The Effects of Vitamin D_3 (1α,25-Dihydroxyvitamin D_3) on the Haematopoietic Differentiation of Stem Cells in Murine *in vitro* Studies and Human *in vivo* Studies

Mayada Al Qaisi

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Director of Study
Prof. Bill Gilmore

Healthcare Science Research Institute
The Manchester Metropolitan University

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Abstract

Previous research using cancer cells has demonstrated that 1α,25-dihydroxyvitamin D_3 (VD_3) can suppress cell proliferation and promote cell differentiation. The research reported in this research ascertains that VD_3 plays a similar role in haematopoiesis. This was investigated using the E14/ OP9 co-culture as it has previously been shown to be a good setting for studying haematopiesis. The effects of VD_3 on E14 and OP9 were tested separately with different VD_3 levels to demonstrate that sufficient levels of VD_3 were crucial in promoting differentiation and suppressing proliferation of both cell lines. The effective dosage of VD_3 was 100 nmol/L. This dosage was then used to test the effect of VD_3 on cell proliferation and differentiation in haematopoiesis in the E14 / OP9 co-culture system through analyses of growth factors, cluster of differentiation (CD) markers, colony-forming cell (CFC) counts, haematopoiesis-specific genes and cytokine expression. VD_3 was shown to promote differentiation thereby accelerating the early appearance of differentiated cells in the co-culture system. The *in vitro* study was followed by an *in vivo* pilot study, in which blood samples were taken monthly from a group of adult volunteers over a three-month period for analysis of VD levels, CD34 expression and blood cell counts. In the *in vivo* pilot study, it was found that there were significant non-random temporal variations in the measurements and there were statistically significant positive correlations between each of 25-hydroxyvitamin D (25(OH)D) level, white blood cell count, platelet count, $CD34^+$ progenitor cell count and VD_3 level. Overall, the research reported in this thesis demonstrated for the first time an anabolic effect of VD_3 in haematopoiesis on murine E14 and OP9 cells *in vitro*. In addition, the pilot study on human volunteers demonstrated that a comparable effect takes place on human cells *in vivo* and these results could be used to inform future studies.

Some of the contributions of this thesis have been published in one journal paper[1] and one chapter of a book[2], and presented at one conference[3].

[1] Alqaisi, M., Al-Shanti, N, Wang, Q., and Gilmore, W. S. 2013. Enhanced Differentiation of Stromal Cells and Embryonic Stem Cells with Vitamin D3, *Journal of Medical, Pharmaceutical Science and Engineering*, Vol. 7, No. 9, pp. 228-235.

[2] Gilmore, W., Al Qaisi, M., and Al-Shanti, N., 2015. Chapter 34: Flow Cytometry Enumeration of Hematopoietic and Progenitor Stem Cells: Identificatioin and Quantification, in M. Slevin, G. McDowell (eds.), *Handbook of Vascular Biology Techniques*, Part IV, pp. 439-451, Netherlands: Springer Science+Business Media Dordrecht.

[3] 15th International Conference on Hematology (ICH 2013), London, UK, September 5-6, 2013, World Academy of Science, Engineering and Technology.

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Author's Declaration

I declare that the work in this thesis was carried out in accordance with the regulations of Manchester Metropolitan University. Apart from the help and advice acknowledged, the work within was solely completed and carried out by the author.

Any views expressed in this thesis are those of the author and in no way represent those of Manchester Metropolitan University and the Institute for Biomedical Research in Human Movement and health.

This thesis has not been presented to any other university for examination either in the United Kingdome or overseas. No portion of the work referred to in this research project has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

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Table of Contents

List of Figures

List of Tables

Abbreviations

Activin A	a member of TGF
BMP4	bone morphogenetic protein 4
Brachyury	a protein encoded by the T gene
CD	cluster of differentiation
CFC	colony-forming cell
EB	embryoid body
E-CFC	erythroid CFC
ESC	embryonic stem cell
FCM	flow cytometry
FITC	fluorescein isothiocyanate
FLK1/KDR	kinase insert domain protein receptor
Flt3	foetal liver kinase
FSc	forward scatter
GATA1	GATA binding factor 1 (globin transcription factor 1)
GATA2	GATA binding factor 2
GEMM-CFC	granulocyte, erythroid, macrophage, megakaryocyte CFC
GM	growth medium
GM-CFC	granulocyte-macrophage CFC
hESCs	human embryonic stem cells
Homeobox protein goosecoid	a protein encoded by the GSC gene
Homeobox protein Hox-B1	a protein encoded by the HOXB1 gene
HSC	haematopoietic stem cell
LIF	leukaemia inhibitory factor
M-CFC	macrophage CFC
Nodal	nodal growth differentiation factor
Oct4	octamer-binding transcription factor 4
OP9	a murine stromal cell line established from new-born B6C3F1 op/op mouse calvaria
PBS	phosphate buffered saline
PS	primitive streak
RM	room temperature

SMAD	mothers against decapentaplegic homolog
SSc	side scatter
STAT	signal transducer and activator of transcription
TAL1 / SCL	T-cell acute lymphocytic leukaemia protein 1 / stem cell leukaemia
TBS	tris buffered saline
TGF-B or TGF-β	transforming growth factor beta
VD_3	1α,25-dihydroxyvitamin D_3
Wnt	wingless-type mouse mammary tumour virus (MMTV) integration site

Chapter 1 Introduction

1.1 Literature Review

1.1.1 Embryonic stem cells

Embryonic stem cells (ESCs), derived from the inner cell mass of the blastocyst-stage of early mammalian embryos, have the potential to undergo unlimited self-renewal by placing them in specific culture conditions, either *in vitro* or *in vivo* (Evans and Kaufman 1981), as shown in Figure 1. Once the cells are released from these conditions and placed into a differentiation-promoting environment (*in vitro* or *in vivo*), the cells differentiate into derivatives of all three primary germs: ectoderm, mesoderm, endoderm and then into many different cell types in the body (Nishikawa *et al.*, 2007). Thus, ESCs have unveiled widespread application potentials in the fields of biomedical research and regenerative medicine (Yu *et al.*, 2007; Keller, 2005).

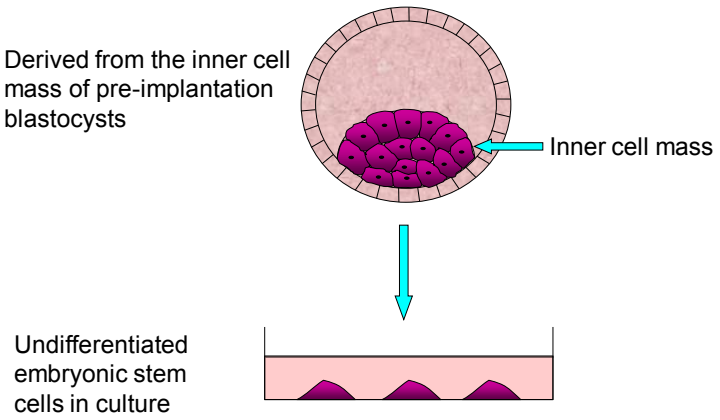


Figure 1. Embryonic stem cells (ESCs) derived from the inner cell mass of the blastocyst-stage of embryo
(Source: personal communitation)

The majority of recent studies into embryonic stem cells suggest that ESCs could be used to cure many cellular deficiency diseases, such as heart failure, stroke, and haematological disorders resulting from the absence of one or more critical populations of cells, and so if cell populations could be regenerated, tissue repair could be realised (Murry and Keller, 2008).

Patient-specific ESC equivalents (Park *et al.*, 2008; Takahashi *et al.*, 2007; Yu *et al.*, 2007) would provide new tools to evade the immune system, thus giving insight into fundamental disease mechanisms and providing drug-discovery screens. The most important potential application of ESCs is the generation of cells that could be used for cell-based therapies, such as transplanting haematopoietic stem cells (HSCs) in leukaemia treatment (Brustle *et al.*, 1999; Tian, *et al.*, 2008), which entails the use of high-quality sources of tissue-matched bone marrow, mobilised peripheral blood or umbilical cord blood. Appropriate bone marrow is often in short supply, whilst cord blood, though bankable, contains fewer HSCs, which makes it less suitable for adult transplantation. Directed differentiation of ESCs towards HSCs offers a potentially attractive alternative to these conventional sources (Ledran *et al.*, 2008).

The pluripotency of ESCs makes them difficult to control, but to enable differentiation of ESCs, three methods have been employed: (1) forming three-dimensional aggregates, known as embryoid bodies (EBs); (2) making ESC cultures as monolayers on extracellular matrix proteins; (3) directly placing ESC cultures on supportive stromal layers, so that differentiation could take place in contact with these cells (Keller 2005; Nishikawa *et al.*, 2007). These will be described in more details in Section 1.1.5 with Figure 6.

Although it is not clear which method is the most efficient one, each method promotes ESCs to differentiate into many different cell types in culture. In early studies, remarkable differentiation has been found using foetal calf serum (FCS) in the protocol, but these protocols were difficult to repeat because of the poorly defined mixture of factors in serum and the variability between different serum lots, and most were not well optimised for any particular lineage. Studies were successful using serum-free medium with specific inducers for differentiation (Kubo *et al.*, 2004; Ng *et al.*, 2005; Yasunaga *et al.*, 2005) and for reporter ESCs to observe and have access to early differentiation stages (Fehling *et al.*, 2003; Gadue *et al.*, 2006; Ng *et al.*, 2005; Tada *et al.*, 2005). These techniques have aided understanding of ESC differentiation through developmental biology by recognising the main phenomena in ESC cultures that facilitate early lineage commitment in the embryo, enabling efficient and reproducible generation in highly differentiated cell populations. The cellular mechanism and target cell affected by microenvironments in augmenting haematopoietic specification from pluripotent embryonic stem cells (ESCs) has yet to be evaluated (Junfeng *et al.*, 2008). This study goes on to focus on the

development and differentiation of haematopoietic lineages, as access to such cell population may provide new therapies for some of the world's most devastating diseases. This is particularly important for the study of human development because the availability and accessibility of normal cells from human embryos is limited.

1.1.2 Embryogenesis in mice

In embryogenesis, gastrulation is the formation of three primary layers: ectoderm, mesoderm and endoderm, as shown in Figure 2. Gastrulation in the mouse begins with the appearance in the epiblast region of the primitive streak (PS), which will go on to form the posterior part of the embryo (Tam and Behringer, 1997). Gastrulation involves uncommitted epiblast cells from the PS becoming either mesoderm or endoderm.

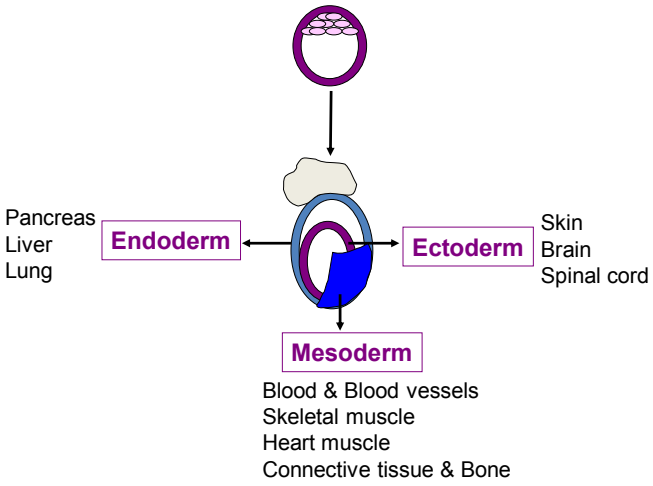


Figure 2. Embryonic stem cells differentiate into derivatives of all three primary germs – ectoderm, mesoderm, endoderm – during the process of gastrulation (Source: personal communication)

Gene expression and developmental potential have been found to vary according to the regions (posterior, mid, and anterior) of the PS. Different genes have been expressed: Brachyury (T) (Kispert and Hermann, 1994) and Mixl1 (Hart *et al.*, 2002; Ng *et al.*, 2005) are expressed throughout the PS; Foxea2 and Goosecoid are typically in the anterior part (Kinder *et al.*, 2001; Seilies *et al.*, 2006); HoxB1 , Evx1 are in the posterior part and early sequential expression of mouse Hox genes is essential for their later function (Forlani *et al.*, 2003).

Specification of mesoderm and endoderm subpopulations is not random in time and space. The first mobilised epiblast cells cross the posterior PS, forming the extra-

embryonic mesoderm, which in turn specialises to form different tissue cells such as haematopoietic and endothelial cells of the yolk sac (Kinder, 1999). With further gastrulation, cells moving through the anterior of the PS give rise to different types of mesoderm, such as cardiac mesoderm, as well as paraxial and axial mesoderm at a later stage. Definitive endoderm comes from epiblast cells that have crossed the anterior part of the PS, whilst epiblast cells that do not enter the PS form the ectoderm (Keller 2008; Tam *et al.*, 1997).

Specific lineages seem to be induced by spatial and temporal factors during gastrulation in and around the PS. The transforming growth factor-beta (TGFß) family are essential for PS formation and germ layer induction. This includes the bone morphogenic protein (BMP4) family (Hogan, 1996), the nodal growth differentiation factor (Nodal) (Shier, 2003) family and the wingless-type mouse mammary tumour virus (MMTV) integration site (Wnt) family (Yamaguchi, 2001). Gadue *et al.* (2005) confirmed that TGF-βs, BMP4, Wnt, Nodal and fibroblast growth factor (Fgf) pathways are essential for the differentiation of ESCs in culture. These genes are required for gastrulation and germ layer induction in the embryo. A combination of different levels of expression of agonists of the pathways and of regionalised expression of inhibitors leads to the formation of signalling domains, which regulate germ layer induction and specification (Gadue *et al.*, 2005). Thus, germ layer development is a dynamic process that is controlled partially by coordinated activation and regional inhibition of the Wnt, Nodal and BMP-signalling pathways.

1.1.3 Translating embryology to ESCs

Some properties of ESC cultures, through the manipulation of BMP, Wnt and Nodal pathways (which are involved in the systematisation of germ layer development in vitro), have been studied in the immediate vicinity of the PS. It has been proved that Brachyury is found to control the construction of the PS region and the onset of mesoderm population (Lerchner *et al.*, 2000; Murry 2008). In the absence of serum in the culture of ESCs, BMP4 was found to induce the forming of a Brachyury-positive population and subsequently the development of foetal liver kinase 1 (FLK1)-expressing ($FLK1^+$) mesoderm (Ng *et al.*, 2005; Nostro *et al.*, 2008). Hence, BMP4 is crucial for the formation of $FLK1^+$ mesodermal cells (Sarah *et al.*, 2001). Disruption of the basic stem cell leukaemia (SCL) gene expression causes a block early in the hematopoietic program,

leading to defects in all hematopoietic lineages. SCL expression is induced by BMP4, and a dominant negative BMP4 receptor inhibits SCL expression in the central region of the embryo (Paul *et al.*, 1998).These findings agree with previous work demonstrating that BMP4 is needed for the generation of $FLK1^+$ and SCL^+ cells mesoderm (Park *et al.*, 2004; Vidricaire *et al.*, 1994).

Furthermore, it was found that the activin and Wnt pathways are required for the formation of cells expressing Brachyury and mesoderm development. Blocking Wnt signalling at an early phase of differentiation in serum-stimulated cultures stopped the establishment of cells expressing Brachyury and mesoderm expansion, indicating that this pathway is required for the generation of the PS (Naito *et al.*, 2006; Ueno *et al.*, 2007). Conversely, adding Wnt to serum-containing cultures at the onset of differentiation accelerates the formation of PS population and enhances mesoderm development.

It was also found that the activation of the Nodal pathway by the addition of Activin A (activin) could induce a PS population and subsequent mesoderm formation (Vidricaire *et al.*, 1994; Kubo *et al.*, 2004). It has also been demonstrated that haematopoiesis from the mesoderm occurs via BMP4-mediated signals and that expansion and/or differentiation of such progenitors is achieved by the interplay of the TGFβ1 family and the Activin A signalling (Park *et al.*, 2004). From these investigations, a partial understanding of signalling pathways in early ESC differentiation can be derived.

The regulation of ESC differentiation is through direct and indirect effects, and the differentiation pathway is intricate. Gadue *et al.* (2006) designed a reporter mouse-ESC cell line to model PS establishment *in vitro*. It contained the green fluorescent protein (GFP) cDNA targeted PS gene Brachyury (T) and CD4 cDNA targeted anterior PS gene Foxa2. The GFP was only activated or reported when cells were undifferentiated and were turned off once they differentiated. Thus, the investigation revealed that Wnt and Nodal signalling are simultaneously required for the development of the GFP-T^+CD4^- $Foxa2^+$ PS cell population. However, Wnt and activin/Nodal individually showed distinct inducing properties. It was also found that BMP4 signalling was not required for PS generation (Nostro *et al.*, 2008), but BMP4 alone does promote the development of the PS and the mesoderm (Ng *et al.*, 2005; Park *et al.*, 2004), an indirect effect mediated by the induction of Wnt and Nodal, as shown in Figure 3 below. Thus, BMP4, Wnt and activin/Nodal signalling pathways that regulate the PS development and the onset of

mesoderm in the early embryo have also been found to be required *in vitro*. These are summarised in Figure 3 below.

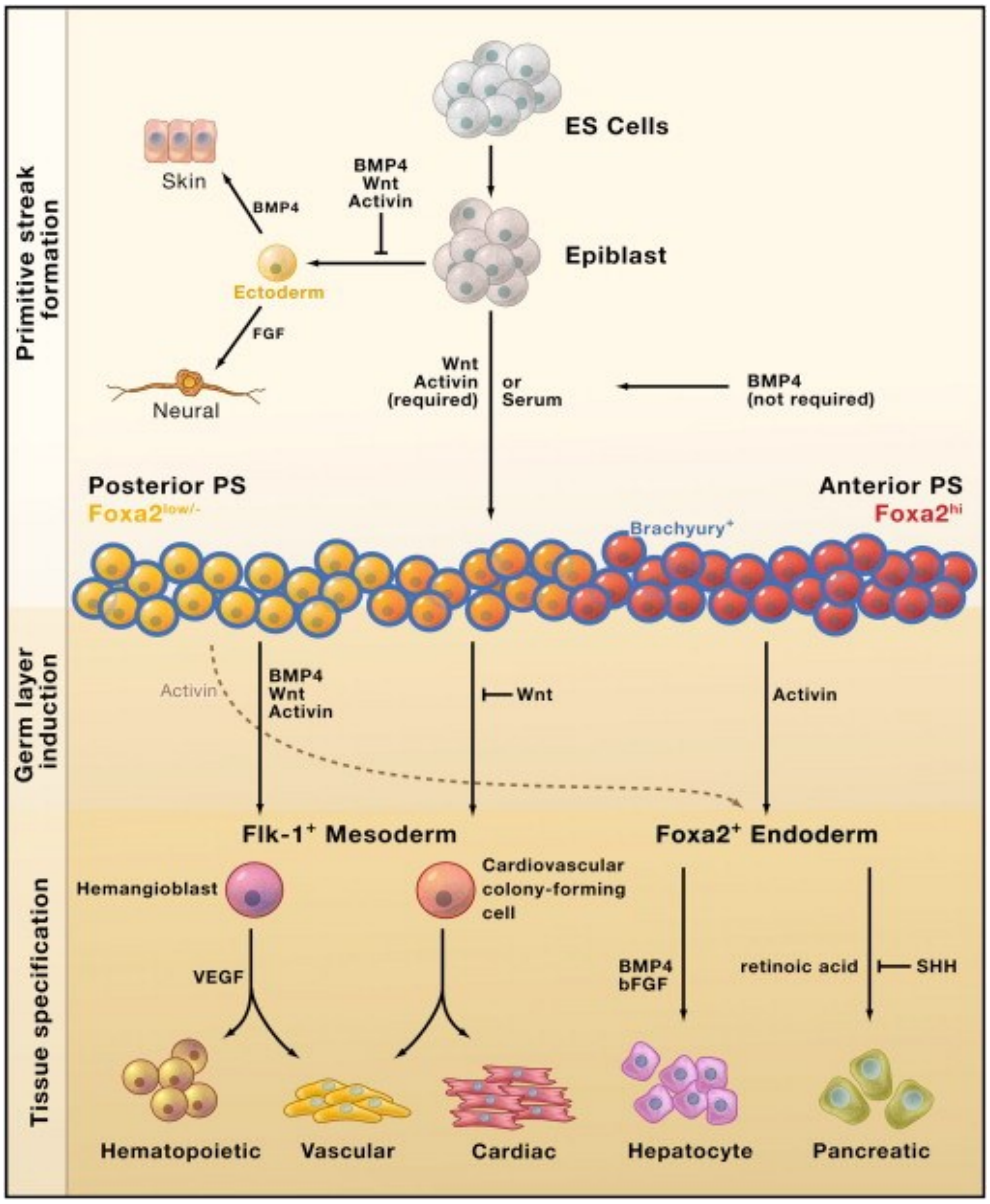


Figure 3. ESC differentiation pathways, showing the roles of BMP4, Wnt and activin (Source: www.sciencedirect.com/science/article)

1.1.4 Cytokines and growth factors

Cytokines and growth factors are cell signalling proteins and glycoproteins, each of which has a matching cell-surface receptor that transmits the signal through subsequent intracellular signalling cascades to alter a function or to cause a response from the cell. Almost all cytokines are pleiotropic, and many cytokines often have overlapping activities so that a single cell can respond to several cytokines in the same manner. In

addition, it is quite often to have a cellular function that is encouraged by a collection of cytokines and growth factors. The same cytokine can play different roles with different types of cells, partly because there are different receptors for the same cytokine for different functions.

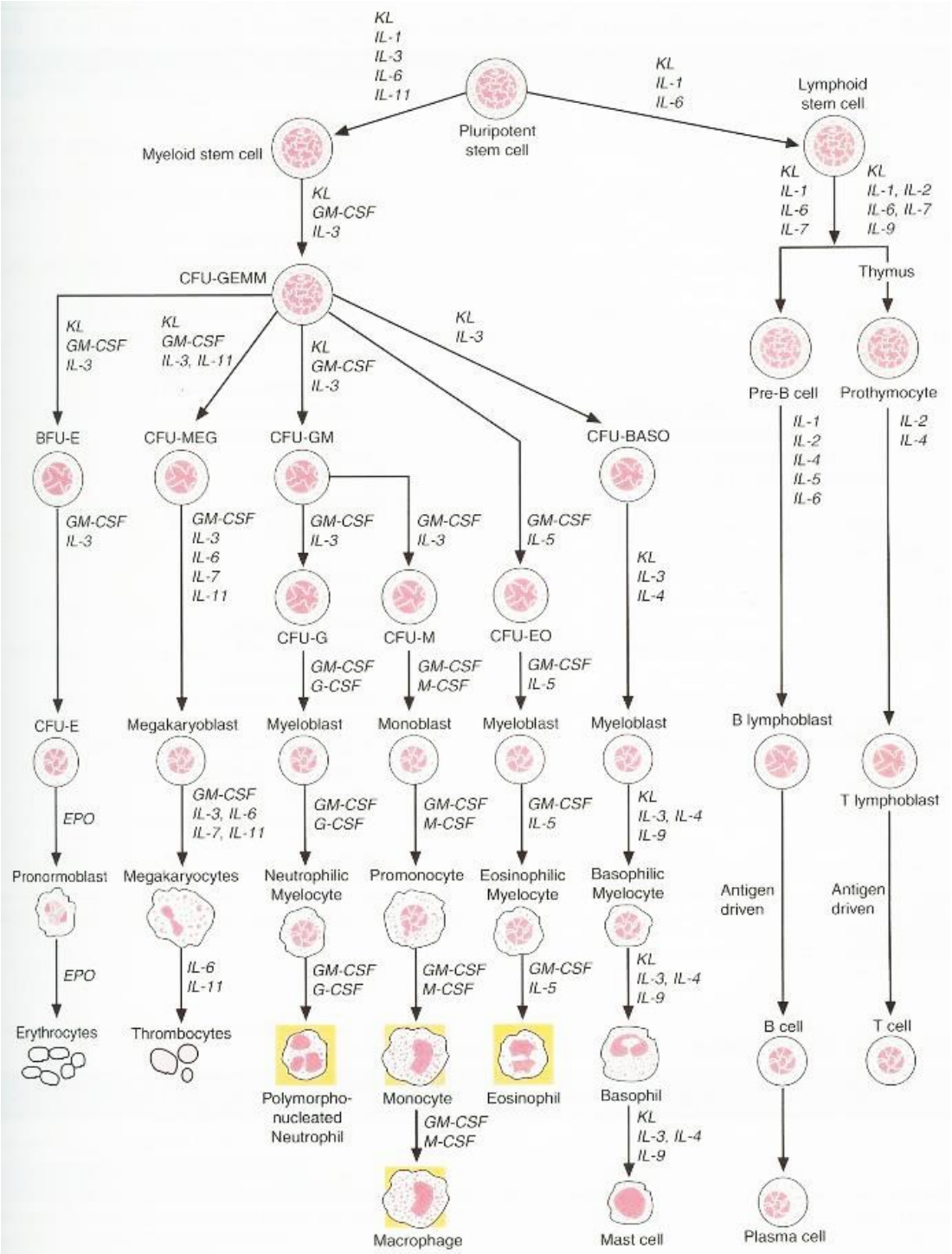


Figure 4. Deviation of haematopoietic cells showing sites of activity of CSFs and ILs (Rodak, Fritsma and Doig, 2008)

Many cytokines have been shown to be important to hematopoietic development, such as erythropoietin (Epo) (Iscove and Sieber, 1975), granulocyte colony-stimulating factor (G-

CSF) (Nicola *et al.*, 1983), macrophage-CSF (M-CSF or CSF-1) (Stanley and Heard, 1977), granulocyte / macrophage-CSF (GM-CSF) (Metcalf *et al.*, 1980), interleukin 1 (IL-1) (Mochizuki *et al.*, 1987), IL-3 (Iscove and Roitsch, 1985), IL-4 (Paul and Ohara, 1987), and IL-6 (Wong *et al.*, 1988). Some of these factors are lineage specific, such as Epo and CSF-1, whilst others act on multiple lineages, such as IL-3 and GM-CSF. Steel factor (SLF) (Anderson *et al.*, 1990; Zsebo *et al.*, 1990) and its receptor, c-kit (Chabot *et al.*, 1988; Geissler *et al.*, 1988), are critical to hematopoietic, germ cell, and melanocyte development. However, their precise functions during development are not clear (Russell, 1979; Silvers, 1979).

1.1.5 Differentiation of ESCs in culture

Given suitable conditions and free of the factors keeping them as stem cell, ESCs will undergo differentiation, yielding progeny made up of the derivatives of the three embryonic germ layers, namely mesoderm, endoderm and ectoderm (Keller, 1995; Smith, 2001). However, there is no differentiation in culture of wild-type ESCs into trophectoderm. Instead, the ESCs form the inner cell mass, which is the foundation of the embryonic population. This is represented in Figure 5 below.

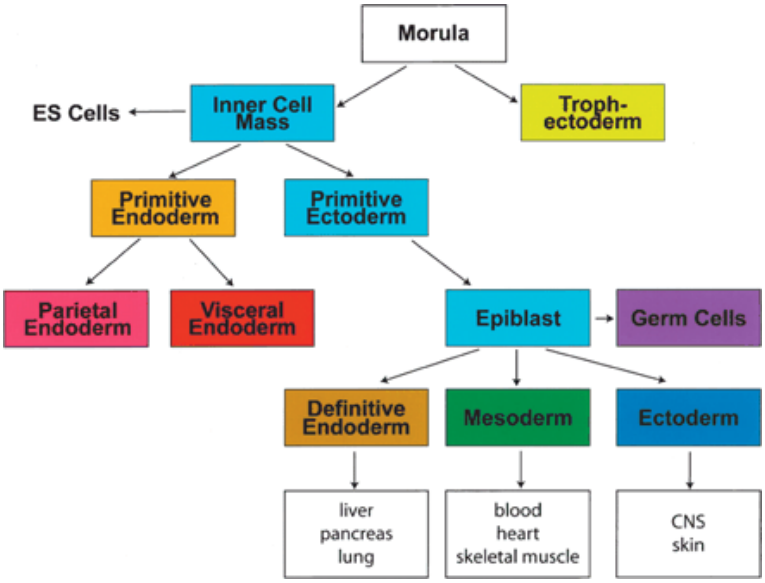


Figure 5. Scheme of early cell populations in relation to primary germ layers in early mouse development
(Source: http://genesdev.cshlp.org/content/19/10/1129.full)

ESC differentiation may be initiated through three general approaches, as shown in Figure 6 below. The first method allows the aggregation of ESCs to form three-dimensional

colonies called embryoid bodies (EBs) (Doetschman *et al.*, 1985; Keller 1995). With the second method, there is direct culturing of ESCs on stromal cells, where differentiation of the former occurs in contact with the latter (Nakano *et al.*, 1994). The most frequently used stromal cell line in this second method is OP9, isolated originally from CSF-1-deficient op/op mice cells (Yoshida *et al.*, 1990). In the third method, extracellular matrix proteins are used, on which an ESC monolayer differentiates (Nishikawa *et al.*, 1998).

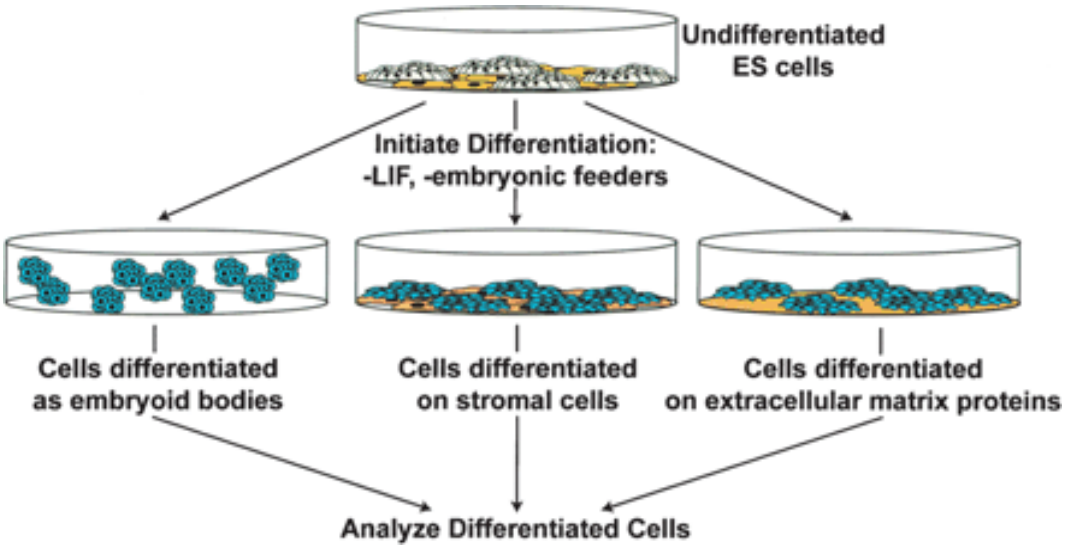


Figure 6. Three different methods for ESC differentiation.
(Source: http://genesdev.cshlp.org/content/19/10/1129.full)

ESCs of a wide variety of cell types have been generated from the three aforementioned differentiation methods (Keller, 1995; Smith, 2001). Detailed study of lineages have shown that these methods have proved to be efficient for differentiation to occur, whereby the events sequence in culture was shown to be a faithful recapitulation of what happens in the early stages of an embryo.

There are particular advantages and limitations to each of the three approaches to ESC differentiation. The three-dimensional structure of EBs is advantageous in that it is favourable to cell-cell interactions that may be significant for some programs of development (Keller, 1995). EBs are complex because of the generation of cytokines and the inducing factors in the structures themselves. These complexities can make it difficult to interpret the results of experiments aiming to gain understanding of the signalling pathways of lineage commitment.

The growth of a particular cell line is promoted in co-culture with stromal cells. The drawback is that stromal cells may produce certain factors whose effects are to influence

the differentiation of ESCs to unwanted cell types. Another disadvantage of this method is that it may be difficult to separate the ESC-derived cells from the stromal cells (Gadue *et al.*, 2005).

Differentiation in monolayers is a simple method, in which the aforementioned complex effects of neighbouring cells and supporting stromal cells can be minimised. However, this method is dependent on the matrix proteins used, as particular proteins may be critical in whether the developing cell types are generated and survive.

There are three criteria to consider in choosing the appropriate ESC model for lineage-specific differentiation (Smith, 2001). First, it is necessary to set up protocols in which the particular cell type may be developed efficiently and reproducibly. It is desirable to combine selection strategies with schemes of optimal differentiation in order that highly enriched cell populations can be isolated. Secondly, the program of development that sets up the lineage of the early embryo should be recapitulated in lineage from ESCs. Thirdly, whether in culture or subsequently transplanted to an animal model, the mature cell populations that have developed in these cultures must exhibit appropriate functional properties. However, the third criterion remains unfulfilled (Keller, 2005).

1.1.6 Mesoderm induction

The surface glycophosphoprotein CD34 is expressed on committed and primitive haematopoietic stem cells (Krause *et al.*, 1996). About 1 to 5% of bone marrow cells and 0.1% of peripheral blood cells express $CD34^+$ in normal individuals (Krause *et al.*, 1996). Therefore, the detection of changes in haematopoietic stem cells can be done by analysing $CD34^+$ cells. Physiological levels of vitamin D were found to promote differentiation of $CD34^+$ hematopoietic progenitors, indicated by all the monomacrophagic imuno-phenotypic and morphological markers in a liquid culture model being induced (Grande *et al.*, 2002).

The development of haematopoiesis is from subpopulations of mesoderm induced temporally in a defined pattern. The FLK1 and platelet-derived growth factor (PDGFR) receptors are largely expressed on subpopulations of this lineage, whereby the early stages of mesoderm induction from the PS can be monitored by the up-regulation of these receptors. Thus, FLK1 and PDGFR expression marks progenitors with broad mesodermal potential (Ema *et al.*, 2006). The ESC-derived $CD34^+$ cells are highly enriched in cells

expressing haematopoiesis associated genes FLK1, GATA1, GATA2, and SCL/TAL1 (Maxim *et al.*, 2005).

GATA-binding factor 1 (GATA1) and GATA-binding factor 2 (GATA2) belong to the GATA transcription factor family that binds to a DNA region with GATA sequence, and are involved in cell growth and cancer (Ko and Engel, 1993). GATA1 is essential for erythroid and megakaryocytic development and is expressed at high levels in erythroid cells, mast cells, and megakaryocytes. It regulates the switch of foetal haemoglobin to adult haemoglobin (Ohneda and Yamamoto, 2003). GATA2 is expressed in hematopoietic progenitors, including early erythroid cells, mast cells, and megakaryocytes, as well as in non-haematopoietic ESCs (Dorfman *et al.*, 1992; Ohneda and Yamamoto, 2003).

Although most studies have not distinguished PS formation from mesoderm induction, several have provided insights into the signalling pathways that regulate this step. It is known that the BMP signalling is necessary to induce $FLK1^+$ haematopoietic mesoderm from Brachyury-expressing populations, which indicates that this pathway is at the level of mesoderm induction (Park *et al.*, 2004). This finding is in agreement with other studies demonstrating that ventral mesoderm can be generated by treating ESCs with a combination of cytokines and bone morphogenetic protein 4 (BMP4) during the development of embryoid bodies (EB) (Chadwick *et al.*, 2003).

It was found that haematopoietic mesoderm generation from a Brachyury-GPF tagged PS population depends on a combination of Wnt, activin/Nodal, and BMP signalling, and that the up-regulation of FLK1 correlates with mesoderm fate (Hidaka *et al.*, 1999; Nostro *et al.*, 2008). These findings confirm previous studies demonstrating clearly that FLK1 gene is essential for early stages of haematopoietic development (Maxim *et al.*, 2005).

With these findings, it is established that subpopulations of mesoderm can be generated in mouse ESC cultures with various concentrations of BMP4, or a combination of BMP4 and activin, and with manipulation of different signalling pathways at relevant developmental stages (Era *et al.*, 2007).

1.1.7 ESCs and haematopoietic lineages

Haematopoiesis has been extensively studied, since many of the first ESC studies concentrated on blood cell development (Keller, 2005; Olsen *et al.*, 2006). The aim of such investigations has often been that of inducing the formation of transplantable HSCs from ESCs. The relationship of various cells related to haematopoiesis is depicted in Figure 7.

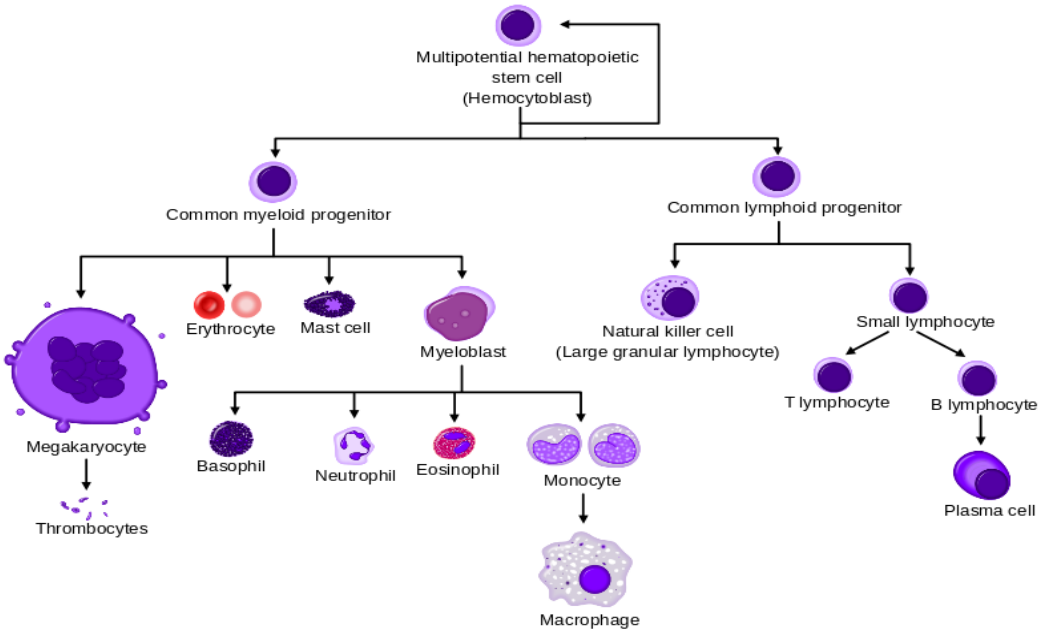


Figure 7. Haematopoiesis maturation and differentiation chart (Source: http://www.dentalarticles.com/images/hematopoiesis.png)

Although it is comparatively easy to generate blood cell lineages from ESCs, the development of HSCs from genetically unmodified ESCs is another matter, possibly due to the complexity of embryonic haematopoietic development, which occurs at different times and at different embryonic sites.

In the early mouse embryo, haematopoiesis commences independently at two different sites, namely the yolk sac and the para-aortic splanchnopleura (P-Sp). The P-Sp is an intra-embryonic region in the caudal part of the embryo where aorta, gonads and mesonephros are subsequently developed (AGM) (Cumano and Godin, 2007).

The yolk sac and the P-Sp show significantly different haematopoietic potential. The yolk sac generates primitive erythrocytes and a subset of other haematopoietic populations including macrophages and progenitors of the definitive erythroid, megakaryocyte, and mast cell lineages, but pre-circulation yolk sac manifests little or no lymphoid or HSCs

potential. On the other hand, the P-Sp-derived haematopoietic population generates both HSCs and multi-potential progenitors that yield myeloid, lymphoid, and definite erythroid lineages *in vitro*. In human embryos, haematopoietic expansion follows a similar model (Keller *et al.*, 2005; Chen *et al.*, 2003). Given these differences, it will be necessary to recapitulate these developmental stages *in vitro* and to identify the equivalent of P-Sp-derived haematopoiesis in order to isolate HSCs from ESCs (Murry *et al.*, 2008). This, in turn, allows for further studies into the basic biology and therapeutic potential of this progenitor.

1.1.8 Factors crucial to the maintenance of ESCs

There is an on-going revision of the main methods and techniques employed for the maintenance of ESCs in undifferentiated proliferative states, even years after the initial discovery of the derivation of ESC lines. Currently, researches indicate that there are a number of variables contributing to growth and differentiation *in vitro*.

ESCs are initially established and maintained in co-culture with mouse embryonic feeder cells (Evans and Kaufman, 1981; Martin, 1981). The leukaemia inhibitory factor (LIF) was identified in later studies as a feeder-cell-derived molecule playing a key part in maintaining these cells (Smith *et al.*, 1988; Williams *et al.*, 1988; Stewart *et al.*, 1992). With recombinant LIF, feeder cell function can be replaced and the growth of undifferentiated ESCs can be supported, given the presence of appropriate batches of FCS (Smith *et al.*, 1988; Williams *et al.*, 1988). BMP4 was found to play a role in ES cell growth and it can replace the need for serum in the presence of LIF (Ying *et al.*, 2003). Therefore, ESCs can be grown with defined factors without serum or feeder cells. LIF acts through the LIFR/gp130 receptor and activates signal transducer and activator of transcription 3 (STAT3), an important regulator of mouse embryonic stem cell self-renewal. STAT3 is known to inhibit differentiation into both mesoderm and endoderm lineages by preventing the activation of lineage-specific differentiation programs (Niwa *et al.*, 1998; Matsuda *et al.*, 1999; Graf *et al.*, 2011), whereas yhe activation of the mothers against decapentaplegic homolog (SMAD) (a transcription factor) and the subsequent induction of the helix-loop-helix Id factors (an inhibitor of differentiation) mediate the effect of BMP4 on undifferentiated ESCs (Katherine *et al.*, 2010). The treatment of ESCs during EB development with a combination of cytokines and BMP4, a ventral mesoderm inducer, strongly promotes hematopoietic differentiation. Addition of BMP4 had no

statistically significant effect on hematopoietic differentiation, but it enables significant enhancement in progenitor self-renewal (Chadwick *et al.*, 2003). Apart from STAT3 and Id, three other transcription factors and enzymes were found to be essential in the maintenance of the undifferentiated state of ESCs. These are Oct3/4 (Niwa *et al.*, 2000) and Nanog (Chambers *et al.*, 2003; Mitsui *et al.*, 2003; Loh *et al.*, 2006). It has been shown that Oct4, Nanog and Sox2 harmoniously work in a combinatorial complex to regulate ESC pluripotency and cellular differentiation (Kashyap *et al.*, 2009; Nishikawa *et al.*, 2007). In the absence of Oct4, pluripotent cells return to trophoblast both *in vivo* and *in vitro*, identifying Oct4 to be an important regulatory molecule in the initial cell fate decisions during mammalian development. Nanog was identified as a factor that can sustain pluripotency in ESCs even in the absence of leukaemia inhibitory factor (LIF) (Loh *et al.*, 2006).

Although stem cells from different origins require different growth conditions for maintenance and display different cell surface markers, the undifferentiated state of ESCs can be characterised by a high level of alkaline phosphatase (AP) expression, which, along with the expression of surface markers including stage-specific embryonic antigens (SSEA-1, -3 and -4), and the transcription factors Oct4, Nanog, and Sox2 indicates a subset of cells with self-renewal potential (Chunhui *et al.*, 2001; O'Connor *et al.*, 2008). The AP enzyme is considered to be the most recognisable stem-cell marker (Chen *et al.*, 2011). This enzyme is capable of catalysing reactions to give a coloured product that is easily detectable by light microscopy.

1.1.9 Recapitulating haematopoiesis in mouse ESC cultures

Whether cultured with serum or serum-free supplemented with inducers, mouse ESCs show rapid and synchronous differentiation to the haematopoietic lineages (Keller, 2005; Murry *et al.*, 2008). It was found that differentiation in these cultures closely parallels that in the early embryo: PS to $FLK1^{+}$; FLT3 mesoderm to yolk-sac-like haematopoietic program. The haemangioblast has been identified as a progenitor that manifests haematopoietic and vascular potential and defines the start of haematopoiesis (Choi *et al.*, 2009). Another progenitor has been identified in the posterior PS region of the early mouse embryo that may signify the yolk sac haemangioblast. Thus, haemangioblast commitment is initiated in the primitive streak of the mouse embryo (Huber *et al.*, 2004). In ESC cultures, primitive erythroid progenitors develop after the appearance of the

haemangioblast, bringing about the primitive erythropoiesis stage of haematopoiesis (Kitajima *et al.*, 2003; Maxim *et al.*, 2005; Murry *et al.*, 2008).

It has been observed in the differentiation cultures that, besides primitive erythrocytes, other progenitors such as those of the macrophage, definitive erythroid, megakaryocyte and mast cell lineages develop with a kinetic model parallel to the yolk sac (Murry *et al.*, 2008). In this early stage of haematopoiesis, HSCs and progenitors of the lymphoid lineage are not generated. The equivalent of yolk sac haematopoiesis is represented in the first haematopoietic population to be developed from the ESCs, as suggested by these patterns of lineage development. It seems that this *in vitro* system recapitulates what is found *in vivo*, given that yolk sac haematopoietic development in ESC cultures and the early embryo are markedly similar.

Although the yolk sac stage is well characterised in mouse ESC cultures, specification and development is relatively unexplored for P-Sp haematopoiesis. A characteristic defining the P-Sp is lymphoid potential, and mouse ESCs can generate these lineages, given the suitable conditions.

$CD34^+$ populations, obtained by differentiation of ESCs in co-culture with OP9 stromal cells in the presence of a combination of growth factors, are enriched in cells with features of lymphohaematopoietic potential (Vodyanik *et al.*, 2005). Moreover, mouse ESCs will yield cells of B lymphoid potential when co-cultured with monolayer MS-5 stromal cells and three cytokines, namely stem cells factor (SCF), interleukins 3 (IL-3) and IL-7 (Taguchi *et al.*, 2007). B cells potential was demonstrated in the co-culture of ESCs with OP9 stromal cells in media containing lymphoid cytokines (Nakano *et al.*, 1994). Holmes and Carlos (2009) demonstrated that differentiation of mouse ESCs into T-lymphocytes can be achieved *in vitro* with the support of OP9-DL1. This is OP9 that ectopically expresses the notch ligand delta-like 1 (DLL1) and the DLL1 gene plays a role in mediating cell fate decisions during haematopoiesis.

Several gene targeting studies demonstrated the role of specific transcription factors including Scl/tal-1 (Begley *et al.*, 1989), Runx1 (Wang and Speck, 1992), GATA1 and GATA2 (Orkin, 1992) in the establishment of the haematopoietic system. Each of these transcription factors functions at definitive stages of blood cell differentiation (Keller, 2005).

In ESCs co-cultured with OP9 stromal cells, GATA1 and GATA2 were detected on days 2 and 3 of ESC differentiation, coinciding with the appearance of CD34+ cells, whilst SCL expression was detected a day later and then gradually decreased, while GATA1 expression gradually increased up to day 10 (Maxim *et al.*, 2005). This confirms earlier studies demonstrating that GATA1 and GATA2 transcription factors seem to regulate the proliferation and differentiation of blood cells lineage (Suwabe *et al.*, 1998).

From these observations, it appears that in these cultures a population equivalent to the P-Sp region is generated. Collectively, these observations indicate that the haematopoietic system commences with the production of a limited number of specialised lineages in the yolk sac and matures over time into a full multi-lineage system with the switch to the P-Sp region (Keller, 2005).

Although it has been successful attempt to obtain HSCs differentiated from ESCs in serum-stimulated cultures (Burt *et al.*, 2004), routine isolation of HSCs by these method is still not achieved. This means that long-term engraftment in host animals of mouse HSCs differentiated from could depend on a particular batch of serum. It is found that, in order to achieve the development, survival and expansion of HSCs derived from mouse ESCs, the expression of HoxB4 and the caudal-related homeobox-containing factor Cdx4 in mouse ESC-derived haematopoietic had to be activated prior to transplantation (Daley *et al.*,; Kyba *et al.*, 2002; Wang *et al.*, 2005b). The expression of Cdx4 in mouse ESC cultures promotes haematopoiesis (Davidson *et al.*, 2003), whilst the expression of HoxB4 induces extensive self-renewal, with expansion of bone marrow-derived HSCs and multi-lineage repopulation in recipient animals (Sauvageau *et al.*, 1995).

Earlier studies investigating the effect of stromal cells and their microenvironments that promote haematopoietic differentiation of ESCs have shown that osteoblastic cells regulate the haematopoietic stem cell niche (Calvi *et al.*, 2003). Moreover, cytokines and BMP4 secreted from stromal cells promote haematopoietic differentiation of human embryonic stem cells (hESCs) (Chadwick *et al.*, 2003). Ralph *et al.* (2000) found that a number of cytokines play a role in the differentiation of blood cells, and the pathway of differentiation is determined by the balance of these cytokines within the microenvironment of the pluripotent stem cell and its subsequent lineage.

Though the earliest stages of haematopoietic development in human embryos are not accessible for research, it is found that the *in vitro* hESCs/OP-9 co-culture system can be

used to recapitulate it (Lanza *et al.*, 2009) as well as supporting both haemogenic precursors and their primitive haematopoietic progeny (Ji *et al.*, 2008). Enhanced characterisation of haematopoietic progeny using the ES/OP-9 system has been achieved in recent studies (Maureen *et al.*, 2011). An understanding of the embryonic origin of HSC and the factors regulating their generation and expansion *in vivo* will provide important information for the manipulation of these cells *ex vivo* (Timothy *et al.*, 2004). To date, characterisation of specific factors capable of influencing haematopoietic cell fate from ESCs remains elusive. There are no protocols for the efficiency of HSCs from ESCs because the factors regulating their specification have not been defined. Moreover, it is still a challenge to isolate the lymphoid progenitors and to identify the signalling pathways involved because the ESCs were differentiated in complex cultures with stromal cells in serum-based medium.

It seems that the complex process of haematopoiesis is regulated locally by the interaction of hematopoietic cells with cytokines secreted by adjacent stromal cells. It is suggested by several lines of evidence that some aspects of normal haematopoiesis can also be regulated by some vitamins such as VD_3, compounds that are biochemically distinct from cytokines, and hematopoietic growth factors might have also been involved (Grande *et al.*, 2002). These compounds are capable of exerting their biological effects through the interaction with specific nuclear receptors of the steroid receptor superfamily (Kumar *et al.*, 1999).

1.1.10 The benefit of using the E14/OP6 co-culture

ESCs derived from the inner cell mass of the blastocyst-stage of early mammalian embryos have the potential to undergo unlimited self-renewal in specific culture conditions either *in vitro* or in *vivo* (Lanza and Klimanskaya, 2009). Interestingly, once released from these conditions and placed into a differentiation-promoting environment *in vitro* or *in vivo*, the cells differentiate into derivatives of all three primary germs: ectoderm, mesoderm, endoderm and then into many different cell types in the body (Nishikawa *et al.*, 2007; Thomas *et al.*, 2009; Lanza *et al.*, 2009).

It has been shown that ESCs have the potential to differentiate into haematopoietic lineage (Vodyanik *et al.*, 2006). ESCs differentiate into endothelial cells with haemangioblastic properties and colony-forming cells (CFCs) when the embryoid body differentiates or placed in S17 bone marrow stromal cell line co-culture (Kaufman *et al.*, 2001; Zhan *et*

al., 2004). During the development of the embryoid body, if both cytokines and BMP4 are added, ESCs haematopoietic differentiation is markedly promoted (Chadwick *et al.*, 2003). It is demonstrable from these results that ESCs as an alternative source of haematopoietic precursors, most useful in studies of haematopoietic ontogeny and haematopoietic cell transplantation. For this purpose, HSCs has to be produced in great quantity from ESCs, and the methods used must be re-producible.

Alternatively, ESCs can be induced *in vitro* to differentiate into myeloid, lymphoid, erythroid, and megakaryocytic lineage cells by the macrophage colony-stimulating factor (M-CSF)-deficient stromal cell line OP9 (Nakano *et al.*, 1994). It is found that, when cultured on OP9 stromal cells, ES cells differentiate into $FLK1^+$ haemangioblasts, hematopoietic progenitors, and finally mature, terminally differentiated lineages (Maureen *et. al.*, 2001). Hence the *in vitro* ESC/OP9 co-culture system can be used to recapitulate the early stages of hematopoietic development and support both haemogenic precursors and their primitive haematopoietic progeny (Vodyanik, 2005). It was originally designed to produce hematopoietic progeny without the over production of macrophages, as the OP9 stromal cell line is derived from the calvaria of osteopetrosis mutant mice that lack functional M-CSF.

The study of Vodyanik *et al.* (2006) confirms other studies that it was possible to observe the haematopoietic differentiation of ESCs, $CD34^+$ cells and CFCs in OP9 co-culture. This shows that the $CD34^+$ cells that the OP9 system generated brought about B, natural killer (NK) and myeloid cell lineages, which indicated that ESCs could yield cells with definitive haematopoietic potential. It was also found that another benefit of OP9 co-culture was that $CD34^+$ cells could be generated in large quantity without the addition of cytokines.

Thus, the use of ESCs/OP9 co-culture may be an excellent *in vitro* model to analyse haematopoietic development in its earliest stages. These include the study of cell populations and charactering the earliest genes involved. There is no need for external cytokines with this model, and its use does not incur the technical and ethical difficulties of experimenting on human embryos.

1.1.11 The pluripotency transcription factors Nanog, Oct4 and Sox2 and ESC differentiation

Three transcription factors are crucial in the regulation of the pluripotency of mouse ESCs, and these are octamer-binding transcription factor 4 (Oct4), Nanog homeobox (Nanog) and sex-determining region Y (SRY)-related high mobility group (HMG) box 2 (Sox2) (Chambers *et al.*, 2008; Niwa *et al.*, 2008; Masui *et al.*, 2008). During cell differentiation, the maintenance of ESCs' pluripotency is reduced due to the down-regulation effect of the Oct4 protein (Masui *et al.*, 2008).

Homeodomain protein Nanog mainly expresses itself in pluripotent cells and is crucial for the development of the early embryo (Chambers *et al.*, 2008). Evidence from some studies indicates that Nanog maintains the self-renewal capability of ESCs and suppresses their differentiation, though it is not necessary for establishing pluripotency (Takahashi *et al.*, 2006; Chambers *et al.*, 2008). Over-expression of Nanog enables mouse ES cells to propagate in an environment free of leukaemia inhibitory factor (Chambers *et al.*, 2008). In contrast, when Nanog is absent or lacking, ESC differentiation occurs (Zaehres *et al.*, 2008; Liang *et al.*, 2008).

Both Nanog (Liang *et al.*, 2008) and Oct4 (Liu 1996) form specific transcriptional re-repressive complexes. However, research has found that Nanog has two powerful transcriptional activating domains (Avillion *et al.*, 2003), suggesting that, as with Oct4 (Ben-shushan *et al.*, 2008), Nanog can be a transcriptional activator.

The Sox2 transcription factor belongs to the SRY-related HMG box (Sox) transcription factor family (Masui *et al.*, 2008; Avillion *et al.*, 2003). Its expression is found in pluripotent cells and multi-potent embryonic and extra-embryonic cells. Whilst Sox2 is still less well characterised than either Oct4 or Nanog (Chambers *et al.*, 2008; Niwa *et al.*, 2008; Masui *et al.*, 2008), it is known to be expressed in pluripotent and multi-potent embryonic and extra-embryonic cells and is of major importance in regulating the fate of cells (Huangfu *et al.*, 2008).

In conclusion, Oct4, Nanog and Sox2 activate and maintain the expression of genes involved in self-renewal, while simultaneously repressing genes that mediate differentiation (Wu *et al.*, 2008; Yang *et al.*, 2009). This is schematically summarised in Figure 8. Thus, the undifferentiated state of ESCs can be characterised by the expression

of Oct4, Nanog, and Sox2, whilst the differentiated state loses the expression of these transcriptional factors.

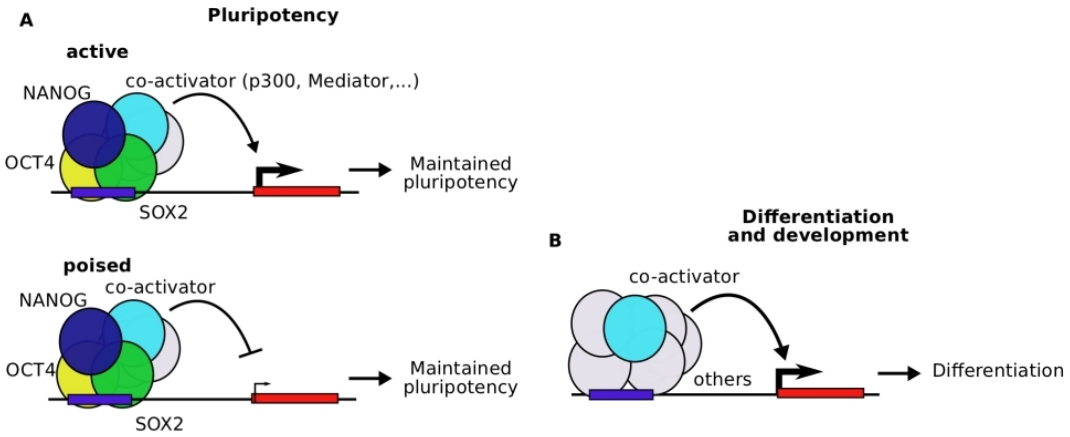


Figure 8. The function of Oct4, Nanog and Sox2 in regulating pluripotency and differentiation (Source: http://openi.nlm.nih.gov/imgs/512/3/3245296/3245296_pcbi.1002304.g009.png)

1.1.12 CD34 marker of haematopoietic progenitor cells

Haematopoietic activity seems to be mostly due to the cell population enriched in $CD34^+$ from marrow or mobilised peripheral blood (Civin *et al.*, 1984; Sutherland *et al.*, 1989; Yabe *et al.*, 1996). There is down-regulation of CD34 expression on primitive cells in their differentiation into mature cells (Sutherland *et al.*, 1992). However, CD34 expression is also detected on clonogenic progenitors and some lineage-committed cells (Andrew *et al.*, 1989). While the exact role of CD34 remains to be defined, its pattern of expression is indicative of an important role in early haematopoiesis (Sutherland *et al.*, 1992). Therefore, CD34 has been regarded as the most crucial marker for HSCs and $CD34^+$ cells can be analysed for detecting changes in HSCs. In fact, the current design of nearly all clinical and experimental protocols related to *in vitro* culture, gene therapy and HSC transplantation are for cell populations enriched for $CD34^+$ cells.

However, CD34 may not be the marker for the most primitive HSC, as it was shown that mouse HSCs could be $CD34^-$ (Osawa *et al.*, 1996). Furthermore, in human $CD34^-$ cells low levels of engraftment and haematopoietic capacity were shown (Bhatis *et al.*, 1998). Additionally, repopulating activity in a $CD34^-$ cell population in foetal sheep was demonstrated in transplantation studies (Zanjani *et al.*, 1998). It has been shown that mouse and human $CD34^+$ cells may both proceed from $CD34^-$ cells (Nakamura *et al.*, 1999; Sato *et al.*, 1999). This means HSCs may possibly be $CD34^+$ or $CD34^-$. Therefore, if the expression of CD34 marker is used as the only criterion for selecting primitive stem

cells, it might run the risk of excluding more primitive stem cells that do not express CD34.

1.1.13 Structure and synthesis of 1α,25-dihydroxyvitamin D_3 (VD_3)

Vitamin D refers to a set of steroid molecules. The active form of vitamin D is 1α,25-Dihydroxy-vitamin D_3 (VD_3), also known as cholecalciferol. Specifically, as shown in Figure 9, VD_3 forms through the absorption ultra-violet (UV) B rays by a precursor molecule 7-dehydrocholesterol in the skin of animals (including human). Since adequate exposure to sunlight may be sufficient to satisfy an individual's vitamin D requirement without need for dietary ingestion, it is not strictly speaking a vitamin. Dietary sources of vitamin D include egg yolk, fish oil and a number of plants. The plant form of vitamin D is called vitamin D_2 (VD_2), known also as ergosterol. Unfortunately, typical diets do not necessarily satisfy an individual's vitamin D requirement. Therefore, exposures to sunlight and/or purposeful dietary supplementation are/is often necessary to prevent vitamin-D deficiency (How *et al.*, 1994).

VD_3 synthesis in the skin is through conversion from 7-dehydrocholesterol, which is the chemical precursor of VD. Until the inactive form of VD_3, cholecalciferal, is metabolised in the body to the hormonally-active form, known as 1,25-dihydroxycholecalciferol, vitamin D is not significantly biologically active. It is transformed in two steps. First, VD_3 is transferred by VD binding protein to the liver, where it is hydroxylated to 25-hydroxycholecalciferol by the enzyme 25-hydroxylase. Then, after being transferred to the kidneys, 25-hydroxycholecalciferol acts as a substrate for 1-alpha-hydroxylase, resulting in 1,25-dihydroxycholecalciferol, which is the biologically active form (Brannon *et al.*, 2008). This enzyme is also found in various extra-renal sites, such as osteoclasts, skin, colon, brain, and macrophages, and this may be a reason for the wide-ranging effects of VD. The short half-life of VD in the liver is only about 3 weeks and therefore the body requires frequent replenishment of this vitamin (Teresa *et al.*, 2009).

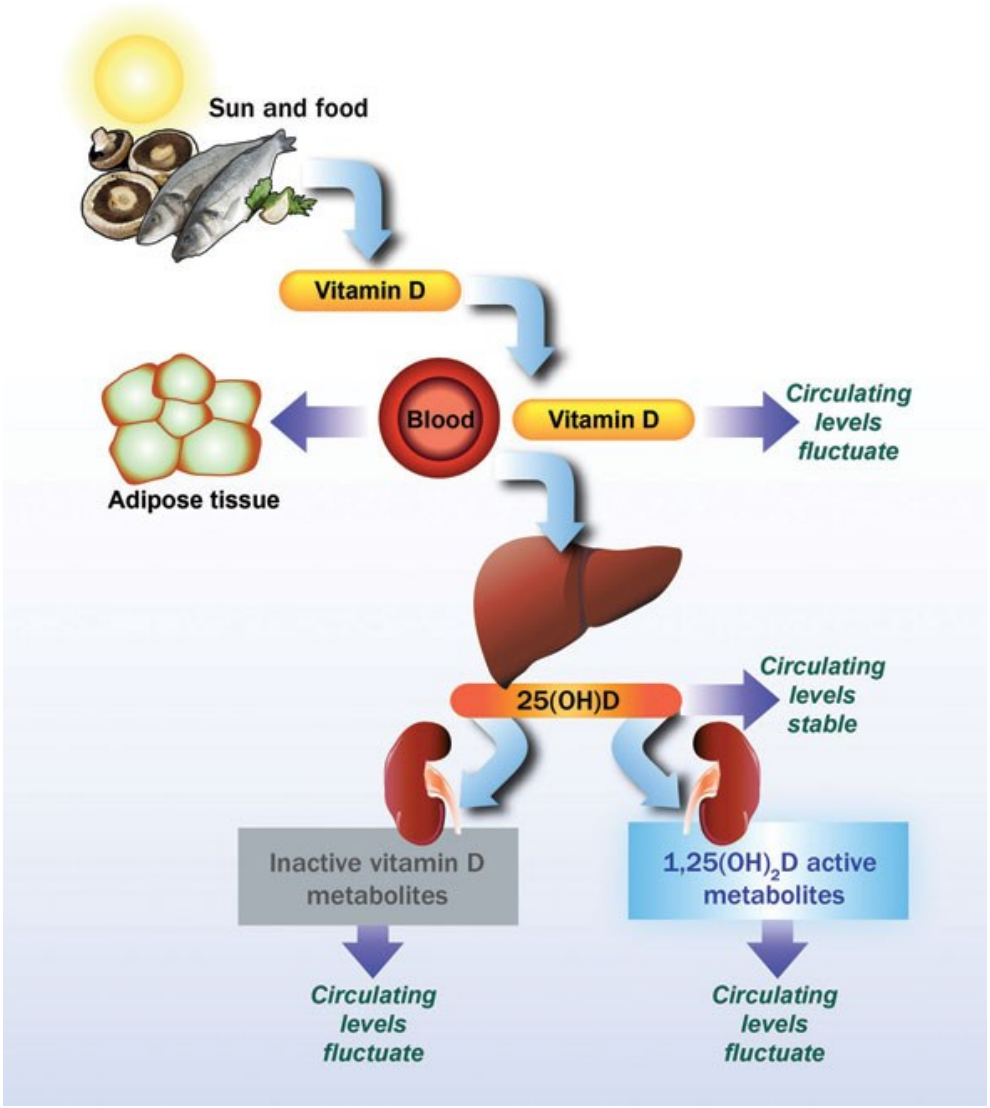


Figure 9. The synthesis and metabolism of Vitamin D (Source: http://www.mayoclinic.org/medicalprofs/endocrinology-vitamin-d-tests-eu0603.html)

VD_2 and VD_3 are hydrophobic, i.e., non-water-soluble, and so in order to be transported in blood, they are bound to carrier proteins. Vitamin D-binding protein is the major carrier. While the half-life of 25-hydroxycholecalciferol is in terms of weeks, that of 1,25-dihydroxycholecalciferol is just hours.

VD_3 synthesised in skin exposed to UVB rays, and UVB rays are sufficiently present only when the sun is sufficiently high in the sky. Therefore, earlier and later in the day, and seasonally in higher latitudes, there may not be sufficient amount of UVB rays for the synthesis of adequate amount of VD_3. Neither do UVB rays penetrate thick cloud. Consequently, the amount of VD_3 synthesis is determined by the strength of the UVB rays, the length of exposure time to UVB rays and the pigmentation of the skin. Tanning

beds are not reliable means for acquiring VD_3 as artificial tanning yields variable levels of UVA and UVB rays.

1.1.14 Control of Vitamin D synthesis

Hepatic synthesis of 25-hydroxycholecalciferol is only loosely regulated, and the level of this molecule in blood generally reflects the amount of vitamin D produced in the skin or ingested. In contrast, the activity of producing 1-alpha-hydroxylase in the kidney is tightly regulated. It is the kidney that serves as the major control point in the production of the active hormone 1,25-dihydroxycholecalciferol. The major inducer of 1-alpha-hydroxylase is parathyroid hormone. 1-alpha-hydroxylase is also induced by low level of phosphate in blood.

Different species differ in their abilities to synthesise vitamin D. Humans, cattle, horses, pigs, rats and sheep have sufficient 7-dehydrocholesterol in their skin to be efficiently converted to cholecalciferol. On the other hand, cats and dogs have much less 7-dehydrocholesterol in their skin and its photochemical conversion to cholecalciferol is inefficient, so these animals rely more dietary intake of vitamin D than other species do (How *et al.*, 1994).

1.1.15 The effect of VD_3 on functional cells

Vitamin D has well-established effects in the regulation of calcium and phosphate homeostasis. VD_3 is widely used for the treatment of metabolic bone diseases such as rickets / osteomalacia, renal osteodystrophy and osteoporosis (Deluca, 2004). Bone density is increased by the treatment with hydroxylated vitamin D (Papadimitropoulos *et al.*, 2002). This is mainly achieved through an increase in calcium uptake in the intestine (Eastell *et al.*, 1991). The differentiation of ESCs into osteoblasts *in vitro* is enhanced to 60% when exposed to VD_3 (Zurnieden *et al.*, 2007).

In addition to its classical function in the maintenance of calcium homeostasis and exerting a wider range of biological activities including the regulation of cellular differentiation and proliferation (Anderson *et al.*, 2003), VD_3 was demonstrated to be a powerful differentiation inducer for a large variety of neoplastic cells, including carcinoma cells of various origins and acute myeloid leukaemia (Grande *et al.*, 2002). It has been shown to have pro-differentiation and anti-proliferative effects on keratinocytes

(Ludered *et al.*, 2010) and prostate cancer cells (Chen *et al.*, 2000), as well as stimulating granulocyte-monocyte committed stem cells into their clonal proliferation *in vivo* (Yetgin *et al.*, 2004).

Vitamin D metabolites may also be involved in immune-regulation and haematopoietic differentiation (Walters, 1992). It is found that VD_3 strongly induces haematopoietic stem cells to differentiate along a myeloid pathway and give rise to $CD14^+$ cells (Weeres *et al.*, 2014). Studies of vitamin-D deficient rickets cases were undertaken regarding the effect on bone remodelling and the haematopoietic system (Yetgin *et al.*, 1982; Yetgin *et al.*, 1989). VD_3 was found *in vitro* to inhibit the proliferation of colonogenic blast cells and induce the differentiation of acute myeloblastic leukaemia (AML) cell lines into macrophage-like cells (Koeffler *et al.*, 1984; Mangelsdorf *et al.*, 1984; Munker *et al.*, 1986). However, $1,25(OH)_2D_3$[7] slightly stimulates normal human granulocyte-monocyte committed stem cells into their clonal proliferation.

Vitamin D receptor (VDR) is a transcription factor that mediates the actions of its ligand, VD_3, in the promotion of monocyte/macrophage differentiation and the inhibition of proliferation and cytokine production by activated T lymphocytes (O'Kelly *et al.*, 2002). VDR is known to be expressed in various tissues including kidney, intestine, bone, macrophages, placenta and skin (Anderson *et al.*, 2003). Expression of VDR has been detected in various normal and leukemic haematopoietic cells, including macrophages and activated T lymphocytes (O'Kelly *et al.*, 2002). An intracellular VDR3 has been detected in numerous tissues and cultured cells ranging from mouse fibroblasts to human breast cancer cells. Therefore, it appears that vitamin D may have a much broader physiological impact in the body (Peehl *et al.*, 1994).

In the target cells, VD_3 acts through binding to VDR as indicated in Figure 10. This combination of vitamin D and receptors then function as transcription factors to modulate gene expression (How *et al.*, 1994) and indirectly activates a family of cell cycle control proteins called cyclin-dependent kinases (CDKs). However, their activity is regulated by another family of proteins called cyclins.

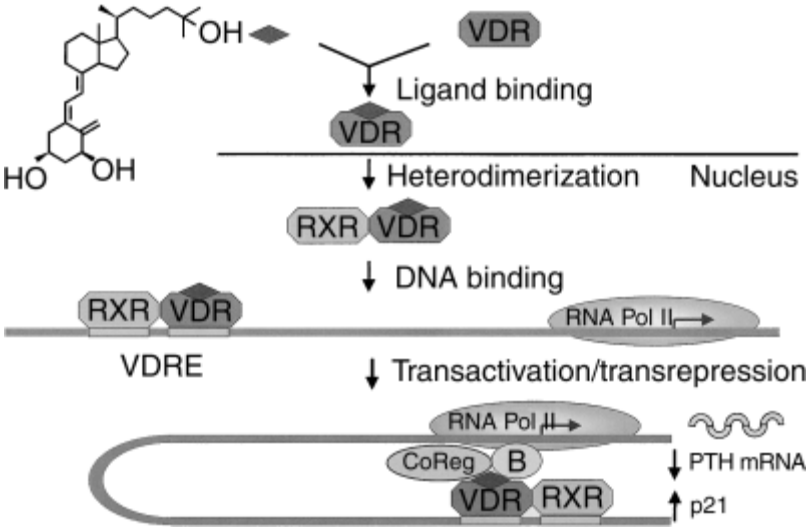


Figure 10. The mechanism of gene regulation by VD_3
(RXR: Thyroid hormone receptor)
(Source: http://www.nature.com/ki/journal/v63/n85s/images/4493809f1.gif)

So far, the effects of VD_3 in different stages of differentiation of various types of cells were examined using *in vitro* techniques. Hiroshi *et al.* (2012) investigated the effect of VD_3 in the proliferating, differentiating and differentiated phases of C2C12 myoblasts, and found that VD_3 supplementation inhibited the proliferation of C2C12 myoblasts, significantly decreased the mRNA expression of neonatal myosin heavy chain (MHC) and increased gene expression of MHC isoforms in the differentiation of C2C12 myoblasts for 8 days.

Systemic or locally produced VD_3 may play a role in modulating cell development processes such as haematopoiesis (Bunce *et al.*, 1997). It has been reported that physiological levels of vitamin D promote a differentiation of $CD34^+$ haematopoietic progenitors characterised by the induction of all the monomacrophagic immunophenotypic and morphological markers in a liquid culture model (Yetgin *et al.*, 2004). Patients receiving treatment for acute myeloid leukaemia (AML) or acute lymphoblastic leukaemia (ALL) may have limited exposure to sunlight and often experience gastrointestinal side effects that may decrease their ability to maintain an adequate vitamin D level (Sprota *et al.*, 2011). These findings support the notion that vitamin D might be involved in cell growth regulation and cancer protection. This might be the reason why the risk of dying from colorectal cancer is the highest in areas with the least amount of sunlight (Tangpricha *et al.*, 2001).

Macrophage differentiation of human normal and leukemic myeloid stem cells has been induced by VD_3 and its fluorinated analogues (Koeffler *et al.*, 1984). Enhancement of VD_3 induced differentiation of human leukaemia HL-60 cells into monocytes by parthenolide via inhibition of the activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (Kang *et. al.*, 2002). The efficacy of VD_3 in leukaemia therapy has been improved by the development of novel vitamin D analogues that have potent antiproliferative activity and low hypercalcemic side effects (Sonoko *et al.*, 2006). The cyclin-dependent kinase inhibitor 1 (p21) is a cyclin-dependent kinase inhibitor (CKI) and is a regulator of cell cycle progression at G_1 / S phase (Gartel and Radhakrishnan, 2005). The cyclin inhibitory protein p27Kip1 (p27) plays a vital role in regulating cell proliferation in response to the extracellular growth environment. Active proliferation requires the suppression of p27 levels throughout the cell cycle (Sa *et al.*, 2004). VD_3 increases the expression of the cell cycle-regulating genes p21 and p27, inducing a G0 / G1 phase arrest in squamous cell carcinoma cell lines (Hager *et al.*, 2001). Thus, the effects of VD_3 may vary according to the type of cell and stage of differentiation at which VD_3 treatment is started.

1.1.16 The effect of VD_3 on the cell cycle

In order for mitosis to start, specific signals are needed by cells so that they can proliferate and be committed to the cell cycle. The mechanism is shown in **Error! Reference source ot found.**.

There is a family of cell cycle control proteins known as cyclin-dependent kinases (CDKs) whose concentration remains constant all through the cell cycle. The activity of CDKs is regulated by another family of proteins known as cyclins. Specific cyclins bind to specific CDKs, and CDKs cannot function without this specific binding. The cyclin-CDK interactions maintain the cell cycle, whilst at the same time there are control mechanisms which inhibit the cyclin-CDK complexes.

Cyclin kinase inhibitors (CKIs) keeps the cell cycle inactive before the start of a new cycle. The CKI-CDK-cyclin complex is formed when CDKs interact with CKIs. When a growth factor or an external signal binds to its binding site or its receptor, the CKI-CDK-cyclin complex is modified, which permits interaction between a subgroup of cyclins and their appropriate CDKs. This is regulated by cell division cycle 25 (CDC25) phosphatase.

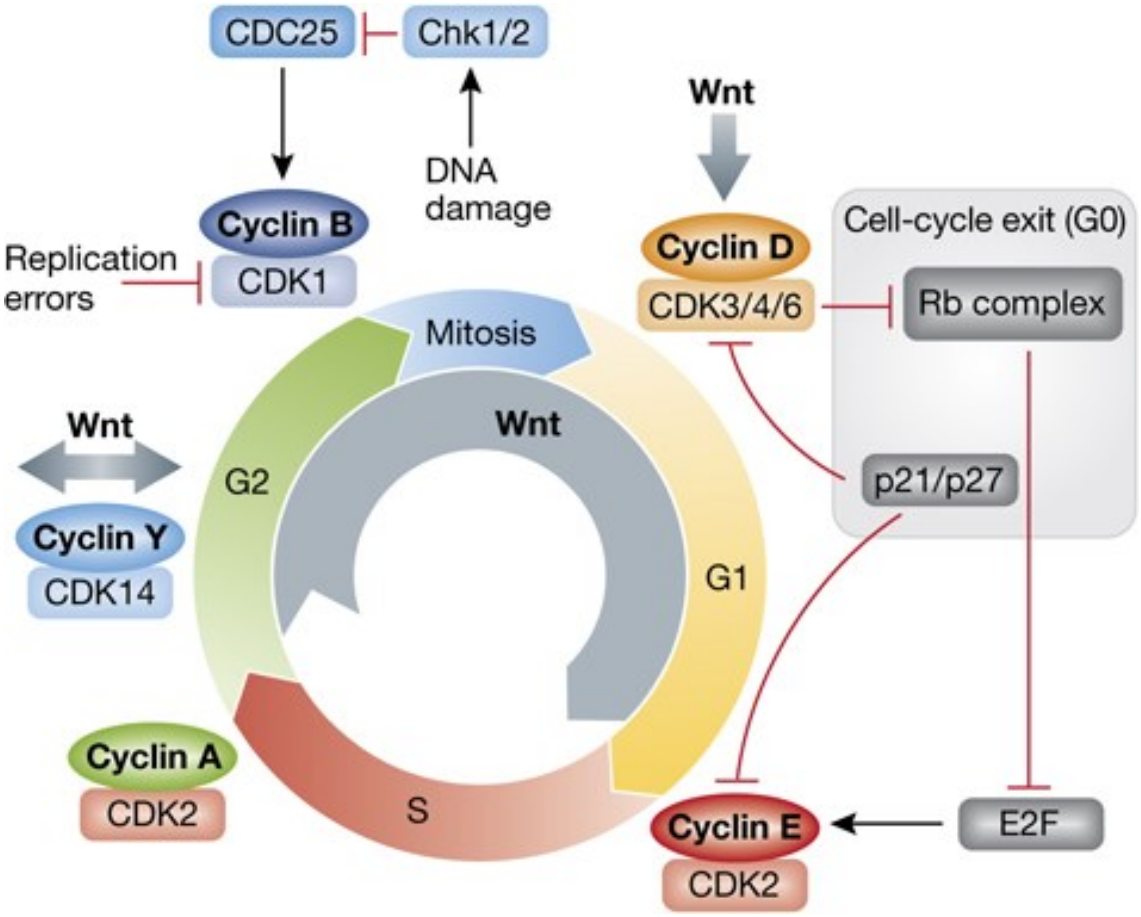


Figure 11. cyclins and their CDKs that control cell-cycle progression (Source: Niehrs and Acebron, 2012)

There is an important regulatory protein known as retinoblastoma (pRb) with which the cyclin D-CDK4 and the cyclin D-CDK6 complexes interact. When CDK4 interacts with cyclin D, the resulting cyclin D-CDK4 complex adds the phosphor group to the pRb. This process is known as phosphorylation. Phosphorylation has the effect of lessening the binding capacity of a transcription factor known as E2F-1, and once E2F-1s are released, E2F-1-dependent genes will be activated. The transcription of a range of genes, notably the gene-encoding cyclin E, is promoted by the release of E2F-1. The enzyme histone deacetylase (HDAC) is another protein bound to pRb.

The mechanism of action of VD_3 was suggested to be mediated via the increase and up-regulation (Okuno *et al.*, 2012) in the expression of p21 and p27 genes. The latter genes were thought to encode cyclin-dependent kinase (CDK) inhibitors (Kanatani *et al.*, 1999) previously associated with the inhibition of G1-cyclin-dependent kinase (Harper *et al.*, 1993; Toyoshima and Hunter, 1994). Cyclins and their associations with CDKs and CDK inhibitors (CKIs) act to regulate cell cycle progression from the G1 to S phase. The production of CDK inhibitory proteins (cip/kip) by p21 and p27 and their subsequent

complex formation with CDKs may inhibit the phosphorylation of retinoblastoma (pRb) and blocks progression of the cell cycle from G1 to S phase (Kanatani *et al.*, 1999; Coqueret, 2003; Aoyagi *et al.*, 2013). With the suspension of phosphorylation, pRb becomes a docking station to prevent the binding of the transcription factor E2F-1 to its target gene, which in turn prevents the production of the cyclin E gene. When cyclin E production is compromised, the cell cycle cannot move along from the G1-phase to the S-phase, thereby causing cells to differentiate and not to proliferate.

1.1.17 Vitamin D deficiency

Vitamin D has been shown to be important for several reasons (Teresa *et al.*, 2009). It controls calcium absorption in the small intestine (Granney *et al.*, 2008). It works in tandem with parathyroid hormone to bring about skeletal mineralisation and maintain bloodstream calcium homeostasis. It has probable anti-inflammatory (Krishnan and Feldman, 2011) and immune-modulating effects (Prietl *et al.*, 2013), and it also has possible effects on cytokine levels. Thus, it is beneficial to have sufficient amount of VD_3, and insufficient amount of VD_3 is linked to many health problems.

Guidelines on daily vitamin D allowance and vitamin D deficiency

The US Institute of Medicine Recommended Dietary Allowance of VD is currently 400 IU per day for children younger than 1 year of age, 600 IU per day for children at least 1 year of age and adults up to 70 years, and 800 IU per day for older adults (IOM, 2011). The US Endocrine Society's Clinical Practice Guideline advised that 400-1000 IU per day may be needed for children aged less than 1 year, 600-1000 IU per day for children aged 1 year or more, and 1500-2000 IU per day for adults aged 19 years or more to maintain 25(OH)D above the optimal level of 30 ng/mL (Pramyothin and Holick, 2012).

The US Institute of Medicine established that serum 25(OH)D of 20 ng/mL or more will cover the requirements of 97.5% of the population (Pramyothin and Holick, 2012). Individuals with level lower than this threshold are considered VD deficient (Sandy *at al.*, 2009).

Furthermore, the National Health Service (NHS) of the United Kingdom (UK) has published descriptions of vtamin D levels of the population related to different stages from VD sufficiency to VD insufficiency and VD depletion (Table 1).

Table 1. Stages from vitamin D sufficiency to vitamin D insufficiency and vitamin depletion (Source: NHS)

VD sufficiency	
	VD supply diminished by lack of dietary vitamin, malabsorption of dietary vitamin or decreased exposure to sunlight
	Lowered concentration of plasma concentration of 25(OH)D and VD_3
	Decreased intestinal absorption of calcium
	Increased synthesis and secretion of parathyroid hormone
	Increased synthesis of VD_3 corrects defective calcium absorption, gives normocalcaemia at the expense of parathyroid hormone
	Further decrease in 25(OH)D through increased turnover and increased consumption
VD insufficiency	Insufficient 25(OH)D to form adequate VD_3
	Failure to absorb calcium; calcium resorbed from bone; bone unable to mineralise
VD depletion	Osteomalacia and rickets

Several health problems associated with low VD level are described in this table. However, there are also other health problems that may be related to low VD levels. Some of the links between VD deficiency and health are discussed below.

Vitamin D and mortality

VD may be one of the factors in reducing the risk of mortality owing to its anti-inflammatory and immune-regulatory roles. VD_3 supplementation is associated with lowered risk of mortality among dialysis patients (Wolf *et al.*, 2007), administered on secondary-hyperparathyroidism patients. There is a correlation between low VD levels in serum and risk of mortality among chronic kidney patients before dialysis (Inaguma *et al.*, 2008). However, no randomised prospective trials of VD on kidney patients have been undertaken (Al-Aly, 2007; Melamed and Thadhani, 2012). In addition, an association between 25(OH)D deficiency and hospital mortality in Medical Intensive Care Unit (MICU) patients has also been demonstrated (Sindhaghstta *et al.*, 2011).

In terms of health effects in general, low VD levels are related to increased inflammation and oxidative load. Low VD amongst more than 3,000 patients due for coronary angiography showed both increased cardio-vascular deaths, as well as multi-cause mortality (Dobnig *et al.*, 2008). A standardised mortality ratio of 1.26 (any figure above 1.0 indicates a higher-than-expected mortality) in the lowest-quartile VD level was found

in a study of over 13,000 individuals (Melame *et al.*, 2008). A meta-analysis[4] (Zheng *et al*., 2013) of 42 randomised controlled trials between January 1960 and January 2013 collected from several databases, including MEDLINE, Embase and The Cochrane Register of Controlled Tirals, showed that supplementation of VD is effective in preventing overall mortality in a long-term treatment that is longer than 3 years, though it failed to identify the efficacy of VD on the mortality of any specific disease.

Vitamin D and cardiovascular disease

The presence of VD receptors in vascular smooth muscle, endothelium, and cardiomyocytes may have an influence on cardiovascular disease. Low VD levels have been found to be related to hypertension, coronary-artery calcification, and existing cardiovascular disease (Stefan *et al.*, 2013). More than 1,700 individuals underwent a cohort study of VD levels and cardiovascular events for a five-year period. It was found that individuals with lower 25-OH D levels had a higher probability of suffering a cardiovascular event, with a hazard ratio of 1.62, a relationship that was significant for hypertension sufferers only (Wang *et al.*, 2008).

Vitamin D and diabetes

It seems that VD_3 may be important in glucose-metabolism homeostasis and in connection with types 1 and 2 diabetes mellitus (DM). From epidemiological data, it has been suggested that there may be a connection between early-life VD_3 exposure and type 1 DM (Mathieu *et al.*, 2005), and it is thought that increased VD_3 intake in infancy may reduce the risk of this disease developing (Danescu *et al.*, 2009). Type 1 DM is linked to VD_3 receptor-gene polymorphisms (Matieu *et al.*, 2004; Teresa *et al.*, 2009). There is an association between VD_3 and a number of factors contributing to type 2 DM, notably defective pancreatic-cell function, insulin sensitivity and systemic inflammation.

[4] Meta-analysis is a statistical technique that combines the findings from independent studies by combining data from two or more randomised control trials. It is most often used to assess the clinical effectiveness of healthcare interventions. A good meta-analysis aims for complete coverage of all relevant studies, looks for the presence of heterogeneity, and explores the robustness of the main findings using sensitivity analysis (Krombie and Davis, 2009).

There have been different physiological explanations on the role of VD_3, such as in insulin secretion, with calcium in insulin action, or in cytokine regulation (Mathieu *et al.*, 2005; Palomer *et al.*, 2008; Danescu *et al.*, 2009).There is evidence in one study for a link between low VD_3 level, calcium or dairy consumption and the occurrence of type 2 DM or metabolic incidence, with a 0.36 prevalence (the portion in the total population with this condition), which occurred most frequently in non-black participants with the lowest 25-hydroxyvitamin D. The study also showed type 2 DM had greatest prevalence of 0.71 in individuals with low dairy consumption. Another study (Dalgård *et al.*, 2011) found that VD sufficiency may provide protection against type 2 DM in subjects aged 70-74. An inverse relationship was found between type 2 DM and metabolic syndrome incidences and the intake of vitamin D and calcium (Pitta *et al.*, 2007).

Vitamin D and osteoporosis

The most frequently occurring metabolic bone disease is osteoporosis, and lack of vitamin D is linked to it. Specifically, insufficient serum vitamin D causes decreased active transcellular calcium absorption. It has been found that combined calcium-vitamin D supplementation is linked to increased bone mineral density (Schnabel *et al.*, 2012) and decreased hip fractures (Rodriguez-Martinez and Garcia-Cohen 2002). It has been demonstrated that a daily vitamin D intake of greater than 700 IU in combination with calcium reduced bone loss in comparison to a placebo, but 300 to 400 IU without calcium had no effect in reducing fractures (Granney *et al.*, 2008). Furthermore, a Cochrane review was unable to determine whether vitamin D supplementation alone reduced bone fractures, but recommended combined calcium-vitamin D supplementation for the frail and elderly nevertheless (Avenell *et al.*, 2009), and a meta-analysis concurred that combined supplementation is necessary (Boonen *et al.*, 2007). Bergman *et al.* (2010) found that the daily dose of 800 IU of VD_3 reduces the incidence of osteoporotic non-vertebral, hip, and non-vertebral-non-hip fractures in elderly women, and VD_3 supplement increases the benefit of calcium supplementation for non-vertebral and non-vertebral-non-hip fractures.

Vitamin D and falls amongst the elderly

Vitamin D is thought to be crucial for the wellbeing of the elderly. Its supplementation evidently reduces the risk of falls, possibly by improving muscle function (Bruyère *et al.*, 2014).

A trial of women who had had at least one fall in a prior 12-month period found that recipients of 1000 IU daily of ergocalciferol (VD_2, i.e., calcium and vitamin D) had a significantly lower risk of falls than those who had received a placebo (Prince *et al.*, 2008). Daily vitamin D doses below 800 IU did not significantly reduce the risk of falls compared to the placebo (Broe *et al.*, 2007). Another study found a significant 22% reduction in fall risk amongst an elderly group in reception of vitamin D compared to other elderly receiving calcium only or a placebo (Bischoff-Ferrari *et al.*, 2004). Daily supplementation of 800 to 1000 IU is recommended for fall prevention function (Bruyère *et al.*, 2014).

Vitamin D and cancer

Vitamin D is thought to be beneficial in preventing and suppressing cancer, perhaps because of its role in regulating cell growth and differentiation (Osborne *et al.*, 2002). It has been demonstrated *in vitro* that dietary levels of VD and VD_3 exhibit equivalent anticancer activity in mouse xenograft models of breast and prostate cancer (Srilatha *et al.*, 2012). In a cohort study analysing the risk factors in a followed-up group of some 1,100 men who do not have a disease, an increment in 25(OH)D level of 25 nmol/L was linked to a 17% reduction in total cancer cases (Giovannucci *et al.*, 2006). However, another study, of nearly 17,000 people, found no such link, but rather an inverse relation between vitamin D level and colorectal cancer, though serum 25(OH)D levels of 80 nmol/L afforded a 72% lowering of colorectal cancer risk in comparison with levels below 50 nmol/L (Freedman *et al.*, 2007). A meta-analysis of 63 studies was done, in which 20 of 30 studies showed a lower frequency or reduced mortality of colon cancer, whilst 9 out of 13 breast-cancer studies and 13 out of 26 prostate-cancer studies similarly showed reduced incidence or mortality (Garland *et al.*, 2006). A meta-analysis of 5 studies (Mohr *et al.*, 2014) found that high serum 25(OH)D was associated with lower mortality from breast cancer and suggested that serum 25(OH)D in all patients with breast cancer should be restored to the normal range (30-80 ng/mL).

Deficiency in Vitamin D may be a cause of osteomalacia, bone pain, muscle weakness, fatigue, and may increase the risk of fracture. It may also precipitate or exacerbate osteopenia and osteoporosis. Patients being treated for acute myeloid leukaemia (AML) or acute lymphoblastic leukaemia (ALL) may have had little exposure to sunlight and frequently suffer gastrointestinal side effects that may reduce their capability of maintaining a sufficient level of VD (Lisa *et al.*, 2011).

It is suggested (Iguchi *et al.*, 2005) that Vitamin K_2 plus VD_3 in combination may be effective for differentiation-based therapy for leukaemia, as well as for leukaemia and myelodysplastic syndrome (MDS) where the cytopenias are mediated though apoptosis.

It has been shown that dietary VD administration has an effect on the innate immune parameters of a gilt-head sea bream. The immunostimulant effect was greater on the cellular innate immune parameters assayed, suggesting that similar receptors to those present in mammals are involved in the action of this vitamin in the fish immune system (Cerezuela *et al.*, 2009).

Projections indicate that by increasing the yearly serum 25(OH)D level to 40 to 60 ng/mL (100–150 nmol/L) would reduce about 58,000 new cases of breast cancer every year, and would also bring down the number of new cases of colorectal cancer by about 49,000 annually. Similarly, three quarters of deaths from these diseases in the United States and Canada could be prevented, according to observational studies in combination with a randomised trial. Taking these quantities of the serum will probably halve the case-fatality rates in breast, colorectal and prostate cancer (Cedric *et al.*, 2009). This could be the result of the anti-inflammatory effects of VD_3. It reduces the gene activation in breast cancer cells by interfering with the transactivation potential of the p65 subunit of the nuclear factor kappa B (NF-κB) (Tse *et al.*, 2010). In another study (Guan *et al.*, 2013), it is shown that VD_3 might have anti-proliferation effects on lung cancer cells by up-regulating the transcription of the hsa-let-7a-2 gene in the latter.

The mechanism of the anticancer action of VD_3 is summarised in Figure 12.

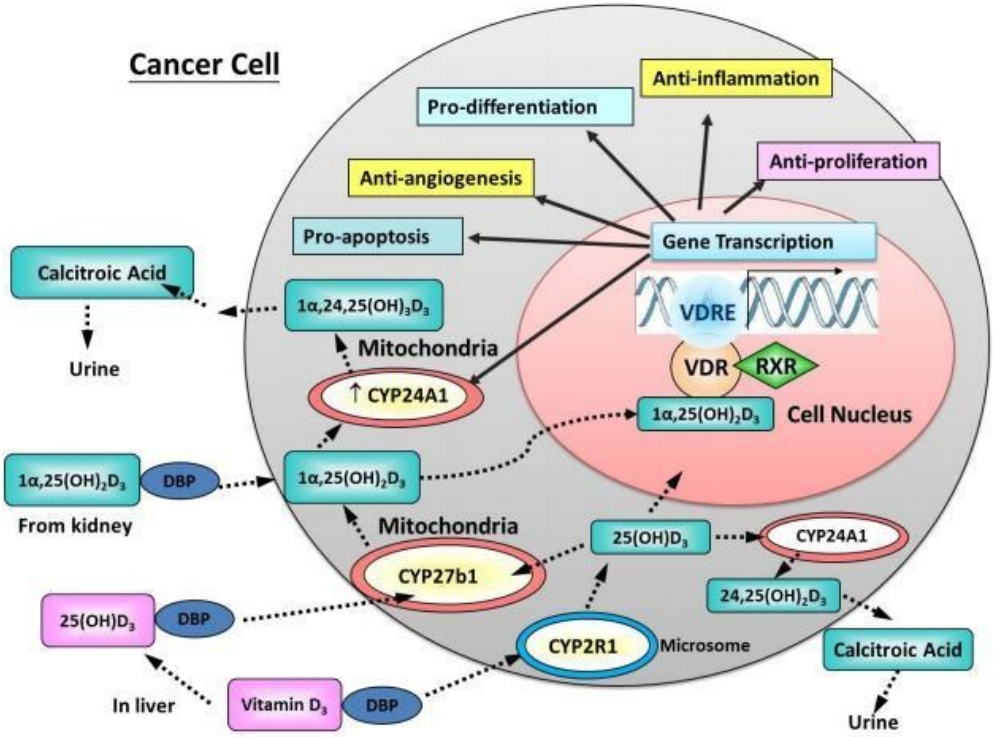


Figure 12. The mechanism of the anticancer action of VD_3 (Source: http://www.vitamindwiki.com/tiki-download_wiki_attachment.php?attId=1783)

1.1.18 Vitamin D and haematological diseases and disorders

VD is a steroid hormone. VD signalling takes place through VDRs. VDR is a receptors in the family of steroid/thyroid hormone-activated transcription factors and is the mediator of VD activity. VD binds with VDR to form dimers. Those dimers can then binds to VD response elements (VDREs) in promoters of target genes. Thus, VD and VDRs bind to specific locations of the human genome, resulting in target gene transcription (Kim *et al.*, 2012; Bogunia-Kubik *et al.*, 2008; O'Kelly *et al.*, 2002; Bunce *et al.*, 1997). Through this mechanism, VD has influence on almost 3,000 genes out of the 25,000 human genes. Thus, it regulates more genes and bodily functions than any other hormone discovered so far, and is one of the most potent hormones in the human body.

VDRs have been identified on hematopoietic and lymphoid cells, amongst other tissues. This indicates that VD may play some important role in blood cell development and immune system function.

In the haematopoietic system, VDR is expressed on various hematopoietic precursors as well as monocytes, some thymocytes, and activated B and T lymphocytes (Luong and Koeffler, 2005). Through these, VD stimulation can influence haematopoietic development. VD_3 treatment of both normal haematopoietic stem cell lines and leukemic cell lines leads to increased monocyte/macrophage differentiation results in increased numbers of these mature cells (Bunce *et al.*, 1997; Grande *et al.*, 2002). This treatment might be primarily affecting cytokine signalling and the final steps in differentiation in these two cell types (Bunce *et al.*, 1997).

As to the effect of VD in immune modulation, it is crucial in the immune response, enabling the production of over 200 antimicrobial peptides that fight off infections. Furthermore, the presence of the VDR on activated lymphocytes suggests a role in immune modulation on differentiated cells. As VD and VDR affects cytokine expression, they not only modulate the Th1 cellular immune response (O'Kelly, 2002), but also favour the Th2 hormonal immune response over Th1 (Luong and Koeffler, 2005; O'Kelly *et al.*, 2002) through their immune-modulatory effect is still unclear. Furthermore, VD and VDR seems to be crucial for proper development of invariant natural killer (iNK) cells, a subset of lymphoid cells involved in the most basic immune responses and also in restricting autoimmunity (Yu and Cantorna, 2011; Yu *et al.*, 2011). Additionally, VD and VDR may also have a role in immune tolerance, the process by which autoimmunity is prevented, as it mediating the homing of lymphoid cells to specific tissues and in attenuating inflammation (Yu *et al.*, 2008).

There is significant interest in studying VD analogues as treatment for myeloid malignancies, such as myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML), as VD promotes differentiation of normal hematopoietic precursors and malignant myeloblasts *in vitro*. Some form of MDS may change to AML, but the exact mechanism by which this change is induced by VDR activation is not fully understood (Hall and Juckett, 2013).

There are concerns of hypercalcemia if supraphysiological doses of VD are needed to induce differentiation (Bunce, Brown and Hewison, 1997; Koeffler, Hirji and Itri, 1985), but it seems that it is more promising to induce differentiation with fractionated dosing that reduces each dosage to within physiological ranges (Grande *et al.*, 2002).

Various approaches of VD therapy for MDS have been studied. Some studies focus on single agent VD therapy (Koeffler, Hirji and Itri, 1985; Harrison and Bershadskiy, 2012; Molnar *et al.*, 2007; Mellibovsky *et al.*, 1998; Motomura *et al.*, 1991) and some use VD_2 analogues (Koeffler *et al.*, 2005; Petrich *et al.*, 2008), whilst some other try combining VD with other differentiation and cytotoxic agents (Siitonen *et al.*, 2007) or using growth factors with differentiating agents (Ferrero *et al.*, 2009), as well as looking at combinations of VD analogues as differentiating agents with cytotoxic chemotherapy (Ferrero *et al.*, 1996).

AML, unlike MDS, tends to be fatal over weeks or months without effective treatment. Therefore, therapeutic plans that include only differentiating agents such as VD have tended to be used only in patients with very treatment resistant disease or those at high risk for side effects of conventional cytotoxic chemotherapy (Harrison and Bershadskiy, 2012). There are trials with low dose cytarabine regimens combined with VD analogue and another agent (Slapak *et al.*, 1992; Ferrero *et al.*, 2004). There is at least one trial combining VD analogue and chemotherapy with a control group (Hellstrom *et al.*, 1990), but most studies do not have control group and are also limited by variable response criteria and unclear end points, so it is difficult to draw conclusions from these studies.

As to VD therapy for non-myeloid haematological cancers, pre-clinical studies have shown that VD has an inhibitory effect on lymphoid neoplastic cells (Luong and Koeffler, 2005), and activity of the VD analogue EB1089 in the myeloma H929 cell line (Park *et al.*, 2002; Park *et al.*, 2000; Puthier *et al.*, 1996) appears to promote apoptosis and induce cell cycle arrest by down-regulation of cyclin dependent kinases (Park *et al.*, 2002).

In addition, VD analogues are well-established therapies for some autoimmune conditions such as psoriasis, likely due to the effects of VD on the signalling, tissue targeting, or immune regulation of activated T and B cells. VDR stimulation is also crucial to the activity of antigen presenting cells and thus is crucial to response to infections. VD and VDR can also be used in immune reconstitution and immune surveillance in patients of transplantation such as allogeneic hematopoietic stem cell transplantation (HSCT) to suppress side effects and improve success rates (Pakkala *et al.*, 2001).

Besides using in direct treatments, it is prudent to maintain normal level of patients particularly vulnerable to bone disease and mineral loss, such as patients after allogeneic stem cell transplant and patients with multiple myeloma.

1.2 Research Motivation

The capacity of ESCs to undergo unlimited self-renewal and differentiation into many different cell types in the body has huge potentials in the fields of biomedical research and regenerative medicine. ESCs have been used for therapeutic intervention in the treatment of a wide range of disease conditions. One of the most important potential applications of ESCs for this purpose is the generation of cells that could be used for cell-based therapies, such as transplanting HSCs in leukaemia treatment. The conventional treatment involves the use of high-quality sources of tissue-matched bone marrow, mobilised peripheral blood or umbilical cord blood. However, appropriate bone marrow is often in short supply and cord blood, though bankable, is less suitable for adult transplantation because it contains fewer ESCs. Thus, direct differentiation of ESCs towards HSCs offers a potentially attractive alternative to these conventional sources.

Additionally, it has been demonstrated that VD_3 is a powerful differentiation inducer for a wide variety of neoplastic cells, so this active form of vitamin D may help to promote cell differentiation in other cases. Previous related researches had been conducted mainly using cancer cells. Thus it is worth finding out if this is true with haematopoiesis using non-cancer ESCs.

The difficulty with such research on ESCs is that the earliest stages of haematopoietic development that are not accessible in human embryos. However, it has been shown *in vitro* that the ESCs / OP9 stromal cells co-culture system can be used to recapitulate the earliest stages of haematopoietic development and support both haemogenic precursors and their primitive haematopoietic progeny (Lynch *et al.*, 2011; Vodyanik *et al.*, 2005). This is due to two important properties of the OP9 stromal cells. First, it does not produce the macrophage colony-stimulating factor (M-CSF) due to mutation, so there would be no M-CSF that would inhibit the haematopoiesis process (Kitajina, 1998). Secondly, it produces various cytokines that could be used to successfully to induce mouse ES cell differentiation into myeloid, lymphoid, erythroid, and megakaryocytic lineage cells

(Lynch, 2011), so no exogenous cytokines would be needed (ATCC, LGC standards, UK).

Furthermore, E14 was shown to be efficiently able to support full development (Wakayama, 1999), so it is useful in researches on haematopoiesis. Hence, the E14 and OP9 cell lines and the E14 / OP9 co-culture are selected as the subjects of the *in vitro* studies in this research.

1.3 Aims, Objectives and Hypotheses

Based on the research motivation, therefore, the **aim** of these studies is to elucidate the effect of VD_3 on the mechanisms of ESC differentiation towards haematopoietic cells and then blood cells. The overall **hypothesis** is that VD_3 enhances and accelerates haematopoiesis. The null hypothesis is that VD_3 has no effect on the differentiation of ESCs into blood cells.

More specifically, VD_3 enhances the differentiation of ESCs in the vicinity of OP9 stromal cells into blood cells of erythroid, myeloid and B cell lineages, even without the influence of exogenous growth factors or complex structures. The null hypothesis is that VD_3 will not enhance the differentiation of ESCs into blood cells when co-cultured with OP9 stromal cells.

Thus, the **first objective** of the research is to ascertain whether or not VD_3 plays an important role in suppressing proliferation and promoting differentiation of cells in general. The first **hypothesis** is that VD_3 is instrumental in promoting cell differentiation and suppressing cell proliferation. The null hypothesis is that VD_3 has no effect in promoting cell differentiation and suppressing cell proliferation.

Then, the **second objective** of the research is to ascertain if VD_3 plays a significant role in haematopoiesis. The second **hypothesis** is that VD_3 encourages haematopoietic development by suppressing proliferation and promoting differentiation of cells in haematopoiesis. The null hypothesis is that VD_3 has no effect on haematopoiesis.

Once the effect of VD_3 on haematopoiesis is investigated with controlled amounts of VD_3, the next step in the study would be to ascertain the effect of the change in VD_3 level on the variations in the products of haematopoiesis. Thus, the **third objective** of the research

is to establish the effect of variable VD_3 level on the haematopoietic progenitor cell count and the total number of blood cells over time. The third **hypothesis** is that the number of haematopoietic progenitor cells and major types of blood cells increase or diminish as the VD_3 level increases or diminishes, i.e. there is positive correlation between the number of cell counts and the level of VD_3. The null hypothesis is that VD_3 level has no effect on the number of haematopoietic progenitor cells and major types of blood cells.

1.4 Overall Design of the Study

To test the hypotheses, three parts of researches involving several sets of studies were designed and conducted. First, to ascertain whether VD_3 is crucial in promoting differentiation and suppressing proliferation, *in vitro* studies using mouse cell line were conducted. Separate investigations were carried out to find out the effects of VD_3 on the proliferation phase of the E14 cell line and on stromal OP9 cells, respectively, as these two types of cells would also be used in the next step of the study. In both cases, VD_3-treated cells were compared to untreated ones. The materials, principles, protocols and methods used in this part of the research, as well as the results and findings obtained from it, are presented in Chapter Two.

Secondly, to ascertain whether VD_3 accelerates and promotes haematopoiesis of ESCs in the presence of OP9 stromal cells, *in vitro* studies using mouse cell lines were done using the co-culture of ESCs and OP9 stromal cells, i.e., the E14 / OP9 co-culture, as this was shown to be a good setting for the study of haematopoiesis. The materials, principles, protocols and methods used in this part of the research, as well as the results and findings obtained, are presented in Chapter Three.

Thirdly, to ascertain if and how variation in VD_3 level affects haematopoiesis, a pilot study was done *in vivo* with human participants, where the VD_3 level was monitored but not controlled by the designs or settings of the experiment. The materials, principles, protocols and methods used in this part of the research, as well as the results and findings obtained, are presented in Chapter Four.

Finally, based on the findings of these experiments, an overall conclusion was drawn. The summary of findings, the novelty, the contributions and the limitations of the research, together with possible directions for further researches, are presented in Chapter Five.

Chapter 2 The Effects of VD_3 on E14 and OP9 Cells *in Vitro*

2.1 Introduction

The aim of this part of the study is to ascertain if VD_3 plays a key role in suppressing proliferation and promoting differentiation of cells in culture. The hypothesis is that VD_3 is instrumental in promoting cell differentiation and suppressing cell proliferation.

Hence, there are two kinds of effects of VD_3 that are under investigation. First, the anti-proliferation effect of VD_3 has studied through cell proliferation assays by cell counting and alkaline phosphate staining. Secondly, the effect of VD_3 on cell differentiation has studied through cell cycle examination by flow cytometric analysis. Both mouse ESC line E14 and mouse stromal feeder OP9 cells in the proliferation stage have been investigated separately *in vitro* with both sets of tests.

The materials and equipment used for the studies, the methods and protocols adopted for experimental preparation and analyses, the results obtained from the experiments, and the discussion based on the results are presented in this chapter.

2.2 Protocols and Methods

2.2.1 Cell culture preparation

The cell cultures used in the study were prepared from thawed frozen stock. These were grown to desired population by inducing cell proliferation. Afterwards, cells were taken from these proliferated populations and transferred or passed to different growth conditions designed for the experiments. Unused cell cultures can be preserved by cryopreservation to be resurrected in the future for later experiments. These procedures used in the preparation of cell cultures for the study are described below.

Inducing proliferation of OP9 and E14 cells

The stromal cells used in the present study were of the OP9 cell line (ATCC, LGC standards, UK) established from the calvaria of new born op/op mice. This cell line was chosen due to the property that they do not produce macrophage colony-stimulating factor (M-CSF). Primarily, OP9 cells do not produce functional M-CSF due to an osteoporotic mutation in the gene encoding M-CSF, and the presence of M-CSF had inhibitory effects on the differentiation of ESCs into blood cells other than macrophages. In fact, it has been reported that M-CSF has some inhibitory effects on the process of haematopoiesis (Kitajima *et al.*, 1998), and OP9 cells have been used to co-culture mouse ESCs to successfully induce the differentiation of ESCs into blood cells of erythroid, myeloid and B cell lineages. Furthermore, co-cultivation with OP9 did not require exogenous growth factors or complex embryoid structures (Kitajima *et al.*, 1998).

To prepare samples for the present study, OP9 cells were induced to proliferate and expand by thawed cell stock vials at room temperature and added to a gelatinised 25 mm culture dish or T75 flask, at a density of 5×10^5 cells per dish or 2×10^6 per flask, in an undifferentiated state in a fully humidified atmosphere with 5% CO_2 at 37 ºC in GM composed of 385 mL alpha modification minimum essential medium (no nucleosides) (αMEM) with 2.2 g/L sodium bicarbonate at pH 7.2, 100 mL heat-inactivated HyClone™ Foetal Bovine Serum, 5 mL non-essential amino acid solution (NEAA), 5 mL L-glutamine (200 mM final), 5 mL penicillin-streptomycin solution (10.000 units/mL penicillin and 10.000 µg/mL streptomycin), and 500 µL 2-Mercaptoethanol (50 mM final). The medium was changed every 2-3 days.

OP9 cultures were never grown beyond 90% confluency as this would result in their differentiation into adipocytes. It is possible to generate stable cell lines because OP9 differentiation is not diminished by maintenance in either high cell-density or long period in continuous culture (Wolins *et al.*, 2005). It was found that it took 6 hours for OP9 cells to settle down.

The ESCs used in the present study are the murine ESCs of E14 cell line passages 1-36. The first successfully derived murine ESC line was achieved by Martin Evans and Matthew Kaufman in 1981 (Lanza *et al.*, 2009) and the ESC line E14 was derived from the inbred mouse strain 129/Ola by Dr. Hooper in Edinburgh in 1985 (Hooper *et al.*, 1985).

As samples for the current study, E14 cells were induced to proliferate and expand by thawed cell stock vials at room temperature and added to gelatinised 25 mm culture dishes or T75 flasks at a density of 5×10^5 cells per dish or 2×10^6 per flask. They were in an undifferentiated state in a fully humidified atmosphere with 5% CO_2 at 37 ºC in growth medium (GM) composed of 450 mL Dulbecco's Modified Eagle's medium (DMEM) with 10% Knockout™ Serum Replacement, 5 mL non-essential amino acid solution (NEAA), 5 mL L-glutamine (200 mM final), 5 mL penicillin-streptomycin solution (10.000 units/mL penicillin and 10.000 µg/mL streptomycin), 50 µL leukaemia inhibitory factor (LIF), and 500 µL 2-Mercaptoethanol (50 mM final) until 90% confluence was attained. The medium was changed every 2-3 days.

Passaging of cells

Passaging of cells was performed by aspirating culture mediums, washing cells with PBS to remove any remaining serum, trypsinising with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) for 3-5 minutes at 37 ºC to detach cells adhered to the substrate and re-suspending the cells in pre-warmed GM (in at least 1:1 ratio of trypsin:GM to deactivate the action of the trypsin. E14 cells were centrifuged at 750 rpm for 5 minutes at room temperature, whilst OP9 cells were centrifuged at 130 × g for 7 minutes at room temperature. The supernatant was discarded and cells were re-suspended in fresh GM. For cell counting, cell suspension was prepared in a 1:1 dilution in trypan blue stain (Bio Whittaker, Wokingham, UK) and cells were counted with a Neubauer haemocytometer (Assistent, Sondheim, Germany). Viable cells excluded the trypan blue dye and only the dead cells were stained, confirming a disruption to membrane integrity. After cells were counted and the numbers adjusted for dilution and stock volumes, cells were seeded with GM in a dish or a multi-well dish, pre-coated with 0.1% gelatine. Working volumes were normally 15 mL of mediums for a T75 flask, 2 mL of medium per well for a 6-well multi-dish and 5 mL of medium for a 25 mm culture dish.

Cell cryopreservation and resurrection

Optimal freezing of cells depends on minimising intracellular ice crystal formation and reducing damage from formation of foci of high-concentration solutes when intracellular water freezes. This is achieved by freezing slowly, allowing water to leave the cell, but not so slowly that ice crystal formation is encouraged, and thawing rapidly to minimise

ice crystal growth and generation of solute gradients formed as the residual intracellular ice melts.

For preparation of cell stocks, confluent cell monolayers were trypsinised and cells counted with a Neubauer haemocytometer. Cell numbers were diluted in GM to 5×10^5 cells/mL. 10% for E14 and 5% for OP9 of dimethyl sulphoxide (DMSO) (BDH, Poole, UK) was added dropwise to the cell suspension and cells were transferred to 1 mL cryovials. DMSO partially solubilises and stabilises the cell membrane, making it less prone to puncture and irreversible damage. This is mainly because it interrupts the lattice structure of ice so that fewer ice crystals would be formed. It also draws water out of the cells, dehydrating them and protecting intracellular components from ice crystal damage.

Subsequently, cryovials of E14 cells were placed into freezing chambers (Mr Frosty™ which contains isopropanol alcohol) purchased from Nalgene (Rochester, NY, USA). These chambers were placed into a box of ice for 15 minutes, then at -20 ºC for 3 hours, and then transferred for overnight storage at -80 ºC. This procedure allows gradual freezing of the cells at a rate of -1 ºC/min, which allows the reduction in metabolism of the cells as well as enhanced survival upon resuscitation. Following overnight incubation at -80 ºC, cryovials were transferred to liquid nitrogen for long-term storage. The growth of ice crystals is reduced in liquid nitrogen, thus enhancing cell survival during long-term storage. On the other hand, cryovials of OP9 cells were placed into a box of ice for 15 minutes then the freezing chambers were transferred for long-term storage at -80 ºC.

For restoring cell stocks from liquid nitrogen, cryovials were placed at room temperature in a cell culture safety cabinet until thawed and cell suspension quickly transferred to a 0.1% gelatine pre-coated flasks or dishes containing warm GM.

Prevention of Cross-Contamination of Cells

It is important to prevent and detect cross-contamination as it will severely affect the outcome of the experiments and invalidate the findings. Hence, there are standard procedures to follow so as to prevent and detect cross-contamination. These are listed in Table 2 on page 62. In this table, cross-contamination is categorised into seven types according to the nature of the contaminants: bacteria ($Gram^+$, $Gram^-$), yeast, fungus, virus, endotoxin, mycoplasma and cellular.

Generally, for the studies of this research, all standard procedures were followed, with special attention on the cellular type of contamination. These include working with only one cell line at a time, thoroughly clean before and after introducing a new cell line into the laminar flow hood, sterilising all equipment and follow all standard procedure in preparation of all materials, etc. More specifically, to remove or limit the chances of cross-contamination, disposable tools were used extensively, whilst non-disposable ones were exclusively designated for this research and were kept in a dedicated cabinet.

Most importantly, alkaline phosphatase staining was used regularly to determine the purity of stem cell samples.

Table 2. Troubleshooting Cell Culture Contamination
Common cell culture contaminants, their sources, detection and prevention. (Corning, 2013)

Type	Source	Detection	Prevention
Bacteria (Gram +, Gram -)	– Lab personnel – Unfiltered air – Humidified incubators – Purified water – Insects – Plants – Contaminated cell stock – Media	– Microbial culture – Gram's stain test – Visual turbidity – pH becomes acidic	– Aseptic technique – Antibiotics – Filtration (< 0.22 µm) – Use sterile products – Disinfection of CO2 incubators – Daily cleaning of hood space with 70% alcohol as well as monthly cleaning with 10% bleach. – Daily, or at minimum weekly emptying of used media traps. – Store cell line stocks in vapour phase of LN2, not in liquid phase
Yeast	– Humidified incubators – Lab personnel – Unfiltered air – Contaminated cell stock	– Microbial culture – Visual turbidity	– Aseptic techniques – Antimycotics – Filtration (< 0.5 µm) – Use sterile products – Disinfection of CO2 incubators – Daily cleaning of hood space with 70% alcohol as well as monthly cleaning with 10% bleach. – Daily, or at minimum weekly emptying of used media traps. – Store cell line stocks in vapour phase of LN2, not in liquid phase
Fungus	– Fruit – Cellulose products (cardboard) – Plants – Unfiltered air – Lab personnel – Contaminated cell stock	– Microbial culture – Visual particulates, visual mycelia	– Aseptic technique – Antimycotics – Filtration (< 0.5 µm) – Use sterile products – Daily cleaning of hood space with 70% alcohol as well as monthly cleaning with 10% bleach. – Daily, or at minimum weekly emptying of used media traps. – Store cell line stocks in vapour phase of LN2, not in liquid phase
Virus	– Original tissues – Serum – Cross-contamination – Lab personnel	– Co-cultivation – PCR – Electron microscopy – In vivo testing – Assays	– Aseptic technique – Ultrafiltration – Chemical treatment – Gamma irradiation or GI serum – Avoid using animal-derived products – Store cell line stocks in vapour phase of LN2, not in liquid phase
Endotoxin	– Serum – Bacterial contamination – Contaminated or not properly maintained water supply	– Limulus amoebocyte lysate (LAL) assay	– Aseptic technique – Ultrafiltration (< 5,000 daltons) – Affinity chromatography – Avoid using animal-derived products
Mycoplasma	– Contaminated cell lines – Serum – Media – Lab personnel	– Hoechst stain – Microbial culture – Specialized kits – PCR	– Aseptic technique – Antibiotics – Ultrafiltration (< 0.04 µm) – Use sterile products – Avoid using animal-derived products – Store cell line stocks in vapour phase of LN2, not in liquid phase
Cellular	– Cross-contamination of cultures – Cross use or sharing of media from a different cell line.	– Cell authentication to determine identity and species of cells	– Work with only one cell line at a time – Thoroughly clean before and after introducing a new cell line into the laminar flow hood – Store cell line stocks in vapour phase of LN2, not in liquid phase

2.2.2 Cell proliferation assay by cell counting

Cell proliferation of OP9 stromal cells and ESCs (E14) was evaluated by both conventional haemocytometer cell counting and alkaline phosphatase staining.

To prepare cell cultures for the experiment to assess the effects exerted by VD_3 on cell proliferation of pluripotential OP9 and E14 cells by conventional haemocytometer cell counting, confluent cultures of OP9 and E14 cells were first formed in separated T75 flasks following the procedures described above. 6-well plates were pre-coated with 2 mL of gelatine. Those prepared for OP9 cells were incubated overnight at 37 ºC, and those for E14 cells were incubated for 45 minutes at room temperature. Excess gelatine was then aspirated.

For cell plating, adherent cells on the T75 flasks were collected following trypsinisation, and were counted using a haemocytometer in the presence of trypan blue dye. Cells were then seeded at 80×10^3 cells in 2 mL GM per well of six-well plates. These were divided into two sets containing separate samples of OP9 and E14 cells. For each set, VD_3 was added to the media in different wells at the concentrations of 1 nM, 10 nM and 100 nM, respectively, for both OP9 and E14 samples. One set of samples were then incubated for 48 hours, and the other set 72 hours, before cell counting was performed. 6-well plates were incubated in a fully humidified atmosphere with 5% CO_2 at 37 ºC. The control samples were prepared in the same way but without the addition of VD_3.

After the respectively designated periods of incubation, the samples were processed for cell counting. First, culture medium of each confluent 6-well plates was aspirated and the medium was saved in separate 15 mL tube (Lynch *et al.*, 2011). Cells were then washed with PBS to remove any remaining serum. The samples were trypsinised with 0.25% trypsin / EDTA for 3-5 minutes at 37 ºC to detach cells adhered to the substrate, and the cells were then re-suspending in pre-warmed GM (in at least 1:1 ratio of trypsin:GM) to deactivate the action of the trypsin. Each medium removed during aspiration was then added back to the same sample (*ibid.*). OP9 cell samples were then centrifuged at 130 × g for 7 minutes at room temperature, whilst E14 cells samples were spun at 750 rpm for 5 minutes at room temperature. The supernatant was discarded and cells were re-suspended in a 1:1 dilution in trypan blue stain to stain the dead cells before being counted with a Neubauer haemocytometer to obtain the results.

2.2.3 Alkaline phosphatase staining for the determination of pluripotency

Principle

Alkaline phosphatase (AP) is a hydrolase enzyme responsible for dephosphorylating molecules such as proteins, nucleotides and alkaloids under alkaline pH condition. In general, alkaline is located at the cell surface and is linked to the cell membrane. Thus, it is widely used as a stem cell membrane marker. Elevated expression of this enzyme is associated with undifferentiated pluripotent cells and stem cells (Macgregor *et al.*, 1995). Cultures used to effectively propagate undifferentiated ESCs contain cells that can form single-cell-derived colonies of AP^+ cells. In addition, it was shown that decreased expression of alkaline phosphatase enzyme is associated with differentiated ESCs (O'Connor *et al.*, 2008). There are various methods for detecting AP, such as methods based on enzymatic reaction following visualisation with fast red violet dye. The dye is a cell-permeable chromogenic substrate for alkaline phosphatase that is non-toxic to cells, diffusing out over the course of one hour. Undifferentiated cells appear red or purple, whereas differentiated cells appear colourless. This feature provides a simple, sensitive, and rapid assay for the visualisation of undifferentiated pluripotent cells and colonies.

Method

To assess the effect of VD_3 on cell proliferation of OP9 stromal cells and ESCs, 6-well plates were used. Separate sets of plates containing OP9 and E14 cells respectively were used. For the samples of each cell, one set was to be incubated for 48 hours and the other 72 hours. Each set contained samples with 1 nM, 10 nM and 100 nM of VD_3, respectively, as well as controls that had no VD_3 added. The experiment was triplicated.

The wells on the 6-well plates were pre-coated with 2 mL of 0.1% gelatine. Those to be containing OP9 were incubated overnight at 37 ºC and those to be plated with ESCs were incubated for 45 minutes at room temperature. Excess gelatine was then aspirated.

For cell plating, the confluent cultures of OP9 and E14 cells were first formed following the procedures described before. The adherent cells on the plates or in the flasks were collected following trypsinisation, and were counted using a haemocytometer in the

presence of trypan blue dye. Cells were then seeded at 80×10^3 cells in 2 mL GM per well with or without VD_3. VD_3 was added to the media at the concentrations of 1 nM, 10 nM, and 100 nM, respectively. 6-well plates were then incubated for either 48 or 72 hours in a fully humidified atmosphere with 5% CO_2 at 37 ºC.

After designated periods of incubation, the medium was aspirated and the cells were washed twice with PBS and fixed with methanol for 10 minutes. Excess methanol was then aspirated. For cell staining, 0.0012 g of Napthol (Sigma, Steinheim, Germany) was added into 30 mL of Tris (pH 9.2), 1200 µL of Dimethylformamide (Sigma) was added to dissolve the Napthol and then 0.0012 g of Fast Red TR salt (Sigma) was added to the solution immediately before use. Sufficient staining solution was added to cover the plates (2–2.5 mL) for 30 minutes. The stain was aspirated, and the plates were washed with H_2O. After drying, alkaline phosphatase colonies were counted. Undifferentiated cells appeared red or purple, whereas differentiated cells appeared colourless. The results were evaluated in conjunction with the control. Optimum staining was obtained by developing the substrate in the dark.

2.2.4 Cell cycle examination by flow cytometric analysis

Principle

Flow cytometric analysis or flow cytometry (FCM) is useful in counting and examining the properties of cells. The instrument for performing flow cytometry is called a flow cytometer, and it consists of the fluidic, optics and electronic systems, as illustrated in Figure 13.

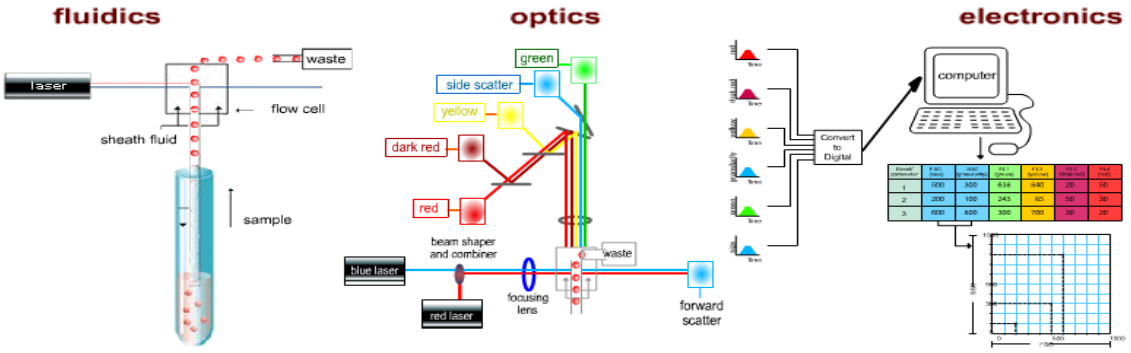


Figure 13. A schematic representation of the mechanisms of flow cytometry (Source: http://www.bdcompany.com/)

As illustrated in the figure, cells are injected into a hydro-focused stream of liquid and passed under a spot hit by a laser beam. The laser beams scattered by the passing cells are then picked up by detectors pointing at the same spot. Forward scatter (FSc) is picked up by the detector situated directly opposite the laser source, and it is an indication of the size of the cell, whilst side scatter (SSc) is picked up by detectors aimed perpendicular to the incoming laser beam, and this provides information on the inner complexity of the cell. Furthermore, fluorescent detectors may also be used to collect information on fluorescent chemicals found in cells, including the ones added to cells as required. For instance, to use FCM for the examination of cells in different phases of the cell cycle, a fluorescent intercalating agent, propidium iodide (PI), is added to the sample. It binds to DNA between the bases, and its fluorescence is intensified. Thus the intensity of the fluorescence of the PI-treated sample observed using FCM can reveal information about its DNA contents, as well as its phase in the cell cycle.

There are different phases in the cell cycle. In the G1 phase, cell division has not yet started. In the S-phase, there is DNA replication, so the cell has more DNA than in the G1 phase. In the G2 phase, the DNA is fully replicated, and in the M phase, mitosis is underway, so a cell in either one of these two phases has twice the DNA it has in the G1 phase. In FCM with PI, the more DNA bases there are, the higher the intensity of fluorescence there will be (Moore *et al.*, 2010). So, if a batch of cells has been induced to differentiate, cells in the G1 phase will accumulate, so their percentage will be higher than those in the other phases, and the intensity of fluorescence will be reduced.

Method

To analyse the effect of VD_3 on the cell cycle distribution of OP9 stromal cells and ESCs (E14), respectively, in the proliferation phase, confluent cultures of separated OP9 and E14 cells were first formed in T75 flasks and monolayer were washed with PBS and cells were detached with trypsin, as described above. Cells were counted then seeded at 500×10^3 cells in 5 mL GM in 25mm plates with or without VD_3 and incubated for either 48 hours or 72 hours. For the treatments, VD_3 was added to the media at the concentrations of 1 nM, 10 nM, and 100 nM, respectively. Plates were incubated in a fully humidified atmosphere with 5% CO_2 at 37 ºC.

For cell cycle analysis, after designated periods of incubation, trypsinsation and neutralisation, cells were harvested in 15 mL centrifuge tubes and pelleted by

centrifugation for 7 minutes at 130 × g for OP9 cells and for 5 minutes at 750 rpm for E14 cells. The supernatant was removed, and cells were washed with PBS and centrifuged. After centrifugation, the PBS supernatant was removed and cells were fixed with 3 mL of 75% ethanol. This was added in a drop wise manner to the cell pellet while vortexing (ethanol facilitates PI to intercalate into double-standard DNA). Samples were stored at -20 ºC overnight before centrifugation and removal of the ethanol supernatant. Cells were washed twice with PBS and 1 mL of PBS was retained with the cell pellet to enable re-suspension. Cells were mixed and transferred to 3 mL BD Falcon flow cytometry tubes (BD Biosciences, San Jose, CA, USA). 50 µl of ribonuclease A (RNase) and 10 µL of PI were added to the cell solution. RNase treatment was necessary as PI is also able to bind to RNA molecules. After 30 minutes of incubation at room temperature, samples were stored at 4 ºC before flow cytometric analysis was performed.

Analysis

Flow cytometric analysis was performed using the Cell Quest Pro software. Cell cycle was analysed using Modfit^{TM} software (Verity Software House, Topsham, ME, USA), which allows analysis of cells in the G1/S/G2/M phases of the cell cycle.

2.2.5 Materials, general equipment and specialist software used

The materials, general equipment and specialist software used in this study are summarised below.

Chemicals, solvents and reagents

Unless otherwise stated, all general equipment, chemicals and solvents (analytical 'AnalaR' or molecular biology/tissue culture grade) were purchased from Sigma (Poole, U.K.), Fisher Scientific (Leicestershire, UK), BDH (Poole, UK) and BD Biosciences (San Jose, CA, USA).

Cell culture

All cell culture experiments were performed under a Class II microbiological safety cabinet (Labcaire SC-R Recirculating Class II, North Somerset, UK). All cell incubations

were performed in Triple Red Laboratory Technology Nuaire DH Autoflow CO_2 Air Jacketed Incubator (Buckinghamshire, UK). Liquid, medium and supernatants were discarded using an IBS Integra Biosciences Vacusafe Comfort (Chur, Switzerland). All solutions used for cell culture were prepared with distilled water from Elgastat Option 4 water purifier (Elga Ltd, High Wycombe, UK).

Cell culture reagents

Sterile cell culture medium and supplements were purchased as follows:

Dulbecco's modified Eagle's medium (DMEM), Knockout Serum replacement, and Non-essential amino acid solution (NEAA) were purchased from Invitrogen (Paisley, Scotland).

Minimum Essential mediums (No Nucleosides), powder (α MEM) was purchased from Invitrogen. Defined Foetal Bovine Serum HyClone, Heat-inactivated, (FBS) was purchased from Thermo Fisher Scientific (UK). Foetal bovine serum was purchased from Invitrogen, 1,25-dihydroxyvitamin D_3 and May-Grunwald Giemsa staining, Napthol, Dimethylformamide Fast Red (TR) salt, and RNase were purchased from Sigma-Aldrich (England). Human LIF was purchased from Millipore (UK).

The supplements L-glutamine, penicillin streptomycin solution and trypsin/EDTA, gelatine type A porcine skin were purchased from Invitrogen, and phosphate buffered saline (PBS) from Oxoid Ltd. (Basingstoke, UK). All solutions were prepared non-sterile in distilled water and were sterilised by autoclaving at 121 ºC in a bench top autoclave from Prestige Medical (Birmingham, UK) before usage in cell culture.

Plastic wares

Tissue culture flasks (T25 and T75), 6-well Nunclon™ Δ surface multi-well dishes were purchased from Nunc Life Sciences, Thermo Fisher Scientific (Rockslide, Denmark). Cryogenic vials, 15 mL and 50 mL sterile centrifuge tubes, cell scrapers and pipette tips for tissue culture, biochemistry and flow cytometry were purchased from Fisher Scientific (Loughborough, UK). 1.5 mL tubes were purchased from Eppendorf (Hamburg, Germany) Syringes were purchased from Terumo (Leuven, Belgium) and syringe filters (0.22 μM) from Corning (Lowell, MA, USA).

3 mL BD Falcon flow cytometry tubes were purchased from BD Biosciences (San Jose, CA, USA). Flow Cytometry was performed on BD FACSCalibur™ (Becton Dickinson, Franklin Lakes, NJ, USA) with Cell Quest Pro Software (Becton Dickinson, Franklin Lakes, NJ, USA). Cell cycle analysis was performed using Modfit™ software (Verity Software House, Topsham, ME, USA). Reagents for Flow cytometry were purchased from BD (BD Biosciences, San Jose, California, USA).

Haemocytometer

Cell proliferation assay was performed using trypan blue from Life Technology, haemocytometers from Abcam plc and the Leica DMI6000B microscope.

Live imaging microscopy

Live imaging microscopy was performed using Leica DMI6000B microscope equipped with an autoflow incubator, CO_2 controller, heating unit and temperature controller, thus allowing maintenance of a fully humidified atmosphere with 5% CO_2 at 37 ºC. Videos were generated and exported using the Leica Application Suite software (Wetzlar, Germany).

Flow cytometry

Flow Cytometry was performed using a BD FACSCalibur™ (Becton Dickinson, Franklin Lakes, New Jersey, USA) with Cell Quest Pro Software (Becton Dickinson). Cell cycle analysis was performed using Modfit™ Software (Verity Software House, Topsham, Maine, USA). Reagents for flow cytometry were purchased from BD (BD Biosciences, San Jose, California, USA).

2.2.6 Statistical Analyses

Statistical analyses and significance of data were determined using GraphPad Prism version 5.00 (GraphPad Software, San Diego, California USA, www.graphpad.com). Statistical significance for the difference between two contrasting measurements was determined with a two-way ANOVA, where $p<0.05$ were considered significant. This was followed up by examining the statistical significance for interactions between more than two groups within the factor with Bonferroni post-hoc analysis.

2.3 Results

2.3.1 Cell Proliferation assay by cell counting

The effect of VD_3 on OP9 cells in the proliferation phase

The anti-proliferation effect of VD_3 on OP9 cells was tested through cell counts of OP9 samples after 48 hours and 72 hours of incubation with various VD_3 levels. The results are presented in Figure 14 below. Values in the figure represent cell numbers for the control and samples with different VD_3 concentrations (1 nM, 10 nM and 100 nM) after 48 and 72 hours of incubation, as well as some statistic results. Data are representative of 3 experiments with 3 measurements each.

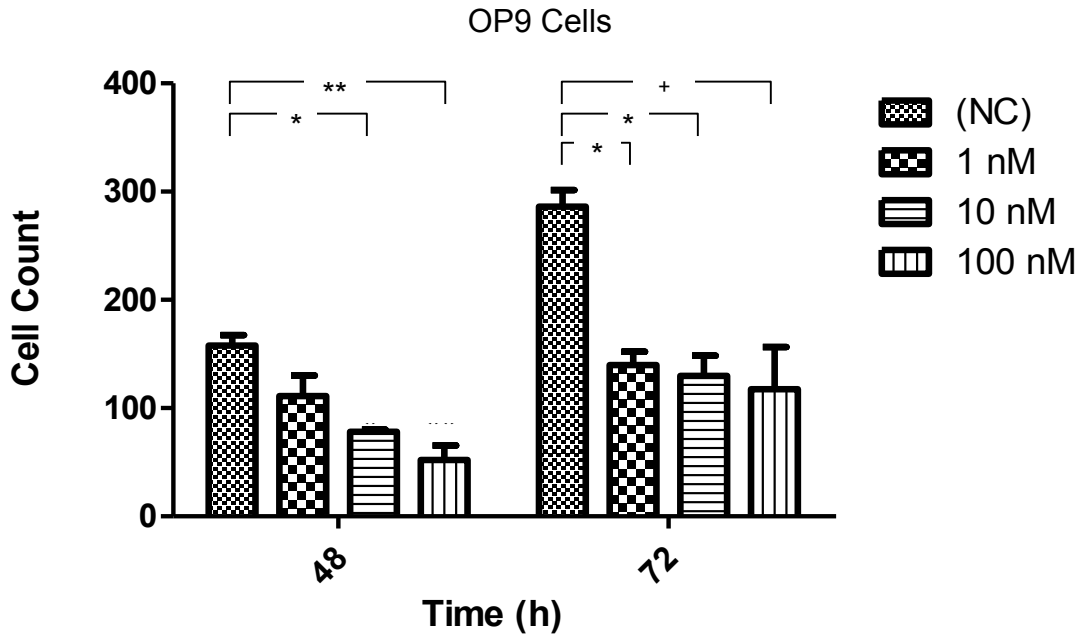


Figure 14. The anti-proliferative effects of VD_3 on OP9 cells
Comparisons between the control and VD_3-treated groups after 48-hour and 72-hour incubations show significant decreases in the number of OP9 cells in both 10 nM ($p<0.01$) and 100 nM ($p<0.001$) of VD_3 whilst significant decrease in the sample with 1 nm of VD_3 only occurs after 72-hour incubation.
N = 9. ($^+$: $p < 0.05$; *: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

As shown in Figure 14 above, the p-values suggest that there was significant decrease in the numbers of OP9 cells after 48-hour incubation in the samples with two higher VD_3 concentrations, of 10 nM and 100 nM, respectively, compared with the control. The p-value for the 1 nM treatment compared to the control was only marginally higher than the 0.05 threshold. Similar results were obtained when the cells were incubated with VD_3 at

the same concentrations for 72 hours. In this case, there was significant decrease in the numbers of OP9 cells in all three levels of VD_3 concentrations compared to the control.

The effect of VD_3 on E14 cells in the proliferation phase

An experiment similar to the one above was also done on E14 cells, and the results are presented in Figure 15 below. Values in the graph represent cell numbers after incubation for the control and VD_3 at different concentrations (1 nM, 10 nM and 100 nM) for 48 and 72 hours, as well as some statistic results. Data are representative of 3 experiments with 3 measurements each.

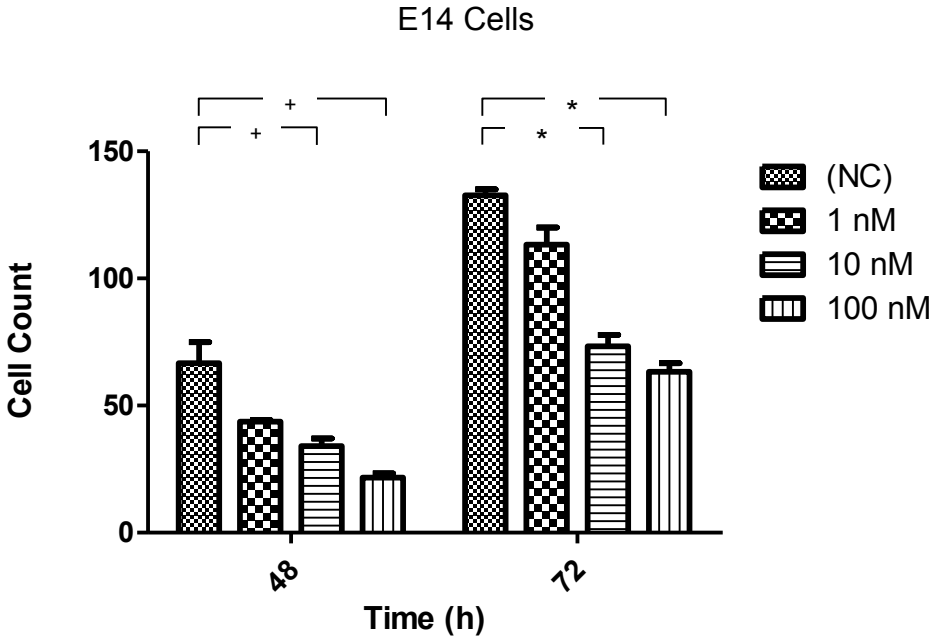


Figure 15. The anti-proliferative effects of VD_3 on E14 cells
Comparisons between the control and VD_3-treated groups after 48-hour and 72-hour incubations show significant decreases in the number of E14 cells in both 10nM ($p<0.01$) and 100nM ($p<0.001$) of VD_3.
N = 9. (+: $p < 0.05$; *: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

As shown in Figure 15 above, the p-values suggest that there was significant decrease in the numbers of E14 cells after 48-hour incubation in the samples with two higher VD_3 concentrations, of 10 nM and 100 nM, respectively, compared with the control. Even the rounded p-value for the 1 nM treatment compared with the control was equal to the threshold. Same results were obtained when the cells were incubated in VD_3 at the same concentrations for 72 hours.

Summary

In both cell proliferation assays on OP9 and E14 cells, there were significant decreases in the numbers of respective cells in the samples with higher concentrations of VD_3 compared with the control, and the decrease was most prominent in the samples with 100 nM of VD_3. In other words, the higher the concentration of VD_3 was, the more suppressed cell proliferation would be, and this applied to both cell lines. This showed that cell proliferation in both cell lines had been suppressed by the presence of VD_3.

2.3.2 Alkaline phosphatase staining for the determination of differentiation

The effect of VD_3 on OP9 cell differentiation

The influence of VD_3 on cellular proliferation and its extent, if any, were qualitatively investigated by the visual staining of alkaline phosphate. Cells were cultivated in a growth medium containing VD_3 at various concentrations (1, 10 and 100 nM) for 48 or 72 hours.

Alkaline phosphatase is associated with undifferentiated cells, and decreased expression of the enzyme is associated with differentiated cells. The specific localisation of the staining inside cells was considered to be indicative of the presence of undifferentiated cells (Macgregor *et al.*, 1995). In contrast, in the absence of such a stain (i.e. colourless cells) it was assumed that these cells have differentiated into other types of cells (O'Connor *et al.*, 2008).

The results of alkaline phosphatase staining of OP9 cells are shown in Figure 16 and Figure 17 below. A reduction in the staining colour was manifest when comparing the effect of VD_3 at 1 nM, 10 nM, and 100 nM concentration as compared to the control at both 48 and 72 hours. The large area of colourless cells demonstrated that VD_3 may have inhibited cell proliferation and increased cell differentiation. This finding was associated with a morphological change in cells displaying reduced proliferation as cells were shown to be more rounded (Wolins *et al.*, 2005).

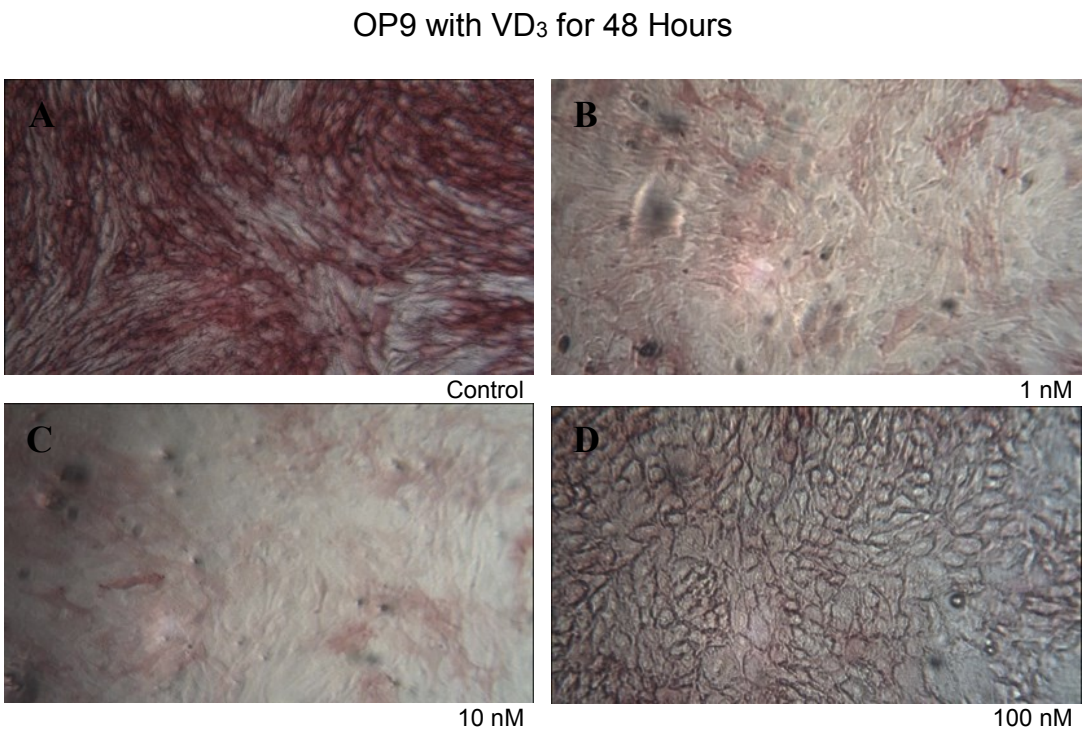


Figure 16. The result of alkaline phosphate staining assay showing the anti-proliferation effect of VD_3 on OP9 cells after 48 hours of incubation
A reduction in the staining colour was manifest when comparing the effect of VD_3 incubation at 1 nM (B), 10 nM (C) and 100 nM (D) concentrations with the control (A). The undifferentiated cells in (A) are shuttle-shaped whilst the differentiated ones in (D) are more rounded. N = 9.

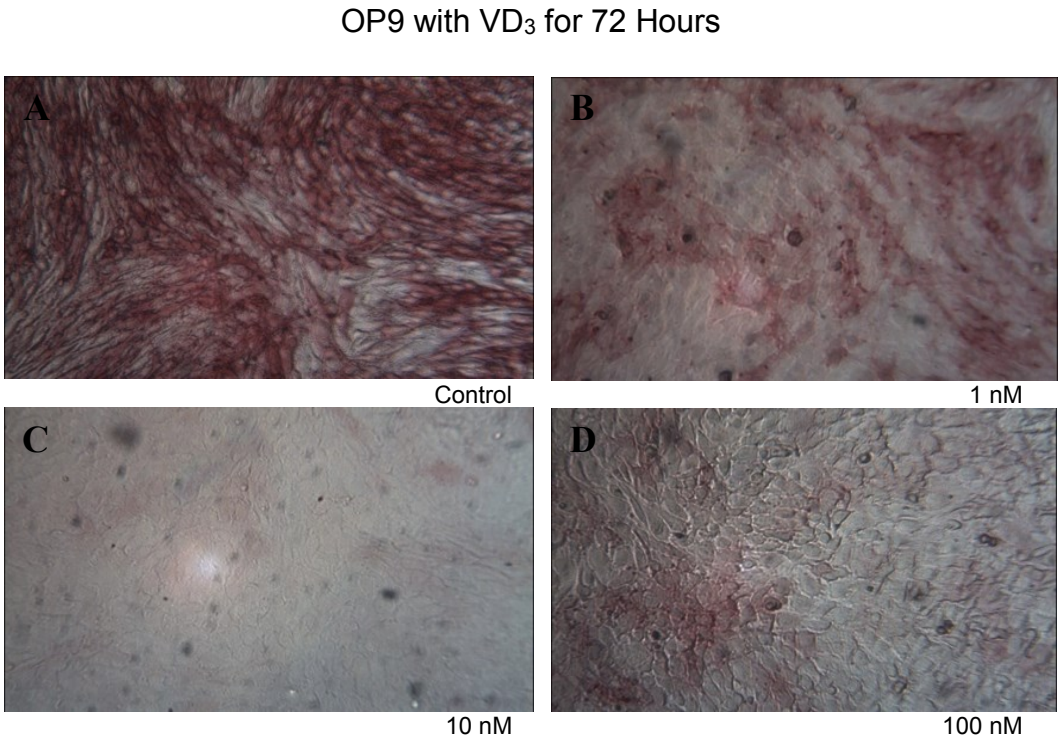


Figure 17. The result of alkaline phosphate staining assay showing the anti-proliferation effect of VD_3 on OP9 cells after 72 hours of incubation
A reduction in the staining colour was manifest when comparing the effect of VD_3 incubation at 1 nM (B), 10 nM (C) and 100 nM (D) concentrations to the control (A). N = 9.

The effect of VD_3 on E14 cell differentiation

As shown in Figure 19 below, a similar trend of staining degree and density was also found in the experiments with E14 cells compared to the ones with OP9 cells described above. This finding was associated with a morphological change of cells displaying reduced proliferation.

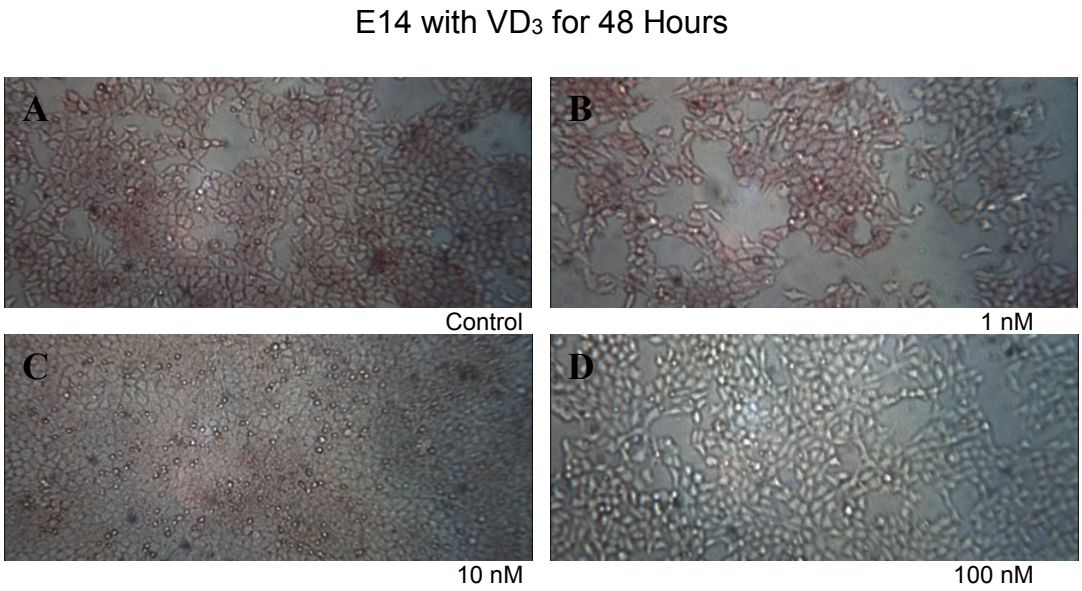


Figure 18. The result of alkaline phosphate staining assay showing the anti-proliferation effect of VD_3 on E14 cells after 48 hours of incubation
A reduction in the staining colour was manifest when comparing the effect of VD_3 incubation at 1 nM (B), 10 nM (C) and 100 nM (D) concentrations to the control (A). N = 9.

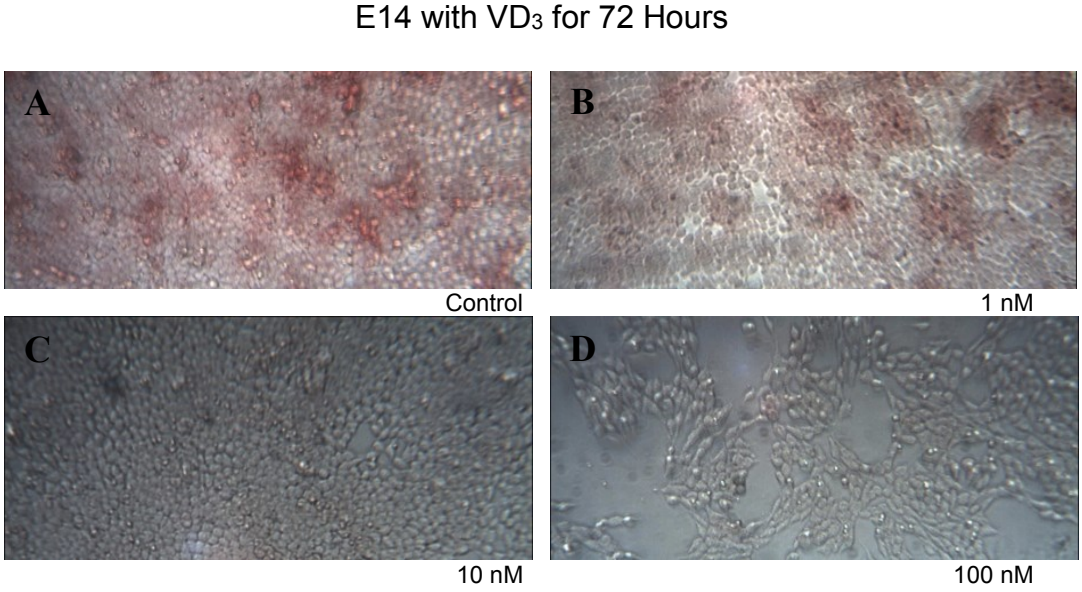


Figure 19. The result of alkaline phosphate staining assay showing the anti-proliferation effect of VD_3 on E14 cells after 72 hours of incubation
A reduction in the staining colour was manifest when comparing the effect of VD_3 incubation at 1 nM (B), 10 nM (C) and 100 nM (D) concentrations to the control (A). N = 9.

Summary

In both experiment with OP9 and E14 cells using alkaline phosphate staining, there were reduction of staining with increased level of VD_3. With the OP9 cell line, there was also decrease in shuttle-shaped cells and increase in more rounded and shortened cells. These effects were most prominent in samples with 100 nM of VD_3. In other words, the higher the concentration of VD_3 was, the more promoted cell differentiation would be, and this applied to both cell lines. This showed that cell differentiation in both cell lines had been promoted by the presence of VD_3.

2.3.3 Cell cycle examination by flow cytometric analysis

The effect of VD_3 on the cell cycle of OP9 cells

To determine the specificity of cell counting and staining methods, and confirm the results obtained, cell-cycle analysis was performed using propidium iodide. As shown in Figure 20 below, there was significant change in the percentage of OP9 cells in the G-phase compared to the control, at the respective concentrations of 1 nM, 10 nM and 100 nM after 48 or 72 hours of incubation as compared to the control. Moreover, the fact that there was a significant increase in the percentage in the G-phase with respect to the S-phase following VD_3 incubation at the two higher concentrations of 10 nM and 100 nM as compared to control confirms that VD_3 promotes cell differentiation rather than division.

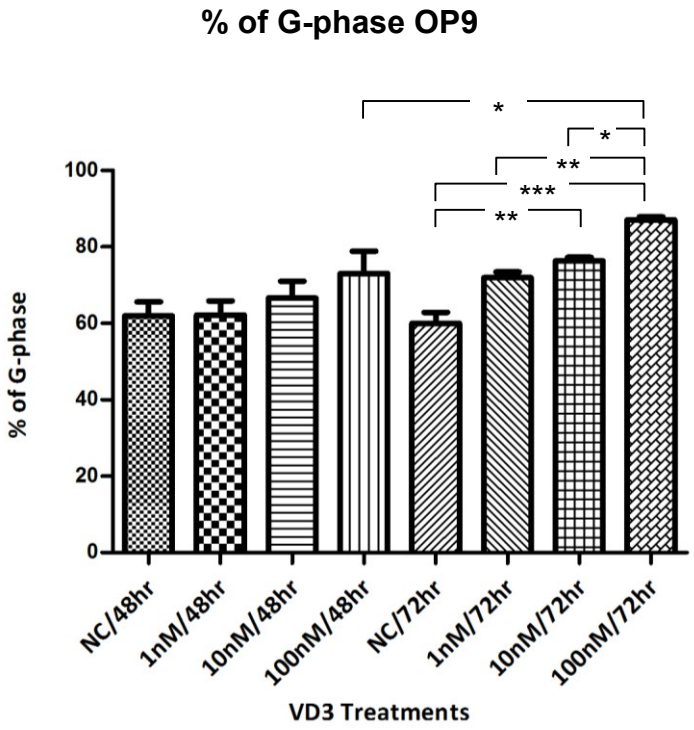


Figure 20. The effect of VD_3 incubation on the percentage of OP9 cells displaying the G-phase of cell cycle using flow cytometric analysis after 48 and 72 hours of incubation. N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

There were significant changes amongst several pairs of measurements with different levels of VD_3 after 72 hours of incubation, i.e., the control vs. 10 nM of VD3, the control vs. 100 nM of VD_3, 1 nM of VD_3 vs. 100 nM of VD_3, and 10 nM of VD_3 vs. 100 nM of VD_3. There was also significant difference between the samples with 100 nM after both 48 and 72 hours of incubation. Data are derived from 3 experiments.

Similarly, results were obtained when cells in the S-phase were incubated for 48 or 72 hours, as shown in Figure 21 below.

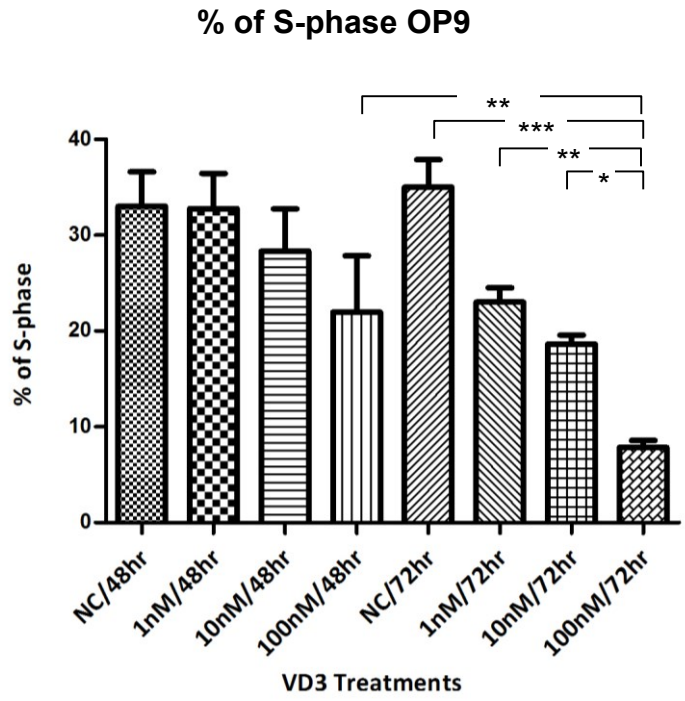


Figure 21. The effect of VD_3 incubation on the percentage of OP9 cells displaying the S-phase of cell cycle using flow cytometric analysis after 48 and 72 hours of incubation. N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

There was significant change in the percentage of cells of the S-phase after 72 hours of incubation with 100 nM of VD_3 as compared to the control and the treatments with 1 nM and 10 nM of VD_3, respectively. There was also significant difference between the samples with 100nM after both 48 and 72 hours of incubation. Data are derived from 3 experiments.

The effect of VD_3 on the cell cycle of E14 cells

As shown in Figure 22, a similar trend was found in the cell cycle analysis of E14 cells to those with OP9 cells after 48 or 72 hours of incubation with 100nM of VD_3. There was a significant increase in the percentage of cells in the G-phase with 100 nM of VD_3 comparing to the respective controls.

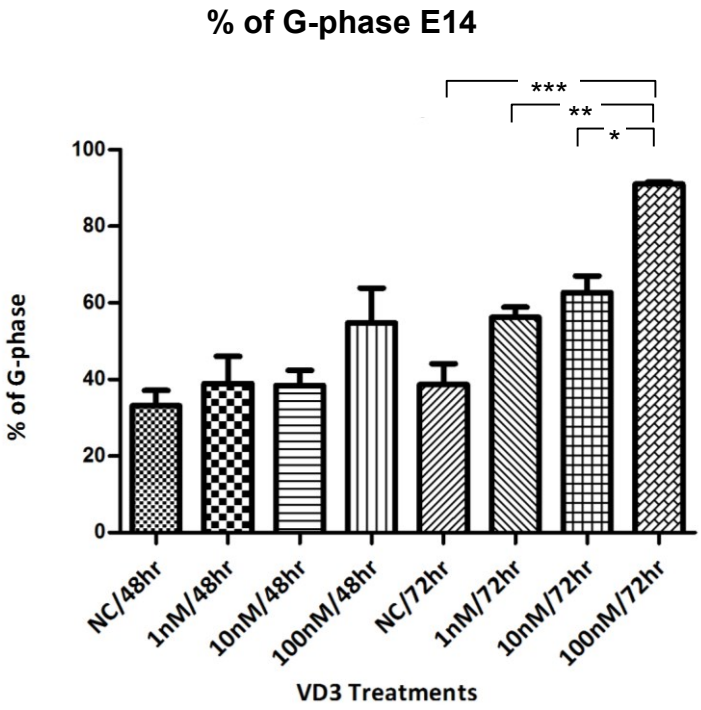


Figure 22. The effect of VD3 incubation on the percentage of E14 cells displaying the G-phase of cell cycle using flow cytometric analysis after 48 and 72 hours of incubation. N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

There was significant change in the percentage of cells of the G-phase after 72 hours of incubation with 100 nM of VD_3 as compared to the control and the treatments with 1 nM and 10 nM of VD_3, respectively. Data are derived from 3 experiments.

Again, as shown in Figure 23 below, a similar trend was found in the cell cycle analysis of E14 cells in the S-phase to the corresponding tests with OP9 cells.

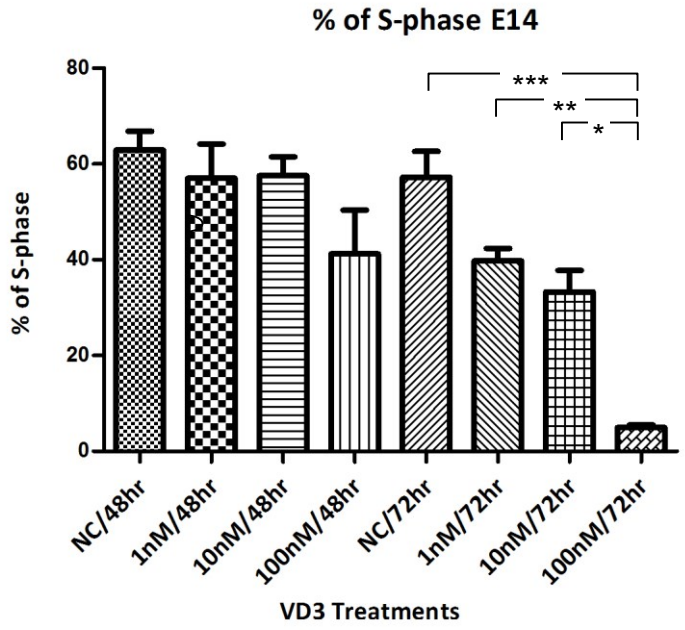


Figure 23. The effect of VD_3 incubation on the percentage of E14 cells displaying the S-phase of cell cycle using flow cytometric analysis after 48 and 72 hours of incubation. N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

There was significant change in the percentage of cells of the S-phase after 72 hours of incubation with 100 nM of VD_3 as compared to the control and the treatments with 1 nM and 10 nM of VD_3, respectively. Data are derived from 3 experiments.

Summary

In both cell cycle examinations with the OP9 and E14 cell lines, there were significant increases in the percentage of cells in the G phase and significant decrease in the percentage of cells in the S phases with the presence of VD_3 compared with the control after 72 hours of incubation, whilst similar trends of changes were not significant with only 48 hours of incubation. These changes were more prominent in the samples with higher concentrations of VD_3.

In other words, after 72 hours of incubation, the higher the VD_3 concentration was, the higher percentage of cells in the G phase and the lower percentage of cells in the S phase there would be, and this applied to both cell lines. This showed that cell differentiation in both cell lines had been promoted by the presence of VD_3

2.4 Discussion

This was the first study into the effects of VD_3 on E14 embryonic stem cells and OP9 stromal cells, respectively, in inhibiting proliferation and encouraging differentiation through cell cycle manipulation.

In the cell proliferation assay, with the results of the separate OP9 and E14 cell counts, it was found that there was a correlation between the decrease of cell counts and the presence of higher level of VD_3 concentration, suggesting that VD_3 was related to the inhibition of the proliferation or the promotion of the differentiation of the OP9 cells. There was also a correlation between the decrease of cell counts and the presence of higher level of VD_3 concentration, suggesting that VD_3 was also related to the inhibition of the proliferation or the promotion of the differentiation of the E14 cells. Therefore, as shown in the results presented in Section 2.3, the statistically significant cell-number decreases for both OP9 and E14 cells suggested that VD_3 promoted cellular differentiation and inhibits cellular proliferation.

In the study on the determination of pluripotency by alkaline phosphatase staining, the level of staining was reduced with increased level of VD_3 in E14 and OP9 cells, respectively. This suggested that the number of cells with pluripotency was reduced with increased level of VD_3, meaning that many of these cells had differentiated into other cells. In other words, cell differentiation had been encouraged by the presence of VD_3. The result from flow cytometric analysis further confirmed with the consistent findings about the role of VD_3 in promoting cellular differentiation, i.e., the likelihood of cellular differentiation depends primarily on VD_3 level and the period of incubation. This also suggested that VD_3 might act directly or indirectly to elicit a cellular arrest in G-phase and drive cells to differentiation.

Overall, these findings of the anti-proliferation and pro-differentiation effect of VD_3 are in agreement with previous *in vitro* studies using squamous cell carcinoma (Hershberger *et al.*, 1999), prostrate adenocarcinoma (Getzenberg *et al.*, 1997), cancer of the ovary (Zhang *et al.*, 2005), cancer of the breast (Colston *et al.*, 1992) and cancer of the lung (Nakagawa *et al.*, 2005), as well as with previous research in apoptosis (Simboli-Campbell *et al.*, 1996), angiogenesis (Mantell *et al.*, 2000) and in the role of VD_3 in cell cycle perturbation and anti-proliferation (Hiroshi *et al.*, 2012). In line with the findings

of these researches, the findings from the study presented in this chapter demonstrated that the proliferation phases of both OP9 stromal cells and ESCs of the E14 cell line were inhibited by VD_3.

This is in agreement with the findings of previous studies showing that VD_3 inhibits cell proliferation by mediating cell cycle arrest. The cell's entry into the S phase is controlled by the activity of CDK2, cyclin E and cell division cycle 25A (CDC25A) phosphatase (Brenner *et al.*, 2014; Santoni-Rugiu *et al.*, 2000; Iavarone and Massague, 1997). VD_3 stops this from happening by preventing the activation of three related complexes, i.e., G1 phase complexes cyclin D1-CDK4 (Brookes *et al.*, 2015; Asghar *et al.*, 2015; Kliewer *et al.*, 1992; Carlberg, 1996) and G1-S governing complexes of cyclin E-CDK2 and cyclin A-CDK2 (Tsai *et al.*, 1993; Tsai *et al.*, 1991). This is because VD_3 reduces the activities of CDC25A phosphatase that regulates CDK2 and CDK4 activities (Busini *et al.*, 2004).

Studies so far have shown that there are three possible ways through which CDK2 activation is reduced and entry to the S phase is prevented. In one scenario, VD_3 deregulates cyclin D1-CDK4 kinase activity. This prevents pRb phosphorylation, and pRb inaction then leads to E2F sequestration and inactivation. This prevents cyclin A and E protein expression (Narasimha *et al.*, 2014; Jenson *et al.*, 2000; Helin, 1998; Nevins, 1998; Zhang *et al.*, 1999), so CDK2 activation is consequently prevented.

In another scenario, CDK2 activation can also be stopped by the induction of p21. VD_3 is known to induce the expression of p21 (Paydas *et al.*, 2014; Cozzolino *et al.*, 2001; Liu *et al.*, 1996; Zhuang and Burnstein, 1998; Verlinden *et al.*, 1998), which is a key molecule in blocking cellular proliferation (Fecteau *et al.*, 2014; Liu, Hu and Chakrabarty, 2009). This is done through the induction of p21 transcript via a mechanism related to VDR (Milczarek *et al.*, 2014; Liu *et al.*, 1996). The increase in p21 protein prevents the activation of CDK2 (Bian and Li, 2012). Additionally, the increased p21 protein contributes to the failure of CDK2 activation and possibly prevents CAK (CDK activating kinase) activation of CDK2 (Mueller *et al.*, 2015; Lolli and Johnson, 2005; Jenson *et al.*, 2000; Hitomi *et al.*, 1998).

Alternatively, CDK2 activity could also be stopped by the reduction in c-Myc gene expression because c-Myc induces CDK2 activity (Jackstadt and Hermeking, 2014; Dang, 1999). Activation of the ERK2 (extracellular-signal-regulated kinase 2) pathway

might cause down-regulation of c-Myc gene expression and thus stops CDK2 activation (Roskoski Jr, 2012; Busini *et al.*, 2004).

Moreover, a similar phenomenon was reported for p27 in inhibition of CDK4 (Kato *et al.*, 1997) and CDK6 and subsequently CDK2 (Wang *et al.*, 1997). This is because p27 potently prevents Rb from being phosphorylation by cyclin E-CDK2, cyclin A-CDK2, and cyclin D2-CDK4 (Polyak *et al.*, 1994). The increase in p27 protein level was dependent on the concentration of VD_3 and was accompanied by an increase in cyclin D and E proteins, which normally peak in mid-G1 and at the G1 to S-phase transition, respectively (Wang *et al.*, 1996). Thus, CDK6 and CDK2 activities are regulated by VD_3 (Wang *et al.*, 1997).

Therefore, exposure to VD_3 induced cell cycle arrest, accompanied by upregulated protein expression of the cyclin-dependent kinase inhibitor (CDKI) p21 and p27 (Verlinden *et al.*, 1998; Campbell *et al.*, 1997). VD_3 treatment leads to marked up-regulation of p21 and p27 and marked down-regulation of cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors. Thus, VD_3 up-regulates p21 and p27 as an early event, which in turn could block the G1 phase to S phase transition (Kawa *et al.*, 1997). This is because p21 and related p27 act as broad spectrum regulators of cyclin dependent kinase function by participating in ternary complexes by efficiently interacting with cyclins D1, D2, D3, E and A, and to a lesser extent with cyclin B (Hall *et al.*, 1995).

In summary, VD_3 deregulates CDK2 and CDK4 activities. The up-regulation of VD_3 level leads to the up-regulation of p27, which leads to the prevention of CDK4 activation and decreased phosphorylation of pRb. This causes sustained E2F sequestration that decreases cyclin A expression. Decrease in cyclin A expression and increase in p21 lead to repression of CDK2 activities. Without CDK2 activation, cell cycle will not transit from the G1 phase to the S phase, leading to a cell cycle block in the G1 phase. Low CDK2 protein level and decreased cyclin E-CDK2 association also contribute to the lack of CDK2 activation. It is through this mechanism that the anti-proliferation and pro-differentiation properties of VD_3 are manifested.

In short, according to related researches, the mechanism of the anti-proliferation and pro-differentiation effects of VD_3 is understood as follows. VD_3 bonds to VDR and promotes the production of p21 and p27 (Hiroshi *et al.*, 2012). This promotes the production of CDK inhibitory protein (Harper *et al.*, 1993; Toyoshima and Hunter, 1994). This inhibits

the phosphorylation of pRb (Kanatani *et al.*, 1999) and reduces its amount. This in turn prohibits the binding of E2F-1 to its target gene, and compromised the production of cyclin E. Thus the cell cannot transit from the G1-phase to the S-phase (Hager *et al.*, 2001; Verlinden *et al.*, 1998), so the cell differentiates rather than proliferates.

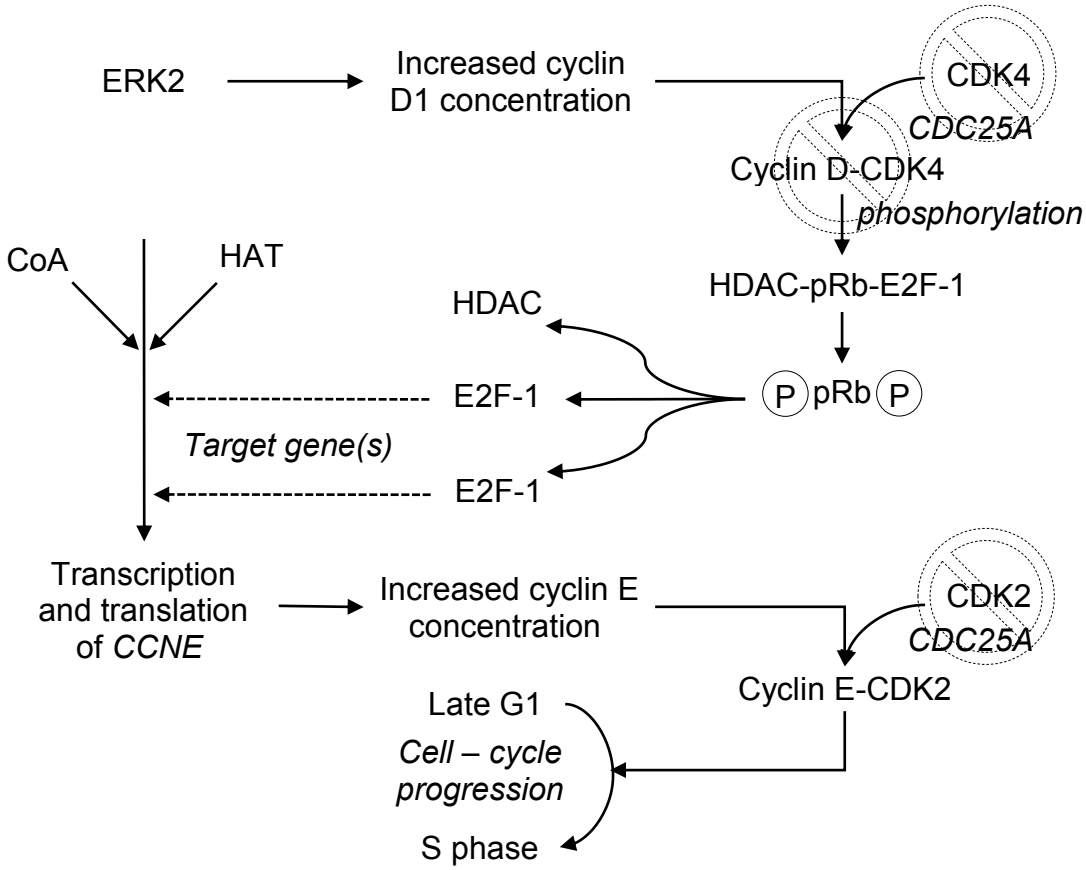


Figure 24. Possible ways through which VD_3 stops the entry to the S phase (Source: Adopted from Moore, Knight and Blann, 2010: 273 with modification)

2.5 Conclusion

As presented and discussed above, separate investigations had been carried out to find out the effects of VD_3 on the proliferation phase of the E14 cell line and on stromal OP9 cells, respectively, and it was found that optimal concentration, 100 nM in these cases, of VD_3 was crucial in promoting differentiation and suppressing proliferation of both isolated E14 and OP9 cells in these *in vitro* studies. This was the first demonstration of the anabolic effect of VD_3 on murine OP9 and E14 cells, respectively, *in vitro*.

Chapter 3 The Effects of VD_3 on E14 / OP9 Co-culture *in Vitro*

3.1 Introduction

The findings from the study presented in the previous chapter have demonstrated that VD_3 has the effect of curtailing the proliferation and promoting the differentiation of E14 and OP9 cells, respectively. Specifically, it was found that the optimal concentration of VD_3 to reduce proliferation and promote differentiation was 100 nM.

However, it remains to be determined whether this vitamin is able to influence both the early and the late phases of haematopoiesis of ESCs (E14) in the co-culture with OP9. Hence further studies need to be conducted to ascertain the validity of the hypothesis that, in the co-culture of ESCs and OP9 stromal cells, the presence of VD_3 enhances the differentiation of ESCs into blood cells of erythroid, myeloid, and B cell lineages without adding exogenous growth factors.

Hence, in this part of the research, the presence of early haematopoietic progenitors in ESC (E14) / OP9 co-culture in the presence of VD_3 was monitored through the evaluation of CD-marker expressions, colony-forming cell (CFC) counts, haematopoiesis-associated gene and cytokine expressions on ESCs at key days between days 2 and 12 of differentiation.

The materials and equipment used for the studies, the methods and protocols adopted for experimental preparation and analyses, the results obtained from the experiments, and the discussion based on the results are presented in this chapter.

3.2 Principles and Methods

3.2.1 Materials and general equipment used

The sources of the cell culture, reagents, solvents, chemicals and plastic wares used in this study are the same as the ones mentioned in the first four parts of Section 2.2.5.

3.2.2 E14 / OP9 Co-culture Preparation

The E14 / OP9 co-culture that was needed for clonogenic progenitor cell assay, flow cytometry and real time RT-PCR was prepared with the following procedures.

Forming of a confluent monolayer of OP9 cells

A confluent layer of OP9 cells was prepared before E14 cells were added to it. After the formation of confluent culture, half of the medium was changed on days 2 and 3, and cells were cultured for an additional 3 to 4 days to obtain the best results.

Obtaining undifferentiated E14 cells

Undifferentiated E14 cells were obtained by detaching them from the substrate and harvested by washing them twice with PBS and trypsinising them with pre-warmed 0.25% trypsin-EDTA at 37ºC. Then the cells were re-suspended in the pre-warmed co-culture differentiation medium composed of 385 mL of Alpha Modification Minimum Essential Medium (No nucleosides, αMEM), 50 mL of Foetal Bovine Serum, 250 µL of monothioglycerol, 5 mL of non-essential amino acid solution (NEAA), 5 mL of L-glutamine (200mM final), 5ml of penicillin-streptomycin solution (10.000 units/mL penicillin and 10.000 mg/mL), 500 µL of 2-Mercaptoethanol, and 2.2 g/L of sodium bicarbonate (in at least 1:1 ratio of trypsin vs. GM) to deactivate the action of the trypsin. E14 cells were centrifuged at 750 rpm for 5-7 minutes at room temperature. The supernatant was discarded and cells were re-suspended in the aforementioned co-culture differentiation medium.

Adding E14 cells to the confluent monolayer of OP9 cells

The E14 cells were added to OP9 cultures at a density of 250×10^3 in the 5 mL co-culture differentiation medium in 25 mm plates with or without VD_3. Cells were incubated at 37° C, in fully-humidified air, at 5% CO_2, for up to 12 days. VD_3 was added to the medium at the concentrations of 100 nM. This concentration was chosen because it is the optimum value found in healthy individuals, and it was found to have significant effect on the differentiation of the cells in the previous experiment.

Cells were harvested on after 2, 5, 8, 10 and 12 days of incubation, respectively, and single-cell suspension was prepared by washing the cells twice with PBS, followed by the treatment with 0.025% trypsin-0.5 mM EDTA for 5 minutes at room temperature. The cells were filtered through a 100 μM cell strainer (BD Biosciences) (Vodyanik *et al.*, 2005), centrifuged, and ready to be used as the control and treatment for clonogenic and flow-cytometric assays as well as gene-expression analysis.

Additional procedures to remove cells of unknown origin

In very unlikely events, adherent cells of unknown origin might appear on some occasions. These cells are disadvantageous for the maintenance of haematopoietic cells, because they cover the OP9 layer and inhibit haematopoiesis.

To avoid this deleterious effect, the induced haematopoietic cells should be processed again on days 5, 8 and 10 (Kitajima *et al.*, 2003) with the following procedures. On each of these days, after taking the sample designated for the experiments for the day, each of the remaining co-culture flasks (for harvesting on subsequent days) was aspirated and the aspirated medium was saved with the haematopoietic cells in suspension (Lynch *et al.*, 2011). The samples were washed twice with PBS, trypsinised with pre-warmed 0.25% trypsin-EDTA, and then the cells were re-suspended in the pre-warmed co-culture differentiation medium to stop trypdinisation, filtered with a 100 μM strainer, added to the differentiation medium saved from aspiration with the suspended haematopoietic cells (Lynch *et al.*, 2011), and centrifuged at 750 rpm for 7 minutes at room temperature. The supernatant was discarded and the co-culture differentiation medium was added to the cells. A 21-gauge blunt-ended needle was used to re-suspend cells to ensure single-cell suspension. Then the cells were counted using a haemocytometer. The cell count is hypothesised to increase 100-150-fold from day 1 to day 5. The cells were re-seeded onto a new layer of confluent OP9 cells in each flask for subsequent days.

The co-culture was kept until day 12 to obtain the information on mature haematopoietic progenitors.

3.2.3 Immunofluorescence

Principle

Immunofluorescence is a specific example of immunochemistry. It uses antibodies labelled with a fluorescent dye, or fluorochrome, to detect target proteins, i.e., antigens. When the fluorescent antibody is combined with the corresponding antigen, the antigen-antibody complex can be visualised using a fluorescent microscope. The commonly used fluorochrome in immunofluorescence is fluorescein isothiocyanate (FITC). It absorbs blue rays of wavelength of 488 nm and emits green light of wavelength of 520 nm.

Sample preparation and fixation

The objectives of the whole procedure described below are to determine the role of Oct4, Nanog and Sox2 as the master regulators of both pluripotency and differentiation of ESC (E14) in OP9 co-culture, and to evaluate the effect of VD_3 as an accelerator and a promoter of early differentiation of E14 in the co-culture. After formation of the confluent culture of OP9 in T25 flask, 24-well plates were pre-coated with 0.5 mL of 0.1% gelatine and incubated overnight at room temperature.

Excess gelatine was then aspirated. In order to prepare for cell-plating, adherent OP9 cells in the T75 flasks were harvested following trypsinisation with pre-warmed 0.25% trypsin-EDTA at room temperature, centrifuged, and then counted using a haemocytometer. The harvested cells were then seeded in quantities of 1×10^4 cells in 1 mL growth medium per well. After formation of confluent cultures of OP9 cells on day 2, half of the medium was changed, and cells were cultured for an additional 2 days to obtain the best results.

To detach cells adhered from the substrate, undifferentiated E14 cells were harvested by washing them twice with PBS, and then trypsinised. After that, the E14 cells were re-suspended in the pre-warmed co-culture differentiation medium. Then the cells were centrifuged at 750 rpm for 5-7 minutes at room temperature. The supernatant was discarded, the cells were re-suspended in the co-culture differentiation medium and added to the OP9 cultures at a density of 1×10^4 cells in 1 mL co-culture differentiation medium in 24-well plates either without VD_3 or with VD_3 at 100 nM concentration. Then the cells were incubated in a fully humidified atmosphere with 5% CO_2 at 37° C for up to 5 days.

Samples were taken from incubation at the end of days 1, 3 and 5. For each well containing these samples, the medium was aspirated and the cells were washed twice with PBS and fixed with 4% paraformaldehyde (Sigma, St. Lois, MO, USA) in 1× PBS for 10 minutes. Excess paraformaldehyde was then aspirated. Cells were washed 3 times with PBS.

Blocking

Diluted blocking serum was prepared with 5% bovine serum albumin (BSA) in PBST, which was 500 μL Tween 20 in 1l PBS. Tween 20 was used to permeabilise the cells to help the antibodies to get into fixed cells. The prepared blocking buffer was added to cover the wells of 24-well plates and incubated for 30 minutes to minimise non-specific adsorption of the antibodies. The blocking serum was aspirated, and the plates were washed with PBST to remove the excess of BSA.

Staining and Detection

Two types of antibodies are used in secondary or indirect immunofluorescence. The primary antibodies are raised against an antigen of interest and are typically unconjugated (unlabelled), whilst the secondary antibodies are raised against immunoglobulins of the primary antibodies. The secondary antibodies are directly conjugated to the fluorochrome molecules. Antibodies can be raised in different species. This mechanism is depicted in the schematic diagram presented in Figure 25.

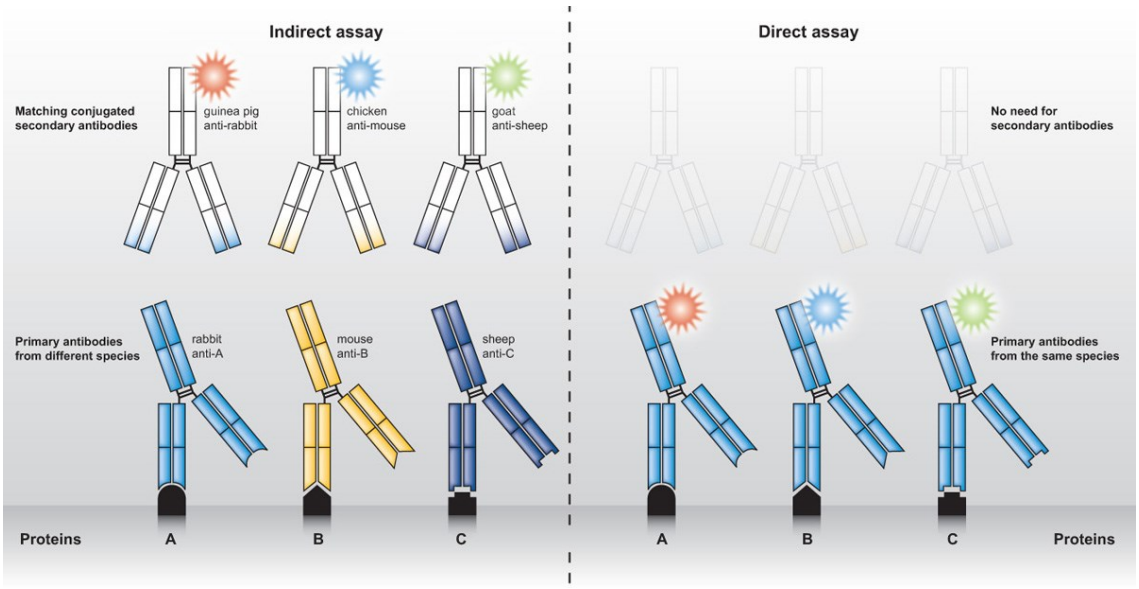


Figure 25. The schematic diagram of the mechanisms of the indirect and direct methods of immunochemical staining
(Source: http://www.abcam.com/ps/CMS/Images/IndirectVsDirect_1200x615.jpg)

The primary antibodies used in this study Oct3/4 (C-10), Nanog (A-11) mouse IgG and Sox2 (D-17) Goat IgG antibodies (Santa Cruz Biotechnology, UK). Appropriately diluted primary antibody solution (in 1:100 ratio of primary antibody vs. 5% BSA/TBST blocking buffer) was added to the samples collected on days 1, 3 and 5 of incubation and left to incubate overnight to allow the antibodies to attach to the corresponding antigens. Afterwards, the excess of primary antibody was aspirated, and the plates were washed with PBST 3 times for 3 minutes to remove unbound primary antibody.

The secondary antibodies used in this study were Goat anti-mouse IgG-FITC and donkey anti-goat IgG-FITC (Santa Cruz Biotechnology, UK). Each of these secondary antibodies is species-specific to a portion of one of the primary antibodies and is conjugated to fluorescent substrate that produces a coloured signal for the identification of the position of the bound protein. For fluorescent secondary antibody binding, cells were incubated with secondary antibodies (in 1:100 ratio of secondary antibody vs. blocking buffer) for 60 minutes in darkness before the plates were washed with PBST 3 times for 3 minutes.

Undifferentiated ESCs, i.e., E14 strictly maintained in an undifferentiated state, were prepared alongside the tests and used as the control against which the results of the tests were evaluated. Images of both tests and controls were obtained using Leica DMI 6000B imaging system and a FITC-fluorescence L5 filter cube (excitation wavelength = 480/40 nm). This microscope is equipped with an autoflow incubator, CO_2 controller, heating unit and temperature controller, allowing maintenance of a fully humidified atmosphere

with 5% CO_2 at 37 ºC. Videos were generated and exported using the Leica Application Suite software (Wetzlar, Germany).

Counterstains

To help the primary stain stand out, a second stain is often applied after immunochemical staining of the target proteins to provide visual contrast. Many of these dyes stain specific discrete cellular compartments or antigens, whilst other ones stain the whole cell. Both chromogenic and fluorescent dyes are available for immunochemical staining to provide a vast array of reagents to fit every experimental design. Commonly used dyes are haematoxylin, Hoechst stain and 4',6-diamidino-2-phenylindole (DAPI).

HardFSet™ Mounting Medium contains DAPI that binds strongly to AT-rich regions in DNA, i.e., a region where the sequence is highly rich in adenine (A) and thymine (T), forming a stable complex which fluoresces approximately 20 times greater than DAPI alone. This product is a substrate (reporter molecule) designed to be used for procedures requiring fluorescent labelling of DNA. DAPI excites at about 360 nm and emits at about 460 nm when bound to DNA, producing a blue fluoresce through a blue/cyan filter of fluorescence microscopy. DAPI can pass through an intact cell membrane, so it can be used to stain both live and fixed cells. However, it passes through the membrane of live cells less efficiently and thus is less effective in staining live cells. In this study, DAPI was used to provide a contrasting background to the blue fluoresce stains of the differentiated cells to make the latter easily recognisable.

An alternative approach of immunofluorescence using an optical microscope

An alternative approach was also adopted for the preparation and detection of immunofluorescence. In this approach, to prepare and fix the samples of the tests and controls, the E14 cells were harvested after incubation of 24 hours, 3 days and 5 days, respectively, and single-cell suspension was prepared by washing the cells with PBS twice, trypsinised, filtered through a 100 μM cell strainer (BD Biosciences) and centrifuged. Then a pellet was obtained for the use of immunocytochemistry of 24-well plates with and without VD_3. Cells from the pellet were placed and smeared on slides, utilising the common procedure of preparing a blood or bacteria smear for easy staining and observation, as shown in Figure 26. Then the slides were dried in the air, and the cells

were fixed with 4% paraformaldehyde (Sigma, St. Lois, MO, USA) in 1× PBS for 10 minutes. Excess paraformaldehyde was then aspirated, and the cells were washed 3 times with PBS.

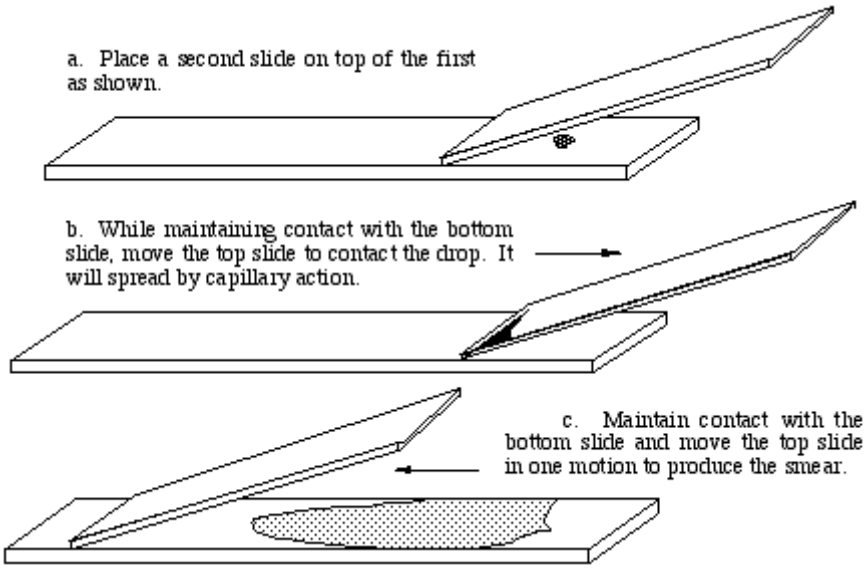


Figure 26. Diagram showing the procedure of preparing a blood smear that was employed to prepare the plates for immunocytochemistry
(Source: http://www.ruf.rice.edu/~bioslabs/bios318/smear.gif)

Afterwards, the fixed samples were stained by dropping staining solutions unto the samples and then rinsing off excessive staining solutions in a jar with running water. The images of the stained samples were obtained using an optical microscope instead of the sophisticated imaging system used in the previous approach.

3.2.4 Morphological detection of different colony-forming cells and CFC counting

Principle

May-Grunwald-Giemsa stains are universally employed for routine staining which are used to display morphological features and very satisfactory results can be obtained. It consists of Methylene Blue (methylthioninium chloride), Azure B (trimethylthionin) and Eosin Y (tetrabromo-fluorescein). The compound variations in binding of the dyes to chemical structures and interactions amongst themselves can influence the mechanism by which definite components of a cell's composition stain only with particular dyes, and every component of the cell is stained in this method. The basic cellular components are

stained red-purple by azure. The acidic components of the cell are stained blue by methylene. The alkaline components of the cell are stained orange-red by eosin. Thus these dyes allow for distinction in shades of staining and for staining granules differentially, and this information is useful to distinguish amongst the various haemopoietic cells.

Morphological detection of different colony-forming cells

Method

A clonogenic progenitor cell assay was performed to identify and confirm the cell content of particular colonies by using May-Grunwald-Giemsa staining. The cells from the ESC/OP9 co-culture (prepared according to the preparation method mentioned in Section 3.2.1) were placed on slides, and the slides were dried in the air. The cells were fixed by immersion in a jar of methanol for 5-10 minutes before being transferred for 10-15 minutes to a staining jar containing May-Grunwald stain that was freshly diluted with an equal volume of buffered water. Then the slides were transferred without washing to a jar containing Giemsa's stain that was freshly diluted with 9 volumes of buffered water to pH6.8 for 10-15 minutes. The slides were then transferred to a jar containing buffered water of pH6.8, rapidly washed in three or four changes of water and finally allowed to stand undisturbed in water for a short time for staining differentiation. Samples were then thus ready for identification. The morphology of different CFC types was captured in images with a Zeiss Axio Imager M1 microscope using the Zen2012 software.

Colony-forming cell counting

Method

To prepare for the sample to be observed after different days of incubation with or without VD_3, cells from the ESC/OP9 co-culture (prepared according to the preparation method mentioned in Section 3.2.1) were seeded at different densities depending on the designated days of incubation before observation: 1 to 5 days, 2×10^5 cells/mL; 7 to 8 days, 5×10^4 cells/mL; and 9 to 10 days, 2×10^4 cells/mL. Undifferentiated ESC cells were plated at densities up to 5×10^5 cells/mL, and no CFCs were found.

Clonogenic progenitor cell assay was performed to identify and confirm the cell content of appropriate colonies. To prepare the samples for counting after designated days of incubation, the culture medium was removed from the dishes and flasks containing E14 / OP9 co-culture with and without VD_3. The remaining colonies were washed once with PBS to remove any residual culture media. Cells were fixed by adding 2 mL methanol for each plate, incubated for 15 minutes at room temperature before methanol was removed and the plate air dried at room temperature for 5 minutes. Then each plate was put in 2 mL of May-Grunwald stain freshly diluted with an equal volume of buffered water. After staining for 10 minutes at room temperature, 2 mL Giemsa staining freshly diluted with 9 volumes of buffered water of pH6.8 was also added, without washing, for 10-15 minutes. Then the staining solution was removed and each plate was rinsed with running distilled water to remove unbound stain until water remains clear. Water was removed and the plate was allowed to dry at room temperature, and the sample was then ready for identification and counting using the Zeiss Axioimager M1 microscope. All clonogenic progenitor assays were performed in duplicate.

CFCs were scored after incubation according to their colony morphology as erythroid (E-CFC), granulocyte, erythroid, macrophage, megakaryocyte (GEMM-CFC), granulocyte-macrophage (GM-CFC), and macrophage (M-CFC). The frequency of CFC was calculated per 10^6 total cells.

3.2.5 Phenotype analysis by flow cytometry

One of the major obstacles to the clinical use of ESCs is the difficulty of visually distinguishing them from other cell types. The discovery of molecule markers resulting from unique gene expression patterns in cells of different types of cells provided a valuable tool in the identification of undifferentiated and differentiated ESCs (Moore, *et al.*, 2011; Zhao *et al.*, 2012).

Cell surface markers

There are specialised proteins expressed on the surface of cells. These proteins have the property of being able to bind or adhere to other signal molecules (Zhao *et al.*, 2012). Since each type of these membrane proteins has a different structure, its affinity for signal molecules is specific. Certain proteins are only present in or expressed by particular cell types, so particular proteins on the cell surface can be used as cell markers, and these cell

markers are the most important non-invasive and non-destructive tools for the identification of ESCs.

CD antigens

Cell surface marker antigens are commonly referred to as cluster of differentiation (CD) antigens. These are specific groups or cluster of epitopes of cell surface proteins belonging to several different classes, such as integrins, which are α/β heterodimeric cell surface receptors that mediate the attachment of a cell to its surrounding tissues, adhesion molecules, glycoproteins and receptors. Different types of cells have different CD antigens, so they can be used as effective tools for the identification and classification of different cell populations (Zhao *et al.*, 2012) using anti-CD monoclonal antibody (mAb) or antibodies.

CD antigens are named with their CD numbers. Some of the key CD markers related to murine haematopoietic studies and the corresponding monoclonal antibodies selected for this study (BD bioscience pharmingen™) are listed in Table 3 below.

Table 3. The monoclonal antibodies used in this study and the corresponding CD markers (Source: BD Biosciences, 2010)

Rat anti-mouse FLK1	FLK1 is monoclonal antibody specifically binds to foetal liver kinase 1, also known as VEGF receptor-2 (VEGF-2), a receptor for vascular endothelial growth factor (VEGF). It is expressed on distinct sets of mesoderm during gastrulation and on endothelial cells in embryonic and adult tissues. *In vivo* and *in vitro* studies indicate that FLK1 is required for the embryonic development of vascular endothelial and haematopoietic cells.
Rat anti-mouse CD31	CD31 is integral membrane protein, a member of the immunoglobulin superfamily, which mediates cell-to-cell adhesion. It is expressed on the surface of adult and embryonic endothelia cells.
Rat anti-mouse CD34	CD34 is a surface glycophosphoprotein expressed on committed and primitive hematopoietic progenitor cells.
Rat anti-mouse CD41	CD41 is integral membrane protein expressed on platelets, megakaryocytes and early haematopoietic progenitors.
Rat anti-mouse CD43	CD43 is a membrane protein expressed on granulocytes, monocytes, macrophages, platelets, natural killer cells, thymocytes and most T helper cells.
Rat anti-mouse CD45	CD45 is a transmembrane glycoprotein expressed at high levels on the cell surface of leukocytes.

Principle

The process of phenotyping involves the use of mAbs directed against extracellular CD antigens. The antibodies are labelled with fluorescent dyes called fluorochromes that will vary in their response to lasers of differing wavelengths. The fluorescence emission is then measured using flow cytometry, and this will indicate which of the labelled antibodies are attached to the cells. The data are electronically stored for analysis and can be displayed in the form of a graph or histogram. The total cells detected were displayed as a dot-plot of red fluorescence intensity vs. green fluorescence intensity, which can then be selectively gated to look at sub-populations in greater detail.

Method

Sets of cell samples incubated with respectively designated period of 2, 5, 8, 10, and 12 days with or without VD_3 were prepared according to the E14 / OP9 co-culture preparation method described in Section 3.2.1. When the supernatant was discarded, single-cell suspensions were prepared with cells from the pellet in 5 mL PBS containing 0.05% sodium azide, 1 mM EDTA, 2% FBS, and 2% normal mouse serum (Sigma), or, alternatively, stain buffer (FBS) ready made-up (BD pharmingen™). This was then divided into 0.5 mL portions for testing against each CD marker under investigation. Each 0.5 mL portion of this stain buffer solution with cells, which had been incubated either with or without VD_3, was allocated to 15 mL tubes and then was incubated with 5 µL of a monoclonal antibody, which binds to any corresponding cell surface antigen that is present. After 30 minutes of incubation at room temperature and in darkness, the cells were then centrifuged at 750 rpm for 5 minutes at room temperature. The supernatant was discarded, single-cell suspensions were prepared in 0.5 mL of sheath fluid solution for each tube, and the samples were ready for flow cytometric analysis. For each monoclonal antibody, control staining with the appropriate isotype-matched control monoclonal antibody (BD pharmingen™) was included to establish thresholds for positive staining.

Flow Cytometry was performed on BD FACSCalibur™ (Becton Dickinson, Franklin Lakes, NJ, USA) with Cell Quest Pro Software (Becton Dickinson, Franklin Lakes, NJ, USA).

Criteria for CD marker selection

The monoclonal antibodies were tested for cross-reactivity with OP9, and only the ones without detectable cross-reactivity with OP9 cells were selected for this study. These we0re: PE rat anti-mouse FLK1, APC rat anti-mouse CD31, PE rat anti- mouse CD34, PE rat ant-mouse CD41, PE rat ant-mouse CD43, and FITC rat anti-mouse CD45 (BD pharmingen™). The percentage of positive cells was calculated as the percentage of positive cells stained with specific antibody subtracted by the percentage of background staining with corresponding isotype control.

3.2.6 RNA extraction

A high purity of RNA solution is needed for Real-time Reverse Transcription Polymerase Chain Reaction (Real time RT-PCR) to be described in the next section. To achieve this, RNA was extracted from cells and isolated. RNA extraction was achieved using Guanidinium thiocyanate-phenol-chloroform extraction, with Trizol® reagent (Invitrogen, Life Technologies, Paisley, UK). Trizol contains phenol and Guanidinium thiocyanate, which have cell lysing and protein denaturing actions, so it also prevents the activity of RNAse and DNAse enzymes. Following cell treatments mentioned in Section 3.2.1, 330 μL of Trizol® per well of a 6-well plate was added to the cell monolayers and plates were incubated for 5 minutes at room temperature. Cell lysates were collected and stored at -20 ºC until further analysis.

For RNA isolation, a mixture of chloroform and Trizol® with the ratio of 1:5 was added to the lysate. The solution was mixed, incubated for 10 minutes at room temperature and centrifuged for 16 minutes at 12,000 × g. Chloroform, along with the phenol contained in the Trizol® reagent, causes the proteins to denature and precipitate, and thus separates the RNA into an aqueous supernatant. After the addition of chloroform and centrifugation, there was a visible separation of the solution into three phases, namely the aqueous phase, interphase and organic phase, as shown in Figure 27. The majority of the RNA was present in the aqueous phase, whilst DNA and proteins were in the interphase and organic phases, respectively. The aqueous phase was transferred to a new tube and equal volume of ice-cold isopropanol was added. Isopropanol precipitates and recovers the RNA from the aqueous phase, forming a visible pellet after 10 minutes of centrifugation at 12,000 × g. The RNA pellet was washed with 75% EtOH, centrifuged

at 8,000 × g for 10 minutes and air dried, and Tris-EDTA (TE) buffer (Applied Biosystems, Ambion, Cheshire, UK) was added to reconstitute the RNA.

Figure 27. After the addition of chloroform to the Trizol® lysate and centrifugation, the solution is separated into 3 phases.
RNA is isolated from the aqueous phase.
(Source: www.openwetware.org)

After reconstitution in TE buffer, RNA concentration and purity were assessed by UV spectroscopy, using a NanoDrop UV Spectrophotometer (NanoDrop, Wilmington, DE, USA). The absorbance of the diluted RNA sample was measured at 260 and 280 nm. Absorbance at 260 nm was used to determine the RNA concentration, as it is known that a 260 reading of 1.0 is equivalent of 40 µg/ml of RNA. A 260/280 ratio was used to assess the purity of RNA, as it is known that a 260/280 ratio of 1.8-2.1 indicates a good purity of RNA solution for the use of Real-time PCR.

Method

Sets of cell samples incubated with respectively designated period of 2, 5, 8, 10, and 12 days with or without VD_3 were prepared according to the E14 / OP9 co-culture preparation method described in Section 3.2.1. From each of these culture samples, total RNA was extracted and isolated following the procedure described in the previous section. These RNA samples were then used in running the TaqMan® PCR-based method by using the TaqMan® RNA-to-C_T™ 1-Step Kit (Applied Biosystems, San Jose, CA, USA), as mentioned in the previous section. The workflow is illustrated in Figure 28:

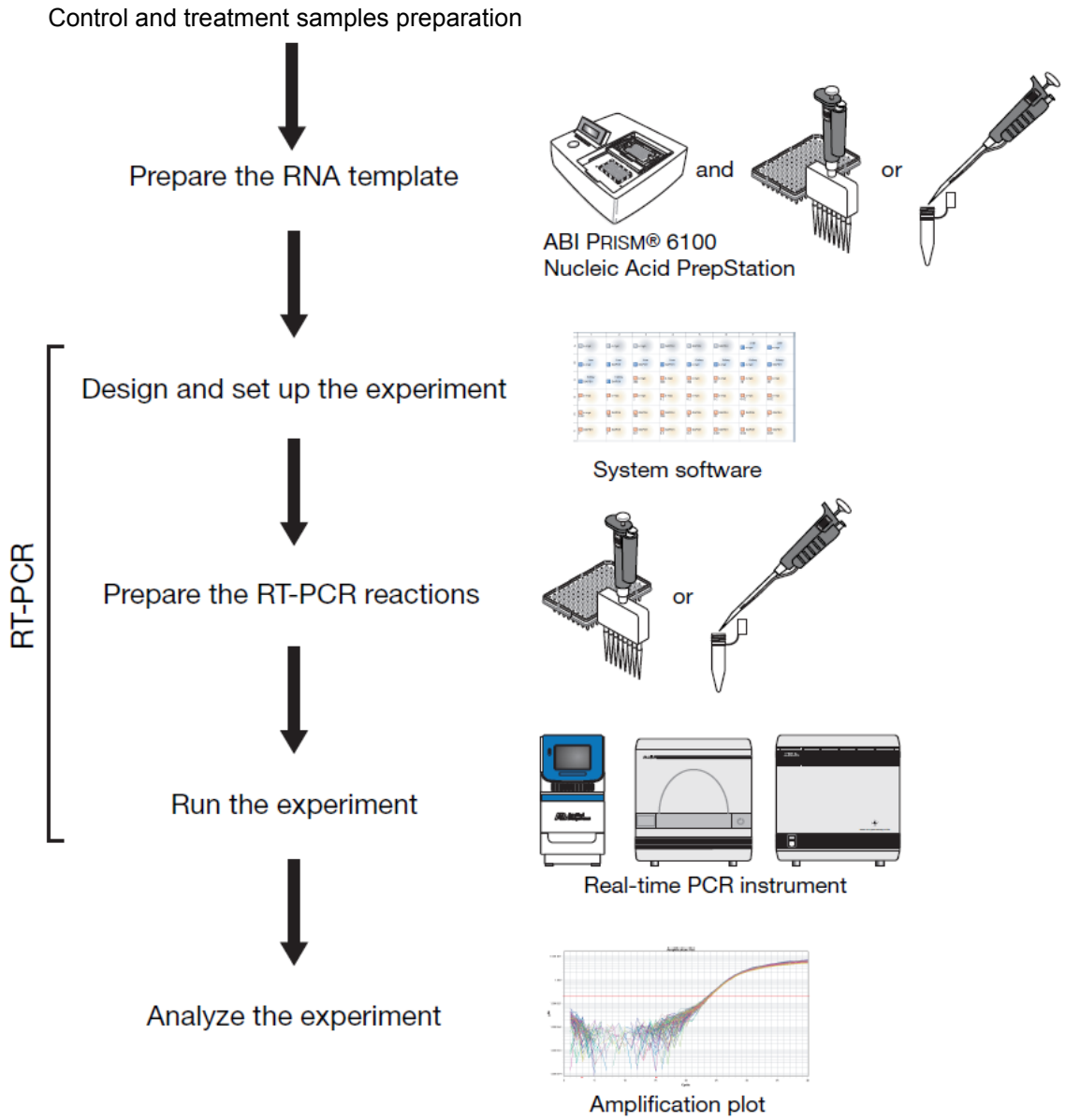


Figure 28. The workflow for one-step RT-PCR using the TaqMan® RNA-to-CT™ 1-Step Kit (Source: Applied Biosystems, 2012: 6)

3.2.7 Real time RT-PCR

Principle

Gene expression can be altered in response to various conditions or by different stages of cell development and is a key step in knowing how many cellular processes, including differentiation, are accomplished. A commonly used method for investigating gene expression is Real-time Reverse Transcription Polymerase Chain Reaction (Real-time RT-PCR) or Quantitative Polymerase Chain Reaction (qPCR). It quantifies the amount of mRNA molecules produced by a specific gene after its transcription. This is achieved by producing complementary DNA (cDNA) through reverse transcribing the previously isolated mRNA (section) and amplifying the resulting cDNA, whilst detecting in real time the amount of amplified cDNA molecules (hence the emphasis of real-time in its name). The detection of amplified cDNA is performed by observing the fluorescence from the fluorescence-emitting molecules used for labelling the cDNA molecules. The intensity of fluorescence is proportionally increased with the amplification of the cDNA molecules with each cycle of the reaction. Two commonly used fluorescent tags in Real-time PCR are SYBR® Green I dye and TaqMan® probes. SYBR® Green I dye is the cheaper alternative. It binds to all double stranded DNA and enables the detection of all amplified double-stranded DNA molecules. However, it may also detect non-specific reaction products. TaqMan® probes are more expensive, but can be synthesised for each unique target sequence and are therefore emitting fluorescence only when the specific PCR product is amplified. A comparison between these two chemical products is presented in Figure 29.

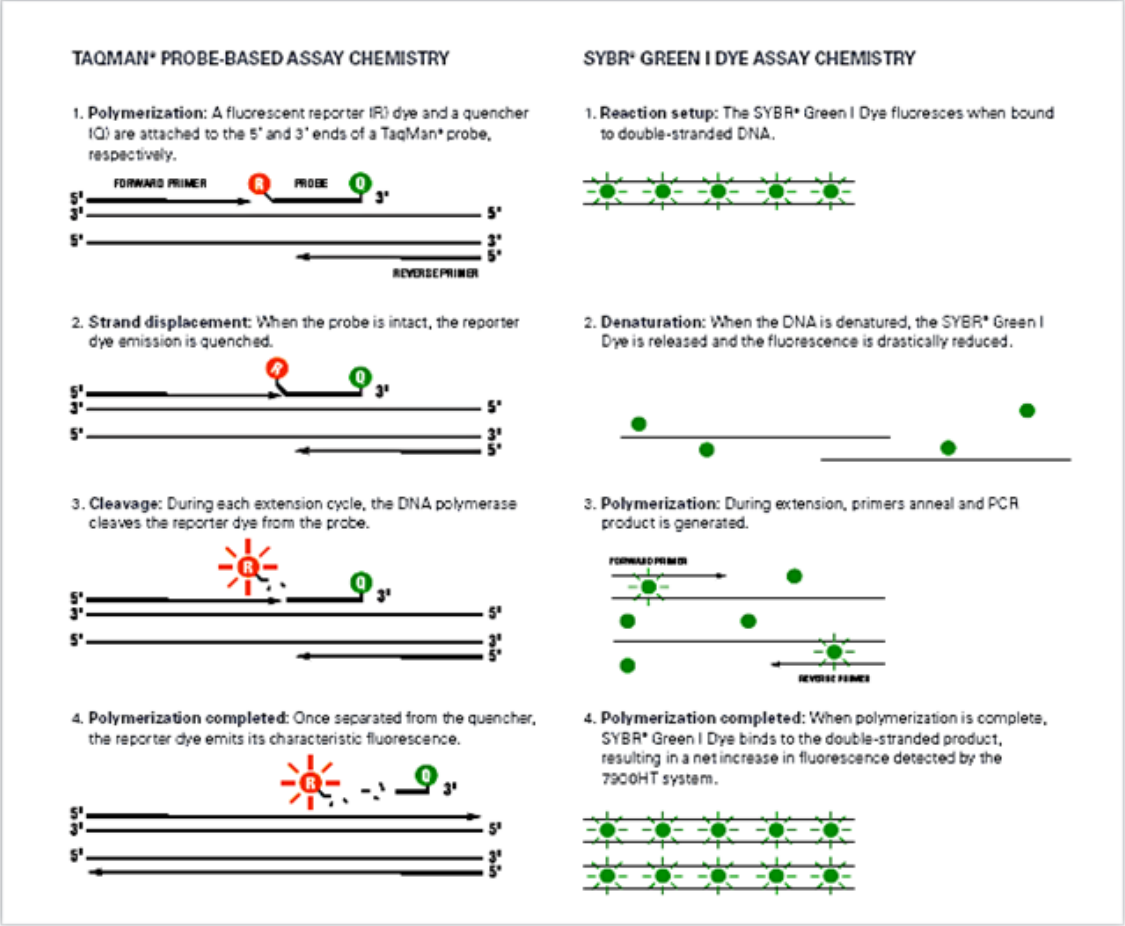


Figure 29. A comparison between SYBR® Green and TaqMan® used in Real-time PCR. TaqMan® detection is more specific as it uses a specially designed probe for each target sequence and emits fluorescence only when the target sequence has been replicated. SYBR® Green dye is less specific, as it binds to any double stranded DNA present in the reaction.
(Source: www.appliedbiosystems.com)

The increase in fluorescence with each cycle is detected. This enables the estimation of the rate of amplification and the abundance of the cDNA molecules generated. The higher the gene expression, i.e., the starting concentration of cDNA, the fewer number of amplification cycles it would take for the levels of fluorescence to reach a certain threshold. Thus the first cycle at which the instrument can detect the amplification generated fluorescence to be above the ambient background signal is denoted as the threshold cycle C_t, or quantification cycle C_q by the new *Minimum Information for Publication of Quantitative Real-Time PCR Experiments* (MIQE) guideline, of the sample. The lower the value of C_t is, the higher the level of gene expression would be. Comparison of C_t values between different samples is therefore indicative of how gene expressions between these samples differ. Figure 30 shows an example of 3 samples with different gene expression levels and how this is represented by their C_t values.

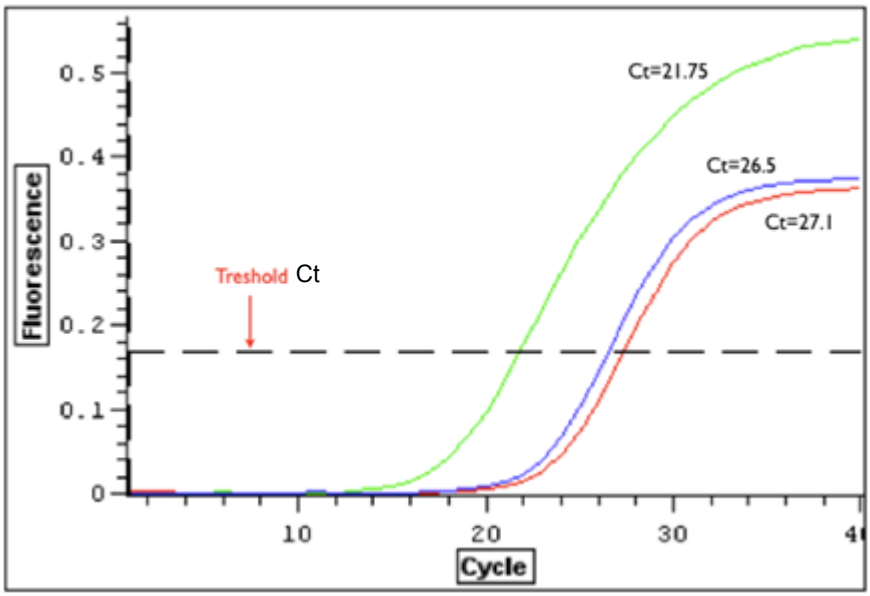


Figure 30. Real-time PCR analysis demonstrates the number of cycles required for 3 different samples to reach a specific fluorescence threshold (represented by their C_t value).

The lower the C_t value or the number of cycles required to reach the fluorescence threshold, the higher the expression of the gene. Thus, in the figure above, the sample represented by the green curve is the one with highest gene expression ($C_t = 21.75$), followed by the blue one ($C_t = 26.5$) and the red sample is the one with the lowest gene expression ($C_t = 27.1$).

The TaqMan® method

The TaqMan® PCR-based method was used in this study. This was done using the TaqMan® RNA-to-C_T™ 1-Step Kit (Applied Biosystems, San Jose, CA, USA). The kit contains both AmpliTaq Gold® DNA polymerase and ArrayScript™ UP (Ultra-Pure) RT (Reverse Transcriptase). The former is the enzyme required for DNA amplification and the latter the reverse transcription of mRNA into cDNA.

Final reaction volume was 20 µL per reaction, composed of 8.5 µL of RNA (5.5 ng/µL), 10 µL master mix containing the DNA polymerase enzyme, 1 µL of probe TaqMan primers mix and 0.5 µL RT enzyme. Applied Biosystems StepOnePlus Real-time PCR system with StepOne Software v2.2.2 (Applied Biosystems, Life Technologies) was used. The housekeeping gene RNA polymerase GAPDH was used as a reference gene. The following program cycles were used:

Table 4. The stages of the program cycles used in RT-PCR with the TaqMan© method (Source: Applied Biosystems, 2012: 10)

Stage	Step	Temperature	Time
Holding	Reverse Transcription	48 ºC	15 minutes
Holding	Activation of TAQ polymerase	95 ºC	10 minutes
Cycling (40 cycles)	Denaturation	95 ºC	15 second
	Annealing/Extension	60 ºC	1 minute

The following predesigned dyes and primers (Applied Biosystems) were used in the TaqMan® detection method:

Table 5. The dyes and primers used in RT-PCR with the TaqMan© method (Source: Applied Biosystems, 2012)

Gene	**Dye or Primer / Assay ID**
FAM	Reporter dye
BHQ1	Quencher dye
GAPDH	mm99999915_m1
Kdr/FLK1	mm01222421_m1
Tall/SCL	mm01187033_m1
GATA1	mm01352636_m1
GATA2	mm00492301_m1
p21/CDKNA	mm04205640_g1
p27	mm00495994_m1

The Livak method

Comparative quantification of the target gene expression in the samples was performed based on C_t normalised to GAPDH for each time point with $\Delta C_t = C_{t\,(experiments)} - C_{t\,(GAPDH)}$ and the Livak method or the $2^{-\Delta\Delta CT}$ method was used to ascertain the fold change using the C_t value for the vehicle as a reference. $\Delta\Delta CT = \Delta CT_{\,(VD3)} - \Delta CT_{\,(vehicle)}$. The fold change in mRNA expression for each time point was plotted in a graph using negative control as a reference as $2^{-\Delta\Delta CT}{}_{\,(vehicle)} = 1$. The formula for the calculation of the normalised expression ratio $2^{-\Delta\Delta CT}$ is

$$\Delta\Delta CT = \Delta Ct_{\,test} - \Delta Ct_{\,calibrator}$$

$$\Delta Ct_{\,test} = \text{Mean } Ct_{\,target\ gene,\ test} - \text{Mean } Ct_{\,reference\ gene,\ test}$$

$$\Delta Ct_{\,calibrator} = \text{Mean } Ct_{\,target\ gene,\ calibrator} - \text{Mean } Ct_{\,reference\ gene,\ calibrator}$$

where

Mean $Ct_{\text{target gene, test}}$ is the average C_t value (sample and triplicate) of the targeted gene in the experimental condition;

Mean $Ct_{\text{reference gene, test}}$ is the average C_t value (sample and triplicate) of reference house-keeping gene in the experimental condition;

Mean $Ct_{\text{target gene, calibrator}}$ is the average C_t value (sample and triplicate) of targeted gene in the calibrator condition; and

Mean $Ct_{\text{reference gene, test}}$ is the average C_t value (sample and duplicate) of reference house-keeping gene in the calibrator condition.

To compare the effect of the presence of VD_3 on E14 in the E14 / OP9 co-culture, the experimental condition was E14 / OP9 co-culture with VD_3 and the calibrator condition was E14 / OP9 co-culture without VD_3. The RNAs isolated from E14 after different days of incubation were used for the calculation of ΔCt_{test} and $\Delta Ct_{\text{calibrator}}$. The targeted genes were FLK, P21, Gata1 and Gata2, etc. whilst GAPDH was used as the reference gene in all RT-PCR assays, and a pooled C_t value (22.31 ± 1.04) for the reference gene was taken from all RT-PCR runs (n = 9) as there was no significant difference in C_t values for the reference gene at different time points and between cell types.

Analysis

Relative quantification of gene expression was determined automatically by Opticon Monitor version 3.1.32 (MJ Geneworks Inc. and Bio-Rad Laboratories, Inc., Hercules, CA, USA) and StepOne Software v2.2 (Applied Biosystems, Life Technologies). The relative quantification is based on analysing changes in gene expression relative to a reference sample (control). One sample from the experiments was set as a calibrator/control and fold change to this calibrator was determined for every sample of the experiment. All samples were normalised to a house-keeping gene (GAPDH) as its expression is known to remain consistent in E14 cells regardless of the treatment (Tricarico *et al.*, 2002).

From this, the C_t values for all genes were tabulated and imported into an Excel spread sheet designed for the application of the Livak Method for determining normalised expression ratios using the $2^{-\Delta\Delta CT}$ formula.

3.2.8 Cytokine expression assay

Better understanding of the growth control of hematopoietic cells during the *in vitro* differentiation of ES cells could be achieved through the investigation of the expression of a number of cytokine related genes involved in the haematopoiesis process. The point at which a particular factor begins to play a role corresponds to a detection of significant expression of the related gene. Conversely, the lack of expression is an indication that the corresponding factor is not critical at that point. As it is quite common for several cytokines to act simultaneously or collectively, it is important to be able to detect several expressions concurrently.

There are several assays available for cytokine detection and analysis. For instance, cytokine bioassays measure biological activity by some sort of proliferation assay of primary cells or cell lines dependent or responsive to the cytokine of interest. Immunofluorescence staining and flow cytometry mentioned before use fluorescently labelled antibodies to detect intracellular cytokines and surface receptors at the single cell level. These and other assays have several drawbacks. It may take a long time to run some of these assays, whilst each analyte needs to be quantitated separately in some other assays.

On the other hand, simultaneous detection of multiple analytes lasting only several hours could be achieved by conducting Luminex multiplex cytokine expression assays using the Luminex kits.

Principle

A specific antibody to the target protein is covalently coupled to microspheres (beads) that are dyed internally with two fluorescent dyes. Up to 100 distinctly colours could be achieved through using precise concentration ratios of the dyes. Beads with each distinct colour are coated with a specific capturing antibody. After an analyte from a test sample is captured by the bead, as illustrated in Figure 31, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with the reporter molecule streptavidin PE conjugate to complete the reaction on the surface of each microsphere.

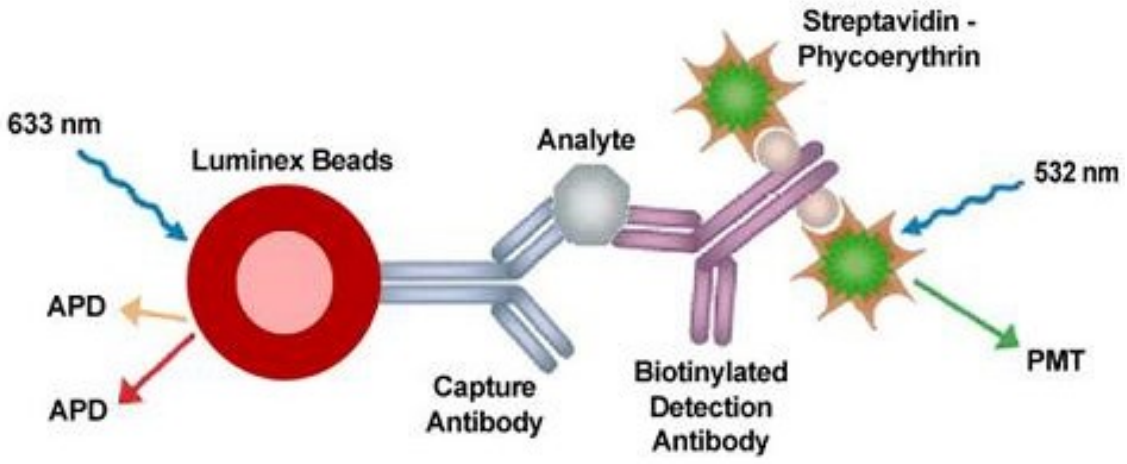


Figure 31. Schematic representation of the principle of the Luminex assay (Source: www.crcjussieu.fr/crc/upload/editor/image/Equipe09/multiplex%20Assay%20Design2.JPG)

As shown in Figure 32, the beads are passed rapidly through a first laser to excite the dyes inside the beads. A second laser excites PE, the fluorescent dye on the reporter molecule. The signals from individual beads are then identified using high-speed digital signal processors and the result quantified based on fluorescent reporter signals.

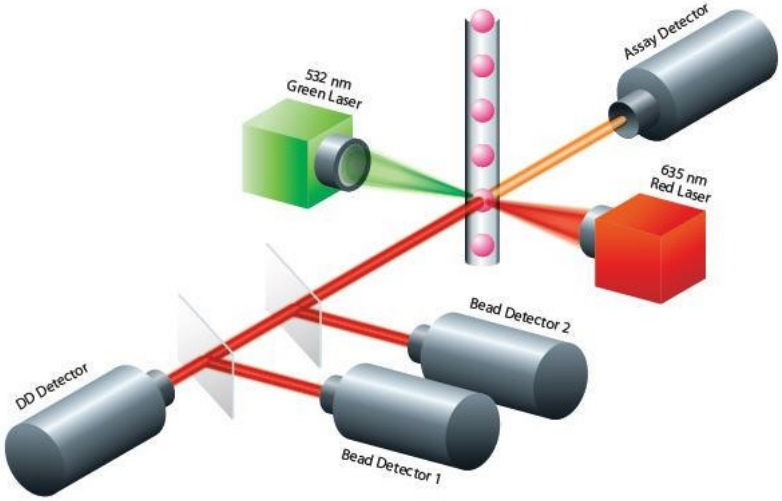


Figure 32. Internal components of the Luminex system (Source: http://ifr125.timone.univ-mrs.fr/biopuces/LuminexPrinciple.jpg)

Alternatively, as illustrated in Figure 33, superparamagnetic beads could be used, and the beads with the captured analyte can be pulled by a magnet to form a monolayer before it is illuminated by red and green LED light sources for the identification and quantification of the analytes.

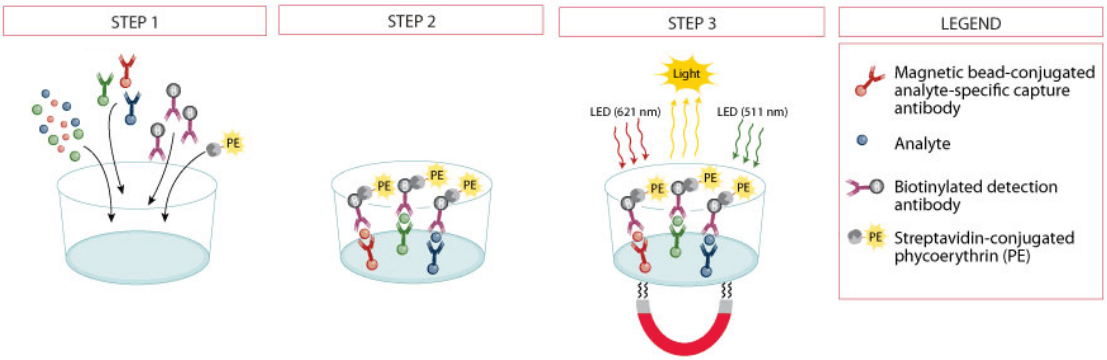


Figure 33. Principle of magnetic bead based multiplex assay (Source: http://www.rndsystems.com/product_detail_objectname_versamap_magnetic_bead_based_multiplex_assay_principle.aspx)

Method

The assays were conducted using the Luminex 200 system (Bio-Plex) running the xPONENT analytical software and the Milliplex MAP Kit of mouse cytokine / chemokine magnetic bead panel for 96-well plate assays (EMD Millipore). Cell samples were E14 / OP9 co-culture incubated for 2, 5, 8, 10 or 12 days with or without 100 nM of VD_3 prepared according to the procedures described previously. The superlative of each of the samples was used in the analysis using the Luminex 200 system. The cytokine of interest in this study are G-CSF, GM-CSF, IL-1α, IL-3, IL-4, IL-5, IL-6, TNF-α and VEGF because their known roles in haematopoiesis, which are briefly described in Table 6.

Table 6. The cytokines studied in this study and their roles in haematopoiesis (Source: Kurzrock, 2000; Gerber *et al,*. 2002; Gerber & Ferrara, 2003; Xu *et al.*, 2000)

G-CSF	Colony stimulating factors (CSFs) are soluble, membrane-bound substances of the hematopoietic microenvironment. It is secreted glycoproteins that bind to receptor proteins on the surfaces of hematopoietic stem cells, activating intracellular signalling pathways that can cause the cells to proliferate and differentiate into a specific kind of blood cell (usually white blood cells). They may be synthesised and administered exogenously. Granulocyte colony-stimulating factor (G-CSF) is a haematopoietic growth factor required for the proliferation and differentiation of haematopoietic precursors of neutrophil granulocytes. It increases the numbers of neutrophils and modulates neutrophil functions, as well as inducing the production of cytokines such as tumour necrosis factor alpha (TNF-alpha).
GM-CSF	Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a protein secreted by macrophages, T cells, mast cells, endothelial cells, and fibroblasts. It functions as a WBC growth factor, stimulating stem cells to produce granulocytes and monocytes.
IL-1	Interleukin-1 has been associated with numerous activities. Some of these include the induction of the IL-2 receptor, the stimulation of pre-B cell differentiation, the augmentation of NK-cell cytotoxicity, the induction of adhesion molecules on endothelial cells, the induction of fever, the stimulation of thymocyte proliferation, the enhancement of collagen production, and the stimulation of the release of other cytokines involved in haematopoiesis.
IL-3	The principle effects of Interleukin-3 haematopoietic growth factor are on early haematopoietic progenitors in which IL-3 induces haematopoiesis and cell differentiation. Administration of IL-3 produces an increase in erythrocytes, neutrophils, eosinophils, monocytes and platelets. It acts synergistically or additively with other haematopoietic growth factors.
IL-4	Interleukin-4 is primarily derived from T cells and its principle site of action is the B cell. It stimulates B cell proliferation and activation. It induces IgG 1 & IgE expression from B cells and class II MHC expression. It also induces the differentiation of eosinophils and the involved in the activity of T cytotoxic cells.
IL-5	The primary effect of Interleukin-5 is on the eosinophilic lineage. It stimulates eosinophil chemotaxis and eosinophil expansion.
IL-6	Interleukin-6 is produced by lymphoid and non-lymphoid cells. It acts on T cells and B cells and stimulates multilineage haematopoiesis, including the maturation of megakaryocytes.
TNF-α	Tumour necrosis factors (TNF) are a group of 19 cytokines which can cause cell death (apoptosis). These cytokines have similar sequences, functions and structures. One of the first members of the TNF family to be identified was Tumour necrosis factor alpha (TNF-α). It is a monocyte derived cytotoxin, implicated in tumour regression, septic shock and cachexia. It stimulates cell proliferation and induces cell differentiation under certain conditions. It is a potent pyrogen, causing fever by direct action or by stimulation of IL-1 secretion. It can cause cytolysis of certain tumour cell lines.
VEGF	Vascular endothelial growth factor (VEGF) is a chemical signal produced by cells that stimulates the growth of new blood vessels. VEGF is a sub-family of growth factors, specifically the platelet-derived growth factor family of cystine-knot growth factors. It acts as an endocrine-like hormone to induce extramedullary haematopoiesis, as well as promoting the formation of terminally differentiated red blood cells.

3.3 Statistical Analyses

Statistical analyses and significance of data were determined using GraphPad Prism version 5.00 for Mac OS X and Microsoft Windows (GraphPad Software, San Diego California USA). As more than one factor was present, two-way ANOVA was performed to investigate the significances of factors or interactions. This was followed up by examining the statistical significance for interactions between more than two groups within the factor with Bonferroni post-hoc analysis. All results are presented as mean ± standard error of the mean (SEM). All values with $p < 0.05$ were considered as significant.

3.4 Results

3.4.1 Immunofluorescence

The purpose of this part of the experiment is to determine the role of Oct4, Sox2, Nanog as master regulators of stem cell pluripotency and differentiation of ESCs, and to evaluate the effect of VD_3 as an accelerator and a promoter of early differentiation of ESCs as illustrated in Figure 8.

These investigations were done by co-culturing E14 cells on OP9 cells in differentiated media with or without VD_3 for up to 5 days. The extent, if any, of the roles of the aforementioned regulators was qualitatively investigated by immunocytochemistry.

The results of immunofluorescence are shown in Figure 34. Compared to untreated co-cultures, it was found that in up to 5 days' incubation of VD_3-treated co-cultures, there was visible reduction of fluorescence. This suggested the reduction of Oct4, Sox2 and Nanog expression. As mentioned before, Oct4, Sox2 and Nanog are associated with undifferentiated cells, and decreased expression of these transcription factors is associated with differentiated cells. Their heightened expression inside cells is therefore considered to be indicative of the presence of undifferentiated cells. On the other hand, reduced or lack of expression of these genes means that cells have differentiated.

Summary

Therefore, the results of immunofluorescence showed that VD_3 was associated with the reduction of Oct4, Sox2 and Nanog expressions, the inhibition of cell proliferation, as well as the acceleration and promotion of cell differentiation. This result agrees with previous studies using other cell cultures that have linked VD_3 to the acceleration and promotion of differentiation.

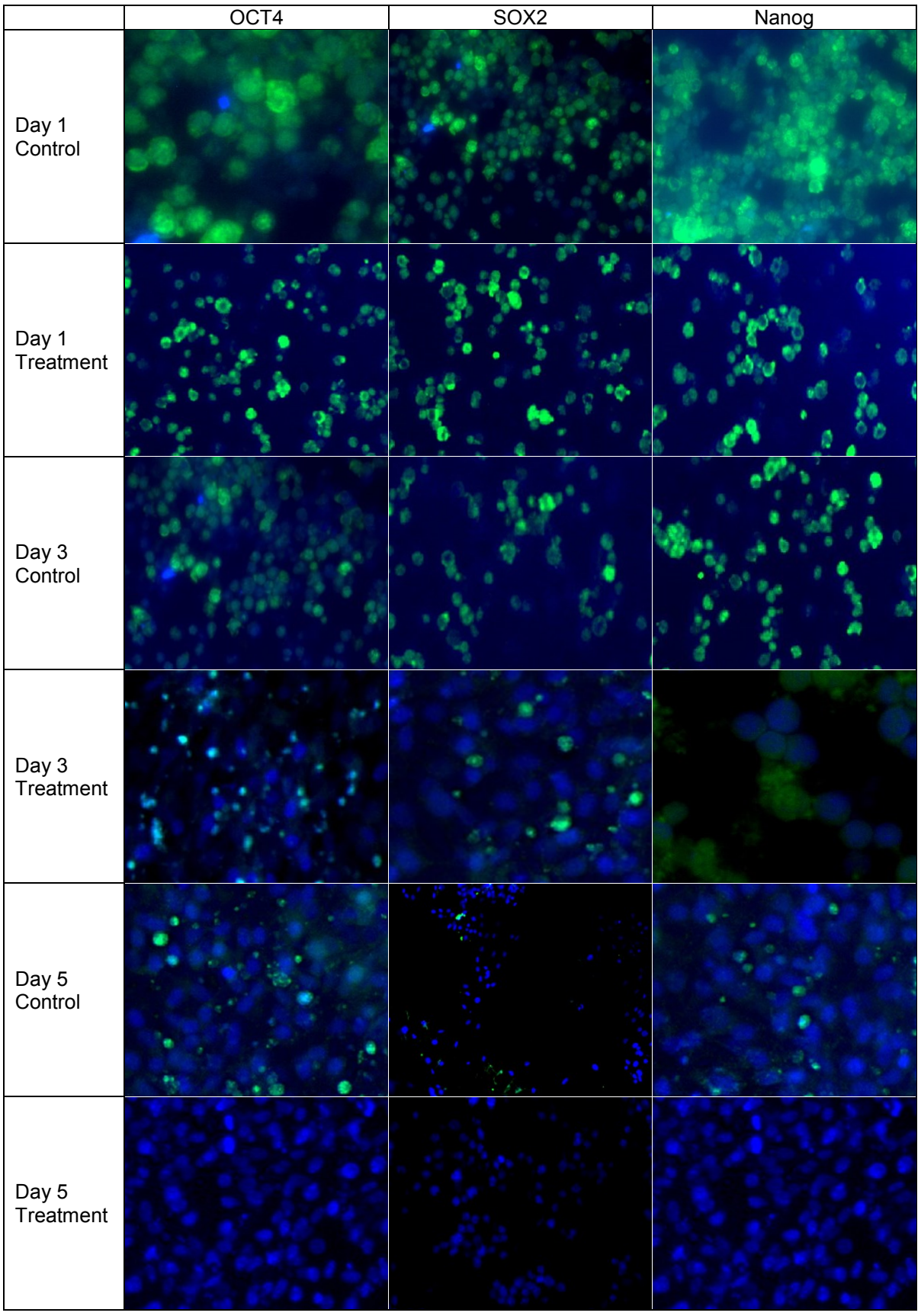
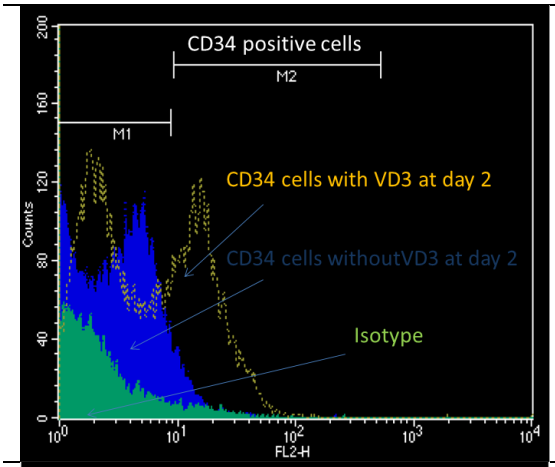


Figure 34. The results of immunofluorescence staining of E14 / OP9 co-culture with or without VD_3 on days 1, 3 and 5.
All cells are marked by the blue stain for easy identification whilst only undifferentiated cells are marked by the florescent green stain. The number of undifferentiated cells is reduced towards Day 5 and is consistently less in treatment than in control. N = 9.

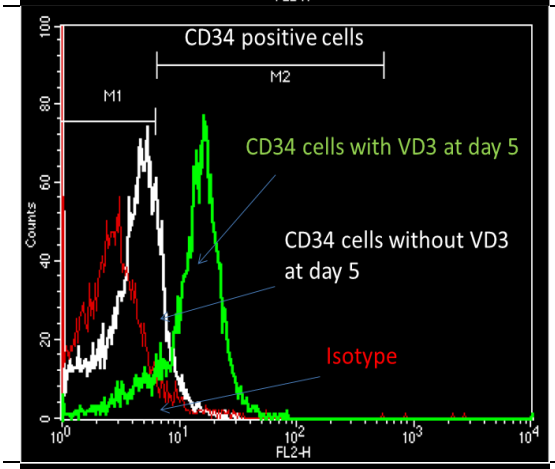
3.4.2 CD-marker expressions on E14 cells in the presence of OP9 cells and VD_3

The data for the expressions of each CD markers of the sample were derived from the output charts of flow cytometry. Some of these examples are shown in Figure 35 below, with some brief explanation of how the data can be read from these charts.



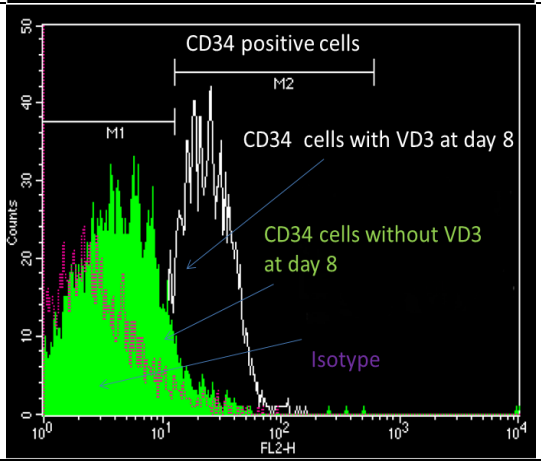
Day 2

The amount of $CD34^+$ progenitor cells in the sample with VD_3 (the area between the yellow curve and the x-axis within the region of M2) is greater than the amount of CD34+ progenitor cells in the sample without VD_3 (control) (the area between the top curve of the dark blue region and the x-axis within the region of M2).



Day 5

The amount of $CD34^+$ progenitor cells in the sample with VD_3 (the area between the green curve and the x-axis within the region of M2) is greater than the amount of CD34+ progenitor cells in the sample without VD_3 (control) (the area between the white curve and the x-axis within the region of M2).



Day 8

The amount of $CD34^+$ progenitor cells in the sample with VD_3 (the area between the white curve and the x-axis within the region of M2) is greater than the amount of $CD34^+$ progenitor cells in the sample without VD_3 (control) (the area between the top curve of the green region and the x-axis within the region of M2).

Figure 35. Some examples of flow cytometry results on the amount of $CD34^+$ progenitor cells on set days

The expressions of different CD markers, FLK, CD31, CD34, CD41, CD43 and CD45, were assessed successively on days 2, 5, 8, 10 and 12, respectively. ESC (E14) cellular differentiation was dependent on the fluctuations in the levels of CD-marker expressions on the given days, starting from day 2, in the E14 / OP9 co-culture that were either untreated or treated with VD_3. These fluctuations are described below and shown in graphical form.

Each assessment procedure was done in triplicate to arrive at an average, given as percentage. Each pair of these averages was statistically analysed to determine if it was statistically significant. For the measurements of each CD marker, the significance of the presence of VD_3 and the duration of incubation were evaluated using two-way ANOVA tests and Bonferroni post-hoc analysis.

FLK Expression

FLK1 is monoclonal antibody specifically binds to foetal liver kinase 1, also known as VEGF receptor-2 (VEGF-2), a receptor for vascular endothelial growth factor (VEGF). It is expressed on distinct sets of mesoderm during gastrulation and on endothelial cells in embryonic and adult tissues. *In vivo* and *in vitro* studies indicate that FLK1 is required for the embryonic development of vascular endothelial and haematopoietic cells (BD Biosciences, 2010).

The mean magnitude of FLK expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 36 below.

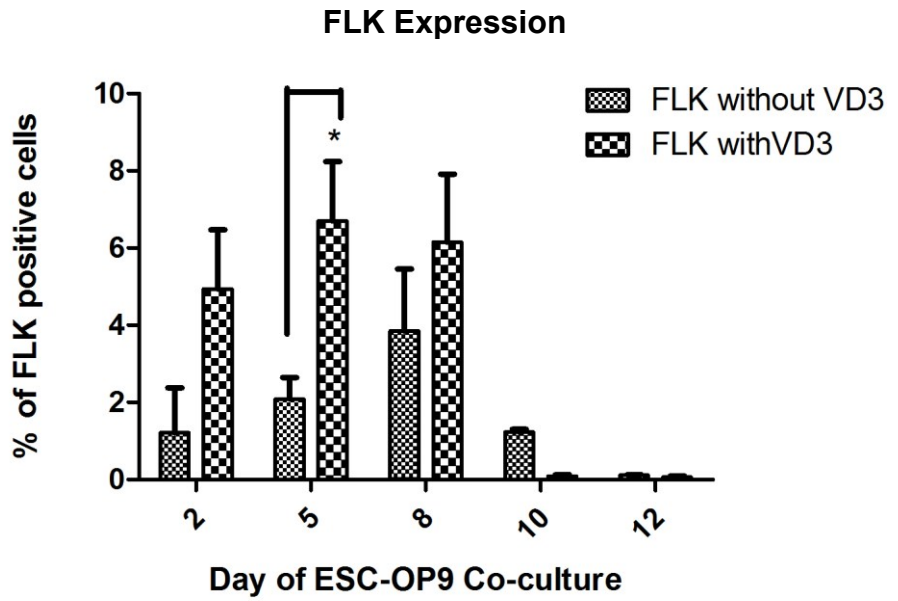


Figure 36. Comparison of the percentages of FLK^+ cells in the untreated and VD_3-treated E14 / OP9 co-cultures after different days of incubation
N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

The changes in the magnitude of FLK expression against time were visible in the bar chart above. The magnitude of FLK expression of untreated E14 cells increased after day 2, to a peak on day 8, and then sharply decreased to the lowest value taken after day 12. The magnitude of FLK expression of treated E14 cells increased after day 2, to a peak on day 5, and only markedly decreased after day 8. The level of FLK expression of treated cells was superior to the magnitude of expression of untreated cells on days 2-8, but inferior after day 8 owing to its marked decrease. The greatest difference in the expressions between the treated and the untreated E14 cells was on day 5 and it was statistically significant ($p < 0.05$). The differences in the level of FLK expression between the treated and untreated cells were not statistically significant with the samples taken at the end of other days.

This suggests that the FLK expression of E14 cells had been hastened by the presence of VD_3 in the co-culture with OP9 cells. In other words, E14 cell differentiation had been encouraged and promoted by the presence of VD_3 in the co-culture with OP9 cells.

Both the presence of VD_3 and the duration of incubation were significant factors to the FLK expression in the experiment, as indicated by the results of the two-way ANOVA test and confirmed by the Bonferroni post-hoc analysis.

CD31 Expression

CD31 is integral membrane protein, a member of the immunoglobulin superfamily, which mediates cell-to-cell adhesion. It is expressed on the surface of adult and embryonic endothelia cells (BD Biosciences, 2010).

The mean magnitude of CD31 expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, together with the results of the two-way ANOVA test, are presented in Figure 37 below.

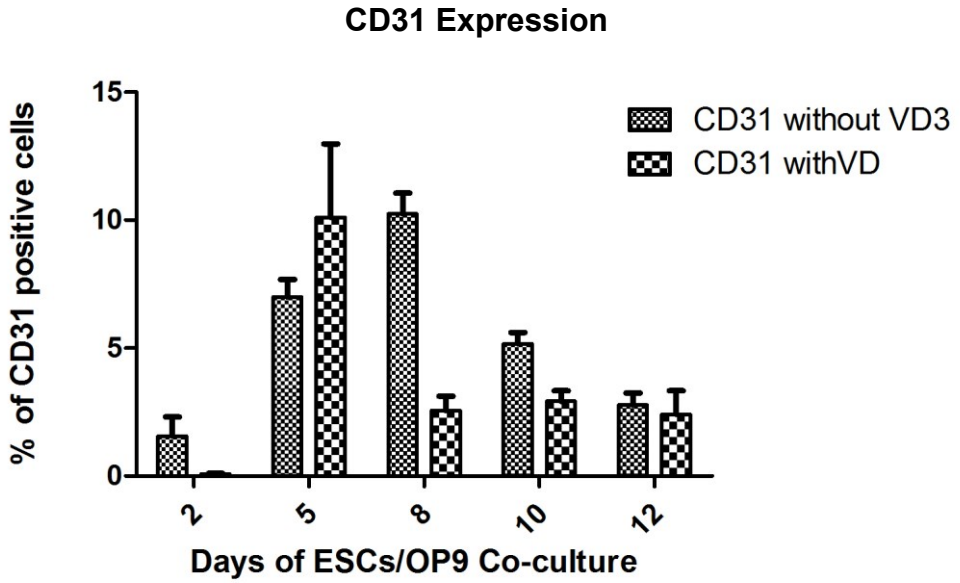


Figure 37. Comparison of the percentages of $CD31^+$ cells in the untreated and VD_3-treated E14 / OP9 co-cultures after different days of incubation
N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

Again, the changes in the level of CD31 expression against time were visible in the bar chart above. The level of CD31 expression of untreated E14 cells reached its peak on day 8, and then decreased afterwards. In comparison, the level of CD31 expression of treated E14 cells increased sharply after a small percentage on day 2 to a peak on day 5, and markedly decreased after day 5, whereupon the level remained relatively unvaried on days 8-12. The level of CD31 expression of treated cells was superior to the level of expression of untreated cells only on day 5, though it was not statistically significant.

Again, the shift of maximum from day 8 to day 5 suggests that the CD31 expression of E14 cells had been hastened by the presence of VD_3 in the co-culture with OP9 cells. In

other words, E14 cell differentiation had been encouraged and promoted by the presence of VD_3 in the co-culture with OP9 cells.

Both the presence of VD_3 and the duration of incubation were significant factors to the CD31 expression in the experiment, as indicated by the results of the two-way ANOVA test and confirmed by the Bonferroni post-hoc analysis.

CD34 Expression

CD34 is a surface glycophosphoprotein expressed on committed and primitive hematopoietic progenitor cells (BD Biosciences, 2010).

The mean magnitude of CD34 expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 38 below.

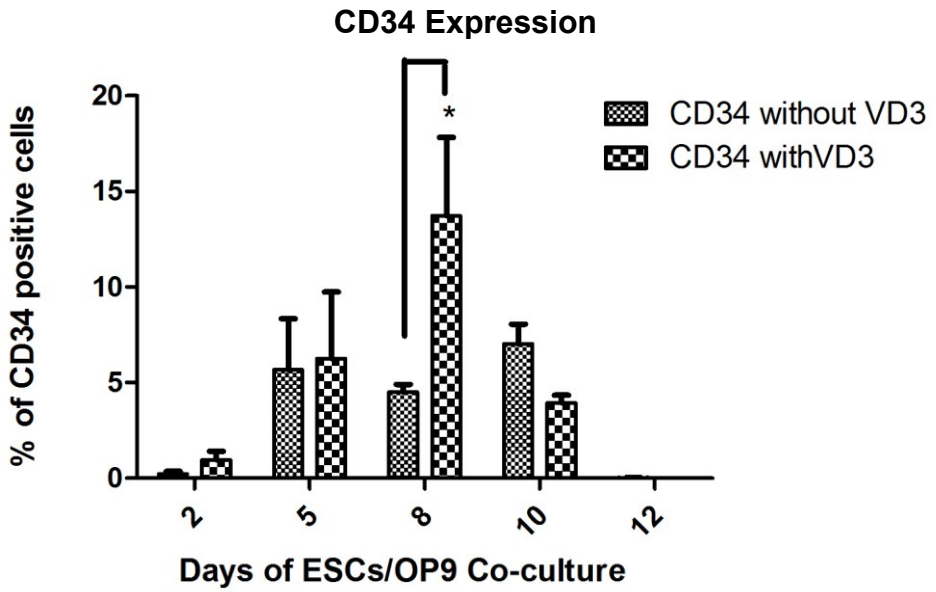


Figure 38. Comparison of the percentages of $CD34^+$ progenitor cells in the untreated and VD_3-treated E14 / OP9 co-cultures after different days of incubation
N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

As shown in the figure above, the level of CD34 expression of untreated E14 cells increased after day 2, then slightly decreased after day 5, slightly increasing again after day 8, to reach a peak on day 10, followed by a decrease after that. Similar to the results of FLK and CD31 expressions, the level of CD34 expression of VD_3-treated E14 cells rapidly increased after day 2, to a peak on day 8, and then rapidly decreased afterwards. The level of CD34 expression of treated cells was superior to the level of expression of

untreated cells on days 2-8. The maximum difference between the expressions of the VD_3-treated and untreated E14 cells was on day 8 and this is statistically significant ($p < 0.05$). Again, the differences between the VD_3-treated and untreated cells were not statistically significant on other days.

Once again, the shift of maximum from day 10 to day 8 suggests that the CD34 expression of E14 cells had been hastened by the presence of VD_3 in the co-culture with OP9 cells. In other words, E14 cell differentiation had been encouraged and promoted by the presence of VD_3 in the co-culture with OP9 cells.

Both the presence of VD_3 and the duration of incubation were significant factors to the CD34 expression in the experiment, as indicated by the results of the two-way ANOVA test and confirmed by the Bonferroni post-hoc analysis.

CD41 Expression

CD41 is integral membrane protein expressed on platelets, megakaryocytes and early haematopoietic progenitors (BD Biosciences, 2010).

The expressions of CD41, CD43 and CD45 displayed transition patterns along time which were different from those of FLK, CD31 and CD34 in the E14 cells in untreated co-culture with OP9 cells.

The mean level of CD41 expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, and the results of the two-way ANOVA test, are presented in Figure 39 below.

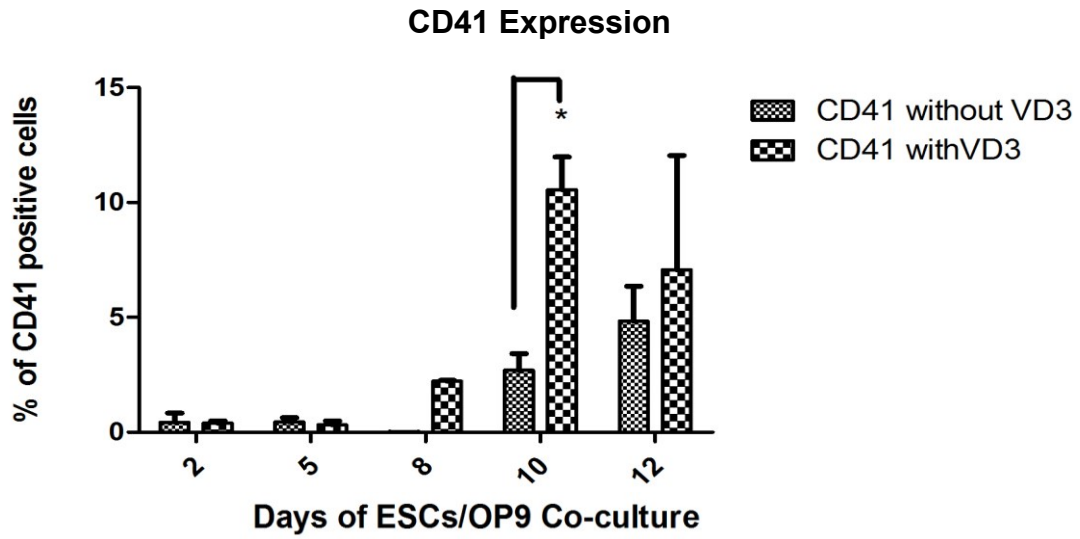


Figure 39. Comparison of the percentages of $CD41^+$ cells in the untreated and VD_3-treated E14 / OP9 co-cultures after different days of incubation
N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

As shown in the bar chart above, the level of CD41 expression of untreated E14 cells increased after day 5 and reached the highest value after day 12. On the other hand, the level of CD41 expression of VD_3-treated E14 cells slightly decreased after day 5 then rapidly increased after day 8 and peaked after day 10. The level of CD41 expression of VD_3-treated cells was superior to the level of expression of untreated cells after days 8, 10 and 12. The maximum difference between the CD41 expressions of the VD_3-treated and untreated E14 cells occurred after day 10 ($p < 0.05$) and that was the only statistically significant difference.

Once again, the shift of maximum from day 12 to day 10 suggests that the CD41 expression of E14 cells had been hastened by the presence of VD_3 in the co-culture with OP9 cells. In other words, E14 cell differentiation had been encouraged and promoted by the presence of VD_3 in the co-culture with OP9 cells.

Both the presence of VD_3 and the duration of incubation were significant factors to the CD41 expression in the experiment, as indicated by the results of the two-way ANOVA test and confirmed by the Bonferroni post-hoc analysis.

CD43 Expression

CD43 is a membrane protein expressed on granulocytes, monocytes, macrophages, platelets, natural killer cells, thymocytes and most T helper cells (BD Biosciences, 2010).

The mean magnitude of CD43 expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, together with the results of the two-way ANOVA test, are presented in Figure 40 below.

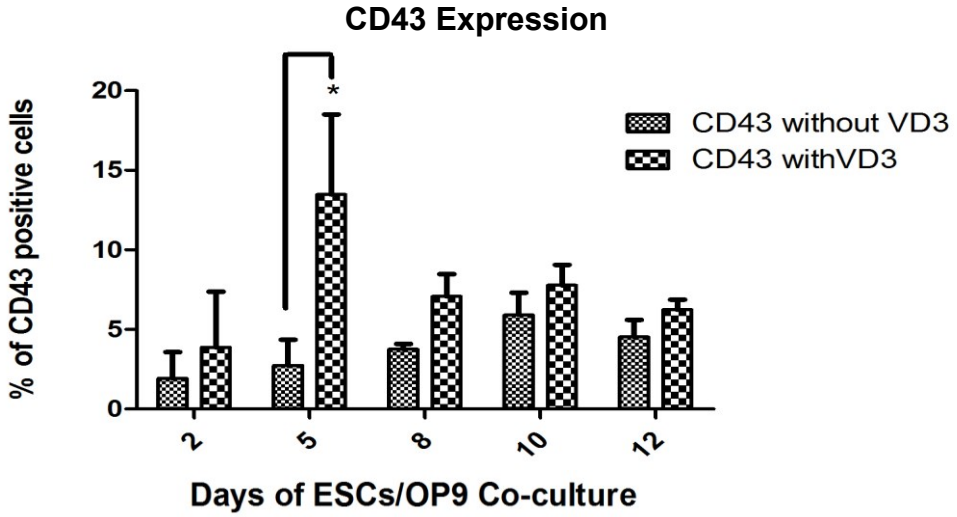


Figure 40. Comparison of the percentages of $CD43^+$ cells in the untreated and VD_3-treated E14 / OP9 co-cultures after different days of incubation
N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

As shown in the bar chart above, the level of CD43 expression of untreated E14 cells increased after day 2, to ultimately peak on day 10; whereas the level of CD43 expression of VD_3-treated E14 cells rapidly increased after day 2, to a peak on day 5, then rapidly decreased after day 5, before virtually levelling out. The level of CD43 expression of VD_3-treated cells was always superior to that of untreated cells. The greatest difference between the CD34 expressions of the VD_3-treated and untreated E14 cells appeared after day 5 and it was statistically significant ($p < 0.05$). The difference was not statistically significant on the samples taken after other days of incubation.

Unlike the results with other gene expressions, only the presence of VD_3 was a significant factor to the CD43 expression in the experiment, as indicated by the results of the two-way ANOVA test. The significance was confirmed by the Bonferroni post-hoc analysis.

CD45 Expression

CD45 is a transmembrane glycoprotein expressed at high levels on the cell surface of leukocytes (BD Biosciences, 2010).

The mean magnitude of CD45 expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 41 below.

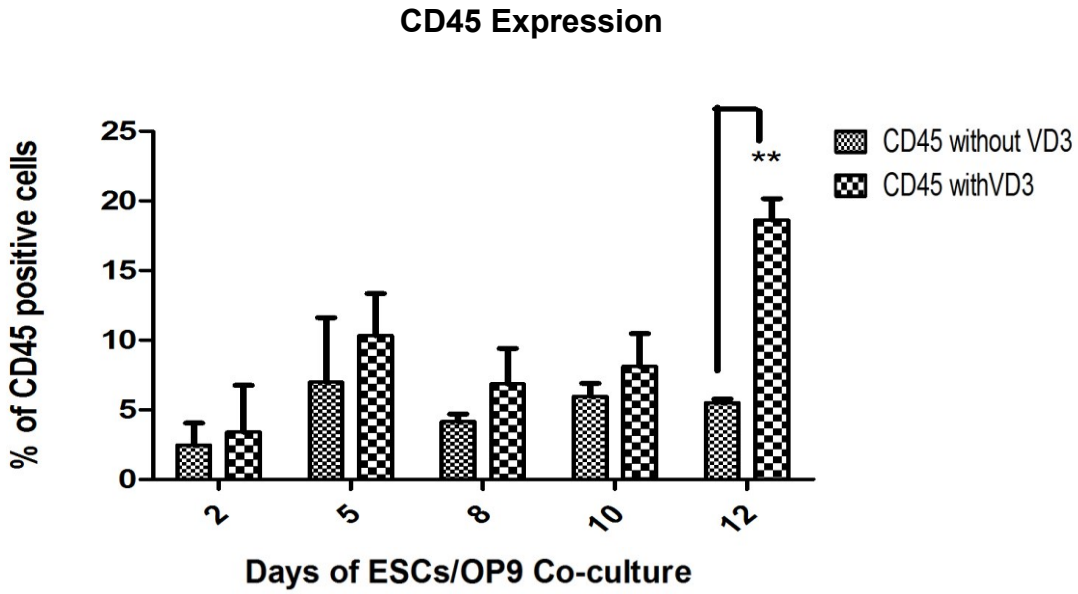


Figure 41. Comparison of the percentages of $CD45^+$ cells in the untreated and VD_3-treated E14 / OP9 co-cultures after different days of incubation
N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

Here, as shown in the bar chart above, the level of CD45 expression of untreated E14 cells increased after day 2, to a peak on day 5, with minor fluctuations afterwards. The level of CD45 expression of VD_3-treated E14 cells peaked first on day 5, but then again and much more pronouncedly on day 12. Again, the level of CD45 expression of VD_3-treated cells was superior to the level of expression of untreated cells on all days. The maximum difference between the expressions of CD45 of the VD_3-treated and the untreated E14 cells was on day 12 and it was statistically significant ($p < 0.01$). The differences in the level of CD45 expressions between the VD_3-treated and untreated cells were not statistically significant on other days.

In this case, both the increase in gene expression in the VD_3-treated samples and the shift of maximum from day 12 to day 10 suggest that the CD45 expression of E14 cells had been hastened by the presence of VD_3 in the co-culture with OP9 cells. In other words, E14 cell differentiation had been encouraged and promoted by the presence of VD_3 in the co-culture with OP9 cells.

Both the presence of VD_3 and the duration of incubation were significant factors to the CD45 expression in the experiment, as indicated by the results of the two-way ANOVA test and confirmed by the Bonferroni post-hoc analysis.

Summary

In the control, FLK^+ cells appeared from the beginning of the duration and the number of cells peaked on day 8 before dropping drastically on day 10. $CD34^+$ cells appeared noticeably on days 5, 8 and 10. The temporal trend of the $CD31^+$ cells closely followed that of $CD34^+$ cells by 1 or 2 days. $CD41^+$ cells appeared 3 days after the $CD34^+$ population and gradually increased by day 10 of culture. $CD43^+$ cells were present throughout the duration, gradually peaked on day 10. The number of $CD45^+$ cells fluctuated around 5% after day 5.

In the treatment, on the other hand, $CD34^+$ cells appeared on day 2 and were more abundant until day 8. $CD31^+$, $CD41^+$ and $CD43^+$ cells followed closely after the appearance $CD34^+$ cells and peaked 2 to 4 days early. FLK^+ cells appeared more prominently in the beginning of the duration between day 2 and day 8 and the peak moved from day 8 to day 5. $CD45^+$ cells appeared more prominently throughout, with substantial peak on day 12.

In other words, compared to the control, the peaks of the expression of the FLK, CD31, CD34 and CD43 markers had appeared earlier, whilst those of the CD41 and CD45 markers had appeared later and greater in the treatment. The early decline of the expression of CD34 marker indicated that differentiation had occurred earlier. The greater expressions of the latter groups of CD markers towards the later days showed the greater build-up of several strands of differentiated cells. More of these cells had been produced because differentiation had started earlier. Thus, these temporal trends indicated that cellular differentiation had happened earlier with the presence of VD_3. Additionally, on most days with every CD marker, there were greater expressions in the treatment than in the control. Both of these were indications that cell differentiation in haematopoiesis had been promoted by the presence of VD_3.

3.4.3 Identification and counting of colony-forming cells

The mode of E14 cell differentiation into different colony-forming cells, namely erythroid (E-CFC), granulocyte, erythroid, macrophage, megakaryocyte (GEMM-CFC), granulocyte-macrophage (GM-CFC), and macrophage (M-CFC), were assessed successively on days 2, 5, 8, 10 and 12, respectively, according to morphology and CD-marker expression. In order to do this assessment, the cells were prepared on slides, and then stained by May-Grünwald Giemsa staining of the cytospins of different CFC types, and morphological examination and image-capture were attained with a Zeiss Axio Imager M1 Microscope using ZEN 2012 software. Each type of cells was identified and counted in the examination. Each assessment procedure was done in triplicate to arrive at an average. These averages were statistically analysed to determine whether or not there was statistical significance. For each set of measurements, the significance of the presence of VD_3 and the duration of incubation were evaluated using two-way ANOVA tests.

Cell count results of erythroid colony-forming cells (E-CFC)

As shown in the microscopic photographs in Figure 42 below, individual E-CFCs can be identified in the cell smears of E14 / OP9 co-cultures after incubation.

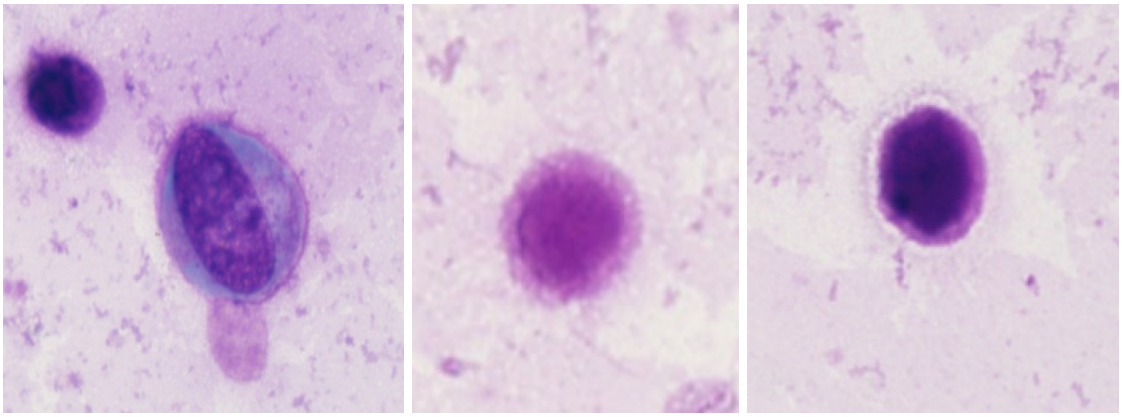


Figure 42. Individual cells of E-CFC identified in cell smears of differentiated E14 cells in co-culture with OP9 cells.
May–Grünwald Giemsa staining was used and images were captured with a Zeiss Axio Imager M1 microscope using ZEN 2012 software. N = 3.

The identified E-CFCs were counted for each sample, and the overall result of cell counts of E-CFC per 10^6 E14 cells in the untreated and VD_3-treated E14 / OP9 co-cultures after 2, 5, 8, 10 and 12 days of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 43 below.

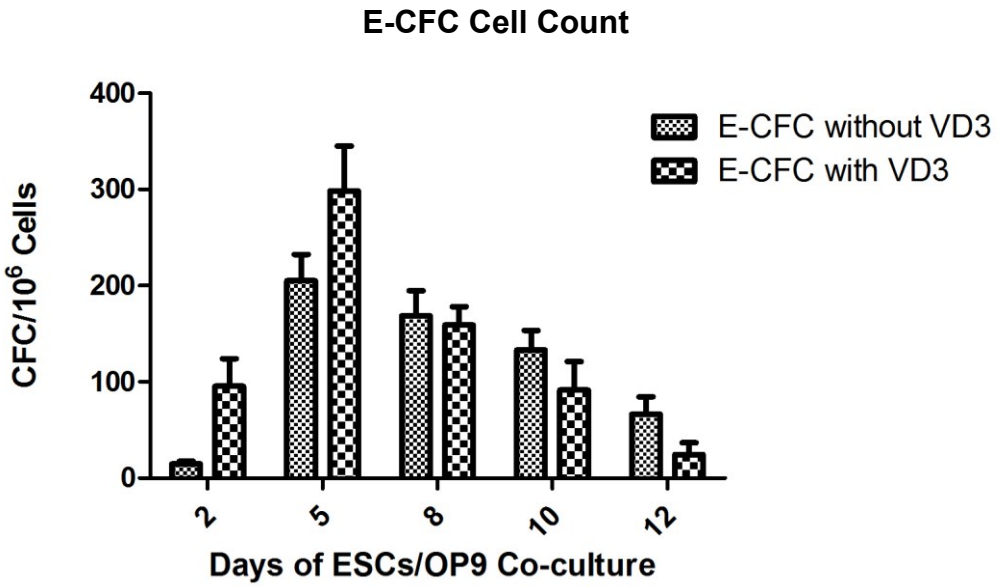


Figure 43. Comparison of changes along time in the number of E-CFCs in untreated and VD_3-treated E14 cells with in co-culture with OP9 cells

As shown in the bar chart above, the numbers of E-CFCs in both treated and untreated co-cultures increased after day 2 to a peak on day 5. The number of E-CFCs in treated co-cultures decreased markedly after day 5. On days 2 and 5, the numbers of E-CFCs in the VD_3-treated samples were higher than that of the untreated controls. In contrast, after day 5, the number of E-CFCs in the treated samples was lower than that of the untreated controls. However, none of these differences in numbers were statistically significant.

According to the result of the two-way ANOVA test, the presence of VD_3 was not a statistically significant factor for the measurements, whilst the time of incubation was, though it is difficult to interpret the findings as there was statistically significant interaction between these two factors in this experiment.

Cell count results of granulocyte, erythroid, macrophage and megakaryocyte colony-forming cells (GEMM-CFCs)

As shown in the microscopic photographs in Figure 44 below, GEMM-CFCs can be identified in the cell smears of E14 / OP9 co-cultures after incubation.

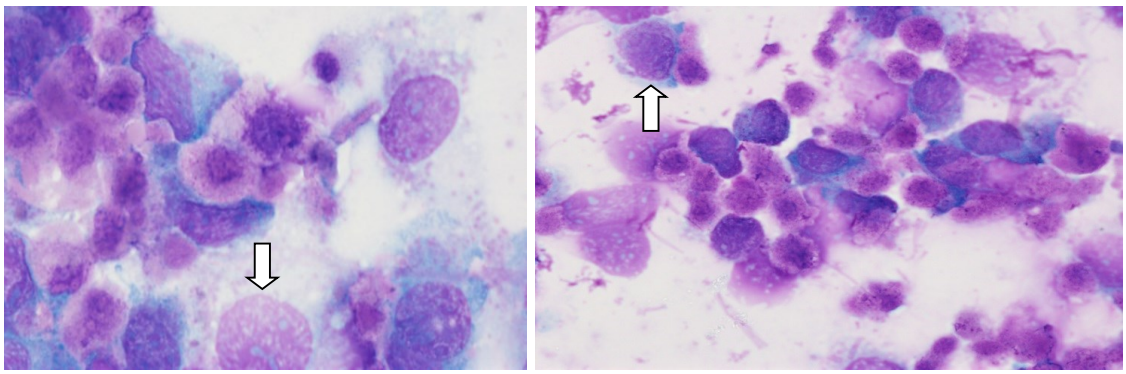


Figure 44. GEMM-CFCs indicated by arrows identified in cell smears of differentiated E14 cells in co-culture with OP9 cells.
May–Grünwald Giemsa staining was used and images were captured with a Zeiss Axio Imager M1 microscope using ZEN 2012 software. N = 3.

The identified GEMM-CFCs were counted for each sample, and the overall result of cell counts of GEMM-CFC per 10^6 E14 cells in the untreated and VD_3-treated E14 / OP9 co-cultures after 2, 5, 8, 10 and 12 days of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 45 below.

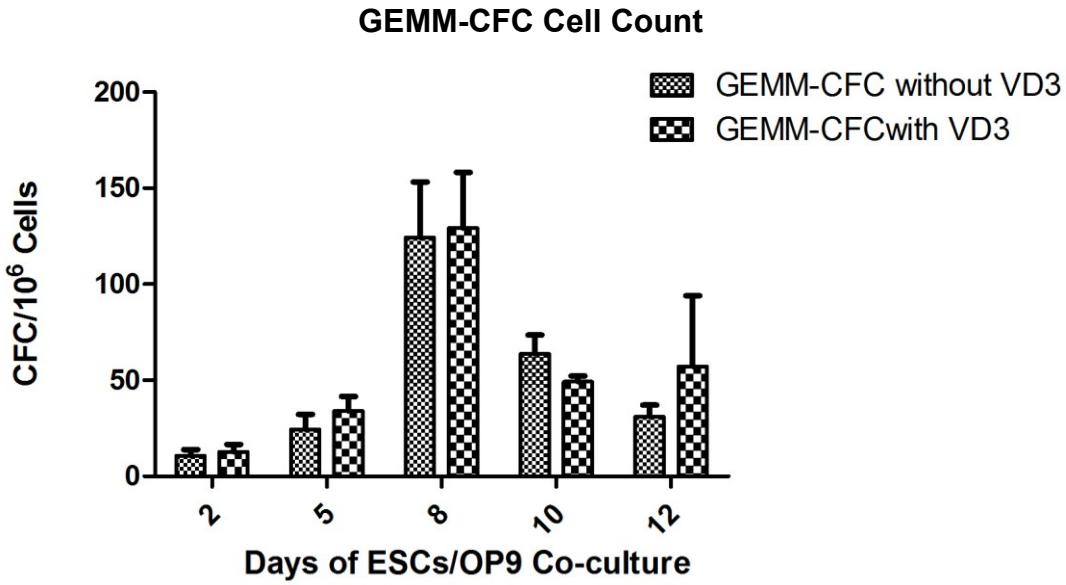


Figure 45. Comparison of changes along time in the number of GEMM-CFCs in untreated and VD_3-treated E14 cells with in co-culture with OP9 cells
N = 3.

As shown in the bar chart above, between all pairs of measurements after the same days of incubation, the number of GEMM-CFCs of VD_3-treated samples was virtually the same as that of untreated controls. The numbers in both treated and untreated cells increased slightly to day 5, then increased rapidly to a peak on day 8, and decreased afterwards. For all pairs of measurements, the differences in the numbers between the treated samples and the corresponding untreated controls were not statistically significant.

According to the result of the two-way ANOVA test, the presence of VD_3 was not a statistically significant factor for the measurements, whilst the time of incubation was, and there was no statistically significant interaction between these two factors in this experiment.

Cell count results of granulocyte-macrophage colony-forming cells (GM-CFCs)

As shown in the microscopic photographs in Figure 46 below, GM-CFCs can be identified in the cell smears of E14 / OP9 co-cultures after incubation.

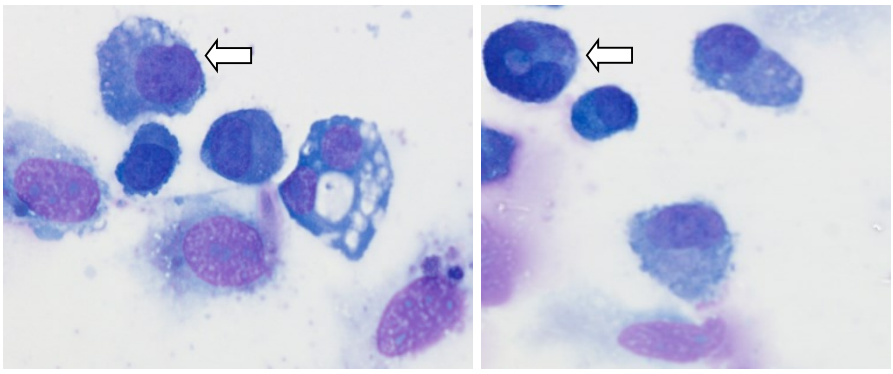


Figure 46. GM-CFCs indicated by arrows identified in cell smears of differentiated E14 cells in co-culture with OP9 cells.
May–Grünwald Giemsa staining was used and images were captured with a Zeiss Axio Imager M1 microscope using ZEN 2012 software. N = 3.

The identified GM-CFCs were counted for each sample, and the overall result of cell counts of GM-CFC per 10^6 E14 cells in the untreated and VD_3-treated E14 / OP9 co-cultures after 2, 5, 8, 10 and 12 days of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 47 below.

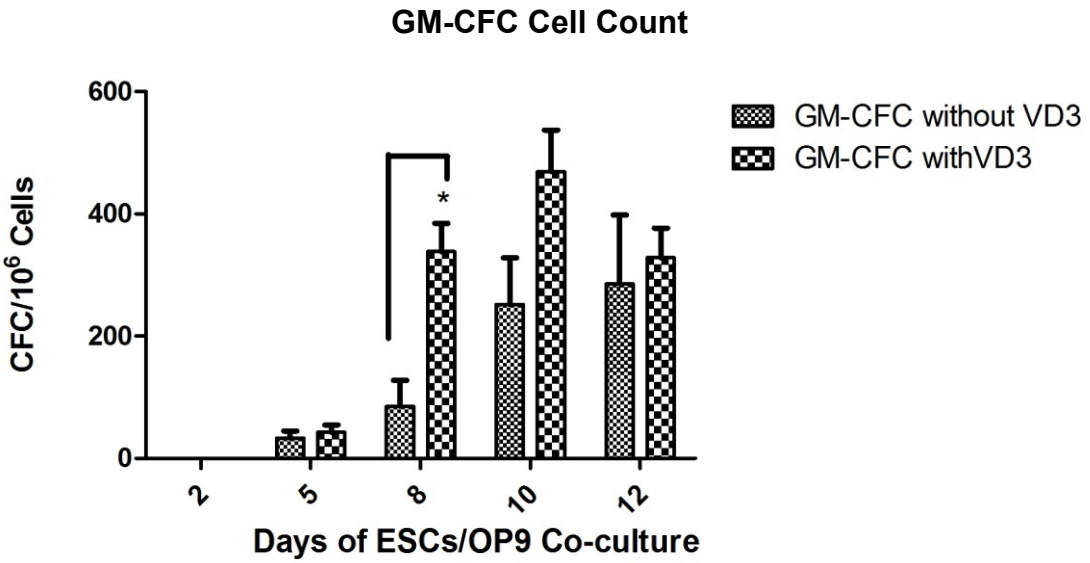


Figure 47. Comparison of changes along time in the number of GM-CFCs in untreated and VD_3-treated E14 cells with in co-culture with OP9 cells
N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

As shown in the bar chart above, the number of GM-CFCs in untreated controls increased after day 5 to a peak on day 12. The numbers of GM-CFCs in VD_3-treated samples increased after day 5 to a peak on day 10. The numbers of GM-CFCs in VD_3-treated samples were higher than those in untreated controls from day 5 onwards. The greatest difference between the treatment and control was on day 8 and this was statistically significant ($p < 0.05$), whereas the differences between the treatment and control for other days were not statistically significant.

According to the result of the two-way ANOVA test, both the presence of VD_3 and the time of incubation were statistically significant factors for the measurements, and there was no statistically significant interaction between these two factors in this experiment. The significance was confirmed by the Bonferroni post-hoc analysis.

Cell count results of macrophage colony-forming cells (M-CFCs)

As shown in the microscopic photographs in Figure 48 below, M-CFCs can be identified in the cell smears of E14 / OP9 co-cultures after incubation.

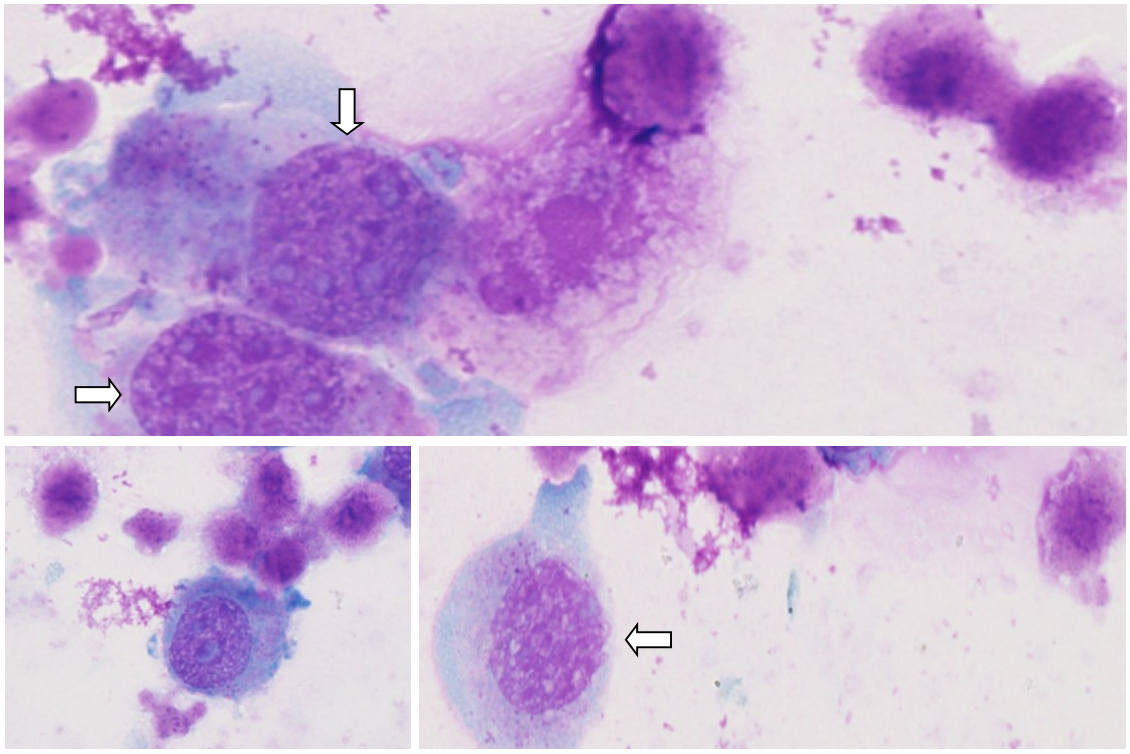


Figure 48. M-CFCs indicated by arrows identified in cell smears of differentiated E14 cells in co-culture with OP9 cells.
May–Grünwald Giemsa staining was used and images were captured with a Zeiss Axio Imager M1 microscope using ZEN 2012 software. N = 3.

The identified M-CFCs were counted for each sample, and the overall result of cell counts of M-CFC per 10^6 E14 cells in the untreated and VD_3-treated E14 / OP9 co-cultures after 2, 5, 8, 10 and 12 days of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 49 below.

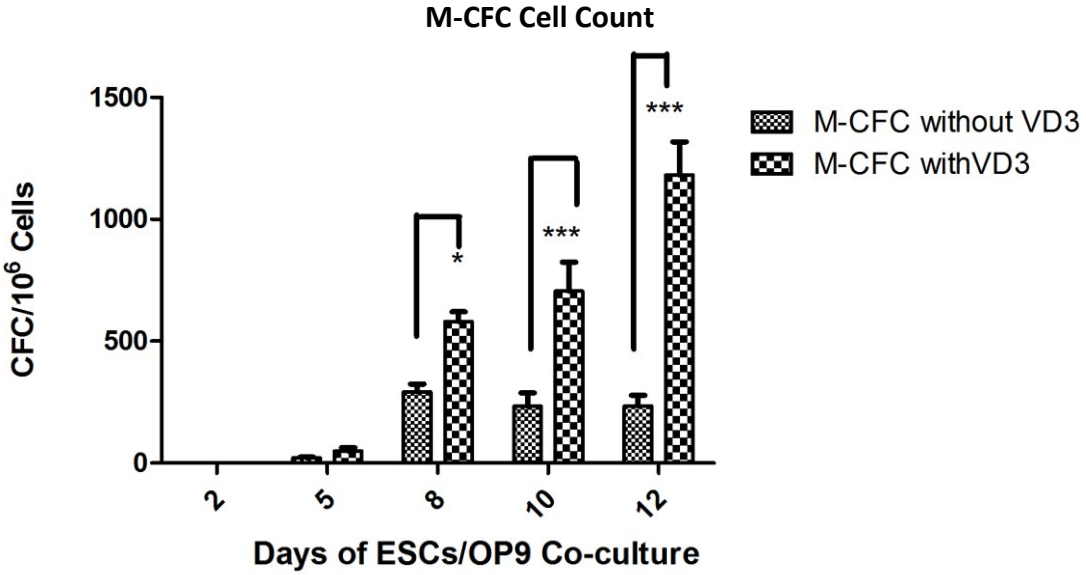


Figure 49. Comparison of changes along time in the number of M-CFCs in untreated and VD_3-treated E14 cells with in co-culture with OP9 cells
N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

As shown in the bar chart above, the number of M-CFCs in untreated controls increased after day 5 to a peak on day 8, whereas the numbers of M-CFCs in VD_3-treated samples increased after day 5 to a peak on day 12. The numbers of M-CFCs in VD_3-treated samples were higher than those of the corresponding untreated controls from day 5 onwards. The difference between the VD_3-treated sample and the corresponding control was statistically significant on day 8 ($p < 0.05$) and statistically extremely significant on days 10 and 12 ($p < 0.001$), whilst the maximum appeared on day 12.

According to the result of the two-way ANOVA test, both the presence of VD_3 and the time of incubation were statistically significant factors for the measurements. However, as there is extremely significant interaction between these two factors, it is difficult to interpret the P values of this test. The significance was confirmed by the Bonferroni post-hoc analysis.

Summary

In the E14 / OP9 co-culture, E14 cells displayed a poly-dispersed cell morphology consisting of single cells and colonies. This differentiation of both untreated and VD_3-treated cells can be detected through monitoring the fluctuations in the levels of CD marker expressions on different days of incubation.

The decreases in number in E-CFCs and GEMM-CFCs, and the contrasting increases in GM-CFCs and M-CFCs towards day 12 of E14 / OP9 co-culture is indicative of a shift in E14 cellular differentiation from haematopoietic cells to different cell lineages.

Overall, from untreated E14 cells, the trends for E-CFCs and GEMM-CFCs showed gradual decreases, while, in contrast, the trends for GM-CFCs and M-CFCs showed gradual increases.

In both untreated and VD_3-treated E14 cells, kinetic analyses of the emergence of CFCs demonstrated that E-CFCs and GEMM-CFCs were induced on day 2 of culture. The former was accompanied by the appearance of $CD34^+$ progenitor cells, whilst the latter was accompanied by the appearance of $CD34^+$, $CD41^+$ and $CD43^+$ cells. GM-CFCs and M-CFCs appeared later, along with the induction of $CD45^+$ cells. Generally, after day 5

of incubation, there was prominent expansion of GM-CFC and M-CFCs as well as gradual decrease of E-CFCs and GEMM-CFCs.

With the presence of VD_3, the measurement of all four types of CFCs had increased on almost all days of observation. This suggested that more cells were formed through cell differentiation.

3.4.4 Real-time quantitative PCR for gene expressions

The expressions of different genes, FLK, GATA1, GATA2, SCL, p21 and p27, were assessed successively on days 2, 5, 8, 10 and 12, respectively. ESC (E14) cellular differentiation was dependent on the fluctuations in the levels of gene expressions on the given days, starting from day 2, in the E14 / OP9 co-culture that were either untreated or treated with VD_3. These fluctuations are described below and shown in graphical form. Each assessment procedure was done independently trice and in triplicate to arrive at an average, given as relative quantification (fold change).

FLK gene expression

The mean level of FLK gene expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 50.

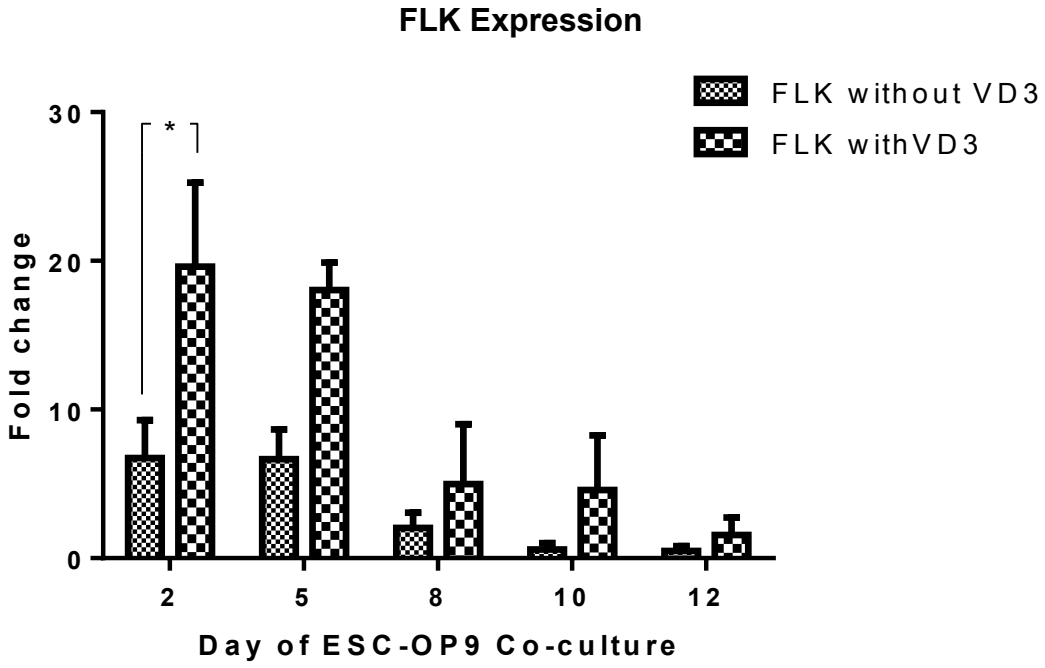


Figure 50. Comparison of the fold-change of FLK^+ cells in the untreated and VD_3-treated E14 / OP9 co-cultures after different days of incubation
N = 9. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

The changes in the level of FLK expression against time and between the control and the treatment were visible in the bar chart above. According to the 2-way ANOVA test, both time and the presence of VD_3 were significant factors. In the control group, the expression

of FLK appeared towards the beginning of the duration. In comparison, the early peak of FLK in the treated co-cultures appeared higher, and the difference between the treatment and the control was significant. This significance was confirmed by the Bonferroni post-hoc analysis.

GATA1 gene expression

The mean magnitude of GATA1 gene expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 51.

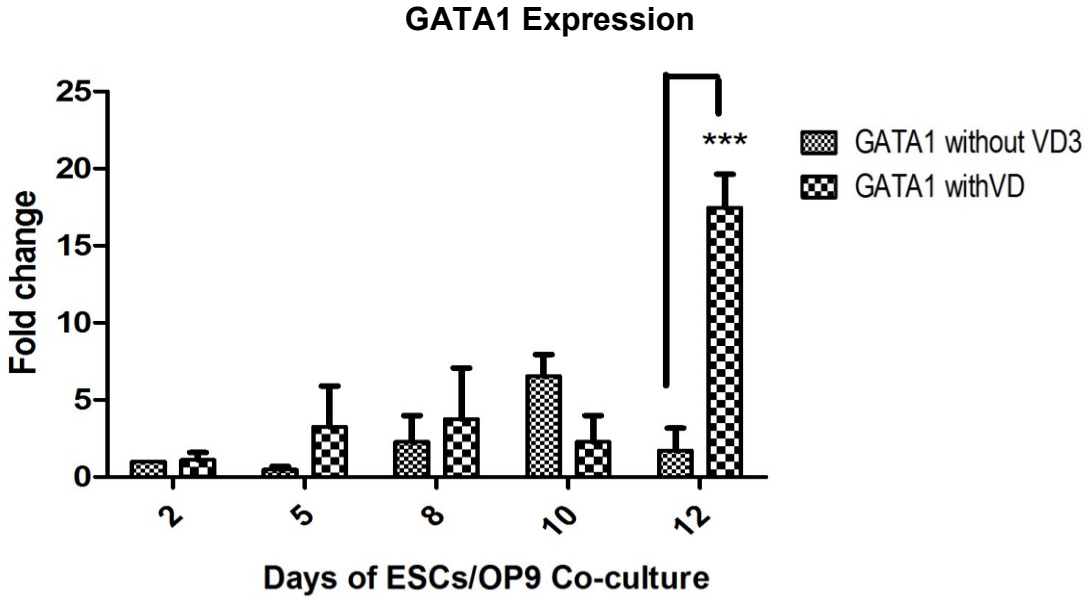


Figure 51. Comparison of the fold-change of $GATA1^+$ cells in the untreated and VD_3-treated E14 / OP9 co-cultures after different days of incubation
N = 9. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

In both treated and untreated groups, there was GATA1 gene expression from day 2, though this expression registered no time-dependent increase in its magnitude until after day 5. For the treated group, the peak of gene expression was not reached until at least day 12, whereas its counterpart in the untreated group appeared around day 9, with noted decrease towards zero afterwards. The most significant difference in gene expressions between the two groups was registered on day 12. This significance was confirmed by the Bonferroni post-hoc analysis.

GATA2 gene expression

The mean magnitude of GATA2 gene expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 52.

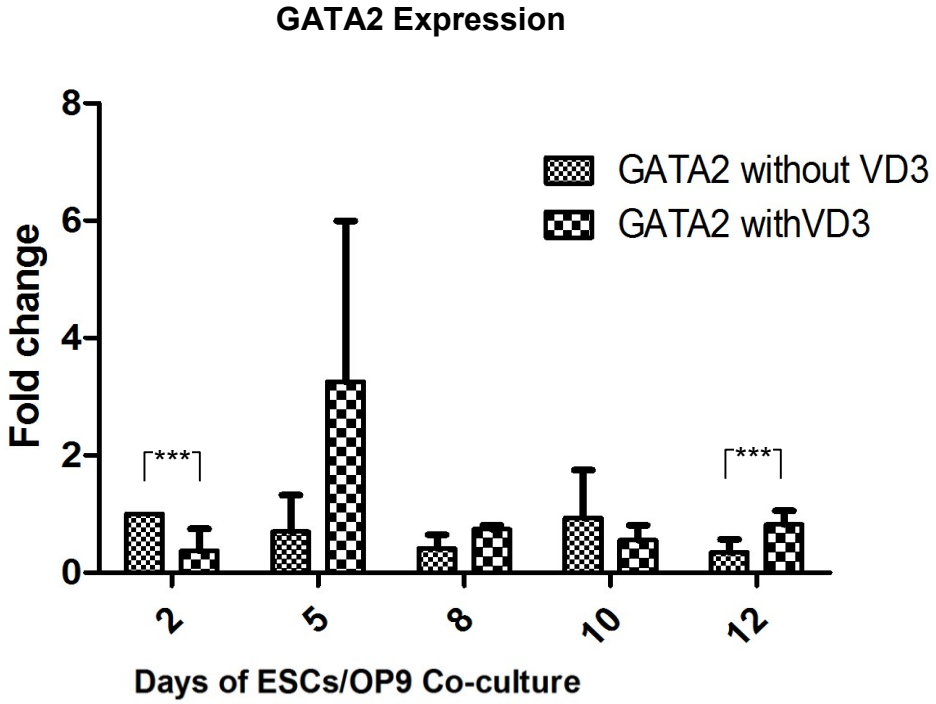


Figure 52. Comparison of the fold-change of $GATA2^+$ cells in the untreated and VD_3-treated E14 / OP9 co-cultures after different days of incubation
N = 9. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

In the untreated control, the magnitude of GATA2 gene expression was small and slowly decreasing throughout the period. In the treated group, however, the expression was notably increased from virtually zero to a peak around day 5 before more slowly decreases to almost zero after day 12. In other words, the expression of GATA2 was more concentrated around day 5 in the treated group. The magnitude of GATA2 gene expression in treated cells was generally superior to that of untreated cells on days 5, 8 and 12. The maximum GATA2 gene expression differential between the treated and the untreated E14 cells was on day 5. The significances of these were confirmed by the Bonferroni post-hoc analysis.

SCL gene expression

The mean magnitude of SCL gene expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation are presented in Figure 53.

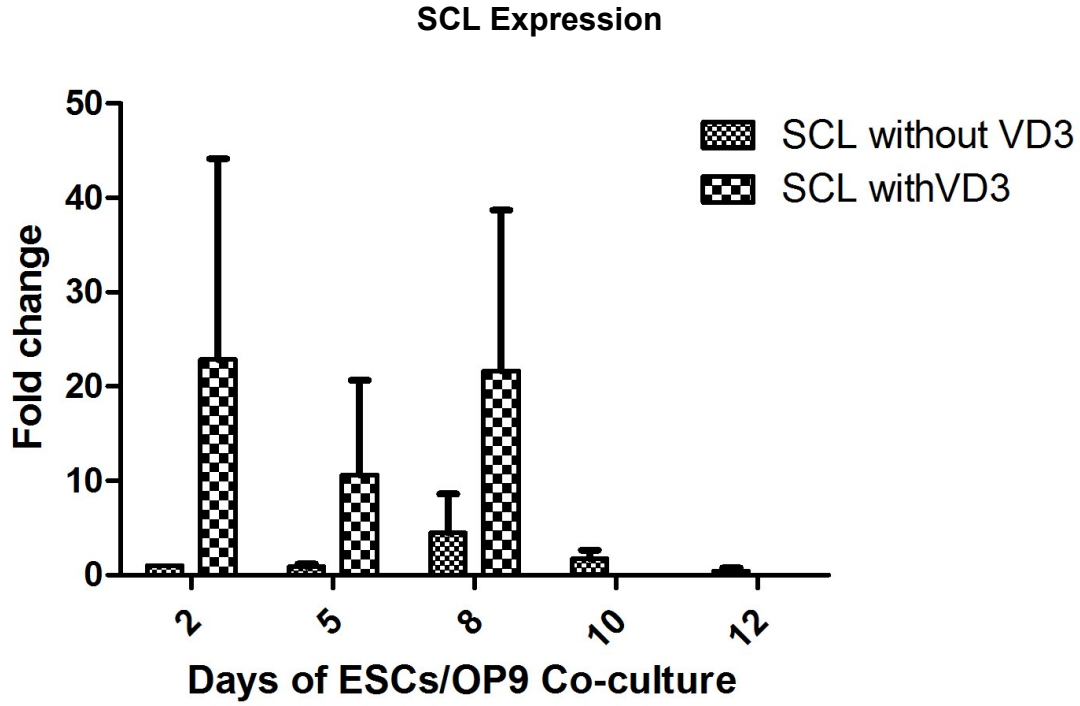


Figure 53. Comparison of the fold-change of SCL^+ cells in the untreated and VD_3-treated E14 / OP9 co-cultures after different days of incubation
N = 9.

The SCL gene expression was very low in the untreated control, increasing slowly from zero to a small peak around day 8 before gradually reduced to nearly zero again after day 12. In contrast, the expression was much higher throughout the period in comparison, slowly increased to a peak around day 4 to a value slightly higher than the initial value. Afterwards, the expression slowly reduced to nearly zero after day 12. The strength of the peak expression for the treated group was about four times of that of the untreated control. The maximum difference between the SCL gene expressions of the two groups appeared on day 2.

p21 gene expression

The mean magnitude of p21 gene expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation are presented in Figure 54.

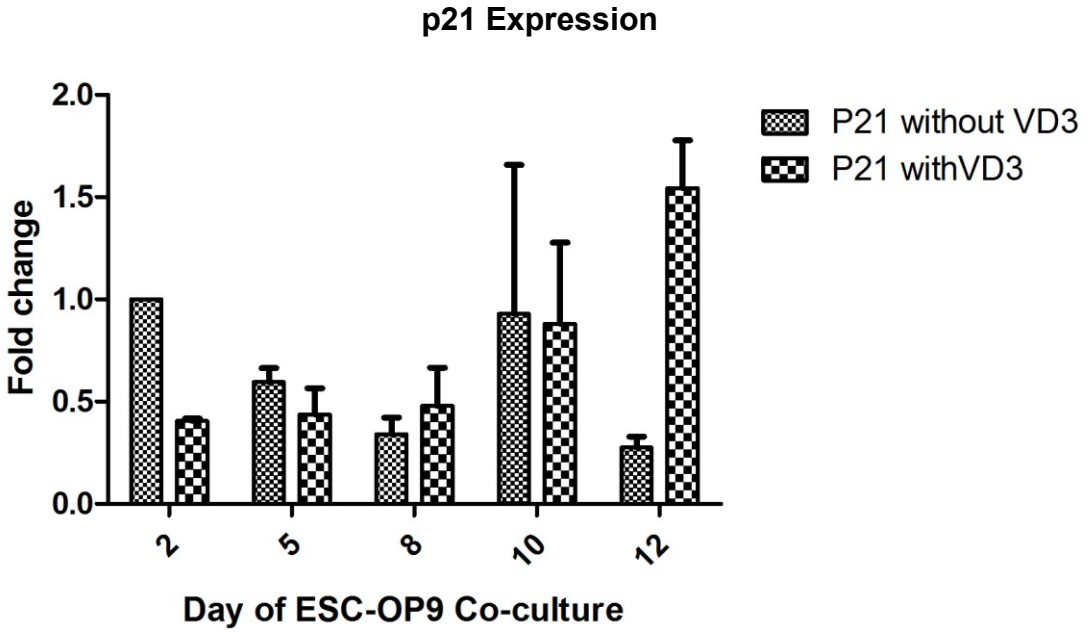


Figure 54. Comparison of the fold-change of $p21^+$ cells in the untreated and VD_3-treated E14 / OP9 co-cultures after different days of incubation
N = 9.

The level of p21 gene expression was small in both control and treated groups. However, the expression was generally decreasing for the untreated control throughout the duration of observation, whereas its counterpart in the treated group was constantly increasing in the same duration. The magnitudes of the p21 gene expression of these two groups were comparable at day 8. The maximum difference between the p21 gene expressions of the treated and the untreated E14 cells was on day 12.

p27 gene expression

The mean levels of p27 gene expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 55.

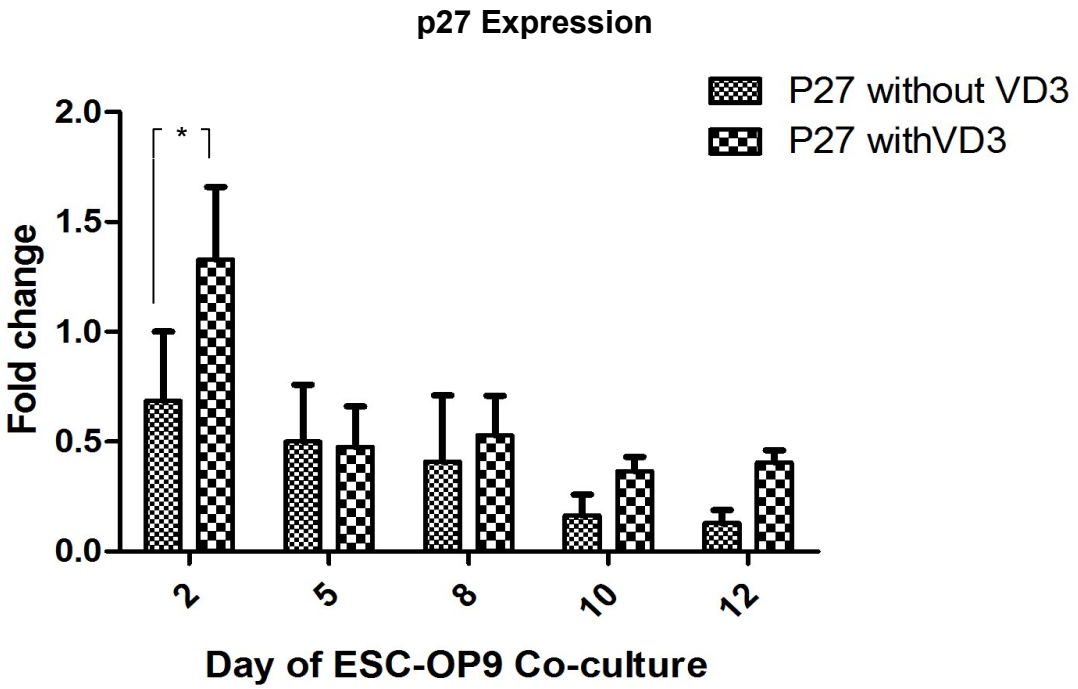


Figure 55. Comparison of the fold-change of $p27^+$ cells in the untreated and VD_3-treated E14 / OP9 co-cultures after different days of incubation
N = 9. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

In both the untreated control and the treated group, the magnitude of the p27 gene expression was small and constantly decreased notably throughout the duration, and the greatest difference between the measurements of both groups was observed in the beginning. The significance of this difference was confirmed by the Bonferroni post-hoc analysis. The initial magnitude was around seven times greater than the terminal measurement in both groups. Throughout the duration, the magnitude in the treated groups was roughly between 1.5 and 2 times of its counterpart in the untreated control.

Summary

In control, the expression of FLK appeared towards the beginning of the duration. GATA1, GATA2 and SCL expressions were detected about the same time as the $CD34^+$ cells. GATA1 expression gradually increased up to day 10 of culture, whilst GATA2 and SCL expression peaked around the middle of the observed duration and then gradually decreased. In comparison, the peaks of FLK, GATA2 and SCL in the treated co-cultures appeared earlier and higher, and GATA1 expression in the treated co-cultures appeared higher and continued increasing till the end of the duration. The higher peaks for VD_3-treated E14 cells suggest that VD_3 promotes cell differentiation.

With the presence of VD_3, the general expression of p21 gradually increased towards day 12. This correlated with the fact that p21 played the role of stopping the cell progressing from the G2 phase to the M phase and thus it supressed proliferation. In other words, the up-regulation this expression indicated that proliferation had been hindered.

On the other hand, compared with the control, the p27 gene expression was constantly greater and had peaked earlier. This correlated with the fact that p27 played the role of stopping the cell progressing from the G1 phase to the S phase and thus enabled differentiation. In other words, the early up-regulation of its expression indicated that differentiation had been encouraged.

3.4.5 Cytokine multiplex assay

G-CSF Expression

The mean levels of G-CSF expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 56.

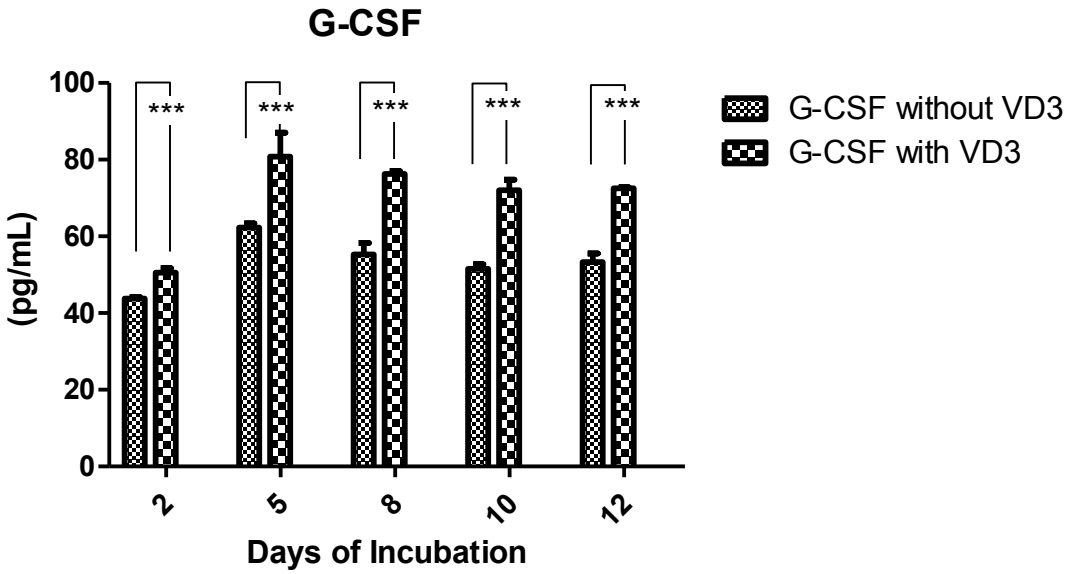


Figure 56. Comparison of the G-CSF expressions in both control and treatment N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

According to the 2-way ANOVA test, both time and the presence of VD_3 were significant factors of the measurement of expression. The significance was confirmed by the Bonferroni post-hoc analysis.

GM-CSF Expression

The mean levels of GM-CSF expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 57.

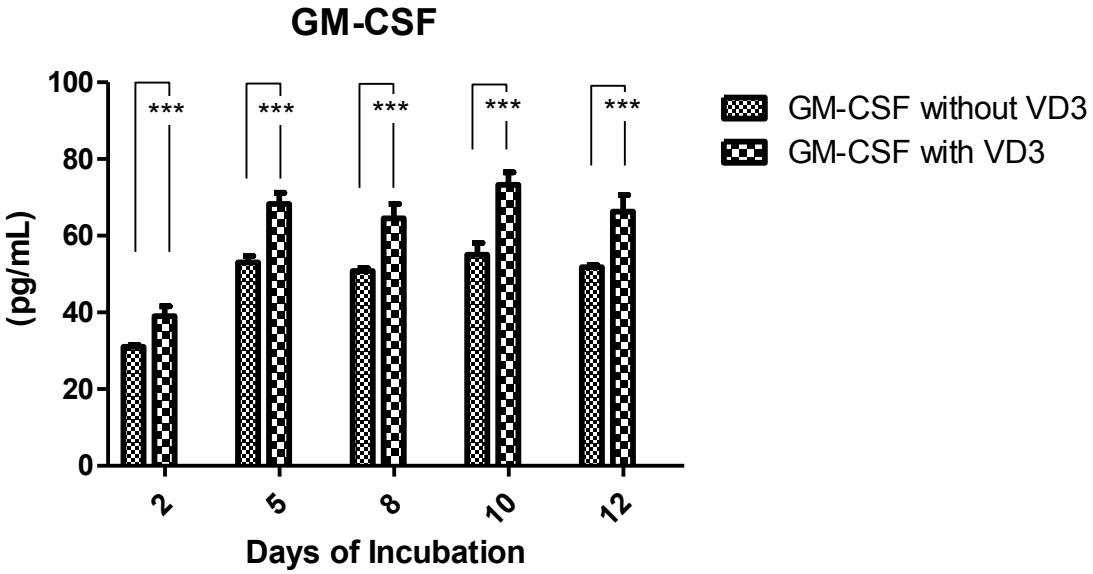


Figure 57. Comparison of the GM-CSF expressions in both control and treatment N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

According to the 2-way ANOVA test, both time and the presence of VD_3 were significant factors of the measurement of expression. The significance was confirmed by the Bonferroni post-hoc analysis.

IL-1α Expression

The mean levels of IL-1α expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 58.

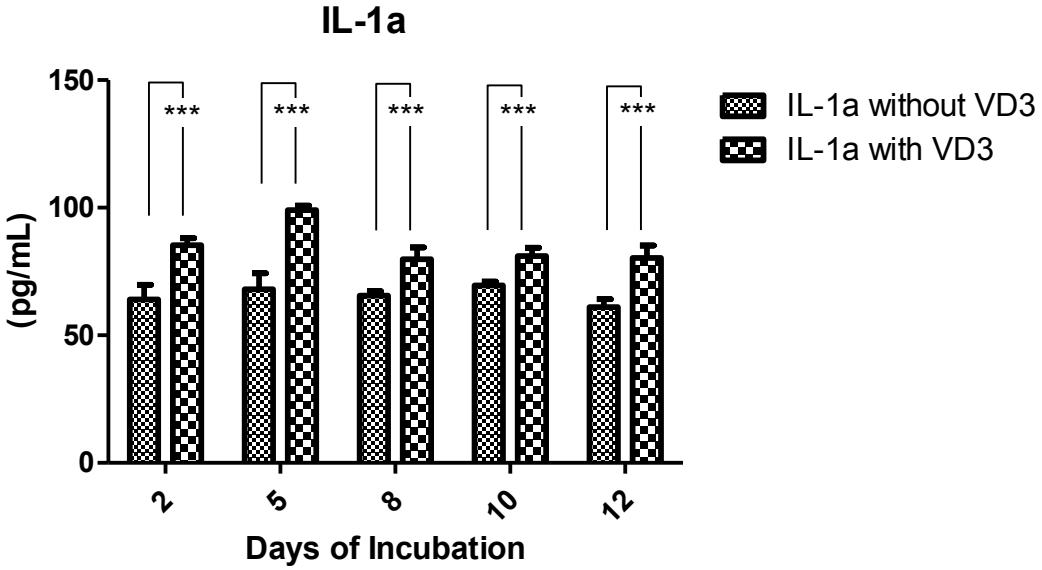


Figure 58. Comparison of the IL-1α expressions in both control and treatment
N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

According to the 2-way ANOVA test, both time and the presence of VD_3 were significant factors of the measurement of expression. The significance was confirmed by the Bonferroni post-hoc analysis.

IL-3 Expression

The mean levels of IL-3 expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 59.

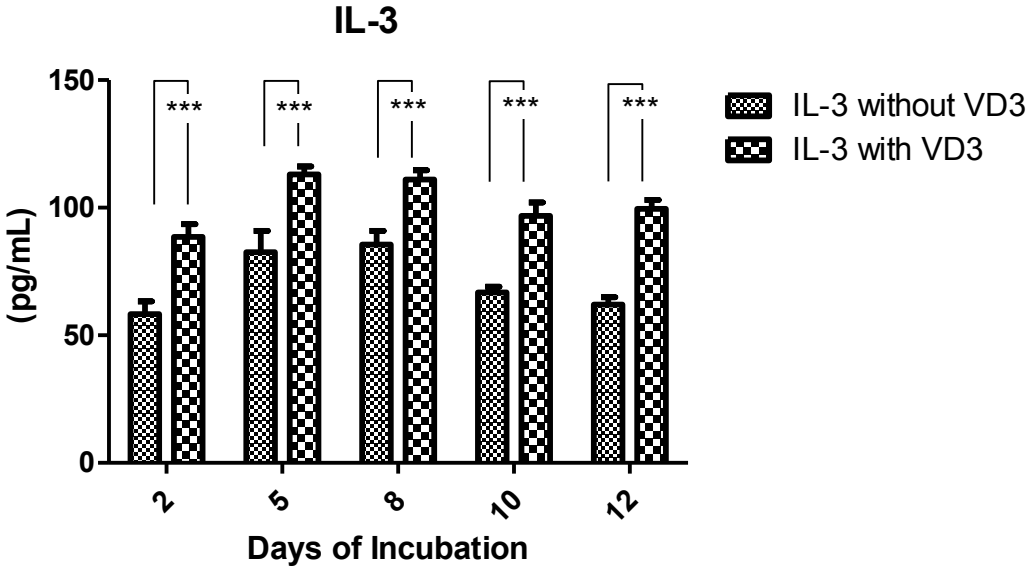


Figure 59. Comparison of the IL-3 expressions in both control and treatment
N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

According to the 2-way ANOVA test, both time and the presence of VD_3 were significant factors of the measurement of expression. The significance was confirmed by the Bonferroni post-hoc analysis.

IL-4 Expression

The mean levels of IL-4 expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 60.

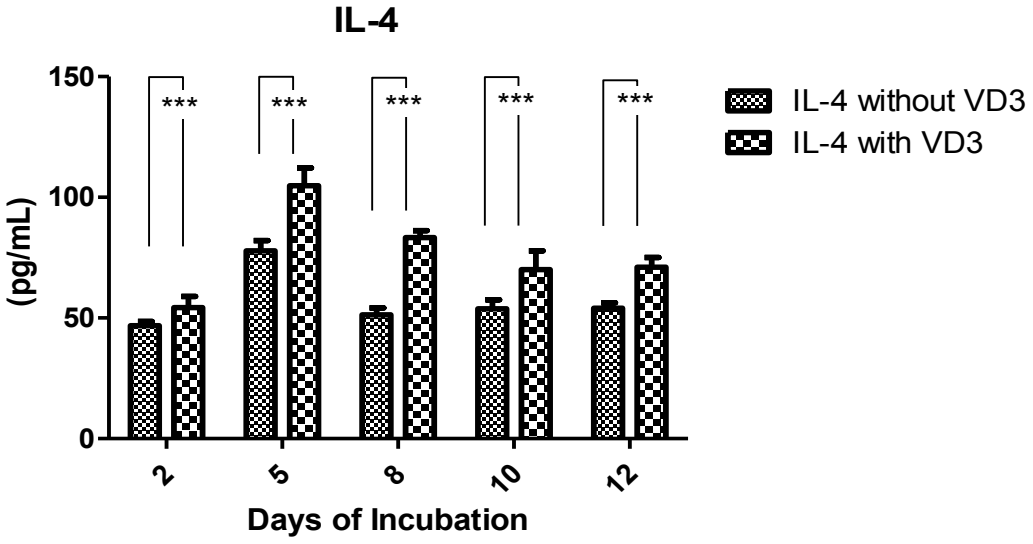


Figure 60. Comparison of the IL-4 expressions in both control and treatment
N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

According to the 2-way ANOVA test, both time and the presence of VD_3 were significant factors of the measurement of expression. The significance was confirmed by the Bonferroni post-hoc analysis.

IL-5 Expression

The mean levels of IL-5 expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 61.

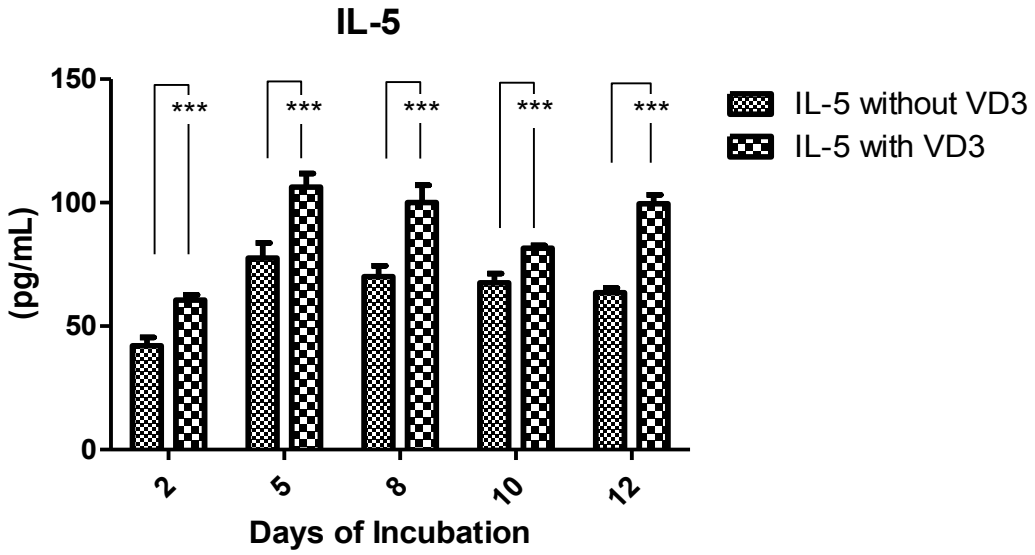


Figure 61. Comparison of the IL-5 expressions in both control and treatment N = 3. (*: p < 0.01; **: p < 0.001; ***: p < 0.0001)

According to the 2-way ANOVA test, both time and the presence of VD_3 were significant factors of the measurement of expression. The significance was confirmed by the Bonferroni post-hoc analysis.

IL-6 Expression

The mean levels of IL-6 expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 62.

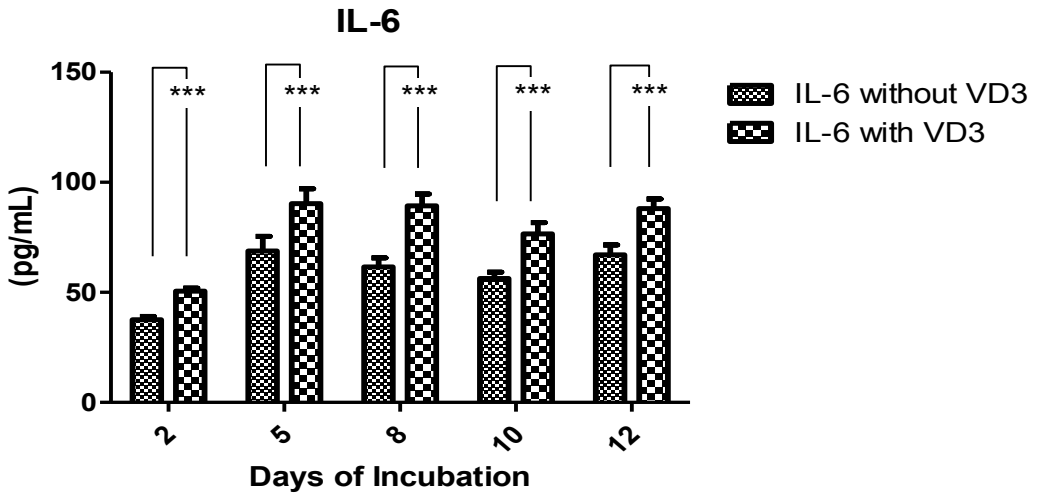


Figure 62. Comparison of the IL-6 expressions in both control and treatment
N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

According to the 2-way ANOVA test, both time and the presence of VD_3 were significant factors of the measurement of expression. The significance was confirmed by the Bonferroni post-hoc analysis.

TNF-α Expression

The mean levels of TNF-α expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 63.

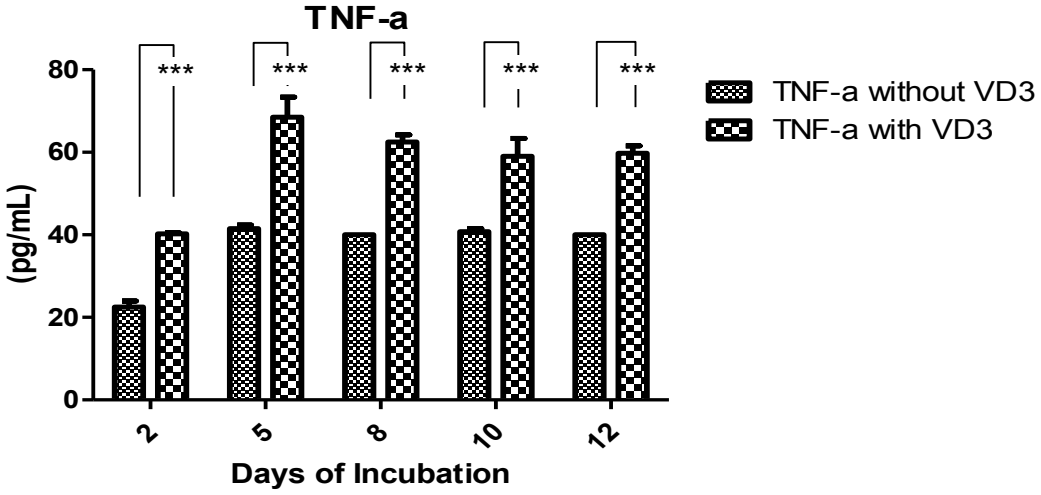


Figure 63. Comparison of the TNF-α expressions in both control and treatment
N = 3. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$)

According to the 2-way ANOVA test, both time and the presence of VD_3 were significant factors of the measurement of expression. The significance was confirmed by the Bonferroni post-hoc analysis.

VEGF Expression

The mean levels of VEGF expression of both untreated control and VD_3-treated E14 cells in co-culture with OP9 cells taken after days 2, 5, 8, 10 and 12 of incubation, as well as the results of the two-way ANOVA test, are presented in Figure 64.

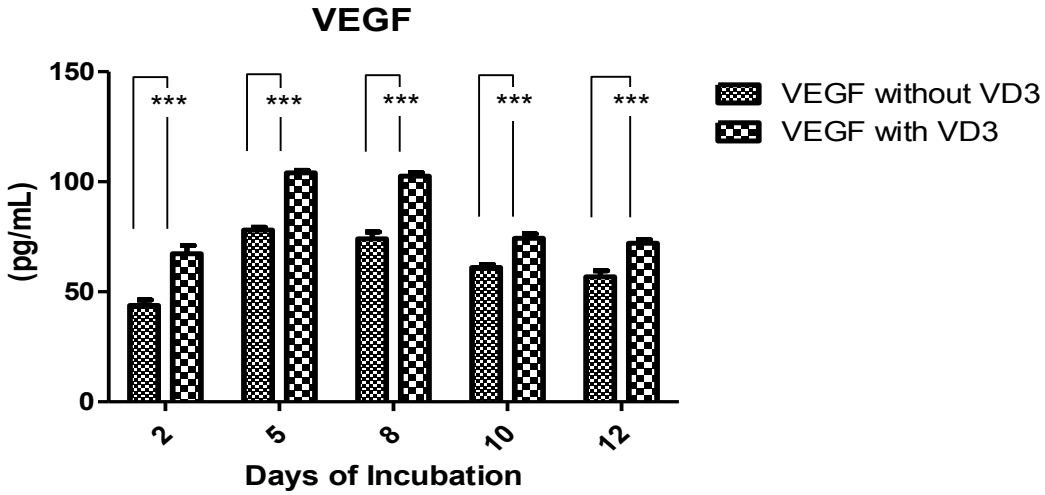


Figure 64. Comparison of the VEGF expressions in both control and treatment N = 3. (*: p < 0.01; **: p < 0.001; ***: p < 0.0001)

According to the 2-way ANOVA test, both time and the presence of VD_3 were significant factors of the measurement of expression. The significance was confirmed by the Bonferroni post-hoc analysis.

Summary

In the control, the measurements of IL-1α expression were constantly high throughout the duration. The temporal kinetic of the VEGF expression showed an early build-up to a minor peak around days 5 and 8 and sustained throughout the duration. The temporal kinetics of the TNF expression followed the initial trend of that of VEGF and remained constant after day 5.

This was followed by the temporal kinetics of both the GM-CSF and the G-CSF expressions, and these were subsequently followed by the temporal kinetics of both the IL-3 and the IL-5 expression.

The temporal kinetics of both the IL-4 and the IL-6 expressions also followed that of the VEGF expression.

On the other hand, compared with the control samples, the expression of each cytokine is statistically significantly higher in the treated samples overall. This suggests that the presence of VD_3 encourages the expressions of these cytokine. As these cytokines are related to the presence of various blood cells, higher levels of these cytokines also suggests higher numbers of those blood cells, which are the products of differentiation from HSCs. Thus, these results suggest that VD_3 encourages HSC differentiation.

3.5 Discussion

In this study, the E14 / OP9 co-culture has been used successfully for haematopoietic differentiation of mouse and non-human primate ESCs and to obtain multilineage haematopoietic progenitors, as well as mature haematopoietic cells, such as lymphocytes and megakaryocytes, which avoid the ethical challenge facing the alternative method using the embryoid body (Vodyanik *et al.*, 2005). The onset of haematopoiesis and the appearance of early haematopoietic progenitors in the E14 / OP9 co-culture were investigated through the evaluation of CFCs and the expressions of haematopoiesis-associated molecules, including CD markers, genes and cytokines, on both undifferentiated and differentiated ESCs during incubation up to 12 days.

3.5.1 Haematopoiesis in the E14 / OP9 co-culture

First, the study demonstrated that it was appropriate to use the E14 / OP9 co-culture for the study on haematopoiesis because signs of various cell differentiations associated with different stages of haematopoiesis had been observed. In the control, FLK^+ cells appeared from the beginning of the duration and the number of cells peaked on day 8 before dropping drastically on day 10. $CD34^+$ cells appeared noticeably on days 5, 8 and 10. The temporal trend of the $CD31^+$ cells closely followed that of $CD34^+$ cells by 1 or 2 days. $CD41^+$ cells appeared 3 days after the $CD34^+$ population and gradually increased by day 10 of culture. $CD43^+$ cells were present throughout the duration, gradually peaked on day

10. The number of $CD45^+$ cells fluctuated around 5% after day 5. This showed that FLK^+ mesoderm (Lynch *et al.*, 2011) appeared first, followed by the appearance of $CD34^+$ haematopoietic progenitor cells (Krause *et al.*, 1994) and $CD31^+$ endothelia cells (Choi *et al.*, 1998), followed by the appearance of $CD41^+$ early haematopoietic progenitors, platelets and megakaryocytes (Ferkowicz *et al.*, 2003). The gradual increase of $CD43^+$ cells suggested the accumulation of various differentiated cells, such as granulocytes, monocytes, macrophages, etc. (Vodyanik, 2005), whereas the presences of lymphohaematopoietic cells were shown by the measurement of CD45 (Saltini *et al.*, 1990). These temporal changes indicated the occurrences of various forms of cell differentiations at different stages of haematopoiesis.

Likewise, in the findings regarding gene expressions, GATA1, GATA2 and SCL expressions in the control group were detected about the same time as the $CD34^+$ cells. GATA1 expression gradually increased up to day 10 of culture, whilst GATA2 and SCL expression peaked around the middle of the observed duration and then gradually decreased. As SCL played an important role in haematopoietic differentiation (Lim *et al.*, 2013), its expression indicated the appearance of haematopoietic differentiation. This was accompanied by the expression of GATA2 as the protein encoded by GATA2 plays an essential role in regulating transcription of genes involved in the development of haematopoietic cell lineages (Gerber *et al,*. 2002). The gradual increase of GATA1 expression was linked to the gradual appearances of various cell lines produced from the haematopoiesis process (Lim *et al.*, 2013).

These were supported by the findings with CFCs. E-CFCs and GEMM-CFCs were induced towards the beginning of the duration. The former coincided with the appearance of $CD34^+$ progenitor cells, whilst the latter concurred with the appearance of $CD41^+$ and $CD43^+$ cells. GM-CFCs and M-CFCs appeared later, in accord with the induction of $CD45^+$ cells.

These were in agreement with the reduction of Oct4, Sox2 and Nanog expressions through the passage of time with immunofluorescence, and the temporal kinetics the appearance of various cells revealed by the expressions of various cytokines. All of these indicated progress in cellular differentiation from haematopoietic cells to different cell lineages.

As to the findings of the study of cytokines, the measurements of IL-1α expression were constantly high throughout the duration. This was in line of the role of IL-1 in helping the induction of the IL-2 receptor and stimulating the release of other cytokines involved in haematopoiesis (Gerber and Ferrara, 2003).

The temporal kinetic of the VEGF expression showed an early build-up to a minor peak around days 5 and 8 and sustained throughout the duration. This early expression was in line with the role VEGF plays in helping the initiation of haematopoietic differentiation and promotes the formation of terminally differentiated red blood cells (Kurzrock, 2000).

The temporal kinetics of the TNF expression followed the initial trend of that of VEGF and remained constant after day 5. This was in line of the role TNF plays in stimulates cell proliferation and induce cell differentiation under certain conditions (Gerber *et al.*, 2002).

This was followed by the temporal kinetics of both the GM-CSF and the G-CSF expressions. This indicated the further differentiation from myeloid stem cells, as the GM-CSF is required for the differentiation of haematopoietic precursors of the granulocyte and monocyte lineages (Xu *et al.*, 2000), and G-CSF is required for the differentiation of haematopoietic precursor of the granulocyte lineage (Kurzrock, 2000).

This was also closely followed by the temporal kinetics of both the IL-3 and the IL-5 expression, because IL-3 works in conjunction with other haematopoietic growth factors to increase the production of erythrocytes, neutrophils, eosinophils, monocytes and platelets, whilst IL-5 stimulates eosinophil expansion (Gerber *et al,*. 2002).

The temporal kinetics of both the IL-4 and the IL-6 expressions followed that of the VEGF expression, but there were also build-up of IL-6 expression towards the end of the duration. This was primarily related to the cell differentiation from lymphoid stem cells, as IL-4 stimulates B cell proliferation and activation, and IL-6 acts on T cells and B cells and stimulates multilineage haematopoiesis, including the maturation of megakaryocytes (Rodak *et al.*, 2008).

Thus, the temporal relations of these findings indicate the process of haematopoiesis was in progression in the E14 / OP9 co-culture throughout the duration of the study.

Therefore, all these observations showed that, in the E14 / OP9 co-culture, E14 cells had not only differentiated, but also differentiated into cells of various haematopoietic lineages. In other words, haematopoiesis had happened. Moreover, the differentiation into myeloid, lymphoid, erythroid, and megakaryocytic lineage cells had all occurred in the co-culture, so it is an appropriate means to study all lineages of haematopoiesis.

These findings confirm with the findings of several prior researches. Nakano *et al.* (1994) showed that ESCs can be induced *in vitro* to differentiate into myeloid, lymphoid, erythroid, and megakaryocytic lineage cells by co-culturing with OP9. Maureen *et al.* (2001) found that, when cultured on OP9 stromal cells, ESCs differentiate into $FLK1^+$ haemangioblasts, hematopoietic progenitors, and finally mature, terminally differentiated lineages. Vodyanik *et al.* (2006) showed that it was possible to observe the haematopoietic differentiation of ESCs, $CD34^+$ cells and CFCs in OP9 co-culture. It was also found that another benefit of OP9 co-culture was that $CD34^+$ cells could be generated in large quantity without the addition of cytokines. These studies and others (Lynch *et al.*, 2011; Lim, *et al.*, 2013) showed that the *in vitro* ESC/OP9 co-culture system can be used to recapitulate the early stages of hematopoietic development and support both haemogenic precursors and their primitive haematopoietic progeny.

3.5.2 The effects of VD_3 on haematopoiesis

Secondly, the effects of VD_3 in promoting cell differentiation and suppressing cell proliferation in the haematopoietic process were studied through the comparison of treatment with control. With immunofluorescence, the more visible reduction of Oct4, Sox2 and Nanog expressions showed that a greater percentage of the cells had differentiated with VD_3 than without, i.e., cell differentiation had been enhanced by the presence of VD_3.

Compared with the control, FLK expression appeared earlier and the expression was greater in the treatment. This meant that the FLK^+ mesoderm (Lynch *et al.*, 2011) had appeared earlier and in greater quantity. The peak of CD34 expression was earlier and significantly higher, shifting from day 10 to day 8. This suggested that the $CD34^+$ haematopoietic progenitor cells (Krause *et al.*, 1994) appeared earlier and in greater quantity. The peak of CD31 expression shifted from day 8 to day 5, suggesting the $CD31^+$ endothelia cells (Choi *et al.*, 1998) appeared earlier. The expression of CD41 was greater and had a significantly great peak on day 10. This meant that $CD41^+$ early haematopoietic

progenitors (Ferkowicz *et al.*, 2003) appeared earlier and in greater quantity. The measurements of CD43 expression were greater on all days and had a significantly greater peak on day 5 shifted early from day 10. This indicated an earlier build-up of various $CD43^+$ differentiated cells (Vodyanik, 2005). The expression of CD45 was greater on all days and had a significant peak on day 12. This showed that greater quantities of lymphohaematopoietic cells (Saltini *et al.*, 1990) were produced through cell differentiation. Thus, these temporal trends indicated that cellular differentiation had happened earlier with the presence of VD_3. The greater measurements suggested that the activity of cell differentiation had been intensified. Both of these were indications that cell differentiation in haematopoiesis had been promoted by the presence of VD_3.

This was supported by the findings with CFCs in which, compared with the control, the quantities of all four types of CFCs were higher in the treatment in general. This suggested that more cells were formed through cell differentiation.

In the findings regarding gene expressions, in comparison with the control, the peaks of FLK, GATA2 and SCL in the treated co-cultures appeared earlier and higher, and GATA1 expression in the treated co-cultures appeared higher and continued increasing till the end of the duration. The higher peaks for VD_3-treated E14 cells suggest that VD_3 promotes and accelerates cell differentiation.

With the presence of VD_3, the general expression of p21 gradually increased towards day 12. This correlated with the fact that p21 played the role of stopping the cell transiting from the G1 phase to the S phase (Fecteau *et al.*, 2014). With the up-regulation of p21, CDK2 activation was reduced. This actuated G1/S cell cycle arrest, so cells could not proliferate.

On the other hand, compared with the control, the p27 gene expression was constantly greater and had peaked earlier. This correlated with the fact that p27 played the role of stopping the cell progressing from the G1 phase to the S phase and thus enabled differentiation (Galderisi *et al.*, 2003). In other words, the early up-regulation of its expression indicated that differentiation had been encouraged.

Thus, the findings on all gene expressions studied indicated that, in haematopoiesis, VD_3 promotes and accelerates cell differentiation and supresses proliferation.

As to the findings of the study of cytokines, overall, the expression of each cytokine is statistically significantly higher in the treated sample than in the control sample. This suggests that the presence of VD_3 encourages the expressions of these cytokine. As these cytokines are related to the presence of various blood cells, higher levels of these cytokines also suggests higher numbers of those blood cells, which are the products of differentiation from HSCs. Thus, these results suggest that VD_3 encourages HSC differentiation.

Therefore, all these comparisons between the treatment and the control showed that, in the E14 / OP9 co-culture, with the presence of VD_3, haematopoietic differentiations in all related cell lineages had been promoted and accelerated, whilst proliferation had been supressed. In other words, it was found in this study that VD_3 promotes and at times accelerates cellular differentiation, as shown by higher and sometimes earlier peaks in CFC numbers, CD-marker expressions, gene expressions and cytokine expressions.

These findings with E14 / OP9 co-culture is in agreement with those of the related prior *in vitro* studies using other cell lines, in spite of the major difference that this study was conducted on non- cancer embryonic stem cells whilst the others were on cancer cells. Significant anti-cancer effects with the addition of VD_3 or its analogues have been shown in these studies using squamous cell carcinoma (Hershberger *et al.*, 1999), prostrate adenocarcinoma (Getzenberg *et al.*, 1997), ovary cancer cells (Zhang *et al.*, 2005), breast cancer cells (Colston *et al.*, 1992) and lung cancer cells (Nakagawa *et al.*, 2005).

3.5.3 Correlation with researches on cell-cycle perturbation by VD_3

Through various related researches, aspects of the mechanism with which VD_3 affects cell proliferation and differentiation had been discovered. It is understood that the anti-proliferation and pro-differentiation activity of VD_3 is through cell-cycle perturbation. It has been demonstrated that the effects of VD_3 and its derivatives operate by means of the VDR to regulate proliferation, apoptosis (Simboli-Campbell *et al.*, 1996) and angiogenesis (Mantell *et al.*, 2000). VDR encodes the proteins p21 and p27 involved in blocking cell cycle progression (Liu *et al.*, 1995). It was found that VD_3-induced cell-cycle arrest by means of the VDR in the proliferating C2C12 cells is indicated by the dose-dependent increase of the VDR mRNA level (Hiroshi *et al.*, 2012).

On the other hand, genes mediating G1 arrest are induced by hormonal agents like $1,25(OH)_2D_3$ to initiate an induction that leads to terminal differentiation. The differentiation pathway can be activated by merely arresting these cells in G1 with p21 and p27 (Liu *et al.*, 1996), which are proteins encoded by the VDR, and the process can be induced by VD_3 (Hiroshi *et al.*, 2012).

Additionally, cyclins and their associations with CDKs and CDK inhibitors (CKIs) all regulate cell cycle progression, in which p21 and p27 (Toyoshima *et al.*, 1994) have been found to be inhibitors of G1 cyclin-dependent kinase. VD_3 treatment inducing a G0 / G1 phase arrest in squamous cell carcinoma cell lines (Hager *et al.*, 2001) and, also, breast-cancer MCF-7 cells (Verlinden *et al.*, 1998) reportedly increased expressions of p21 and p27. The gene expressions of p21 and p27 were up-regulated by VD_3 treatment in the C2C12 myoblast cell line (Hiroshi *et al.*, 2012).

Furthermore, VD_3 inhibits the phosphorylation of retinoblastoma (pRb) and blocks progression of the cell cycle from G1 to S phase. Significant decreases in the amount of pRb and significant increased expression of p21 and p27 were also observed in monocytes leukaemia, which preceded the appearance of dephosphorylated pRb (Kanatani *et al.*, 1999). Therefore, the findings from the studies mentioned above suggest that VD_3 treatment up-regulates p21 and p27 gene expressions, through which CKIs influence cell-phase arrest.

The findings of this study as discussed so far suggested that these processes had occurred in haematopoiesis.

3.6 Conclusion

With optimal dosage, VD_3 was found to inhibit the proliferation of E14 and OP9 cells. This inhibition was evident in terms of decreased cell number. Visually, alkaline-phosphatase staining revealed significant differences between VD_3-treated and untreated cells, characterised by decreased enzyme expression (resulting in colourless cells). Cell-cycle analyses using propidium-iodide showed no significant percentage change in VD_3-treated E14 and OP9 cells compared to the untreated controls within their G and S-phases.

With optimal dosage, VD_3 was also found to promote and accelerate the differentiation of the cells in the treated E14 / OP9 co-cultures, whereby GM-CFC number, in

concurrence with FLK, CD31, CD34 and CD43 markers, together with FLK, GATA2 and SCL gene expressions, manifested earlier (accelerated) and higher (promoted) peak values, which then markedly decreased. In contrast, M-CFC number, in concurrence with CD45 marker, together with GATA1 and p21 gene expressions, peaked with later and higher values towards day 12, compared with the untreated co-cultures.

Thus, the findings demonstrated that VD_3 enhanced cellular differentiation in E14 / OP9 co-cultures, in which ESC-derived $CD34^+$ progenitor cells became highly enriched in colony-forming cells that expressed haematopoiesis-associated genes GATA1, GATA2, SCL, and FLK, such that the phenotype of primitive haematopoietic progenitors and definitive hematopoietic stem cells were displayed.

The findings of the research on separate E14 and OP9 cultures as well as on the E14 / OP9 co-culture indicate that VD_3 treatment up-regulates p21 and p27 gene expressions. Although cyclin-dependent kinase inhibitor protein (CDK-CDI) through p21 and p27 is thought to influence cell-phase arrest, adequate VD_3 concentration could still enhance cellular differentiation and inhibits proliferation. Furthermore, this is supported by the fact that the expressions of various cytokines associated with various blood cells are also higher when VD_3 is present.

This was the first demonstration of the anabolic effect of VD_3 on mouse embryonic stem cells (E14 cell line) co-cultured on OP9 stromal cells *in vitro*, confirming similar researches using other cells suggesting potential clinical effects of VD_3 treatment. In other words, this research demonstrates that OP9 cells support both haemogenic precursors and their primitive hematopoietic progeny, making it possible to generate hematopoietic stem cells from ESC lines.

Chapter 4 A Pilot Study into the Effects of VD_3 on Human Haematopoiesis *in Vivo*

4.1 Introduction

It is found through the studies described in the previous two chapters that VD_3 is crucial in promoting the differentiation and suppressing the proliferation of both E14 and OP9 cells, and accelerates and promotes haematopoiesis of E14 cells in the presence of OP9 cells *in vitro*. Previous researches by others also reveal that VD_3 regulates bone health and many features of the immune system, and it has been shown to be a powerful differentiation inducer for a wide variety of neoplastic cells. Therefore, the next step in the study would be to ascertain whether a lower level of VD_3 hinders haematopoiesis. To achieve this, *in vivo* studies have been done with human participants during winter when the chance of exposure to sunlight for VD_3 synthesis is limited.

The objective of this part of the research is to establish the effect of VD_3 level on the stem cell count and the numbers of three major types of blood cells. It is hypothesised that the stem-cell count and the numbers of three major types of blood cells decline over time as the VD_3 level diminishes. The null hypothesis will be that VD_3 level will have no effect on the number of stem cell and the total number of blood cells.

Thus, to achieve this objective, it is necessary to accomplish the following three tasks first:

1. Recruit normal healthy participants;
2. Collect blood samples from the participants monthly over the period of study;
3. Measure the VD_3 level of blood samples;
4. Measure the stem-cell count by determining circulating $CD34^+$ haematopoietic progenitor cells;
5. Measure the total number of blood cells and the various differentiated cells.

Once these tasks are accomplished, the results would be analysed and compared with one another to test the hypothesis.

The materials, equipment, principles and methods used for the experiments to obtain these measurements, the results obtained from the experiments, and the finding from the analysis of these obtained results are presented in the subsequent sections of this chapter.

4.2 Principles and Methods

4.2.1 Ethical approval

Before the commencement of the study, the standard ethics procedure of the university (Appendix A.2) was followed and the ethical approval for the study was obtained (Appendix A.3).

4.2.2 Demographics of the participants

Initially, sixteen healthy participants of aged between 35 and 45, eight men and eight women, were recruited amongst the research students of the university to take part in this study. Eventually, four of the original eight men did not stay with the study through to the end, so only the samples of twelve of these participants, four men and eight women, were used for this study. None of the participants were VD deficient at the beginning of the experiment or throughout its duration according to the US national guideline as serum 25(OH)D levels greater than 20 ng/mL were generally considered adequate for healthy individuals (IOM, 2011). The demographic characteristics of the participants are shown in Table 7.

Table 7. Demographic characteristics of the participants

Participant Code	Sample Code	Age	Gender	Baseline VD Status		Comments
				25(OH)D (ng/mL)	$1,25(OH)_2D$ (pg/mL)	
PN_001	MMU_001	43	F	21.1	31	
PN_002	MMU_002	45	M	27.0	49	
PN_003						*withdrawn*
PN_004						*withdrawn*
PN_005	MMU_003	36	F	23.5	48	
PN_006	MMU_004	35	F	21.5	24	
PN_007						*withdrawn*
PN_008	MMU_005	41	F	29.8	41	
PN_009	MMU_006	40	F	22.2	52	
PN_010	MMU_007	43	M	31.4	34	
PN_011	MMU_008	43	M	21.1	77	
PN_012	MMU_009	45	M	20.6	46	
PN_013	MMU_010	43	F	24.7	47	
PN_014	MMU_011	35	F	31.4	52	
PN_015	MMU_012	43	F	24.3	48	
PN_016						*withdrawn*

4.2.3 Recruitment of participants

Advertisements (Appendix A.4) had been put up in the university campus for recruitment. During the recruitment process, potential participants were provided with the details of this study. Information of this study was provided in verbal and written formats (Appendix A.5) in order for potential participants to make an informed decision on whether or not to participate. Participants were encouraged to understand this information and make a considered decision on their own, away from the researchers. Each participant was asked to sign a copy of the consent form to give their consent (Appendix A.6), whilst they had the right to withdraw from the experiment at any time, too. They were also asked to complete a medical questionnaire (Appendix A.7) with which they needed to answer several questions about their general health as well as any medication or supplement that they took regularly. All recorded details from these questionnaires were completely anonymous (free from any potential identification). This was achieved by allocating each participant with a non-identifiable participant number (PN). Based on these details, only the ones stated negatively to all of these questions were included in the study.

4.2.4 Sample collection and management

Each of the participants contributed blood samples in a procedure taking no more than 15 minutes each time. The samples were taken by a phlebotomist by inserting a needle into an antecubital vein. Each time, three baseline blood samples of 5 mL each were taken. After obtaining the blood, the needle was removed and gauze applied at the site of venepuncture. The participants were then free to leave the laboratory.

Two of these three samples collected were kept in 5 mL serum tubes (without anti-coagulant) and the other in a 5 mL EDTA tube (with anticoagulant). These two types of tubes are shown in Figure 65. The samples in the serum tubes were for Vitamin D measurements, and the samples in the EDTA tubes were for the various blood cell counts.

(a)  (b) 

Figure 65. (a) 5 mL Serum tube (without anti-coagulant) and (b) 5 mL EDTA tube (with anti-coagulant) used for blood sample collection

All raw samples and data were stored anonymously under a participant number (PN) rather than by name. Data storage was complied with the Data Protection Act (1998). If a participant had decided to withdraw from the study at any time after the experimental procedure commenced, all that participant's samples and data stored in whatever format would be destroyed.

The blood samples thus collected were then used in the three experiments described in the following sections.

4.2.5 Tests for Vitamin D measurement

Serum Preparation

After collection, the blood samples kept in serum tubes were allowed to clot by leaving it undisturbed at room temperature. This usually took 15-30 minutes. The clot was removed by centrifuging at 1000-2000 × g for 10 minutes in a refrigerated centrifuge. Following centrifugation, the liquid component (serum) was transferred into a clean polypropylene tube using a Pasteur pipette. Serum is the blood extract which contains all the proteins not used in blood clotting (coagulation) and all the electrolytes, antibodies, antigens and hormones. The samples were maintained at -80° C whilst in transit to the NHS. Special couriers were used to transfer the serum samples to the NHS laboratories at the Manchester Central Hospitals.

Liquid chromatography

At the NHS, examination was done by means of liquid chromatography mass spectroscopy (LCMS). It is liquid chromatography used in conjunction with tandem mass spectrometry. This is a potent technique in providing selectivity, sensitivity and reliability in results. It is used to separate and measure the concentration of VD_3 from the midst of other hormones and protein.

Chromatography refers to the use of any analytical technique with which components of mixtures of molecules could be separated out for identification, and possibly for their concentrations in the original mixture to be estimated. A substance for investigation in a sample is known as an analyte. However, other substances in a biological or clinical sample may interfere or be confused with the analyte, making it necessary to separate and isolate it. Chromatography techniques are mainly used to remove contaminants from the analyte under investigation, to make it possible to identify unknown analytes, and to determine analyte concentration.

Generally, the fundamental principle of all chromatography techniques is that of the differences between the relative affinities of the polar molecules in a mixture of two distinct and immiscible phases, with one mobile and the other stationary. Irrespective of the particular type of chromatography, each substance in a mixture can be separated out from the rest by its different affinities to the two phases (Glencross *et al.*, 2011; James 2009). This is demonstrated schematically in Figure 66.

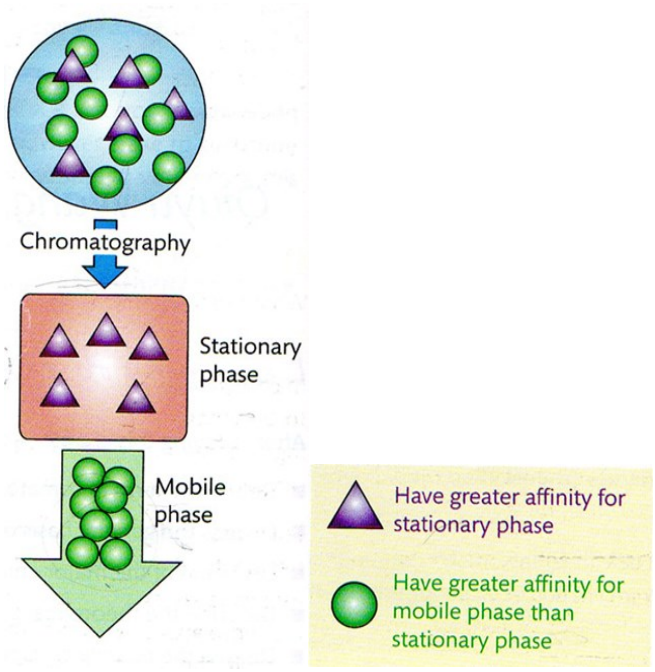


Figure 66. Schematic representation of chromatography in which two substances separated out using differences in their relative affinities to two phases
(Source: Glencross, Ahmed & Wang, 2011)

The way a substance distributes at equilibrium between the immiscible mobile and stationary phases is described by the partition coefficient (Kd). As shown in Figure 67, for a substance X distributing between the mobile and stationary phases, denoted as A and B, respectively, at respective concentrations of X[A] and X[B], Kd is defined as

$$Kd = X[A] / X[B]$$

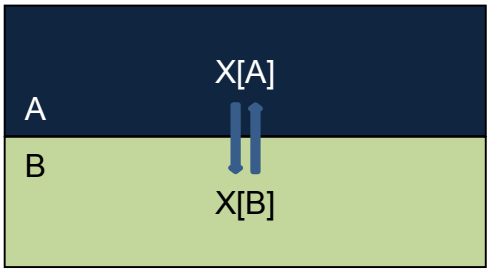


Figure 67. Schematic illustration of a substance distributing at equilibrium between two immiscible phases in chromatography

High-performance liquid chromatography (HPLC)

Adsorption and partition effects are generally involved in the methods of high-performance liquid chromatography. Partition chromatography works by using the differences in solubility and partition coefficients of polar substances to separate them

between the mobile and stationary phases. The two partition-chromatography techniques that tend to be used are the normal-phase and reverse-phase liquid chromatography, and the latter is the commonest type of HPLC. In the normal phase, the stationary phase is a polar substance and the mobile phase a non-polar solvent in the normal phase. On the other hand, the contrary occurs in reverse phase liquid chromatography, where the stationary phase is a non-polar substance, and the mobile phase a polar solvent. The major components of a high performance liquid chromatography system are shown in Figure 68.

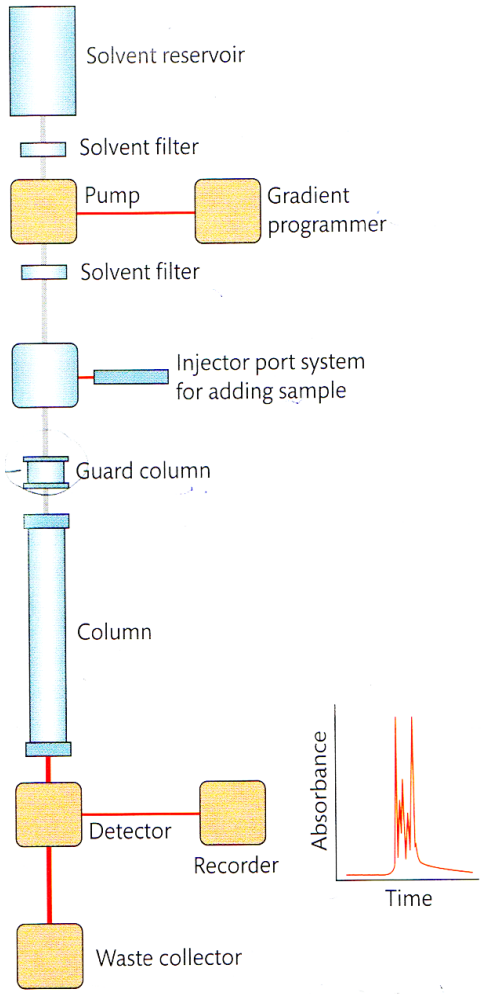


Figure 68. The major components of a high-performance liquid chromatography system (Source: Glencross *et al.*, 2011)

Mass spectrometry

Mass spectrometry in general involves a spectrometer consisting of an ioniser or source region, a mass analyser, and a detector, as shown in Figure 69.

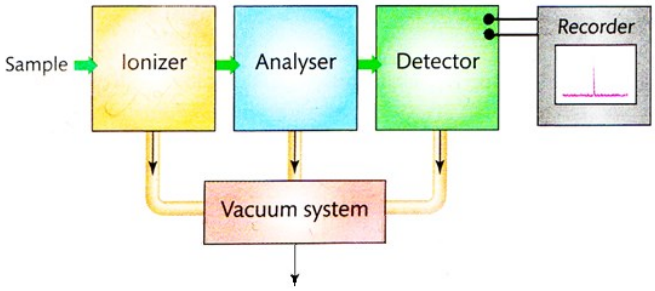


Figure 69. The general organisation of a mass spectrometer
(Source: Glencross *et al.*, 2011)

Each of these three parts is kept at high vacuum so as to minimise collisions between analyte and residual air molecules, which could diminish accuracy in analysis. For atoms or molecules in a sample, it is necessary to add or remove a H^+ ion to ionise them, giving a positive ($[M\text{-}H]^+$) or a negative ($[M\text{-}H]^-$) ion, respectively. The site of ionisation is the source region.

Once ionisation has taken place, a potential difference is applied to transfer the sample into the analyser. The ions produced are separated from the different components of the initial sample by using static or dynamic electric or magnetic fields in the analyser. Since each ion has a characteristic movement through the analyser, dependent on its mass, separation of the ions takes place (Glencross *et al.*, 2011), as shown in Figure 70.

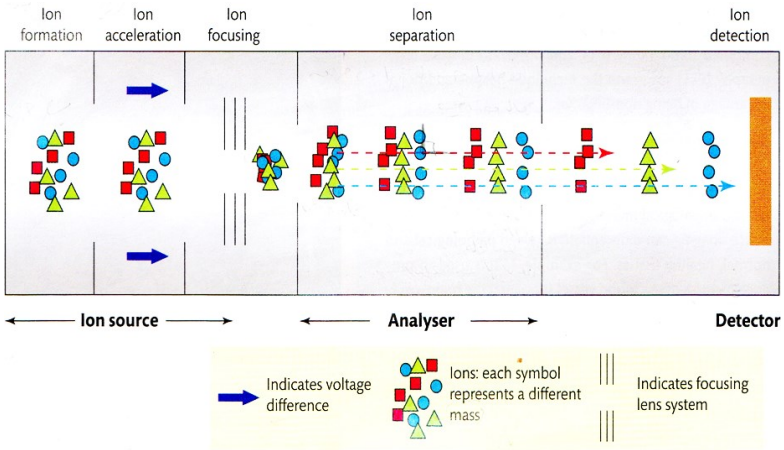


Figure 70. The overview of the operation of a mass spectrometer.
An electric potential difference is used to accelerate ions through the system.
(Source: Glencross *et al.*, 2011)

Tandem mass spectrometry

Tandem mass spectrometry, used to separate mixtures, involves a spectrometer consisting of an ioniser or source region, two mass analysers in sequence with a collision-induced

dissociation (CID) cell between them, and a detector. This is illustrated in Figure 71. After introducing the sample into the ioniser, separation one from another of the individual components of the mixture occurs in the first analyser and a selected ion is transmitted into the CID cell. The ion is fragmented in the CID cell. The second analyser analyses fragments of ions from an individual component to yield an MS / MS spectrum that represents the specific compound. Fragmentation of ions from any given compound of the sample tends to occur in a predictable way. Therefore, fragment sizes produced can be pieced together like a jigsaw puzzle to reveal information about the structure of the component (Glencross *et al.*, 2011).

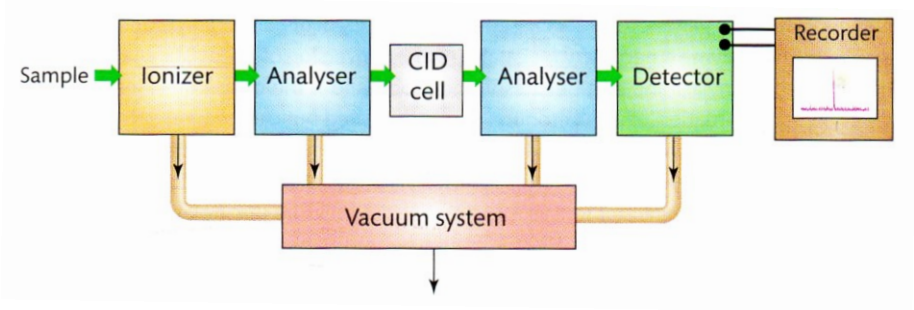


Figure 71. The general organisation of a tandem mass spectrometer (Source: Glencross *et al.*, 2011)

4.2.6 Phenotype analysis by flow cytometry

As mentioned above, a major difficulty in using ESCs is the problem of identifying them amongst other types of cells. One of the means of distinguishing the identity of a particular cell type is by molecular cell markers. These markers result from unique gene expression patterns within cells. In this way, such markers can be most useful in identifying undifferentiated and differentiated ESCs (Zhao *et al.*, 2012; Moore, *et al.*, 2011).

Cell Surface Markers

Specialised proteins expressed on the surface of cells. These proteins are able to bind or adhere to other signal molecules (Zhao *et al.*, 2012). Since each type of membrane protein has a different structure, the affinity for signal molecules is specific. Some proteins are only found or secrete in specific cell types, so the presence of specific proteins on the cell surface can act as cell markers. Cell-membrane proteins are the most important type of marker for identifying ESCs with the membrane remained intact.

Cluster of Differentiation (CD) Antigens

Cell-surface marker antigens are surface proteins, known as cluster of differentiation molecules or CD molecules. This refers to specific cluster of epitopes on cell-surface antigens. The CD number indicates the cell-surface antigen and anti-CD monoclonal antibodies used to determine cell expression of individual CDs.

CD antigens belong to various different classes. One class of these are integrins, which are α/β heterodimeric cell surface receptors that mediate the attachment of a cell to surrounding tissues. Some other classes are adhesion molecules, glycoproteins, and receptors. Since CD antigens vary according to cell types, they can be used as effective tools to identify and characterise different cell populations (Zhao *et al.*, 2012).

Principle

The phenotyping process involves using a monoclonal antibody or antibodies (mAbs) directed against extracellular CD antigens. Fluorescent dyes, known as fluorochromes, are used to label the antibodies. The fluorochromes will vary in their response to lasers at different wavelengths. By forcing a diluted cell suspension through a fine tube, the cells go through the laser beam in a flow-cytometer. The extent of light scattering is detected by sensitive photomultiplier tubes. Cell size can be obtained from the extent of forward scattering and cell granularity from side scattering. By measuring the fluorescence emission, those fluorochrome-labelled antibodies attached to the cells are indicated. The electronically stored data can be displayed as a graph or a histogram for analysis. A dot-plot can display the total cell detected. In order to analyse sub-populations in greater detail, the dot-plot can be selectively gated.

Figure 72. The BD FACSVerse™ flow cytometer (Source: www.bd.com)

The BD stem cell enumeration kit

Using the BD Stem Cell Enumeration Kit (BD Biosciences, Oxford Science Park, Oxford, UK), assays are carried out with the samples being stained with suitable reagents in separate BD Trucount™ tubes. Fluorochrome-labelled antibodies attach themselves to specific cell-surface antigens when a blood sample is added to the reagent, whilst the DNA and RNA of all nucleated cells are stained by the nucleic-acid dye. A particular number of fluorescent beads are released when the lyophilised (frozen) pellet is dissolved in the BD Trucount™ tube. The overall procedure is illustrated in Figure 73.

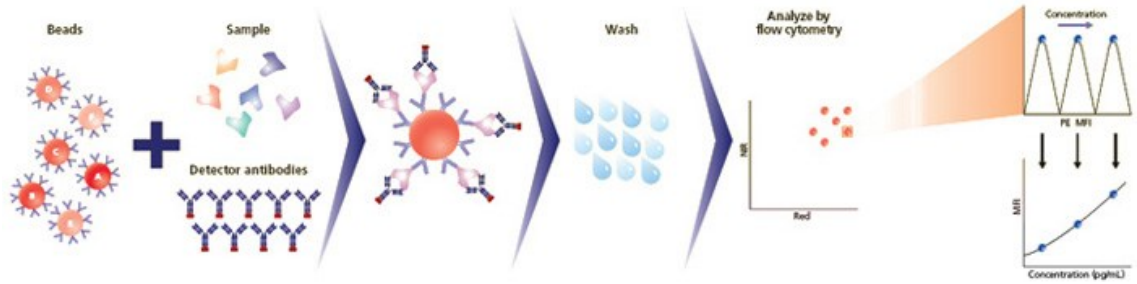


Figure 73. Overview of the procedure of phenotype analysis by flow cytometry (Source: www.bdbiosciences.com/wcmimages/facscalibur_features_beadsflowchart_lrg.jpg)

The extent of non-specific binding is assessed by means of the control agent. Amongst such bindings, especial attention is given to the ones brought about by fragment crystallisable (Fc) receptors, which are the proteins on the surface of particular cells, such as lymphocytes, natural killer cells, macrophages and neutrophils. Before the sample is obtained on flow cytometry, erythrocytes are lysed using BD FACS™ lysing solution.

During analysis, the respective absolute numbers of $CD34^+$ and $CD45^+$ progenitor cells in the sample can be determined by dividing the respective numbers of CD34 and CD45 cellular events by the number of fluorescent bead events, then multiplying by the bead concentration.

The reagents (BD Biosciences, Oxford Science Park, Oxford, UK) supplied with the BD stem cell enumeration kit are listed in Table 8 below.

Table 8. The reagents supplied with the BD stem cell enumeration kit (Source: BD Sciences, 2011)

BD stem cells reagent (CD45/CD34)	This is prepared in phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) and 0.1% sodium azide. The reagent contains FITC and PE.
CD45 fluorescein isothiocyanate (FITC)	CD45 recognises human leucocyte antigen that is a member of the leucocyte common antigen (LCA) family, which is present on all human leucocytes and is weakly expressed on haematopoietic progenitor cells.
CD34 phycoerythrin (PE)	CD34 recognises the class III human progenitor cell antigen (HPCA). The CD34 antigen is present on immature haematopoietic precursor cells and all haematopoietic colony-forming cells in blood, including unipotent and pluripotent progenitor cells.
7-aminoactinomycin-D (7-AAD)	This is a nucleic-acid dye used to identify dead cells
10x ammonium chloride lysing solution	This is a fixative-free solution for red blood cell lysis
50 BD Trucount™ tubes	Each single-use tube contains a freeze-dried pellet of fluorescent beads.

Method

Circulating blood stem cells measurements

The BD stem cell enumeration kit was used to determine the absolute counts of circulating $CD34^+$ and $CD45^+$ haematopoietic progenitor cells. This kit made it possible to enumerate $CD34^+$ and $CD45^+$ haematopoietic progenitor cells accurately and reproducibly. CD34 and CD45 marker expressions were measured by means of a flow cytometer. To 100 µL of whole blood, 20 µL of CD45 FITC/CD34 PE reagent and 20 µL of 7-AAD reagent were added to a Trucount™ tube. Tubes of the samples were vortexed and then kept in the dark at room temperature for 20 minutes before 2 mL of 1x ammonium chloride lysing solution was added. Then the tubes containing the samples were vortexed and kept in the dark at room temperature for 10 minutes. Thus the samples were ready within 1 hour after lysis, and measurements were made using the BD FACSVerse™ flow cytometer.

4.2.7 Blood cells counting

Blood cell counts were obtained using the XS-1000i/XS-800i Sysmex blood-counting machine, as explained in the sections below.

Principle

There are three broad types of blood cells, namely white blood cells, red blood cells and platelets. They range in diameter from about 15 μm (micrometres) in the case of white blood cells to about 2 μm for platelets. The basic form of measuring blood-cell number, and distinguishing their types according to size, is by sheath-flow DC detection using the XS-1000i/XS-800i Sysmex blood-counting machine. There are twee processes in this method, namely achieving accurate dilution, passing cell one by one through the detection aperture, and counting cells by the electric signals, as highlighted in Figure 74. These are described in the sections below.

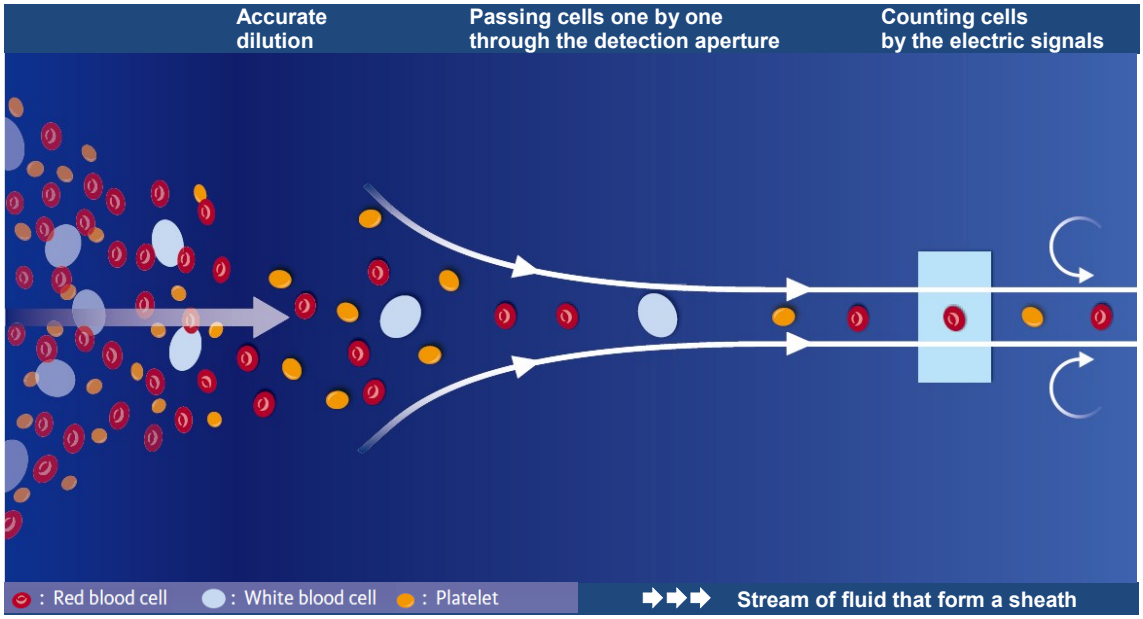


Figure 74. Three processes in counting blood cells (Source: Sysmex, www.sysmex.co.jp)

Achieving accurate dilution

In order to reduce errors, it is necessary to achieve accurate dilution of the blood samples to avoid blood cells piling up together. The measuring chamber must exhibit micro-level precision. Degradation and abrasion are kept to a minimum by the apparatus used.

Passing cells one by one through the detection aperture

The process of counting the cells is aided by prior alignment. As illustrated in Figure 75, there is a stream of fluid forming a sheath around the flowing blood cells to keep them aligned and guided through the centre of the detection aperture to be counted. Backward

flow through the aperture is avoided by the flow of the sheath solution to ensure the flow of blood cells is unidirectional so that each cell will not be counted more than once.

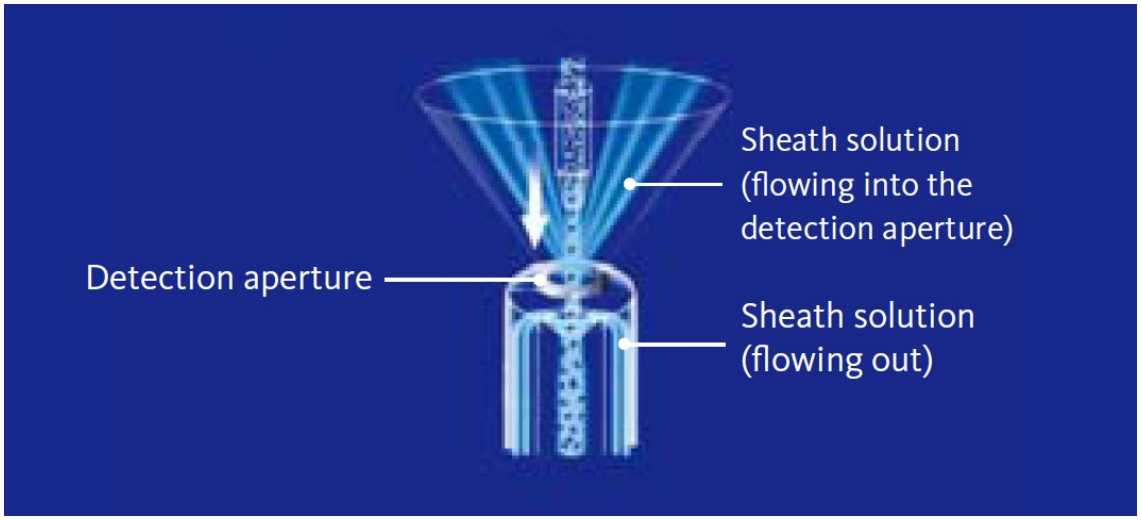


Figure 75. Passing cells one by one through the detection aperture
(Source: Sysmex, www.sysmex.co.jp)

Counting cells by the electric signals

Electric current does not pass through blood cells easily. This means there is resistance when electric current is passed through a cell. Alternatively, as presented in Figure 76, an electric field (voltage) and its measurement (current) are set to go through a solution. When a cell goes through this current solution, the measurement of current is changed. Resistance is defined by the ratio between voltage and current. Because voltage is kept constant, resistance changes according to the change in current. In this way, cells can be counted according to how many times an electric resistance spike is read. Additionally, the larger the cell is, the greater the resistance will be, so cell types may be distinguished by the measurement of resistance.

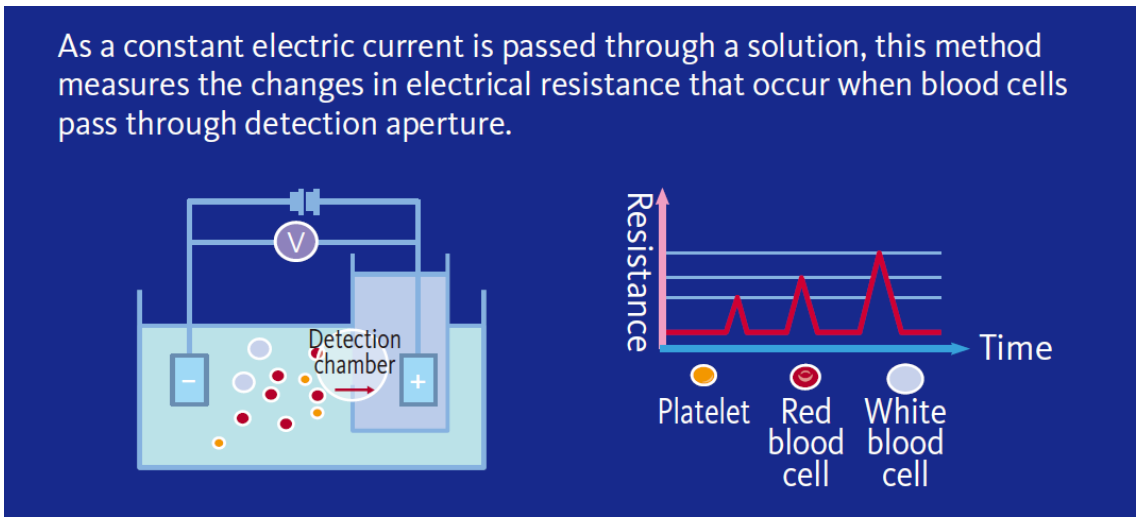


Figure 76. Counting cells by the electric signals
(Source: Sysmex, www.sysmex.co.jp)

Acquiring more information using flow cytometry

Flow cytometry can be used to reveal more information about blood cell counts because it can be used to measure the sizes of the cells, to examine the internal structures of the cells, and to analyse the chemicals within these cells.

To do so, the red blood cells and platelets in the sample are first shrunk using a surfactant, and the white blood cells undergo nucleic-acid staining before being used in the flow cytometer. Once inside the flow cytometer, the cells in the sample are irradiated with a laser diode to produce forward-scattered light, side-scattered light and side-fluorescence.

Analysis of the light signals enables the categorisation of the cells, as illustrated in Figure 77. Cell size is indicated by forward-scattered light, internal cellular structure is shown by side-scattered light, and some information about the chemicals inside the cells is revealed by side-fluorescence. With the ability to obtain these additional details, therefore, this technique can be used to not only distinguish amongst the three main types of blood cells, but also classify the five different types of white blood cell, namely neutrophil, lymphocyte, monocyte, eosinophil and basophil.

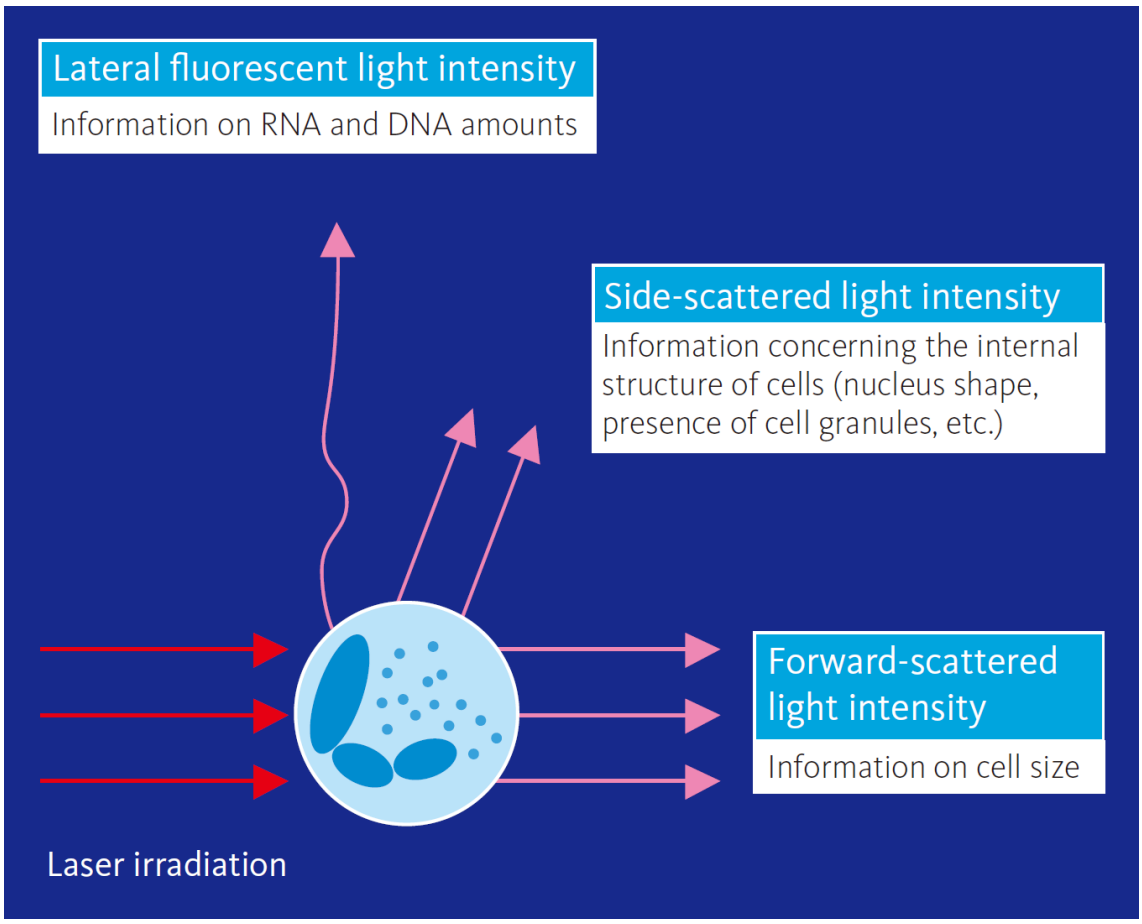


Figure 77. Information captured through flow cytometry (Source: Sysmex, www.sysmex.co.jp)

Method

From each of the blood samples collected from the participants, 2 mL of fresh blood was placed in an EDTA tube to stop it from clotting and was placed in a Sysmex XS-1000i/XS-800i blood-counting machine shown in Figure 78 to ascertain the total blood-cell count and the counts of the various differentiated cells.

Figure 78. General components of Sysmex XS-1000i/XS-800i blood-counting machine (Source: www.dotmed.com)

Reagents for Sysmex blood-counting machine (Sysmex, UK) are listed in Table 9.

Table 9. Sysmex blood-counting machine reagents (Source: Sysmex, www.sysmex.co.jp)

Cell-pack	isotonic diluent
Stromatolyser 4DL	lyse reagent in differential analysis
Stromatolyser 2DS	dye used in differential analysis
Sulfolyser	cyanid free haemoglobin reagent

This same procedure for each participant, initially carried out in September, was repeated successively in the months of October, November and December.

4.3 Statistical Analyses

Statistical analyses and significance of data were determined using GraphPad Prism version 5.00 for Mac OS X and Microsoft Windows (GraphPad Software, San Diego California USA).

One-way ANOVA was used to analyse the data as the factors of time and VD_3 level were considered in separately analyses. All results are presented as mean ± standard error of the mean (SEM). All values with $p < 0.05$ were considered as significant.

The results of one-way ANOVA were further evaluated with various post-hoc tests. In the study of the correlation between time and other measurements, Tukey's and Bonferroni's multiple comparisons tests were used to perform pair-wise comparison to see if the differences between subsets of data were statistically significant. The former is more powerful with larger number of means whilst the latter is more powerful with smaller number of means, but it is recommended that both tests should be performed and the smaller of the two intervals be taken.

Tukey's and Bonferroni's multiple comparisons tests assume that variances are equal across groups or samples. The Bartlett test was used to verify that assumption. ANOVA assumes normality, i.e., samples are taken from a population with Gaussian distribution. As the Bartlett test is sensible to deviation from normality, it could also be used to test non-normality.

In the study of the correlation between VD_3 level and other measurements, Deming (1943) linear regression was applied to the data pairs to take into account of the measurement errors in both quantities. Pearson correlation and Spearman's correlation were used to ascertain the correlation between the independent and dependent variables. The former makes the assumption that data are taken from a population with normal distribution, whilst the latter does not make that assumption and is considered to be more suitable when the sample size is small.

To decide the result of which of these two is more relevant, normality tests were done on each sample data set to assess the probability that the random varialbe underlying the data set is normally distributed. If the data set passes the normality tests, the result of the

parametric Pearson correlation is used, otherwise that of the nonparametric Spearson's correlation is used instead.

There are three normality tests provided by GraphPad Prism. These are the D'Agostino-Pearson omnibus test, the Shapiro-Wilk test and the Kolmogorov-Smirnov test, with the Dallal-Wilkinson-Lilliefor corrected P value. All three normality tests were used for these analyses. The D'Agostino-Pearson omnibus test is recommended by GraphPad Prism as it works well even if there are duplicated data and the mathematics basis is simpler, whereas the Shapiro-Wilk test works very well only if every value is unique and the mathematical basis is complex. The Kolmogorov-Smirnov test is weaker than the other two but can still be useful in situations where the performances of the other two are conpromised.

4.4 Results

4.4.1 Correlations with time

VD_3 level vs. time

The statistics of monthly measurements of VD_3 levels from all participants, including the results of one-way ANOVA, was well as Tukey's and Bonferroni multiple comparisons tests, are presented in Figure 79.

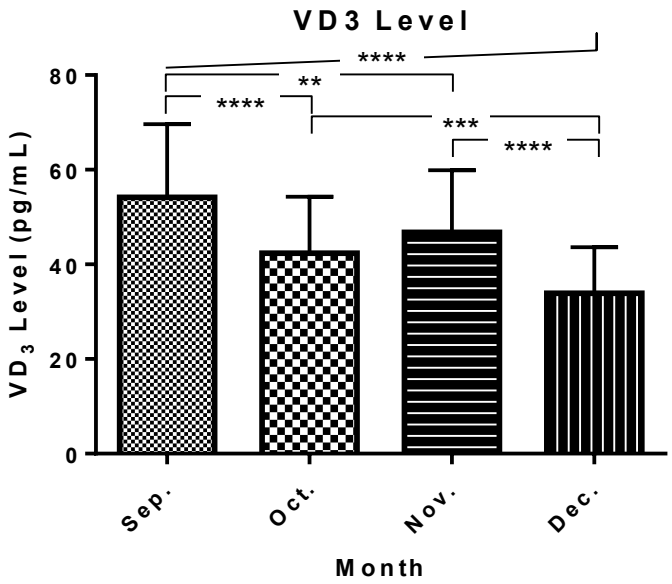


Figure 79. VD_3 level vs. month for all participants
N = 12. (*: p < 0.01; **: p < 0.001; ***: p < 0.0001; ****: p < 0.00001)

The monthly average of 25(OH)D level rose from 57.667 pg/mL in September to 45.167 pg/mL in October, rising slightly to 49.350 pg/mL in November before dropping to 35.333 pg/mL in December. The result of one-way ANOVA indicated that the differences amongst these measurements were significant. This was supported by both Tukey's and Bonferroni's multiple comparisons tests except October vs. November.

25(OH)D level vs. time

The statistics of monthly measurements of 25(OH)D levels from all participants, including the results of one-way ANOVA, was well as Tukey's and Bonferroni multiple comparisons tests, are presented in Figure 79.

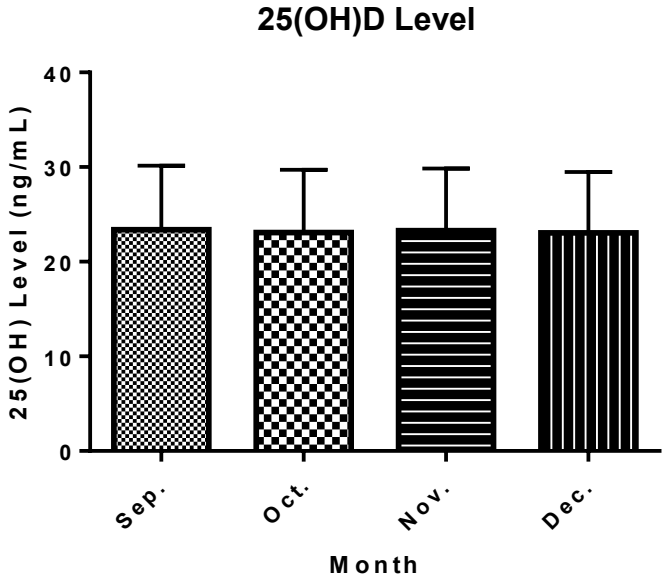


Figure 80. 25(OH)D level vs. month for all participants
N = 12.

The monthly average of 25(OH)D level dropped from 24.896 ng/mL in September to 24.604 ng/mL in October, rising slightly to 24.858 ng/mL in November before dropping to 24.636 ng/mL in December. The result of one-way ANOVA indicated that the differences amongst these measurements were not significant. This was confirmed by both Tukey's and Bonferroni's multiple comparisons tests.

White blood cell count vs. time

The statistics of monthly measurements of white blood cell counts from all participants, including the results of one-way ANOVA, was well as Tukey's and Bonferroni multiple comparisons tests, are presented in Figure 81.

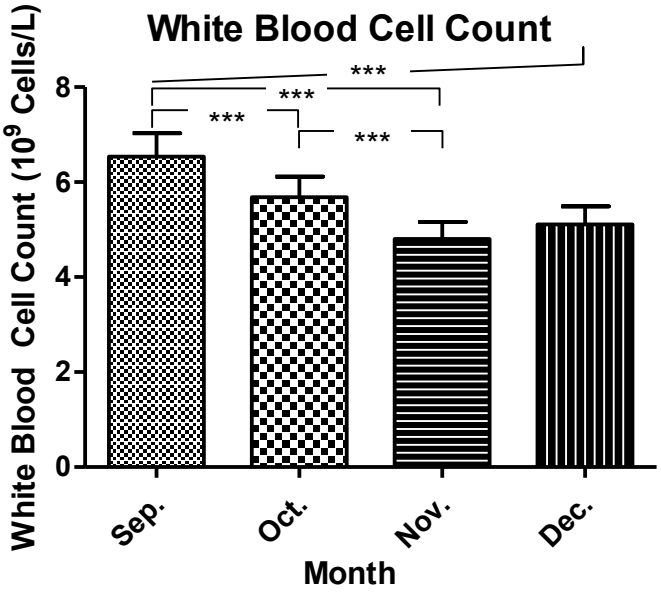


Figure 81. White blood cell count vs. month for all participants
N = 12. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$; ****: $p < 0.00001$)

The monthly average of white blood cell count dropped from 6.969×10^9 cells/L in September to 6.060×10^9 cells/L in October and further to 5.113×10^9 cells/L in November before rising slightly to 5.459×10^9 cells/L in December. The result of one-way ANOVA indicated that the differences amongst these measurements were significant. This was confirmed by both Tukey's and Bonferroni's multiple comparisons tests except October vs. December and November vs. December.

Red blood cell count vs. time

The statistics of monthly measurements of red blood cell counts from all participants, including the results of one-way ANOVA, was well as Tukey's and Bonferroni multiple comparisons tests, are presented in Figure 82.

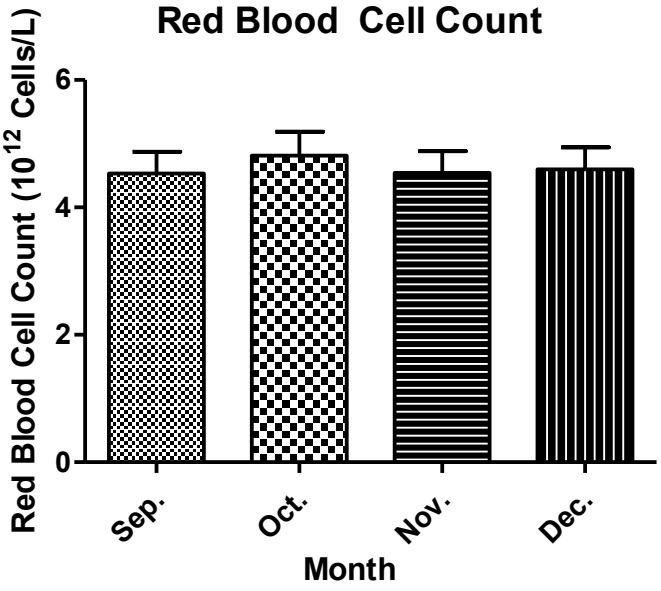


Figure 82. Red blood cell count vs. month for all participants
N = 12.

The monthly average of red blood cell count was 4.837×10^{12} cells/L in September, rising slightly to 5.121×10^{12} cells/L in October and dropping back to 4.850×10^{12} cells/L in November and 4.901×10^{12} cells/L in December. The result of one-way ANOVA indicated that the differences amongst these measurements were not significant. This was confirmed by both Tukey's and Bonferroni's multiple comparisons tests.

Platelet count vs. time

The statistics of monthly measurements of red platelet counts from all participants, including the results of one-way ANOVA, was well as Tukey's and Bonferroni multiple comparisons tests, are presented in Figure 82.

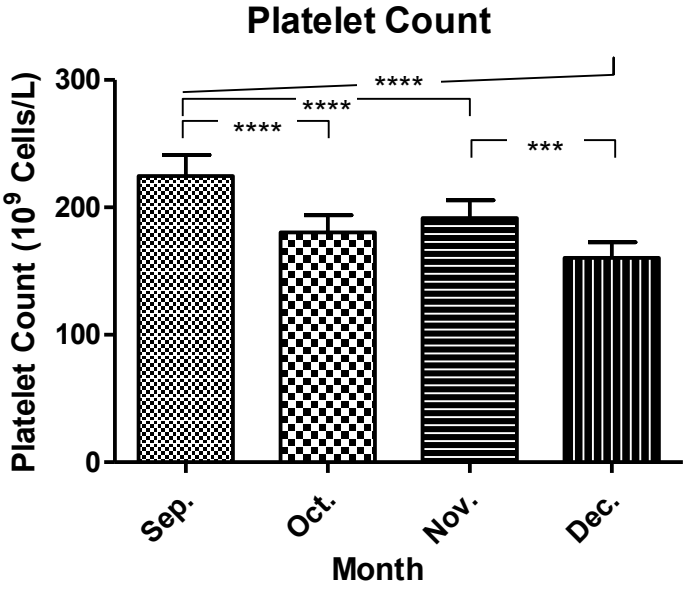


Figure 83. Platelet count vs. month for all participants
N = 12. (*: p < 0.01; **: p < 0.001; ***: p < 0.0001; ****: p < 0.00001)

The monthly average of platelet count dropped from 239.917 × 10^9 cells/L in September to 192.075 × 10^9 cells/L in October, rising to 204.567 × 10^9 cells/L in November before dropping to 170.933 × 10^9 cells/L in December. The result of one-way ANOVA indicated that the differences amongst these measurements were significant. This was confirmed by both Tukey's and Bonferroni's multiple comparisons tests except October vs. November and October vs. December.

$CD34^+$ progenitor cell count vs. time

The statistics of monthly measurements of $CD34^+$ progenitor cell counts from all participants, including the results of one-way ANOVA, was well as Tukey's and Bonferroni multiple comparisons tests, are presented in Figure 84.

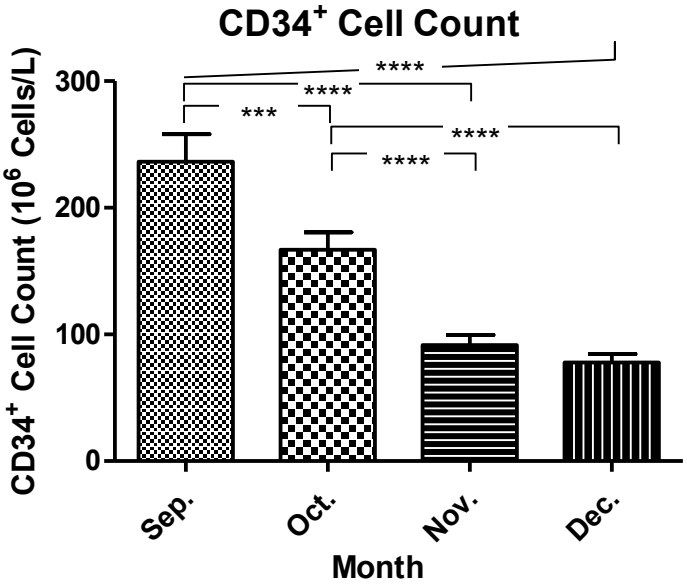


Figure 84. $CD34^+$ progenitor cell count vs. month for all participants
N = 12. (*: $p < 0.01$; **: $p < 0.001$; ***: $p < 0.0001$; ****: $p < 0.00001$)

The monthly average of $CD34^+$ progenitor cell count dropped from 249.417×10^6 cells/L in September to 176.833×10^6 cells/L in October and further to 96.833×10^6 cells/L in November and 82.333×10^6 cells/L in December. The result of one-way ANOVA indicated that the differences amongst these measurements were significant. This was confirmed by both Tukey's and Bonferroni's multiple comparisons tests except November vs. December.

Summary

In the study of the correlations between various observations and time, the results of one-way ANOVA indicated that the temporal variations of VD_3 level, white cell count, platelet count and $CD34^+$ progenitor cell count were all statistically significant, whereas the temporal variations of 25(OH)D level and red blood cell count were statistically insignificant. All these findings were generally confirmed by both Tukey's and Bonferroni's multiple comparisons tests.

4.4.2 Correlations with VD_3 level

25(OH)D level vs. VD_3 level

The relation between 25(OH)D level (ng/mL) and VD_3 level (pg/mL) for all participants are shown in Figure 87, together with the best-fit line according to Deming linear regression. The equation of the best-fit line is

$$y = 0.1020\,x + 19.97$$

Its slope (0.1020 ± 0.04858) was positive but was not to be considered significantly non-zero ($p = 0.0412$).

The sample data of VD_3 passed all three normality tests (for the D'Agostino and Pearson test, $p = 0.4015$; for the Shapiro-Wilk test, $p = 0.2325$; for the Kolmogorov-Smirnov test, $p > 0.10$). The sample data of 25(OH)D passed the D'Agostino and Pearson test ($p = 0.1141$) and the Kolmogorov-Smirnov test ($p > 0.10$) but did not pass the Shapiro-Wilk test ($p = 0.0198$). Therefore, it is justified to rely on the result of Pearson correlation rather than that of the Spearson's correlation, and the correlation between 25(OH)D level and VD_3 level was significant according to Pearson correlation ($r = 0.2958$, $p = 0.0412$).

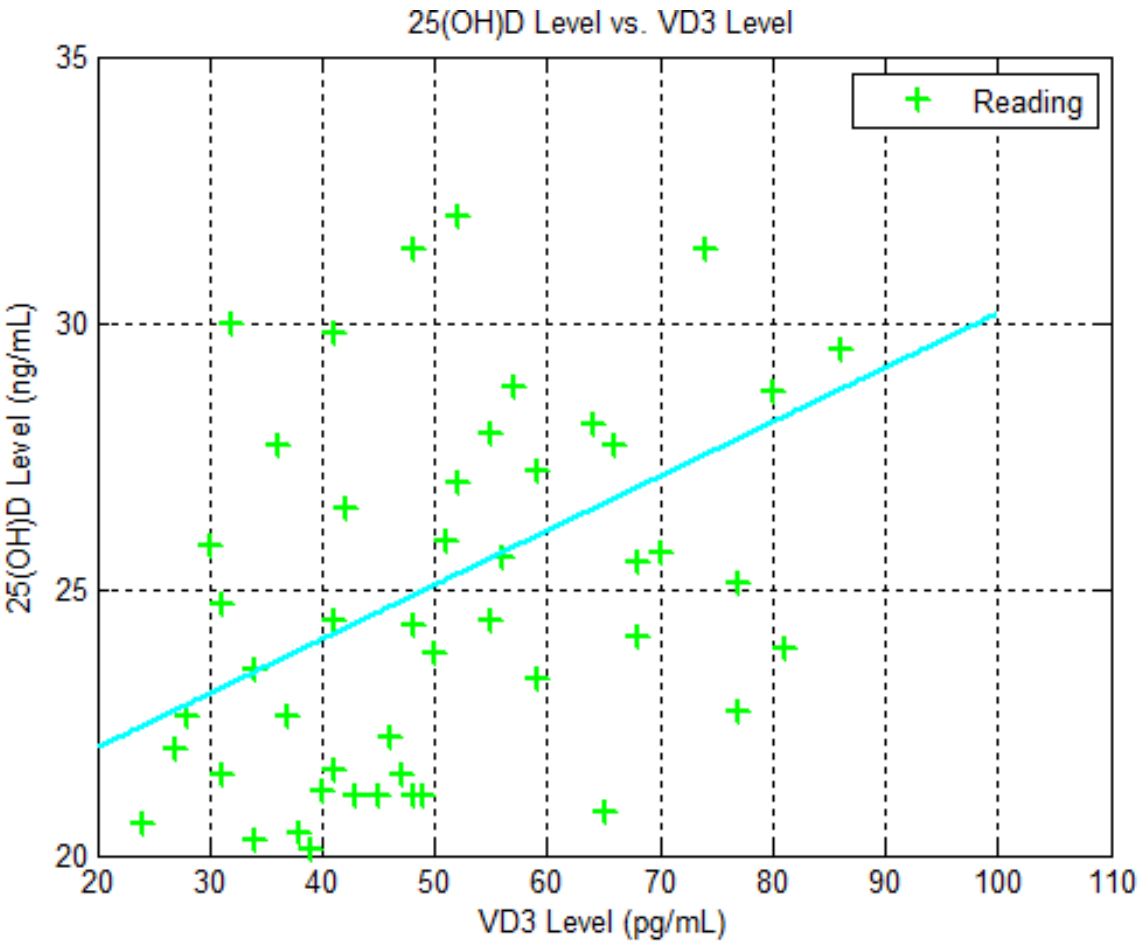


Figure 85. 25(OH)D level (ng/mL) vs. VD_3 level (pg/mL) for all participants and the best-fit line according to Deming linear regression
N = 48.

White blood cell count vs. VD_3 level

The relation between the white blood cell count (10^9 cells/L) and VD_3 level (pg/mL) for all participants are shown in Figure 86, together with the best-fit line according to Deming linear regression. The equation of the best-fit line is

$$y = 0.03727\,x + 4.153$$

Its slope (0.03727 ± 0.01356) was positive and was great enough to be considered significantly non-zero (p = 0.0085).

The sample data of VD_3 passed all three normality tests (for the D'Agostino and Pearson test, p = 0.4015; for the Shapiro-Wilk test, p = 0.2325; for the Kolmogorov-Smirnov test, p > 0.10). The sample data of white blood cell count also passed all three normality tests (for the D'Agostino and Pearson test, p = 0.2305; for the Shapiro-Wilk test, p = 0.0827;

for the Kolmogorov-Smirnov test, $p > 0.10$). Therefore, it is justified to rely on the result of Pearson correlation rather than that of the Spearson's correlation, and the correlation between white blood cell count and VD_3 level was significance according to Pearson correlation ($r = 0.3756$, $p = 0.0085$).

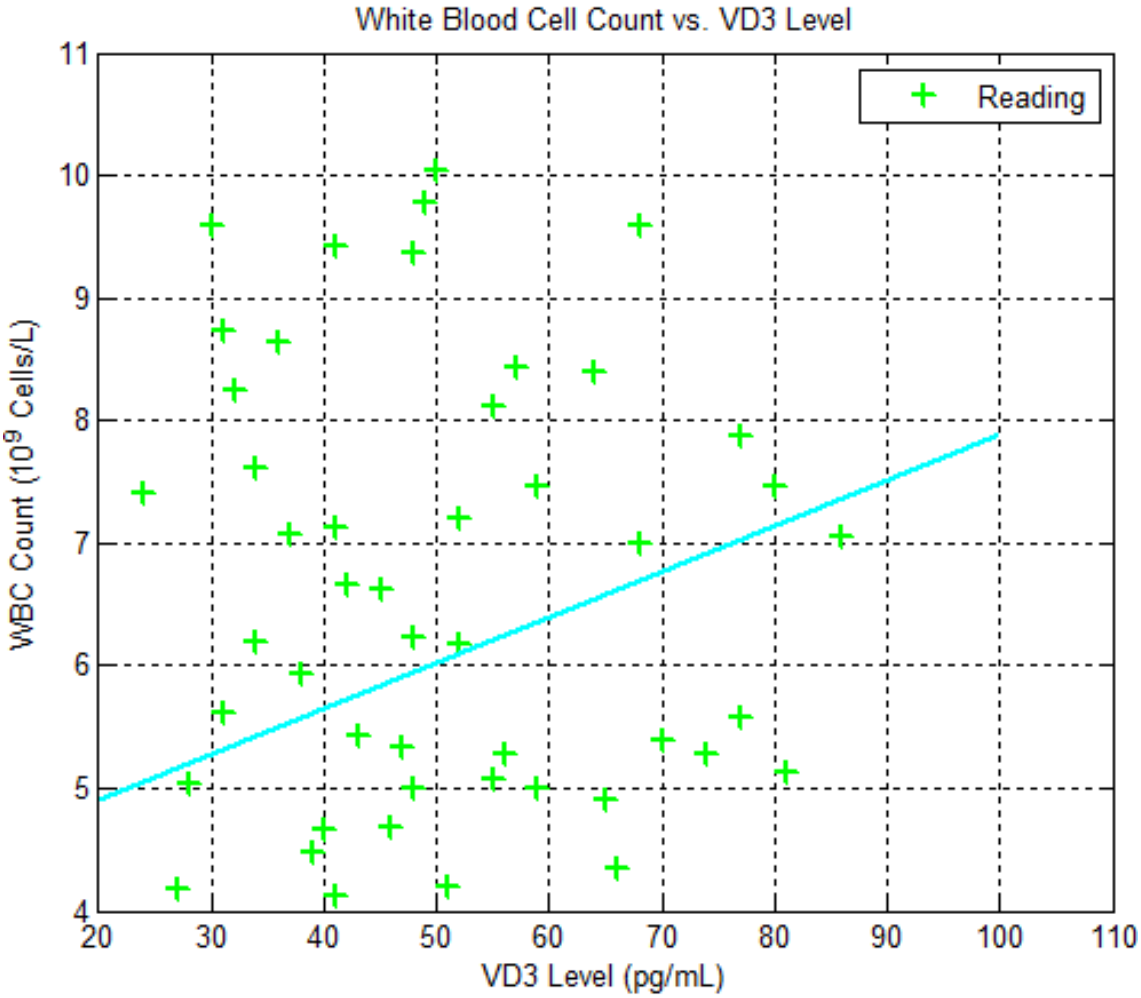


Figure 86. Total white blood cell count (10^9 cells/L) vs. VD_3 level (pg/mL) for all participants and the best-fit line according to Deming linear regression
N = 48.

Red blood cell count vs. VD_3 level

The relation between the red blood cell count (10^9 cells/L) and VD_3 level (pg/mL) for all participants are shown in Figure 87, together with the best-fit line according to Deming linear regression. The equation of the best-fit line is

$$y = -0.005886\,x + 5.203$$

Its slope (–0.005886 ± 0.09266) was marginally smaller than zero and was not to be considered significantly non-zero ($p = 0.5285$).

The sample data of VD_3 passed all three normality tests (for the D'Agostino and Pearson test, $p = 0.4015$; for the Shapiro-Wilk test, $p = 0.2325$; for the Kolmogorov-Smirnov test, $p > 0.10$). However, the sample data of red blood cell count did not pass any of the three normality tests (for the D'Agostino and Pearson test, $p = 0.0255$; for the Shapiro-Wilk test, $p = 0.0003$; for the Kolmogorov-Smirnov test, $p = 0.0002$). Therefore, it is justified to rely on the result of Spearman's correlation rather than that of the Pearson correlation, and the correlation between red blood cell count and VD_3 level was not significant according to Spearman's correlation ($r = –0.01393$, $p = 0.9251$).

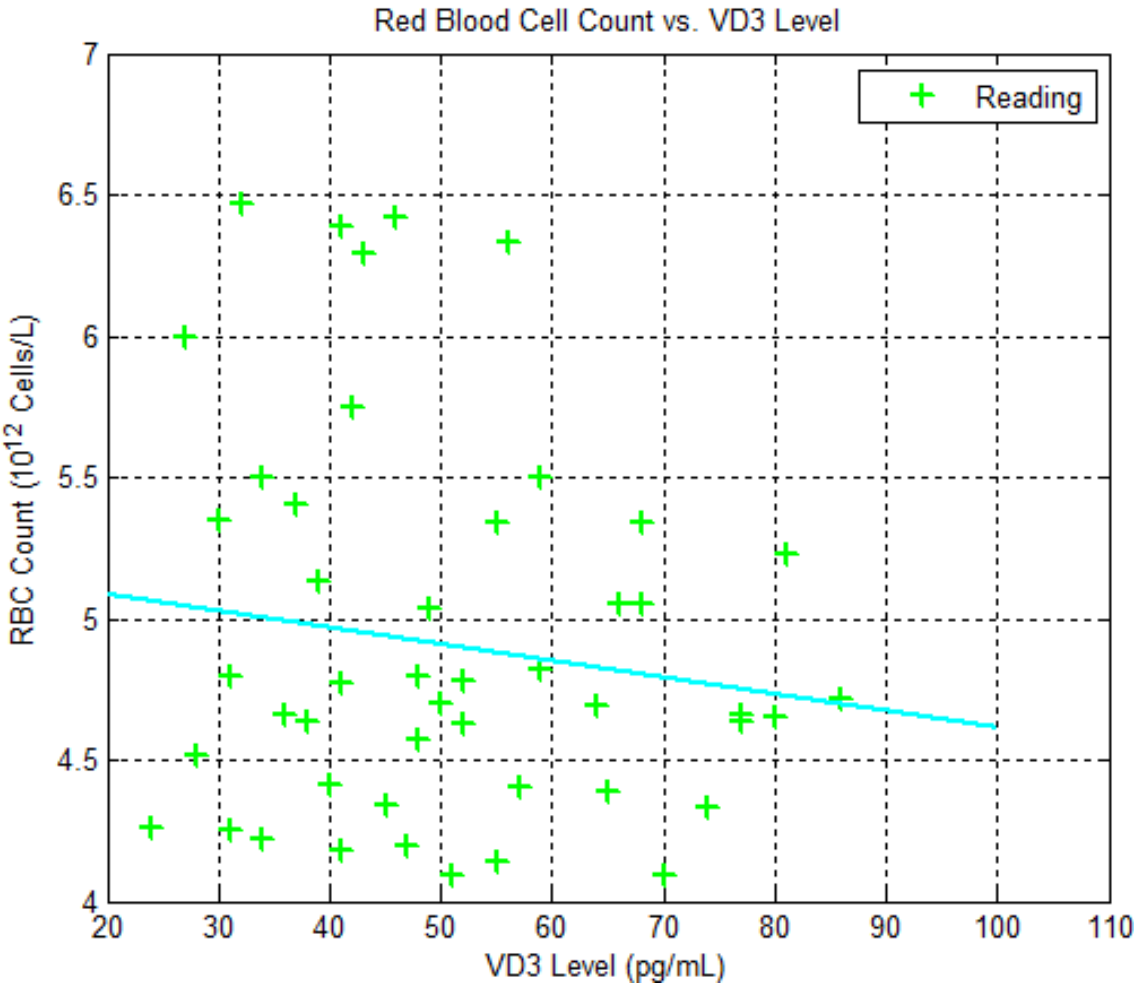


Figure 87. Total red blood cell count (10^9 cells/L) vs. VD_3 level (pg/mL) for all participants and the best-fit line according to Deming linear regression
N = 48.

Platelet count vs. VD_3 level

The relation between the platelet count (10^9 cells/L) and VD_3 level (pg/mL) for all participants are shown in Figure 88, together with the best-fit line according to Deming linear regression. The equation of the best-fit line is

$$y = 6.806\,x - 117.2$$

Its slope (6.806 ± 1.977) was positive and was great enough to be considered significantly non-zero ($p = 0.0012$).

The sample data of VD_3 passed all three normality tests (for the D'Agostino and Pearson test, $p = 0.4015$; for the Shapiro-Wilk test, $p = 0.2325$; for the Kolmogorov-Smirnov test, $p > 0.10$). The sample data of platelet count also passed all three normality tests (for the D'Agostino and Pearson test, $p = 0.6641$; for the Shapiro-Wilk test, $p = 0.6393$; for the Kolmogorov-Smirnov test, $p > 0.10$). Therefore, it is justified to rely on the result of Pearson correlation rather than that of the Spearson's correlation, and the correlation between platelet count and VD_3 level was significant according to Pearson correlation ($r = 0.4527$, $p = 0.0012$).

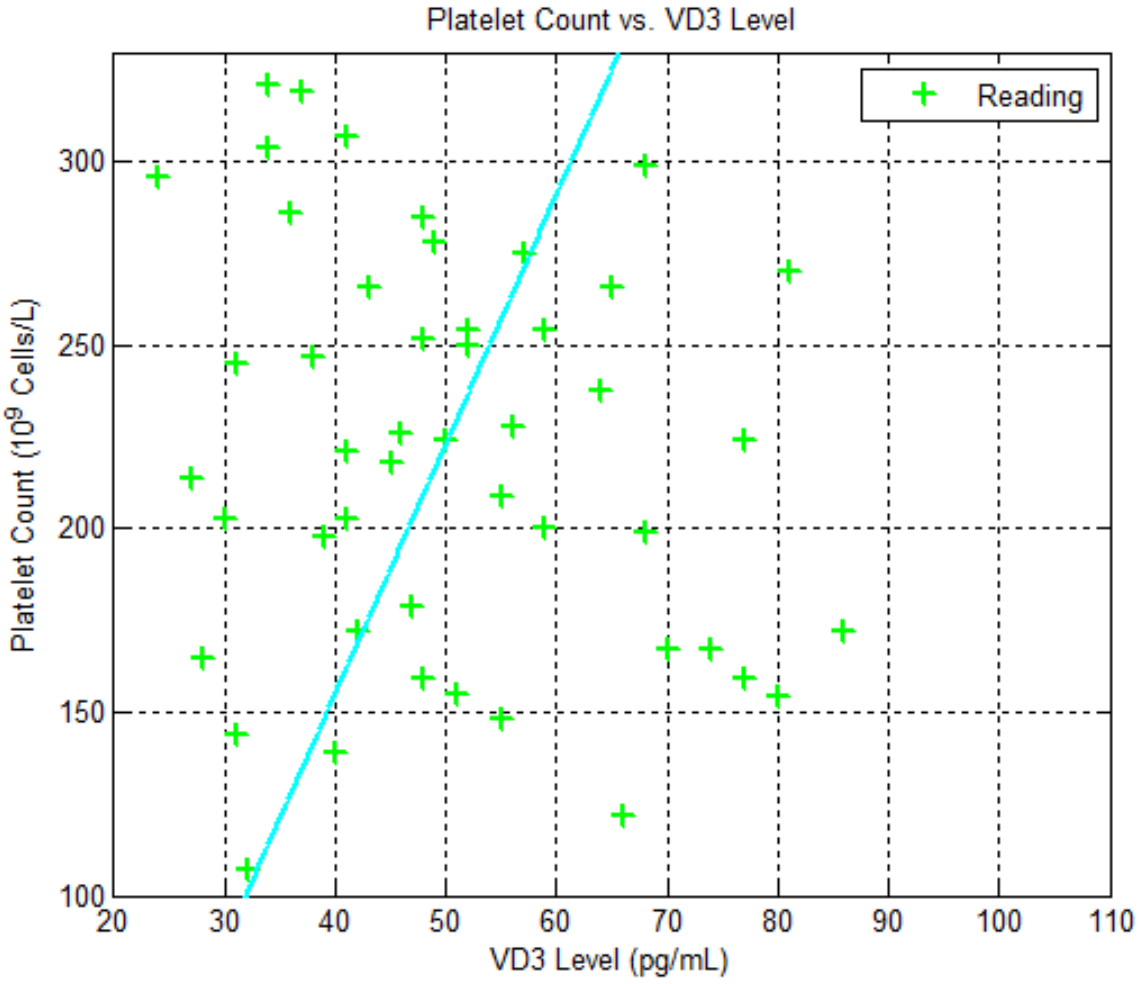


Figure 88. Total platelet count (10^9 cells/L) vs. VD_3 level (pg/mL) for all participants and the best-fit line according to Deming linear regression
N = 48.

$CD34^+$ progenitor cell count vs. VD_3 level

The relation between the $CD34^+$ progenitor cell count (10^6 cells/L) and VD_3 level (pg/mL) for all participants are shown in Figure 89, together with the best-fit line according to Deming linear regression. The equation of the best-fit line is

$$y = 16.71\,x - 631.9$$

Its slope (16.71 ± 5.021) was positive and was great enough to be considered significantly non-zero (p = 0.0017).

The sample data of VD_3 passed all three normality tests (for the D'Agostino and Pearson test, p = 0.4015; for the Shapiro-Wilk test, p = 0.2325; for the Kolmogorov-Smirnov test, p > 0.10). However, the sample data of $CD34^+$ progenitor cell count did not pass any of

the three normality tests (for the D'Agostino and Pearson test, $p = 0.0216$; for the Shapiro-Wilk test, $p = 0.0007$; for the Kolmogorov-Smirnov test, $p = 0.0078$). Therefore, it is justified to rely on the result of Spearman's correlation rather than that of the Pearson correlation, and the correlation between $CD34^+$ progenitor call count and VD_3 level was significant according to Spearman's correlation ($r = 0.4440$, $p = 0.0016$).

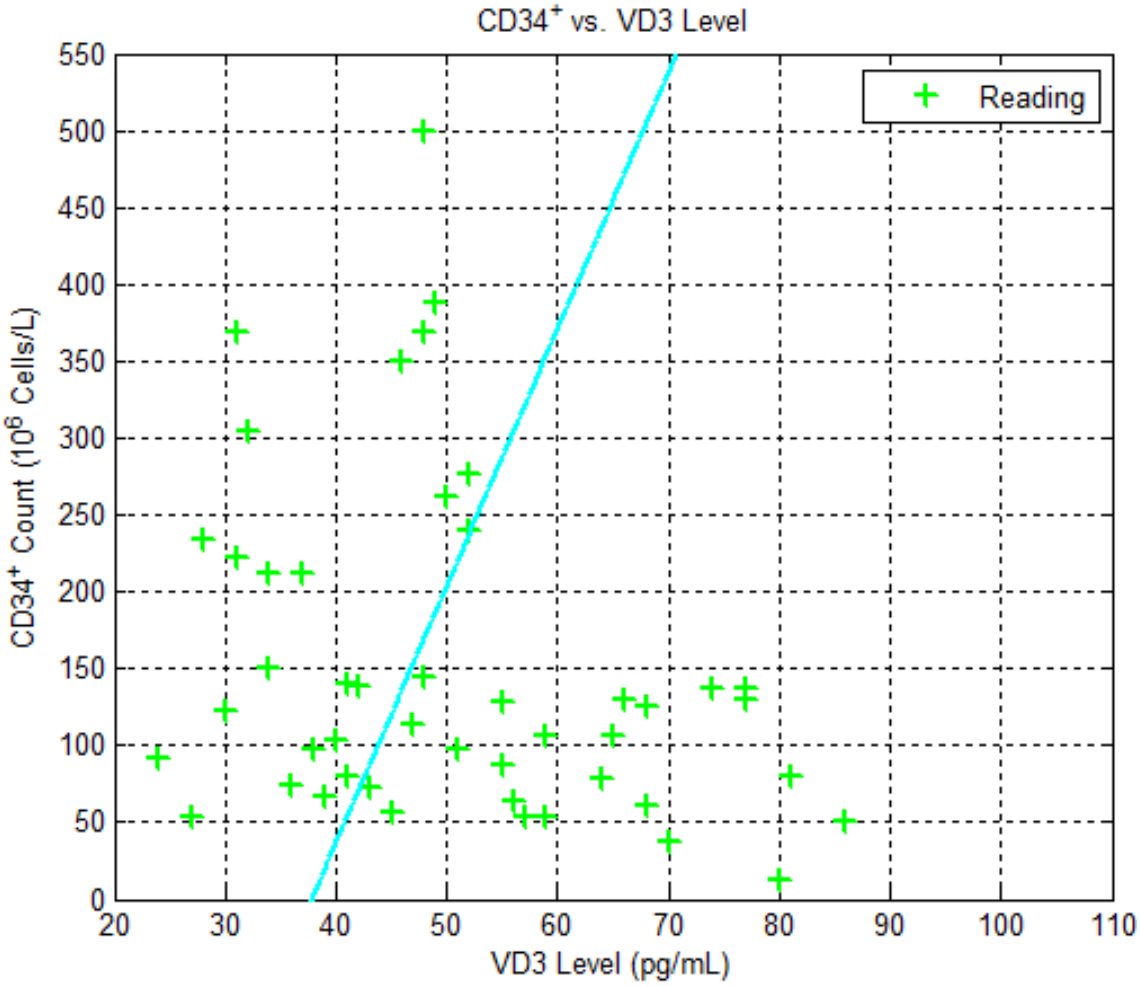


Figure 89. Total $CD34^+$ progenitor cell count (10^6 cells/L) vs. VD_3 level (pg/mL) for all participants and the best-fit line according to Deming linear regression
N = 48.

Summary

In the study of the correlations between various observations and VD_3 level, the results of Deming linear regression indicates that there was statistically significant positive correlation between VD_3 level and each of 25(OH)D, white blood cell count, platelet count and $CD34^+$ progenitor cell count. The only exception was that there was no statistically significant correlation between red blood cell count and VD_3 level. These

results were confirmed by the results of both Pearson correlation and Spearman's correlation.

4.5 Discussion

There are two stages in the analysis of the findings. It is necessary to ascertain that the temporal changes to the measurements are statistically significant before it is possible to check if there are positive correlations between the various cell counts, etc. and VD_3 level.

4.5.1 Correlation with time

The first task in interpreting the findings was to establish if there were significant non-random changes in the measurement along time. In the study of the correlations between various observations and time, the results of one-way ANOVA indicated that the temporal variations of VD_3 level, white cell count, platelet count and $CD34^+$ progenitor cell count were all statistically significant, whereas the temporal variations of 25(OH)D level and red blood cell count were statistically insignificant. All these findings were generally confirmed by both Tukey's and Bonferroni's multiple comparisons tests. This meant that there were significant non-random changes in the measurements along time. On the other hand, the 25(OH)D level and red blood cell count remained virtually constant.

The small sample size did not seem to cause much problem as both Tukey's and Bonferroni's multiple comparisons tests confirmed the significance found.

Because the duration of this pilot study did not cover the four seasons of a year, it was not possible to conclusively ascertain the seasonal variation of these measurements, though it did indicate non-random variation in most of these measurements. Nevertheless, it was not surprising to note that recent researches had found seasonal variation of VD_3 status, particularly in colder climates with reduced sunlight exposure during autumn and winter (Bose *et al.*, 2013), and it was not surprising, either, to note that recent researches had identified this to be responsible for the high prevalence of VD_3 insufficiency amongst populations residing at higher latitudes (Lips, 2010).

4.5.2 Correlation with VD_3 level

As mentioned in Section 4.2.2, none of the participants were VD deficient at the beginning or during the course of the experiment according to the US national guideline as serum 25(OH)D levels greater than 20 ng/mL were generally considered adequate for healthy individuals (IOM, 2011).

Once it was established that there were significant correlations between the measurements and time, which meant that there were significantly non-random changes in the measurements along time, the next focus was on finding if there were positive correlations between the various cell counts and VD_3 level.

In the study of the correlations between various observations and VD_3 level, the results of Deming linear regression indicates that there was statistically significant positive correlation between VD_3 level and each of 25(OH)D, white blood cell count, platelet count and $CD34^+$ progenitor cell count. The only exception was that there was no statistically significant correlation between red blood cell count and VD_3 level. These results were confirmed by the results of both Pearson correlation and Spearman's correlation.

There was positive correlation between 25(OH)D and VD_3 level. That meant the more plasma 25(OH)D there was, the more VD_3 could be synthesised. Conversely, the less plasma 25(OH)D there was, the less VD_3 could be synthesised.

There were positive correlation between VD_3 level and each of white blood cell count, platelet count and $CD34^+$ progenitor cell count. That meant the more VD_3 there was, the more each of these types of cells there would be. Conversely, the less VD3 there was, the less each of these types of cells there would be.

On the other hand, there was no significant correlation between red blood cell count and VD_3 level. In other words, red blood cell count was independent of VD_3 level.

The small sample size did not seem to cause much problem as both Pearman's correlation and Spearman's correlation confirmed the significance of correlation found.

Most of the research into the effects of VD_3 on the growth and differentiation of blood cells concentrated on the cells related to immunology and its potential in treating

autoimmune or inflammatory diseases. Recent studies had shown that VD_3 could influence the growth and differentiation of immune cells and their functions (Wang *et al.*, 2014; Bikle, 2009; Adams and Hewison, 2008; Mora *et al.*, 2008). Moreover, correlation had been found between the seasonal variation of VD_3 level and the variation of human T cells (Khoo *et al.*, 2012). Furthermore, the seasonal variation of VD_3 level had also been found to be linked to the occurrence or the risk of developing various diseases. It had been found that low VD_3 levels in winter contributed to the seasonal peak in the occurrences of influenza and upper respiratory tract infection (Sabetta *et al.*, 2010; Cinde *et al.*, 2009; Cannell *et al*., 2006), and the risk of developing autoimmune diseases such as multiple sclerosis and insulin-dependent diabetes mellitus (Ascherio *et al.*, 2014; Simpson *et al.*, 2011; Zold, Barta and Bodolay, 2011; Moltchanova, Schreier, Lammi and Karvonen, 2009; Correale, Ysrraelit and Gaitan, 2009; de Abreu *et al.*, 2009; Willer *et al.*, 2005).

Thus, the pilot study was unique in being the first attempt to investigate the possible correlation between VD_3 level and a wider range of measurements including 25(OH)D level, white blood cell count, red blood cell count, platelet count and $CD34^+$ progenitor cell count. Its findings further our understanding on the anti-proliferative and pro-differentiative effects of VD_3 in the human body, and support the basis for conducting larger population-based studies to investigate the benefits of VD_3 supplementation at higher latitudes during autumn and winter.

4.5.3 The limitation of short experiment duration

Ideally, the experiment should have been conducted over a longer period such as one whole year to investigate the seasonal variations. For instance, the general downward trends of the measurements might have been shown clearer if data had been collected over a longer period. There were also concerns that the data collected in autumn and winter would be too poor for the study due to limited exposure to sunlight during this period. However, this did not seem to be too problematic as significant temporal variations in most of the measurements had been detected.

One instance where the short period of investigation might have caused problem was the insignificant results related to red blood cell count. VD_3 lasts only for hours after being converted from the inactive form by the body (Bowen, 2011), but red blood cells last 100 to 120 days (Thomé and Petz, 2002: 90), whereas the duration of the experiment from the

first measurements in September to the last ones in December was less than 120 days. This meant that all the red blood cell counts had included the effects of VD_3 before the experiment started, and the full result of the VD_3 level during the experiment on red blood cell count could not be detected before the experiment was over. Thus, the recorded red blood cell counts could not truly be paired with the recorded VD_3 levels and thus could not have truly reflected the outcome of the effect of the recorded VD_3 levels. Therefore, the effect of changing VD_3 level on red blood cell count might have been delayed for three or four months and thus not fully recorded in the four-month experiment, so correlation could not be detected.

Alternatively, due to the relative long-lasting of red blood cells, it might be intrinsically difficult to detect any effect of VD_3 on red blood cell count unless it was kept under the same condition for a long time, and this might be the reason behind the relatively constant measurement of red blood cell count and its lack of correlation with VD_3 level.

Similarly, the seemly constant 25(OH)D level might have been due to the fact that it lasts for weeks in the body (Bowen, 2011). Hence, unless the body was deprived of any dietary VD or exposure to sunlight for a prolonged period, the detrimental effect on plasma 25(OH)D level might not have shown.

The short duration of experiment might have also reduced the correlation between the measurements of white blood cell count and VD_3 level. White blood cells generally last for 5.4 days (Pillay *et al.*, 2010). Most types of white blood cells and platelet lasts no more than a few weeks. Amongst the different types of white blood cells, for neutrophil, it is 6 hours to a few days; for eosinophil, it is 8–12 days, but it circulates for 4–5 hours; for basophil, it is a few hours to a few days; lymphocyte lasts for weeks, except memory cells which last for years; whilst monocyte lasts for hours to days (Daniels *et al.*, 1979). Because some white blood cell could last for weeks, each month's measurement was not independent of the ones in the adjacent months, e.g., the effect of VD_3 on white blood cell count in September could affect the white blood cell count in October as some of the white blood cells generated in September were still present in October.

In comparison, Platelet (thrombocyte) lasts for 8 to 9 days (Harker *et al.*, 2000). Thus, assuming that there was no great fluctuation in VD_3 level, even though there might be a delay of a few days or weeks between the change in VD_3 level and the corresponding change in white blood cell count, platelet count and $CD34^+$ progenitor cell count, these

data were still likely detectable in the data recorded in the same month, and the correlations were still detectable.

4.5.4 The limitation of small sample size

Ideally, more data should have been collected by recruiting more participants, but there are difficulties in recruitment to clinical research in general, and ways to improve such recruitment remain elusive (Newington and Metcalfe, 2014). There was concern that the sample size was too small for any meaningful analysis. Nevertheless, this concern was at least partially relieved by the results of both Tukey's and Bonferroni's multiple comparisons tests in the investigation of correlation with time, and by the results of both Pearson correlation and Spearman's correlation in the investigation of the correlation with VD_3 level. Amongst these tests, Bonferroni's multiple comparisons test and Spearman's correlation were designed to work with small sample sizes.

4.5.5 Difference between *in vitro* and *in vivo* studies

In the *in vitro* experiment, the level of VD_3 in each sample was given, and was fixed initially. This was an independent variable in the experiment. Whatever other data recorded after the experiment were the dependent variables. In other words, the level of VD_3 set at the beginning of the experiment was the cause of whatever other data observed and collected. Furthermore, these data were the results of some reactions related to VD_3 and, even though it was not recorded at the end of the experiment, at least some of the VD_3 in the sample would have been used by these reactions and the level of VD_3 in each sample would have been reduced at the end of the experiment.

In contrast, in the *in vivo* experiment, the amount of VD_3 in each of the blood sample was not given or fixed. It was up to the body to convert some of the available 25(OH)D into VD_3.

Finally, it was worth noting that there were other factors in the body that could influence the cell counts and VD level measurements. For instance, blood cell counts could have been affected by vitamin B6, B12 or foliate (vitamin B9) deficiency, as well as internal bleeding, kidney disease or malnutrition (Weiss, 2013); whilst VD_3 could be consumed by calcium metabolism (Easttel *et al.*, 1991; Lappe *et al.*, 2007; Pittas *et al.*, 2007). The

consequences of these factors could potentially obscure *in vivo* the effects of VD_3 in experiments such as this.

4.6 Conclusion

This is the first attempt at conducting studies of this kind. Despite its limitations, the findings of this study do confirm those of the aforementioned *in vitro* studies that VD_3 suppresses the proliferation of haematopoietic progenitor cells and encourages the latter to differentiate into various blood cells. Additionally, in this *in vivo* study, it was found that there were statistically significant correlations between VD_3 level and $CD34^+$ progenitor cell count, white blood cell count and platelet count, whereas it was not successful in finding significant correlation with red blood cell count.

There was correlation between the amount of serum 25(OH)D and serum VD_3, but only a small amount of the former is converted to the latter. The exact mechanism that dictates the occurrence of this conversion has not been revealed by this study. Nevertheless, it did suggest the existence of some mechanism to keep the level of storage of the inactive form of VD_3 when it was not deemed necessary to be used.

In view of these findings, it is worth extending this study to investigate the effects of VD_3 on these cell counts with more extensive investigations involving more participants and for a longer duration. Taking into considerations of the half-lives of VD_3 and various blood cells under study, as well as the seasonal variation of sunlight, it would be more appropriate to collect weekly samples in a period of at least one year / 52 weeks in order to obtain more conclusive findings and gain better insight into this subject.

Chapter 5 Conclusion

5.1 Summary of Findings

In the first part of the research, it is found that:

- The presence of VD_3 *in vitro* suppresses the proliferation of murine E14 ESCs in isolation.
- The presence of VD_3 *in vitro* promotes the differentiation of murine E14 ESCs in isolation.
- The presence of VD_3 *in vitro* suppresses the proliferation of murine OP9 stromal cells in isolation.
- The presence of VD_3 *in vitro* promotes the differentiation of murine OP9 stromal cells in isolation.
- The effects are most significant in the samples with 100 nM of VD_3 *in vitro*

In the second part of the research, it is found that:

- The presence of VD_3 *in vitro* suppresses the proliferation of murine E14 ESCs in haematopoiesis in the proximity of murine OP9 stromal cells.
- The presence of VD_3 *in vitro* promotes the differentiation of murine E14 ESCs in haematopoiesis in the proximity of murine OP9 stromal cells.

In the third part of the research, it is found that:

- The presence of VD_3 *in vivo* could encourage haematopoiesis by suppressing the proliferation and promoting the differentiation of adult human HSCs, though there are complex mechanisms and other influential factors in the human body that would also compete with this process for the VD_3 available in the body.

5.2 Validation of Hypotheses

The mouse study in the first part of the research ascertained *in vitro* that VD_3 was crucial in suppressing proliferation and promoting differentiation. This was done by investigating

separately the effects of VD_3 on the proliferation phase of the E14 cell line and on stromal OP9 cells. In both studies, VD_3-treated cells were compared to untreated ones. The results showed that VD_3 inhibited the proliferation of the cells in a dose-dependent manner, quantitatively by decreased VD_3-treated cell number, and qualitatively by alkaline-phosphatase staining that revealed colourless VD_3-treated cells indicating decreased enzyme expression. Propidium-iodide cell-cycle analyses showed that by directly comparing VD_3-treated E14 cells with untreated E14, and, likewise, VD_3-treated OP9 cells with untreated OP9 cells, there were significant increase in percentage of cells in the G-phase and significant decrease in percentage of cells in the S-phase in a dose-dependent manner. This meant that there was significant G/S cell cycle arrest that resulted in suppressed proliferation and promoted differentiation.

Thus, the results from separate E14 and OP9 cell cultures indicated that adequate VD_3 level inhibited proliferation and enhanced differentiation. In other words, the first hypothesis of the research had been validated.

The study then went on to ascertain *in vitro* whether VD_3 accelerates and promotes haematopoiesis in mouse ESC co-cultured on OP9 stromal cells (E14 / OP9 co-culture). The E14 / OP9 co-cultures were tested for colony-forming-cell (CFC) numbers, as well as concurrent CD markers, gene expressions, and cytokine expressions on days 2, 5, 8, 10 and 12, respectively, and some general trends were observed. In co-cultures treated with an optimal 100nM VD_3 level, earlier (accelerated) and higher (promoted) peak values had shown with E-CFC and GEMM-CFC numbers, FLK, CD31 and CD34 markers, and FLK, SCL, GATA1 and GATA2 gene expressions. These values then markedly decreased. On the other hand, later and higher peaks had shown with GM-CFC and M-CFC numbers, CD41, CD43 and CD45 markers, and GATA1 gene expressions. These peaks had shifted towards day 12 compared with measurements of the untreated co-cultures.

Hence, the results from E14 / OP9 co-cultures showed that treatment with optimal VD_3 accelerates and promotes differentiation of ESCs towards haematopoiesis, i.e., into haematopoietic stem cells and then into blood cells. This was shown in observation that ESC–derived $CD34^+$ progenitor cells become more enriched in colony-forming cells expressing the haematopoiesis-associated genes GATA1, GATA2, SCL and FLK, as well as expressions of related cytokines, so that the phenotypes of primitive haematopoietic progenitors and definitive hematopoietic stem cells were displayed. These suggested that

greater quantities of various differentiated cells had appeared earlier, which meant cell differentiation had been accelerated and promoted. Likewise, p21 and p27 expressions had been up-regulated by the presence of VD_3, resulting in G/S cell cycle arrest whereby proliferation was suppressed and differentiation was accelerated and promoted.

In other words, it was found that VD_3 played a key role in suppressing proliferation and promoting differentiation of cells in haematopoiesis and, in the co-culture of E14 embryonic stem cells and OP9 stromal cells, VD_3 enhances the differentiation of E14 cells into blood cells of erythroid, myeloid, and B cell lineages without adding exogenous growth factors or complex embryoid structures. Hence, the second hypotheses of the research had also been validated.

In the pilot study that was the third part of the research, it was shown that, first, the numbers of white blood cells, platelets and $CD34^+$ progenitor cells, as well as plasma VD3 levels, had significant non-random variation along time during the observation period. Secondly, it showed that there was statistically significant positive correlation between plasma VD_3 level and plasma 25(OH)D level, white blood cell count, platelet count and $CD34^+$ cell count.

Thus, these findings largely support the hypothesis, despite its limitation on small sample size due to the small number of participants stayed with the study and the short observation duration. Hence, it indicated that it would be worth to further conduct more extensive research to investigate.

Hence, all three hypotheses of the research had been validated, and all three objectives had been met. Therefore, the overall objective was also met and the aim of the research achieved.

5.3 Novelty

Previously, the effects of VD_3 on suppressing proliferation and promoting differentiation had been studied using mainly cancer cells. On the other hand, instead of using cancer cells, this research was conducted to study first the similar effects of VD_3 on ESCs and stromal cells, using the E14 and OP9 cell lines in the *in vitro* studies, and then its effects on the haematopoiesis process using the E14 / OP9 co-culture in the *in vitro* studies and live participants in the *in vivo* study.

In this respect, the first two parts of the research was collectively the first study to investigate *in vitro* the anti-proliferation and pro-differentiation effects of VD_3, especially in haematopoiesis, using E14 and OP9 cells and the E14 / OP9 co-culture. Through its findings, this was the first demonstration of the anabolic effect of VD_3 on murine E14 and OP9 cells *in vitro*.

The third part of the research was also the first attempt at *in vivo* human studies of the effects of VD_3 in enhancing cell differentiation in haematopoiesis that provided useful information to inform further studies.

5.4 Contributions

The findings of the *in vitro* researches on E14 / OP9 co-culture indicate that VD_3 treatment inhibits cellular proliferation and enhances cellular differentiation through cell cycle regulation by up-regulating p21 and p27 gene expressions. These suggest potential clinical applications of VD_3 treatment through cell cycle regulation.

The capacity of ESCs to undergo unlimited self-renewal and differentiation into many different cell types in the body has unveiled widespread application potentials in the fields of biomedical research and regenerative medicine. Furthermore, ESCs have been used for therapeutic intervention in the treatment of a wide number of disease conditions. In particular, the most important potential application of ESCs is the generation of cells that could be used for cell-based therapies, such as transplanting HSCs in leukaemia treatment entailing the use of high-quality sources of tissue-matched bone marrow, mobilised peripheral blood or umbilical cord blood. Appropriate bone marrow is often in short supply and cord blood, though bankable, contains fewer HSCs, which makes it less suitable for adult transplantation. Directed differentiation of ESCs towards HSCs offers a potentially attractive alternative to these conventional sources.

Since this research showed that VD_3 could be used to promote cell differentiation in haematopoiesis, it could be used to improve the success rate of these cell-based therapies, by either increase *in vitro* the quantity of the blood cells needed for later transplantation, or enhancing the differentiation of ESCs towards HSCs and haematopoiesis *in vivo*.

Overall, this thesis furthers the advancement in knowledge of the function of VD_3 in suppressing cell proliferation and enhancing differentiation, specifically in stem cells and

related to haematopoiesis. The effect of VD_3 in accelerating and promoting differentiation of stem cells into blood cells has been established, revealing the importance of VD_3 in human health. This study paves the way for future research into the development of therapeutic strategies for the treatment of leukaemia, in particular. More widely, it indicates that VD_3 plays a crucial role in disease prevention related to cancers and other chronic illnesses. To maintain a healthy state, it is necessary to acquire adequate amount of vitamin D by synthesis through sufficient exposure to sunlight, normal dietary intake, and/or supplementation to achieve an optimal vitamin D level.

5.5 Study Limitations

As mentioned in the discussions of the *in vivo* study, it was not possible to undertake further statistical analyses that were meaningful and more detailed owing to the scarcity of the data, which partly was the result of limited resources and funding. Despite this, as well as the complex nature of the living body, the study did yield some encouraging findings and new contribution to the body of knowledge in this field that could provide directions for further investigation.

Furthermore, one third of the participants of the study were men and the rest women, despite the initial design of having equal number of participants of either gender. Again, due to the limited number of participants and the varied personal health conditions and histories amongst them, it was not possible to investigate any possible gender variations in the *in vivo* study.

One of the reasons of having only limited number of participants was due to the difficulty in recruitments. It might be beneficial to explore in different settings new and diverse strategies to ensure that all potentially eligible patients are invited to participate. It might also be help to establish integrated clinical and academic teams with shared responsibilities for recruitment. Recruitment might have also been hindered by language barriers and long journey times.

Finally, this study does serve as a reminder the extent of complexity such an *in vivo* study would entail and should be taken into consideration in the design of the experiments.

Since the focus of this study was on the changes in proliferation and differentiation, the study of apoptosis was not included. The reduction of undifferentiated cells was assumed

to be due to differentiation. Some of these were supported by the appearance of differentiated cells, but it might be possible that some of the reductions were the result of cell death. Steps had been taken to retain and identify dead cells in some of the procedures, including the retention and inclusion of aspirated media and identification by trypan blue staining. However, this could still be enhanced in further researches by including apoptosis assays to address this issue.

5.6 Possible Further Research Directions

On the basis of the finding of this research, it would be useful to conduct *in vivo* mouse experiments in a condition where the level of VD_3 and other measurements are more constantly controlled and monitored. This might reveal a more detailed mechanism and the temporal and casual relations between different parameters could be established.

This could be followed by *in vitro* study on human cells, though it would be subject to more stringent ethical check.

Afterwards, the study could be furthered by conducting *in vivo* human tests, such as more extensive researches based on the pilot study presented here, running through a longer duration covering a whole year and recruiting more participants.

Alternatively, longer term clinical trials of VD_3 treatments on VD_3 deficient patients could also be conducted, as it is possible that more directed casual relations between certain parameters could be more clearly revealed in the studies of abnormal or extreme circumstances.

5.7 Conclusion

In summary, the findings of this research point out that VD_3 can indeed suppress the proliferation and promote the differentiation of haematopoietic stem and progenitor cells. This suggests potential benefit from the using VD in the prevention and treatment of haematological disorders and diseases, as well as in immune modulation, although the importance of this in various disease states remains poorly understood. Continued scientific investigations and well-controlled trials will likely be necessary to advance the understanding of the mechanisms underneath these physiological effects of VD and confirm any clinical benefit of VD therapy.

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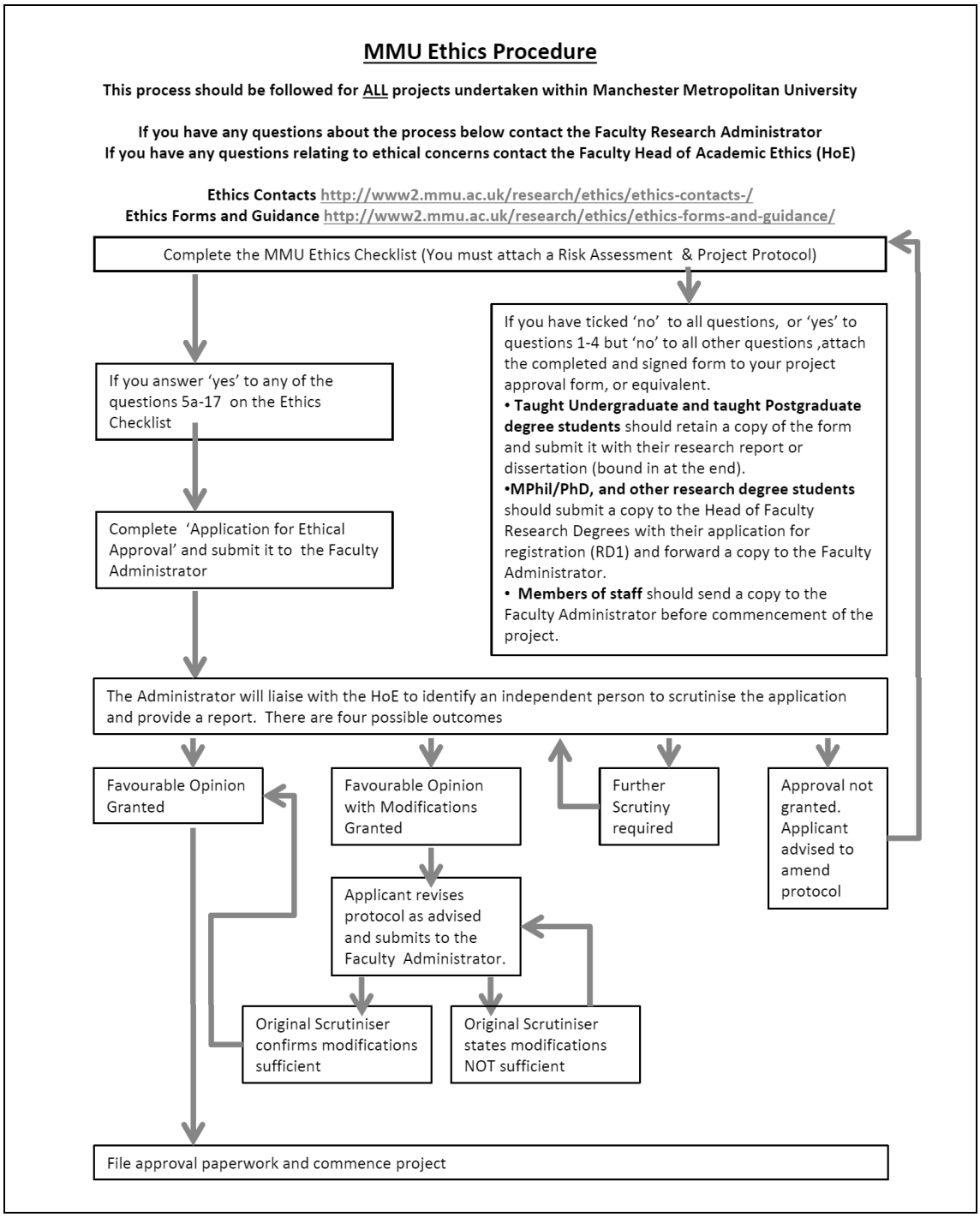
Appendices

A.1 Overview of Research

Table 10. Overview of the design of the studies included in this research, together with the experiments conducted, the results obtained and the key findings from these experiments

in vitro / in vivo	Part	Subject	Tests	Results		Key Findin
				E14 alone	OP9 alone	
in vitro	1	Mouse E14 alone OP9 alone	Cell proliferation assay by cell counting	The number of E14 cells decreases significantly with more than 10 nM of VD3 compared with the control (due to promoted differentiation).	The number of OP9 cells decreases significantly with more than 10 nM of VD3 compared with the control (due to promoted differentiation).	The presence promotes the diffe and suppresse proliferation of E1 the presence o
			Alkaline phosphatase staining for the determination of differentiation	Fewer cells are stained with VD_3 (due to differentiation).	Fewer cells are stained and more are rounded with VD_3 (due to differentiation).	The presence promotes the diffe and suppresse proliferation of OP the presence o
			Cell cycle examination by flow cytometric analysis	The percentage of cells in the G phase is significantly higher with VD_3 compared to the control (due to differentiation). The percentage of cells in the S phase is significantly lower with VD_3 compared to the control (due to differentiation).	The percentage of cells in the G phase is significantly higher with VD_3 compared to the control (due to differentiation). The percentage of cells in the S phase is significantly lower with VD_3 compared to the control (due to differentiation).	The effect is most in the samples wit of VD_3
	2	Mouse E14/OP9 co-culture	Immunofluorescence	The expressions of the growth factors Oct4, Sox2 and Nanog are reduced after incubation due to differentiation, and this reduction is enhanced by the presence of VD_3. These growth factors regulate the pluripotency of the embryonic stem cells.		The presence promotes haemato suppressing prolife promoting differen the E14/OP9 co-
			CD-marker expressions on E14 cells in the presence of OP9 cells and VD_3	The expressions of the CD-markers are generally earlier and with higher peaks with the presence of VD_3 compared with the controls (due to promoted differentiation).		
			Identification and counting of colony-forming cells	The numbers of most colony-forming cells are increased with VD_3. This indicates that VD_3 promotes differentiation.		
			Real-time quantitative PCR for gene expressions	Overall, the gene expressions indicate the presence of VD_3 affects the composition of the culture. This indicates that various differentiated haematopoietic cells appear earlier and are increased in number in treatment than in control. This shows that the presence of VD_3 promotes differentiation.		
			Cytokine multiplex assay	The expressions of selected cytokines are significantly higher in the treatment with VD_3 than in the control. This indicates the higher numbers of the differentiated blood cells in treatment. This means VD_3 promotes the differentiation of stem cells.		
in vivo	3	Human	Tests for Vitamin D_3 measurement	The non-bioactive VD concentration is affected by the degree of exposure to sunlight.		VD3 promotes diff and suppresses pr in human haemat
			Phenotype analysis by flow cytometry	Deming regression is used to take into account the observational variations in both the VD_3 level and the cell counts. There is significant correlation between the VD_3 level and the numbers of white blood cells, platelets and $CD34^+$ (stem) cells.		
			Blood cells counting			

A.2 MMU Ethics Procedure



(http://www2.mmu.ac.uk/research/ethics/ethics-forms-and-guidance/)

A.3 Ethical Approval of the *in vivo* Study

FACULTY OF SCIENCE AND ENGINEERING

Manchester Metropolitan University

M E M O R A N D U M

TO Mayada AlQaisi

FROM AnneMarie Walsh

DATE 23rd January 2013

SUBJECT Application for Ethical Approval **(SE131401)**

On the 11/15/2013 the Head of Ethics for Science & Engineering considered your application for Ethical Approval (SE131401) entitled "The effect of 1,25-dihydroxyvitamin D3 on the total blood-cell count and on the counts of circulating stem cells". The application has been granted Favourable Opinion and you may now commence the project.

MMU requires that you report any Adverse Event during this study immediately to the Head of Ethics (Prof Bill Gilmore) and the Administrator (AnneMarie Walsh). Adverse Events are adverse reactions to any modality, drug or dietary supplement administered to subjects or any trauma resulting from procedures in the protocol of a study.

An Adverse Event may also be accidental loss of data or loss of sample, particularly human tissue. Loss of human tissue or cells must also be reported to the designated individual for the Human Tissue Authority licence (currently Prof Bill Gilmore).

If you make any changes to the approved protocol these must be approved by the Faculty Head of Ethics. If amendments are required you should complete the attached form and submit it to the Administrator.

Regards

AnneMarie Walsh
Research Degrees Group Officer
All Saints North

Page **1** of **1**

A.4 Advertisement for Participant Recruitment

Manchester Metropolitan University

Participants wanted

The effect of 1,25-dihydroxyvitamin D3 (VD3) on the total blood-cell count and on the counts of circulating stem cells.

Healthy volunteers, (young 35-45 years) are needed to participate in a study which will investigate the effect of VD_3 levels on total blood count and circulating haematopoietic progenitor cells. We need in this study to get 15 mL of whole blood from each participant once every month for four months. The process will take up to 10 minutes each time then you will be free to leave the room. The purpose of taking blood samples is to measure VD_3 level, total blood count and circulating haematopoietic progenitor cells. VD_3 is able to mobilise stem cells from bone marrow into blood circulation which may differentiate into blood cells (blood cells renewal) and increase repair of vascular lesions of heart muscle.

The blood will be taken by a Dr AL-SHANTI at a phlebotomy room on the first floor of John Dalton Tower.

For more information, please contact: Mayada Al Qaisi: **09989750@stu.mmu.ac.uk**

All information will be confidential and securely stored in password-protected folder. The name of participants will be coded in compliance with the Data Protection Act 1998.

A.5 Information about the *in vivo* Study

Manchester Metropolitan University
School of Healthcare Science

Volunteer Information

Manchester Metropolitan University

Project title: The effect of 1,25-dihydroxyvitamin D3 (VD3) on the total blood-cell count and on the counts of circulating haematopoietic progenitor cells

This information pack is compiled to provide you with details of this study to help you make an informed decision on whether or not to participate. You are encouraged to understand this information and make a considered decision on your own, away from the researchers, but you can ask the researchers for further clarification and explanation if you have any question.

If you decide to participate, you will need to sign the consent form given to you by the researchers. Please read this information pack and the consent form carefully before signing. You will also need to complete a medical questionnaire in which all recorded details are completely anonymous (free from any potential identification). This will be achieved by allocating each participant with a non-identifiable participant number (PN).

Once you are selected to participate in this study, you are free to withdraw at any time without giving any reason.

Background

The capacity of embryonic stem cells (ESCs) to undergo unlimited self-renewal and differentiation into many different cell types in the body has unveiled widespread application potentials in the fields of biomedical research and regenerative medicine. Embryonic stem cells (ESCs) are being used for therapeutic intervention in the treatment of a wide number of disease conditions. In particular, the most important potential application of ESCs is the generation of cells that could be used for cell-based therapies, such as transplanting haematopoietic stem cells (HSCs) in leukaemia treatment, entailing the use of high-quality sources of tissue-matched bone marrow, mobilised peripheral blood or umbilical cord blood. Appropriate bone marrow is often in short supply and cord blood, although bankable, contains fewer HSCs, which makes it less suitable for adult transplantation. Directed differentiation of ESCs towards HSCs offers a potentially attractive alternative to these conventional sources. Vitamin D_3 was demonstrated to be a powerful differentiation inducer for a wide variety of neoplastic cells. Thus, 1α, 25-Dihydroxyvitamin D_3 could enhance differentiation of embryonic stem cells into blood cells of erythroid, myeloid, and B cell lineages. Exposure to VD_3 is thought to increase the blood-cell counts of differentiated cells. The implications of using VD_3 in cancer prevention and treatment are exciting.

Study protocol

Sixteen healthy participants will be recruited to take part in this study. The same procedure mentioned below will be repeated with each participant once every month between September and December.

Sample collection and management

If you are selected as one of the participants, you will be asked to give blood samples once every month for four months. The blood samples will be taken by a phlebotomist. A needle will be inserted in an antecubital vein to obtain 3 tubes of 5 mL baseline blood samples. After the blood samples are taken, the needle will be removed and gauze applied at the site of venepuncture. This method of blood sampling is routinely used in clinical and research settings. You will then be free to leave the laboratory. This procedure will take no more than 15 minutes.

As mentioned above, all raw data will be stored anonymously and referred to by participant numbers rather than by names. Data storage will comply with the Data Protection Act (1998). If you decide to withdraw from the study at any time after the experimental procedure commences, all your samples and data stored in any form will be destroyed.

Vitamin D_3 measurement test

To prepare the serum for VD_3 measurement, one of the collected blood samples will be allowed to clot by leaving it undisturbed at room temperature. This usually takes 15-30 minutes. The clot will be removed by centrifuging at 1,000-2,000 × g for 10 minutes in a refrigerated centrifuge. Following centrifugation, the liquid component (serum) will be transferred into a clean polypropylene tube using a Pasteur pipette. The samples will be maintained at 2-8°C while handling. Then, the samples will be sent to the NHS for examination of the level of VD_3.

Circulating $CD34^+$ haematopoietic progenitor cells measurements

The BD stem cell enumeration kit will be used to determine the amount of circulating $CD34^+$ haematopoietic progenitor cells. This kit will enable us to enumerate $CD34^+$ haematopoietic progenitor cells accurately and reproducibly. CD34 and CD45 marker expressions will be measured by means of a flow-cytometer machine. To 100 µL of whole blood, 20 µL of CD45 FITC/CD34 PE reagent and 20 µL of 7-AAD reagent will be added to a Trucount tube. The tubes of the samples will be vortexed and then kept in the dark at room temperature for 20 minutes. 2 mL of 1× ammonium chloride lysing solution will be added. After that, the tubes of the samples will be vortexed and kept in the dark at room temperature for 10 minutes. The samples will be ready for analysis within 1 hour after lysis.

Count Blood Cells

2mL fresh blood in an EDTA tube will be placed in an XS-1000i/XS-800i (Sysmex) blood-counting machine in order to ascertain the total blood-cell count and the counts of the various differentiated cells.

A.6 Participant Consent Form

Participant Consent Form

Manchester Metropolitan University

Project Title:	'The effect of 1,25-dihydroxyvitamin D_3 (VD_3) on the total blood-cell count and on the counts of circulating haematopoietic progenitor cells'
Principal Investigator:	Mayada Alqaisi

NAME OF PARTICIPANT:____________________________________

1. I agree to take part in the above research. I have read the Participant Information Sheet. I understand what my role will be in this research, and all my questions have been answered to my satisfaction.

2. I understand that I am free to withdraw from the research at any time, for any reason and without prejudice.

3. I have been informed that the confidentiality of the information I provide will be safeguarded.

4. I am free to ask any questions at any time before and during the study.

5. I agree to the Manchester Metropolitan University processing personal data which I have supplied. I agree to the processing of my supplied data for any purposes connected with the research project as outlined to me

6. I agree that the tissue donation(s) I have given can be stored after completion of this research study/project and can be used in any future, as yet undetermined, study /project. (I have the right to ask for these donated tissue(s) to be destroyed or returned to me at any time).

7. I agree that the tissue donation(s) I have given can be stored after completion of this research study/ project and can be used on any future research project/study that is related to the study/project in which I have taken part.

8. (I have the right to ask for these donated tissue(s) to be destroyed or returned to me at any time).

Name of participant (print) ____

Signed____ Date______________________

- -

If you wish to withdraw from the research, please complete the form below and return to the principal investigator named above.

Project Title:	

I WISH TO WITHDRAW FROM THIS STUDY

Signed____ Date______________________

A.7 Medical Screening Questionnaire

'The effect of 1,25-dihydroxyvitamin D3 (VD3) on the total blood-cell count and on the counts of circulating haematopoietic progenitor cells'

Participant number:

Manchester Metropolitan University

Medical Screening Questionnaire

It is important that the investigators are aware of any health conditions before participation in this research study. This is to ensure that the study protocol will not exacerbate any existing conditions of the participant. Please answer the following questions as accurately as possible.

Are you currently taking any prescribed medication?	YES/NO
Are you currently attending your GP?	YES/NO
Have you ever suffered from a cardiovascular problem? ***for example***: high blood pressure, anaemia, heart attack etc.	YES/NO
Have you ever suffered from a neurological disorder? ***for example***: epilepsy, convulsions etc.	YES/NO
Have you ever suffered from an endocrine disorder? ***for example***: diabetes etc.	YES/NO
Have you ever suffered from a chronic gastrointestinal disorder? ***for example***: Crohn's disease, irritable bowel syndrome etc.	YES/NO
Have you ever suffered from a skin disorder? ***for example***: eczema etc.	YES/NO
Do you suffer from any allergies? ***for example***: medications, foods etc.	YES/NO
Are you aware of any other medical condition that may prevent you from participating in this investigation? ***for example***: immunological disorders, numbness in extremities, asthma etc.	YES/NO
Are you currently taking any supplements ***for example***: creatine	YES/NO

If you have answered "yes" to any of these questions, please provide details below: