases

The EGFR is a 170 kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity. The receptor tyrosine kinase is activated by binding with EGF, transforming growth factor-α (TGF-α) and several other ligands. Upon ligand binding, EGFR undergoes autophosphorylation and initiates multiple intracellular signalling pathways, leading to induction of cellular growth.

The EGFR family has four members: EGFR (or ErbB1), ErbB2, ErbB3 and ErbB4 (Bondzi & Allison, 2013). The four receptor tyrosine kinases are widely expressed in epithelial, mesenchymal and neuronal tissues, and play critical roles during development and growth (Roskoski, 2014). EGFR is involved in a variety of intracellular signalling cascades and has been shown to have regulatory functions in many human cancer cells (Akca et.al., 2006; Lafky et.al., 2008; Runkle et.al., 2012; Bondzi & Allison, 2013). The actions of the ErbB receptors are complex and most of their functions are related to a large family of ligands, EGF-related peptides. By binding the extracellular domain of the receptors, EGF-related peptides form heterodimers with other cell cycle regulatory proteins, which affect cell growth and division (Puccinelli et.al., 2010; Zheng et.al., 1993).

The ErbB receptors consist of a glycosylated extracellular domain, a single hydrophobic transmembrane segment and an intracellular protein tyrosine kinase domain (Roskoski, 2014). Seven ligands bind to EGFR, including EGF and TGF-α, whereas none of these ligands bind to ErbB2, two ligands bind to ErbB3 and all seven ligands bind to ErbB4 (Roskoski, 2014).

EGFR is a target gene of calcitriol, with its expression suppressed in some human cancers. Calcitriol is able to inhibit *EGFR* mRNA and protein expression as well as EGFR function (Shen

et.al., 2011). Mitogens such as EGF are known to exert their effects on cell growth by regulating cell cycle progression through the G1/S checkpoint. Calcitriol was shown to impair EGF-induced EGFR nuclear translocation (Cordero et.al., 2002). Vitamin D interacts with activation of the RAS–RAF–mitogen-activated protein kinase (MAPK) pathway to modulate transcription factor activation and cell mitogenesis (Luangdilok et.al., 2011). Cutaneous tumours are known to exhibit elevated expression of tumour-promoting proteins such as EGF and its receptor (EGFR).

EGFR is the most intensively studied and well-understood driver of cell proliferation. However, the mechanism of calcitriol and calcidiol regulation of EGFR – a protein that boosts the development of skin cancer and an important target for cancer therapy – is not completely understood. EGFR is an essential protein promoting cell growth and division while simultaneously inhibiting cell differentiation (Puccinelli et.al., 2010; Runkle et.al., 2012).

Calcitriol is a potent modulator of EGF–EGFR activity in primary and malignant keratinocytes, to regulate cell growth, division and differentiation (Segaert et.al., 2000; Cordero et.al., 2002; De Haes et.al., 2005). Calcitriol is known to inhibit the expression of EGFR (Zheng et.al., 1993; Dusso et.al., 2004; Shen et.al., 2011). Furthermore, cancer cell survival is fundamentally depending on cell growth; EGFR activation in neoplastic keratinocytes could represent cellular effort to promote survival in the face of the apoptotic vitamin D stimulus (Zheng et.al., 1993; Segaert et.al., 2000; Cordero et.al., 2002).

It is well established that EGFR signalling regulates the proliferative phases of the epidermis, ensuring constant self-renewal whilst controlling differentiation (Fraguas et.al., 2011). Abrogation of ErbB signalling in malignant keratinocytes induced hyperproliferation and uncontrolled cell division (Runkle et.al., 2012). The effect of vitamin D on malignant keratinocyte function involves the activation of multiple pathways that promote differentiation and apoptosis.

Activated EGFR undergoes a transition from an inactive monomeric form to an active homodimer. In addition to forming homodimers, EGFR may pair with other members of the ErbB receptor family, such as ErbB2 (also known as HER2), to create an activated heterodimer (Fraguas et.al., 2011; Mehra et.al., 2011; Roskoski, 2014). EGFR dimerisation stimulates its intrinsic intracellular tyrosine kinase activity, resulting in autophosphorylation of several tyrosine (Y) residues in the carboxyl-terminal domain of EGFR. This autophosphorylation causes downstream activation and initiates several signal transduction cascades, principally the MAPK and associated AKT and JNK pathways, leading to DNA synthesis and cell proliferation (Luangdilok et.al., 2011; Uribe & Gonzalez, 2011). These proteins are known to be major regulators of cell migration, adhesion and proliferation.

The role of calcidiol and calcitriol on the expression of EGF and EGFR in NMSCs requires a deeper understanding as it may contribute to NMSC prevention and treatment.

1.6.3 Vitamin D effects on fibroblast growth factor receptor 3 (FGFR3)

Members of the fibroblast growth factor (FGF) family possess broad mitogenic and cell survival activities. They are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis and tissue repair, as well as tumour growth and invasion. The *FGF*-encoding gene was identified as a proto-oncogene, activated in virally induced mammary tumours in the mouse. Frequent overexpression of this gene has been observed in a variety of human cancers, suggesting that it may be important for neoplastic transformation and tumour progression.

Currently, 22 FGF family members have been identified. FGF3, also known as int-2, is a proto-oncogene that has homology with angiogenesis-promoting FGF, and it plays an important role in various cancer types (Mansour et.al., 1993; Copigny et.al., 1995; Schuuring, 1995; Chaffer et.al., 2007; Hu et.al., 2007; Weisinger et.al., 2010). The *FGF3* gene was identified as a proto-oncogene activated in virally induced mammary tumours in the mouse (Antoine et.al., 2005). Overexpression of this gene is noted in human cancers, which may be important for cutaneous neoplastic transformation and tumour progression (Copigny et.al., 1995; Javle et.al., 2014). The evidence suggests that this proto-oncogene may also play a role in human breast and lung cancers (Copigny et.al., 1995; Arao et.al., 2013).

FGFs signal through four high-affinity transmembrane protein tyrosine kinases, FGF receptors 1–4 (FGFR1–4). FGFR binding to its ligands results in receptor dimerisation and autophosphorylation of the intracellular region, resulting in receptor activation. Genetic alterations of FGFR3 were detected in human cancers such as multiple myeloma and urothelial carcinoma, breast and skin cancers (Oers 2002). These mutations occur somatically and drive the tumourigenetic process of the mutant cells. FGFR3 mutations have been identified in keratinocytes origination from seborrheic keratosis, epidermal nevus and solar lentigo, which are all benign skin lesions (Hafner & Hartmann, 2010).

FGF3 and its receptor may play a role in skin cancers; however, this needs to be further investigated. The functional effects of FGFR3 signalling in keratinocytes that lead to benign skin tumours seem to progress from hyperplasia to low-grade non-invasive superficial tumours. In low-grade skin tumours, FGFR signalling has been recognised by activation of the receptor tyrosine kinase–RAS pathway (Weisinger et.al., 2010). The best-characterised signalling pathway downstream of FGFR is the MAPK pathway (Weisinger et.al., 2010). It has been reported that

FGFR3 is linked to activation of MAPK signalling in conferring resistance to the melanoma suppressing agent, vemurafenib (PLX4032), in melanoma cells (Yadav et.al., 2012). The level of expression of FGF3 and FGFR in NMSCs could be associated with tumour aggressiveness and metastatic potential. It is not clear whether vitamin D metabolites (both, calcidiol and calcitriol) have any influence on FGF3/FGFR3 signalling. However, it has been noted that calcitriol modulates the MAPK pathway and causes cell cycle arrest, inhibits proliferation and triggers cell death. The metabolic pathways of calcidiol and calcitriol and its involvement in the regulation of FGF3 and FGFR3 in cutaneous carcinogenesis warrants further examination. The crosstalk of vitamin D and FGF3/FGFR3 pathway may have inhibitory effects on tumour progression and metastatic spread.

1.6.4 Vitamin D effects on vascular endothelial growth factor (VEGF)

Angiogenesis is tightly regulated by cellular signals that can be inhibited by calcitriol (Ben-Shoshan et.al., 2007). The angiogenic switch is crucial for cutaneous tumour metastasis. Studies have shown that calcitriol inhibits tumour angiogenesis and prevents metastasis, as outlined below.

Calcitriol and calcidiol may have an inhibitory effect on growth factors in the tumour microenvironment of skin cancers (Gabison et.al., 2009; Smola et.al., 1993). For instance, calcitriol suppresses VEGF expression in human colon cancer cells, epidermal keratinocytes and vascular smooth muscle cells by inhibiting its transcriptional activity (Bachelor & Bowden, 2004; Ben-Shoshan et.al., 2007; Cardus et.al., 2009; Micali et.al., 2010).

VEGF overexpression is a hallmark of malignant tumours and has a strong association with angiogenesis and tumour metastasis (Mueller & Fusenig, 2002). Suppression of VEGF signalling causes inhibition of tumour angiogenic capacity and results in a dramatic decrease in malignant tumours (Bachelor & Bowden, 2004; Ben-Shoshan et.al., 2007; Cardus et.al., 2009; Lichtenberger et.al., 2009; Micali et.al., 2010). Moreover, in mice tumour cell-derived VEGF promotes angiogenesis and tumour growth (Lichtenberger et.al., 2009). Deletion of epidermis-specific VEGF in mice results in inhibition of tumourigenesis and impairment of cell proliferation (Lichtenberger et.al., 2009). These stromal–epithelial interactions play an important role in the development, proliferation and metastasis of skin tumours, as well as driving tumour neovascularisation (Lou et.al., 2003; Micke & Ostman, 2004; Koivisto et.al., 2006; Ramirez, et.al., 2009; Bremnes et.al., 2011). Studies have shown that malignant cutaneous keratinocytes undergo accelerated epidermal cell turnover, where VEGF protein overexpression is a major source of pro-angiogenic cytokines (Mackenzie et.al., 2011).

VEGFR and EGFR have a synergistic effect on neoplastic cells (Smola et.al., 1993; Ben-Shoshan et.al., 2007; Lichtenberger et.al., 2009; Mackenzie et.al., 2011). The inhibitory effect of calcitriol on EGFR expression via VDR was shown in human ovarian cancer cells (Shen et.al., 2011). In addition, vitamin D treatment triggers growth arrest of hyperplastic thyroid cells by suppressing EGFR growth-promoting signals (Dusso et.al., 2004). Crosstalk can occur between the three pathways, as activation of VDREs in the VEGFR and EGFR promoters can suppress tumour growth and development (Lichtenberger et.al., 2009).

Regulation of angiogenesis is one method used in cancer therapy; therefore, vitamin D regulation of the molecular interactions between epithelial and stromal cells that drive tumour stromal growth and invasive behaviour should be further investigated.

1.6.5 Vitamin D and proliferating cell nuclear antigen (PCNA)

Proliferating cell nuclear antigen (PCNA) is involved in DNA replication, playing a role in the cell cycle late G1 and S phases and therefore influencing cellular proliferation. It is suggested that PCNA is involved in neutralisation of inhibitors of DNA synthesis. PCNA expression has an association with poor survival and advanced stages in cervical cancer and gliomas, hence, representing an interest as a diagnostic biomarker (Lv et.al., 2015). Interestingly, intratumoral PCNA expression is greater in VDR knockout mice compared to wild type mice (Mordan-McCombs et.al., 2010). The inhibitory effects of calcitriol on PCNA expression in mouse mesenchymal multipotent cells has been demonstrated (Artaza et.al., 2010). In contrast, in a human randomised controlled trial there was no effect of calcitriol (0.5 μg/kg) in surgically excised early stage prostate cancer patients (Beer et.al., 2004). However, at high calcitriol doses, there was clear inhibition of VDR expression (Beer et.al., 2004). This suggests that PCNA expression has VDR–dependent regulation. In studies using human squamous carcinoma cell lines, a reduction of PCNA expression was noted in response to vitamin D analog EB1089 (Akutsu et.al., 2001). In contrast, calcitriol is synergystic with 17β-estradiol-induced and has dose dependent effects on *PCNA* mRNA expression, as well as on proliferation and differentiation of osteoblastic MC3T3-E1 (Song et.al., 2011). The relationship between vitamin D enzymes and receptor and PCNA needs to be assessed in non melanoma skin cancers. The mechanism of potential VDR regulatory actions on PCNA in skin tumours requires further investigations.

1.7 The role of vitamin D in NMSC

The high prevalence of NMSC in Australia makes it imperative to focus research on its detection and prevention. Early detection of lesions significantly contributes to minimising morbidity, cost of treatment and mortality. The role of vitamin calcitriol and calcidiol in keratinocyte-derived

tumours requires further studies, as the possible role of vitamin D in the prevention of skin cancer could result in a significant improvement in NMSC patient morbidity and mortality.

1.7.1 Actinic keratosis

Solar irradiance causes DNA damage and malfunction in apoptotic pathways in cultured human keratinocytes (Assefa et.al., 2005). However, some apoptosis-resistant keratinocytes are able to undergo clonal expansion, which eventually leads to the formation of actinic keratosis (AK). In the past, the management of AK was frequently taken for granted, due to the simplicity and ease of treatment (Torsten & Cockerell, 2005). However, the association of SCC with AK has directed a great degree of focus on this disease. AK is an indicator of cumulative solar exposure, which gives rise to initial photodamaged skin lesions that may progress to invasive cutaneous SCC (Feldman, 2011). At least 20 % of AK will undergo transformation into invasive SCC (Aroni et.al., 2007).

Normal human skin exposed to UV radiation develops apoptotic nuclei in suprabasal keratinocytes and within two days of continued exposure, apoptotic cells are identified in the middle to upper epidermal layers (Norris et.al., 1997). However, most keratinocytes are resistant to UV-induced apoptosis, and apoptosis is not observed in basal cells. Human keratinocytes cultured in full growth factor-supplemented medium are completely resistant to UV-induced apoptosis (Norris et.al., 1997). Conversely, withdrawal of growth factors from keratinocytes rapidly decreases cell survival following irradiation and increases the induction of apoptosis (Norris et.al., 1997). This suggests that AK lesions are dependent on the availability of growth factors, and that formation of AK is subject to the clonal expansion and survival of apoptosis-resistant keratinocytes. In support of these findings, phosphorylation of tyrosine kinase family members has been detected in photodamaged skin, as well as mutations in EGFRs, with consequent activation of RAS and RAF that results in expedited proliferation, leading to AK (Zheng et.al., 1993; Ratushny et.al., 2012).

Detailed examination of AK lesions is required to establish whether the progression of these neoplastic cells to more advanced forms of skin cancer is due to epidermal–dermal pathway interactions. Also, it has yet to be determined whether apoptotic resistance occurs only due to epidermal signalling or whether the signals for further alteration of neoplastic cells derives from epidermal–dermal cell crosstalk.

Apoptotic resistance in cultured keratinocytes is dependent on the presence of growth factors. Keratinocyte defences against apoptosis within the epidermis are altered when growth factors are withdrawn or when cells undergo differentiation (Norris et.al., 1997). AK lesions prior

to transformation to SCC have a high component of inflammatory cells (Stockfleth & Kerl, 2006). The inflammatory processes in AK lesions are associated with increases in T lymphocytes (Berman & Cockerell, 2013). Vitamin D is recognised as an important immunomodulatory agent (Peelen et.al., 2011). It is not surprising that vitamin D metabolic enzymes and VDR are found to be broadly expressed in cells of the immune system (Gottfried et.al., 2006; Peelen et.al., 2011). Further research will shed light on the biological and protective roles of immune cells in various forms of skin neoplasms. It is also crucial to establish if the prevalence of AK and the predisposition to sunburn are vitamin D-dependent.

1.7.2 Basal cell carcinoma

Cancer of the skin is the most common cancer in Caucasians and BCCs account for 90 % of all skin cancers. The vast majority of BCC cases are sporadic, although there is a rare familial syndrome, basal cell nevus syndrome (or Gorlin syndrome), which predisposes to development of BCC (Greenlee & Joshi, 2004). In addition, there is strong epidemiological and genetic evidence that UV exposure is a risk factor of prime importance. The development of BCC is associated with constitutive activation of sonic hedgehog (SHH) signalling (Daya-Grosjean & Couve-Privat, 2005). The SHH signalling pathway is highly conserved in vertebrates and invertebrates. Mutations in the SHH pathway proteins smoothened (SMO), patched 1 (PTCH1) and SHH in BCCs result in continuous activation of target genes (Teichert et.al., 2011). At a cellular level, SHH signalling promotes cell proliferation. VDR plays an essential role in activation of the SHH pathway (Teichert et.al., 2011). Mutations in TP53 are also found with high frequency (> 50%) in sporadic BCC (Kim et.al., 2002).

The mutated clones of cells that survive apoptosis and progress further as AKs often convert to cutaneous tumours in the form of BCC. The name of this disease is purportedly derived from its perceived origin: the cells found in the basal areas of the epidermis and epidermal appendages, such as hair follicles and sebaceous glands (Carr et.al., 2007; Sidoroff & Thaler, 2010). While BCC is rarely a life-threatening disease, deeply invasive, infiltrative or morphea subtypes can progress to malignancy via the lymphatic system or the circulation (Seo et.al., 2011). Less than 300 cases of metastatic BCC have been reported worldwide (von Domarus & Stevens, 1984; Sidoroff & Thaler, 2010). The risk factors for the development of aggressive metastatic BCC include extensive tumour size (> 2 cm), long-standing duration, histological composition and patient's unresponsiveness to treatment (Sidoroff & Thaler, 2010). Other factors may also influence the progression from AK to BCC.

1.7.3 Squamous cell carcinoma

SCC arises in the squamous cells in the epidermis, and is predominantly caused by cumulative UV exposure. SCC can occur on all areas of the body, but it is most common in areas frequently exposed to the sun. SCC is typically characterised by overexpression of the tumour suppressor gene *p53* (*TP53*), which is believed to be the most frequently mutated gene in human oncological conditions. p53 negatively regulates the cell cycle by causing cell cycle arrest at the G1 phase (Nijhof et.al., 2007). p53 protein accumulation is common in sun-exposed cutaneous SCC, thus it is hypothesised that the nature of the mutagenic environment in which SCC develops directly affects the incidence of immunohistochemically-detectable p53-positive cells (Coulter et.al., 1995).

In UV-induced initiation and progression of SCC, the following events take place: DNA damage is induced by solar irradiance; aberrant molecular signalling ensues, culminating in apoptotic cell death. Damaged DNA fragments that fail to repair are passed on to progeny cells, and if there is mutation of the *TP53* gene, this leads to apoptotic resistance. *p53* mutations are common in SCC and are almost always associated with tumour aggressiveness. The inhibition of *p53* function in the epidermis leads to the spontaneous development of SCCs (Chanchal et.al., 2010; Seraj et.al., 2011).

UV light triggers the inhibition of nuclear factor kappa B (NF-κB) (Bharti & Aggarwal, 2002; Vicentini et.al., 2011; Gonzalez-Pardo et.al., 2012; Hwang et.al., 2012). The NF-κB family consists of transcription factors that play an important regulatory role in inflammation (Caicedo-Granados et.al., 2012). It has been reported that nuclear activity of NF-κB is inhibited by the inhibitor of NF-κB α (IκBα). IκBα binds to NF-κB and blocks its signalling (Wu et.al., 2010). Interestingly, VDR interacts directly with NF-κB in human osteoblasts and mouse embryonic fibroblasts (Wu et.al., 2010). In human airway smooth muscle cells treated with calcitriol, IκBα1 inhibits NF-κB and thereby cell proliferation (Song et.al., 2013b). Calcitriol also increased the IκBα1-mediated down-regulation of NF-κB in endothelial cells (Gonzalez-Pardo et.al., 2012).

The addition of vitamin D (particularly calcitriol) to cultured SCC cells inhibits proliferation, induces apoptosis and cell cycle arrest, and down-regulates several angiogenic and apoptotic factors (p21, p53, Bax, survivin, VEGF and basic fibroblast growth factor) (Satake et.al., 2003). A study that tested the efficacy of vitamin D in patients diagnosed with head and neck SCC demonstrated that patients treated with calcitriol had a longer time-to-tumour recurrence compared to those who were not treated before surgery (Walsh et.al., 2010). In addition, clinical study found that the circulatory calcidiol plays a role in pathogenesis and progression of SCC (Gugatschka

et.al., 2011). Disease-free survival and overall survival times were strongly associated with serum calcidiol levels (Gugatschka et.al., 2011). Thus, the incidence and further progression of SCC, as well as mortality, seem to be dependent on calcidiol status.

1.7.4 Squamous cell carcinoma in situ

AK is usually a precursor for SCC in situ (SCCIS), and invasive SCC (Zalaudek et.al., 2012). From a histopathological standpoint, AK, SCCIS and SCC are indistinguishable in the epidermal layer. These tumours consist of atypical keratinocytes with nuclear pleomorphism, poor differentiation and a high mitotic index (Stockfleth & Kerl, 2006). SCCIS typically is a slow-growing, scaly plaque, while invasive SCC is a fast-growing, tender, indurated papule or nodule, where differentiation of early lesions from AK on clinical grounds alone can be difficult (Stockfleth & Kerl, 2006). SCCIS, also known as intra-epidermal carcinoma or Bowen's disease, is a cutaneous malignancy found in the most commonly sun-exposed areas of the skin (such as head, neck, and extremities) (Veness 2006). The risk factors for Bowen's disease include fair skin, long-term sun damage, radiation exposure, immunocompromised status and human papillomavirus infection.

SCCIS is characterised by hyperkeratotic lesions confined to the epidermis, with abnormal keratinocytes replacing the full thickness of the epidermis (Böer-Auer et.al., 2012). The severe dysplasia, with the loss of structural organisation and stratification, occurs through epidermal layers without invasion to the dermis. A lack of large-scale dermatological clinical trials places limitations in finding the cause and hence, cure, for numerous dermatopathological conditions, including skin-based neoplasms.

While it is not clear whether calcidiol and calcitriol are involved in cutaneous neoplasm formation and progression, all roads appear to lead to VDR regulation of gene expression via the MAPK pathway, which is a potential target of calcidiol and calcitriol.

1.8 Vitamin D regulation of signalling pathways

1.8.1 Vitamin D regulation of the MAPK cascade

MAPK activation is achieved through multistep kinase cascades, which include a MAPK kinase (MAPKK or MEK) and a MAPKK kinase/MEK kinase (MAPKKK/MEKK). These cascades modulate numerous transcription factors and cellular regulatory proteins, thus allowing extracellular signals to influence the expression of tumour regulatory genes. Biochemical and genetic analyses have revealed that VDR is an important factor in the regulation of the activities of MAPKs. The existing studies do not completely elucidate the complex mechanism of VDR regulation of the MAPK pathway. However, some of these interactions may involve the

calcitriol/VDR (or calcidiol/VDR) complex rapid response activation of the MAPK pathway, which results in genomic and non-genomic crosstalk regulating tumour behaviour (Pardo et.al., 2006; Rosso et.al., 2012; Tse et.al., 2007b). For example, the antiproliferative effect of calcitriol treatment on osteosarcoma cells suggested the involvement of MAPK/AP-1/$p21^{waf1}$ pathways via non-genomic VDR-dependent regulation (Wu et.al., 2007). In malignant keratinocytes, both endogenous and exogenous EGFR activation was found to induce VEGF expression, which led to activation of the MAPK signalling pathway (Luangdilok et.al., 2011).

1.8.2 The role of vitamin D in other signalling pathways

VDRE and VDR binding sites are responsible for the control of vitamin D target gene expression, whereas many other sites could be temporary anchorage places for unligated/inactivate VDR. From the classical point of view, VDR binds DNA as a heterodimer with a retinoid X receptor (RXR-α, -β, or -γ) and upon ligand binding, changes the transcriptional fate of neighbouring genes (Figure 1.6). Calcitriol alters the expression of many genes that do not contain *VDREs* (Larriba et.al., 2014). In addition, calcitriol can also regulate genes via changes in protein stability or modifications in the phosphorylation process (Lin et.al., 2003; Li et.al., 2004).

Calcitriol activity modulates signalling pathways triggered by other agents at the plasma membrane. Several studies have reported regulatory actions of calcitriol on growth factors and cytokines by altering their intracellular signalling pathways. Wnt signalling pathways play crucial roles during development and are also critical for many tumourigenic processes. There are two main Wnt signalling pathways: the canonical or Wnt/β-catenin pathway, and the non-canonical or β-catenin–independent pathways (Clevers and Nusse, 2012).

Several studies have reported that calcitriol antagonises Wnt/β-catenin signalling in colon cancer cells by reducing β-catenin transcriptional activity and the induction of its relocation from the nucleus to the plasma membrane (Bernard et.al., 2009; González-Sancho et.al., 2011, Larriba et.al., 2011). The activation of β-catenin target genes is attenuated by calcitriol, which indicates the importance of vitamin D in the regulation of β-catenin, a hallmark of malignant cancers. It was shown that calcitriol inhibits β-catenin/TCF transcriptional activity in colon and other cancer cells (Luderer et.al., 2011). The up-regulation of the Wnt/β-catenin pathway by unligated VDR has been described in keratinocytes, where ligand-activated VDR, in contrast to unligated VDR, associated with inhibitory actions on the cellular fate. It was shown that liganded VDR promotes cell differentiation (Shah et.al., 2006; Jiang et.al., 2013; Bikle, 2004). However, further studies are required, as there are some controversial points: whilst VDR enhanced Wnt signalling through direct binding to lymphocyte enhancer-binding factor (LEF) independently of ligand and β-catenin

(Luderer et.al., 2011), ligand-activated VDR is believed to inhibit Wnt/β-catenin signalling (Bikle et.al., 2011a; Jiang et.al., 2013; Larriba et.al, 2013). Interestingly, it has been shown that high levels of β-catenin can potentiate calcitriol transcriptional activity via interactions of activator function (AF-2) domain of the VDR and C terminus of β-catenin (Shah et.al., 2006). The ability of calcitriol to inhibit β-catenin is largely dependant on AF-2 domain and not dependent on a specific promoter (Shah et.al., 2006).

Vitamin D also has inhibitory effects on proliferation and the Sonic Hedgehog (SHH) pathway through inactivation of the Smoothened (SMO) receptor in cultured mouse pancreatic adenocarcinoma cells, but failed to exert its anti-carcinogenic functions in vivo (Brüggemann et.al., 2010).

Vitamin D metabolites may involve in intracellular signalling important for cutaneous tumours growth, mechanism of which is shown in Figure 1.6.

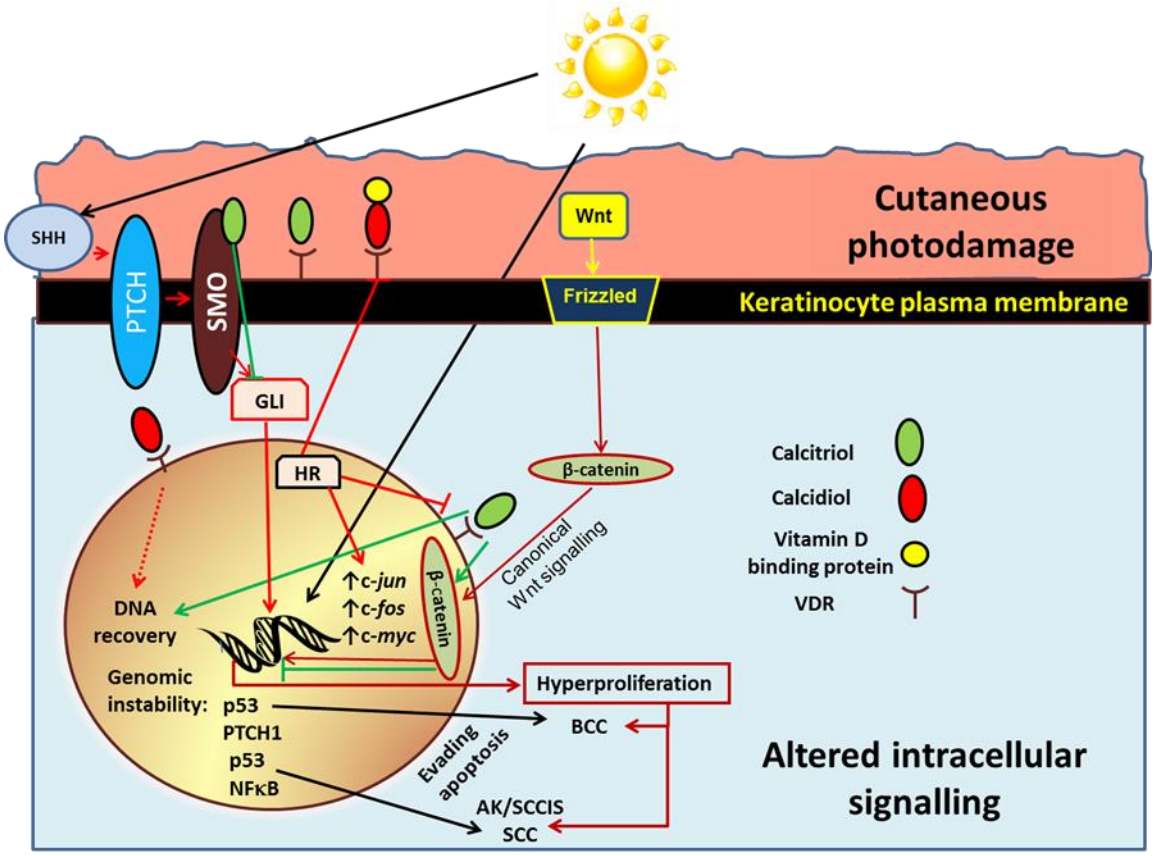


Figure 1.6. Vitamin D regulation of intracellular signalling.

A simplified diagram of molecular signalling in UV-exposed keratinocytes. Vitamin D (calcitriol, and possibly, calcidiol) has an inhibitory effect on the SHH, HR and Wnt/β-catenin signalling pathways. Genetically altered Sonic Hedgehog (SHH) protein binding to its receptor Patched1 (PTCH) triggers Smoothened (SMO) protein signalling, which results in GLI transcriptional mediation and the activation of downstream modulators associated with cell proliferation (*p53, PTCH1, NF-κB*). HR protein repression of VDR activity results in transcriptional activation of genes (*c-jun, c-fos, c-myc*). Wnt binding to its transmembrane receptors (Frizzleds) transmits signalling through a cytoplasmic protein network to cytoplasmic and nuclear β-catenin. Activated VDR prevents β-catenin transcriptional regulation of oncogenes and inhibits proliferation.

Mice with metabolic alterations (in particular, VDR deficiency) display hyperproliferation in the hair follicle and epidermis and decreased epidermal differentiation (Teichert et.al., 2011). In addition, VDR-deficient mice have an increased expression of oncogenes that promote cutaneous tumour development (Teichert et.al., 2011). Several genes that modulate cutaneous malignancies such as BCC and AK were found to be up-regulated in skin cancers, including *SHH*, tumour suppressor protein *PTCH*, transmembrane signalling protein *SMO* and its transcriptional modulator *GLI1*, which regulates *SHH* signalling from the cytoplasm to nucleus (Ohki et.al., 2004; Teichert et.al., 2011). VDR plays an essential role in activation of the SHH pathway in mouse keratinocytes, predisposing the skin to the development of both malignant and benign epidermal neoplasms (Teichert et.al., 2011).

The most common mutation in skin tumours occurs in the Patched (*PTCH*) gene, one of the downstream mediators belonging to the SHH pathway (Daya-Grosjean & Couve-Privat, 2005; Kim et.al., 2002). Binding of SHH to PTCH triggers release of SMO. Genetic alterations in the *PTCH* and *SMO* genes cause activation of the SHH signalling pathway, which influences the genes that control the cell cycle and differentiation (Teichert et.al., 2011). It has been demonstrated in animal models and in humans that calcitriol can inhibit the SHH signalling pathway by binding to SMO (Yu et.al, 2011; Banerjee et.al, 2012).

In keratinocytes of VDR-deficient mice, overexpression of the SHH gene was noted. VDR expression in wild-type mice was detected in the outer root sheath, sebaceous glands and basal layer of the interfollicular epidermis, corresponding to the location of BCCs. VDR deficiency causes a significant hyperproliferative response in keratinocytes and decreased epidermal differentiation (Teichert et.al., 2011). This study also indicated that the increased susceptibility of the epidermis to malignant transformation in VDR-deficient mice is due to a loss of calcitriol and/or VDR regulation of SHH signalling (Teichert et.al., 2011).

Vitamin D metabolic hydroxylases belong to the mitochondrial and microsomal CYP450 family (Guzey et.al., 2002). It was shown that mitochondrial CYP27B1 (encoded by the gene *CYP27B1*) expression correlated with skin tumour phenotype and behaviour, and prognosis in melanoma patients (Brożyna et.al., 2013). Furthermore, calcitriol stimulate cytochrome c release in various cancer models in animals and humans (Narvaez & Welsh, 2001; Guzey et.al., 2002). Released into the cytosol from the mitochondrial intermembrane space during apoptosis, cytochrome c binds and activates *Apaf1*, forming the apoptosome complex responsible for activating pro-caspase-9. This triggers a cascade of proteolytic events, culminating in the activation of caspase-3 and full-blown apoptosis (Guzey et.al., 2002; Robertson et.al., 2012).

In studies of dermal alteration, efforts have generally been focused on dermal fibroblasts. There are limited studies demonstrating the contribution of epidermis and keratinocytes to dermal alteration (Baba et.al., 2005). Fibroblasts are the major component of the dermis, significantly contributing to skin cancer development. The progression of skin carcinogenesis is largely dependent on the epidermal–dermal relationship, as was demonstrated in a number of studies (summarised in Figure 1.8). UV light exposure causes an alteration in the dermal matrix and is a major causative agent for genetic alterations in the skin (Obayashi et.al., 2008). Matrix metalloproteinase-1 (MMP-1) plays a central role in epidermal health and its activity as a collagenolytic enzyme in the dermis is up-regulated upon UV exposure. Fibroblasts cultured in conditioned medium from UVB-irradiated cultured keratinocytes had increased *MMP-1* mRNA levels. Moreover, when fibroblast cultures were irradiated, increased activity of MMP-1 was noted (Obayashi et.al., 2008). This study suggested that the signalling pathways associated with aging and carcinogenesis may be initiated from irradiated keratinocytes, causing changes to cells in the deeper layers of the skin. Calcitriol is shown to have a regulatory effect on the expression of MMP-1 and numerous other proteins involved in epidermal homeostasis (Kobayashi et.al., 2005). The molecular crosstalk of the epidermal cells and the mechanism of vitamin D interactions with MMP-1 is shown in figure 1.8.

1.9 Vitamin D signalling pathways in skin cancer

Vitamin D (both calcidiol and calcitriol), as determined from in vivo and in vitro studies, is associated with the prevention and treatment of various types of malignancies, such as breast, lung and skin cancers (Narvaez & Welsh, 2001; Tang et.al., 2010; Krishnan et.al., 2013; Zhang et.al., 2013). Indeed, vitamin D has a protective role against cutaneous carcinogenesis (Dixon et.al., 2011; Bikle et.al., 2013; Krishnan et.al., 2013). It is clear that vitamin D metabolism is connected to the molecular pathways in the development and prevention of skin cancer. The effects of both vitamin D metabolites on cutaneous cells is shown in Figure 1.7.

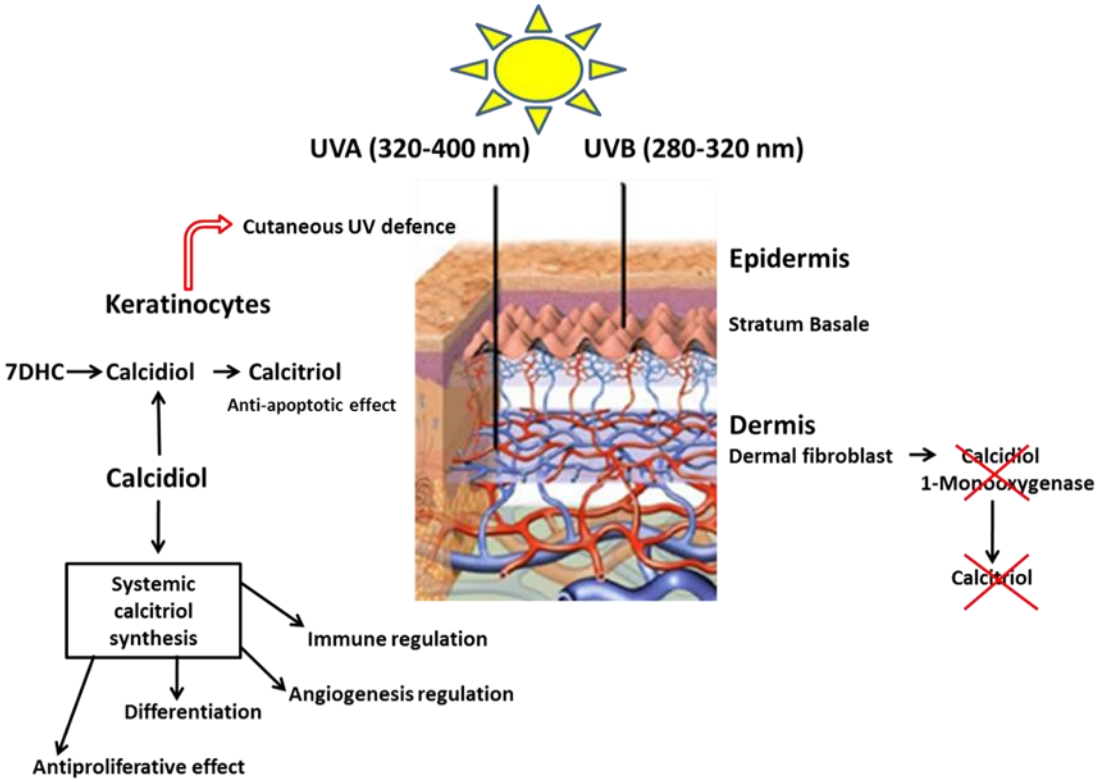


Figure 1.7. Proposed role of vitamin D in skin cancer prevention.

UV light induction of photodamage to cutaneous cells depends on the dose and wavelength of the UV. This can lead to DNA damage, inflammatory responses, cell death, skin ageing and oncogenesis. Several studies have demonstrated photoprotective effects, when calcitriol was used topically prior to irradiation. Documented effects of vitamin D metabolites in the skin cells include decreased DNA damage, reduced apoptosis and increased cell survival. Calcidiol may have a protective effect on keratinocytes and dermal tissue. CYP27B1 is down-regulated in the dermis; Calcidiol supplementation will result in local and systemic synthesis of calcitriol, which could consequently result in photoprotection.

7DHC - 7-dehydrocholesterol

(Adapted from Nemazannikova et.al., 2014; Munetsuna et.al., 2014; Gugatschka et.al, 2011)

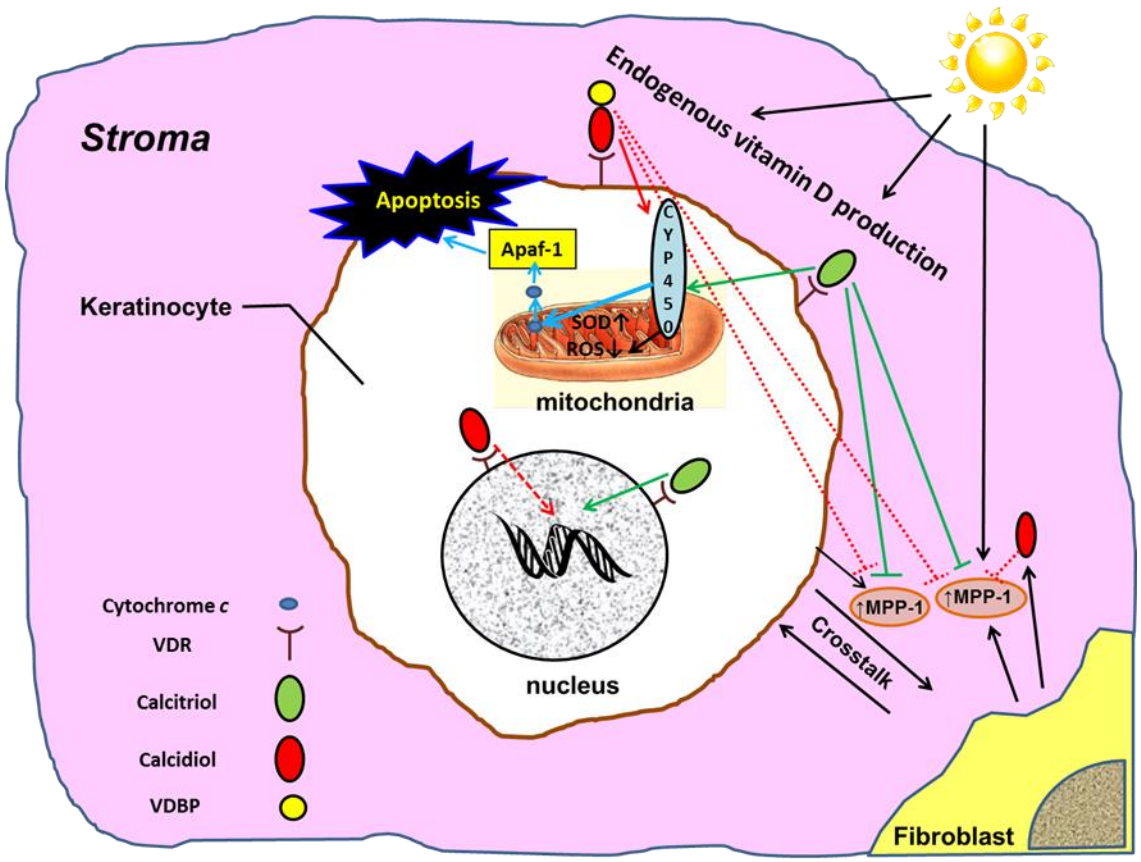


Figure 1.8. Molecular crosstalk between epidermal cells.

UV exposure stimulates endogenous vitamin D synthesis in keratinocytes. The rapid response generated by calcitriol (via nuclear VDR) and calcidiol (via membrane VDR) enhances mitochondrial SOD activity and reduces accumulation of reactive oxygen species (ROS) through mitochondrial CYP450. Numerous CYP450 enzymes (as well as those encoded by the genes CYP27A1 and CYP27B1) enzymes stimulates cytochrome c release, which results in inactivation of *Apaf1* and consequential events culminating in apoptosis. Calcitriol regulation of biological responses occurs via both VDR nuclear and membrane receptors, which signal rapidly through cross-talk of modulatory proteins in the cytosol and the nucleus. Calcitriol, and possibly calcidiol, take part in crosstalk between epidermal keratinocytes and fibroblasts while inhibiting the expression of the UV-induced collagenolytic MMP-1 (matrix metalloproteinase-1).

Epidemiological and laboratory studies suggest that cutaneous carcinogenesis depends on the individual's genetic makeup and their exposure to environmental risk factors (van der Pols et.al., 2013; Bikle et.al.; 2013). Hormones, cytokines, local and systemic immune responses, congenital, genetic as well as environmental factors are predispositions for the development of cutaneous neoplasm (Lee & Miller, 2009). Some individuals have genetic predisposition towards increased risk of skin cancer, while in others an increased risk of ultraviolet exposure causing the mutations accumulation. These factors share some similar elements in initiation and propagation pathways of skin cancer formation with differences in disease progression.

Calcidiol has increasingly been associated with cancer prevention in epidemiological, laboratory, animal and clinical studies. The evidence for calcidiol levels association with prevention of cancer is strongest for colorectal, prostate and breast cancer, but cancers of the skin have a smaller amount of available clinical findings. It is unknown whether calcidiol exerts anticancer effects in its inactivated form, or, perhaps, the conversion to the active secosteroid hormone calcitriol, results in these effects. Whilst calcitriol is known as a global regulator of gene expression and signal transduction in the vast majority of tissues, in cutaneous cells VDR and its ligand contribute to maintenance of the differentiated phenotype and promote pathways that defend cells against endogenous and exogenous stresses – actions that translate to reduced risk for carcinogenic transformation.

Despite the cumulative evidence linking calcidiol to a reduction in cancer development and progression, large randomised studies are required to identify its influence on NMSC. In addition, studies to determine anticancer protective effects of calcidiol levels are still not clear.

The established calcitriol influence on carcinogenesis occurs via regulation of various genes that are involved in differentiation, cell cycling and migration, as presented in Table 1.4.

NMSC is closely linked to abnormalities in epidermal and dermal cell differentiation, and accompanied by aberrations in intercellular communication and apoptosis (Fusenig et.al., 1994; Haug et.al., 1998). The roles of vitamin D pathways in cell differentiation have been demonstrated in animal and human studies for this neoplasia (Christakos et.al. 2003; Lips 2006; Dixon et.al., 2007; Haussler et.al., 2011).

Table 1.4. Vitamin D regulation of gene expression in cancer.

Gene(s) involved	Alteration in gene expression	References
c-fos/c-jun/c-myc	Calcitriol down-regulates activity of proto-oncogenes, inducing differentiation and antiproliferative effects in keratinocytes in animals and humans	(Sebag et.al., 1992; Chatterjee, 2001; Deeb et.al., 2007)
Transforming growth factor-beta (*TGF-β*)	Calcitriol up-regulates expression of *TGF-β*, which suppresses tumour formation in animal models and humans	(Jung et.al., 1999)
p21	Increases expression in animal models and humans; involved in cell cycle regulation	(Hager et.al., 2001; Zenmyo et.al., 2001)
Insulin-like growth factor I (*IGF-I*)	Calcitriol and its analogues inhibit *IGF-I* signalling pathways to promote apoptosis in breast cancer cells	(Xie et.al., 1999)
Peroxisome proliferator-activated receptor gamma (*PPAR-γ*)	Calcitriol up-regulated *PPAR-γ* in in vitro and in vivo models in animals and humans promotes differentiation of malignant cells and/or regression of tumours	(Nicol et.al., 2004; Sertznig et.al., 2010)
p120	Strong inhibition of protein expression in in vitro models by cholecalciferol and calcitriol leading to disruption of cell adhesion and cellular communication	(Stahl et.al., 1994; Clairmont et.al., 1996; Luegmayr et.al., 2000)
Nuclear factor kappa B (*NF-κB*)	Calcitriol inhibits transcriptional activity of *NF-κB* in breast cancer cells, resulting in suppression of cell proliferation	(Tse et.al., 2007b)
B-cell lymphoma-2 (*BCL-2*)	Calcitriol blocks gene expression in animal models and humans, thereby increasing apoptotic cell death	(Biauer et.al., 2009; Sharan et.al., 2011)
p53	Calcitriol increases gene expression after irradiation, which may favour DNA repair over apoptosis in human keratinocytes	(Dixon et.al., 2005; Mason et.al., 2010)
Vascular endothelial growth factor (*VEGF*)	Calcitriol regulates *VEGF* expression in various human cancer cells and its transcriptional activity via *VDREs*	(Ben-Shoshan et.al., 2007; Cardus et.al., 2009)

Epidermal growth factor (*EGF*)	EGF-treated HaCaT keratinocytes amplified the production of calcitriol when calcidiol was used as a substrate; antiproliferative activity of calcitriol in cultured normal human keratinocytes are greatly enhanced by EGF	(Chen et.al., 1995; Lehmann, 1997)
Epidermal growth factor receptor (EGFR)	Calcitriol suppresses EGFR growth signals in rats, resulting in inhibition of parathyroid hyperplasia	(Dusso et.al., 2004)

1.10 Clinical studies using vitamin D and its analogues in skin cancer

The pharmaceutical industry has a considerable interest in the development and production of compounds that mimic some of the biological actions of calcitriol that operate via VDR-mediated mechanisms (Carlberg & Campbell, 2013). This has been particularly true of the class of molecules known as low-calcaemic vitamin D analogues, which supposedly have reduced calcaemic actions and enhanced antiproliferative activity. Analogues of calcitriol are known to inhibit the growth of cancer cell lines in vitro and cancer cell growth in vivo. Some vitamin D analogues have inhibitory effects in SCC carcinogenesis. For example, analogue EB1089 treatment causes cell growth arrest, induces cell cycle regulation and expression of DNA damage gene *GADD45a* (Akutsu, 2001). The biochemical structure of this compound is shown in Figure 1.9. In addition, 22-oxa-1,25$(OH)_2D_3$ (22-oxacalcitriol; see Figure 1.9) has antiproliferative effects on malignant epidermal cells (Koike et.al., 1998).

Calcitriol is used clinically in various applications, including treatment of calcium metabolism disorders, renal osteodystrophy and dermatological applications (Hsu, 1997; Goodman, 2002; Ortonne et.al., 2003). However, hypercalcaemic side effects are often associated with the therapeutic applications of vitamin D in the form of calcitriol (Bechtel et.al., 1995). Vitamin D is a highly flexible molecule, allowing the design of multiple analogues to provide similar anticancer effects but with low calcaemic activity. In addition to naturally occurring metabolites, many synthetic vitamin D analogues have been developed. These analogues have included chemical modifications in the A-ring, central CD-ring region or side chain of calcitriol structure calcitriol, to counteract the adverse hypercalcaemic side effect (Leyssens et.al., 2013; Brown & Slatopolsky, 2008).

Some vitamin D analogues are clinically approved in conditions such as osteoporosis or secondary hyperparathyroidism. Yet none of the existing vitamin D analogues are used in

anticancer treatment. The effects of 22-oxa-1,25$(OH)_2D_3$ were demonstrated on basal human keratinocytes (KB), where was found that the KB cell line is deficient in retinoic acid receptor-β (RAR-β) (Satake et.al., 2003). It was shown in a different study that binding of *RXR/VDR* heterodimers to a single promoter region can give different transcriptional outcomes, demonstrating the complexity and flexibility of vitamin D–triggerred responses (Jimnez-Lara & Aranda, 1999). The lack of RAR-β observed in KB cells could be explained by this flexible VDR response.

Moreover, studies of prostatic stromal and epithelial cells demonstrated that the inhibitory effect of vitamin D metabolites (calcitriol and calcidiol) exerted via RAR-α but not by RAR-β. Moreover, this study demonstrated that in combination RAR-α and calcitriol have strong growth suppressive effects (Lou, 2005). In difficult and treatment resistant cancers, vitamin D meabolites have shown to be effective when applied alone or in combination with another therapeutic agent. For example study of human hepatocytes carcinoma cells (HepG2) and (HepG2-*CYP27B1* cells with CYP27B1 knockout) were found to be responsive to calcidiol treatment when treated at concentrations ranging from 0.001 – 10 μM. This data suggests that calcidiol could be used as a therapeutic agent in hepatic carcinomas (Chiang et.al., 2015). The development of vitamin analogues with aniproliferative, apoptic and promoting differentiation attributes represents an interest in anticancer research, and some of the analogues are presented in figure 1.9.

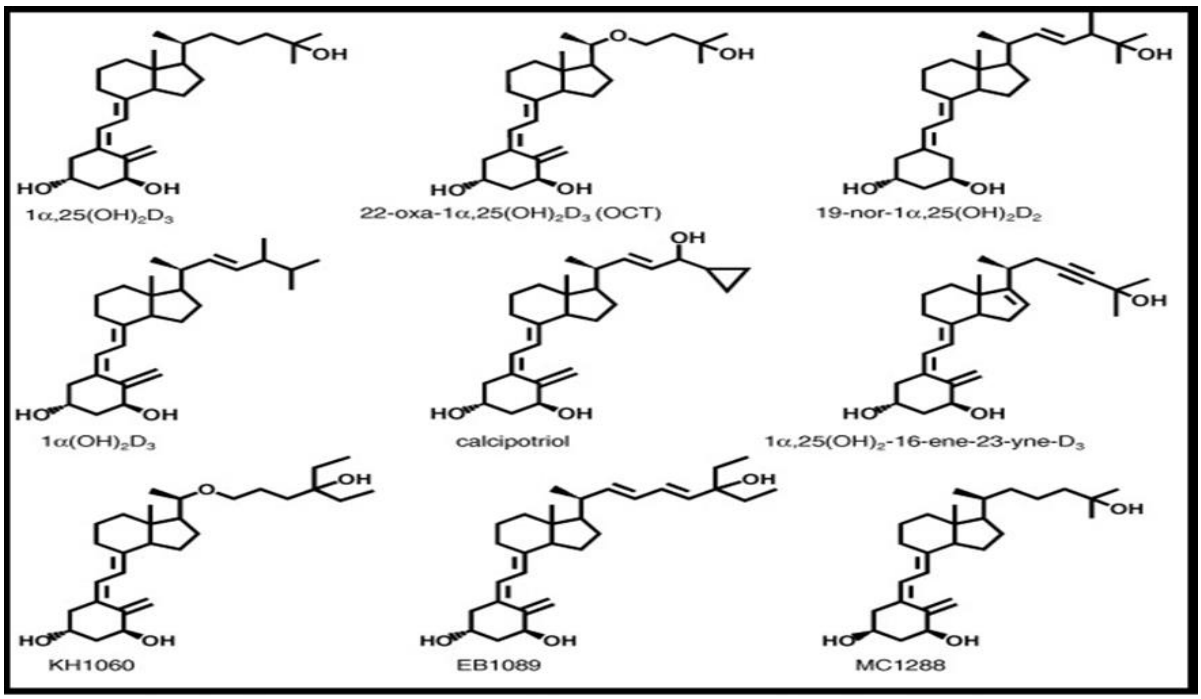


Figure 1.9. Representative vitamin D analogues.
Adapted from Leyssens et.al. (2014) and Brown & Slatopolsky (2008).

22-oxa-1,25$(OH)_2D_3$, an active hormone analogue, when used at a high concentration, suppressed cell proliferation, and induced apoptosis and cell cycle arrest (Jimnez-Lara & Aranda, 1999). While the analogue was more inhibitory than an equivalent dose of calcitriol on cell growth, these result are not conclusive due to the lack of solvent controls in this study (Jimnez-Lara & Aranda, 1999). However, if adequate controls were applied, the implications of these findings are that vitamin D and its derivatives are useful for preventing and/or treating patients with the most aggressive and highly metastatic type of NMSC, SCC. Moreover, it was shown that vitamin D analogues can be used in human skin inflammatory conditions, such as psoriasis (Lippens et.al., 2004; Reichrath et.al., 1997). Other studies show successful use of vitamin D analogues in other cancer types, such as breast, prostate and parathyroid cancers (Garland et.al., 2007; Leyssens et.al., 2013; Golovko et.al., 2005). For instance, synthetic vitamin D analogue, CB1093, has been shown to control cell proliferation with reduced calcemic effects and high capacity to inhibit leukaemic, breast and prostate cancer cell growth with apparent apoptotic results in cancer cells (Golovko et.al., 2005).

However, despite the development of synthetic vitamin D analogues to overcome hypercalcaemic effects, knowledge of their secondary effects and therapeutic efficacy remains limited. Furthermore, the efficacy of calcidiol has not yet been explored in primary and malignant keratinocytes, despite its tumour-suppressive effect at high physiological concentrations on other cancer cell types. Exploration of VDR regulation of skin cancers may aid in the treatment of difficult forms of cancer, which may prevent tumour progression and spread.

1.11 Research aims

1.11.1 Knowledge gap

Solar ultraviolet exposure is an essential factor for adequate circulatory vitamin D levels, however excessive solar ultraviolet exposure initiates melanoma and NMSC. There is little information available on the effects of calcidiol on NMSC in relation to its most metastatic and lethal type, SSC.

A large body of evidence exists for the involvement of vitamin D metabolic enzymes and its receptor in the regulation of cancer-induced pathways; however, their role in NMSC is not clear. The role of the principal metabolic vitamin D enzymes and receptors (CYP27A1, CYP27B1, CYP24A1and VDR) in primary and malignant epidermal cells is also not fully characterised. The effect of calcidiol on regulatory tumour proteins has not been tested in human highly metastatic keratinocytes, despite its tumour suppressive effect in other cancers (Lou et.al., 2003; Grant and Mohr, 2009; Herranz and Hernández, 2015).

The exact role of vitamin D (calcidiol and calcitriol) in skin carcinogenesis is still to be elucidated. This thesis highlights the need for further research into the role of vitamin D in the development and progression of NMSC. It is unknown whether vitamin D metabolites have anticancer benefits in inhibiting tumour progression and thereby, decreasing the risk of malignancy in NMSC. The molecular pathways involved in vitamin D regulation of tumour microenvironment and tumour regulatory proteins, including, epidermal growth factor and its receptor (EGF/EGFR), fibroblast growth factor 3 and its receptor (FGF3/FGFR3) and vascular endothelial growth factor (VEGF) need to be further studied. Clarifying the role of vitamin D in the regulation of cutaneous cancers and its role in the improvement to skin cancer recovery and survival may lead to the development of new drugs to prevent or minimise the occurrence of NMSC. The effects of calcidiol and calcitriol in SSC-4 cell line were investigated.

1.11.2 Hypothesis

1. Calcidiol inhibits neoplastic cell proliferation and migration, and the activity of tumourigenic proteins EGFR and FGFR3, via VDR-mediated activities.
2. The level of expression of vitamin D metabolic enzymes and VDR in neoplastic cells are altered.

1.11.3 Overall aims

1. To determine whether vitamin D metabolites, calcidiol and calcitriol, have an effect on tumour progression into malignant neoplastic lesions in NMSC.
2. In particular: To investigate the relationship between vitamin D metabolic key players (anabolic and catabolic vitamin D hydroxylases: CYP27A1, CYP27B1, CYP24A1 and VDR) and the expression of tumour regulatory proteins EGF/EGFR, FGF3/FGFR3 and VEGF, in healthy and malignant cultured cutaneous cell lines and in human NMSC tissues.

1.11.4 Specific aims

1. To determine the effects of calcitriol and calcidiol on malignant keratinocyte proliferation and migration, using cultured SCC-4 cells (Chapter 2)
2. To determine the effect of calcidiol and calcitriol on the expression of vitamin D hydroxylases (CYP27A1, CYP27B1 and CYP24A1) and VDR, as well as the tumour regulatory proteins EGFR and FGFR3, in cultured primary and malignant epidermal cells (Chapter 3)
3. To determine the expression of vitamin D hydroxylases (CYP27A1, CYP27B1 and CYP24A1) and VDR, as well as EGFR, FGFR3 and VEGF, in NMSC human tissue samples (Chapter 4).

1.12 Summary

1. The elucidation of calcidiol effects on vitamin D hydroxylases and VDR in primary and malignant epidermal cells will add significantly to its role in anti-neoplastic effects in SSC.
2. The investigation of vitamin D metabolic enzyme interactions in healthy and malignant cells will provide insights into vitamin D metabolism in pathological conditions. It will also lead to understanding its potential role in the prevention of cutaneous neoplasm and contribute to a better understanding of the development and progression of NMSC.

CHAPTER 2.

EFFECTS OF CALCIDIOL ON MIGRATION AND PROLIFERATION OF SQUAMOUS CELL CARCINOMA CELLS (SCC-4)

2.1 Introduction

Epidermal neoplastic growth involves dysregulation in the cell cycle and growth of keratinocytes (Afaq et.al., 2005). Epithelial cells in the skin are present in the interfollicular epidermis, in hair follicles and in sebaceous and sweat glands (Smola et.al., 1993). Conditions such as BCC, SCC, SCC in situ and AK involve mutations in keratinocytes; the extent of these mutations are associated with the degree of malignant transformation. Calcitriol and its analogues have antiproliferative properties when cultured with keratinocytes (as shown in Table 1.3, Chapter 1) (Chen et.al., 1995; Jung et.al., 1999; Rossi et.al., 2004; Shannan et.al., 2007; Wu et.al., 2007; Chung et.al., 2009; Geng et.al., 2011). For example, human normal (HPKlA) and malignant keratinocytes (HPKlA-ras cells transfected with a plasmid carrying an activated H-ras oncogene) incubated with calcitriol, shows inhibition of cell growth in a dose-dependent manner (Sebag et.al., 1992). Calcitriol at a dose of 10 nM was inhibitory to HPKIA cells at 72 h, whilst higher concentrations of calcitriol (100 nM) were required to inhibit HPKIA-ras cells (Sebag et.al., 1992). Likewise, the prostate cancer cell line (LNCaP), transfected with an empty vector (LN/C) or transfected with an over-expressing clusterin (protein that protects cells against apoptosis) (LNT-1), the antiproliferative effects of calcitriol was evident at doses of 1000 nM (53 % and 40 % respectively) and 100 nM (36 % and 2 % respectively); lower dose 0.1 nM had no obvious effect in LNT-1 cell proliferation (Shannan et.al., 2007).

Furthermore, inhibition of SCC cell lines (including SCC-4) expressing a functional VDR (Ratnam et.al., 1996), was not apparent at 10 nM calcitriol dose. It was also suggested that SCC cells partially resistant to calcitriol may occur due to an inability of VDR to bind to vitamin D response elements in some genes (Ratnam et.al., 1996). Hence, SCC-4 cell line has a malfunction in the vitamin D pathway regulating cell differentiation and growth (Ratnam et.al., 1996).

Interestingly, the inhibitory effect of calcitriol analogue (EB1089) in the human myeloid leukemia cell line HL-60 was more potent at much lower concentrations of the analogue (0.1 nM) compared to calcitriol 13 nM. The inhibition was dose-dependent only after the addition of TGF-β1 to calcitriol treated HL-60 cells (Jung et.al., 1999). In addition, the vitamin D analogue SM-10193 inhibits growth and differentiation 0.01 nM – 1,000 nM in primary keratinocytes and is more effective than calcitriol (Kobayashi et.al., 1994). Furthermore, calcitriol and its analogues

(EB1089 and CB1093) showed inhibition of insulin growth factor 1 (IGF-1) dependent stimulatory growth of breast cancer cell line (MCF-7) via activation of apoptotic pathways (Xie et.al., 1999).

Increased levels of plasma calcidiol have been reported to have an inverse association with "all cancers risk", including prostate, breast, colon and non-melanoma skin cancers (NMSC) (Lappe et.al., 2007; Grant, 2010; Tang et.al., 2010). Hence, calcidiol may have an inhibitory effect in vivo on malignant cells. However, data is limited on the effects of calcidiol on NMSC as an antitumourigenic agent. Whilst most studies use calcitriol and its analogues, calcidiol represents an interesting metabolite in anticancer research. Importantly, calcidiol status is associated with disease-free survival and overall survival time in patients with head and neck SCC (such as oral cavity, tongue, tonsils, mouth floor, larynx and hypopharynx) (Gugatschka et.al., 2011). In 88 newly diagnosed patients with head and neck SCC higher levels of circulatory calcidiol were associated with disease free survival; with an apparent association between tumour size and disease-free survival (Gugatschka et.al., 2011).

Furthermore, raising blood levels of calcidiol through dietary cholecalciferol uptake enhances local production of calcitriol that exerts inhibitory effects on breast tumours in mice (Krishnan et.al., 2013). Where cholecalciferol (5,000 IU/kg), precursor of calcidiol, given to MMTV-nude mice (MMTV - mouse mammary tumour virus) bearing MCF-7 breast cancer xenografts (in the flanks) resulted in over 50 % tumour size reduction, when fed a standard diet supplemented with 1,000 IU cholecalciferol/kg (Krishnan et.al., 2013). Elevated circulatory calcidiol in these mice had similar tumour suppressive effects as in parallel mice treated intraperitoneally with calcitriol (50 ng/mouse, three times a week). Hence, calcidiol precursor, cholecalciferol, has tumour inhibiting properties in mice (Krishnan et.al., 2013). Whether similar effects can be achieved in skin cancers is not clear.

Interestingly, a clinical study using high-dose (35,000 IU daily for six months) vitamin D therapy in patients with vitiligo and psoriasis showed improved re-pigmentation (25-75 %) and PASI (Psoriasis Area and Severity Index) score (Finamor et.al., 2013). In another nested case–control study involving 5,995 community-residing men, 65 years or older, showed reduced incidence of NMSC and high circulatory calcidiol levels amongst subjects (Tang et.al., 2010).

Since several studies indicate the connection between inhibitory effects of calcidiol on various tumour cells, it is important to investigate the effect of calcidiol on cultured malignant keratinocytes (Lou et.al., 2003; Garland et.al., 2007; Gugatschka et.al., 2011; Krishnan et.al., 2013). As reviewed in Chapter 1 (section 1.5.1), the non-genomic vitamin D complex pathways

could involve the membranous VDR activation pathway, regulation of malignant keratinocyte migration and proliferation and cellular responsiveness to calcidiol. Calcidiol has an inhibitory effect on cell proliferation in human marrow stromal cells by triggering cell cycle arrest, whereas the action of calcitriol involves increased apoptosis (Geng et.al., 2011). Indeed, the inhibitory effect of calcitriol on cell proliferation in a number of malignant cells is cell cycle dependent, with triggering of G0/G1 phase arrest and is mediated by the VDR (Gedlicka et.al., 2006; Geng et.al., 2011). The metastatic capacity and potential of neoplastic cell reflected in their migration abilities.

Herein, the effects of calcidiol *in vitro* on cell proliferation and migration using SCC-4 malignant keratinocyte cell line was determined. The effect of calcitriol was also assessed and compared to calcidiol.

2.2 Materials and methods

2.2.1 SCC-4 cell line

Human SSC cell line SCC-4 (CRL-1628) was obtained from American Type Culture Collection (ATCC), USA. SSC-4 was established from fresh tumour tissue as described previously (Beckett & Rheinwald, 1981). SCC-4 is a progressive squamous carcinoma cell line, derived from the tongue of a 55-year-old male who had radiation and methotrexate treatment for the tumour for 16 months before a biopsy was taken (Beckett & Rheinwald, 1981). SCC-4 cells contain a network of keratin filament bundles in a pattern similar to that found in normal keratinocytes (Beckett & Rheinwald, 1981). Thus, this keratinised epithelial cell line was used as a model for malignant keratinocytes investigation. Primary human neonatal keratinocytes were purchased from American Type Culture Collection (ATCC), USA.

2.2.2 Materials

General tissue culture reagents were purchased from Sigma-Aldrich, USA: Dulbecco's modified Eagle's medium (DMEM; D6429), Ham's F12 nutrient mixture (51651C), fetal bovine serum (FBS; F2442), antibiotic/antimycotic solution (10,000 units/mL penicillin, 10 mg/mL streptomycin and 25 μg/mL amphotericin B), calcidiol (H-1440), calcitriol (D1530), trypsin-EDTA (59417C) and 10x phosphate-buffered saline (PBS; 051M8422). CellTiter-Blue™ (G3580) was obtained from Promega, Australia; Thiazolyl Blue Tetrazolium Bromide (M5655-5X1G) and N,N-Dimethylformamide (D4551-500ML) was purchased from Sigma-Aldrich, Australia.

2.2.3 Methods

2.2.3.1 Proliferation assay

Rapid colorimetric assay for cellular growth and survival, Thiazolyl Blue Tetrazolium Bromide (MTT):

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) forms purple formazan in the mitochondria of living cells in the reduction reaction, which takes place only when mitochondrial enzymes are active, and therefore conversion can be directly related to the number of viable cells. The absorbance of the purple solution can be quantified by measuring a wavelength by a spectrophotometer. The amount of purple formazan produced by cells treated with calcidiol or calcitriol is compared with the amount of formazan produced by untreated cells, to determine the amount of cell death. Higher number of viable cells, similarly to CT Blue assay (based also on mitochondrial enzymatic conversion of resazurin to resofurin), results in greater amount of MTT formazan formation which triggers an increase in absorbance. 3×10^4 cells/well were seeded in 96 well plates. Cultures were grown in humidified incubator 5 % CO_2 and 95 % air atmosphere at 37 ^{0}C. Cellular density was optimised and shown in appendix B – (MTT assay optimisation). SCC-4 cells were allowed to attach for 24 h, and then treated with varying concentrations of calcidiol and calcitriol over 24 - 96 h: calcidiol at 100 nM, 1,000 nM, 10,000 nM, 100,000 nM, 1,000,000 nM and 10,000,000 nM) or calcitriol (at 10 nM, 100 nM, 1000 nM and 10,000 nM). Untreated cells and corresponding to each dose ethanol controls for calcidiol (0.000004 % - 0.004 %) and calcitriol - (0.0004 % - 0.4 %) treatment were also applied. Cellular proliferation was assessed via spectrophotometry (Biorad microplate reader, 6.0) using wavelength 570 nm. Three independent experiments were conducted in triplicates.

To be consistent with the earlier investigations, it was decided to conduct the assessment of proliferation using the MTT assay. MTT assay is robust and reliable benchtop test that can be readily adapted for a wide range of instruments and platforms to acquire the signal of mitochondrial performances, hence cellular viability. The advantage of MTT assay instead of other methods (such as ^{3}H-thymidine) have been shown previously (Gieni et.al., 1995). The use of ^{3}H-thymidine, similarly to BrdU test, would be helpful in the assessment of DNA damage in response to the treatment, however not easily comparable to previously acquired data with lower doses of calcidiol and calcitriol, as cellular viability measured by a different cellular features (DNA damage vs mitochondrial enzymatic performances). The experiments with calcidiol and calcitriol at lower concentrations were conducted using Cell-Titer Blue Cell viability detection

method. The protocol of this method is also based on the mitochondrial biogenesis and described in Appendix B.

2.2.3.2. Migration assay

SCC-4 cells were cultured at 37 °C in a humidified 5 % CO_2 incubator, in DMEM/F12 (1:1) media containing 10 % FBS and supplemented with 1 % penicillin–streptomycin (Lu et.al., 2008). Cells were maintained in 175 cm^2 tissue culture flasks. The optimal seeding density for 24-well plates was optimised. SCC-4 cells at 5 x 10^5 cells per well were plated in 24-well plates for 24 h. Cells in individual wells were wounded by scratching with a 1000 µl size pipette tip, washed with 1x PBS three times and incubated with DMEM/F12 medium containing FBS and antimicrobial solutions.

The cells were allowed to attach for 24 h, prior to treatment with varying concentrations of calcidiol (16 nM - 100,000 nM) or calcitriol (0.1 nM - 100 nM) for 1 h, 4 h, 24 h, 48 h and 72 h. Untreated cells and corresponding to each dose ethanol controls for calcidiol (0.000004 % - 0.004 %) and calcitriol - (0.0004 % - 0.4 %) treatment were also applied. All experiments were conducted 3 times in triplicates. Higher (calcidiol at 1,000,000 nM and calcitriol at 1,000 nM) concentrations were also investigated. Images were taken at all concentrations (data not shown). To create working solutions, both calcidiol and calcitriol were dissolved in 100 % ethanol as per the manufacturer's instructions. The SCC-4 cells were grown as a monolayer. Ethanol-treated (equivalent to the volume of calcidiol or calcitriol) SCC-4 cells were used as a vehicle control and untreated cells were used as a negative control. The cells were photographed using phase-contrast microscopy (20x magnification). The distance of closure was measured using Carl Zeiss, Axio-Vision, LE 54 software (Carl Zeiss, Germany). The experiment was conducted three times in triplicate. See Appendix A for the method used to calculate scratch closure.

2.2.3.3 Light microscopy imaging and growth pattern of cell cultures

SCC-4 cells were seeded at concentration of 10,000 cells per well in 24 wells plates. SCC-4 cells were cultured in DMEM/F12 (1:1) media supplemented with 10 % FBS and 1 % penicillin–streptomycin (Lu et.al., 2008). Cells were allowed to adhere for 24 h prior treatment with the calcidiol or calcitriol. The cell growth and colony formation was monitored and images of the calcidiol and calcitriol treated SCC-4 were captured using phase contrast microscopy at high magnification (100X) at different time points (24 – 72 h). The experiment was conducted three times in triplicates. See Appendix C for the images of all doses and timepoints of representative experiments.

2.2.4 Statistical analysis

Statistical analysis of proliferation and migration assays was performed using Student's t-test. The mean of three independent experiments was considered significantly different when compared to vehicle control groups if $p<0.05$ in proliferation and migration assays.

2.3 Results

2.3.1 Calcidiol and calcitriol inhibits proliferation of SCC-4 cells

Antiproliferative attributes of calcitriol and calcidiol on different cell lines has been shown, however their effects on SCC-4 cells is not clear (Munetsuna et.al., 2014; Geng et.al., 2011; Biauer et.al., 2009; Sharan et.al., 2011).

The effect of calcidiol on SCC-4 cell proliferation was evaluated at various concentrations (100-10,000,000 nM) and time (24, 48, 72 and 96 h). Ethanol treated SCC-4 cells were used as vehicle control at the appropriate concentration (0.004 % - 0.000004 %) (Figure 2.1).

Using calcidiol at 100-10,000,000 nM range no antiproliferative effects were apparent within 24 h. However, partial inhibition of proliferation was noted at 48 h. A dose response was evident with 100,000 nM and above being statistically significant ($p<0.005$ at 100,000 nM and $p<0.0005$ at 10,000,000 nM). The dose dependent inhibition was more evident at 72 h with statistical significance at all doses above 100,000 nM ($p<0.05$ at 100,000 nM; $p<0.005$ at 1,000,000 nM; $p<0.05$ at 10,000,000 nM). Inhibition of proliferation subsided at 96 h with no significant difference (Figure 2.1). These data agree with reports on the antiproliferative effects of calcidiol in immortalised prostate cells, however the inhibition of prostate cell line was observed at much lower concentrations (10-100 nM), where significant reduction in cell numbers were noted (Munetsunaa et.al., 2014). The hazardous effect of calcidiol was shown in oral human immortalised OKF6/TERT2 cells by MTT assay (Wang et.al., 2013). The incubation over 24-48 h with calcidiol at concentrations of 10,000-20,000 nM had detrimental effects on cellular survival, whereas at lower doses (1,000 nM and below) cytotoxic effects were not detected (Wang et.al., 2013).

The inhibitory effects of calcitriol treatment was noted in SCC-4 cell cultures when compared to corresponding ethanol controls (dashed line). In particular this is obvious at 48-72 h at doses 100 nM and above. However, at 96 h the antiproliferative effects of calcitriol was noted only at high doses 1,000 nM ($p<0.005$) and 10,000 nM ($p<0.05$) (Figure 2.2). Although calcitriol only partially inhibits cell proliferation when compared to the corresponding ethanol controls a dose response is apparent at 72 h (10,000 nM) compared to 10-1,000 nM ($p<0.005$). The data are supported by a number of studies where calctriol partially inhibits cell proliferation. Calcitriol (at

doses below 10 nM) appear to stimulate HaCaT cell proliferation (Hill et.al., 2015). Interestingly, the resistance to calcitriol was reported in retinoid resistant HL-60 leukemia cell line, where 100 nM failed to inhibit cell proliferation up to 120 h (Atkins and Troen, 1995). Likewise, partial resistance of SCC-4 cells to calcitriol treatment (1-100 nM) was only noted after an incubation period of 10 days (Akutsu et.al, 2001). Our data is supportive towards calcitriol inhibitory doses in resistant SCC-4 cells. We demonstrate that with elevated concentrations, calcitriol exerts partial suppression of proliferation at 72 h. In studies conducted up to 240 h (data not shown) no further inhibition was observed.

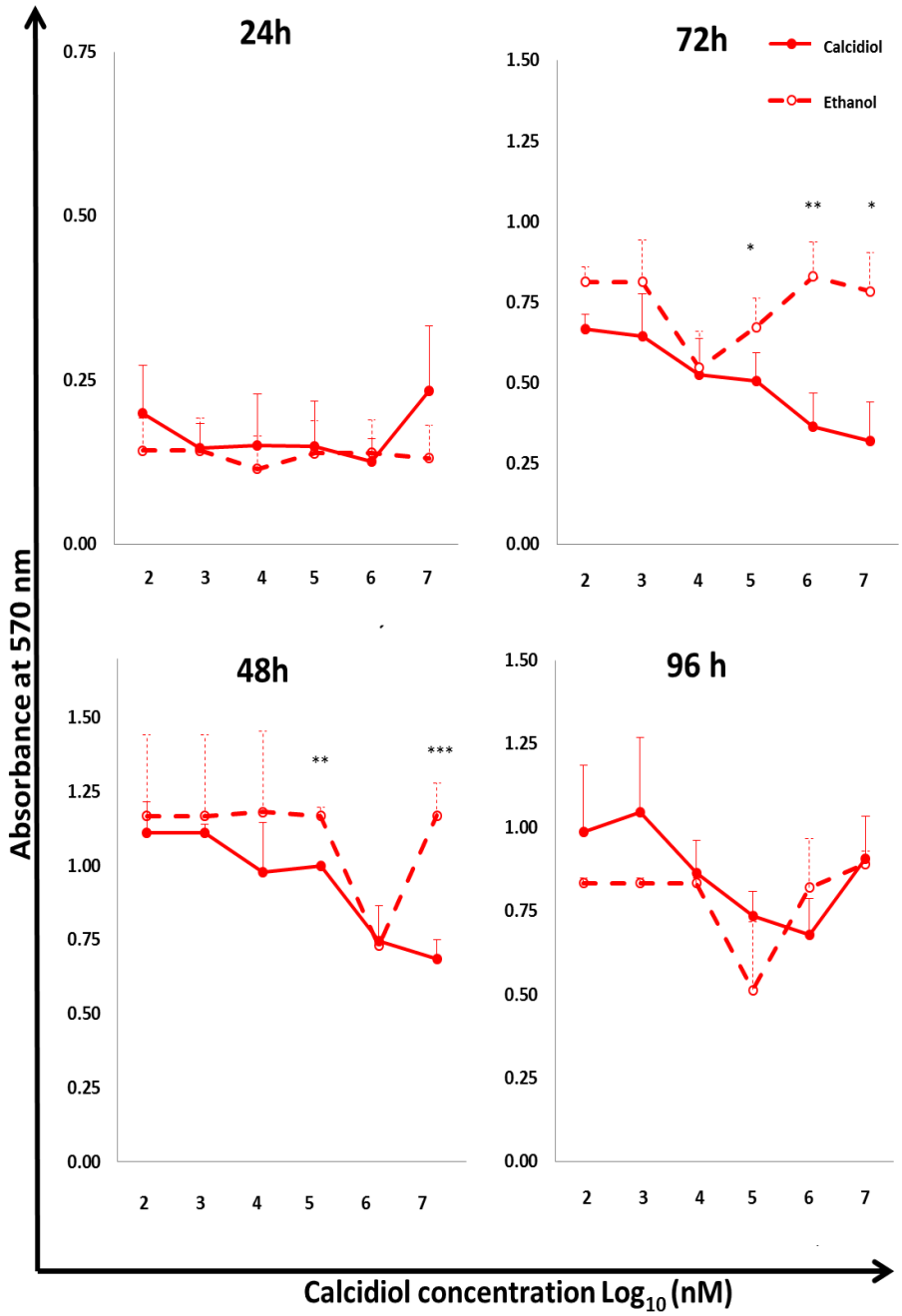


Figure 2.1 Effect of calcidiol on SCC-4 cell proliferation.
SCC-4 cells were treated with calcidiol (100 – 10,000,000 nM) for 24 - 96 h. Ethanol treated SCC-4 cells were used as vehicle control at the corresponding concentrations. Cell proliferation was measured using MTT assay and read at 570 nm. The data represents the mean ± standard deviation (SD). Statistically significant differences were determined using Student's t-test. All results are representative of at least three independent experiments conducted in triplicate. Data was considered significant when p values were below 0.05. * denotes $p<0.05$, ** $p<0.005$, *** $p<0.0005$. P values at each point are shown in Appendix B, Table 2.1.

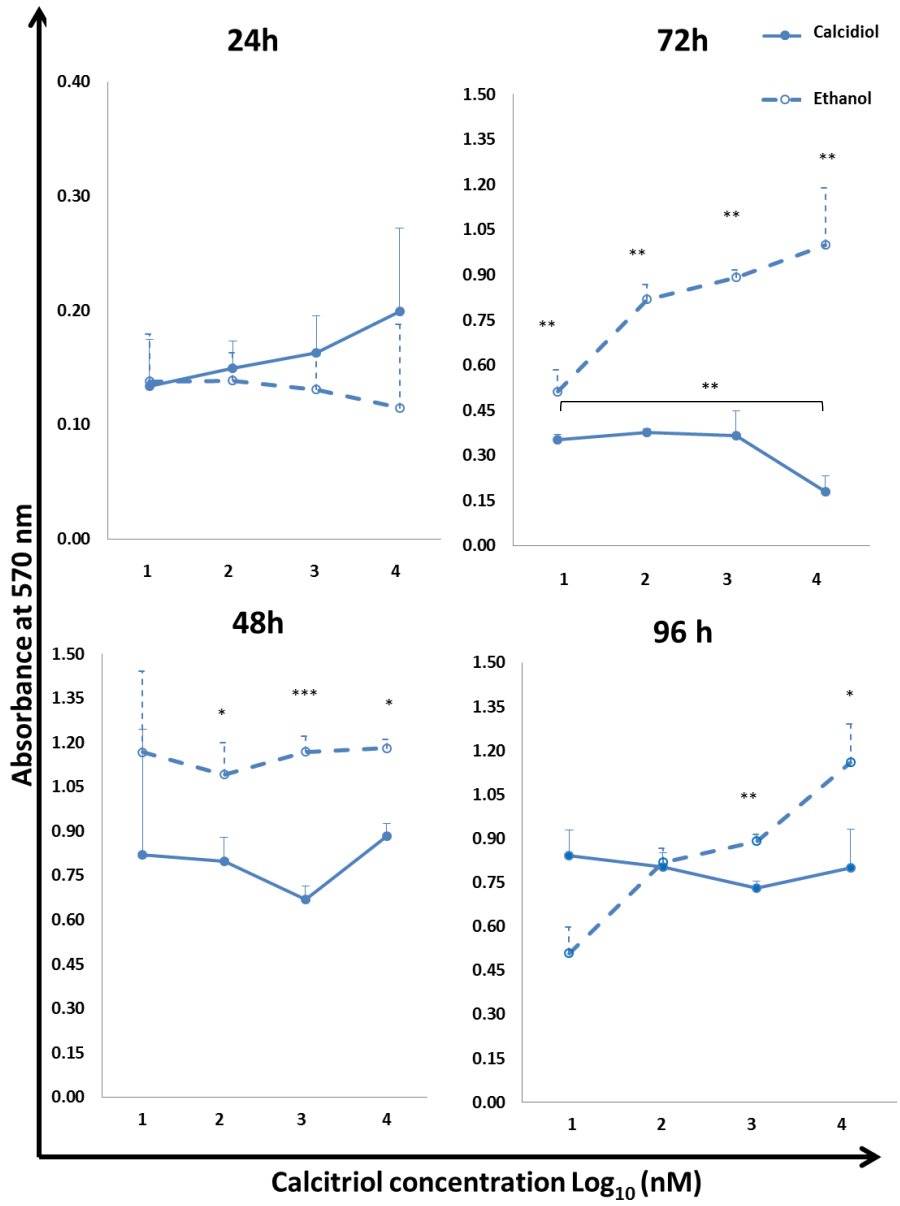


Figure 2.2 Effect of calcitriol on SCC-4 cell proliferation.
SCC-4 cells were treated with 10 – 10,000 nM calcitriol for 24 - 96 h. Ethanol treated SCC-4 cells were used as vehicle control at the corresponding concentrations. Cell proliferation was measured using MTT assay and read at 570 nm. The data represents the mean ± standard deviation (SD). Statistically significant differences were determined using Student's t-test. All results are representative of at least three independent experiments conducted in triplicate. Data was considered significant when p values were below 0.05. * denotes $p<0.05$, ** $p<0.005$, *** $p<0.0005$. P values at each point are shown in the Appendix B, Table 2.2.

2.3.2 Calcidiol inhibits SCC-4 cell migration

Growth patterns of SCC-4 cell cultures indicated that the initial cell number of 5 x 10^5 cells is an optimal seeding density (optimisation process is shown in Appendix B), allowing cellular movement within a short timeframe at low concentrations of calcidiol (16-250 nM) and calcitriol (0.01-10 nM). The effect of calcidiol on malignant SCC-4 cell migration abilities have not been investigated. Calcitriol was used to compare the effects of calcidiol. The effects of lower concentrations of calcidiol and calcitriol were examined in migration assays and shown in Appendix A, Figure A.2.a and Figure A.2.b. When calcidiol treated cells were compared to the corresponding ethanol controls as a ratio, inhibitory effect in dose and time dependent manner were obvious at all calcidiol concentrations. In calcidiol treated cells dose response was apparent, where noticeable inhibition of wound closure was observed as calcidiol concentration increased. At 16 nM calcidiol the percentage of wound openness was less than 250 nM concentration (p<0.05), suggesting an inhibitory trend in the presence of calcidiol (Figure 2.3).

Conversely, in calcitriol treated SCC-4 cells (0.1 and 10 nM) a stimulatory effect of the wound closure was apparent at 24 h when compared to corresponding ethanol controls (p<0.05). However, at 48 and 72 h rapid wound closure was clearly noticable at all doses (Figure 2.4) with no clear dose response effect. The effect of calcitriol and calcidiol, however, was investigated further and its influence on cellular motility at higher concentrations is shown in Appendix C.

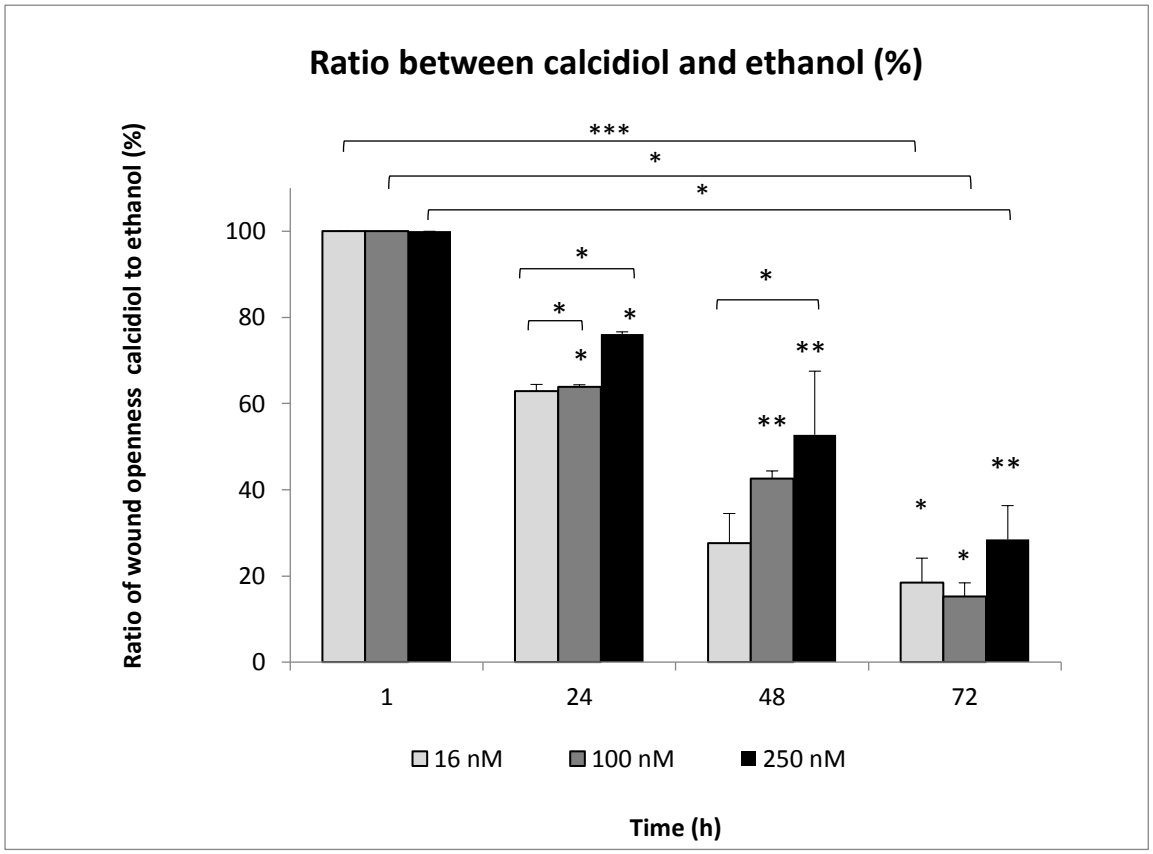


Figure 2.3 Effect of calcidiol on SCC-4 cell migration.
The wound openness of SCC-4 cells treated with calcidiol at 16 nM, 100 nM or 250 nM was measured in three independent experiments at 1, 24, 48 and 72 h in triplicates. At each time point the ratio between the wounded area of calcidiol treated SCC-4 cells to the corresponding ethanol controls were compared to the ratio at time 1 h (taken as 100 % wound openness). Statistically all data points are significant when compared to initial 100 % wound opening. Statistical significance was calculated by comparing calcidiol treated cells to corresponding ethanol controls using student t-test. Statistical comparison also was performed between different time points and doses (p<0.05). Data was considered significant when p values were below 0.05. * denotes p<0.05, ** p<0.005, *** p<0.0005. P values at each point are shown in Appendix B, Table 2.1.

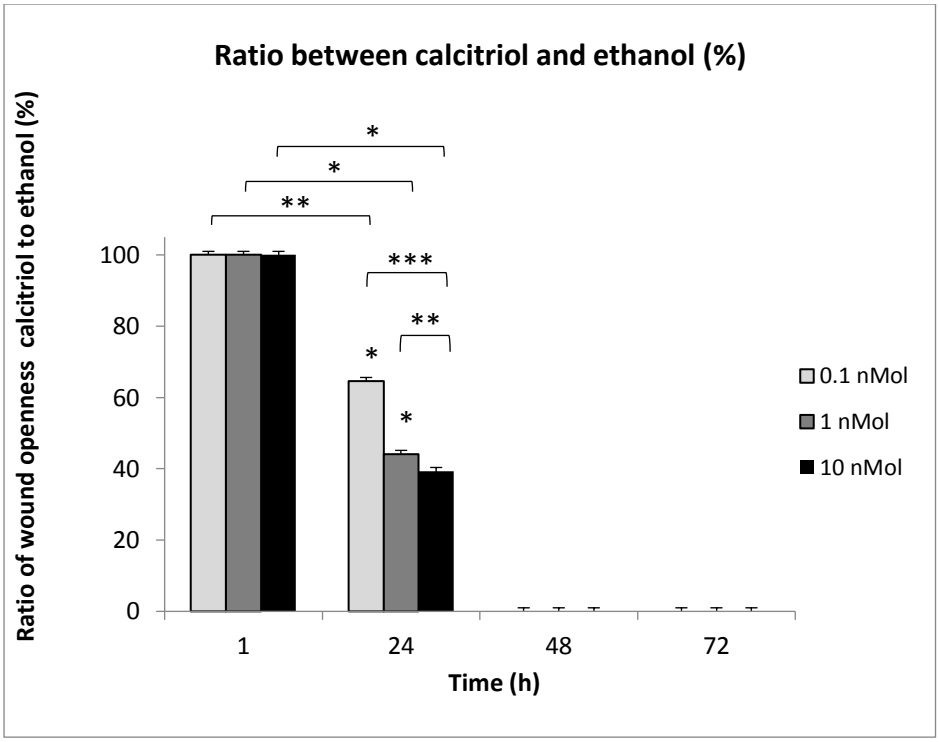


Figure 2.4. Effect of calcitriol on SCC-4 cell migration.
The wound openness of SCC-4 cells treated with calcitriol at 0.1 nM, 1 nM or 10 nM was measured in three independent experiments at 1, 24, 48 and 72 h in triplicates. At each time point the ratio between the wounded area of calcitriol treated SCC-4 cells to corresponding ethanol controls were compared to the ratio at time 1 h (taken as 100 % wound openness). Statistically all data points are significant when compared to initial 100 % wound opening. Statistical significance was calculated by comparing calcitriol treated cells to corresponding ethanol controls using student t-test. Statistical comparison also was performed between different time points and doses (p<0.05). Data was considered significant when p values were below 0.05. * denotes p<0.05, ** p<0.005, *** p<0.0005. P values at each point are shown in Appendix B, Table 2.2.

2.4 Discussion

Cutaneous cells are clearly the primary target of vitamin D metabolites (calcidiol and calcitriol) (Bikle et.al., 2013), however, their effects in vitamin D resistant neoplastic keratinocytes is not very well understood.

Tumour suppressive effects of calcitriol and calcidiol have been reported in a number of cell types (Sharan et.al., 2011; Lou et.al., 2013; Hill et.al., 2015; Chiang et.al., 2013; Bhatia and Falzon, 2015). Recently, it was demonstrated that proliferation of immortalised human keratinocytes HaCaT cells, was inhibited at 100 nM calcitriol, but not at 10 nM (Hill et.al., 2015). In SCC-25 cells, calcitriol suppressed growth of cultured keratinocytes via cell cycle arrest at G0/G1 phase and inhibition of proliferation marker, ki-67, expression (Chiang et.al., 2013). Conversely, lack of inhibitory calcitriol effects on migration and proliferation was evident in colon cancer cells. Calcitriol at dose ranging 1 – 100 nM had no effects in vitamin D resistant colon cancer cell proliferation (Bhatia and Falzon, 2015). Interestingly, the combination of calcitriol and

silibinin (plant extract), restored vitamin D responsiveness, whereas silibinin treatment alone did not (Bhatia and Falzon, 2015). Here, it was shown that antiproliferative qualities of cacitriol and calcidiol in SCC-4 cells were apparent at high concentrations (at 100 nM and 100,000 nM, respectively). In proliferation assays, treatment of SSC-4 cells with low concentrations of both metabolites (calcitriol and calcidiol), had no statistically significant inhibitory effects, when compared to corresponding ethanol controls (Appendix B). However, dose dependent inhibition of SCC-4 cell proliferation was evident at much higher concentrations for calcitriol and calcidiol in our study. Similarly, calcitriol antiproliferative effects were also shownin in uterine fibroid cells only at 10 nM, while lower calcitriol doses had no effect (Halder et.al., 2013). Calcitriol dosage differentially affects SCC-4 cell migration and proliferation: calcitriol dose dependent antiproliferative effects at 72 h was apparent, while in the migration assay a lack of inhibitory effect is noted. In fact, there was stimulation of migration. This is with agreement with a previous report (Hill et.al., 2015), which showed that at lower concentrations of calcitriol the inhibition of SCC cells is not obvious (Hill et.al., 2015), whereas at higher concentrations inhibitory effects were apparent. The effects of lower doses of calcitriol and calcidiol on proliferation are shown in Appendix B.

Antiproliferative effects of calcidiol in some cell types has been shown (Munetsuna, et.al., 2014; Lou et.al., 2003; Geng et.al.2011). Dose dependent growth-inhibitory actions of calcidiol in concentration (1–100 nM) of prostate epithelial cells (PZ-HPV-7) depends on calcidiol itself, regardless of intracellular calcitriol synthesis (Munetsuna, et.al., 2014). Where transfection of VDR siRNA blocked the calcidiol-induced inhibition of proliferation. Hence, it was suggested that calcidiol promoted VDR-ligand cell growth inhibition directly (Munetsuna, et.al., 2014). Furthermore, the inhibitory effect of calcidiol in human marrow stromal cell proliferation was found to be dependent on CYP27B1, where the lack of CYP27B1 expression had no inhibitory responses to calcidiol (at concentrations of 1-100 nM) (Geng et.al., 2011). We note that the inhibitory effect of calcidiol on SCC-4 cells at concentration of 100,000 nM at 48 and 72 h was most apparent (Figure 2.1). The results of calcidiol effect on vitamin D resistant SCC-4 cell proliferation is supported by the data on cultured oral human keratinocyte cell line (OKF6/TERT2), where inhibitory effect of calcidiol can be seen only at substantially high doses (10,000 nM – 20,000 nM); lower concentrations had no effect on cytotoxicity (Wang et.al., 2013). The absence of calcitriol inhibitory effects in proliferation assays at low concentrations is supportive towards earlier published literature on SCC-4 cells (Akutsu et.al., 2001; Ratnam et.al., 1996). Our data demonstrates inhibitory effects of SCC-4 cells at higher calcidiol and calcitriol

doses (100,000 nM and 100 nM, respectively); at lower concentrations the inhibitory effects were not as apparent (Figure 2.3 and 2.4). This observation is supported by other published literature (Wang et.al., 2013).

Correspondingly, the lack of inhibitory effect of lower calcidiol doses (10 nM and 100 nM) was demonstrated in myometrial and leiomyoma cultures, whilst treatment at higher dose (1,000 nM) inhibited the growth of both cell types (Biauer et.al., 2009; Wang et.al., 2013). In addition, we show that statistically significant inhibition of SCC-4 cell proliferation was detected only post 24 h incubation with calcidiol and calcitriol. Interestingly, the inhibition of primary keratinocyte proliferation by calcitriol at much lower doses 0.1-10 nM requires longer period of incubation (14 days) (Smith et.al., 1986). Whilst the inhibitory effects of calcitriol on cell migration have been reported in a number of cancer cell lines (Chen & Holick, 2003; Leyssens et.al., 2013). The SCC cell lines have been reported to have partial resistant to inhibitory actions of calcitriol (Bikle et.al., 1991). However, herein, at high concentrations of calcitriol and calcidiol treatment, inhibition of cell proliferation was noticeable in SCC-4 cultures.

We demonstrated inhibition of highly metastatic SCC-4 keratinocytes proliferative and migrative abilities by calcidiol when compared to untreated and ethanol treated SCC-4 cells. As time progressed the inhibition was more apparent. Contrastingly, stimulation of the wound openness in calcitriol treated cultures was detected only at some doses at 24h. This indicates that calcitriol treated SCC-4 had mild stimulation in migration: although, inhibitory effects on cell proliferation were noted. These findings are in agreement with the latest report on calcitriol effect in HaCaT keratinocytes (Hill et.al., 2015).

Taking together the results of proliferation and migration assays indicate that keratinocyte response to vitamin D (both calcidiol and calcitriol) are dose-time and cell type dependent (normal vs mutated cells). Further studies are required to determine calcidiol and calcitriol signaling mechanism in tumour cells. The lack of effect of both vitamin D metabolites at lower concentration in proliferation assays lead to further investigations of the cellular growth with the presence of calcidiol and calcitriol at high doses. Only high doses of calcidiol and calcitriol exerted inhibitory effects of SCC-4 cell proliferation.

2.5 Conclusion

Treatment of SCC-4 cells with both vitamin D metabolites (calcidiol and calcitriol) have inhibitory effects on cell proliferation at high doses. To our knowledge this is the first report demonstrating

efficacy of calcidiol in inhibition of SCC-4 cell migration and proliferation. The mechanism of this inhibition needs to be further investigated to assist in possible development of tumour inhibitory treatment using calcidiol or analogues thereof.

CHAPTER 3.

VITAMIN D REGULATION OF PROTEIN EXPRESSION IN PRIMARY AND MALIGNANT KERATINOCYTES

3.1 Introduction

It was shown in chapter 2 that calcidiol (100,000 nM) and calcitriol (100 nM) partially inhibited malignant SCC-4 cell proliferation. The supplementation of these metabolites at lower concentration had no obvious inhibitory effects on SCC-4 cell proliferation. Interestingly, at lower calcidiol concentration (250 nM) antimigrative effects were noted despite no antiproliferative effects. Given the inhibitory effects of calcidiol and calcitriol, it was important to assess the levels of vitamin D enzymes and receptor expression in SCC-4 cells and primary keratinocytes in response to calcidiol and calcitriol. Interestingly, failure of calcitriol and calcidiol to completely inhibit SCC-4 cell proliferation may be associated with poor functionality of VDR (Ratnam et.al., 1996). However, studies using other cell lines and human immortalised keratinocytes (HaCaT) showed antiproliferative actions at high pharmacological calcitriol concentrations (Hager et.al., 2001; Hill et.al., 2015). This suggests that calcitriol and calcidiol may influence cell proliferation via VDR-mediated mechanisms (Hager et.al., 2001). Resistance to calcium, for instance, in SCC-4 cells was shown to be dependent on the availability of the VDR/DRIP complex at VDREs (Bikle et.al., 2010). Earlier reports suggested that proliferation of SCC cells induced by vitamin D was mediated by VDR via VDREs (Köstner et.al., 2012; Walsh et.al., 2010). Interestingly, the major tumour drivers appear to be mediated via VDR-controlled signalling (Cardus 2009). The connection between VDR and tumour growth regulation, as well as progression into invasive metastatic forms of skin cancer need to be established. Therefore, it is important to gain insights into the mechanism of SCC-4 cell inhibition and the expression of VDR in vitamin D treated SCC-4 cells (Figure 3.1).

Intracellular vitamin D metabolism is tightly controlled by anabolic and catabolic hydroxylases (CYP17A1, CYP27B1 and CYP24A1, encoded by the corresponding genes *CYP27A1, CYP27B1, CYP24A1*) and vitamin D receptor (*VDR*) (Bikle et.al., 2011a). Herein, the expression of mitochondrial vitamin D enzymes (CYP27A1, CYP27B1 and CYP24A1) were analysed in order to ascertain whether they play a role in intratumoural vitamin D signalling and in tumour inhibition.

In addition, calcidiol and calcitriol may influence MAPK pathway. In fact, inhibition of MAPK activity with specific ERK1/2 inhibitors is enhanced by calcitriol in acute myeloid leukemia cells (Wang at al, 2013). Conversely, calcitriol has been shown to have inhibitory

activity on the MAPK pathway, with suppression of ERK-1 and ERK-2 MAP kinases in breast cancer cells (Rossi et.al., 2004).

The inhibition of SCC-4 cell migration and proliferation by calcidiol and inhibiton of proliferation by calcitriol (Chapter 2) could have an impact on MAPK VDR-mediated signalling. This inhibition is suggested to be a result of VDR modulatory activity (Figure 3.1) (Rossi et.al., 2004). Currently, the mechanism of MAPK pathway inhibition by vitamin D is unknown. The effects of calcidiol on the expression of vitamin D metabolic mitochondrial hydroxylases and VDR, as well as on MAPK pathway proteins, in primary and malignant human keratinocytes requires testing. Furthermore, VDR-mediated crosstalk between non-genomic and genomic signalling pathways at the level of MAPK activation was suggested to be responsible for inhibition of cell migration and proliferation in osteosarcoma cells (Wu et.al., 2007). VDR signalling mechanism that regulates the expression of tumour promoting proteins in keratinocytes, is most likely responsible for inhibitory effects observed in proliferation and migration of primary and malignant keratinocytes.

The crosstalk of vitamin D metabolites, calcidiol and calcitriol, mediated pathways, results in inhibition of cell proliferation and invasive capacity of malignant cells, and involves major tumour driving proteins, such as, epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor 3 (FGFR3) (Wang 2013; Weisinger 2010). The activity of EGFR and FGFR3 is regulated by the MAPK pathway (Weisinger et.al., 2010; Luangdilok et.al., 2011). In stromal cells that are involved in tumour formation, such as neoplastic keratinocytes and fibroblasts, these proteins may have VDR-mediated expression. It is not known whether vitamin D CYP450 oxidoreductases are involved in tumour cell development, or whether the anabolic CYP27A1 has a role in inhibiting carcinogenic transformation. However, it has been demonstrated that expression CYP27A1, in particular, is responsible for VDR activation in human keratinocytes (Endo-Umeda et.al., 2014). In fact, CYP27A1 plays an important role in catabolism of 7DHC and further VDR transcriptional activity via modulatory liver X receptor (LXR) actions (Endo-Umeda et.al., 2014). Moreover, the synthesis of intracellular calcidiol in keratinocytes appears to be CYP27A1-dependent (Schuster, 2011). The role of CYP27A1 in malignant keratinocytes and its influence on NMSC is not clear. The expression of other mitochondrial vitamin D hydroxylases CYP27B1 and CYP24A1 in neoplastic keratinocytes may aid in a better understanding in the process of neoplastic cell transformation.

To better understand the data from chapter 2, it is also important to determine the expression of proliferative cancer antigen protein PCNA in calcidiol and calcitriol treated primary and

malignant keratinocytes, which may aid in mechanistic insights of how vitamin D metabolites influence keratinocyte proliferation.

To evaluate the involvement of vitamin D metabolic enzymes expression in NMSC, the protein expression of VDR, CYP27A1, CYP27B1, CYP24B1, EGFR, FGFR3 and PCNA were evaluated in primary and malignant keratinocytes, following incubation with calcitriol or calcidiol; primary keratinocytes were used as a control.

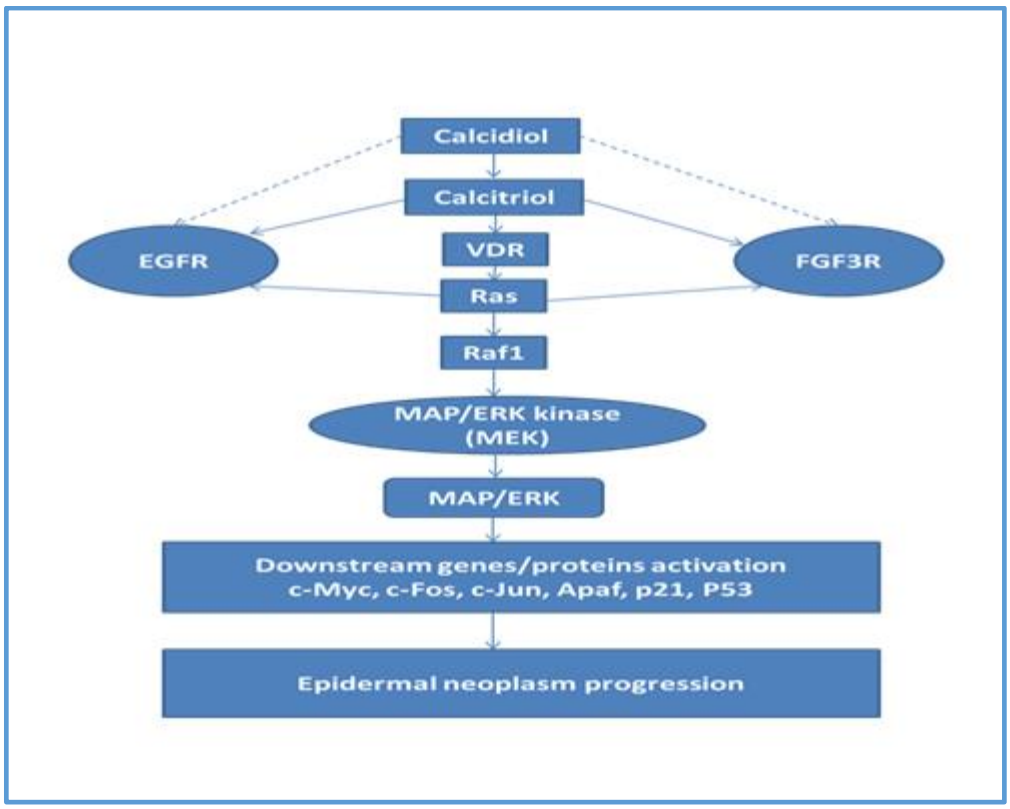


Figure 3.1. Proposed activation by calcidiol mitogen-activated protein kinase (MAPK) cascade.
Calcidiol-mediated VDR-activated MAPK cascade influences incoming signals that regulate proliferation, differentiation and apoptosis. Pathway regulation is mediated by available serum calcidiol, following its conversion to the active hormone, calcitriol. The regulatory activity of VDR results in signal transmission via RAS, RAF1, MEK and ERK, and the regulation of downstream genes/proteins: *c-Myc*, *c-Fos*, *c-Jun*, *Apaf1*, *p21* and *p53*, which are MAPK pathway dependent. The progression of neoplastic transformation and further tumour growth appear to be regulated by calcidiol (with or without its transformation to calcitriol) via VDR–MAPK pathway signalling. Adapted from (Balasubramanian 2007); and KEGG Pathway Database (<http://www.kegg.jp/kegg-bin>, viewed 30 April 2011

3.2 Materials and methods

3.2.1 Cell culture

Human SSC cell line SCC-4 (CRL-1628) was obtained from American Type Culture Collection (ATCC), neonatal keratinocytes were purchased from (102-05-N) Sigma-Aldrich, Australia. Normal adult human primary epidermal keratinocytes (PCS-200-011) were obtained from ATCC. Primary keratinocyte cultures were used as an additional control.

3.2.2 Cell culture reagents

Dulbecco's modified Eagle's medium (DMEM; D6429), Ham's F12 nutrient mixture (51651C), Foetal Bovine Serum (FBS; F2442), antibiotic/antimycotic solution (10,000 units/mL penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B), calcidiol (H-1440), calcitriol (D1530) trypsin-EDTA (59417C) and 10X phosphate-buffered saline (PBS; 051M8422).

Normal keratinocytes were grown in HAMS/DMEM (50/50), supplemented with 10 % FBS, and 0.5 % Penicillin–Streptomycin–Amphotericin Solution. Primary keratinocytes were grown in Dermal Cell Basal Medium (ATCC; PCS-200-030) supplemented with Growth Kit reagents (bovine pituitary extract, 0.4 %; recombinant human (rh) TGF-α, 0.5 ng/mL; L-glutamine, 6 mM; hydrocortisone, 100 ng/mL; insulin, 5 µg/mL; epinephrine, 1 µM; apo-transferrin, 5 µg/mL; ATCC, PCS-200-040) and Penicillin–Streptomycin–Amphotericin B Solution (penicillin 10,000 units/mL, streptomycin 10 mg/mL, amphotericin B 25 µg/mL; ATCC, PCS-999-002).

3.2.3 Cell culture conditions

All cultures were grown at 37 °C in a humidified 5 % CO_2 incubator in 175 cm^2 tissue culture flasks. The cultured SCC-4 cells and primary keratinocytes were treated with 100,000 nM calcidiol and 100 nM calcitriol, over 72 h period. As calcidiol and calcitriol were dissolved in ethanol, treated cells were compared with corresponding ethanol controls at concentrations of 0.000004 % and 0.004 %, correspondingly. For protein analysis, cultured primary and malignant keratinocytes were used only as exponentially growing cells, below passage number 5. Primary human normal keratinocytes and SCC-4 cells were grown until they reached total confluency.

3.2.4 Harvesting cells for expression analysis

Normal human keratinocytes and malignant squamous keratinocytes (SCC-4) were treated with calcidiol (100,000 nM) and calcitriol (100 nM) for 72 h, where the antiproliferative effects were mostly noted. Cells were harvested and total cellular proteins extracted using modified RIPA buffer with protease inhibitor cocktail, pH 7.4.

3.2.5 Western blot reagents

Trypsin-EDTA (59417C), 10x PBS (051M8422) and RIPA lysis buffer (R0278) were obtained from Sigma-Aldrich.

Western Blot reagents were obtained from Life Technologies: MOPS Novex SDS running buffer (NP0001), NuPAGE Novex 10 % Bis-Tris gels (NP0316Box), NuPAGE Novex transfer buffer (NP00061), Novex prestained protein standard (LC5800), NuPAGE Sample Reducing Agent (NP0009).

Protease inhibitor cocktail was obtained from Roche Diagnostics, Australia. Clarity Western ECL Substrate (170-5060) was obtained from Bio-Rad, Australia. Polyvinylidene diflouride (PVDF) transfer membrane (0.45μm**;** 88518) and filter paper (0.83 mm thick; PIE88600) were purchased from Thermo Scientific, Australia.

The primary unconjugated antibodies were obtained from Santa Cruz Biotechnology, USA and used at the optimised dilutions (1:1,000). The antibodies were supplied at the amount of 200 μg and with the specifications as described in Table 3.1

Table 3.1 Specificity of primary antibodies

Primary antibody	**Size of protein**	**Specificity**	**Cross-reactivity**	**Product code Manufacturer**
Polyclonal goat anti-human CYP27A1 (IgG)	60 kDa	Recognises epitope mapping near the N-terminus of CYP27A1	Human, mouse, rat	(P-17; sc-14835), Santa Cruz Biotechnology, USA
polyclonal rabbit anti-human CYP27B1(IgG)	56 kDa	Recognises amino acids 221-310 mapping within an internal region of CYP27B1	Human, mouse, and to a lesser extent rat, porcine	(H-90; sc-67261), Santa Cruz Biotechnology, USA
polyclonal rabbit anti-human CYP24A1 (IgG)	65 kDa	Recognises amino acids 351-437 mapping near the C-terminus of CYP24	Human, mouse, rat	(H-87; sc-66851), Santa Cruz Biotechnology, USA
polyclonal rabbit anti-human VDR (IgG)	53 kDa	Recognises amino acids 344-424 of VDR	Human, mouse, rat	(H-81; sc-9164), SantaCruz Biotechnology, USA
polyclonal rabbit anti-human EGFR (IgG)	170 kDa	Recognises a peptide mapping at the C-terminus of EGFR	Human, mouse, rat	(1005; sc-03), SantaCruz Biotechnology, USA

polyclonal rabbit anti-human FGFR3	97 kDa	Recognises an epitope mapping at the C-terminus of FGFR3	Human, mouse, rat	(C-15,sc-123), SantaCruz Biotechnology, USA
monoclonal mouse anti-human PCNA (IgG2a)	36 kDa	Rat PCNA protein A fusion protein was produced in pC2T bacterial vector and used to generate the antibody. The antibody recognises a polypeptide within the PCNA sequence, although the exact epitope is not clear.	Human	(PC10; M0879) Dako, Australia
polyclonal rabbit anti-human α-tubulin antibody (IgG)	50 kDa	Recognises C-terminus of human tubulin α protein	Human	(OAEC01862) Aviva Systems Biology, Australia

The secondary antibodies: polyclonal rabbit anti-goat Ig–horseradish peroxidase (HRP), polyclonal goat anti-rabbit Ig–HRP (1:2,000) and polyclonal rabbit anti-mouse Ig-HRP (1:2,000) were obtained from Dako, Australia.

3.2.6 SDS-PAGE gels

Separation of proteins from cell lysates was performed using NuPAGE Novex 10 % Bis-Tris SDS-PAGE gels, as per the manufacturer's instructions (NuPAGE Bis-Tris Acetate Gel Protocol; Life Technologies). The approximate molecular weight of proteins was estimated using Novex prestained protein standards.

3.2.7 Chemiluminescence-based Western blot analysis

Western blot analysis was performed according to (Filiz & Dass, 2012). Proteins were separated by electrophoresis on SDS-PAGE gels. The gel, PVDF membrane and pre-cut filter paper were pre-activated in ice-cold transfer buffer containing methanol. The gel and PVDF membrane were placed between filter papers, placed in a cassette and transferred using the Bio-Rad Western transfer system at 150 V for 1 h. The PVDF membrane was blocked for 1 h at room temperature in blocking solution (5 % (w/v) skim milk powder in Tris-buffered saline/0.05 % Tween-20 (TBST), pH 7.5) and incubated with specified primary antibodies diluted in TBST according to the manufacturer's recommendations. The PVDF membrane was washed in TBST three times for 20 min each and incubated with HRP-conjugated secondary antibody, according to the manufacturer's recommendations, in 5 % skim milk/TBST solution for 1 h at room temperature

with gentle rocking. PVDF membrane was washed three times for 20 min each and proteins were detected using Clarity Western ECL Substrate on a Chemidoc (Bio-Rad).

PVDF membranes were stripped in a modified mild antibody removal solution, pH 2.2, at room temperature for 30 min and re-probed with alpha-tubulin for protein loading normalisation. Densitometric analysis was performed in triplicates of each sample using ImageJ analysis software, to compare each of the samples. The methods used for cell lysate preparation and Western blot analysis were as described previously (G. Filiz 2012; Tan et.al. 2014), and are shown in full in Appendix F.

3.2.8 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Data representative of 3 experiments conducted in triplicate. The data represents mean ± SEM of triplicate blots. Differences were determined by an independent Student's t-test and results were considered statistically significant if p-values were < 0.05.

3.3 Results

As calcidiol and calcitriol were dissolved in ethanol, ethanol-treated SCC-4 cells were used as a control at the appropriate concentration (amount equivalent to ethanol-diluted calcidiol or calcitriol). The experiments were conducted using 100,000 nM calcidiol and 100 nM calcitriol, as these concentrations showed antiproliferative effects at 72 h. In addition, at lower calcidiol concentration (250 nM) antimigrative effects were noted, hence this dose was also included. Primary keratinocytes were also used for comparison.

The difference between calcidiol (or calcitriol) treated cells and ethanol was obtained from each triplicate of three independent experiments conducted under the same experimental conditions.

3.3.1 Effects of calcidiol and calcitriol on CYP27A1 expression in primary and malignant keratinocytes

Mitochondrial CYP27A1 is a cytochrome P450 member that hydroxylates vitamin D at $^{25-}$carbon (Chatterjee 2001; Tieu et.al., 2012). CYP27A1 activity is important for metabolic and endocrine homeostasis in humans (Chatterjee 2001). Determination of its function has been investigated in various cell types, mostly due to its involvement in bile acid metabolism. CYP27A1 is also a crucial factor for catabolism of 7-DHC that results in activation of VDR, via selective LXR modulation in human keratinocytes (Endo-Umeda et.al, 2014).

CYP27A1 acts as an active anabolic enzyme in epidermal keratinocytes and is critical in cutaneous vitamin D metabolism (Wong et.al., 2004). It was demonstrated that calcitriol had no effect on the expression of CYP27A1 in human dermal fibroblasts (Lehmann, 1997). Interestingly, CYP27A1 expression was up-regulated in human prostate epithelial cell lines treated with calcidiol precursor, cholecalciferol (Tokar & Webber, 2005a). Obviously, the regulation of this enzyme is very specific for cell and tissue type. In the experiments presented here, no statistically significant differences between CYP27A1 expression in primary and malignant keratinocytes were noted, when treated with calcidiol (100,000 nM) or calcitriol (100 nM) (Figure 3.2.a,b; Table 3.2). Interestingly, at lower calcidiol dose (250 nM) there was no difference between calcidiol treated SCC-4 cells and corresponding ethanol control (Figure 3.2.c), however upregulation of CYP27A1 was detected in primary keratinocytes in response to 250 nM calcidiol ($p<0.05$) (Figure 3.2.c; Table 3.2).

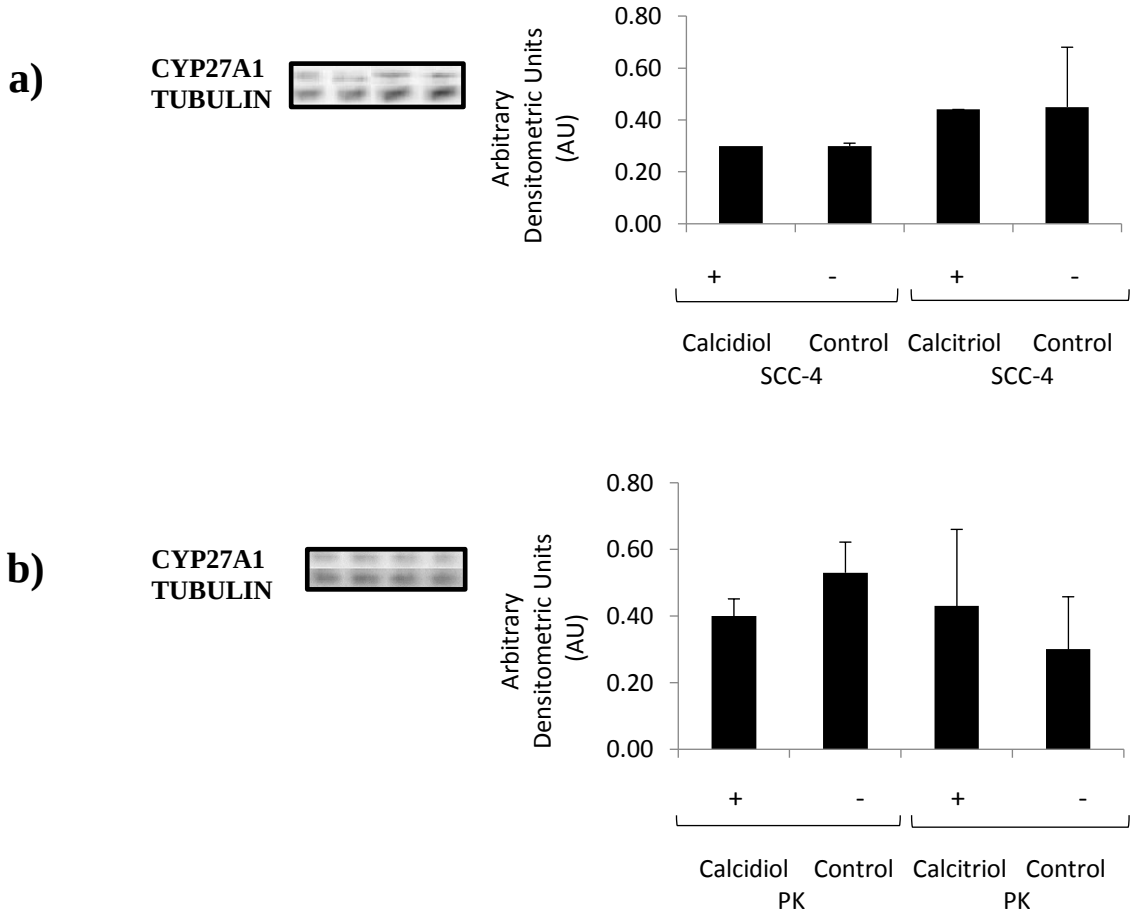


Figure 3.2(a, b) The effect of high dose calcidiol or calcitriol on CYP27A1 levels in SCC-4 cells and primary keratinocytes.

a) SCC-4 and **b)** primary keratinocytes (PK) were treated with 100,000 nM calcidiol or 100 nM calcitriol for 72 h. Total protein ran on SDS page gel electrophoresis and transferred to PVDF membrane. Isolated protein was probed with anti CYP27A1 antibody, following incubation with corresponding HRP-conjugated secondary antibody. Proteins were visualised using Clarity Western ECL Substrate. Protein bands from western blots were assessed by densitometric scanning of the bands and by statistical analysis of the resulting band intensities. Protein levels were normalised using α-tubulin control. Comparisons between data sets were made using Student's t-test. Data presented as a graph is from a representative of 3 experiments conducted in triplicate. The data represents mean ± SEM of triplicate blots. * indicates $p<0.05$. +, with calcidiol or calcitriol; -, corresponding ethanol control.

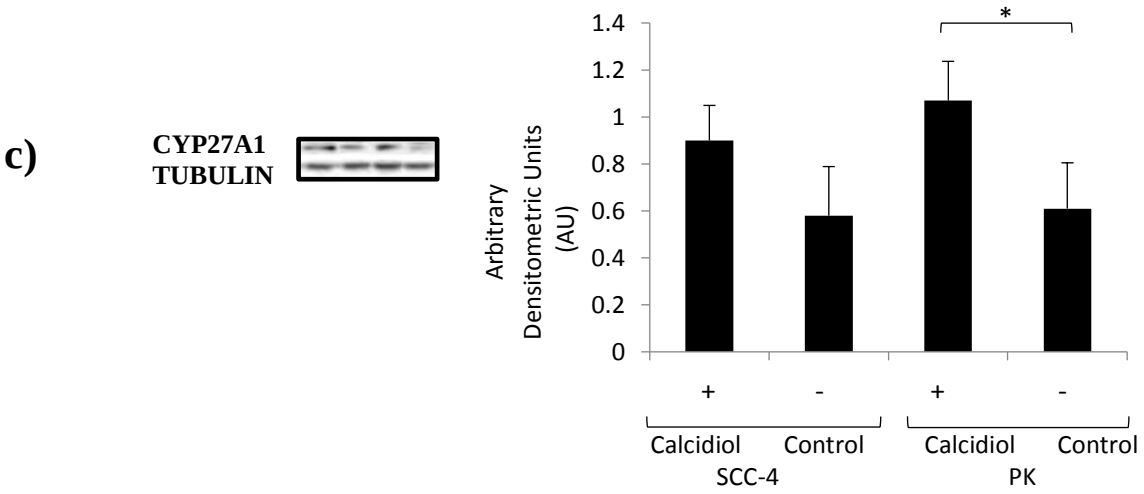


Figure 3.2(c) The effect of calcidiol (250 nM) on CYP27A1 levels in SCC-4 cells and primary keratinocytes. SCC-4 and primary keratinocytes (PK) were treated with 250 nM calcidiol for 72 h. Total protein ran on SDS page gel electrophoresis and transferred to PVDF membrane. Isolated protein was probed with anti CYP27A1 antibody, following incubation with corresponding HRP-conjugated secondary antibody. Proteins were visualised using Clarity Western ECL Substrate. Protein bands from western blots were assessed by densitometric scanning of the bands and by statistical analysis of the resulting band intensities. Protein levels were normalised using α-tubulin control. Comparisons between data sets were made using Student's t-test. Data presented as a graph is from a representative of 3 experiments conducted in triplicate. The data represents mean ± SEM of triplicate blots. * indicates $p<0.05$. +, with calcidiol; -, corresponding ethanol control.

3.3.2 Effects of calcidiol and calcitriol on CYP27B1 expression in primary and malignant keratinocytes

CYP27B1 expression has been associated with nuclear VDR activity (Geng et.al., 2011). Low levels of CYP27B1 have been noted in colorectal cancer and melanoma (Tangpricha et.al., 2001; Brożyna et.al., 2013; Jacobs et.al., 2013). In addition, in melanoma tissue samples, no obvious increase in CYP27B1 was reported (Reichrath et.al., 2004). Similarly, in cultured primary keratinocytes and SCC-4 treated cells with high dose of calcidiol (100,000 nM), there were no noticeable effects on CYP27B1 expression. However, calcitriol treatment caused significant downregulation in CYP27B1 in SCC-4 cultures ($p<0.005$) (Figure 3.3a). Surprisingly, no statistical differences were noted in response to calcitriol treatment in primary keratinocytes (Figure 3.3b).

Interestingly, using lower concentration of calcidiol (250 nM) on SCC-4 cells, the expression of CYP27B1 was significantly increased compared to its corresponding ethanol control ($p<0.005$). Likewise, the expression of CYP27B1 in primary keratinocytes was also increased ($p<0.05$) (Figure 3.3.c; Table 3.2).

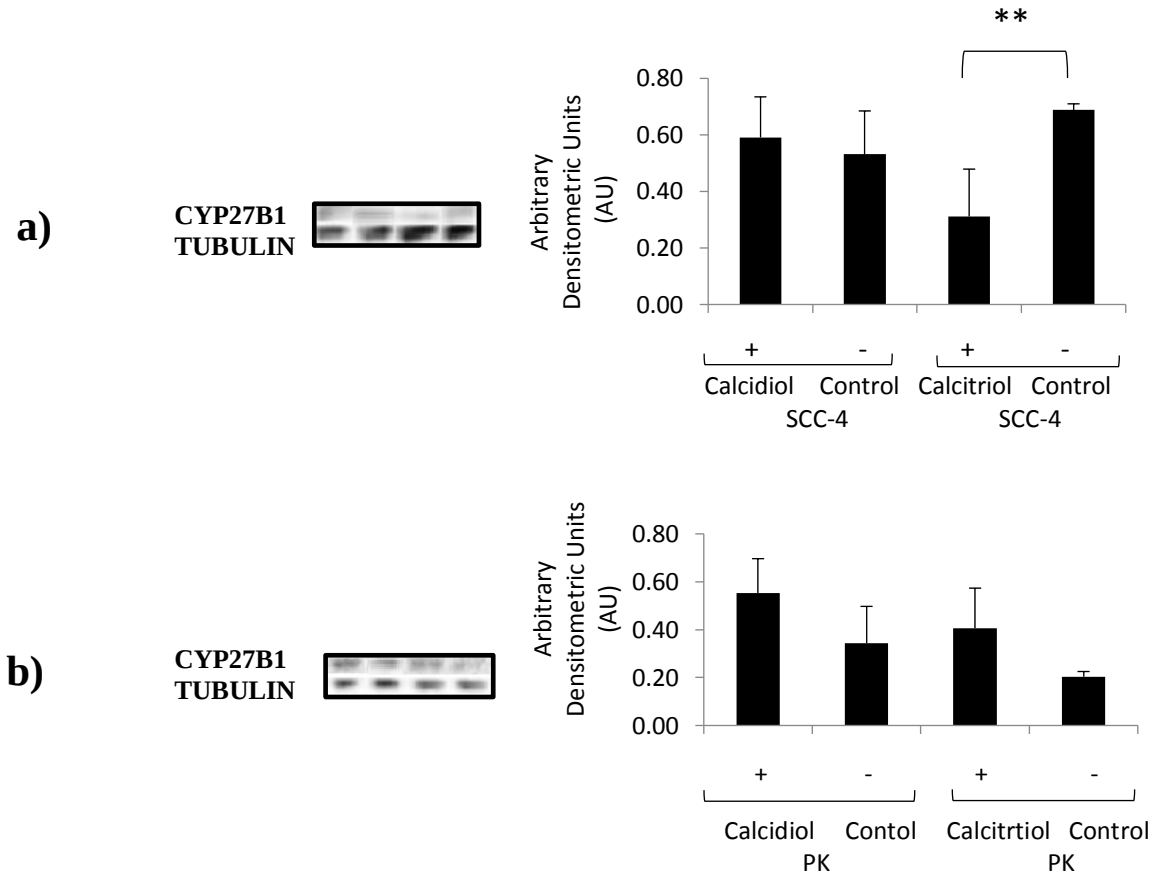


Figure 3.3(a, b) The effect of high dose calcidiol or calcitriol on CYP27B1 levels in SCC-4 cells and primary keratinocytes.

a) SCC-4 and **b)** primary keratinocytes (PK) were treated with 100,000 nM calcidiol or 100 nM calcitriol for 72 h. Total protein ran on SDS page gel electrophoresis and transferred to PVDF membrane. Isolated protein was probed with anti CYP27B1 antibody, following incubation with corresponding HRP-conjugated secondary antibody. Proteins were visualised using Clarity Western ECL Substrate. Protein bands from western blots were assessed by densitometric scanning of the bands and by statistical analysis of the resulting band intensities. Protein levels were normalised using α-tubulin control. Comparisons between data sets were made using Student's t-test. Data presented as a graph is from a representative of 3 experiments conducted in triplicate. The data represents mean ± SEM of triplicate blots. * indicates $p<0.05$. +, with calcidiol or calcitriol; -, corresponding ethanol control.

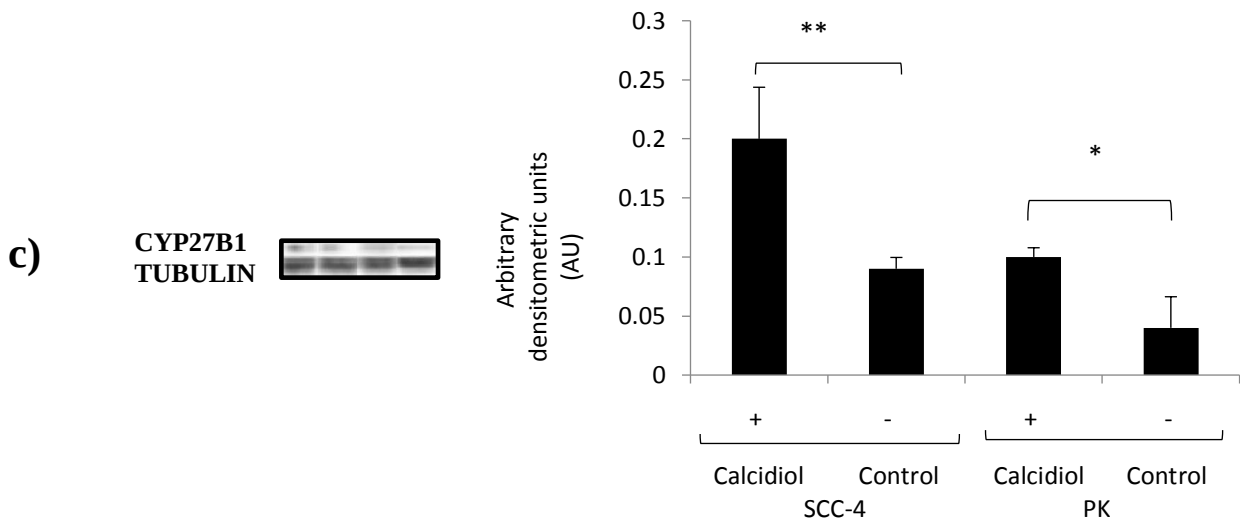


Figure 3.3(c) The effect of calcidiol (250 nM) on CYP27B1 levels in SCC-4 cells and primary keratinocytes. SCC-4 and primary keratinocytes (PK) were treated with 250 nM calcidiol for 72 h. Total protein ran on SDS page gel electrophoresis and transferred to PVDF membrane. Isolated protein was probed with anti CYP27B1 antibody, following incubation with corresponding HRP-conjugated secondary antibody. Proteins were visualised using Clarity Western ECL Substrate. Protein bands from western blots were assessed by densitometric scanning of the bands and by statistical analysis of the resulting band intensities. Protein levels were normalised using α-tubulin control. Comparisons between data sets were made using Student's t-test. Data presented as a graph is from a representative of 3 experiments conducted in triplicate. The data represents mean ± SEM of triplicate blots. * indicates $p<0.05$. +, with calcidiol; -, corresponding ethanol control.

3.3.3 Effects of calcidiol and calcitriol on CYP24A1 protein expression in primary and malignant keratinocytes

CYP24A1 catalyses the side-chain oxidation of calcitriol (Annalora et.al., 2010). Up-regulated expression of CYP24A1 attenuates vitamin D signalling associated with calcium homeostasis and cellular growth regulation (Annalora et.al., 2010). The development of therapeutics for some oncological conditions have focussed on CYP24A1, as it has been proposed as a predicative marker in several malignancies (Chen et.al., 2011; Luo et.al., 2012). Overexpression of CYP24A1 has been described in melanoma and in colon and lung carcinomas (Seifert et.al., 2004; Chen et.al., 2011; Cross et.al., 2011; Luo et.al , 2012; Yao & Ambrosone, 2012; Jacobs et.al., 2013).

SCC-4 cells treated with calcidiol (100,000 nM) significantly increased CYP24A1 expression compared to its corresponding ethanol control ($p<0.05$) (Figure 3.4.a). Similarly, calcitriol (100 nM) treated SCC-4 cells increased expression of CYP24A1 ($p<0.05$) (Figure 3.4.a; Table 3.2). In primary keratinocytes an increased expression trend was apparent but was not statistically significant (Figure 3.4.b). Similar trend was observed in SCC-4 cells and primary keratinocytes treated with 250 nM calcidiol although not significant (Figure 3.4.c).

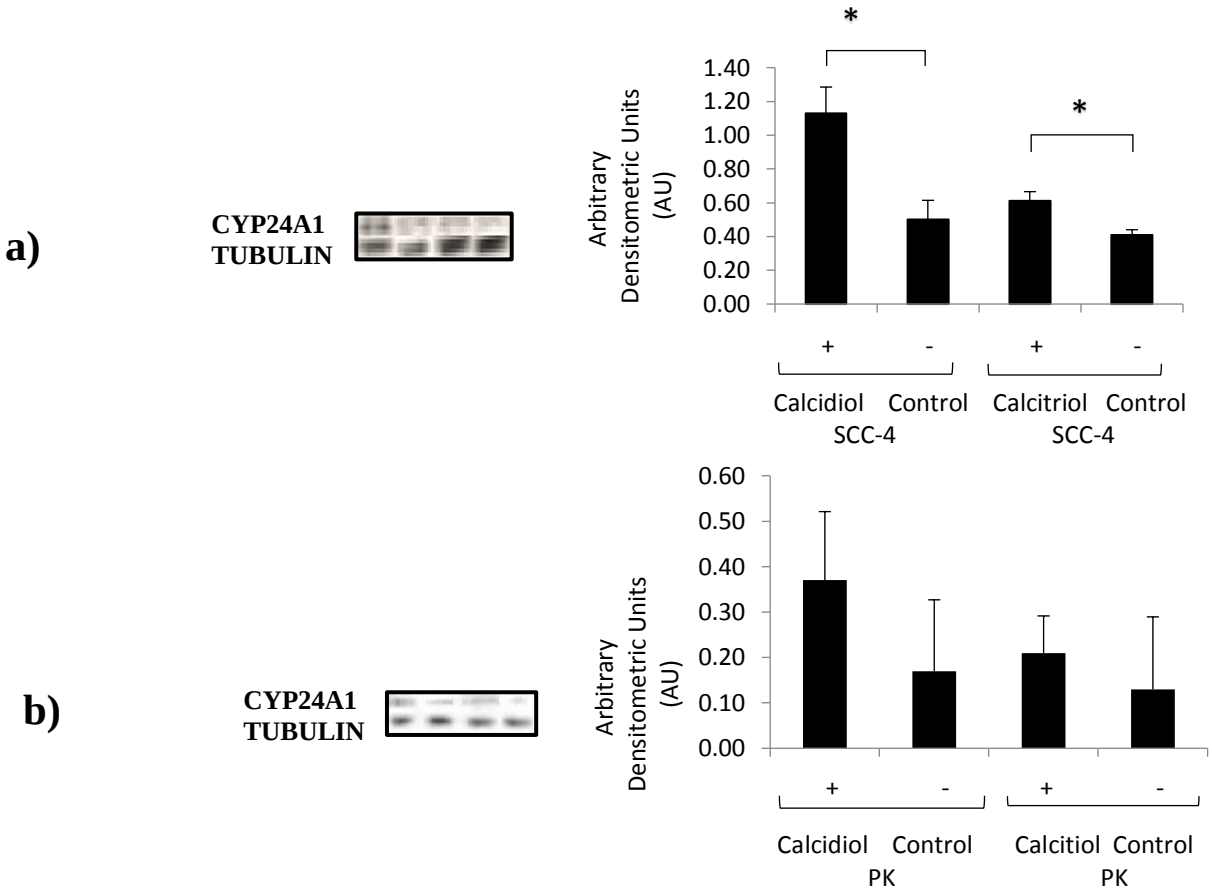


Figure 3.4(a, b) The effect of high dose calcidiol or calcitriol on CYP24A1 levels in SCC-4 cells and primary keratinocytes.

a) SCC-4 and **b)** primary keratinocytes (PK) were treated with 100,000 nM calcidiol or 100 nM calcitriol for 72 h. Total protein ran on SDS page gel electrophoresis and transferred to PVDF membrane. Isolated protein was probed with anti CYP24A1 antibody, following incubation with corresponding HRP-conjugated secondary antibody. Proteins were visualised using Clarity Western ECL Substrate. Protein bands from western blots were assessed by densitometric scanning of the bands and by statistical analysis of the resulting band intensities. Protein levels were normalised using α-tubulin control. Comparisons between data sets were made using Student's t-test. Data presented as a graph is from a representative of 3 experiments conducted in triplicate. The data represents mean ± SEM of triplicate blots. * indicates $p<0.05$. +, with calcidiol or calcitriol; -, corresponding ethanol control.

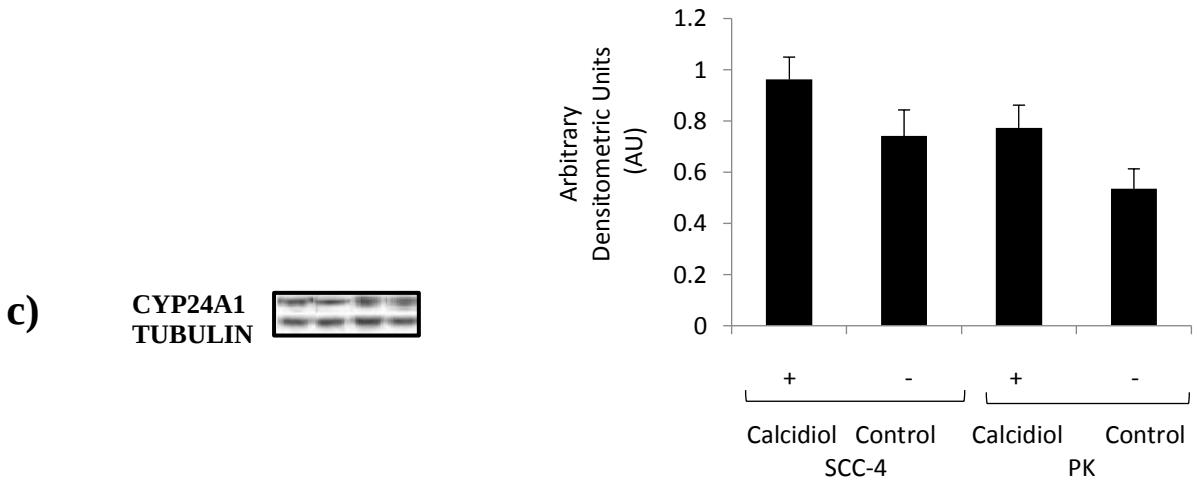


Figure 3.4(c) The effect of calcidiol (250 nM) on CYP24A1 levels in SCC-4 cells and primary keratinocytes

SCC-4 and primary keratinocytes (PK) were treated with 250 nM calcidiol for 72 h. Total protein ran on SDS page gel electrophoresis and transferred to PVDF membrane. Isolated protein was probed with anti CYP24A1 antibody, following incubation with corresponding HRP-conjugated secondary antibody. Proteins were visualised using Clarity Western ECL Substrate. Protein bands from western blots were assessed by densitometric scanning of the bands and by statistical analysis of the resulting band intensities. Protein levels were normalised using α-tubulin control. Comparisons between data sets were made using Student's t-test. Data presented as a graph is from a representative of 3 experiments conducted in triplicate. The data represents mean ± SEM of triplicate blots. * indicates $p<0.05$. +, with calcidiol; -, corresponding ethanol control.

3.3.4 Effects of calcidiol and calcitriol on vitamin D receptor (VDR) expression in primary and malignant keratinocytes

The principal function of VDR, which belongs to the same family of hormone receptors as the steroid and thyroid hormone receptors, is as a vitamin D-inducible transcription factor (Ondková et.al., 2006). Full functionality of VDR in the form of a heterodimer with retinoid X receptor is essential for biological effects of calcitriol (Ondková et.al., 2006). The role of VDR has been extensively studied in malignant cells and its function is to modulate downstream gene expression (Rehan et.al., 2002; Ramirez et.al., 2009; Guan et.al., 2013; Jiang et.al., 2013). The effects of calcidiol on epidermal cells is not clear. VDR expression has been detected in mutated and normal keratinocytes (Midorikawa et.al., 1999). The regulation of neoplastic transformation has been linked to VDR signalling (Ramirez et.al., 2009).

SCC-4 cells and primary keratinocytes treated with either calcidiol (100,000 nM) or calcitriol (100 nM) showed no statistically significant difference in VDR expression compared to their corresponding ethanol controls (Figure 3.5a,b). However, in SCC-4 cells treated with calcidiol at lower concentration (250 nM), the expression of VDR was significantly increased compared to the corresponding ethanol treated cells ($p<0.05$); no effect was noted in primary keratinocytes (Figure 3.5.c; Table 3.2).

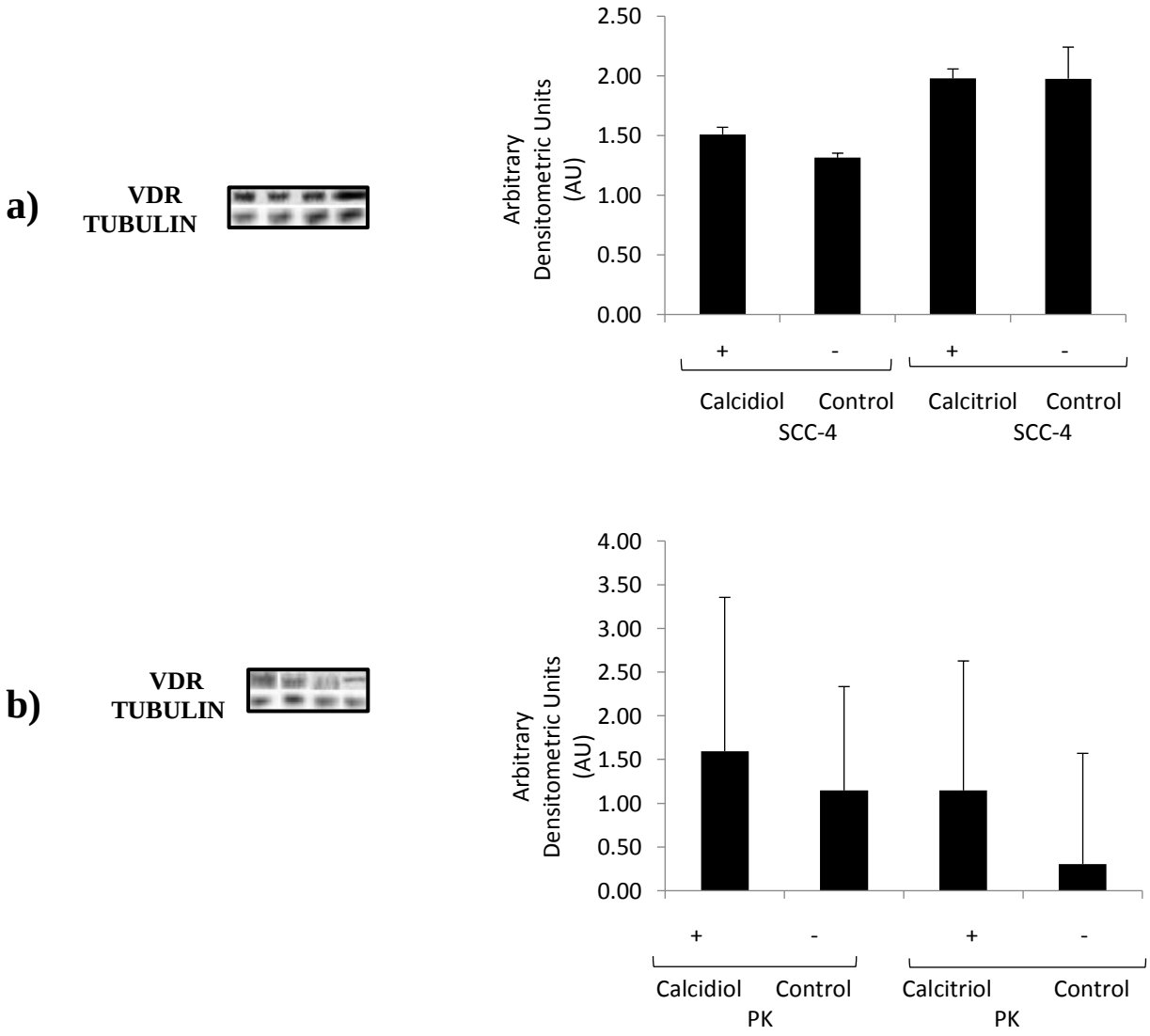


Figure 3.5(a, b) The effect of high dose calcidiol or calcitriol on vitamin D receptor (VDR) protein levels in SCC-4 cells and primary keratinocytes.

a) SCC-4 and **b)** primary keratinocytes (PK) were treated with 100,000 nM calcidiol or 100 nM calcitriol for 72 h. Total protein ran on SDS page gel electrophoresis and transferred to PVDF membrane. Isolated protein was probed with anti VDR antibody, following incubation with corresponding HRP-conjugated secondary antibody. Proteins were visualised using Clarity Western ECL Substrate. Protein bands from western blots were assessed by densitometric scanning of the bands and by statistical analysis of the resulting band intensities. Protein levels were normalised using α-tubulin control. Comparisons between data sets were made using Student's t-test. Data presented as a graph is from a representative of 3 experiments conducted in triplicate. The data represents mean ± SEM of triplicate blots. * indicates $p<0.05$. +, with calcidiol or calcitriol; -, corresponding ethanol control.

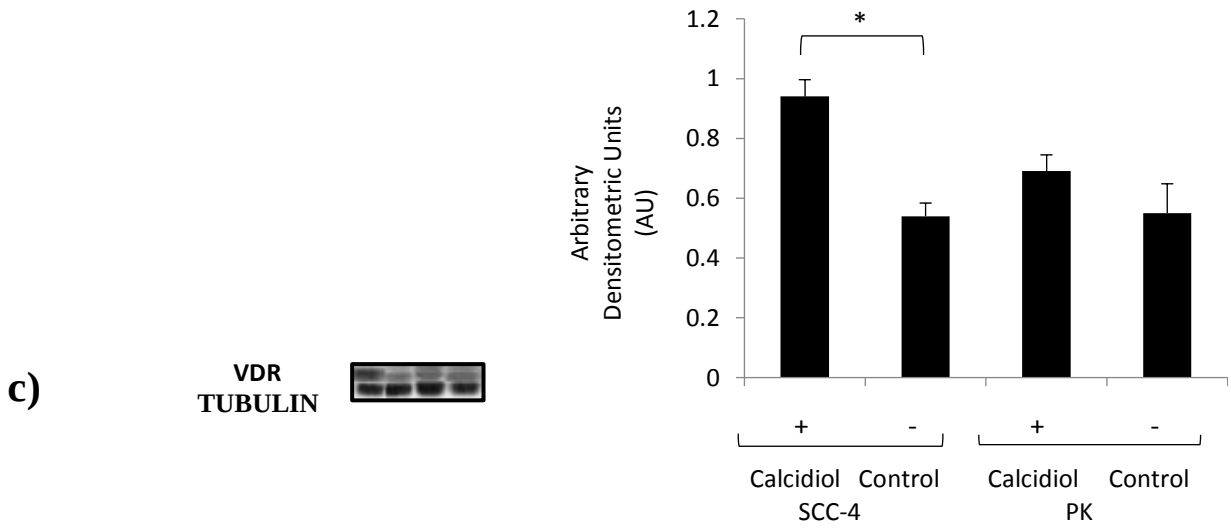


Figure 3.5 (c) The effect of calcidiol (250 nM) on VDR protein levels in SCC-4 cells and primary keratinocytes SCC-4 and primary keratinocytes (PK) were treated with 250 nM calcidiol for 72 h. Total protein ran on SDS page gel electrophoresis and transferred to PVDF membrane. Isolated protein was probed with anti VDR antibody, following incubation with corresponding HRP-conjugated secondary antibody. Proteins were visualised using Clarity Western ECL Substrate. Protein bands from western blots were assessed by densitometric scanning of the bands and by statistical analysis of the resulting band intensities. Protein levels were normalised using α-tubulin control. Comparisons between data sets were made using Student's t-test. Data presented as a graph is from a representative of 3 experiments conducted in triplicate. The data represents mean ± SEM of triplicate blots. * indicates $p<0.05$. +, with calcidiol; -, corresponding ethanol control.

3.3.5 Effects of calcidiol and calcitriol on epidermal growth factor receptor (EGFR) protein expression in primary and malignant keratinocytes

Epidermal regeneration involves a complex process of cell proliferation that is regulated by EGF family members. EGFR plays a critical role in cell proliferation and differentiation (Roskoski, 2014). Elevated EGFR protein expression has been detected in the majority of aggressive NMSCs, and, is associated with reduced survival, radiotherapy resistance and localised tissue-specific failure (Mehra et.al., 2011). EGFR has been consistently reported as a target of VDR regulation (Cordero et.al., 2002; Lafky et.al., 2008; Fraguas et.al., 2011; Zhang et.al., 2013). VDR–EGFR heterodimerisation and control of intracellular trafficking may depend on the availability of calcidiol (Dusso et.al., 2004).

Induction of keratinocyte migration is conveyed through EGFR (Koivisto et.al., 2006). Calcitriol inhibition of EGFR protein expression has been noted in human epidermoid carcinoma cells (Cordero et.al., 2002). The overexpression of EGFR was recognised as a major mitogenic signal that was inhibited by calcitriol (Cordero et.al., 2002). Furthermore, calcitriol changed the cell localisation of EGFR. These results demonstrated that calcitriol alters EGFR membrane trafficking and down-regulates EGFR proliferation signalling (Cordero et.al., 2002).

However, it is shown herein, that primary keratinocytes and SCC-4 cells treated with calcidiol (250 nM or 100,000 nM) or calcitriol (100 nM), had no effect on the expression of EGFR (Figure 3.6; Table 3.2).

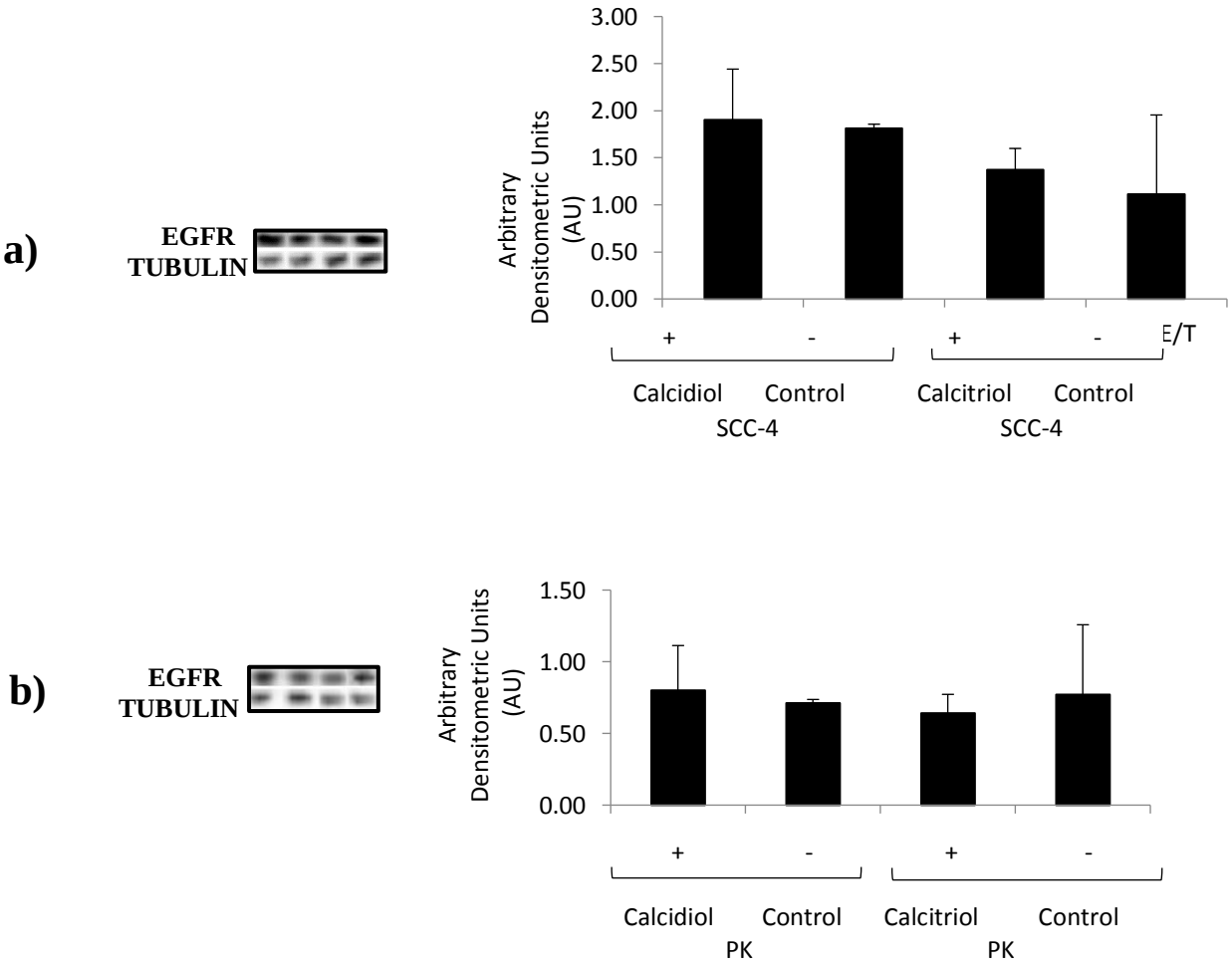


Figure 3.6(a, b). The effect of high dose calcidiol or calcitriol on epidermal growth factor receptor (EGFR) protein levels in SCC-4 cells and primary keratinocytes.

a) SCC-4 and **b)** primary keratinocytes (PK) were treated with 100,000 nM calcidiol or 100 nM calcitriol for 72 h. Total protein ran on SDS page gel electrophoresis and transferred to PVDF membrane. Isolated protein was probed with anti EGFR antibody, following incubation with corresponding HRP-conjugated secondary antibody. Proteins were visualised using Clarity Western ECL Substrate. Protein bands from western blots were assessed by densitometric scanning of the bands and by statistical analysis of the resulting band intensities. Protein levels were normalised using α-tubulin control. Comparisons between data sets were made using Student's t-test. Data presented as a graph is from a representative of 3 experiments conducted in triplicate. The data represents mean ± SEM of triplicate blots. * indicates $p<0.05$. +, with calcidiol or calcitriol; -, corresponding ethanol control.

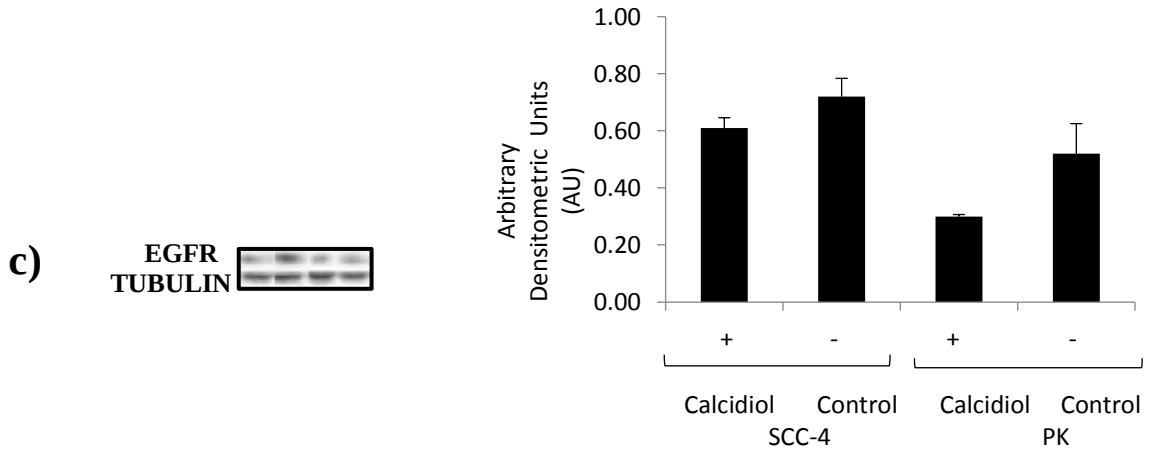


Figure 3.6(c) The effect of calcidiol (250 nM) on EGFR protein levels in SCC-4 cells and primary keratinocytes
SCC-4 and primary keratinocytes (PK) were treated with 250 nM calcidiol for 72 h. Total protein ran on SDS page gel electrophoresis and transferred to PVDF membrane. Isolated protein was probed with anti EGFR antibody, following incubation with corresponding HRP-conjugated secondary antibody. Proteins were visualised using Clarity Western ECL Substrate. Protein bands from western blots were assessed by densitometric scanning of the bands and by statistical analysis of the resulting band intensities. Protein levels were normalised using α-tubulin control. Comparisons between data sets were made using Student's t-test. Data presented as a graph is from a representative of 3 experiments conducted in triplicate. The data represents mean ± SEM of triplicate blots. * indicates $p<0.05$, ** $p<0.005$. +, with calcidiol; -, corresponding ethanol control.

3.3.6 Effects of calcidiol and calcitriol on fibroblast growth factor receptor 3 (FGFR3) protein expression in primary and malignant keratinocytes

FGF3 was originally identified as a proto-oncogene in virally-induced mouse mammary tumours (Antoine et.al., 2005). FGFR3 overexpression has been detected in breast cancer tissue when compared to normal tissue samples (Valladares et.al. 2006). FGFR3 overexpressed in metastatic hepatocellular carcinoma is associated with disease recurrence (Hu et.al., 2007). Furthermore, FGFR3 protein plays a role in melanoma, and, its overexpression has been associated with regulation of the MAPK pathway via inhibition of MEK activities (Yadav et.al., 2012). Targeting the FGFR3/RAS pathway was proposed for the development of new therapeutic agents to improve the outcome of melanoma patients (Yadav et.al., 2012). Hence, as FGFR3 is activated by MAPK signalling and VDR is known to have an impact on MAPK activity, the study of calcidiol-treated keratinocytes would be valuable in NMSC prevention and treatment.

SCC-4 cells and primary keratinocytes showed no effect in FGFR3 protein expression in the presence of calcidiol (100,000 nM) and calcitriol (100 nM) (Figure 3.7.a). However, significant inhibition of FGFR3 expression was noted in primary keratinocytes treated with calcidiol (100,000 nM) ($p<0.05$) when compared to corresponding ethanol control (Figure 3.7.b; Table 2). However, at lower calcidiol concentration (250 nM) the expression of FGFR3 was significantly increased in both SCC-4 cells and primary keratinocytes, compared to corresponding ethanol controls ($p<0.05$) (Figure 3.7c; Table 3.2). The inhibitory effects of calcidiol (100,000 nM) on FGFR3 expression in primary keratinocytes indicates that, antiproliferative effects (noted in Chapter 2) involve inhibition of FGFR3, whereas lower concentration (250 nM) promote the expression of FGFR3 (in both, primary keratinocytes and SCC-4 cells), which modulates cellular replication, growth and survival.

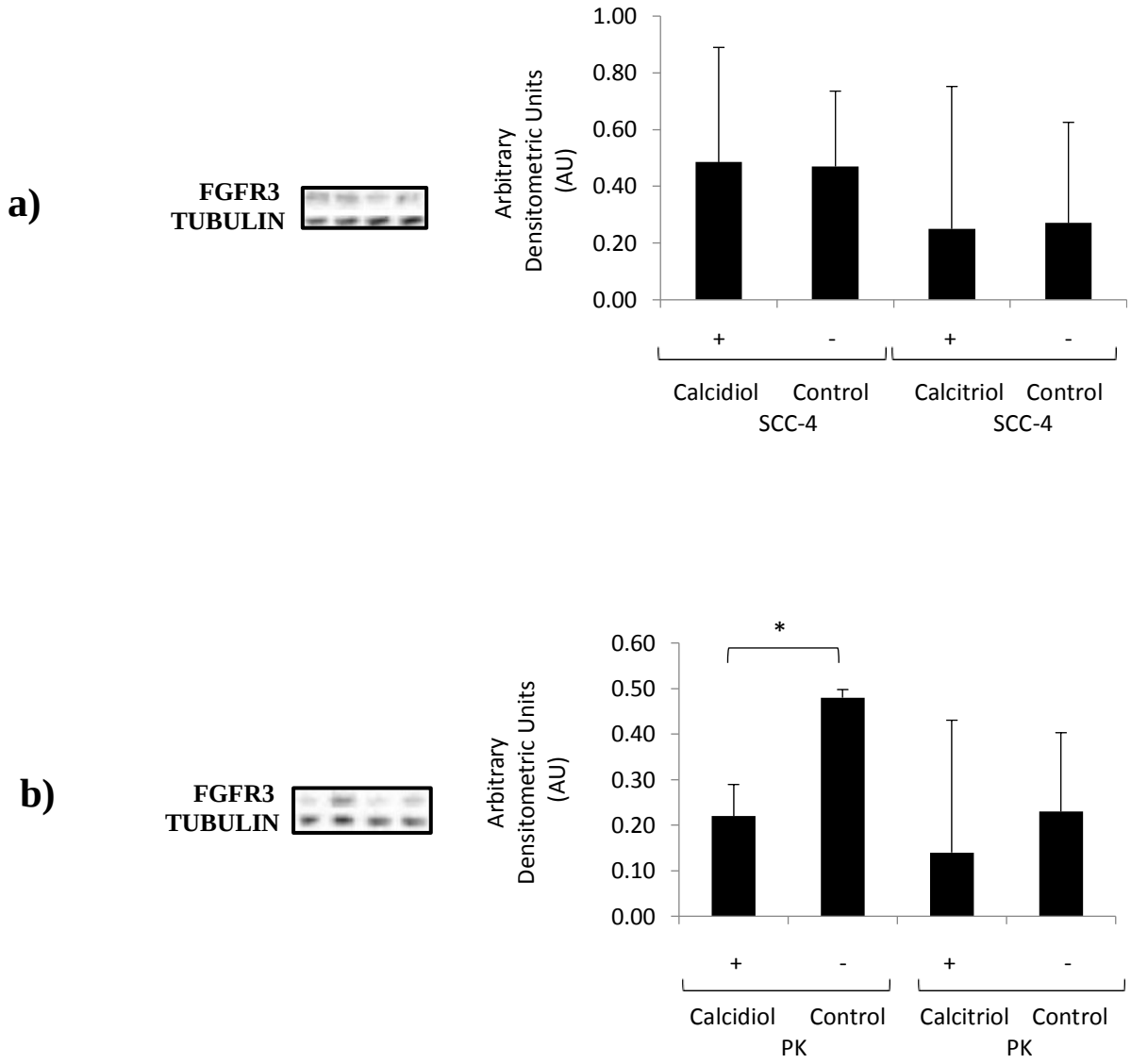


Figure 3.7(a, b) The effect of high dose calcidiol or calcitriol on fibroblast growth factor receptor 3 (FGFR3) protein levels in SCC-4 cells and primary keratinocytes.

a) SCC-4 and **b)** primary keratinocytes (PK) were treated with 100,000 nM calcidiol or 100 nM calcitriol for 72 h. Total protein ran on SDS page gel electrophoresis and transferred to PVDF membrane. Isolated protein was probed with anti FGFR3 antibody, following incubation with corresponding HRP-conjugated secondary antibody. Proteins were visualised using Clarity Western ECL Substrate. Protein bands from western blots were assessed by densitometric scanning of the bands and by statistical analysis of the resulting band intensities. Protein levels were normalised using α-tubulin control. Comparisons between data sets were made using Student's t-test. Data presented as a graph is from a representative of 3 experiments conducted in triplicate. The data represents mean ± SEM of triplicate blots. * indicates $p<0.05$. +, with calcidiol or calcitriol; -, corresponding ethanol control.

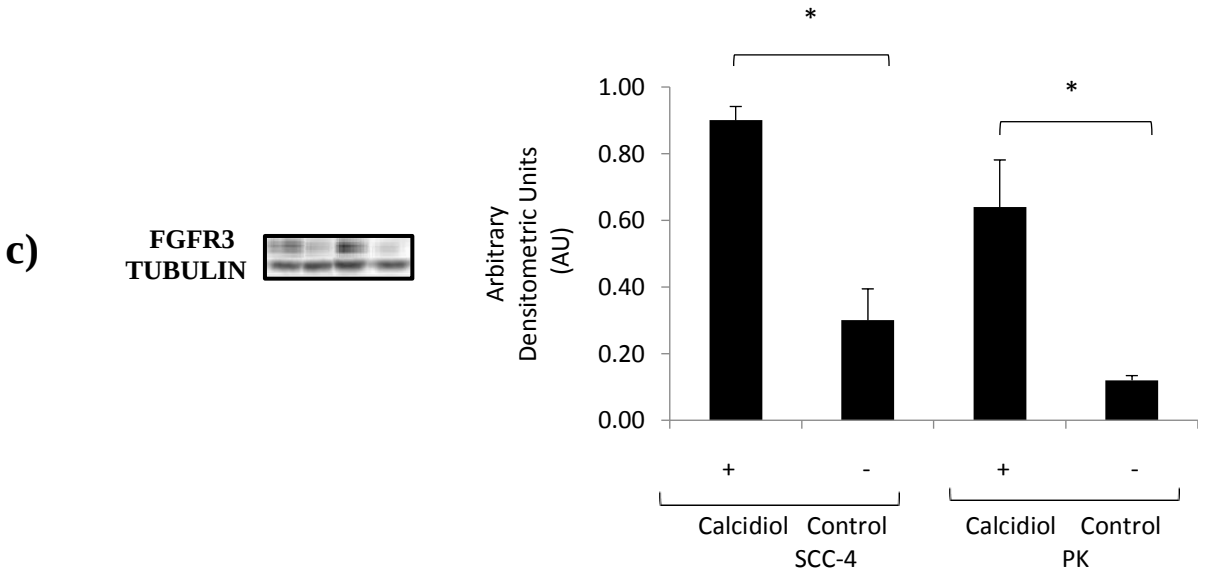


Figure 3.7(c) The effect of calcidiol (250 nM) on FGFR3 protein levels in SCC-4 cells and primary keratinocytes SCC-4 and primary keratinocytes (PK) were treated with 250 nM calcidiol for 72 h. Total protein ran on SDS page gel electrophoresis and transferred to PVDF membrane. Isolated protein was probed with anti FGFR3 antibody, following incubation with corresponding HRP-conjugated secondary antibody. Proteins were visualised using Clarity Western ECL Substrate. Protein bands from western blots were assessed by densitometric scanning of the bands and by statistical analysis of the resulting band intensities. Protein levels were normalised using α-tubulin control. Comparisons between data sets were made using Student's t-test. Data presented as a graph is from a representative of 3 experiments conducted in triplicate. The data represents mean ± SEM of triplicate blots. * indicates $p<0.05$. +, with calcidiol; -, corresponding ethanol control.

3.3.7 Effects of calcidiol and calcitriol on proliferating cell nuclear antigen (PCNA) protein expression in primary and malignant keratinocytes

Proliferating cell nuclear antigen (PCNA) systematic name derived due to its discovery, as a protein was initially detected in the nucleus of dividing cells (Miyachi et.al, 1978; Essers et.al., 2005). PCNA is very well known for its role in DNA replication, particularly in PCNA tethers the polymerase catalytic unit to the DNA template for rapid DNA synthesis (Kelman, 1997). Whilst regulation of PCNA expression by vitamin D metabolites is largely dependent to the cell type and specifications, PCNA protein expression in response to calcidiol and calcitriol in malignant keratinocytes is not clear.

Herein, it is demonstrated that SCC-4 cells and primary keratinocytes treated with calcidiol (100,000 nM) or calcitriol (100 nM) showed no statistically significant difference in PCNA expression compared to their corresponding ethanol controls (Figure 3.8.a,b; Table 3.2).

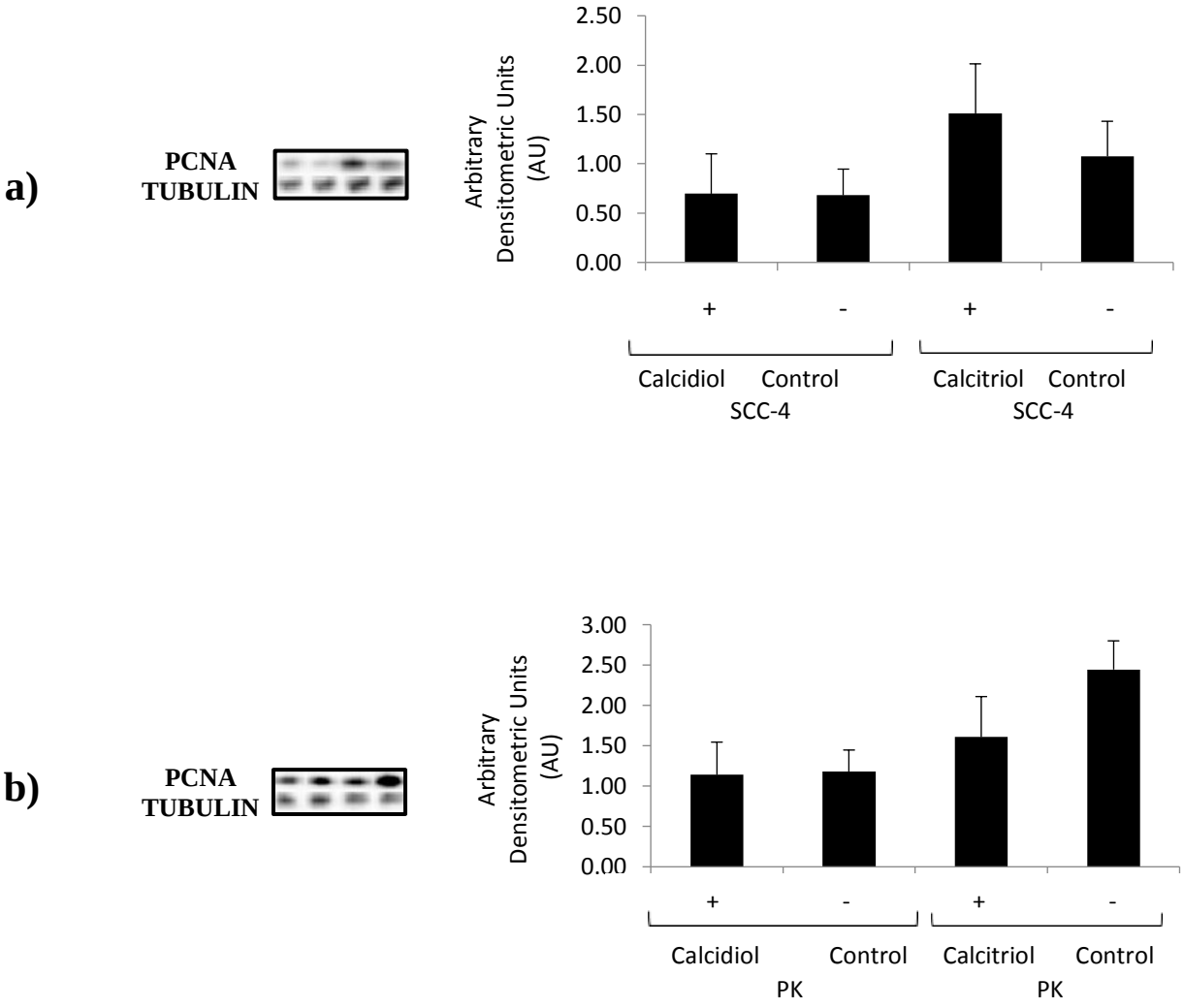


Figure 3.8(a, b) The effect of high dose calcidiol or calcitriol on Proliferative Cancer Antigen (PCNA) protein levels in SCC-4 cells and primary keratinocytes.

a) SCC-4 and **b)** primary keratinocytes (PK) were treated with 100,000 nM calcidiol or 100 nM calcitriol for 72 h. Total protein ran on SDS page gel electrophoresis and transferred to PVDF membrane. Isolated protein was probed with anti PCNA antibody, following incubation with corresponding HRP-conjugated secondary antibody. Proteins were visualised using Clarity Western ECL Substrate. Protein bands from western blots were assessed by densitometric scanning of the bands and by statistical analysis of the resulting band intensities. Protein levels were normalised using α-tubulin control. Comparisons between data sets were made using Student's t-test. Data presented as a graph is from a representative of 3 experiments conducted in triplicate. The data represents mean ± SEM of triplicate blots. * indicates $p<0.05$. +, with calcidiol or calcitriol; -, corresponding ethanol control.

3.4 Discussion

The immunoblotting studies demonstrated that calcidiol (and calcitriol) effects on the expression of vitamin D metabolic enzymes is largely dependent on the metabolite concentration as well as cell specificity (malignant vs normal keratinocytes). Our results also show that these effects are not exclusively controlled via VDR-mediated manner. The effects of calcidiol and calcitriol on the expression of vitamin D metabolic enzymes (CYP27A1, CY27B1, CYP24A1), VDR and tumour driving proteins (EGFR, FGFR3, PCNA) in primary and malignant keratinocytes are summarised in Table 3.2.

Table 3.2. Summary of calcidiol and calcitriol treatments in primary keratinocytes and SCC-4 cells.

	Calcitriol 100 nM		**Calcidiol 100,000 nM**		**Calcidiol 250 nM**	
	SCC-4	PK	SCC-4	PK	SCC-4	PK
CYP27A1	-	-	-	-	-	↑
CYP27B1	↓↓	-	-	-	↑↑	↑
CYP24A1	↑	-	↑	-	-	-
VDR	-	-	-	-	↑	-
EGFR	-	-	-	-	-	-
FGFR3	-	-	-	↓	↑	↑
PCNA	-	-	-	-	N/A	

PK, primary keratinocytes; SCC-4, malignant keratinocytes; (-) no effect observed; (↑) protein upregulation (p<0.05); (↑↑) protein upregulation (p<0.005); (↓) protein downregulation (p<0.05); N/A, not available

3.4.1. The effects of calcidiol and calcitriol on the expression of VDR in human primary keratinocytes and SCC-4 cells.

The results from the immunoblot assays indicate that in cultured human keratinocytes the effects of calcidiol (or calcitriol) are not exclusively modulated via VDR-dependent manner. It was noted that VDR expression is changed only in SCC-4 cells in response to 250 nM calcidiol; without obvious changes in protein expression in primary keratinocytes and SCC-4 cells when treated at higher doses of calcitriol (100 nM) and calcidiol (100,000 nM). It appears that this regulation in response to high concentrations of metabolites may be bypassing VDR-related pathways and may exert antiproliferative effects via alternative molecular signalling. Further studies however are required to confirm this.

These findings did not come as a total surprise, as it was suggested earlier that calcitriol inhibitory effect on cultured keratinocytes is independent of the VDR regulation (Tang et.al., 2011). Similarly, the observed effects of calcidiol and calcitriol may not involve VDR mediated

signal transduction in normal keratinocytes. Whilst the responsiveness of SCC-4 cells in VDR expression could simply be due to malfunction in VDR signalling.

These lower doses showed absence of an antiproliferative effect in malignant SCC-4 cells. Perhaps, this finding may be explained in parts by the alternative mechanism of calcidiol cell entry, such as via heat shock 70kDa protein (HSP70) that was shown in lymphoblastoid cell line (Adams, 1998). It was demonstrated that the affinity of calcidiol for HSP70 is much higher than for VDR (Adams & Gacad, 1998). HSP70 is a member of the molecular chaperone protein family that is present in all eukaryotic cells (Kiang et.al., 1998). In vivo and in vitro studies have shown that various stressors increase production of HSPs as a protection against harmful insults (Kiang et.al., 1998). Increased levels of HSP70 in neoplastic keratinocytes may occur after exposure to calcidiol. Interestingly, it has been reported that cellular uptake of calcidiol requires vitamin D binding protein which may only be true for the kidney, which expresses megalin. Megalin mediated uptake of calcidiol has not been demonstrated in keratinocytes, however in mammary-derived cells megalin (and cubilin) contributes to the cellular calcidiol uptake and activation of the VDR pathway (Rowling et.al., 2006). Since megalin was detected in human keratinocytes of the scalp and hair follicles (Adly et.al., 2010), its may have a role in calcidiol uptake in keratinocytes. The molecular mechanism of calcidiol uptake in keratinocytes should be further investigated as megalin mediated endocytosis occurs in verity of cells and could be useful in better understanding cutaneous vitamin D pathways. These mechanisms of vitamin D metabolic interactions most likely occur in vitro, when cells are treated without the addition of vitamin D binding protein as a vehicle for intracellular delivery of calcidiol, as illustrated in Figure 1.3.

The VDR involvement in inhibitory effects observed in proliferation and migration need to be investigated further, as the results of immunoblot showed the lack of VDR mediatory activity.

3.4.2 The effects of calcidiol and calcitriol on the expression of CYP27A1 in human primary keratinocytes and SCC-4 cells.

In immunoblotting experiments the expression of mitochondrial CYP27A1 was investigated. In primary keratinocytes and SCC-4 cells treated with high concentrations of calcidiol and calcitriol no obvious effects in expression of CYP27A1 were noted; whilst stimulatory cellular responsiveness to 250 nM calcidiol was evident in primary ($p<0.05$), but not malignant keratinocytes. This finding is in agreement with other studies where the expression of CYP27A1 had no effect in human gingival fibroblast cells treated with 1,000 nM calcidiol precursor, cholecalciferol (Liu et.al., 2012). The lack of effect on CYP27A1 expression at higher calcidiol concentrations, could be triggered by the lack in demand in enzymatic activity in both (primary

and malignant keratinocytes) where the abundance of calcidiol substrate results in no effect on cellular expression of CYP27A1.

The effect of calcidiol on CYP27A1 expression is demonstrated for the first time in malignant SCC-4 keratinocytes. These findings suggest that in neoplastic keratinocytes cellular responsiveness in CYP27A1 protein expression to calcidiol and calcitriol treatment is altered. Whilst normal keratinocytes are only responsive at lower calcidiol dose (250 nM), this suggests that higher calcidiol concentration does not affect CYP27A1 expression in keratinocytes.

This lack of effects at high doses was expected due to our earlier findings of VDR protein expression in response to calcidiol and calcitriol. Moreover, it was difficult to predict the CYP27A1 expression in SCC-4 cells due to the aberrant cellular nature in response to vitamin D treatment (Ratnam et.al., 1996).

Meanwhile, it is well known that CYP27A1 plays an important role in catalysis of multiple oxidation reactions in bile acid synthesis in converting dietary vitamin D to active metabolite, mostly microsomal and mitochondrial hydroxylation by several CYP450 hydroxylases (CYP2C11, CYP2D25, CYP3A4, CYP2J1, CYP27A1, and CYP2R1). Whilst, microsomal CYP2R1 was identified to be an important hydroxylase that regulates the process of calcidiol synthesis in human keratinocytes (Cheng et.al., 2004), it was reported that mitochondrial hydroxylation by CYP27A1 represents a driving role in vitamin D metabolism (Endo-Umeda et.al., 2014); where via CYP27A1 activity catabolism of 7-DHC into metabolites that activate nuclear receptor signaling. Interestingly, it was demonstrated that knock down of CYP27A1 in human gingival fibroblasts cells, calcidiol synthesis was significantly decreased, when compared to control cells. However, knock down of CYP2R1 had no effect on calcidiol production (Liu et.al., 2012).

CYP27A1 is an interesting molecular target that may help to establish vitamin D efficacy in cutaneous neoplasm prevention and treatment.

3.4.3. The effects of calcidiol and calcitriol on the expression of CYP27B1 in human primary keratinocytes and SCC-4 cells.

A statistically significant increase in CYP27B1 expression in response to calcidiol (250 nM) treatment in primary and malignant SCC-4 cells was noted; the same effect was not observed in both cells with higher calcidiol concentration (100,000 nM). Therefore, the lack of this negative feedback regulation may explain the increased CYP27B1 protein expression observed in keratinocytes when treated with calcidiol (250 nM), with unexpected withdrawal of apparent effect at higher dose (100,000 nM). The lack of effect in response to higher concentrations may be due to oversaturation of the substrate-enzyme complex and subsequent lack of responsiveness in

CYP27B1 protein expression. Whilst, CYP27B1 expression in human keratinocytes was shown not to be regulated by calcitriol 0.01 – 10 nM concentrations (Xie et.al., 2002). However, decreases in CYP27B1 expression ($p<0.005$) was noted in malignant keratinocytes in response to 100 nM calcitriol treatment, surprisingly without noticeable effects in primary keratinocytes. Inhibition of CYP27B1 expression by 100 nM calcitriol, is supported by earlier observations reported by (Lechner et.al., 2006), where similar effects on expression of *CYP27B1* mRNA in colon, prostate and mammary gland-derived cells were demonstrated.

Although, further studies are required to assess the CYP27B1enzyme kinetic in response to calcidiol in malignant SCC-4 keratinocytes. Extra-renal CYP27B1 protein expression (in both normal and cancer cells) appear not to be regulated by endocrine mediators, such as, parathyroid hormone (PTH) (Young et.al., 2004). Hence, in cultured keratinocytes, the extent of calcidiol conversion to calcitriol was not regulated by the normal feedback system mediated by renal CYP27B1 (Haussler t al., 2010).

3.4.4. The effects of calcidiol and calcitriol on the expression of CYP24A1 in human primary keratinocytes and SCC-4 cells.

As expected, immunoblotting studies demonstrated that expression of CYP24A1 is significantly upregulated in neoplastic SCC-4 cells in response to high concentrations of calcidiol and calcitriol, but not in primary keratinocytes. Hence, calcidiol and calcitriol may exert influence on CYP24A1 protein expression in malignant keratinocytes. It was indicated that CYP24A1 is strongly induced by calcitriol and often serves as a marker of cellular responsiveness to calcitriol (Bikle, 2014).

Moreover, the transcriptional activation of *CYP24A1* mRNA levels induced by calcidiol and calcitriol were shown to be very similar in prostate cells (Munetsuna et.al., 2014). The upregulation of CYP24A1 was reported to be VDR-mediated and dependent on VDR translocation (Munetsuna et.al., 2014). This is indicative of decreases in bioavailable calcidiol degradation by CYP24A1. In SCC-4 cells, higher levels of CYP24A1 could represent a compensatory cellular response to calcidiol and calcitriol treatment, in order to catabolise the potentially toxic substances to neoplastic cells (Jones et.al., 2012).

Surprisingly, upregulation in CYP24A1 expression in response to high concentrations of calcidiol (100,000 nM) in SCC-4 cells, appears not to be dependent on VDR; whilst at lower calcidiol concentration (250 nM), no effects on CYP24A1 expression were apparent. These results indicate that catabolic CYP24A1 may be required in the regulation of malignant keratinocytes and may further contribute to cancer progression by degradation of vitamin D metabolites.

The mechanism of this process remains elusive, as it has not been established whether calcidiol exerts its actions via nuclear, membrane or cytoplasmic VDR actions, or via alternative pathways (Figure 3.9) (Srinivasan et.al., 2011).

3.4.5. The effects of calcidiol and calcitriol on the expression of FGFR3, EGFR and PCNA in human primary keratinocytes and SCC-4 cells.

The results of immunoblot experiments demonstrate that FGFR3 expression is stimulated following calcidiol (250 nM) treatment of primary keratinocytes and SCC-4 cells. Contrastingly, the lack of responsiveness to calcidiol at higher concentrations was noted in SCC-4 cells. However, inhibition of tumour promoting protein FGFR3 expression was observed in primary keratinocytes treated with 100,000 nM calcidiol. This suggests that in primary keratinocytes calcidiol exerts its actions via molecular mechanism that is impaired in SCC-4 cells.

Interestingly, calcitriol 100 nM had no effect on FGFR3 expression in primary or malignant keratinocytes. This is indicative that calcidiol (100,000 nM) inhibition of FGFR3 expression in primary keratinocytes mediated via a different pathway compare to SCC-4 cells (possibly, without involvement MAPK classical signalling). However, this needs to be further investigated. To the student's knowledge, the effects of calcidiol on FGFR3 have not been previously described in highly metastatic SCC-4 keratinocytes and compared to primary epidermal keratinocytes.

FGFR3 is well recognised for its involvement in underlying mechanisms of tumour initiation, growth and migration in number of cancers (Zhou et.al., 2015). Paradoxically, it was suggested that low FGRR3 expression has an association with better survival in patients with urothelial bladder cancer (UBC) (Amaral et.al., 2012). Where it was suggested, that mutations in FGFR3 have been identified as a positive factor in cancer survival, it is not surprising that increases in mutated FGFR3 expression led to decreased tumour survival. However, this study also indicated that the overexpression and FGFR3 mutation also associated with better patient outcome, and low levels of circulatory calcidiol are indicative of a high risk of more aggressive forms of UBC. Conversely, in pancreatic cancer it was found that high FGFR3 nuclear expression is associated with poor overall and disease-free survival (Zhou et.al., 2015).

Furthermore, the influence of calcidiol and calcitriol on tumour modulatory markers EGFR and PCNA were also examined. It was apparent that both metabolites (calcidiol and calcitriol) had no influence on the EGFR and PCNA protein expression in primary and malignant keratinocytes. This is indicative that vitamin D mediated pathways may have alternative mechanisms of action.

Moreover, using an animal model (Sprague-Dawley rats) of advanced renal failure the mechanisms parathyroid hyperplasia, combined calcitriol/anti-EGFR tyrosine kinase inhibitors

(TKI) therapy showed effectively to inhibit parathyroid hyperplasia (Dusso et.al., 2004). Where 100 nM calcitriol (as well as non-antiproliferative calcitriol doses) was shown to potentiate anti-EGFR TKI therapy. It was also demonstrated that calcitriol-induced decreased proliferation was corresponding to decreases in PCNA protein expression in these cells. In contrast, PCNA protein expression examination has shown lack of apparent changes in primary and malignant keratinocytes. This corresponds to other published literature, suggesting the lack of PCNA responsiveness to calcitriol in keratinocytes (Penneys et.al., 1994; Einspahr et.al., 1999; Moor et.al., 2004).

All together, these finding warrants further investigations of the molecular events associated with calcidiol and calcitriol interactions with MAPK signalling pathways and its influence on EGFR, FGFR3 and PCNA.

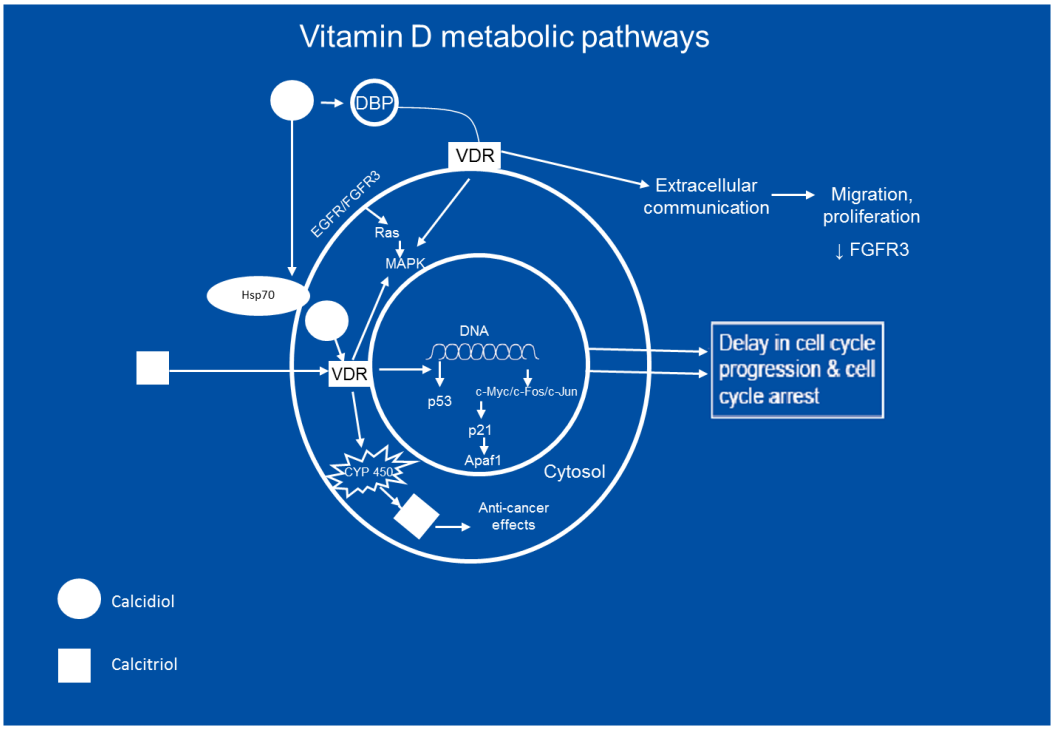


Figure 3.9. Proposed schematic representation of calcidiol and calcitriol signalling.

Calcidiol or calcitriol enters the cell via HSP70 (or via the classical pathway via VDR) following mitochondrial multistep CYP450 hydroxylation. The conversion of calcidiol to the active hormone, calcitriol resulting in antiproliferative, apoptotic and anti-migratory actions in neoplastic cells via modulation of oncogene expression (c-Myc, c-Fos, c-Jun), resulting in activation of p21, which regulates the cell cycle, and Apaf1, which controls cell death. Calcidiol and calcitriol also play a role in the regulation of extracellular communication. Calcidiol regulates the MAPK pathway via direct cytosolic signalling. Three cellular VDRs are expressed in keratinocytes: cytosolic, peripheral and nuclear receptors. The mechanism of VDR activation in neoplastic keratinocytes has never been established experimentally. HSP70 appears to be another channel for calcidiol intracellular delivery (Bachelor & Bowen, 2004; Barsony et.al., 1997; Huhtakangas 2004; Rossi et.al., 2004; Yadav et.al.,2012); KEGGS Pathway Database (<http://www.genome.jp/kegg/pathway>, viewed January 2016).

3.5 Conclusions

In summary, the findings demonstrate:

1. For the first time in one study the effects of vitamin D metabolites (calcidiol and calcitriol) on expression of vitamin D metabolic enzymes, VDR and tumour driving proteins in primary and malignant SCC-4 keratinocytes
2. It was shown that primary and neoplastic cells exhibit selective sensitivity to alteration in vitamin D enzymes (particularly, CYP27B1 and CYP24A1) in response calcidiol and calcitriol treatment. To the authors knowledge this is the first time the changes in CYP27B1 and CYP24A1 protein expression are shown in response to calcidiol in SCC-4 cells.
3. It was evident that EGFR and PCNA expression are not affected by calcidiol or calcitriol.
4. We demonstrate calcidiol dose dependent effects in primary and malignant keratinocytes, where at lower concentration (250 nM) stimulation in FGFR3 expression was noted in both cell types. Whilst at higher calcidiol dose (100, 000 nM) an inhibitory effects was observed only in primary keratinocytes. This finding suggests that calcidiol may exert a suppressive effects on tumour promoting markers at high concentrations and the mechanism of this inhibition can have a role in development of therapeutic agents against neoplastic formation.
5. These data may indicate that antiproliferative effects of calcidiol in SCC-4 keratinocytes do not involve EGFR/FGFR3 modulation in MAPK pathway signalling. The antiproliferative effects (observed in chapter 2) could have an alternative molecular mechanism excluding MAPK. Further investigations are required to understand the molecular interactions of vitamin D metabolites in neoplastic and primary keratinocytes

CHAPTER 4.

VITAMIN D METABOLIC ENZYMES AND RECEPTOR IN NON-MELANOMA SKIN CANCERS: A ROLE IN THE DERMAL–EPIDERMAL RELATIONSHIP

4.1 Introduction

The relationship between intacrine vitamin D metabolic enzymes status and non-melanoma skin cancers (NMSCs) is not entirely clear. There are some inconsistencies and contradictory reports regarding circulatory calcidiol levels and NMSC: whilst some studies show beneficial effects of high circulatory calcidiol levels, others present contrary observations (Tang et.al., 2010; Afzal et.al., 2013). Limited clinical studies and several in vitro reports indicate a connection between vitamin D metabolic key players and NMSC by demonstrating inhibitory effects on tumour cells and positive effects of higher circulatiory vitamin D (calcidiol) in skin cancer prevention (Satake et.al., 2003; Akutsu et.al., 2001; Gibson et.al., 1998; Gedlicka et.al., 2006; Tang et.al., 2010; Köstner et.al., 2012; Liang et.al., 2012).

The role of vitamin D metabolites and its tumour suppressive effects has been shown in a number of cancer types, such as breast, colorectal, endometrial and lung cancers (de Lyra et.al., 2006; Spina et.al., 2007; Zhang et.al., 2007; Ramirez et.al., 2009; Guan et.al., 2013; Bergada et.al., 2014). Limited reports on the relationship between some types of skin cancers and vitamin D metabolites, indicate a need for further investigation of vitamin D metabolic enzymes and their receptor expression in NMSC (Köstner et.al., 2012; Tang et.al., 2010). It was reported that decreased circulatory calcidiol inversely associated with the risk of NMSC (Tang et.al., 2010). In addition, CYP27B1 expression is reduced in more advanced melanoma skin lesions, and there is an inverse association with proliferative marker Ki-67 expression. It was shown that lack of CYP27B1 is linked to decreased melanoma aggressiveness in humans and plays a role in disease pathogenesis and progression (Brożyna et.al., 2014a). Surpisingly, authors also report a positive correlation between CYP27B1 expression with that of CYP24A1 and VDR (Brożyna et.al., 2014b). This may be explained, in part, by upregulation of CYP24A1 by calcitriol (the product of CYP27B1 activity) via VDR activation (Jones et.al., 2011). However, if this is so, the lack of both (CYP27B and CYP24A1) expression with decreased free survival is hard to comprehend. Hence, the role of vitamin D metabolism in cutaneous carcinogenesis is still poorly understood. There is a clear demand for further studies to examine the expression of vitamin D metabolic enzymes in NMSC.

Vitamin D synthesis is mediated via mitochondrial cytochrome P450 family hydroxylase enzymatic reactions: anabolic and catabolic hydroxylases (CYP27A1, CYP27B1 or CYP24A1).

There have been reports showing the importance of vitamin D enzymes and their receptors, however their role in NMSC is limited (Bergada et.al., 2014; de Lyra et.al., 2006; Spina et.al., 2007; Jiang et.al., 2014, Zhang et.al., 2007; Ramirez et.al., 2009; Guan et.al., 2013). Low circularoty calcidiol levels have been linked to decreases in VDR acivity in endometrial cancers. Increase of intratumoural calcidiol was proposed to be due to an increase in CYP27A1 expression and increased VDR-antiproliferative actions (Bergada et.al., 2014). Interestingly, it was shown that lower circulatory calcitriol (but not calcidiol), was found in breast cancer patients (de Lyra et.al., 2006). It was also shown that CYP27B1, CYP24A1 and *VDR* mRNA were found to have the same level of expression of mRNA in breast tumours and normal mammary tissue (de Lyra et.al., 2006). Futhermore, the importance of VDR in skin cancer development was demonstrated in mouse keratinocytes. Deletion of VDR resulted in decreased tumor suppressor genes (*H19, HOTTIP* and *Nespas, Kcnq1ot1, lincRNA-p21, Foxn2-as, Gtl2-as, H19-as*) and increased expression of oncogenes (*mHOTAIR, Malat1* and *SRA*) (Jiang et.al., 2014). As discussed in Chapter 3, VDR and vitamin D metabolic enzymes interact with tumour regulatory proteins in malignant cells thereby influencing tumour formation, growth and preventing angiogenic spread (Garland et.al., 2009; Vanoirbeek et.al., 2011). Tumour promotion and progression is strongly connected to VDR and its metabolic enzymes.

Vitamin D hydroxylases may play an important role in tumourigenesis. Their functions are expanded far beyond vitamin D synthesis and degradation. CYP27A1 induction has been observed in human prostate epithelial cell lines in response to the vitamin D precursor, cholecalcifrol, treatment, where inhibition of the cellular changes associated with malignant transformation and invasion was detected (Tokar and Webber, 2005). Reduced enzymatic activity of activating the vitamin D enzyme, CYP27B1 (encoded by *CYP27B1*) is noted in aggressive types of melanomas (Brożyna et.al., 2013). More recently the crucial role of CYP27A1 in modulation of VDR activation suggest that CYP27A1 plays a vital role in cancinogenesis (Endo-Umeda et.al., 2014).

NMSCs can be seen as a spectrum of skin pathologies, with neoplastic formation with or without signs of inflammation (Emoto & Nakamura, 2008). Human skin consists of two tissue layers, namely a keratinised stratified epidermis and an underlying thick layer of collagen-rich dermal connective tissue. The dermal layer is mainly composed of an extracellular connective tissue matrix, and plays an important role in tumour formation (Staiano-Coico & Higgins, 1992; Teichert et.al., 2011). The initiation of skin tumours is associated with low serum levels of calcidiol (Oberyszyn, 2008; Tang et.al., 2010). Furthermore, calcitriol has inhibitory effects on

tumour promoting genes in various cells, including keratinocyes (Chantalvan et.al., 2004; Palmer et.al., 2008; Tokar & Webber 2005a).

Indeed, calcitriol can inhibit growth of malignant cells both in vitro and in vivo (Xie et.al., 1999; Ben-Shoshan et.al., 2007; Biauer et.al., 2009; Vinceti et.al., 2011; Song et.al., 2013a). Interestingly, treatment with calcitriol is able to protect keratinocytes against solar UV radiation-induced mutagenesis, which is considered to be the principal cause of cutaneous neoplasm formation (Trémezaygues et.al., 2010).

The preventative effects of high endogenous vitamin D levels on the development of neoplastic growth and its aggressiveness has been suggeted by several studies (Tang et.al., 2010; Gugatschka et.al., 2011). Vitamin D receptor regulatory role in cancer is shown in numerous reports, including suppression of oncogenes, induction of tumour suppressor genes, inhibition of proliferation and migration of malignant cells, prevention of metastasis and angiogenesis (Jiang et.al., 2013; Köstner et.al., 2012; Haussler et.al., 2011; Lopes et.al., 2010; Essa et.al., 2010; Chung et.al., 2009; Leyssens, 2013).

It is hypothesised that VDR and vitamin D metabolic enzymes that are involved in synthesis and degradation of vitamin D may be involved in the regulation of the pathophysiological progression of cutaneous tumourigenesis.

The main goal of this study was to analyse vitamin D metabolic enzymes (CYP27A1, CYP27B1 and CYP24A1) expression and VDR in AK, BCC, SCC, SCCIS and normal human skin. In addition, the expression of tumour-driving proteins, such as EGFR, FGFR3, EGF, FGF3, VEGF and PCNA was examined.

4.2 Materials and methods

4.2.1 Tissue samples

NMSC (actinic keratosis (AK), basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and squamous cell carcinoma in situ (SCCIS)) specimens were provided from the archival collection of the Department of Dermatology, St Vincent's Hospital, Fitzroy, Australia, with the support of Professor Rod Sinclair. Specimens were prepared at the HistoLab Laboratory, Melbourne, Australia, with the assistance of a certified pathologist, Dr Andrew Ryan. Normal skin tissue sections were obtained from commercial sources with consideration of age and skin colour (Resolving Images Pty Ltd., Victoria, Australia). Ethics approval was obtained from Department of Dermatology, St Vincent Hospital and Victoria University, Melbourne, Australia.

The characteristics of the tumour specimens and patient demographics are shown in Table 4.1: 20 BCCs (n = 14), 15 AKs (n = 12), 12 SCCIS (n = 9), 8 SCCs (n = 8) and normal skin (n=5) from patients aged 41–93 years.

Table 4.1. Specimen characteristics.

	AK		**SCCIS**		**BCC**		**SCC**		**Normal skin**	
	Male	**Fem**	**Male**	**Fem**	**Male**	**Fem**	**Male**	**Fem**	**Male**	**Fem**
Tumour specimens (number)	15		12		20		8		5	
Patients (number)	6	6	4	5	13	1	7	1	3	2
Patient mean age (years)	62	68	72	73	68	72	60	64	51	39

The original dermatopathology reports were reviewed to verify the diagnosis. All tumours were coded; the patient's age and sex were provided after immunohistochemical analysis of the sections. The patient number does not always match the tumour specimen number, as some tumours were obtained as a result of multiple excisions from the same patient. Tumour tissue sections were compared to sections of AK, a condition well known as a precursor for BCC and SCC. Additionally, protein expression of normal skin tissue samples were used as a secondary control in these experiments.

4.2.2 Reagents

Biotinylated universal antibody streptavidin–biotin (LSAB+) System–HRP **(**K4063) and diaminobenzidine (DAB) were purchased from Dako, Australia.

Xylene, histological grade (FSBX 0250/17), Myer's Haematoxylin Solution (FNNII007) and DePex mountant (FNII023) were obtained from Lomb Scientific, Australia.

The primary unconjugated antibodies were obtained from Santa Cruz Biotechnology, USA and used at the optimised dilutions (1:1,000). The antibodies were supplied at the amount of 200 µg and with the specifications as described in Table 4.2

Table 4.2 Specificity of primary antibodies

Primary antibody	Size of protein	Dilution Factor	Specificity	Cross-reactivity	Product code Manufacturer
Polyclonal goat anti-human CYP27A1 (IgG)	60 kDa	1:100	Recognises epitope mapping near the N-terminus of CYP27A1	Human, mouse, rat	(P-17; sc-14835), Santa Cruz Biotechnology, USA
polyclonal rabbit anti-human CYP27B1(IgG)	56 kDa	1:100	Recognises amino acids 221-310 mapping within an internal region of CYP27B1	Human, mouse, and to a lesser extent rat, porcine	(H-90; sc-67261), Santa Cruz Biotechnology, USA
polyclonal rabbit anti-human CYP24A1 (IgG)	65 kDa	1:100	Recognises amino acids 351-437 mapping near the C-terminus of CYP24	Human, mouse, rat	(H-87; sc-66851), Santa Cruz Biotechnology, USA
polyclonal rabbit anti-human VDR (IgG)	53 kDa	1:50	Recognises amino acids 344-424 of VDR	Human, mouse, rat	(H-81; sc-9164), SantaCruz Biotechnology, USA
polyclonal rabbit anti-human EGFR (IgG)	170 kDa	1:50	Recognises a peptide mapping at the C-terminus of EGFR	Human, mouse, rat	(1005; sc-03), SantaCruz Biotechnology, USA
polyclonal goat anti-human EGF	6 kDa	1:100	Recognises a peptide mapping at the C-terminus of EGF	Human, mouse, rat	(sc-1343), SantaCruz Biotechnology, USA
polyclonal rabbit anti-human FGFR3	97 kDa	1:50	Recognises an epitope mapping at the C-terminus of FGFR3	Human, mouse, rat	(C-15,sc-123), SantaCruz Biotechnology, USA
polyclonal goat anti-human FGF3	31 kDa	1:100	Recognises an epitope mapping at the N-terminus of FGF3	Human, mouse, rat	(sc-33962), SantaCruz Biotechnology, USA
polyclonal goat anti-human VEGF (A-20)	42 kDa	1:100	Recognises a peptide mapping at the N-terminus of VEGF-A	Human, mouse, rat	(sc-152), SantaCruz Biotechnology, USA
monoclonal mouse anti-human PCNA (IgG2a)	36 kDa	1:100	^{35S}-methionine-labelled extract of CV-1 cells (an immortalised cell line of monkey kidney epithelial cells) reacts primarily with a polypeptide corresponding to PCNA protein	Human	(PC10; M0879) Dako, Australia

The secondary antibodies: polyclonal rabbit anti-goat Ig (1:500) and polyclonal goat anti-rabbit Ig (1:500) polyclonal were obtained from Dako, Australia.

4.2.3 Tissue preparation

10 % formalin-fixed, paraffin-embedded block specimens of AK, BCC, SCC, SCCIS and normal skin tissue were sectioned using a rotary microtome at 3 μm. The tissue sections were transferred to a flotation bath of distilled water (dH_2O) at 50 ºC, followed by fixation onto glass slides (Superfrost (+); Thermo Scientific, Australia). Tissue sections were affixed to the slides by further incubation overnight at 60 ºC.

Tissue samples were dewaxed using organic solvent (xylene) and a series of graded ethanol incubations (100 %, 70 % and 30 %).

4.2.4 Immunohistochemical detection of vitamin D metabolic enzymes and receptor expression in human BCC, SCC, SCCIS, AK and normal tissues

Antigen retrieval was performed with 10 mM sodium citrate buffer, pH 6.0, for 10 min at 95 ºC. Tissue endogenous peroxidase activity was blocked using 3 % hydrogen peroxide and sections were blocked with 10 % bovine serum albumin (BSA) for 1 h at room temperature. Samples were incubated with the primary antibody diluted in 10% BSA/PBS. For negative control sections, the primary antibody step was omitted. Biotinylated universal antibody (Labelled Streptavidin Biotin (LSAB) + System– Horseradish Peroxidase (HRP)) was used as a secondary antibody. Subsequently, samples were treated with streptavidin conjugated to HRP at room temperature. To visualise staining, sections were incubated with the substrate-chromogen solution 3, 3 – diaminobenzidine (DAB). Tissues were counterstained in haematoxylin, followed by a series of washes with ethanol (30 %, 70 %, 100 %) and xylene. Slides were coverslipped with DePex mountant.

4.2.5 Assessment of immunostaining and statistical justification

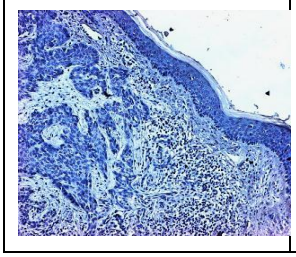
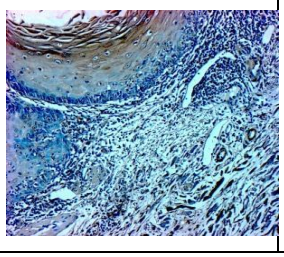
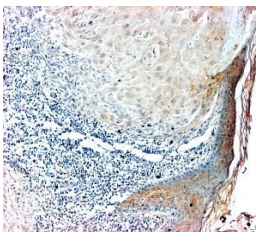
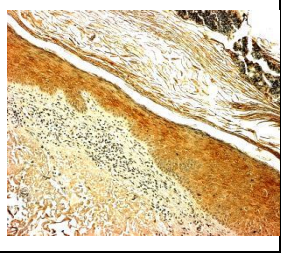
All stained sections were reviewed independently by 2-3 investigators. Staining intensity was graded as poor, moderate or strong. This scoring system was adapted as previously described (Table 4.2) (Atkins et.al., 2004; Zhou et.al., 2014).

In the optimisation experiments for immunohistochemical (IHC) detection of VDR protein, use of the monoclonal antibody sc-13133 was not very successful. The decision was made to use a broader spectrum polyclonal antibody that would provide a better signal of the target protein, even though on occasion there was some non-specific labelling.

It is important to establish here the definitions of tumour and tumour stroma. As in many tissues, the stromal cells in tumours are non-malignant cells, including fibroblasts and numerous types of immune cells, which are intermixed with tumour cells. In the past century, the seed and the soil hypothesis has given rise to a more comprehensive current understanding of the malignant

tumour cell (seed) and the surrounding microenvironment (soil) (Bremnes et.al., 2011). The stromal environment affects the progression of tumours and helps determine the aggressiveness of malignant cancers. It was shown that stromal fibroblasts can enhance tumour cell motility by stimulating the NF-kB pathway and activating MMP-9 (Ikebe et.al., 2004). Hence, the stroma is made up of the non-malignant cells surrounding the tumour, such as carcinoma-associated fibroblasts, epidermal mesenchymal cells, innate and adaptive immune cells and vascular endothelial cells, as well as the extracellular matrix structural proteins: collagen, fibrillin, fibronectin, elastin and proteoglycans (Bremnes et.al., 2011).

Table 4.2. Protein intensity evaluation, according to the score of DAB development in tissue sections.

Level of protein expression			
Negative control (Not expressed)	Poor (low)	Moderate	High
No staining (less than 0.5%) visible on the tissue section	Less than 5 % of cells in the tissue stained	Approximately 5–10% of the tissue stained	More than 10% of the tissue stained very extensively
A) 	B) 	C) 	D) 

The neoplastic skin lesions are representatives of the staining intensity scoring system for protein expression:
A) Negative control or non-specific staining, where the DAB stain was not developed due to an absence of primary antibody.
B) Poor/low staining intensity, where less than 5 % of tissue took up the stain, and staining is weak and incomplete.
C) Moderate staining intensity, where 5 – 10 % of tissue is stained.
D) High staining intensity, where more than 10% of tissue is stained.

DAB reagent was applied to all experimental tissues, resulting in various degrees substrate–chromogen development. (As per protocol of IHC detection by the immunoperoxidase/DAB system; see Appendix G for details.)

4.2.6 Statistical justification

The relationship between vitamin D metabolic proteins and tumour modulatory protein expression in tumour sections and normal skin was determined by IBM Statistical Package for the Social Sciences (SPSS) Statistics software using Multivariable Cross-tabulation Chi-Square test. The correlation was considered statistically significant with $p<0.05$. Only the key correlative data was included in the thesis. Extensive analysis of this data has been calculate and will be available on request.

4.3 Results

4.3.1 Evaluation of vitamin D metabolic enzymes and VDR expression in human tissue samples of AK, BCC, SCC, SCCIS and normal skin

The immunohistochemical experiments were conducted using cancerous and normal skin tissues. Of note, donor' age and gender of the commercially prepared normal tissues were matched to human tumour tissues. Unfortunately, some of the normal tissue were lost during the staining process during the multiple serial washes, leading to reduction in the sample numbers. Despite a decrease in the sample numbers the normal tissue immunoreactivity of vitamin D metabolic enzymes and receptor was compared to precancerous and cancerous skin lesions.

Staining of NMSC sections showed the expression of vitamin D enzymes (CYP27A1, CYP27B1 and CYP24A1) and VDR protein dependent on the cell location (predominantly in epidermal keratinocytes) and type in normal, precancerous and cancerous human skin.

CYP27A1 detection in human NMSC demonstrated differential staining intensity in epidermal keratinocytes and dermal fibroblasts. More intense staining was observed in keratinised layers, with moderate (AK and SCCIS) to high (SCC and BCC) expression observed in epidermal keratinocytes (Figure 4.2). Interestingly, high staining intensity of CYP27A1 was also observed in normal skin. Similarly, IHC detection of vitamin CYP27A1, has been shown in colon cancer (Matusiak & Benya, 2007), endometrial carcinomas and normal endometrial tissues (Bergada et.al., 2014).

The detection of CYP27B1 (CYP27B1) cutaneous tumours revealed minimal staining intensity in the tissues, indicating decreased level of expression of CYP27B1 in the majority of NMSC (Figure 4.3). In contrast, CYP27B was highly expressed in normal skin (Figure 4.3).

The expression of catabolic CYP24A1 expression in normal skin samples was limited. It has been shown that normal skin has limited expression of the catabolic CYP24A1. Contrary, CYP24A1 is up-regulated in all neoplastic skin lesions. Protein expression is observed predominantly in epidermal keratinocytes, with strong expression in neoplastic cells (Figure 4.4). Weaker 24-hydroxylase expression was noted in AK. These observations are supported that in melanoma tissues, where normal skin has diminished CYP24A1 expression, compared to melanoma affected skin (Brożyna et.al., 2014). In these studies there was no correlation amongst staining intensity and melanoma aggressiveness, however it was shown that elevated levels of CYP24A1 are associated with melanoma progression at early stages of disease.

VDR expression was detected in all cutaneous tumours and normal skin (Figure 4.5). This supports earlier reports on VDR detection in some types of skin tumours (BCC and SCC) (Mitschele et.al., 2004; Reichrath et.al., 2004).

Interestingly, the infiltration with immunoregulatory cells was clearly observed in the areas of neoplastic keratinocyte localisation in SCC, and around blood vessels in SSC, BCC and SCCIS, which is shown in the presented images, where applicable. This is well documented and an expected feature of cutaneous tumours (Freeman et.al., 2014). Lower amount of immune cell infiltration in SCC was compared with abundant infiltration of the immune markers of intra epidermal carcinomas (Freeman et.al., 2014). Abundant immune cell infiltrates are involved in critical contribution in tumour responsiveness to treatment. It was indicated that the infiltrative immune markers (particularly, ratio of CD4+ T-cells to CD8+ T-cells) can be used as an indicator of invasive tumors, such as SCC, development and further SCC progression (Freeman et.al., 2014).

Prior to highlighting details of neoplastic cutaneous tissue, it is important to display the quality of normal skin. The definition of the epidermis and dermis, with clearly intact basal membrane without poorly differentiated keratinocytes. The normal skin tissue is demonstrated in Figure 4.1.a).

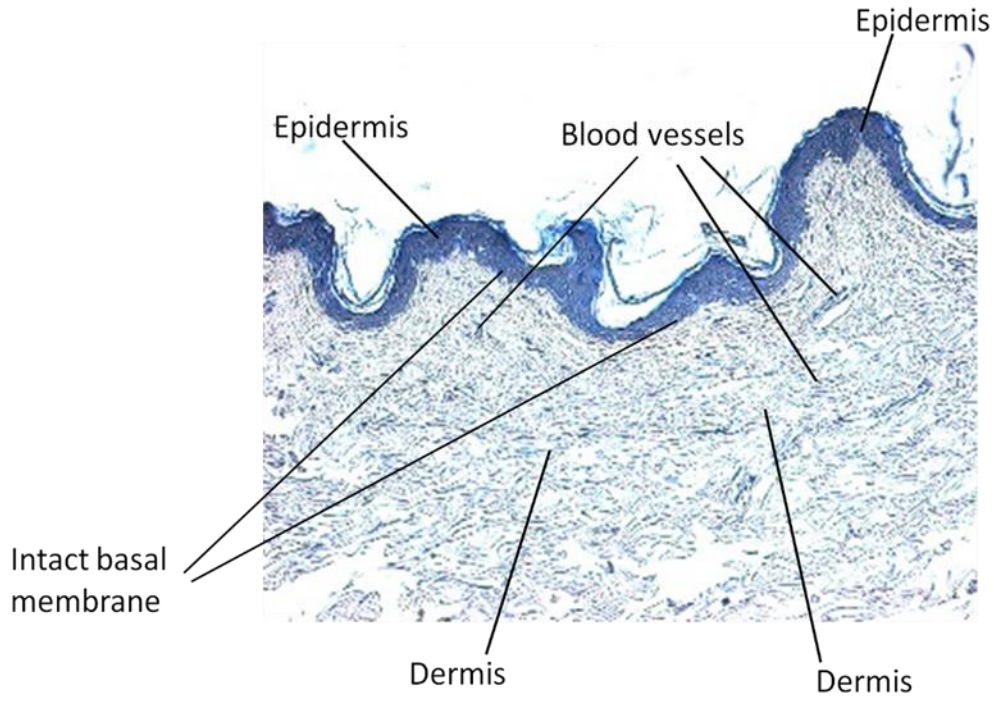


Figure 4.1(a) Representative image of normal human skin

Keratinocytes of the epidermal layer can be seen within the border of stratum basale (basal membrane); the progeny keratinocytes (or epidermal mesenchymal cells) that reside in the basal membrane of the epidermis have essential regenerative qualities, that keeps epidermal layer regeneration from the bottom of the basal membrane, while the top layer of epidermal cells are constantly being 'sloughed' off the body.

It is also important to establish the tumour structural characteristics, so that the detection of abnormal structural features will be possible. In healthy skin, the keratinocytes of the basal membrane do not invade the dermal structural compartments, but reside strictly in the epidermal layer (Koster, 2009). When apoptosis-resistant keratinocytes invade the dermal layer by disrupting the basal membrane, the formation of a tumour can be seen in the various tissue samples (Melnikova, 2005). The image shown in Figure 4.1(b) is representative of this process.

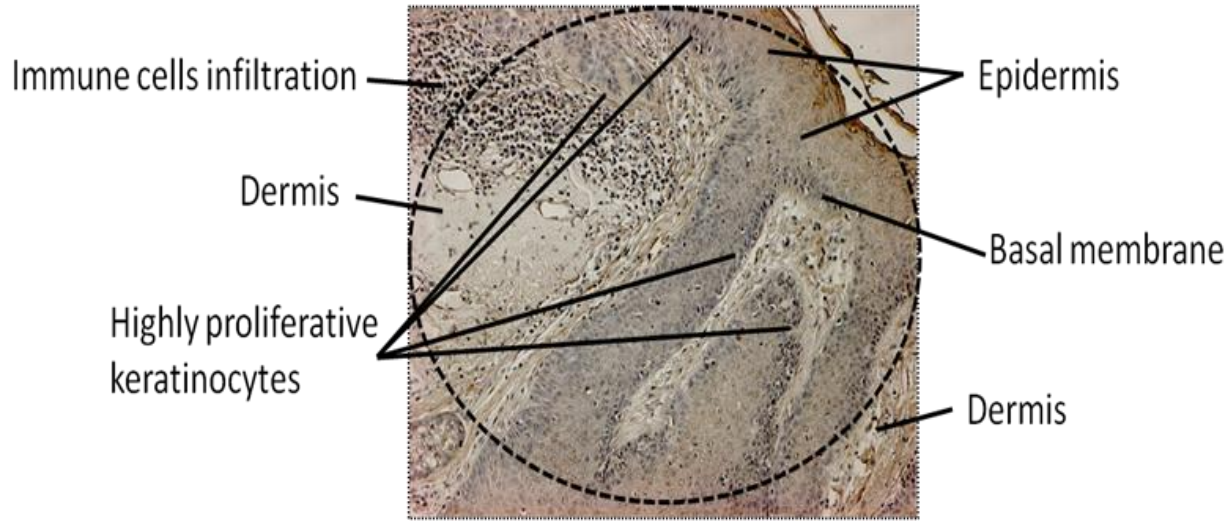
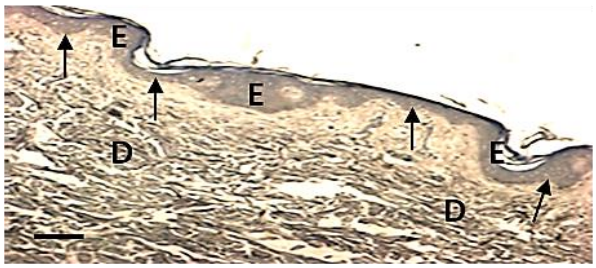
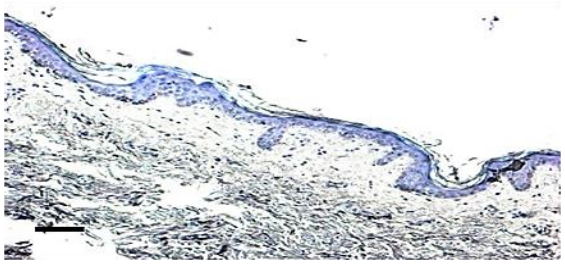


Figure 4.1(b) Representative image of an invasive SCC tumour.

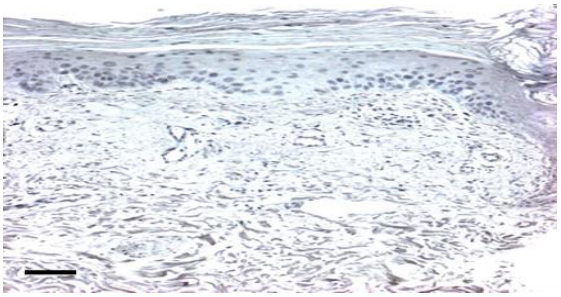
Highly proliferative keratinocytes can be seen in the dermal layer; the progeny keratinocytes (or epidermal mesenchymal cells) that reside in the basal membrane of the epidermis have essential regenerative qualities. The nucleus of basal membrane keratinocytes appears enlarged due to cell division, and these cells are quite prominent along the stratum basale. This keeps the epidermis regenerating from the bottom epidermal basal membrane, while the top layer of epidermal cells are constantly being 'sloughed' off the body. However, mutated apoptosis-resistant keratinocytes can invade the dermal layer and form a cluster of uncontrollably dividing cells (a tumour) in any cutaneous area that is not specifically for epidermal regeneration, and in cases of malignant SCC extend beyond cutaneous tissue. The tumour stroma (circled area) requires a mixture of cells that may include healthy and mutated cells, as well as lymphocytes, neutrophils, macrophages, and many other immune regulators. The stromal cells of the dermal layer are adjacent to the epidermal layers of the skin, and produce a range of growth factors that stimulate tumour cell division.

A) NSK

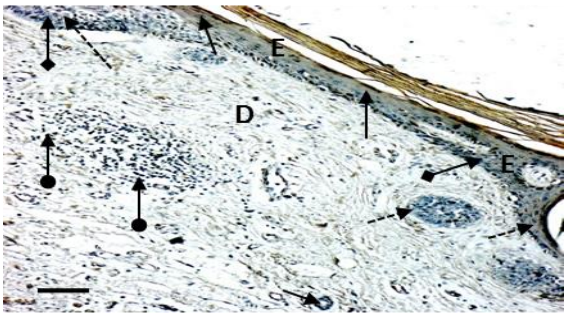


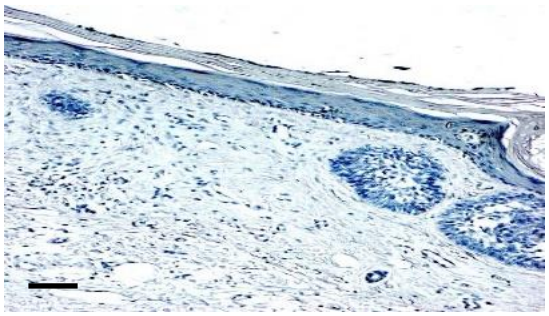


B) AK

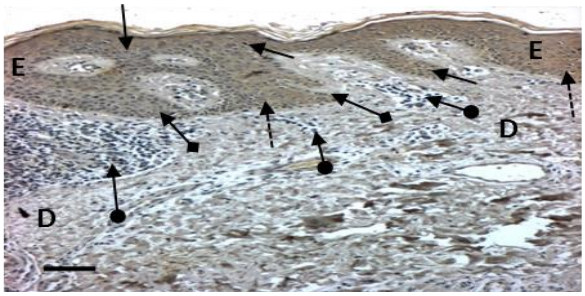


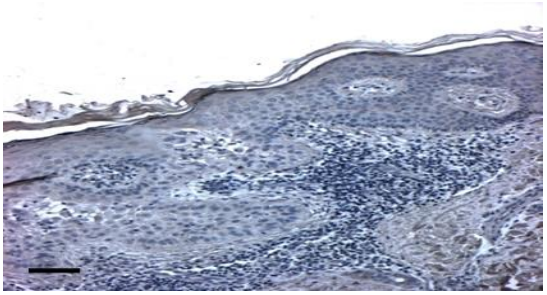
C) BCC



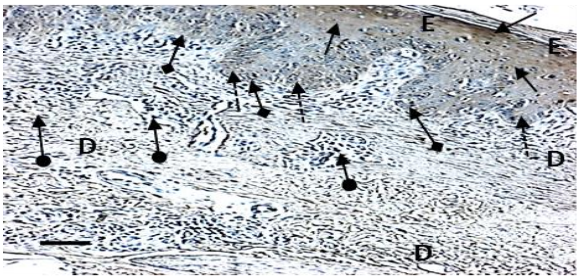


D) SCC





E) SCCIS



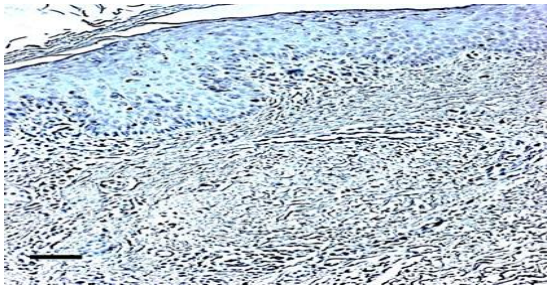


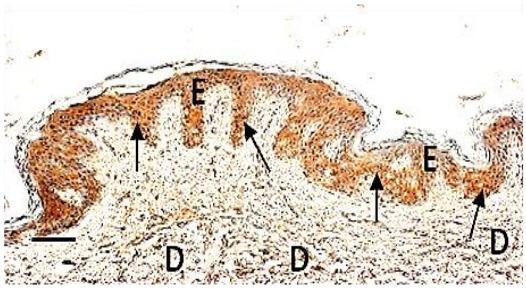
Figure 4.2. Immunohistological detection of vitamin D CYP27A1 expression in human AK, BCC, SCC, SCCIS and normal skin.

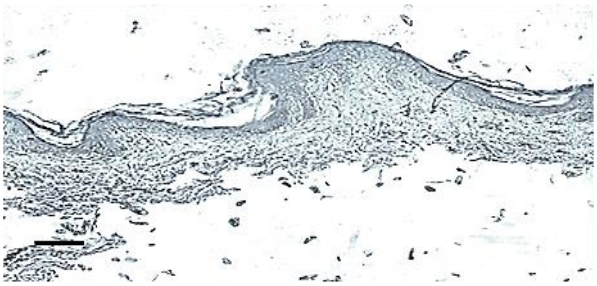
The left panel shows IHC detection of vitamin D CYP27A1 in human A) NSK (normal skin), B) AK, C) BCC, D) SCC and E) SCCIS. The right panel is a negative control that was stained without the primary antibody and corresponds to the tumour shown in the left panel. Disruptions of the basal membrane by neoplastic keratinocytes are indicated with 'diamond-ended' arrows. Immune cell infiltration is shown with 'circled-ended' arrows. The positively stained neoplastic, premalignant and normal keratinocytes are shown with solid black arrows and are indicative of the tissue staining intensity described in Table 4.2. Poorly differentiated keratinocytes are shown with black dashed arrows, where applicable. Scale bars: 20 µm.

E, epidermal layer; D, dermal layer.

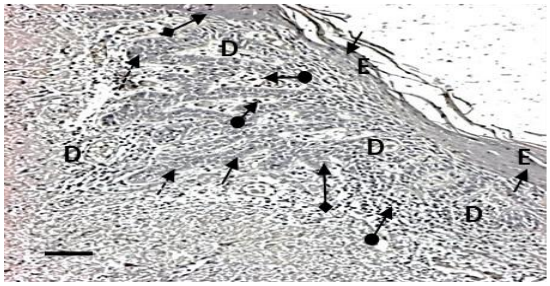
Stromal keratinocytes showed lower expression of CYP27A1 in BCC and SCCIS sections, whereas SCC had high levels of CYP27A1 protein expression. Due to the use of polyclonal antibodies that have a wider binding capacity, some negative control tissue developed negligible non-specific staining.

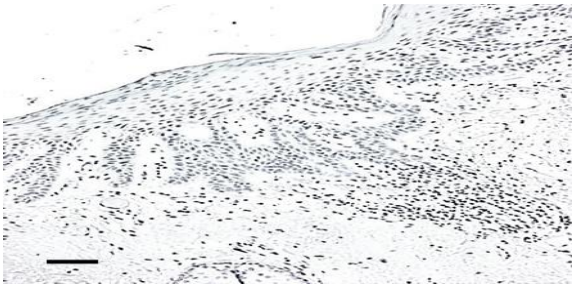
A) NSK



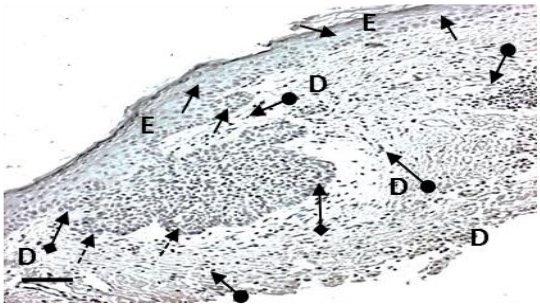


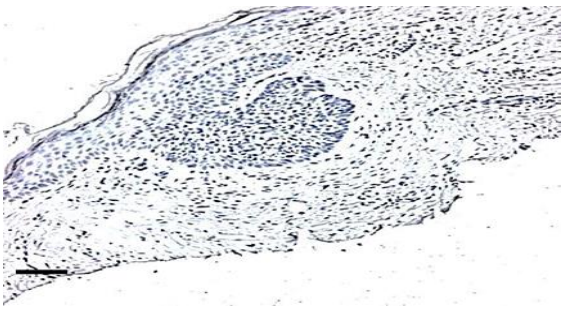
B) AK



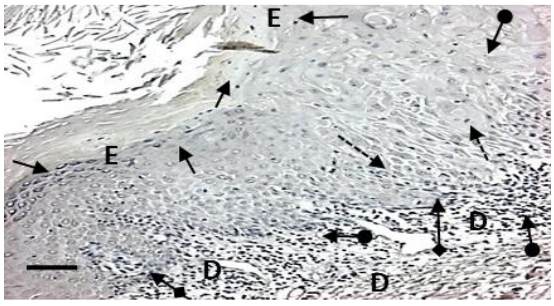


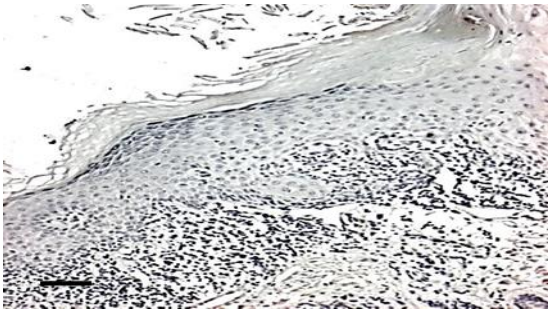
C) BCC



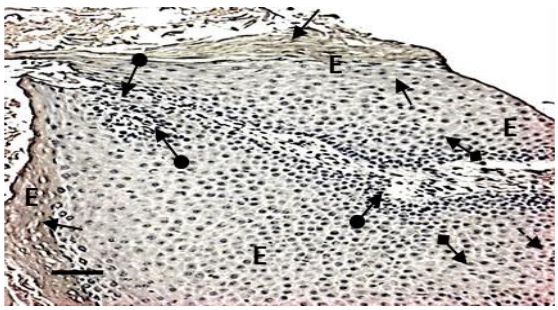


D) SCC





E) SCCIS



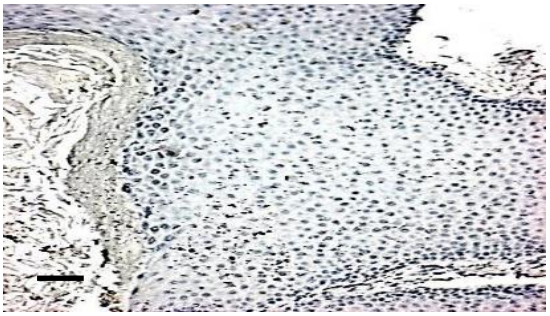
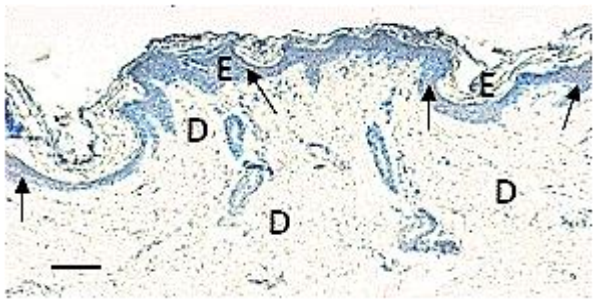


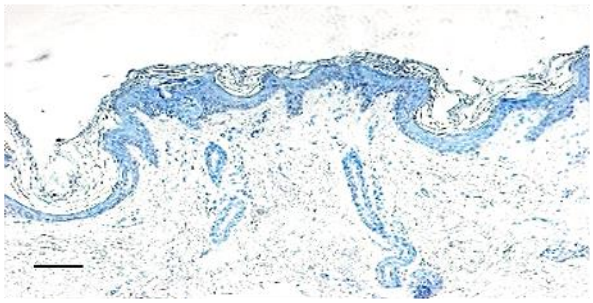
Figure 4.3. Immunohistological detection of CYP27B1 expression in human AK, BCC, SCC, SCCIS and normal skin.

The left panel shows IHC detection of CYP27B1 in human A) NSK (normal skin), B) AK, C) BCC, D) SCC and E) SCCIS. The right panel is a negative control that was stained without the primary antibody, and corresponds to the tumour shown in the left panel. Disruptions of the basal membrane by neoplastic keratinocytes are indicated with 'diamond-ended' arrows. Immune cell infiltration is shown with 'circled-ended' arrows. The positively stained neoplastic, premalignant and normal keratinocytes are shown with solid black arrows and are indicative of the tissue staining intensity described in Table 4.2. Poorly differentiated keratinocytes are shown with black dashed arrows, where applicable. Scale bars: 20 µm. E, epidermal layer; D, dermal layer.

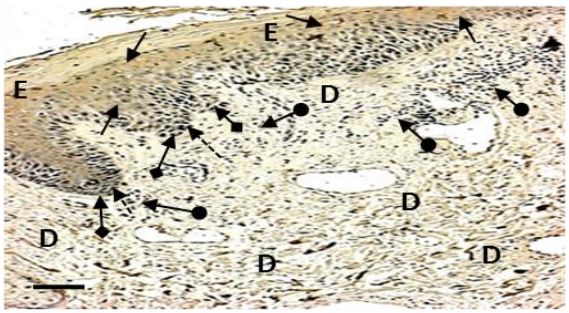
CYP27B1 detection shows poor levels of expression throughout all the epidermal layers and extending down to stratum basale.

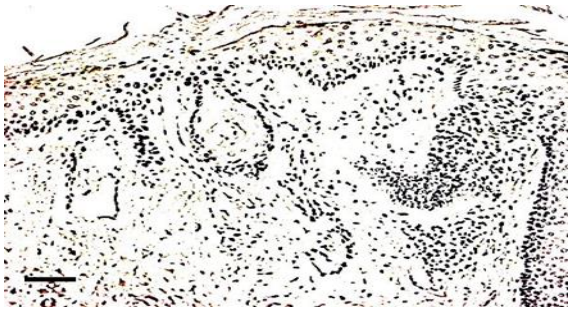
A) NSK



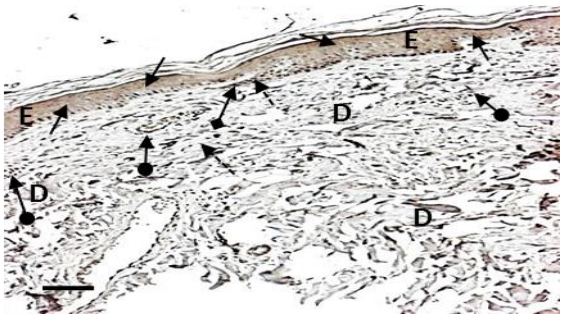


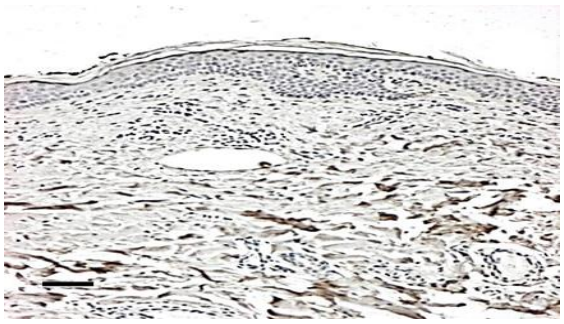
B) AK



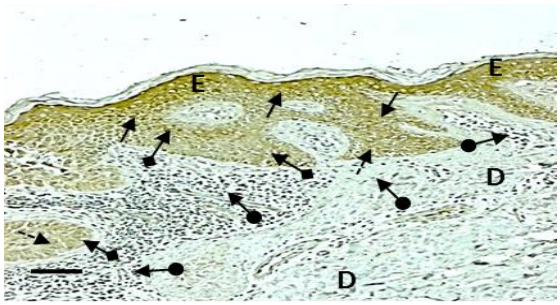


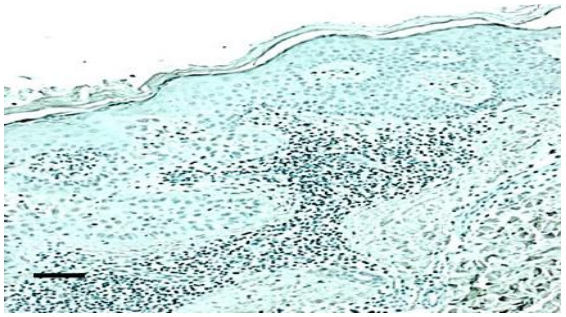
C) BCC



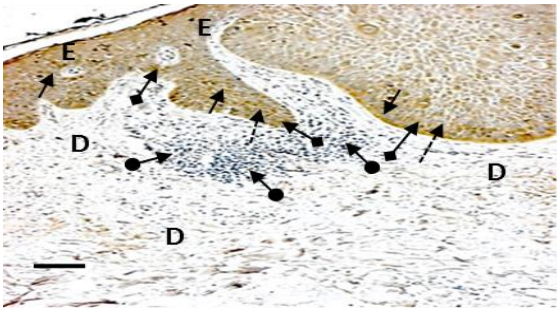


D) SCC





E) SCCIS



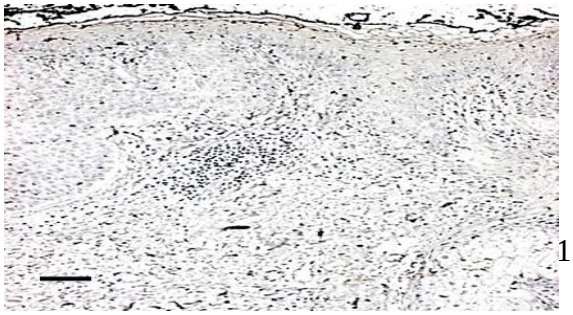
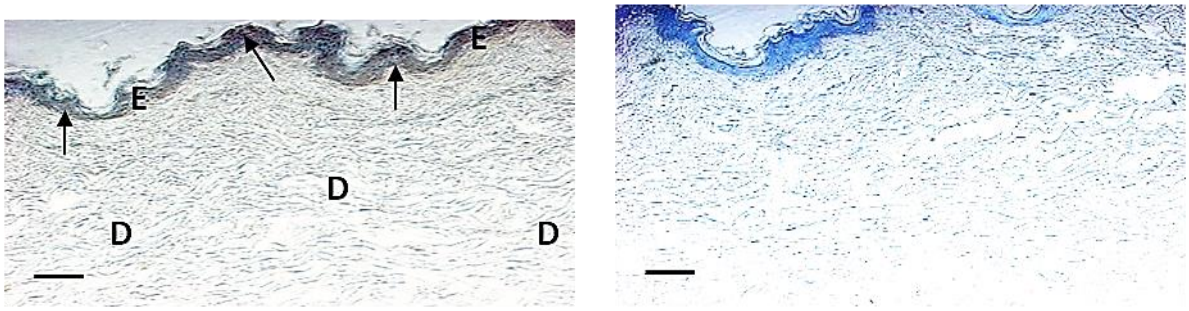


Figure 4.4. Immunohistological detection of CYP24A1 expression in human AK, BCC, SCC, SCCIS and normal skin.

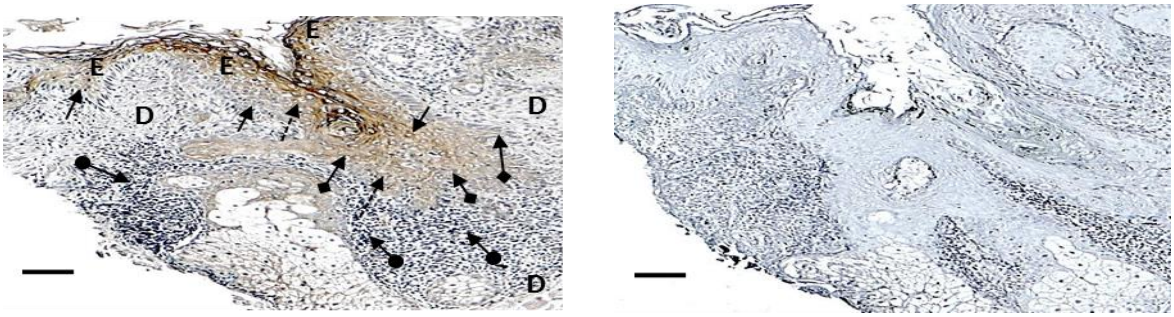
The left panel shows IHC detection of CYP24A1 in human A) NSK (normal skin), B) AK, C) BCC, D) SCC and E) SCCIS. The right panel is a negative control that was stained without the primary antibody, and corresponds to the tumour shown in the left panel. Disruptions of the basal membrane by neoplastic keratinocytes are indicated with 'diamond-ended' arrows. Immune cell infiltration is shown with 'circled-ended' arrows. The positively stained neoplastic, premalignant and normal keratinocytes are shown with solid black arrows and are indicative of the tissue staining intensity described in Table 4.2. Poorly differentiated keratinocytes are shown with black dashed arrows, where applicable. Scale bars: 20 µm. E, epidermal layer; D, dermal layer.

CYP24A1 staining showed prominent expression in BCC, SCC and SCCIS, with less staining observed in AK.

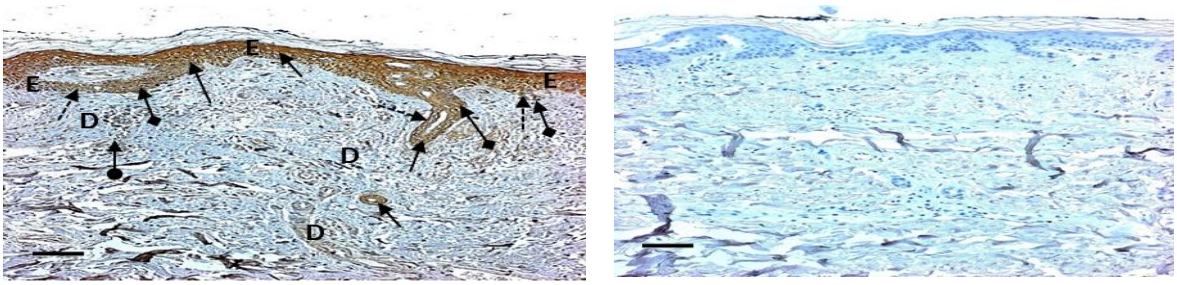
A) NSK



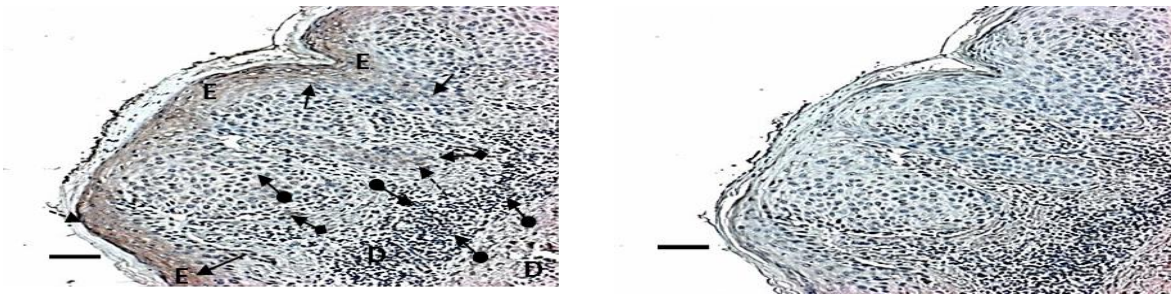
B) AK



C) BCC



D) SCC



E) SCCIS

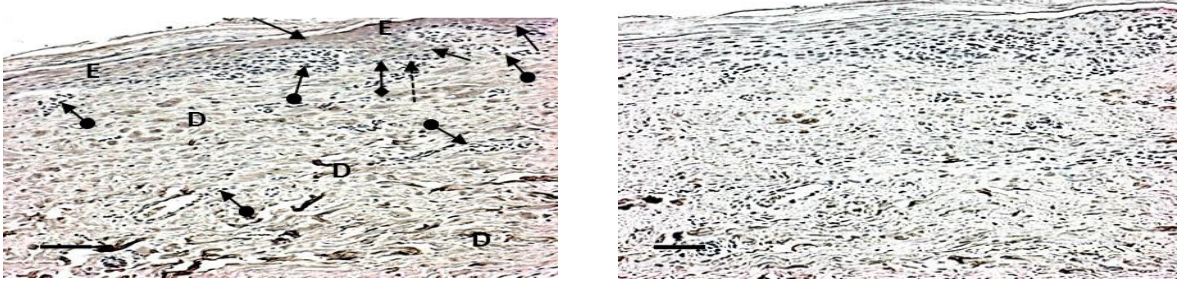


Figure 4.5. Immunohistological detection of VDR expression in human AK, BCC, SCC, SCCIS and normal skin.

The left panel shows IHC detection of VDR in human A) NSK (normal skin), B) AK, C) BCC, D) SCC and E) SCCIS. The right panel is a negative control that was stained without the primary antibody, and corresponds to the tumour shown in the left panel. Disruptions of the basal membrane by neoplastic keratinocytes are indicated with 'diamond-ended' arrows. Immune cell infiltration is shown with 'circled-ended' arrows. The positively stained neoplastic, premalignant and normal keratinocytes are shown with solid black arrows and are indicative of the tissue staining intensity described in Table 4.2. Poorly differentiated keratinocytes are shown with black dashed arrows, where applicable. Scale bars: 20 µm. E, epidermal layer; D, dermal layer.

VDR – vitamin D receptor

4.3.2 Evaluation of EGFR and FGFR3 expression in human tissue samples of AK, BCC, SCC, SCCIS and normal skin.

The epidermal growth factor (EGF) family is known to regulate keratinocyte regeneration, differentiation and apoptosis (Puccinelli et.al., 2010). Numerous reports suggest a regulatory role for EGF in neoplastic transformation in different cancers (Lafky et.al., 2008; Lichtenberger et.al., 2009; Luangdilok et.al., 2011; Roskoski, 2014). The activity of EGF in epidermal cells regulates cell cycle progression, and plays a vital role in mid to late G1 phase cell cycle activation in animal models (Santiskulvong et.al., 2001). Interestingly, it was reported that UVB has an inhibitory effect on EGF activity in human keratinocytes, by decreasing EGF binding abilities to prevent the expression of downstream regulatory genes (Zheng et.al., 1993).

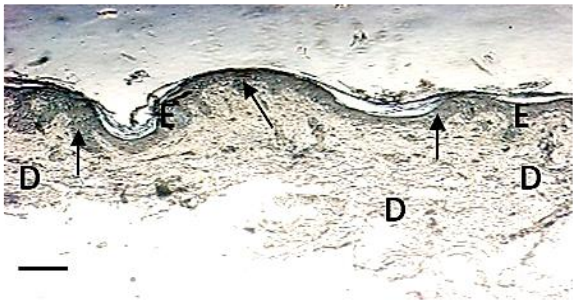
EGFR functions as a survival factor in oncogenic transformation and plays an important role in normal epidermal homeostasis (Sibilia et.al., 2000). EGFR studies in human SCCs show abnormalities in the expression of EGFR. However, vitamin D effects on the expression of EGFR in human NMSCs has not been reported.

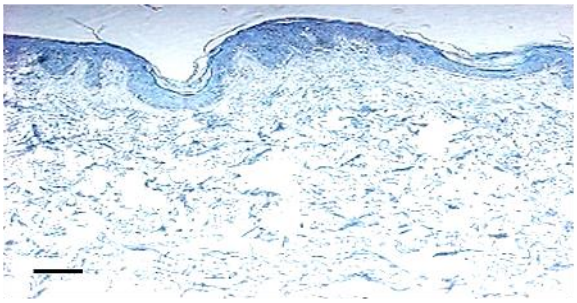
Fibroblast growth factor (FGF) proteins are required for maintaining skin homeostasis, and when dysregulated they play a role in tumourigenesis (Eswarakumar et.al., 2005). FGFR3 has been shown to play a tumour promoting role in many cancers, including breast, prostate, bladder and bone (Sanak et.al., 1997; Chaffer et.al., 2007; Hu et.al., 2007; Naumov et.al., 2012). In addition, FGFR3 activation is a major cause of benign epidermal tumours in mice. Overexpression of FGFR3 is associated with epidermal neoplastic formation in animal models and in humans (Logié et.al., 2005; Yadav et.al., 2012). Furthermore, studies have demonstrated that pharmacological or genetic inhibition of *FGFR3* expression restores the sensitivity of vemurafenib-resistant human melanocytic cells. The underlying mechanism of FGFR3 action against vemurafenib, which is used clinically for the treatment of melanoma, is that the enhanced activation of FGFR3 is linked to RAS and MAPK activation, therefore conferring vemurafenib resistance (Yadav et.al., 2012).

IHC experiments showed that EGFR protein was expressed in NMSC and normal skin. The majority of skin tumours displayed increased expression of EGFR in constantly regenerated keratinocytes. Interestingly, EGFR inteinsive staining was found in the areas where the inflammatory processes and infiltrating immune cells were noted (Figure 4.6). In normal skin sections, EGFR expression was prominent in keratinocytes, as EGFR plays a vital role in epidermal regeneration. Amplified EGFR staining intensity was also detected in keratinocytes of AK, BCC, SCC and SCCIS (Figure 4.6).

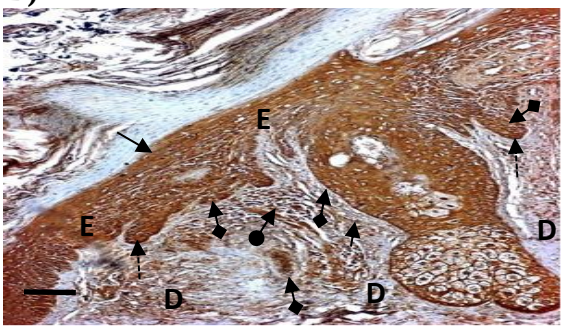
IHC detection of FGFR3 showed strong tissue staining of malignant and premalignant and normal keratinocytes, with a large amount of infiltrating immune cells at the stromal sites (Figure 4.7).

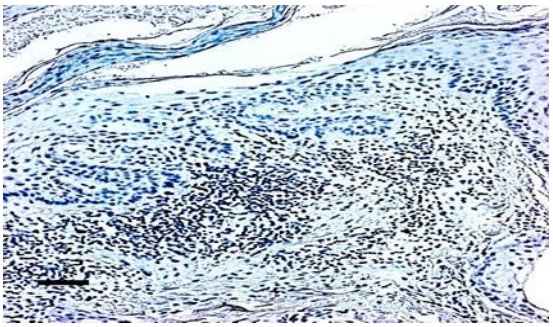
NSK



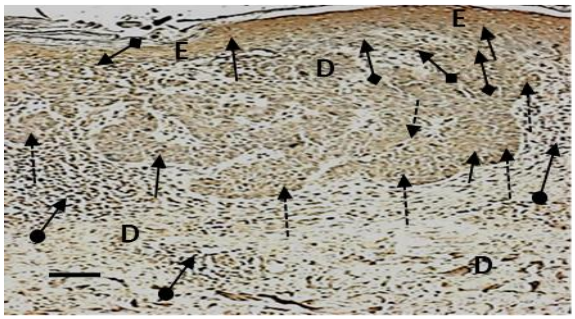


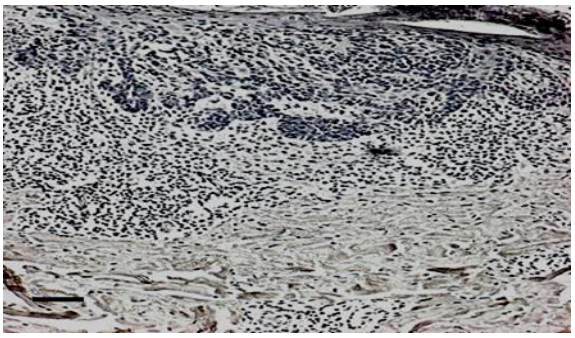
B) AK



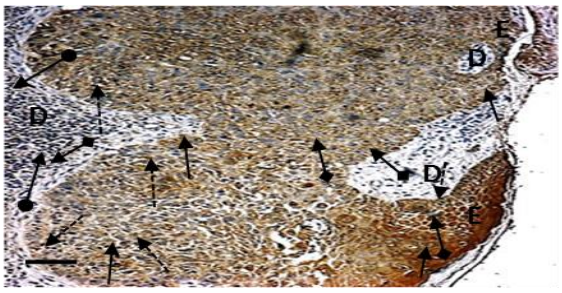


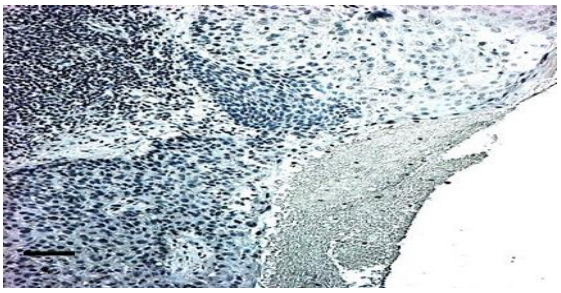
C) BCC



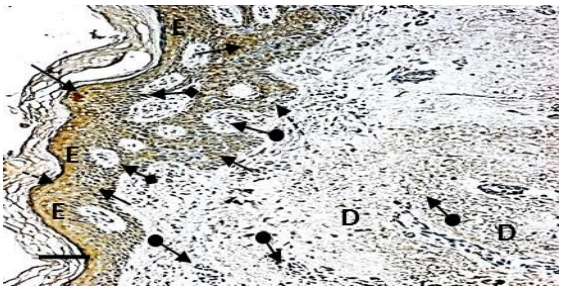


D) SCC





E) SCCIS



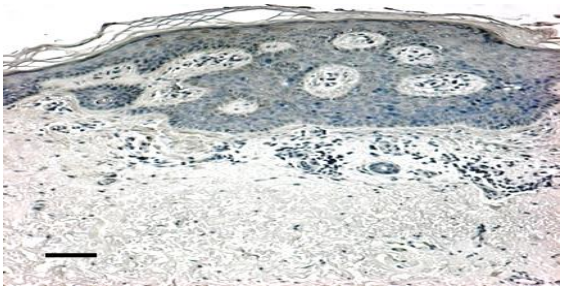
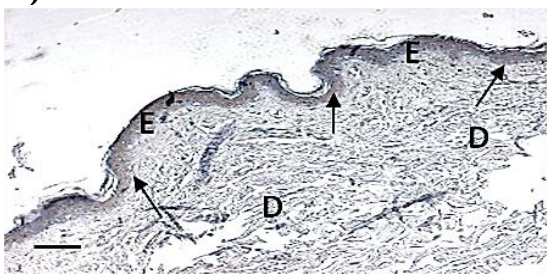
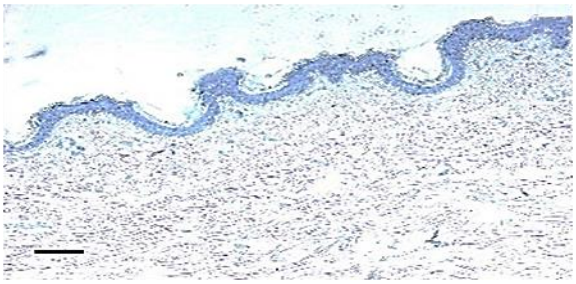


Figure 4.6. Immunohistological detection of EGFR expression in human AK, BCC, SCC, SCCIS and normal skin.

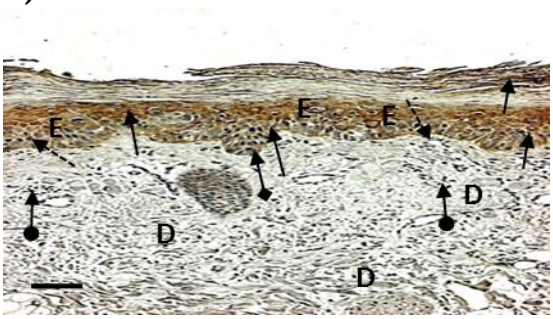
The left panel shows IHC detection of EGFR in human A) NSK (normal skin), B) AK, C) BCC, D) SCC and E) SCCIS. The right panel is a negative control that was stained without the primary antibody, and corresponds to the tumour shown in the left panel. Disruptions of the basal membrane by neoplastic keratinocytes are indicated with 'diamond-ended' arrows. Immune cell infiltration is shown with 'circled-ended' arrows. The positively stained neoplastic, premalignant and normal keratinocytes are shown with solid black arrows and are indicative of the tissue staining intensity described in Table 4.2. Poorly differentiated keratinocytes are shown with black dashed arrows, where applicable. Scale bars: 20 µm. E, epidermal layer; D, dermal layer.

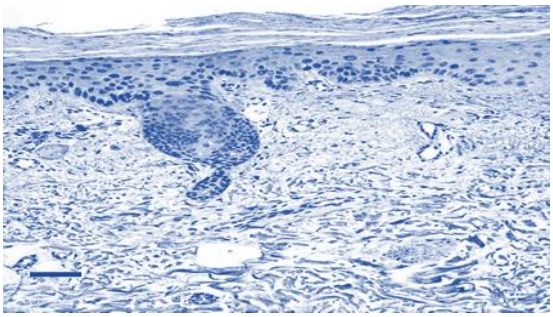
A) NSK



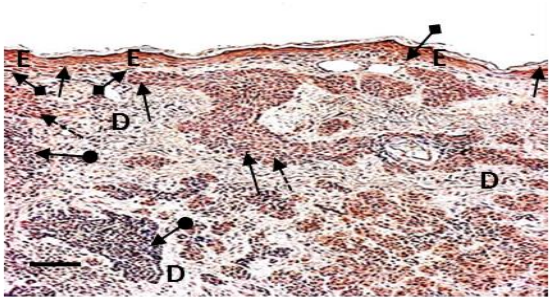


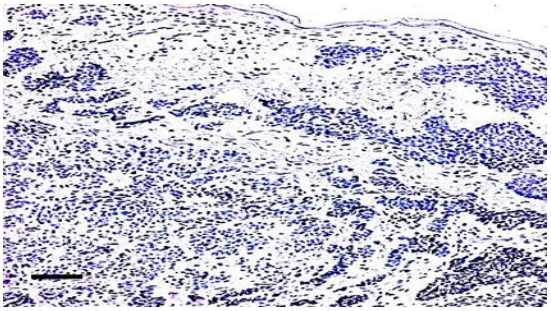
B) AK



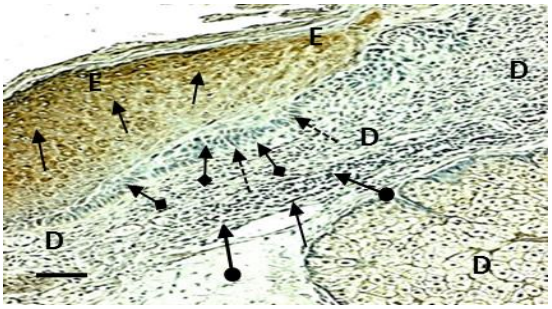


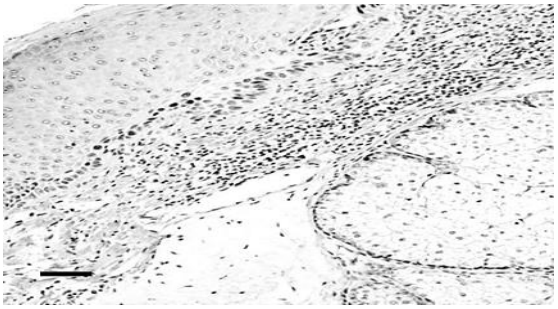
C) BCC



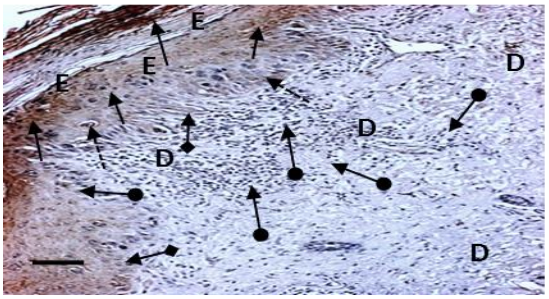


D) SCC





E) SCCIS



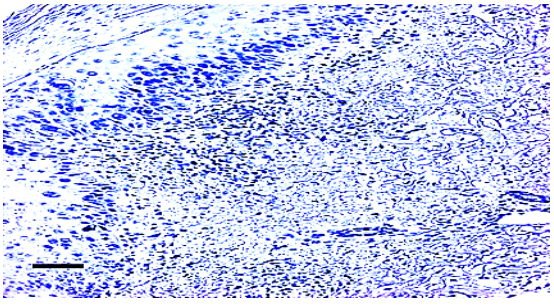


Figure 4.7. Immunohistological detection of FGFR3 expression in human AK, BCC, SCC, SCCIS and normal skin.

The left panel shows IHC detection of FGFR3 in human A) NSK (normal skin), B) AK, C) BCC, D) SCC and E) SCCIS. The right panel is a negative control that was stained without the primary antibody, and corresponds to the tumour shown in the left panel. Disruptions of the basal membrane by neoplastic keratinocytes are indicated with 'diamond-ended' arrows. Immune cell infiltration is shown with 'circled-ended' arrows. The positively stained neoplastic, premalignant and normal keratinocytes are shown with solid black arrows and are indicative of the tissue staining intensity described in Table 4.2. Poorly differentiated keratinocytes are shown with black dashed arrows, where applicable. Scale bars: 20 µm. E, epidermal layer; D, dermal layer.

4.3.3 Evaluation of growth factor expression (EGF, FGF3 and VEGF) in human tissue samples of AK, BCC, SCC, SCCIS and normal skin.

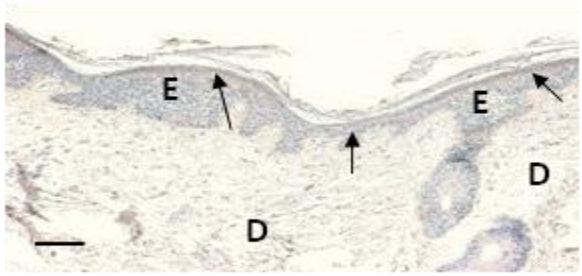
To determine the role of growth factors in NMSC, the expression of EGF, FGF3 and VEGF in the cutaneous lesions was investigated. The importance of growth factor binding to its receptor in ligand-activated phosphorylation was reviewed earlier (see section 1.6.2). However, the possibility of ligand activation via a different molecular pathway was not considered. These immunohistological investigations provided an overview of the major tumour-promoting growth factors.

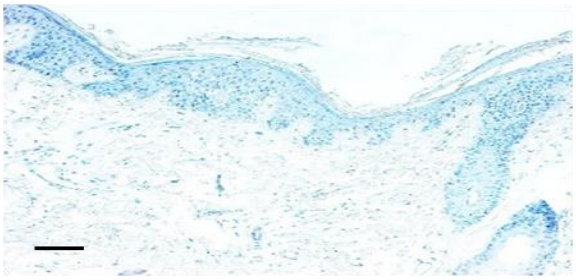
Immunohistochemical investigation of EGF expression in human NMSCs has shown that EGF has a moderate to high level of expression in the epidermal layer of all the tumours (Figure 4.8). The immunolabelling of FGF3 showed high level of expression among neoplastic and pre-malignant lesions. It was noted that in normal skin, AK lesions and surprisingly, in BCC tumours, FGF3 expression was predominantly found in epidermal keratinocytes (Figure 4.9). SCC and SCCIS tumours had scattered FGF3 protein expression amongst epidermal–dermal cells.

Overexpression of VEGF was noted in all the tumour types. Staining was observed in dermal areas around the blood vessels and sebaceous glands, as well as in epidermal keratinocytes and tumour keratinocytes (Figure 4.10).

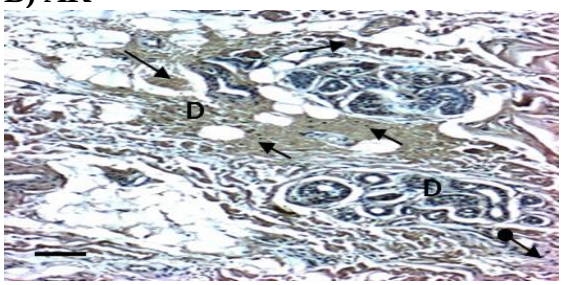
Evaluation of the staining for vitamin D metabolic enzymes, growth factors and growth factor receptors in normal skin and all the NMSC tumours is shown in Table 4.3.

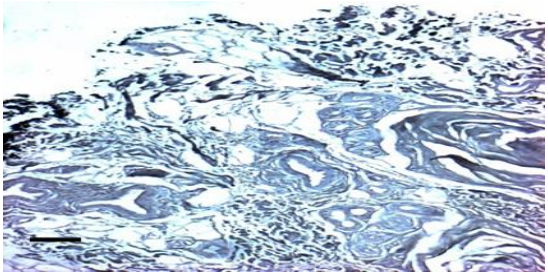
A) NSK



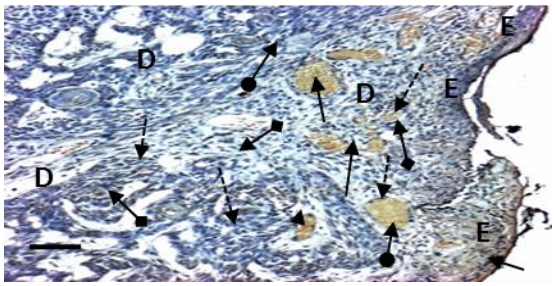


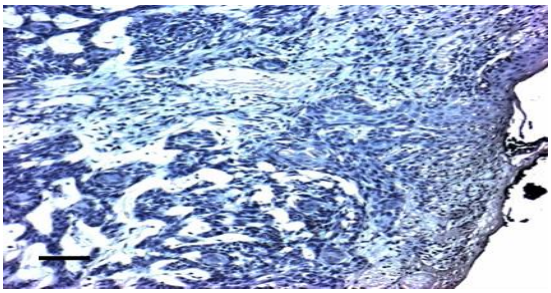
B) AK



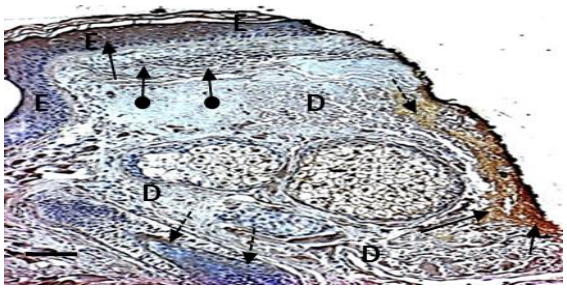


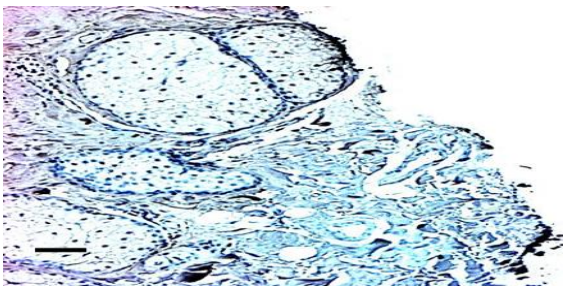
C) BCC



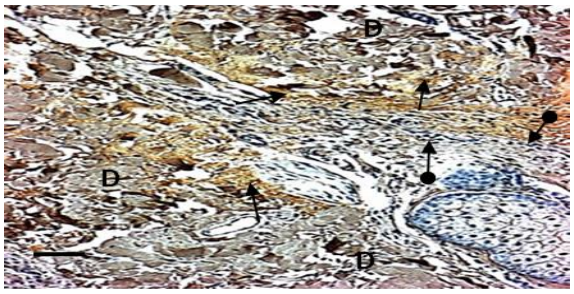


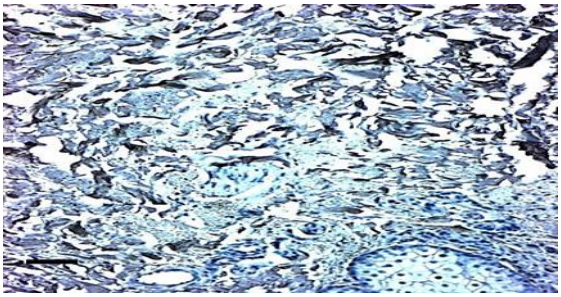
D) SCC





E) SCCIS

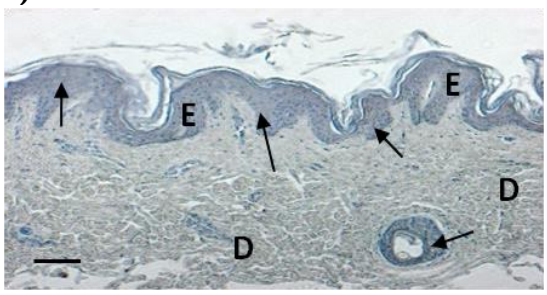


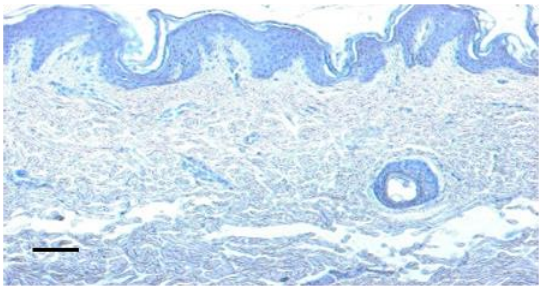


4.8. Immunohistological detection of EGF expression in human AK, BCC, SCC, SCCIS and normal skin.

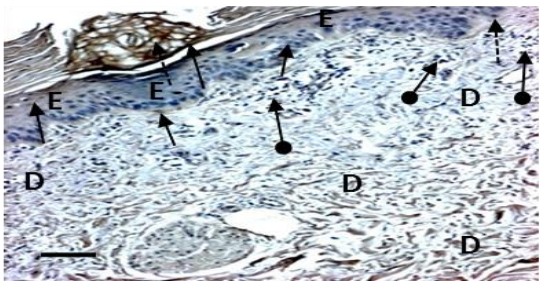
The left panel shows IHC detection of EGF in human A) NSK (normal skin), B) AK, C) BCC, D) SCC and E) SCCIS. The right panel is a negative control that was stained without the primary antibody, and corresponds to the tumour shown in the left panel. Disruptions of the basal membrane by neoplastic keratinocytes are indicated with 'diamond-ended' arrows. Immune cell infiltration is shown with 'circled-ended' arrows. The positively stained neoplastic, premalignant and normal keratinocytes are shown with solid black arrows and are indicative of the tissue staining intensity described in Table 4.2. Poorly differentiated keratinocytes are shown with black dashed arrows, where applicable. Scale bars: 20 μm. E, epidermal layer; D, dermal layer.

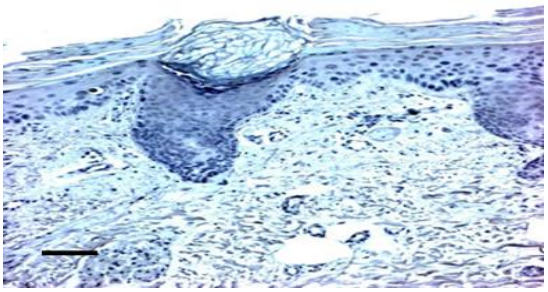
A) NSK



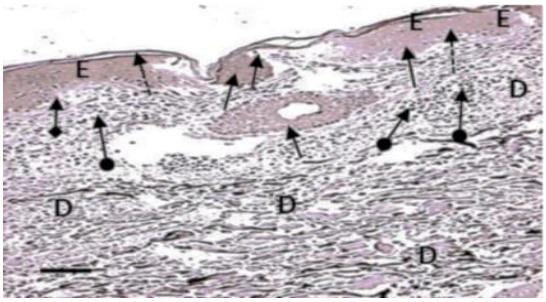


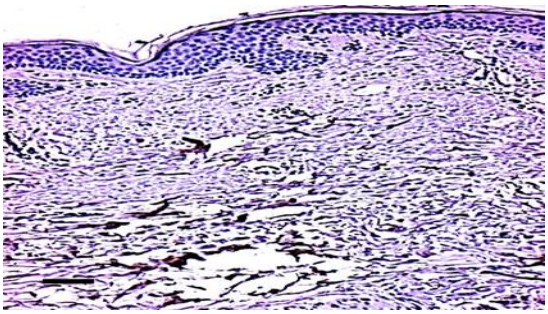
B) AK



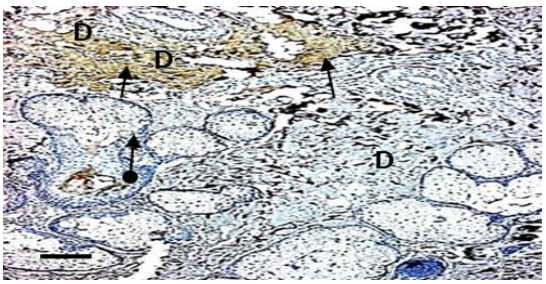


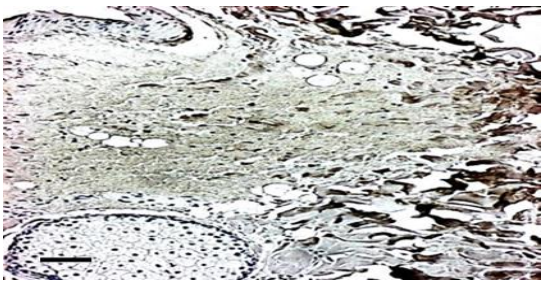
C) BCC



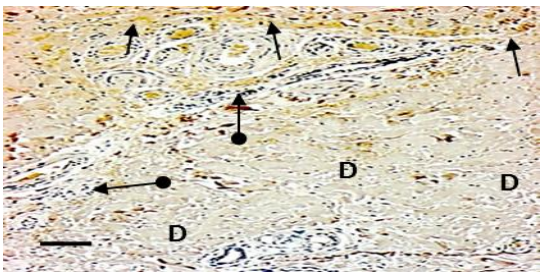


D) SCC





E) SCCIS



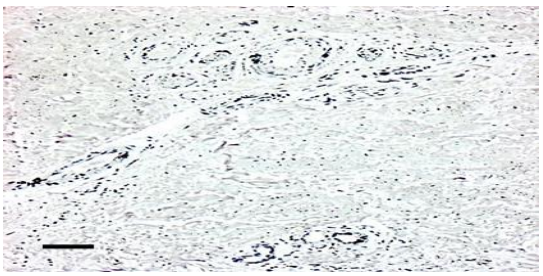
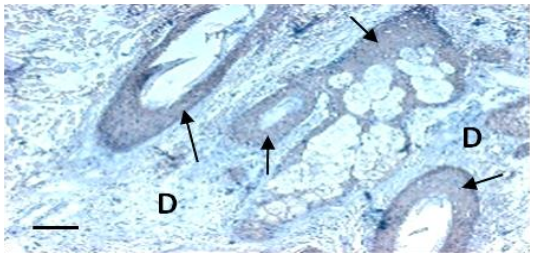
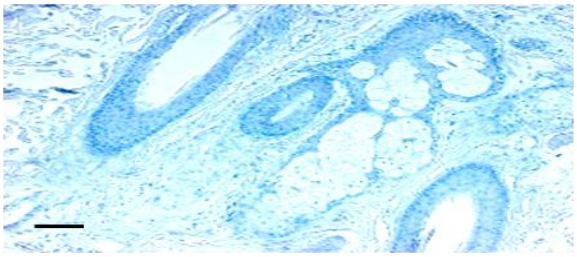


Figure 4.9. Immunohistological detection of FGF3 expression in human AK, BCC, SCC, SCCIS and normal skin.

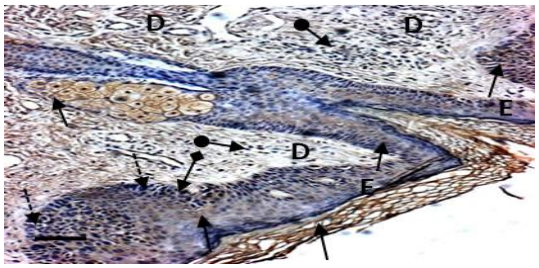
The left panel shows IHC detection of FGF3 in human A) NSK (normal skin), B) AK, C) BCC, D) SCC and E) SCCIS. The right panel is a negative control that was stained without the primary antibody, and corresponds to the tumour shown in the left panel. Disruptions of the basal membrane by neoplastic keratinocytes are indicated with 'diamond-ended' arrows. Immune cell infiltration is shown with 'circled-ended' arrows. The positively stained neoplastic, premalignant and normal keratinocytes are shown with solid black arrows and are indicative of the tissue staining intensity described in Table 4.2. Poorly differentiated keratinocytes are shown with black dashed arrows, where applicable. Scale bars: 20 µm. E, epidermal layer; D, dermal layer.

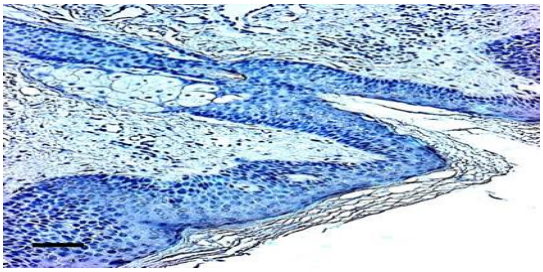
A) NSK



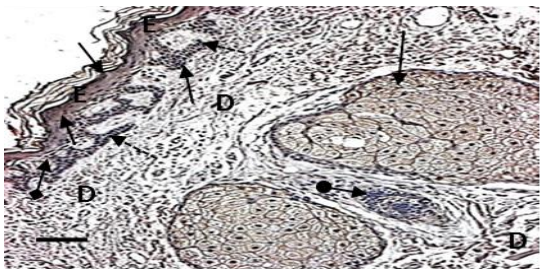


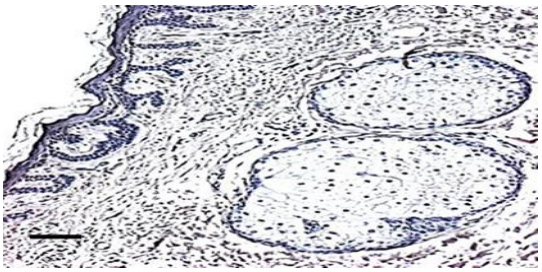
B) AK



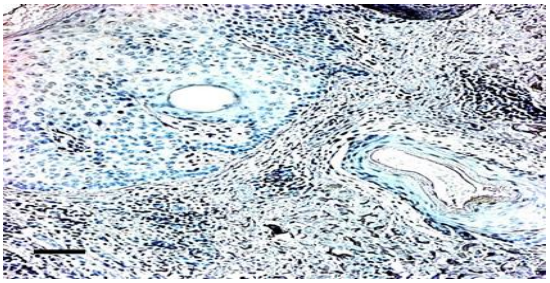


C) BCC

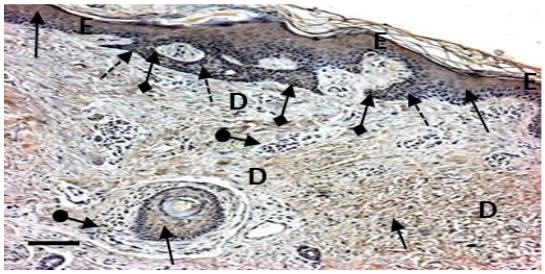




D) SCC



E) SCCIS



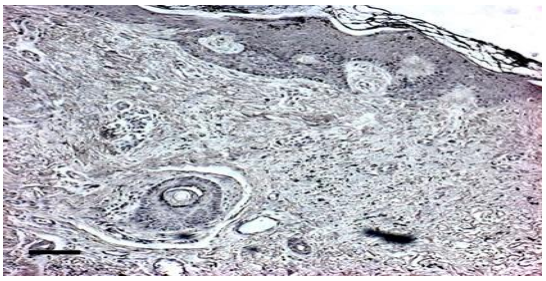


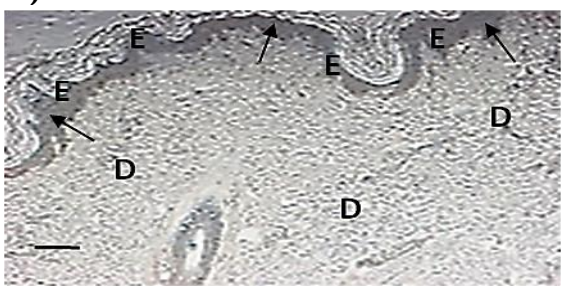
Figure 4.10. Immunohistological detection of VEGF expression in human AK, BCC, SCC, SCCIS and normal skin.

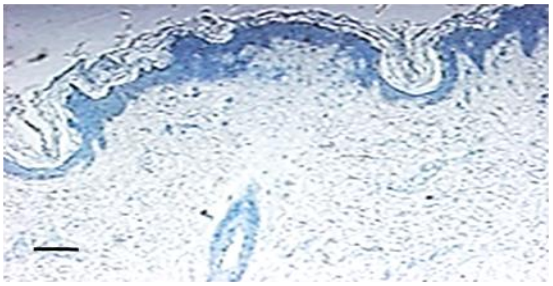
The left panel is IHC detection of VEGF in human A) NSK (normal skin), B) AK, C) BCC, D) SCC and E) SCCIS. The right panel is a negative control that was stained without the primary antibody, and corresponds to the tumour shown in the left panel. Disruptions of the basal membrane by neoplastic keratinocytes are indicated with 'diamond-ended' arrows. Immune cell infiltration is shown with 'circled-ended' arrows. The positively stained neoplastic, premalignant and normal keratinocytes are shown with solid black arrows and are indicative of the tissue staining intensity described in Table 4.2. Poorly differentiated keratinocytes are shown with black dashed arrows, where applicable. Scale bars: 20 μm. E, epidermal layer; D, dermal layer.

4.3.4 Evaluation of Proliferating Cell Nuclear Antigen (PCNA) in human tissue samples of AK, BCC, SCC, SCCIS and normal skin.

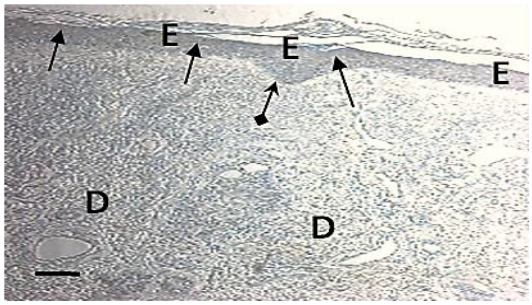
To better understand intratumoural proliferative capacities of neoplastic lesions, analysis of PCNA expression was assessed in human tissues. PCNA expression is associated with actively proliferative cells and is indicative of DNA replication. The prominent detection of PCNA expression was found in highly regenerative epidermal keratinocytes of all tumours and normal skin. Interestingly, poorly differentiated keratinocytes had lower staining intensity, while mutated and migrated beyond the basal membrane keratinocytes with well differentiated morphology had extensive staining. PCNA protein expression in normal skin was predominantly in the epidermal layer, whilst keratinocytes of neoplastic tissues showed intensive staining in various dermal and epidermal locations. Epidermal layers encompasses stem cells within stratum basale that enable tissue regeneration. Due to high regenerative abilities of the normal epidermal cells the moderate to high level of PCNA expression was detected in normal human skin. Interestingly, poorly differentiated keratinocytes of SCC had diminished level of PCNA expression, whereas well differentiated keratinocytes of other types of neoplastic tissues displayed quite high intensity staining in suprabasal layers and in the perturbed basal membrane (Figure 4.11). This observation agrees with the literature on PCNA expression in keratinocyte, and may suggest that PCNA has a function in keratinocyte proliferation and may not be involved in differentiation (Penneys et.al., 1994; Einspahr et.al., 1999; Moor et.al., 2004).

A) NSK

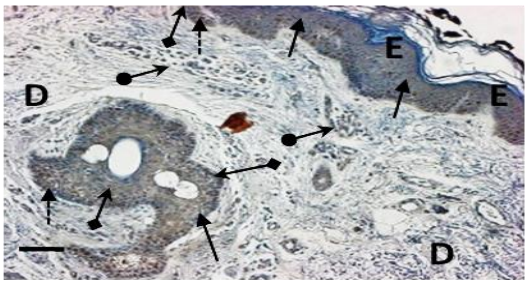


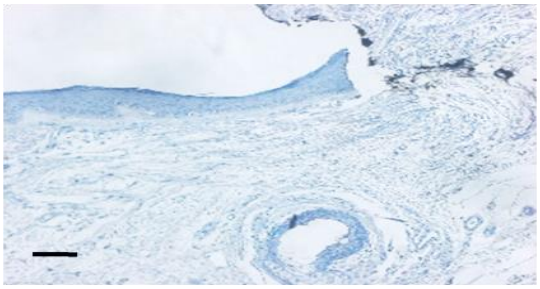


B) AK

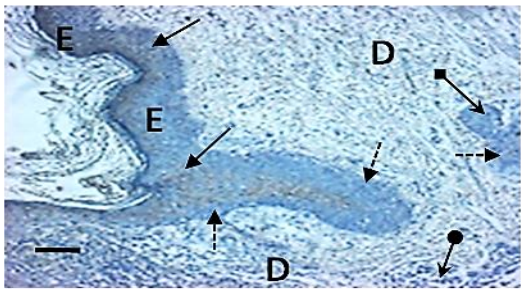


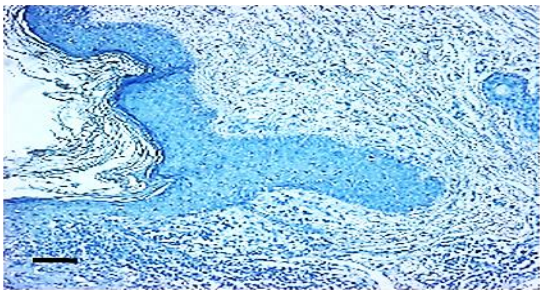
C) BCC



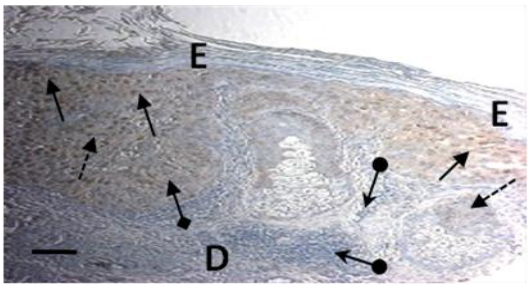


D) SCC





D) SCCIS



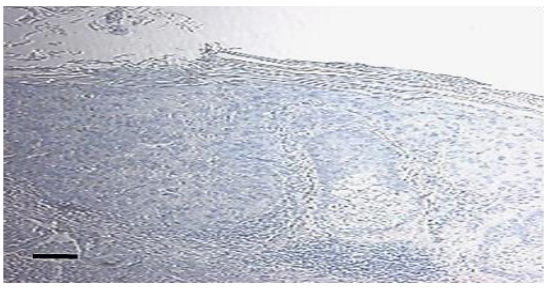


Figure 4.11 Immunohistological detection of PCNA expression in human AK, BCC, SCC, SCCIS and normal skin.

The left panel is IHC detection of PCNA in human A) NSK (normal skin), B) AK, C) BCC, D) SCC and E) SCCIS. The right panel is a negative control that was stained without the primary antibody, and corresponds to the tumour shown in the left panel. Disruptions of the basal membrane by neoplastic keratinocytes are indicated with 'diamond-ended' arrows. Immune cell infiltration is shown with 'circled-ended' arrows. The positively stained neoplastic, premalignant and normal keratinocytes are shown with solid black arrows and are indicative of the tissue staining intensity described in Table 4.2. Poorly differentiated keratinocytes are shown with black dashed arrows, where applicable. Scale bars: 20 μm. E, epidermal layer; D, dermal layer.

Protein	Low levels of expression* (0-5%)	Moderate levels of expression* (5-10%)	High levels of expression* (more than 10%)
Normal skin			
Vitamin D CYP27A1 (CYP27A1)	**0**	**0**	**5**
CYP27B1 (CYP27B1)	**1**	**1**	**3**
Vitamin D3 24-hydroxylase (CYP24A1)	**3**	**1**	**1**
VDR	**0**	**0**	**5**
EGFR	**1**	**2**	**1**
EGF	**2**	**3**	**0**
FGFR3	**1**	**2**	**2**
FGF3	**1**	**1**	**2**
VEGF	**1**	**1**	**2**
PCNA	**0**	**1**	**3**
Actinic keratosis neoplastic lesions			
Vitamin D CYP27A1 (CYP27A1)	**0**	**2**	**13**
CYP27B1 (CYP27B1)	**9**	**2**	**4**
Vitamin D3 24-hydroxylase (CYP24A1)	**0**	**0**	**15**
VDR	**1**	**2**	**12**
EGFR	**1**	**2**	**12**
EGF	**1**	**5**	**9**
FGFR3	**1**	**1**	**13**
FGF3	**1**	**4**	**10**
VEGF	**0**	**3**	**12**
PCNA	**0**	**1**	**4**
Basal cell carcinoma			

Vitamin D CYP27A1 (CYP27A1)	**5**	**6**	**10**
CYP27B1 (CYP27B1)	**12**	**5**	**4**
Vitamin D3 24-hydroxylase (CYP24A1)	**1**	**3**	**17**
VDR	**1**	**2**	**18**
EGFR	**1**	**2**	**18**
EGF	**2**	**3**	**16**
FGFR3	**1**	**1**	**19**
FGF3	**1**	**3**	**17**
VEGF	**1**	**2**	**18**
PCNA	**0**	**1**	**4**
Squamous cell carcinoma			
Vitamin D CYP27A1 (CYP27A1)	**2**	**4**	**2**
CYP27B1 (CYP27B1)	**4**	**4**	**0**
Vitamin D3 24-hydroxylase (CYP24A1)	**1**	**2**	**5**
VDR	**1**	**4**	**3**
EGFR	**1**	**2**	**5**
EGF	**1**	**6**	**1**
FGFR3	**0**	**4**	**4**
FGF3	**2**	**2**	**4**
VEGF	**1**	**1**	**6**
PCNA	**0**	**0**	**4**
Squamous cell carcinoma in situ			
Vitamin D CYP27A1 (CYP27A1)	**2**	**2**	**8**
CYP27B1 (CYP27B1)	**3**	**9**	**0**
Vitamin D3 24-hydroxylase (CYP24A1)	**1**	**2**	**9**

VDR	**2**	**2**	**8**
EGFR	**2**	**1**	**9**
EGF	**0**	**2**	**10**
FGFR3	**1**	**1**	**10**
FGF3	**2**	**1**	**9**
VEGF	**0**	**2**	**10**
PCNA	**1**	**2**	**2**

***** Number of tissue samples analysed. See Table 4.1 for tumour specimen characteristics and Table 4.2 for determination of staining intensity. Staining intensity was evaluated by 3 independent (blinded) scoring.

4.4 Discussion

The cancer inhibitory effects of vitamin D has been consistently reported in breast, prostate, lung, colon and endometrial cancers (Chen & Holick 2003; Grant 2010; Peehl 2004; Zeleniuch-Jacquotte, Gallicchio & Hartmuller 2010; Zhang et.al. 2013). As such, we determined whether there is an association between vitamin D metabolic enzymes and VDR expression with skin cancer progression, in BCC, SCC, SCCIS, AK and normal skin. In addition, we assessed the relationship between vitamin D metabolism and tumour-promoting proteins, EGFR, FGFR3, EGF, FGF3, VEGF and PCNA.

CYP27A1, CYP27A1, is a well conserved enzyme that also expresses 27-hydroxylase activity toward cholesterol and is involved in cholestrerol hydroxylation and bile acid biosynthetic pathways (Prosser et.al. 2006). Recent studies have shown the importance of microsomal hydroxylation in activation of nuclear VDR in human keratinocytes (Endo-Umeda et.al. 2014). In particular, CYP27A1 is involved in the formation of calcidiol that activates VDR, whilst cholecalciferol itself does not. Calcidiol is a 25-hydroxy metabolite of vitamin D metabolism produced via CYP27A1 hydroxylation. Calcidiol has a lower affinity for VDR, however has agonistic action in cells by a mechanism independent from its conversion to the active hormone (Endo-Umeda et.al. 2014). Importantly, there is VDR-dependent inhibition of numerous cancers and oncogenes (Chung et.al. 2009; Guan et.al. 2013; S. Ondková 2006; Wu 2007). We demonstrated that anabolic CYP27A1 expression was highly expressed in normal skin. By comparison to normal skin, there was moderate staining in most SCC, and reduced CYP27A1 expression in AK and SCCIS tissues. This data may suggest that patients with decreased enzymatic expression of CYP27A1 may have, as a consequence, lower circulatory levels of endogenous

calcidiol. Similar findings have been noted in colon cancer (Matusiak & Benya, 2007), endometrial carcinomas and normal endometrial tissues (Bergada et.al., 2014).

CYP27B1 is one of the key enzymes regulating both systemic and tissue levels of calcitriol; it appears to be greatly down-regulated in most tumours. Levels of serum calcitriol largely relies on the enzymatic activity of CYP27B1 (or hydroxyvitamin D 1α-hydroxylase) (Jones, 2008). CYP27B1 in normal skin was highly expressed, however, there was minimal staining intensity in poorly differentiated tissues, as well as decreased expression in AK, BCC, SCC and SCCIS. The low expression of CYP27B1 has been reported in melanoma cells (Brożyna et.al., 2013) and in normal and malignant breast tissues (Suetani et.al., 2012). The diminished staining of CYP27B1 throughout the entire epidermis, extending down to the basal layer, in all tumours. This finding is indicative that the synthesis of CYP27B1 is diminished in neoplastic skin lesions; this was confirmed by the detection of intensive expression of CYP27B1 in the normal skin (Figure 4.3). CYP27B1 hydroxylation plays an important role in anticancer activities of dietary vitamin D in the form of cholecalciferol. It has been demonstrated that dietary supplementation of cholecalciferol (calcidiol precursor) significantly inhibits breast tumour growth in mice (Krishnan et.al., 2013). Perhaps, the mechanism of neoplastic cell survival involves downregulation of CYP27B1 activity and is associated with its intracrine accumulation of calcitriol. This finding provides evidence of the concept that intracrine vitamin D metabolic enzymes, including CYP27B1, may regulate growth characteristics of cutaneous carcinomas.

CYP24A1 is the key enzyme which converts calcitriol to calcitroic acid, hence contributes to acidic intratumoural environment (Gröschel, et.al., 2015; Luo et.al., 2012). It was shown that normal skin had limited expression of catabolic CYP24A1, and, was upregulated in all neoplastic skin lesions. It was noted that in neoplastic tissues CYP24A1 expression was distributed amongst the epidermal cutaneous layers and also was detected in dermal fibroblasts. The level of CYP24A1 expression in SCCIS was elevated, mainly in neoplastic keratinocytes and in AK skin lesions there is low levels of CYP24A1 expression in well-differentiated keratinocytes. The intense and broad distribution of CYP24A1 staining observed in all four tumour types suggests that extensive degradation of both vitamin D metabolites (calcidiol and calcitriol) take place in these tumours. CYP24A1 has a positive correlation ($p<0.05$) with the levels of expression of other vitamin D metabolic enzymes and VDR, but not with the expression of CYP27B1 (Table 4.3). Decreased CYP24A1 expression in normal skin, compared to neoplasic lesions, has also been observed in melanomas (Brożyna et.al., 2014); although there was no correlation between staining intensity and melanoma aggressiveness. However, it was shown that elevated levels of CYP24A1 are

associated with melanoma progression at early stages of tumor development. In our studies, there was a positive correlation (p<0.05) betwen VDR expression in SCC and expression of CYP24A1. Similarly, CYP24A1 may have a role in non melanomas and transformation of AK to more aggressive cancer type (such as an invasive and metastatic SCC). Interestingly, in in vitro studies, using calcitriol analog (Eldecalcitol, ED-71) showed induction of expression of CYP24A1 in oral cancer cell lines and in human gingival fibroblasts in a dose-dependent manner; high doses of ED-71 (0.5 μg/kg once every 4 days) significantly suppressed tumour formation in athymic nude mice, where as low doses (0.1 μg/kg) had no effect (Shintani et.al., 2015).

Vitamin D receptor (VDR) is an endocrine ligand that plays an important function in regulation of endogenous vitamin D status. VDR has versatile functions: regulation of placental development, regulation of numerous genetic pathways that involve in health and disease state and its involvement in neoplastic cells regulation (Buckberry et.al., 2015; Sherman et.al., 2014; Jiang et.al., 2013; Hibleret.al., 2010; Campbell et.al., 2010). The detection of VDR in normal skin indicated that it is highly expressed. The intensive VDR immunoreactivity was noted also in the majority of AK, BCC, SCCIS and in some SCC. In BCC, overexpression of VDR was predominantly in keratinised cells. VDR expression in epidermal layer, sebaceous glands and keratinocytes surrounding blood vessels in the normal skin were found to be quite prominent. In AK lesions, the expression of VDR was detected in granular and horny epidermal layers, as well as in the sebaceous glands and distal areas of hair follicles with reduced expression, compared to normal skin. In SCCIS, decreased expression of VDR was detected in granular and horny epidermal layers. The reported lack of metastatic ability of BCC may be associated with VDR mediation of neoplastic development and progression (Carr et.al., 2007). It was noted that VDR was distributed amongst the epidermal and dermal cutaneous layers. This supports ealier reports on VDR detection in some types of skin cancers (BCC and SCC) (Mitschele et.al., 2004; Reichrath et.al., 2004). Interestingly, it was shown that VDR polymorphisms are of importance for the development of BCCs and cutaneous SCCs (Köstner et.al., 2012).

Interestingly, there was low expression levels of CYP27A1 and CYP27B1 in AK lesions, compared to normal tissue. An inverse association was established between CYP27B1 and CYP24A1 in all tumours (p<0.05), but not in normal skin, where poor immunoreactivity for CYP24A1 was observed. These findings suggest that CYP27B1 is diminished in NMSC, compared to normal skin, whilst the enzymatic activity of the vitamin D-degrading CYP24A1 was elevated in most tumours, but not in normal skin.

The status of protein expression in non-cancerous skin surrounding the tumour was not investigated due to the limited amount of presumably normal tissue without the neoplastic infiltration. Many of the specimens used in this study were from biopsies that are very small (2 mm), which were obtained from delicate areas surrounding the eyes, nose and ears in some patients. Ostensibly normal tissue adjacent to the tumour was negligible in many samples. Due to this fact, it was impossible to use the unaffected skin from the same sample as a negative control in this study. Therefore, analysis was conducted using normal skin tissue samples and compared to precancerous and cancerous skin.

IHC studies have shown that skin cancer is a disease associated with dysregulated cell survival and death signalling pathways that contribute to neoplastic formation (Evan & Vousden 2001). Most cancers share a common pathogenesis, where DNA damage and mutagenesis triggers the initiation of tumourigenesis. In cutaneous carcinogenesis, modifications in several signalling pathways are caused by ultraviolet irradiation. The normal epidermal or dermal cells that in this way accumulate damaged or mutated DNA then amplify, and emerge as a neoplastic growth.

IHC studies of human neoplastic lesions revealed the status of intratumoural vitamin D enzymes and receptors. It was shown that expression of the vitamin D anabolic enzyme CYP27B1 was diminished in human melanoma cancers (Brożyna 2013). This reduced expression was correlated with melanoma phenotype and behaviour, and was also found to affect the survival rates in melanoma patients. These results showed the important role of vitamin D in the pathophysiological manifestation of melanoma (Brożyna 2013). Interestingly, in study of vitamin D system in squamous cell carcinoma, the mRNA expression of *CYP27B1* was detected to have an elevated expression by real time PCR (Reichrath et.al., 2004).

Various epidemiological, clinical and animal studies have suggested the involvement of vitamin D in the regulation of NMSC. IHC staining for vitamin D metabolic enzymes has been reported in different cancers. It was shown that VDR is highly expressed in human precancerous lesions and oesophageal carcinomas (Zhou et.al. 2014). Interestingly, in human endometrial cancers there was significantly higher expression of VDR in patients with cancer compared to healthy controls (Agic et.al. 2007). Herein, VDR expression was noted in all NMSCs and in precancerous AK lesions, and was found to be well defined, but not as extensive as in normal skin samples.

Significance of growth factors and it receptors in epidermal health

Epidermal growth factor receptor (EGFR) has been a hallmark of malignant phenotype of many cancers. It is often used as a diagnostic marker in histopathological examination (Roskoski, 2014). Both EGF and EGFR are involved in tumour growth, promotion and signalling (Lafky, 2008). Morever, *EGFR* shares the same domain and has synergistic mechanism of activation on tumour growth with *VEGFR*. EGFR prevents mutated cells from apoptosis, while VEGF activates VEGFR, that controling angiogenesis and promotes cellular proliferation (Lichtenberger, 2009). These proteins are therefore considered as potent growth factors in epidermal tumours.

Calcitriol inhibits ligand-dependent phosphorylation of EGFR and alters its cellular localisation. Furthermore, calcitriol has an impact on EGFR activation by EGF, resulting in transcriptional activation of the cyclin D1 promoter. In fact, vitamin D (in the form of calcitriol) has an inhibitory effect on EGFR growth signalling (Cordero et.al., 2002). Growth factors and their receptors play a vital role in regulating cancer growth and progression (Smola et.al., 1993). In the studies in this chapter, it was evident that there was strong expression of EGFR and FGFR3 in NMSCs and in normal skin. These findings agree with other reports of EGFR and FGFR3 expression in cancerous tissues (Chaffer 2007; Hu 2007; Lichtenberger 2009; Mehra 2011; Naumov 2012; Roskoski Jr 2014; Sanak 1997; Santiskulvong et.al., 2001), and, by human epidermal cells (Luangdilok et.al., 2011; Simper et.al., 2015). EGFR and FGFR3 activation results in MAPK-mediated regulation of neoplastic growth (Yadav et.al., 2012; Luangdilok et.al, 2011), suggeting that vitamin D inhibition of neoplastic cell may follow this pathway (shown earlier in chapter 3. Figure 3.1).

The studies of EGFR and FGFR3 staining showed that the overexpression of both growth factor receptors corresponded to diminished expression of the anabolic vitamin D enzymes, CYP27B1 in AK, BCC, SCC and SCCIS ($p<0.05$), but not in normal skin, where there was extensive expression of CYP27B1. Moreover, the high expression of CYP24A1 in neoplastic keratinocytes also corresponds to the expression of FGFR3 and EGFR in cutaneous neoplastic lesions and has an inverse association with CYP24A1 expression in normal skin ($p<0.05$).

The status of expression of several MAPK modulatory factors, EGF, FGF3 and VEGF, was investigated in normal, precancerous and cancerous skin. Normal skin had low to moderate EGF immunoreactivity predominately within epidermal layers. EGF is an essential factor for normal tissue regeneration, where constant cell renewal takes place. Indeed, it was noted in this study that EGF was expressed in all tumour types at moderate to high levels. Interestingly, not all tumours had EGF expression in cancer-associated fibroblasts (CAFs). In contrast, in highly regenerative

epidermal keratinocytes the expression of EGF was detected in most of the cancerous samples with preserved integrity (some of the obtained tissues had only a small fraction of the epidermal layer and an extensive dermal layer).

Similarly, FGR3 immunodetection in normal skin showed moderate to high intensity staining, whereas high levels of FGF3 expression were observed in most neoplastic lesions, which suggests that this growth factor may contribute to enhancement of neoplastic growth. The expression of FGF3 was also present in CAFs in the dermal layer. Detection of VEGF expression in pre-neoplastic, neoplastic and malignant tissues has shown a high level of expression in most tissues. This suggests that VEGF plays an important role in neoplastic growth. The growth factor expression findings support earlier reports that neoplastic growth utilises all possible mechanisms for its survival, including growth factor signalling and overexpression of CYP24A1, to promote a suitable microenvironment for neoplastic growth (Smola et.al., 1993; Mueller 2002; Johnson et.al., 2010).

The neoplastic keratinocyte disruption of the basal membrane allows spread of malignant cells into distal tissues with the assistance of tumour-promoting factors (Mueller 2002; Smola et.al., 1993; Staiano-Coico 1992). It is well-established that growth factor activation of its cognate receptor triggers the activation of the MAPK pathway, which mediates downstream signalling (Luangdilok 2011; Weisinger 2010). The MAPK pathway has been reported to be dependent on VDR (Daniel 2004; Luangdilok 2011; Rossi et.al.,2004; Rosso et.al.,2012; Wang et.al.,2009; Wang et.al., 2013; Wu et.al., 2007).

PCNA expression showed that keratinocytes of normal and neoplastic skin have similarly high proliferative qualities, with the exception of SCC. In SCC, differentiated keratinocytes have extensive level of immunoreactivity for PCNA, whereas in poorly differentiated keratinocytes observed decline in staining intensity. PCNA expression significantly correlated with VDR and CYP24A1 expression in BCC ($p<0.05$), with VDR expression in SCC and CYP24A1 in AK tissues. Interestinly, PCNA in normal skin had statistically significant correlation with EGF and FGF3 in AK lesions ($p<0.05$)

Vitamin D metabolic enzymes and VDR expression in human NMSC were found to have statistical significant correlation amongst CYP27A1, CYP24A1 and VDR, with, EGFR, FGFR3 ($p<0.05$), but not CYP27B1.

4.5 Conclusions

The main goal of this chapter was to determine the expression in vitamin D metabolic enzymes and VDR expression in BCC, SCC, SCCIS, AK. In addition, to determine the status of intratumoural expression of growth-promoting proteins: EGFR, FGFR3, EGF, FGF3, VEGF and PCNA.

The major finding of this study is the immunohistochemical appearance of the enzyme (CYP27B1) that relates to vitamin D metabolism. The expression of CYP27B1 was found to have a significant inverse correlation with the tumour-promoting proteins, FGFR3 and EGFR expression. In addition, for the first time in a single study, we demonstrate the expression status of VDR and key vitamin D metabolic enzymes (CYP27A1, CYP27B1 and CYP24A1), as well as tumour regulatory proteins (FGFR3 and EGFR) in human NMSC (AK, BCC, SCC and SCCIS) and normal skin. These findings may contribute to a better understanding of vitamin D metabolism in cutaneous carcinogenesis and possibly be used as an aid in management of NMSC.

CHAPTER 5.

GENERAL DISCUSSION

5.1 The effects of calcidiol and calcitriol on keratinocyte proliferation and migration

Calcitriol (<10 nM) has been shown to decrease proliferation in a number of tumours, such as breast, colon, prostate (Leyssens, 2013). However, at low concentrations (<10 nM) of calcitriol the inhibitory effects are not always seen. For example, higher doses (100 nM) of calcitriol were required to inhibit immortalised human keratinocytes (HaCaT) and cutaneous squamous cell carcinoma cells (SCL-1) (Trémezaygues et.al., 2010). Likewise, calcitriol only partially inhibited SCC-4 cell line at the same dose (Akutsu et.al., 2001). In addition, squamous cells have been reported to develop strong multidrug resistance, which is a major challenge for the clinical treatment of epithelial cancers (Zhu et.al. 2014). For instance, it has been shown that prostate and glioblastoma cells exhibit resistance to calcitriol treatment (Bao et.al., 2010; Reichrath et.al., 2010). Various mechanisms of this resistance have been proposed in different cells, but no clear signalling mechanism has been confirmed. In SCC cells, the proposed mechanism of resistance to vitamin D signalling involves DRIP protein subunits. DRIP205 directly binds to VDR; silencing DRIP (steroid receptor co-activator) leads to keratinocyte hyperproliferation (Oda et.al., 2004). Moreover, DRIP localisation is mostly present in basal and suprabasal epidermal layers, where keratinocytes undergo constant regeneration (Oda et.al., 2004). However, the mediation of vitamin D activity associated with DRIP complex may also involve other protein influence on tumour regulation. Aberrant nuclear expression of tumour suppressor p53 protein and cytoplasmic expression of Bcl-2 are noted in cervical lesion biopsies, compared to normal tissue (Grace et.al., 2003).

In resistant melanoma cell lines, dose and time dependent antiproliferative properties are apparently stimulated by both calcidiol and calcitriol (Gruber & Anuszewska, 2002). In addition, the effects of calcitriol in various cancer cells have been studied in vitro. Malignant and non-malignant cultured cholangiocytes, incubated with calcitriol (100 nM) results in decreased cell proliferation (Kennedy et.al., 2013). Indeed, calcitriol dependent effects seems to be dependent on the culture conditions, incubation time and dose, to provoke calcitriol- modulatory effects of cell proliferation, cell cycle regulation, lipid content and interleukin (IL)-6/IL-8 secretion in vitro (Krämera et.al., 2008). These conditions were also considered in proliferation and migration assays and immunoblotting experiments in this thesis using primary and neoplastic keratinocytes.

It was demonstrated that calcitriol was able to exert partial inhibitory effects, when used in high concentrations (100,000 nM and 100 nM respectively) especially after 72 h (Kennedy

et.al., 2013). Interestingly, higher calcitriol dose (100 nM) had to be used in achieving inhibition of MCF-7 cells (Narvaez & Welsh, 2001). Correspondingly, it was noted in chapter 2, that in partially resistant SCC-4 malignant keratinocytes, calcitriol inhibitory effects occured using similar concentrations (>100 nM) and time (72 h). However, calcidiol and calcitriol had differential effects on cell migration, where calcidiol (16 – 10,000,000 nM) suppressed malignant keratinocyte movement, whilst calcitriol showed stimulatory effect at low doses (0.01 – 10 nM); at higher dose (>100 nM), inhibition of migration was noted (Chapter 2, Appendix 2.B, Table 5.1). Interestingly, both calcidiol and calcitriol at higher concentrations showed partial anti-proliferative effects to SCC-4 cells in a dose dependent manner.

Table 5.1 Summary of proliferation and migration of SCC4 cells in the presence of calcidiol or calcitriol

	Calcitriol 10 nM	**Calcitriol 100 nM**	**Calcidiol 250 nM**	**Calcidiol 100,000 nM**
Proliferation	↑ Mild stimulatory	↓ Partial Inhibitory	-	↓ Partial inhibitory
Migration	↑ Mild stimulatory	↓	↓ Partial Inhibitory	↓

↓ - significant inhibitory effects, Partial ↓ - partial inhibitory effects, ↑ Mild Stimulatory effect – the stimulatory trend with some significance were noted, (-) no apparent effects were seen.

These findings provoked further questions, on whether these effects were VDR dependent. Additionally, the effects of calcidiol and calcitriol on tumour promoting proteins that may influence cellular proliferation and migration were tested in primary and neoplastic keratinocytes. Hence, protein expression analysis were investigated in immunobloting experiments in response to calcidiol and calcitriol treatment. The expression of these proteins were also evaluated in human tissue samples from patients with NMSC.

5.2 The effects of calcidiol and calcitriol on expression of vitamin D enzymes and receptor in human primary keratinocytes and SCC-4 cells; expression in human neoplastic skin tissues.

Vitamin D enzymes and receptor expression in human skin tissue samples and in cultured neoplastic and normal keratinocytes were determined. In immunoblots of primary keratinocytes and SCC-4 cells (cultured with calcitriol (100 nM) or calcidiol (100,000 nM)) and expression of vitamin D metabolic enzymes (CYP27A1, CYP27B1 and CYP24A1) and its receptor (VDR) were evaluated. In addition, expression of CYP27A1, CYP27B1, CYP24A1 and VDR were analysed using human tissues of normal, precancerous (AK) and neoplastic (BCC, SCC and SCCIS) skin.

Calcidiol and calcitriol effects on the expression profile of CYP27A1, CYP27B1 and CYP24A1 in cultured keratinocytes (malignant and primary) appears not to be VDR-dependent. Supportive to the in vitro findings, it was noted that the expression of CYP27B1 appears not to have a connection with VDR in advanced neoplastic tissues (such as SCC) (Table 5.2). Understanding the influence of vitamin D metabolites on the expression of its metabolic enzymes in malignant and non-malignant cells may aid in a better understanding of cutaneous neoplasms.

Table 5.2 Summary of the expression of vitamin D enzymes and receptor in human keratinocytes treated with calcidiol or calcitriol and expression in human normal skin NMSC tissues

	Calcitriol 100 nM		**Calcidiol 100,000 nM**		**Calcidiol 250 nM**		**Normal skin (NS) and NMSC**				
	SCC-4	PK	SCC-4	PK	SCC-4	PK	**NS**	**AK**	**BCC**	**SCC**	**SCCIS**
CYP27A1	-	-	-	-	-	↑	↑	↑	↑	↓↑	↑
CYP27B1	↓↓	-	-	-	↑↑	↑	↑	↓	↓	↓	↓and ↑mild
CYP24A1	↑	-	↑	-	-	-	↓	↑	↓	↑	↑
VDR	-	-	-	-	↑	-	↑	↑	↑	↑	↑
EGFR	-	-	-	-	-	-	↓↑	↑	↑	↑	↑
FGFR3	-	-	-	↓	↑	↑	↑	↑	↑	↑	↑
PCNA	-	-	-	-	N/A		↑	↑	↑	↑	↑↓

PK, primary keratinocytes; SCC-4, malignant keratinocytes; (-) no effect observed; (↑) protein upregulation (p<0.05); (↑↑) protein upregulation (p<0.005); (↓) protein downregulation (p<0.05); N/A – not available
Normal skin and NMSC: ↑ - intensive DAB staining intensity was detected in majority of tissue, ↓ - poor 3,3′-Diaminobenzidine (DAB) staining intensity was detected in majority of tissues, ↓↑ - variable staining intensity was detected in the given samples, ↓and ↑mild – low to mild staining intensity was detected.

It is apparent that vitamin D plays a role in NMSC by influencing the proliferation and migration of malignant cells. However, a wider array of the target genes need to be examined to understand the molecular mechanism of action. It is also apparent that these effects do not involve VDR mediatory activities in SCC-4 cells. The details of the influence of calcidiol and calcitriol on vitamin D enzymes and receptor and its involvement in tumour regulation are discussed below.

5.2.1. The effects of calcidiol and calcitriol on vitamin D receptor (VDR) expression in cultured neoplastic (SCC-4) and primary keratinocytes; expression levels in normal human skin and NMSC tissues

VDR is a well-recognised mediator of oncogenic processes (Golovko et.al., 2005; Tse et.al., 2007b; Wang et.al., 2009). It is involved in the regulation of cell proliferation, differentiation, apoptosis, cell cycle, migration and cell communication. Hence, the expression levels of VDR in cancer cells is of great interest (Segaert et.al., 2000; Golovko et.al., 2005). It has been

demonstrated in a number of studies that calcitriol-dependent regulation in tumours occurs via nuclear VDR–ligand transcriptional regulation (Haussler et.al., 2010; Srinivasan et.al., 2011; Jiang et.al., 2013).

Protein expression levels by IHC analysis tissue specimens showed an increase in VDR in normal skin, precancerous AK lesions and BCC (that are known to very rarely undergo metastatic spread). Interestingly, VDR expression was not restricted to the epidermal keratinocyte layer, but was also present in dermal layers, particularly in sebocytes, dermal papillae, blood vessels and proliferating keratinocytes (Chapter 4). In addition, a noticeable decline in VDR expression of stromal keratinocytes in SCC, but not in BCC may be one reason for the low metastatic capacities of BCCs. Similarly, VDR expression in malignant melanoma is decreased (Chen et.al., 2009). The proposed mechanism of reduced VDR signalling was reported to be related to melanogenesis (synthesis of pigment intermediates), which affects cellular responsiveness to vitamin D and thus VDR signalling in melanomas (Brożyna et.al., 2013). Furthermore, successively increased levels of VDR expression from normal tissue to precancerous lesions to well-differentiated colonic tumours, and decreased expression in poorly differentiated tumours is established (Murillo et.al., 2007). Similar findings were noted in NMSC tissues, where in well-differentiated AK, BCC, SCC and SCCIS epidermal keratinocytes, prominent levels of VDR immunoreactivity was detected. Additionally, staining of the normal skin showed noticeably high immunoreactivity in VDR.

Moreover, the effects of calcidiol and calcitriol on VDR expression in cultured primary or malignant keratinocytes has been found to have no modulatory activity at high doses calcidiol and calcitriol, whilst at lower calcidiol dose (250 nM) stimulation of VDR was noted only in SCC-4 cells. This is indicative that VDR-modulatory activity had stimulatory effects on proliferation at low doses of calcidiol (< 250 nM). Interestingly, nuclear VDR translocation in calcidiol (100 nM) treated immortilised prostate cells appears to depend on calcidiol but not calcitriol, where nuclear import of VDR is closely linked to the transcriptional activation (Munetsuna et.al., 2014). Moreover, in MCF-7 breast cancer cells vitamin D resistance has been shown not to be dependent on VDR expression (Ooi et.al., 2010). Where vitamin D-resistant MCF-7 cells did not have apoptotic morphology, DNA fragmentation or up-regulation of apoptosis-related proteins after treatment with calcitriol, they did express VDR (Ooi et.al., 2010). Furthermore, vitamin D resistance in malignant melanocytes was shown to be associated with poor VDR expression (Seifert et.al., 2004). Indeed, VDR plays an important role in cancer; however, the mechanism of its effects remains elusive, especially in NMSC. More studies are needed to establish its mode of

action and interactions with other cell signalling pathways in various cancers, including cutaneous malignancies, such as NMSC.

5.2.2 The effects of calcidiol and calcitriol on CYP27A1 expression in cultured neoplastic (SCC-4) and primary keratinocytes; expression levels normal huan skin and NMSCtisssues.

As a P450 enzyme, CYP27A1 is known as a cholesterol-metabolising molecule (Holick, 2010). The CYP27A1-knockout mice, which have normal serum cholesterol levels, show alterations in various tissues (ocular organs, lungs, liver and spleen) with activation of macrophages and high levels of oxidative stress markers (Saadane et.al., 2014). Cholesterol metabolism is tissue specific, and its significance has not yet been established in many tissues, in which CYP27A1 may also be expressed and may involve in calcidiol synthesis. Hence, CYP27A1 plays a role in cholesterol homeostasis, and has been proposed to have important effects in cancer (Bergada et.al., 2014).

It has been shown that vitamin D has a positive regulatory influence on immune responses (Orgaz-Molina et.al., 2012). It was reported that the availability of calcidiol to immune cells in patients with psoriasis may be the primary determinant of the amount of calcitriol produced for intracrine and paracrine effects at sites of cutaneous inflammation (Finamor et.al., 2013). In mice, it has been reported that topical application of calcitriol induced atopic dermatitis-like syndrome in the epidermal keratinocytes of transgenic RXR-α/-β mice (Li et.al., 2006). These finding also may indicate the possible vitamin D signalling mechanisms in which VDR/RXR signalling could be involved in the pathogenesis of cutaneous inflammation (Li et.al., 2006).

The principal function of CYP27A1 in cancers has been poorly understood. In endometrial cancer tissues elevated CYP27A1 levels, suggest potential defensive effects by cells through increased intratumoural calcidiol synthesis by CYP27A1. This in turn enhances autocrine/paracrine antiproliferative actions (Bergada et.al., 2014). Another theory is that during intracellular synthesis of calcidiol, pathways that mediate tumour regulation are de-activated, hence directing the fate of mutated neoplastic cells to decrease proliferation. This mechanism of CYP27A1 regulatory antitumour activity was shown (Bergada et.al., 2014).

Moreover, it was suggested that CYP27A1 may be involved in a regulatory role in human prostate cancer, where prostate epithelial cells produce appreciable levels of CYP27A1 when treated with cholecalciferol (a derivative of 7-DHC), which was suggested to act by blocking or reversing premalignant changes (Tokar & Webber 2005a). Further, in vitro findings showed the effects of CYP27A1 enzymatic activity in neoplastic prostate cells treated with cholecalciferol, via production of inhibitory intracrine calcidiol and calcitriol that subsequently lead to tumour suppression (Tokar & Webber, 2005b).

Expression CYP27A1 was examined in both in vitro experiments and in human tissues derived from normal, precancerous and neoplastic skin. Protein expression of CYP27A1 in malignant SCC-4 cells and primary keratinocytes were analysed by western blot. Elevated levels of CYP27A1 expression were noted in primary keratinocytes, when cells were treated with 250 nM calcidiol, whilst effects of calcidiol at higher concentrations on CYP27A1 expression were not detected in malignant SCC-4 cells; calcitriol had no effect. This is suggestive that at higher concentrations of calcidiol and calcitriol the responsiveness of CYP27A1 is diminished.

Interestingly, IHC analysis demonstrated high CYP27A1 staining intensity in human AK, BCC, SCC and SCCIS neoplastic skin lesions. However, CYP27A1 detection showed low staining intensity in tumour stromal cells, which may indicate a malfunction of vitamin D associated signalling in the tumour stroma itself. Interestingly, in some SCC tissues the expression of CYP27A1 was found to be low, compare to normal skin. This triggers the question of whether CYP27A1 has other (non-metabolic) functions in metastatic skin tumours (such as SCC).

Increased CYP27A1 levels in cancer tissues are believed to be a possible compensatory mechanism for its low levels of CYP27B1 expression (Bergada et.al., 2014). However, the results can, perhaps, be explained by another theory that was suggested previously: that CYP27A1 overexpression in neoplastic tissues is a cellular attempt to compensate for low systemic calcidiol levels that possibly directly (or indirectly, via calcitriol conversion) exerts antitumourigenic effects (Tokar & Webber 2005a; Sharan et.al., 2011; Bergada et.al., 2014).

Indeed, clinical reports on circulatory calcidiol status in NMSC are limited and contradictory (Tang et al., 2010; Liang et.al., 2012). Interestingly, some identified mutations of CYP27A1 have been associated with coronary heart disease, haemoglobin binding defects, cerebrotendinous xanthomatosis (a lipid storage disorder), abnormal synthesis of bile acids, development of cataracts, tendon xanthomas and progressive neurologic deterioration (Guptaet.al., 2007; Inanloorahatloo et.al., 2013; Prosser et.al., 2006). It is not known whether mutations of CYP27A1 are associated with skin cancer, and if so, the effects they may have on the development of NMSC.

Whilst, CYP27A1 appears to be not regulated by high doses of calcidiol or calcitriol, in most human skin (including neoplastic lesions) it certainly has elevated expression, with exception to stromal keratinocytes. CYP27A1 involvement in tumour growth and progression requires further investigations.

Hence, the activity of CYP27A1 and its link to cancer progression requires further studies. There is a lack of clinical studies reporting the level of circulatory and localised synthesis of

calcidiol, suggesting a need for further investigations to determine whether CYP27A1 reflects the progression of cutaneous carcinogenesis.

5.2.3 The effects of calcidiol on CYP27B1 in cultured neoplastic (SCC-4) and primary keratinocytes; expression levels in normal skin and tumours from NMSC patients

CYP27B1 is an enzyme that is involved in the synthesises of the active vitamin D hormone calcitriol. The activity of this protein is associated with the intracellular and systemic levels of calcitriol. It has been shown in enzymatic studies of the substrate specificity of CYP27B1 that vitamin D 25-hydroxyl group is essential for CYP27B1 function (Inouye & Sakaki, 2001). Numerous reports have shown that CYP27B1 is expressed in human non-malignant and malignant cells in different tissues (Zehdner et.al., 2000; Diesel et.al., 2004; Young et.al., 2004; Tokar & Webber 2005a; Bergada et.al., 2014).

It has been shown that disruption or deficiency of CYP27B1 is associated with longer recovery from infection, greater susceptibility to disease and lower immune responses in CYP27B1-knockout mice with chlamydial infections (He et.al., 2013). In addition, mice lacking CYP27B1 results in decreased epidermal differentiation and barrier function (Bikle et.al., 2004). Hence, epidermal CYP27B1 enzyme activity is required for normal differentiation of keratinocytes in vivo (Bikle et.al., 2004). Interestingly, extra-renal CYP27B1 activity, has been reported to cause excessive serum calcitriol (Jones, 2013). Calcitriol has been reported to have a major regulatory role in calcium homeostasis in differentiated keratinocytes in vivo (Bikle et.al., 2004).

It was shown in human endometrial carcinomas elevated levels of CYP27B1 expression is present in the early stages of neoplastic development, and decreased with cancer progression (Bergada et.al., 2014). In colon cancers, CYP27B1 is strongly expressed in normal, premalignant and malignant colonic epithelial cells, irrespective of tumour cell differentiation (Murillo et.al., 2007). Of interest, calcitriol antiproliferative properties (at high concentration, 100 nM) in cholangiocarcinoma patients demonstrated that CYP27B1 expression is similar in non-malignant versus malignant tumours (Kennedy et.al., 2013).

Interestingly, reduced expression of CYP27B1 was noted in malignant keratinocytes in response to 100 nM calcitriol treatment, without noticeable effects in primary keratinocytes (Chapter 3). This is supportive towards published literature showing similar CYP27B1 reduction (Bikle et.al., 2002). In contrast, calcidiol (250 nM) exerted stimulatory effects on CYP27B1 levels in primary and malignant keratinocytes. The lack of antiproliferative effects noted in SCC-4 cells

treated with 250 nM, suggests that elevated CYP27B1 expression may have a regulatory role in SCC-4 cell survival.

These findings were confirmed by IHC evaluation of CYP27B1 expression in normal skin and NMSC tissues. It was demonstrated that CYP27B1 ranging from poor to moderate expression in cancer tissue, however, decreased CYP27B1 expression was noted in advanced stages of cutaneous neoplastic leisions (such as SCC), where only minimal CYP27B1 expression was detected. Contrastingly, in normal skin, expression of CYP27B1 was prominent. This is indicative of the importance of CYP27B1 involvement in normal skin homeostasis and its involvement in regulation of neoplastic progression.

Whilst it is not established whether the inhibitory effects on cancer cells occur via VDR/CYP27B1 pathways, studies have demonstrated that SCC-4 cells treated with a berberine-derived compound showed antitumour activity via inhibition of NF-κB activation (Ho et.al., 2009). This decrease in *NFKB1* gene expression suggests that anticancer activity may be due to ERK1/2 MAPK and NF-κB signalling pathways. This is indicative that the antiproliferative effects observed in SCC-4 cells could also recruit alternative modulators when treated with calcidiol or calcitriol and requires further investigations.

5.2.4 The effects of calcidiol and calcitriol on CYP24A1 in cultured neoplastic (SCC-4) and primary keratinocytes; expression levels in normal human skin and NMSC tissues

CYP24A1 is a cytochrome P450 enzyme, and is known to catalyse hydroxylation reactions at C-23 and C-24 of the side chain of both calcidiol and its hormonal form, calcitriol (Jones et.al., 2012). The principal role of CYP24A1 is not only the 24-oxidation of vitamin D to produce calcitroic acid, also known as biliary catabolite, but also the catalyst is of a similar pathway that is initiated from 23-hydroxylation and culminates in the production of calcitriol–26,23-lactone (Ramsay et.al., 2008). The degradation of calcidiol and calcitriol by CYP24A1 results in by-product formation (lactone and calcitrolic acid) that alters the tumour microenvironment to create more favourable environment for neoplastic cell growth conditions. It is important not to underestimate the significance of CYP24A1, as it is involved in homeostatic regulation of vitamin D in normal cells and has an altered activity in neoplastic cells (Guoan et.al., 2011; Luo et.al., 2012). It is unclear, whether the neoplastic cell attempts to compensate for the inhibitory actions of vitamin D (calcidiol and calcitriol) by overexpressing CYP24A1, that produces inactive intermediates (1,24,25-trihydroxyvitamin D3, 24-oxo-1,25-dihydroxyvitamin D3, 24-oxo-1,23,25-trihydroxyvitamin D3 and tetranor-1,23-dihydroxyvitamin D3) of C24-oxidation pathway and calcitriolic acid that are easily excreted (Tieu et.al., 2014). Moreover, lactone overexpression

is associated with increased malignant progression and altered pH of the tumour microenvironment (Ramsay et.al., 2008). Interestingly, it was demonstrated that 24-hydroxylation metabolic product 24R,24,25-(OH),D when used at high concenrations in chicks, was capable of minor stimulation of intestinal calcium absorption. Whilst, in treated rats the effect was not noticed, in contrast to that calidiol, shown marked stimulation of intestinal calcium absorption. A comparison of the metabolism of 25-hydroxy [26,27- 3H] vitamin D3 and 24,25-dihydroxy [26,27-3H] vitamin D via HPLC, in the rat and chick shows that 24,25-dihydroxyvitamin D, and 1,24,25-trihydroxyvitamin D, disappear at least 10 times more rapidly from the blood and intestine of chicks. These results demonstrate that 24,25-dihydroxyvitamin D, is significantly less biologically active in the chick than in the rat, suggesting on more rapid metabolism and excretion (DeLuca et.al., 1976).

It has been reported that overexpression of CYP24A1 is associated with attenuation of vitamin D signalling and the more advanced stages of malignant conditions (Guoan et.al., 2011; Annalora et.al., 2010).

CYP24A1 expression has been studied in a number of cancers. For example, CYP24A1 is strongly expressed in breast carcinomas (Mimori et.al., 2004). It was suggested that overexpression of CYP24A1 may be associated with systemic and intratumoural, or local tissue specific, vitamin D metabolic status and enzymatic expression (Mimori et.al., 2004). Indeed, CYP24A1 activity has been associated with cancer progression. However, there are some inconsistencies in the expression of CYP24A1. While the published studies agree on profound overexpression of CYP24A1 in colon, ovary and lung carcinoma tissues, contrastingly, in breast tumours *CYP24A1* has decreased transcription of mRNA relative to normal analogous tissues (Anderson et.al., 2006). Furthermore, increased CYP24A1 expression has been reported in human prostate cancers (poorly differentiated and highly advanced stages), which was correlated with a parallel increase in the tumour proliferation rate (Tannour-Louet et.al., 2014). Moreover, it was shown in non-small cell lung cancer cells and primary human lung tumours that CYP24A1 has an aberrant expression that restricts transcriptional regulation and growth control by calcitriol in these cells (Zhang et.al., 2013). It could be assumed that similar mechanism of this enzyme activity may exist in malignant keratinocytes in response to calcitriol (or calcidiol).

IHC analysis of human cutaneous cancers revealed that CYP24A1 expression is significantly enhanced in most NMSC (Chapter 4). This has also been noted in colon, prostate and breast cancers (Guzey et.al.,2002; Mimori et.al., 2004; Leyssens et.al., 2013). Similar effects have also been noted in other cancer cells (Anderson et.al., 2006; Luo et.al., 2012). Intense staining for

CYP24A1 was also shown in human NMSC tissues (Chapter 4). Interestingly, the more aggressive tumours had the most intense staining for CYP24A1, whilst in normal skin CYP24A1 expression were noted to be mostly diminished. It seems that CYP24A1 has a definite role in tumour persistence and is a hallmark of most malignancies. It is not clear whether the inhibition of CYP24A1 would be associated with a better clinical outcome and hence a reduction in patient morbidity.

It was reported that in normal human keratinocytes the inhibition of CYP24A1 activity triggered an increase in and stabilisation of both vitamin D intracellular metabolites: calcidiol and calcitriol (Schuster et.al., 2001). In contrast, a significant elevation of CYP24A1 activity was observed in prostatic neoplastic cells when treated with high doses of cholecalciferol, calcidiol precursor (Tokar & Webber 2005b).

Interestingly, the immunoblotting results (Chapter 3) showed that treatment of SCC-4 with calcidiol (100,000 nM) stimulates the expression of CYP24A1 in malignant (but not in normal) keratinocytes. The stimulation of CYP24A1 expression in neoplastic cells is in agreement with published literature (Tokar & Webber 2005b). The contrasting lack of effect at selected calcidiol and calcitriol doses in normal keratinocytes in CYP24A1 expression indicates that SCC-4 cells have a higher sensitivity in CYP24A1 expression to calcidiol and calcitriol treatment. This sensitivity, perhaps, may relate to neoplastic cell survival, tumour growth and progression, that has been proposed by several studies (Guoan et.al., 2011; Luo et.al., 2012).

Our cumulative data (from IHC and in vitro studies) suggest that neoplastic keratinocytes have altered CYP24A1 expression that may contribute to further tumour progression. It is imperative to understand the role of CYP24A1 in cutaneous neoplasm and its regulation by calcidiol and calcitriol. It was indicated that development of a molecule that has antitumour effects without activating CYP24A1 may lead to positive outcomes (Luo et.al, 2012). Trying to mimic the cancer-inhibitory effects that are exerted by vitamin D metabolites has led to the development of vitamin D analogues, which have inhibitory effects with minimal activation of CYP24A1. Indeed, QW-1624F2-2, a synthetic analogue of calcitriol, was shown to suppress the invasiveness of human breast cancers by having an inhibitory effect on CYP24A1 expression (Sundaram et.al., 2006).

5.3 The effects of calcidiol and calcitriol effects on EGFR expression in cultured neoplastic (SCC-4) and primary keratinocytes; expression levels in normal human skin and NMSC tissues

The activation of EGF membrane receptor (EGFR) by serum EGF binding, is essential for physiological functions of the skin (Sibilia et.al., 2000). Epidermal keratinocyte regeneration is largely dependent on the EGFR signalling pathway (Fraguas et.al., 2011). It has been shown that EGFR is a major mitogenic signalling entity in calcitriol metabolism in keratinocytes (Garach-Jehoshua et.al., 1999). The EGFR signalling that leads to cell hyperproliferation and angiogenesis, is highly expressed in a variety of malignant tumours, and its expression has been correlated with disease progression and poor survival in many cancers (Bondzi& Allison, 2013; Han & Lo, 2012).

In IHC experiments, detection of EGFR in invasive SCC showed strong expression in well-differentiated keratinocytes. Surprisingly, similarly high levels of EGFR staining intensity were seen in AK tissues, where EGFR expression was detected in cutaneous sebocytes. Less intense staining for EGFR was noted in poorly differentiated keratinocytes in BCC. Interestingly, in normal skin the expression of EGFR had mostly moderate staining (Chapter 4), which is obviously an essential protective factor for highly regenerative tissue (Pastore et.al., 2008).

The ability of EGFR to affect the cell cycle and differentiation has been shown to be different in healthy and neoplastic cells (Puccinelli et.al., 2010; Runkle et.al., 2012). Calcitriol shown to inhibit autocrine EGFR activation in neoplastic EGFR-overexpressing cells, which resulted in reduced phosphorylation of ERK1/2, the main growth signal of the MAPK pathway. In aggressive malignant tumours driven by enhanced EGFR expression, prevention of nuclear phosphorylated ERK1/2 translocation was used as an indicator of the efficacy of anti-EGFR therapy (Dusso et.al., 2004).

Calcitriol has been reported as an agent that regulates EGFR and related ErbB family member activities. This could represent a valuable treatment in many cancers that respond to EGFR targeted therapy. For instance, in patients with head and neck cancers, where tumour location would compromise the choice of treatment, many tumours displayed intrinsic or acquired resistance to EGFR inhibitory drugs. In metastatic SCC, EGFR inhibitory agents are currently moving through pre-clinical and clinical development (Cohen, 2014).

In the human immortalised keratinocytes HaCaT, treatment with calcitriol has been found to increase autonomous cell proliferation, in the absence of exogenous growth factors such as EGF. It was shown that this stimulation is calcitriol and EGF dependent (Garach-Jehoshua et.al., 1999).

Results of immunoblot studies demonstrated that calcitriol and calcidiol at antiproliferative doses (100 nM and 100,000 nM respectively) had no significant effects on EGFR expression in keratinocytes (both, malignant and primary). Perhaps keratinocytes may have selective sensitivity to calcidiol and calcitriol in their expression of EGFR, as these metabolites may interfere with major tumour pathways and bypass the inhibition of the essential growth promoting marker in highly regenerative keratinocytes.

Interestingly, the development of drugs with the targeted suppression of EGFR has resulted in an anti-EGFR tyrosine kinase inhibitor (TKI), erlotinib, which is currently in phase III trials for oral SCC prevention (Cohen, 2014). Numerous other drugs are in earlier stages of development for head and neck SCC treatment, including novel anti-EGFR monoclonal antibodies (MEHD7945A, necitumumab and RO5083945), small-molecule TKIs (vandetanib, icotinib and CUDC-101), EGFR antisense, various add-on therapies to radiation and chemotherapy (bevacizumab, interleukin-12, lenalidomide, alisertib and VTX-2337) and drugs (temsirolimus, everolimus, OSI-906, dasatinib and PX-866) intended to overcome resistance to anti-EGFR agents (Cohen, 2014).

5.4 The calcidiol effects on FGFR3 expression in cultured neoplastic (SCC-4) and primary keratinocytes; expression levels in normal human skin and NMSC tissues

FGFR3 plays a significant role in mouse skin carcinogenesis, where the expression of FGFR3 showed a strong correlation with neoplastic development in transgenic mice (Li et.al., 1998). Transgenic mice, with targeted activation of mutant FGFR3 in basal cells of the epidermis develop benign epidermal tumours without metastatic morphology (Logié et.al., 2005). This study also performed a screen for FGFR3 mutation in seborrheic keratosis lesions, and noted that a large proportion of tumours had FGFR3 mutations (Logié et.al., 2005). In human Kaposi's sarcoma tissues, expression of FGFR3 correlates with tumour progression. FGF3 binding to FGFR3 results in receptor activation, with consequential activation of MAPK and further potential downstream signalling (Iwata & Leung, 2012; Weisinger et.al.,2010).

In breast tumours, FGFR3 has been classified as a mammary oncogene together with Wnt1 (Hollmann et.al., 2001). Interestingly, Wnt1 signalling is involved in SHH activation of downstream mediators and has been shown to play a role in SCC and BCC tumourigenesis (Miyazaki et.al., 2005). Furthermore, it was shown in a rat chondrosarcoma cell line that FGFR3 is involved in the inhibition of cell proliferation and FGFR3 regulates bone development in rodents (Sahni et.al. 1999). Aberrant signalling of FGFR3 in neoplastic cells was associated with enhanced proliferation and cellular resistance to apoptosis (Chaffer et.al., 2007). It has also been shown that

FGFR3 can activate different signal transduction pathways, and regulates the early stages of tumourigenesis in mammary epithelial cell lines (Fioravanti et.al. 1997).

The immunoblotting results demonstrated elevated FGFR3 protein expression in responsive to calcidiol (250 nM) treatment in primary and malignant keratinocytes (Chapter 3). Interestingly, calcidiol inhibitory effects (100,000 nM) were obvious only in primary, but not in neoplastic keratinocytes (Chapter 3), whilst, lack of effect was noticed in both, primary and neoplastic keratinocytes, treated with 100 nM calcitriol (Chapter 3).

This is indicative that SCC-4 cells have dysfunction in regulation of epidermal homeostasis, where proteins responsible for tissue regeneration have aberrant responses to calcidiol, compared to normal cells. It is obvious that calcidiol is involved in the regulation of FGFR3; however, the mechanism of this signalling is not clear.

The results of IHC study showed that FGFR3 expression is an important aspect in epidermal keratinocytes. It was noted in stromal keratinocytes of SCCIS and keratinocytes of AK lesions elevated expression of FGFR3; however, the strongest levels of expression were noted in BCC and SCC (Chapter 4). Interestingly, in the majority of normal skin tissues moderate to high levels of FGFR3 expression was detected, suggesting a role of FGFR3 role in normal skin homeostasis.

Taken together, these studies suggest a connection between neoplastic formation and tissue FGFR3 expression. The possible mechanism of calcidiol and calcitriol involvement in the regulation of FGFR3 expression in human skin has not been investigated (to our knowledge). The FGFR3 expression in NMSC and in calcidiol or calcitriol treated keratinocytes is shown for the first time as per authors knowledge.

5.5 Determination of PCNA expression in cultured neoplastic and primary keratinocytes; expression levels in normal human skin and NMSC tisssues

The expression of proliferating cell nuclear antigen (PCNA) represents an interest for this study as PCNA is predominantly synthesised in the late G1 and S phase of the cell cycle and considered to be the a useful marker for proliferating cellular capacity. The evaluation of PCNA expression in human NMSC lesions and normal skin tissues demonstrated that PCNA is prominently expressed in highly proliferative epidermal keratinocytes of the vast majority of NMSC samples and in normal tissue. This suggests that cellular responsiveness to the growth promoting factors is similar in malignant and primary keratinocytes. This is supported by existing literature (Penneys et.al., 1994; Einspahr et.al., 1999; Moor et.al., 2004). Furthermore, the expression of PCNA was examined in response to calcidiol and calcitriol treatment in immunobot experiments.

Surprisingly, lack of differential effects were shown in primary and neoplastic keratinocytes. The results of these studies suggest that PCNA is an important protein in the epidermis, and that is not influenced by either of the vitamin D metabilotes (calcidiol or calcitriol). These findings indicate that the mechanism of calcidiol and calcitriol molecular pathways may not involve PCNA signalling in human keratinocytes.

CHAPTER 6.

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Concluding remarks

Whilst vitamin D metabolites are regarded as tumour inhibitory agents in some cancer types, it was shown that its intracrine metabolism is involved in the regulation of neoplastic cell growth and proliferation (Bikle et.al., 2011a; Bikle et.al., 2013). Numerous studies demonstrate the anticancer properties of calcidiol and calcitriol (Campbell et.al., 2009; Leyssens et.al., 2013; Krishnan et.al., 2013; Jiang et.al., 2013; Guan et.al., 2013).

Prior to summarising the major findings of in vitro and immunohistochemical studies, it is important to emphasise that the main focus of this thesis was to determine the effects of calcidiol on neoplastic keratinocytes in NMSC. The effect of cacitriol was investigated in order to examine whether the observed effects were due to calcidiol itself (or possibly due to its intracellular conversion to calcitriol). It was concluded that calcidiol has an independent mechanism of action from calcitriol, where calcidiol inhibitory effects on neoplastic cell migration (Chapter 2), and differential effects on protein expression (Chapter 3), are convincing evidence in support to this observation.

The major findings of the experiments are:

1. Protein detection studies in cultured human cutaneous cells by immunoblotting showed for the first time in one study that CYP27B1 and FGFR3 in human primary and neoplastic epidermal cells are responsive to calcidiol, which is in agreement with the current literature (Schuster, 2011). It was noted that CYP24A1 had elevated expression in response to calcidiol and calcitriol treatment in neoplastic keratinocytes, but not in primary. The molecular mechanism of this mediation was not investigated; however, calcidiol (250 nM) treatment stimulates expression of VDR and metabolic vitamin D enzymes in SCC-4 cells, which are in agreement with CYP450 enzymatic levels detected in human NMSCs.
2. The effect of calcidiol on the expression FGFR3 was shown to be calcidiol (at 250 nM) dependent in cultured human primary and malignant keratinocytes
3. In NMSC human tissues CYP24A1 expression is greatly enhanced, which may contribute to progression of carcinogenesis and possible metastatic spread (Luo et.al., 2012).
4. In human tissue specimens, vitamin D enzyme (CYP27B1 and CYP24A1) expression may be involved in NMSC progression to more aggressive cancer types. Study of the growth

factors expressed in human NMSC tissues suggests that the protein expression of the vitamin D metabolic enzyme CYP27B1 has an inverse relationship with the expression of growth factors (EGF, FGF3 and VEGF). This is a crucial factor for the progression from the skin cancer precursor, AK, to malignant SCC, which also agrees with the current literature (Pietras & Östman, 2010).

5. Further to this, it was demonstrated that calcidiol and calctriol treatment exert partial antiproliferative effects in malignant keratinocytes (SCC-4 cells).
6. Finally, FGFR3 protein expression was shown in AK, BCC, SCCIS and SCC. FGFR3 expression was found to be a critical mode of action in highly proliferative neoplastic keratinocytes of NMSC. However, the mechanism of FGFR3 regulation by calcidiol remains unclear.

These findings all together indicate that calcidiol exerts antiproliferative effects in malignant keratinocytes. These effects appear not to have VDR-mediatory regulation. Considering these findings, the development of novel calcidiol-based anti-skin cancer therapies or preventative strategies should be based on an understanding of calcidiol interactions in NMSC prevention and treatment.

6.1 Future directions

The dilemma of sun exposure and skin cancer still exists. The promising prospects of supplementary sources of vitamin D have been shown in animal models (Bikle et.al., 2011). It was demonstrated that supplementation using the vitamin D precursor cholecalciferol caused the reduction and regression of skin tumours in mice (Bikle et.al., 2011). However, animal research is not always in agreement with human studies, due to the variation of genomic responses between different species to the treatment.

However, while animal research does not always agree with the human situation, NMSC remains a leading malignancy in Australia, which creates a need for futher clinical studies to aid in a better understanding whether supplementation of the ‘sunshine’ vitamin, or controlled solar exposure, will help reduce the incidence of NMSC and help to improve the morbidity and mortality of this disease.

1. This study attempted to determine the role of calcidiol in the development and progression of NMSC. There is a need to further investigate the influence of this

metabolite on intracrine pathways in the transformation of precancerous lesions to malignant types.

2. Further studies are also required in order to determine the efficacy of vitamin D and its role in skin cancer prevention, as its application alone or in combination with other tumour inhibitory compounds may improve the condition.
3. The particular focus should be directed in investigating the molecular mode of action of calcidiol. Herein, the high expression of CYP24A1 protein was inversely associated with low intracrine expression of the anabolic vitamin D enzyme CYP27B1. It is well known that some NMSCs, such as SCC, are classified as some of the most lethal metastatic tumours (Hoekstra, 2008; Ratushny et.al., 2012). The ablation of CYP24A1 activity in tumour cells could lead to a reduction in metastatic capacity and permit the inhibitory activity of calcidiol. Therefore, the recruitment of possible therapeutic agents that inhibit CYP24A1 activity in neoplastic cells would represent a milestone in NMSC.
4. Antimigrative effects of calcidiol were apparent. However, it would be interesting to explore whether similar effects would be observed in assays using boyden chambers or in automated quantification of cell migration and invasion in real time under physiological conditions. Further experiments would also include investigations of calcidiol effect with siRNA inhibition of CYP27B1 (or VDR) in malignant SCC-4 keratinocytes, to determine whether the antiproliferative effects of calcidiol are induced by calcidiol itself or whether intracellularly generated calcitriol is responsible for this effect.
5. In addition, confirming the expression of VDR in response to inhibitory and stimulatory doses of calcidiol and calcitriol by flow cytometry in SCC-4 cells compared to primary keratinocytes is required. This may help to determine the role of VDR in skin cancers.
6. Furthermore, it would be informative to evaluate the effects of calcidiol in animal studies using calcidiol (either as dietary supplementation or as intratumoural injection) and determine its antiproliferative properties in vivo.
7. Moreover, future studies are required to determine the preventative effects of calcidiol and calcitriol on neoplastic transformation and progression to malignancy and in NMSC in animal models and in human clinical studies.

CHAPTER 7.

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APPENDIX A. CELL MIGRATION PROTOCOL

To determine the effects of calcidiol and calcitriol on SCC-4 cell migration abilities, scratch assays were conducted. SCC-4 cells were seeded into 24-well tissue culture plates at a density of 5 x 10^5 cells per well. During the optimisation of SCC-4 cell density, the sporadic cell growth in monolayers with frequent cell detachment and the abundance of floating cells in the medium was observed. An example of colony formation by SCC-4 cells can be seen in Figure A.1.

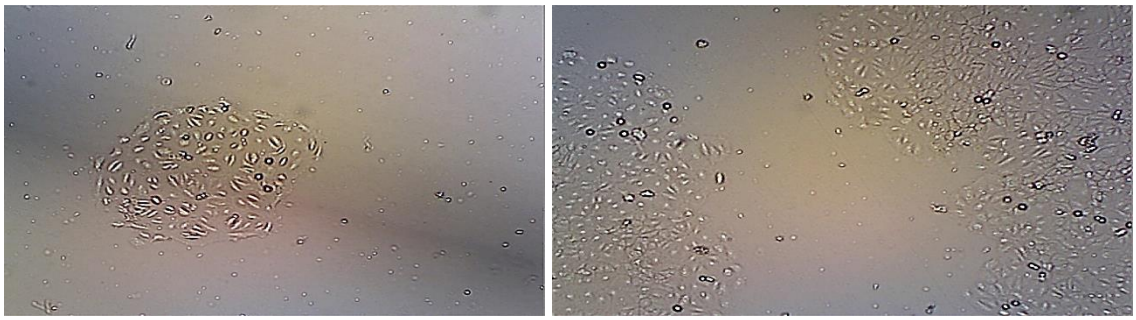


Figure A.1. Cultured SCC-4 cell line growth patterns.

SCC-4 cells were grown in complete culture medium as described in the materials and methods section of Chapter 2 (section 2.2.4). Cells were frequently detached from the colony, causing excessive floating of cellular debris and dead cells. The initiated growth of the neighbouring colonies would have a patchy pattern, and complete confluency of SCC-4 cultures largely depends on an even cell distribution at the time of culture inoculation. Immediately after seeding, the cultures of SCC-4 cells were placed on a rotary platform (Solid State Control Multimix Platform; Ratek, Melbourne, Australia) for brief and gentle agitation, to ensure that the tissue culture flask or plate surface has an even distribution of cells, with subsequently even colony formation.

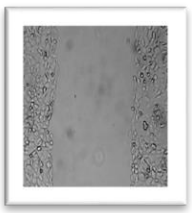
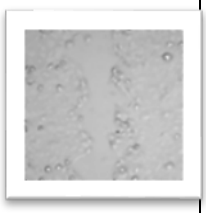
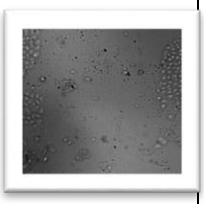
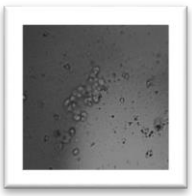
To ensure complete cell attachment, 5 x 10^5 SCC-4 cells per well were allowed to adhere over a 24 h period. Untreated SCC-4 cultures were used as a control in these experiments. After 24 h of cell settlement, each well was gently and slowly scratched with a 1000 µl pipette tip across the centre of the well. While scratching across the surface of the well, the pipette tip was held perpendicular to the bottom of the well, to ensure that the distance between the scratch edges is equal across the scratch length, and the scratch was always applied in one direction. After scratch application, each well containing SCC-4 cells was gently washed three times with 1x PBS (pH 7.4) to remove the detached cells.

The medium was replenished in the untreated control wells with fresh complete medium; in vehicle control wells with complete medium supplemented with ethanol at the appropriate concentrations; and in treatment wells with medium contained calcidiol or calcitriol at various concentrations. At the indicated time points (1 h, 4 h, 24 h, 48 h and 72 h) the cells were photographed using an inverted microscope (Labomed TCM) at 20x magnification using the same

microscope configurations at each time point. The gap distance in each well was quantitatively evaluated using Carl Zeiss AxioVision, LE 54 software (Carl Zeiss, Germany). To reduce variability in the results, each experiment was repeated three times

Table A.1 Effect of vitamin D metabolites on the migration abilities of SCC-4 cells)

A.1.a) Effect of calcidiol on SCC-4 cell migration

Time (h)	Untreated	Calcidiol 250 nM	Corresponding ethanol control	Calcidiol 100,000 nM	Corresponding ethanol control
1					
24					
48					
72					

P values to Figure 2.3 presented in Table 2.3 (main text):

1. Statistical significant p values for calcidiol treated SCC-4 cells compared to corresponding ethanol controls in SCC-4 cell migration at time points 24, 48 and 72 h is shown in Table 2.3:

Table 2.3.1

	Time (h)		
Calcidiol dose (nM)	24	48	72
16	0.159	0.063	0.011
100	0.027	0.002	0.009
250	0.039	0.0019	0.003

2. Statistical comparison between time points in calcidiol treated cells at each dose p values in migration assay is shown in Table 2.4

Table 2.3.2

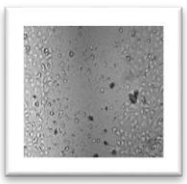
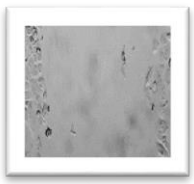
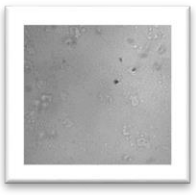
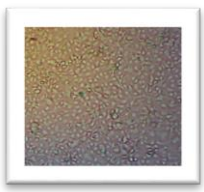
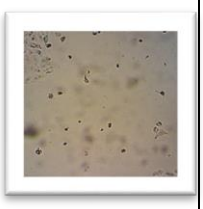
	Time (h)		
Calcidiol dose (nM)	24	48	72
16	0.001987	0.012439	0.00071
100	0.005548	0.04871	0.044042
250	0.006511	0.024236	0.039279

3. Statistical comparison p values between doses in calcidiol treated cells at each time point in migration assay is shown in Table 2.5

Table 2.3.3

	Time (h)		
Calcidiol dose (nM)	24	48	72
16/100	0.0113	0.0142	0.0635
16/250	0.0124	0.0426	0.0472

A.1.b) Effect of calcitriol on SCC-4 cell migration

Time (h)	Untreated	Calcitriol 10 nM	Corresponding ethanol control	Calcitriol 100 nM	Corresponding ethanol control
1					
24					
48					
72					

P values to Figure 2.4 (main text):

1. Statistical significant p values for calcitriol treated SCC-4 cells compared to corresponding ethanol controls in SCC-4 cell migration is shown in Table 2.4.1:

Table 2.4.1

Calcitriol dose (nM)	Time (h)
	24
0.1	0.032
1	0.075
10	0.037

2. Statistical comparison between time points 1-24 h in calcitriol treated cells at each dose p values in migration assay is shown in Table 2.4.2

Table 2.4.2

	Time (h)
Calcitriol dose (nM)	P values between 1 and 24 h
0.1	0.003406
1	0.018443
10	0.01542

3. Statistical comparison p values between doses in calcitriol treated cells at time point 24 h in migration assay is shown in Table 2.4.3

Table 2.4.3

	Time (h)
Calcitriol dose (nM)	24
0.1/10	0.00033
1/10	0.00002

Statistically significant stimulation by calcitriol treatment was noted in migration assay (Figure 2.4, main text)

APPENDIX B. CELL PROLIFERATION PROTOCOL

CellTiter-Blue Cell Viability detection

The seeding density of SCC-4 cells for 96-well plates was optimised. Due to the sporadic and slow nature of SCC-4 cell growth and the extended incubation time with CellTiter-Blue Cell Viability active reagent required for generation of a fluorescent signal, it was decided to use 3 x 10^4 cells per well. SCC-4 cells at 3 x 10^4 cells/well were plated in 96-well plates for 24 h, followed by treatment with or without calcidiol (at 16 nM, 50 nM, 100 nM and 250 nM) or calcitriol (at 0.01 nM, 0.1 nM, 1.0 nM and 10 nM) for 1 hour (h), 4 h, 24 h, 48 h and 72h. To create working solutions, both vitamin D compounds were dissolved in 100% ethanol, as per the manufacturer's instructions. The SCC-4 cells were grown as a monolayer. Ethanol-treated (equivalent to the volume of vitamin D) SCC-4 cells were used as a vehicle control, and untreated cells were used as a negative control.

To measure the number of viable vitamin D (calcidiol and calcitriol) treated cells, the fluorescence-producing CellTiter-Blue reagent was used. SCC-4 cells were grown as per the materials and methods section described in Chapter 2. Due to different cell type metabolic rates, this assay requires a different amount of incubation time for fluorescent signal generation for each cell line/type. The CellTiter-Blue Cell Viability assay was used to determine cellular viability, which involves measurement of the reduction rates of resazurin (CellTiter-Blue Cell Viability active reagent) into resorufin. A fluorescent signal occurs only in viable cells with high metabolic capacity, whereas non-viable cells, lacking metabolic capacity, are not able to reduce the indicator dye resazurin to produce a fluorescent signal. The expected linear relationship between cell number and fluorescence intensity signal was not achieved in these experiments, due to the uniqueness of SCC-4 cell line growth. The linear range and lower limit of detection are dependent on the cell type and the ability to reduce resazurin, thus directly indicating cellular metabolic capacity. At the selected cell density, a linear relationship was not achievable due to the high cell density, which was required for adequate fluorescent signal generation within the optimal time frame of 4 h. The amount of fluorescence produced in cells after incubation with CellTiter-Blue Cell Viability Reagent was measured using a FLUOStar Galaxy microplate reader (BMG Labtech, Germany), using a fluorescence plate mode at 590 nm for excitation and 610 nm for emission. Three independent experiments were conducted in triplicates.

To optimise cell density, cells were counted using a coulter counter and seeded at between 1 x 10^4 and 5 x 10^4 cells/well. A seeding density of less than 3 x 10^4 required at least 11 h of incubation with the CellTiter-Blue reagent for slight colour changes to be observed in the medium.

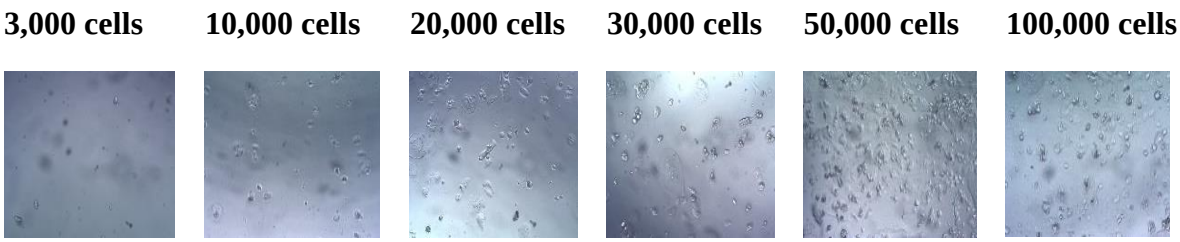
The 3 x 10^4 cells had quantifiable changes in medium colour after 4 h of incubation with the CellTiter-Blue reagent (see Figure A.10).

Optimisation of cell density for CellTiter-Blue assay

For optimisation of cell density, SCC-4 cells were plated at a density of 1 x 10^4–5 x 10^4 cells/well and incubated at 37 °C, in a humidified 5% CO_2 and 95% air atmosphere, in complete medium for 24 h in a 96-well plate. Due to the nature of SCC-4 growth and cell detachment from monolayers, some floating cellular debris or detached cells were observed, which could interfere with the fluorescence reading. To ensure the accuracy of fluorescent signals, cells were washed with 1x PBS (pH 7.4) three times to remove detached cells. 90 µl of complete medium was added to each well prior to addition of 10 µl CellTiter-Blue Reagent. After the addition of CellTiter-Blue directly to each well, SCC-4 cells were incubated for 4 h at 37 °C, to allow the cells to convert resazurin to resorufin.

After 4 h incubation with CellTiter-Blue Reagent, the fluorescent signal was measured using a FLUOstar Galaxy microplate reader (BMG Labtech, Germany), at the following wavelengths: excitation at 590 nm and emission at 610 nm. The gain setting was maintained at the same level at all time points.

Prior to conducting the experiments the cell density that would provide adequate optical density (OD) was determined. Due to sporadic and patchy growth patterns of SCC-4 cells it was essential to establish the seeding density for MTT assay. Cell number below 30,000 were not sufficient for obtaining optical density acceptable for this cell line (approximate OD = 0.1).



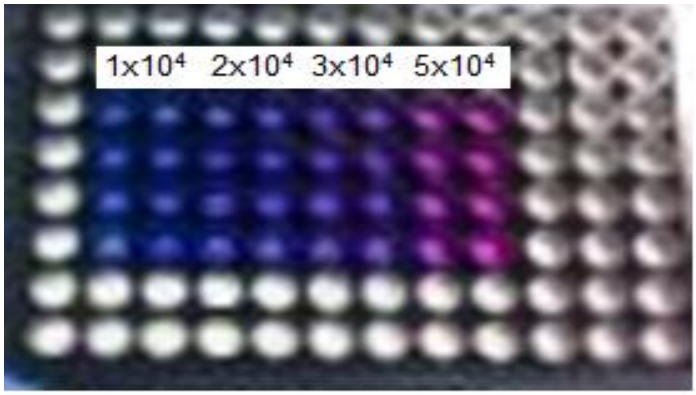


Figure B.1. The optimisation of cell density in proliferation assays.

SCC-4 cells were plated in eight wells at a density of 1 x 10^4, 2 x 10^4, 3 x 10^4 and 5 x 10^4 in a black 96-well clear, flat bottom microplate. Assays using low SCC-4 cell numbers (1 x 10^4 and 2 x 10^4) produced fluorescent signal only 11 h and 23 h post incubation with resasurin; whereas increasing the cell density to 3 x 10^4 led to increased fluorescent detection and a decreased incubation period to 4 h, which showed the optimal fluorescent detection.

In the experimental setup, untreated SCC-4 cells were used as an untreated control. Each plate had an additional test compound control, consisting of only media with the vehicle or vitamin D metabolite without cells, which was used as an indication of the non-specific background and to determine if the readings required correction.

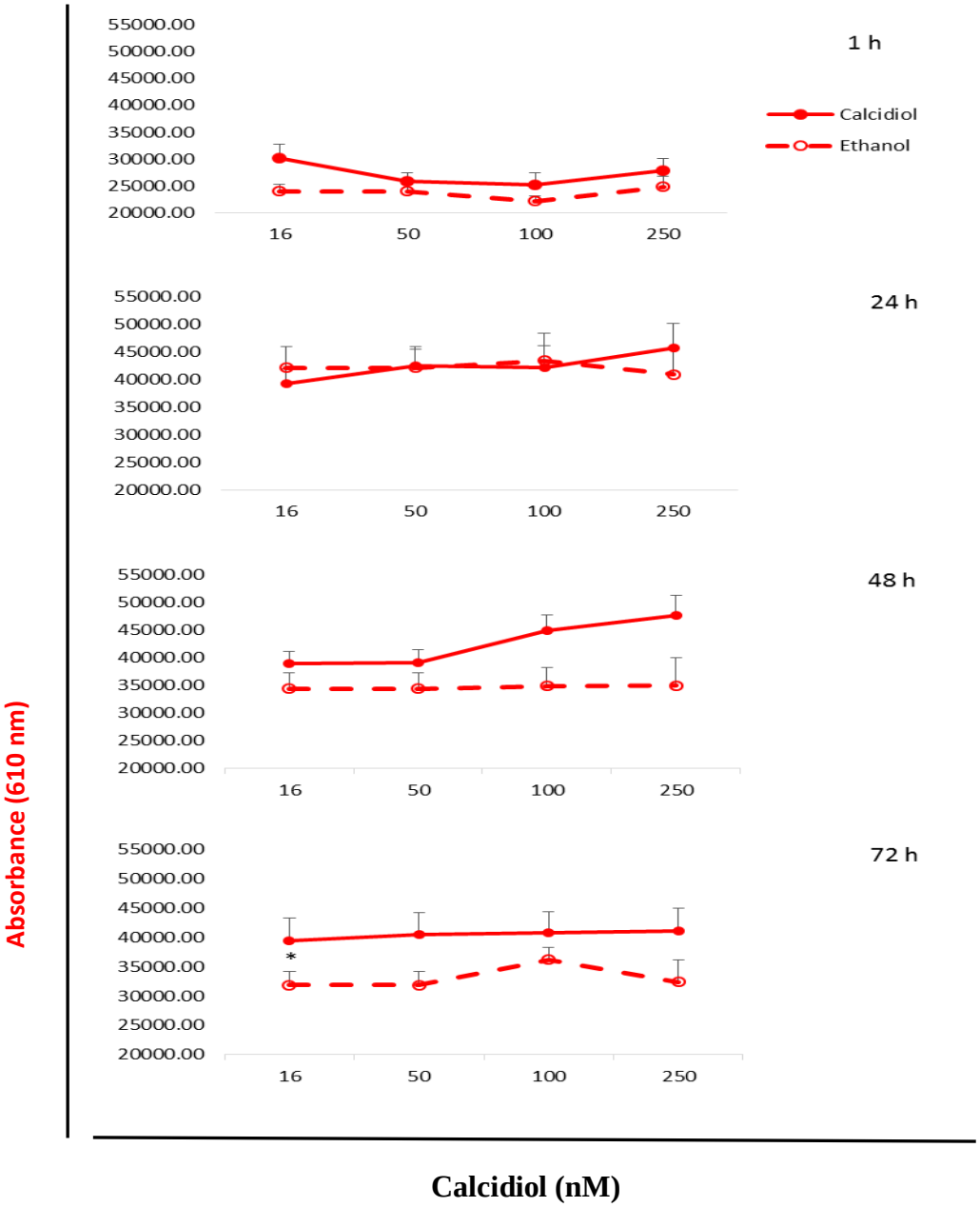


Figure B.2.a. Effect of calcidiol on SCC-4 cell proliferation.
Cell proliferation was measured using CT Blue assay at the wavelength 610 nm. All results are representative of at least three independent experiments conducted in triplicate. Statistically data was considered significant when p values were below 0.05.

Table B.2.a Statistical significance of calcidiol treated SCC-4 cells over the period of 1-72 h p values

Calcidiol	16 nM	50 nM	100 nM	250 nM
1 h	0.226	0.381	0.225	0.309
24 h	0.092	0.946	0.841	0.480
48 h	0.213	0.209	0.067	0.056
72 h	0.013	0.408	0.661	0.894

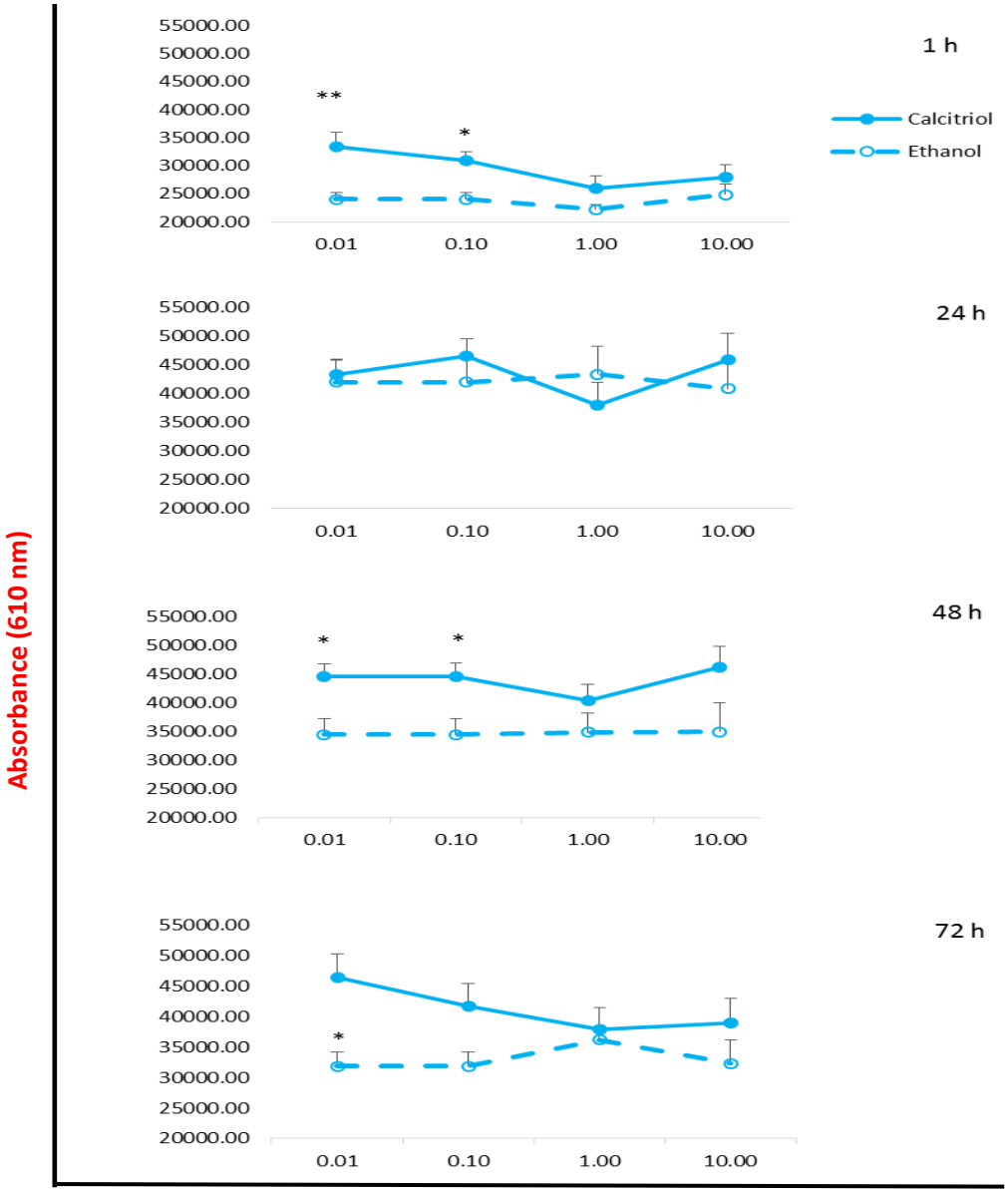


Figure B.2.b Effect of calcitriol on SCC-4 cell proliferation.
Cell proliferation was measured using CT Blue assay at the wavelength 610 nm. All results are representative of at least three independent experiments conducted in triplicate. Statistically data was considered significant when p values were below 0.05, which is shown in the table B.2.b.

Table B.2.b Statistical significance of calctriol treated SCC-4 cells over the period of 1-72 h p values

Calcitriol	0.01 nM	0.1 nM	1 nM	10 nM
1 h	0.002	0.010	0.332	0.154
24 h	0.797	0.151	0.417	0.645
48 h	0.023	0.025	0.236	0.110
72 h	0.033	0.060	0.812	0.777

Supplementary materials to MTT assay:

Table 2.1 Statistical significance p values of calcidiol treated SCC-4 cells over 24 – 96 h

Calcidiol	100 nM	1,000 nM	10,000 nM	100,000 nM	1,000,000 nM	10,000,000 nM
24 h	0.276	0.892	0.865	0.630	0.678	0.169
48 h	0.227	0.165	0.188	0.002	0.495	0.000531
72 h	0.792	0.462	0.073	0.011	0.005	0.019
96 h	0.253	0.182	0.625	0.144	0.247	0.854

Table 2.2 Statistical significance p values of calctriol treated SCC-4 cells over 24 – 96h

Calcitriol	10 nM	100 nM	1,000 nM	10,000 nM
24 h	0.903	0.504	0.323	0.159
48 h	0.121	0.038	0.0001	0.017
72 h	0.001	0.001	0.006	0.009
96 h	0.061	0.898	0.003	0.036

APPENDIX C. MORPHOLOGY AND GROWTH OF SCC-4 CULTURES

Colonogenic and morphological assessment of vitamin D treated SCC-4 cells. Light microscopy imaging and growth pattern of cell cultures

SCC-4 cells were seeded at concentration of 10,000 cells per well in 24 wells plates. SCC-4 cells were cultured in DMEM/F12 (1:1) medium supplemented with 10 % FBS and 1 % penicillin–streptomycin (Lu et.al., 2008). Cells were allowed to adhere for 24 h prior supplementing the treatment. The cell growth and colony formation was monitored, the images of the calcidiol and calcitriol treated SCC-4 were captured using phase contrast microscopy at high magnification (100X) at different time points (24 – 72 h). The experiment was conducted three times in triplicates. See Appendix C for the images of other doses and time points of representative experiment.

Using light microscope, images of migrating SCC-4 cells were captured at the time points of 1, 24, 48 and 72 h in the presence of calcidiol and calcitriol (Table 2.4). Captured images of representative SCC-4 treated cells with calcidiol (250 nM and 100,000 nM) and calcitriol (10 nM and 100 nM) and corresponding ethanol controls are shown (Table 2.5a). The media was changed at every time point.

Untreated cells show rapid wound closure, whereas 250 nM calcidiol exerts an inhibitory effect on the wound closure. At high calcidiol doses (100,000 nM) considerable cellular detachment with floatation was noted, which made the wounded area patchy in appearance with uneven cell distribution. This made acquisition of measurement at the higher dose 100,000 nM unattainable.

Calcitriol at 10 nM dose caused more rapid wound closure compared to untreated cells and corresponding ethanol control (Table 2.5b). High calcitriol dose (100 nM) caused detrimental effects compared to ethanol treated and untreated SCC-4 cells. Enormous cell detachment and floatation was also noted in the calcitriol treated wells, while unevenly distributed remaining patches of adherent cells were impossible to measure and compare with the previously obtained measurements at lower calcitriol doses.

Treatment of SCC-4 cells with high calcidiol and calcitriol doses resulted in aberrant cellular detachment and scattered colonies distribution. Further analysis of the cellular morphology and colony formation in response to the vitamin D (calcidiol and calcitriol) treatment was conducted.

Changes in cellular morphology in response to calcidiol and calcitriol

The most extensively studied mechanism of vitamin D antineoplastic action is an induction of cellular differentiation and associated with its antiproliferative effects (Beer & Myrthue, 2004). It was noted that calcidiol and calcitriol treatment at various concentrations have an effect on cellular morphology and colony formation. Prior to demonstrating the effects of calcidiol and calcitriol, it is essential to show the untreated malignant keratinocyte at high magnification (100X) with definition of cellular appendages. Cellular pseudopodia release is apparent when cells seek a suitable space to launch. Images were captured using a phase contrast microscope (Figure 2.5). While most published papers do not show these features of malignant keratinocytes in images at higher resolution, it is important to emphasise that cellular pseudopodia release was not obvious as soon as media temperature (37 ^{0}C) was decreased.

A) **B)** **C)**

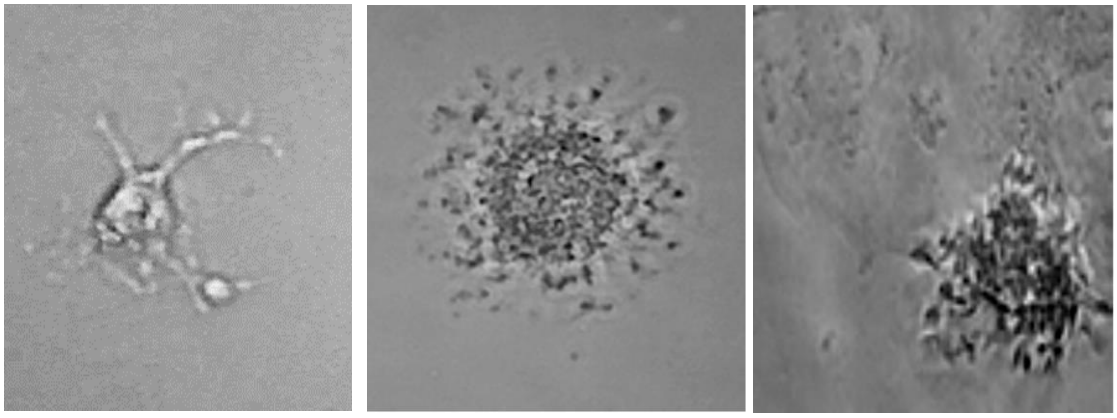
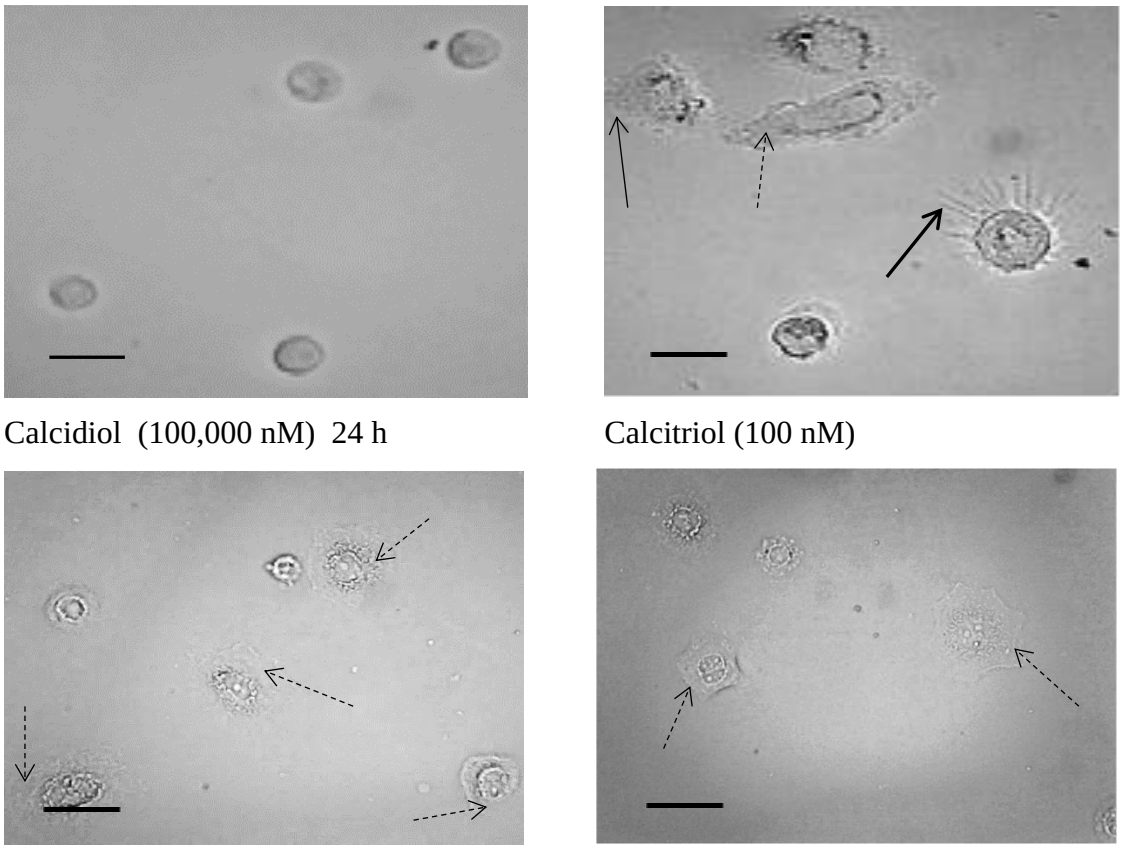


Figure C.1. Malignant keratinocyte SCC-4 morphology.
The single keratinocyte movement was captured using 100X magnification. The keratinocyte pseudopodia release is obvious from the captured images. A) Cellular features were only observed via light microscopy while the culture media was maintained warm at temperature 37 ^{0} C. B) Cell adherence to the established colony, where cell is actively seeking for the space to launch. C) Cellular pseudopodia is involved in the process of adherence and formation of a stable extracellular matrix. Once cell culture media reached ambient temperature the pseudopodia were no longer detectable.

Light microscopy images demonstrate the effects of calcidiol and calcitriol treatment compared to its corresponding ethanol controls. The representative images captured after 24 h incubation: SCC-4 cells treated with calcidiol 100,000 nM and calcitriol 100 nM. The cellular morphological changes (such as absence of pseudopodia and extracellular matrix) were not noted in ethanol treated contols and in calcitriol treated SCC-4 cells (Figure 2.6.) The effect of other calcidiol and calcitriol doses are shown in Appendix C.

Treated cells exhibited sporadic and patchy cellular detachment in response to vitamin D (both, calcidiol and calcitriol) at higher doses. The detrimental effects of calcidiol on pseudopodia, destruction of extracellular matrix, inhibition of cell division and colony formation were observed. Cultured cell exposure to high doses of calcidiol and calcitriol resulted in alteration of cell morphology and colony formation. Further studies are needed to explain this observation.



Calcidiol (100,000 nM) 24 h | Calcitriol (100 nM)

Ethanol control to calcidiol | Ethanol control to calcitriol

Figure C.2. Calcidiol (100,000 nM) and calcitriol (100 nM) treated SCC-4 cells colony formation and morphology.
Calcidiol treatment at high concentrations (shown dose of 100,000 nM) have an impact on SCC-4 cells pseudopodia and extra-cellular matrix (ECM), whereas calcitriol (at 100 nM dose) has inhibitory effect on SCC-4 without obvious inhibitory influence on pseudopodia or ECM formation. Furthermore, such changes were not noted in corresponding ethanol controls. Pseudopodia are shown with black arrors and ECM – with dashed arrors. Images were taken at 100X magnification, Bar scale 50 µm.

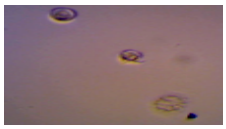
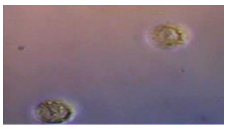
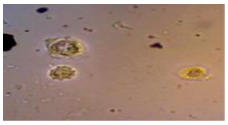
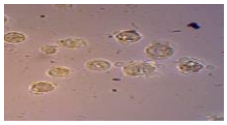
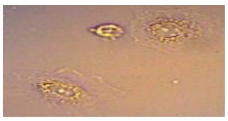
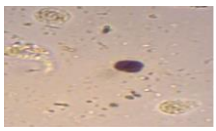
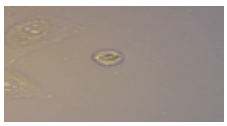
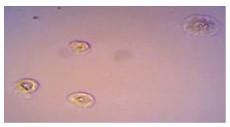
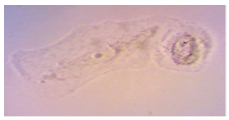
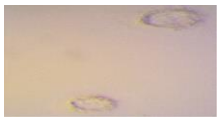
	Concentration		
Time	**100 nM**	**100,000 nM**	**1,000,000 nM**
0.5 h			
Ethanol control			
24 h			
Ethanol control			
48 h			
Ethanol control			
72 h			
Ethanol control			

Figure C.3. Calcidiol treated SCC-4 cells colony formation and morphology.
Calcidiol inhibits cell migration by destroying SCC-4 cells pseudopodia at high concentrations (1000 - 1,000,000 nM), whereas 100 nM dose has some inhibitory effects on pseudopodia, without an impact on extracellular matrix formation. Ethanol treatment at all doses have stimulatory effects on SCC-4 cell growth and elongated morphological changes, without apparent effects on colony formation.

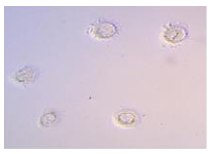
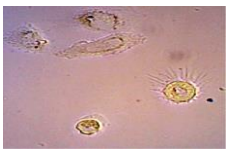
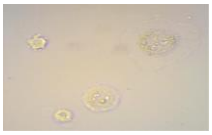
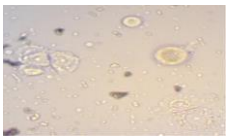
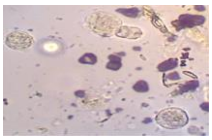
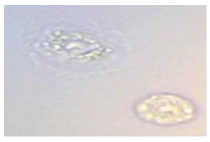
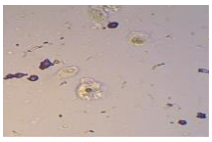
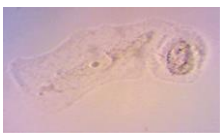
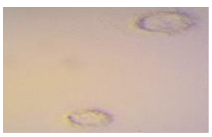
	Concentration		
Time	**10 nM**	**100 nM**	**1,000 nM**
0.5 h			
Ethanol control			
24 h			
Ethanol control			
48 h			
Ethanol control			
72 h			
Ethanol control			

Figure C.4. Calcitriol treated SCC-4 cells colony formation and morphology.
Calcitriol treatment caused cell detachment and obvious cell destruction. Calcitriol destroys cellular pseudopodia at high concentrations (1,000 nM), whereas dose of 10 nM calcitiol exerts a minor effect on cellular filaments without obvious influence on extracellular matrix (ECM). Ethanol treatment at all doses have stimulatory effects on SCC-4 cell growth and elongated morphological changes, without apparent effects on pseudopodia.

APPENDIX D. STATISTICS

Statistical comparison of p values to corresponding vehicle control in calcidiol or calcitriol treated SCC-4 cells and primary keratinocytes

CYP27A1

Figure 3.2.a,b	MK calcidiol (100,000 nM)	MK calcitriol (100 nM)	PK calcidiol (100,000 nM)	PK Calcitriol (100nM)
p values	0.973609	0.974214	0.503115	0.707396

Figure 3.2.c	MK calcidiol (250 nM)	PK calcidiol (250 nM)
p value	0.062858	0.021222

CYP27B1

Figure 3.3 a,b	MK calcidiol (100,000 nM)	MK calcitriol (100 nM)	PK calcidiol (100,000 nM)	PK Calcitriol (100nM)
p values	0.789161	0.003483	0.568333	0.526132

Figure 3.3c	MK calcidiol (250 nM)	PK calcidiol (250 nM)
p values	0.001411	0.042552

CYP24A1

Figure 3.4 a,b	MK calcidiol (100,000 nM)	MK calcitriol (100 nM)	PK calcidiol (100,000 nM)	K Calcitriol (100nM)
p values	0.0122	0.0321	0.1065	0.1115

Figure 3.4c	MK calcidiol (250 nM)	PK calcidiol (250 nM)
p values	0.0925	0.0689

VDR

Figure 3.5.a,b	MK calcidiol (100,000 nM)	MK calcitriol (100 nM)	PK calcidiol (100,000 nM)	K Calcitriol (100nM)
p values	0.0532	0.0619	0.6240	0.751957

Figure 3.5.c	MK calcidiol (250 nM)	PK calcidiol (250 nM)
p values	0.0174	0.2913

EGFR

Figure 3.6.a,b	MK calcidiol (100,000 nM)	MK calcitriol (100 nM)	PK calcidiol (100,000 nM)	K Calcitriol (100nM)
p values	0.347198	0.424831	0.119314	0.289324

FGFR3

Figure 3.7.a,b	MK calcidiol (100,000 nM)	MK calcitriol (100 nM)	PK calcidiol (100,000 nM)	PK Calcitriol (100nM)
p values	0.437708	0.800373	0.029903	0.12105

PCNA

Figure 3.8.a,b	MK calcidiol (100,000 nM)	MK calcitriol (100 nM)	PK calcidiol (100,000 nM)	PK Calcitriol (100nM)
p values	0.652069	0.411188	0.976058	0.154789