Effects of Diethylstilbestrol on Murine Early Embryonic Stem Cell Differentiation Using an Embryoid Body Culture System

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ABSTRACT

Objectives: The effects of estrogens on immune system formation and function are well documented. Diethylstilbestrol (DES), a synthetic estrogen, has been linked to neoplasia and immune cell dysfunction in humans and animals exposed *in-utero*. *In-vitro* effects of DES exposure of murine embryonic stem (ES) cells on the early embryonic immune system development and the expression of cellular surface markers associated with common hemangioblastic and hematopoietic precursors of the endothelial, lymphoid & myeloid lineages were investigated.

Hypothesis: Early ES cell expression of CD45 a marker common to lymphoid lineage hematopoietic stem cells and differentiation of lymphoid lineage precursors are affected by *in-vitro* exposure to DES.

Methods: Murine ES cells were cultured using a variety of techniques: an OP9 co-culture system, and formation of embryoid bodies (EBs) in a liquid medium and hanging drop system. The OP9 co-culture system did not appear to give rise to well differentiated lymphoid lineage cells during 12 days of differentiation. The hanging drop EB culture system, previously shown to promote differentiation of endothelial and lymphoid precursor cells, was chosen for further studies of ES cell differentiation. ES cells were harvested at five time points: undifferentiated (day 0), and differentiated (days 3, 8, 12 and 16). Differentiating ES cells were treated with DES beginning on day 3. The synthetic estrogen, DES, was chosen as a treatment because of its similar potency to 17β estradiol and documented association with neoplasia in women exposed *in-utero*. Surface marker expression, measured by real-time RT-PCR amplification, was recorded using fluorogenic TaqMan® probes designed specifically for the surface proteins of interest: oct4, c-Kit, Flk1, ERα, ERβ, CD45, Flt1, & VE-cadherin.

Analysis & Results: Changes in surface marker gene expression between day 0 and day 16 of differentiation were analyzed using the RT-PCR threshold counts (C_T) and the $2^{-\Delta\Delta CT}$ method. The expression of each target mRNA was normalized internally to a housekeeping gene (18s rRNA) and calculated relative to day 0. ANOVA (Type 3 fixed-effects analysis, SAS) was performed using the unexponentiated $\Delta\Delta C_T$ values. The effects of DES, time, and the interaction between DES and time were evaluated for days 8, 12 and 16. Additionally, the effects of DES on the expression of each marker were evaluated for day 16. Expression of estrogen receptor α & β (ERα & β*)* in the EBs was established, and did not appear to be affected at any time by treatment with DES. ER*α* was expressed in significant levels on day 16, while ERβ was expressed in low levels throughout the period of differentiation. The expression of the cell surface marker, c-Kit was significantly ($P<0.0001$) altered by the presence of DES between the three time points sampled. The expression of the VEGF receptor, Flt1, and the adhesion molecule, VE-cadherin, markers of endothelial cells, were also significantly ($P<0.026$) altered by treatment with DES on day 16 of differentiation. Treatment with DES appeared to have no effect on the expression of CD45, a marker common to lymphoid precursor cells.

Conclusions: These results indicate the presence of estrogen receptors in differentiating ES cells as early as day three *in-vitro* (ERβ) until day 16 (ERα). DES alters expression of common hemangioblastic and hematopoietic precursor, as well as endothelial lineage markers, but has no effect on expression of the marker of lymphoid lineage development before day 16. These effects coincided with the expression of ERα. The enduring effects of DES exposure *in-utero* may not be manifest in this ES model, or may occur at later stages of differentiation or in selected subpopulations of $CD45^+$ cells.

ACKNOWLEDGEMENTS

This thesis is dedicated to my parents and Chris: without their patience, support, encouragement, love and understanding, this would not have been possible. In addition, I would like to thank Dr. Bill Huckle for all his optimism, enthusiasm, friendship, patient help and teaching throughout this project. I also would like to thank my committee members, Dr. Sharon Witonsky, Dr. Will Eyestone, and Dr. Virginia Maxwell, for their mentorship, constructive input, patience and assistance over the last three years.

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ABBREVIATIONS

DES…….Diethylstilbestrol
CD……...Cluster of Differentiation
ES……Embryonic Stem (cell)
EB……Embryoid Body
RT-PCR……...Real Time Polymerase Chain Reaction
C_T……Threshhold Count
ANOVA……..Analysis of Variance
ER……Estrogen Receptors
VEGF……..Vascular Endothelial Growth Factor
HSC…….Hematopoietic Stem Cell
SCF……..Stem Cell Factor
M-CSF…….Macrophage Colony Stimulating Factor
GM-CSF……..Granulocyte-macrophage Colony Stimulating Factor
G-CSF……..Granulocyte Colony Stimulating Factor
Flk1……..VEGF Receptor 2
Flt1……...VEGF Receptor 1
MIP 1α…….Macrophage Inflammatory Protein
TGF-β……..Transforming Growth Factor Beta
ICM……..Inner Cell Mass
POU……..Pit-oct-unc Transcription Factor Family
PORE……Palindromic Oct-factor Recognition Element
MORE……...More PORE
PDGF……Platelet Derived Growth Factor
PDGFRα……...Platelet Derived Growth Factor Receptor alpha
TCR……..T-Cell Receptor
Thy1……..CD90: Thymocyte marker
LFA1…….CD11a: Leukocyte Function Associated Molecule
VE…….Vascular Endothelial
DP……..Desmoplakin
MAP……..Mitogen-activated Protein
NOD……..Non-Obese Diabetic
ERE……...Estrogen Response Element
BRCA1……..…Breast Cancer 1 Factor
ERKO……....Estrogen Receptor Knock-Out
IFNγ……...Interferon Gamma
Flt3L…… Flt3 Ligand
IL……...Interleukin
LIF…….Leukemia Inhibitory Factor
DMSO……...Dimethylsulfoxide
AMEM……..Alpha Modification of Eagle's Medium
DMEM……..Dulbecco's Modification of Eagle's Medium
βME……...Beta Mercaptoethanol
PBS……Phosphate Buffered saline

CHAPTER I

INTRODUCTION & LITERATURE REVIEW

Introduction: ***Embryonic Stem (ES)*** *cells are essential for origin and function of the immune system*

In the 1950s, it was noted by C.E. Ford, et. al., that lethal irradiation of the murine bone marrow destroyed its cell population, and those animals which received transplants of healthy bone marrow cells not only survived but expressed lymphoid cell types consistent with the donor. Approximately 30 mouse bone marrow **hematopoietic stem cells (HSC)** were sufficient to repopulate and save ~50% of lethally irradiated mice, reconstituting all blood cell types in the surviving mice.(1) This discovery was the foundation for understanding the immune system, the renewing and repopulating potential of stem cells, and the origin of technology for adult hemopoietic stem cell transplants (bone marrow and cord blood) that have saved countless lives since the first bone marrow transplant in 1968.(2) The first real stem-cell-only transplants to successfully treat diseases which were, until this time, invariably fatal, were performed using T cells removed from an MHC non-identical donor marrow or spleen cell suspension, which eliminated transplantation of factors that usually resulted in graft versus host disease (3)(4).

Differentiation of stem cells to their respective terminally mature cell-type is a complex process involving an intricate sequence of steps. Differentiation is regulated by developmental genes and their protein products, the cells' micro-environment, cytokines secreted by neighboring cells, contact and migration signals.(5-7) Mechanisms of stem cell lineage differentiation are not completely understood, but pose great potential for therapeutic application in cell and tissue replacement therapy. Characterization and understanding of

factors influencing and driving differentiation of HSC in early embryo and adult tissues are vital to understanding immune function and development. Additionally, the short and long-term effects of certain pharmacological agents on the differentiation pattern of ES cells are relatively unexplored. *In-utero* estrogen exposure has been linked to neoplasia in humans, abnormalities of the reproductive tract of mice, rats and humans, as well as implicated in the pathogenesis of autoimmune disease in humans, and immune dysfunction in mice.(8-18)

The immune system is made up of four basic groups of functional immune cells: B & T lymphocytes, macrophages, dendritic cells and natural killer cells, and the granulocytes. One percent of the human body's weight is composed of lymphocytes.(19) Within a human lifetime, the number of lymphocytes produced is equivalent to a person's body weight; this high production rate is necessary to provide a constant supply of immune cells to face the widely variable antigenic challenges faced each day.(19) This process of constant differentiation and resupply is known as hematopoiesis, and involves common hemangioblastic and hematopoietic precursors.

Adult hematopoiesis is the evolution of eight different cell lineages from a common hematopoietic precursor.(1, 5, 6, 20, 21) (Figure 1A) In the embryo, ES cells differentiate through a common hemangioblast which gives rise to endothelial lineage cells as well as hematopoietic precursors to lymphoid and myeloid lineage cells (Figure 1B).(22) In the early murine embryo, hematopoietic precursors are evident in blood islands as erythropoietic stem cells by day 8 – 10 of embryogenesis.(5, 23, 24) ES cell differentiation is under the influence of the microenvironment (cell-cell interactions with stromal elements) and soluble factors. Cytokines and growth factors, such as **stem cell factor** (SCF or c-Kit ligand), **macrophage colony stimulating factor** (M-CSF), **granulocyte-macrophage colony stimulating factor**

(GM-CSF) and **fetal liver kinase 2 and 3 ligands** (Flk2/Flt3 ligand), are provided by the local supporting stroma and common precursor cells of the yolk sac and blood islands, in the early embryo, by the fetal spleen and liver in later embryogenesis, and finally, by the bone marrow in the adult. These factors act in synergy to regulate differentiation and maturity of the various lineages, turning on and off transcription of genes, and guiding the complex set of steps in a specific order for the proper development of the embryo. Inhibitors of differentiation include factors such as interleukin 8 (IL-8), MIP-1α, TGF-β and interferons, which antagonize cytokines and activate terminal maturation of stem cells.(6) On-going investigations are attempting to elucidate the inter-relationships between all factors involved in this process.

Figure 1A: Overview of Adult Hematopoiesis

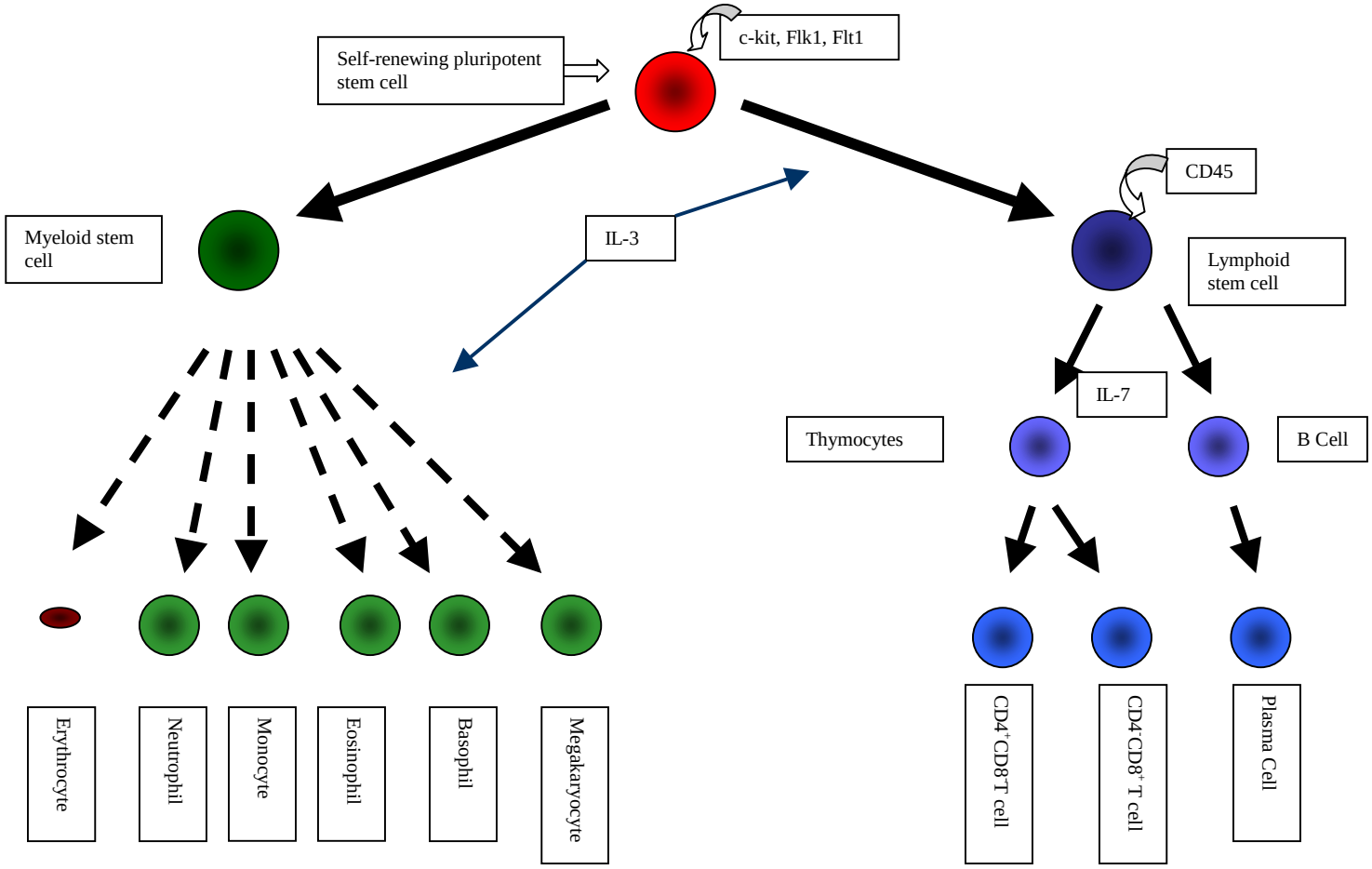
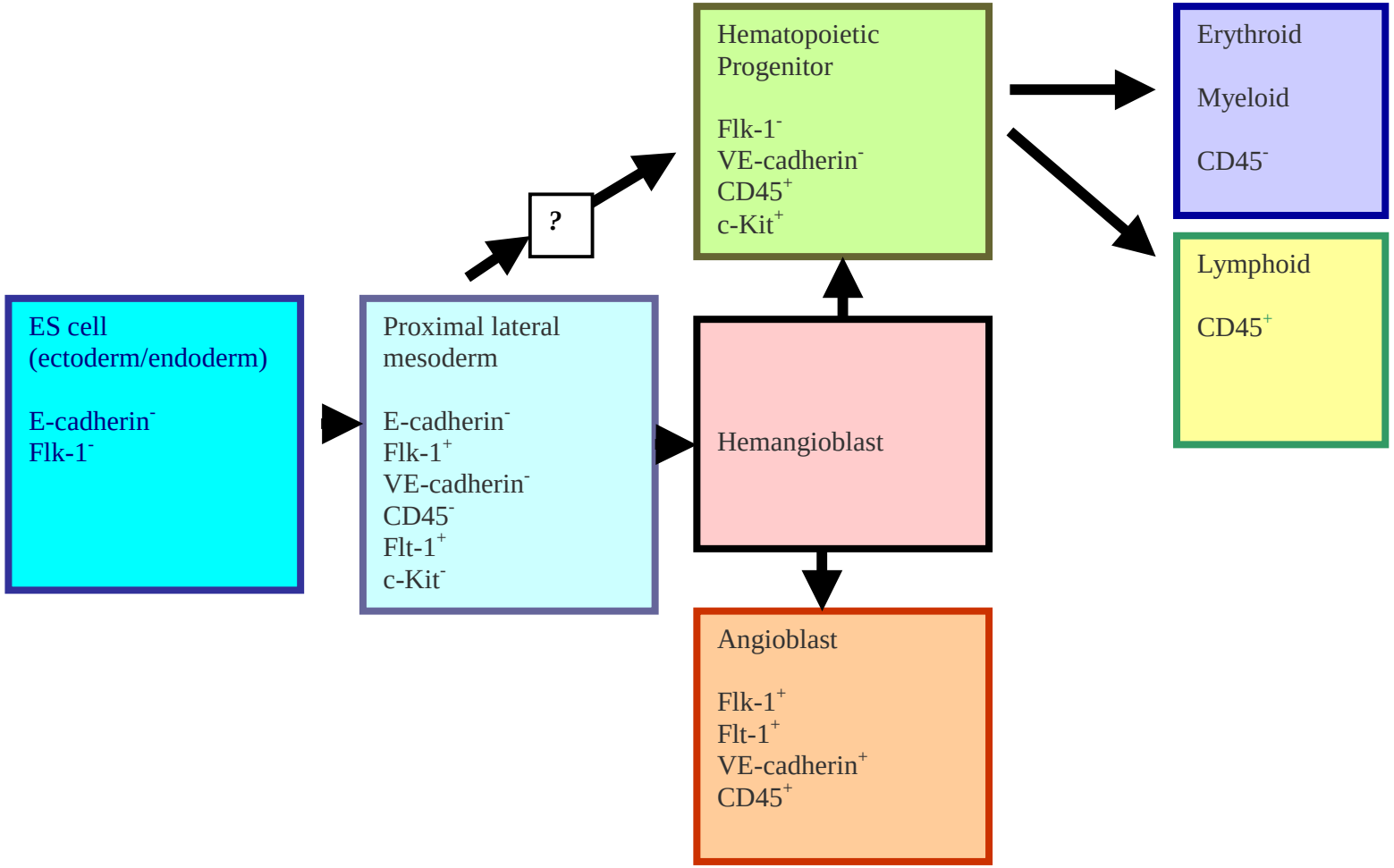


Figure 1B: Embryonic Stem cell differentiation from ES cell to Lymphoid, Myeloid and Endothelial cells.



LITERATURE REVIEW

Embryogenesis and ES cell generation

ES cells were first isolated and described in the 1980s.(7, 25-27) They are harvested from the pluripotent **inner cell mass (ICM)** of pre-implantation blastocyst stage embryos at about days 3 – 4 of gestation. When maintained under proper conditions, such as in the presence of a feeder layer of stromal cells and inhibitory factors, they have the potential to remain undifferentiated, while continuing to self-renew their population indefinitely. Totipotency and pluripotency are hallmark characteristics of stem cells: they can differentiate into one of many lineages of cells. Totipotency indicates a capacity to generate an entirely new organism, while pluripotency describes some degree of limitation as compared to totipotency; pluripotency is the result of initial stages of commitment by cells to become a specific cell-type. Differentiation of each cell lineage and formation of the tissues and organs within the embryo are determined by cellular interactions and inter/intra-cellular signaling which triggers gene expression and directs the commitment of each cell to its lineage.(28, 29) All factors necessary for reliable experimental vascular and hematopoietic differentiation are yet to be determined.

During normal murine embryogenesis, the fertilized zygote divides through a 4 and 8 cell stage, to become a morula (16 cells). The cells in the outer layer of the morula form attachments to each other and function as a trophoblast, while the inner cells are destined to become the ICM of the blastocyst at around days 3.5 of gestation.(30, 31) The *in vivo* blastocyst procedes through gastrulation, the germ layers are formed, and the embryo is implanted.

At the time of implantation, three cell types exist in the embryo: trophoectoderm, which eventually becomes the fetal chorion and placental tissue, primitive endoderm, that will become the yolk sac cavity and exocoelem, and an ICM that contains the pluripotent epithelial cells that become ectoderm, mesoderm, and endoderm. The ectodermal cells are neural crest progenitors. The mesodermal cells are endothelial, hematopoietic, myocardial, bone, cartilage, and muscle precursors. The endodermal cells eventually develop into the liver and pancreas.(28)

During normal *in-vivo* embryogenesis, the mesoderm is a sheet of cells between the endo and ectoderm. Differentiation of cells within the mesoderm leads to formation of blood islands, or clumps of epithelioid cells. The outer layer of the blood island forms endothelial cells, while the inner cells are destined to be hematopoietic cells. Because of their close proximity, hematopoietic and endothelial cells may originate from a common hemangioblastic precursor.(24, 32-34)

Cellular markers identify ES cell lineage precursors

A wide variety of surface proteins expressed by stem cells have a role in cell signaling, adhesion and migration of the stem cell to an appropriate microenvironment that fosters specific lineage development.(28, 29) Surface proteins have been characterized with respect to structure, function, expression, and effect on embryogenesis *in vivo*; they may be used to identify specific lineage precursor cells. Undifferentiated totipotent stem cells express the transcription regulator oct4, which dictates ES cell self-renewal and limits differentiation, while common pluripotent precursors to the endothelial and hematopoietic lineages express Flk1 and c-Kit (CD117). (35-37) Differentiation of this common precursor gives rise to

endothelial cells expressing VE-cadherin (CD144) and Flt1, and lymphoid cells, expressing CD45.

Oct4: A POU (Pit-Oct-Unc) Transcription Regulator& Marker Common to Early ES Cells

Oct4 (Figure 2) is a 352 amino acid protein in the class V family of POU proteins. The POU transcription regulators are DNA binding proteins that activate transcription of target genes containing the octameric sequence motif AGTCAAAT. The POU domain is bipartite: it contains two independent subdomains: a 75 amino acid amino-terminal POU-specific (POUs) and a 60 amino acid carboxy-terminal POU homeo-domain (POUhd) connected by a flexible linker that varies in length. Two subdomains of oct4 can bind DNA, independently of each other, through a helix-turn-helix structure, or they can form homo- or hetero-dimers that bind specific binding motifs: Palindromic oct-factor recognition Elements (PORE) or More PORE (MORE).(37)·(38) The amino-terminal domain of oct4 has a role in transactivation, as does the carboxy-terminal domain. Additionally, the carboxy-terminal domain is cell-type-specific and regulated by phosphorylation.(35) This specificity may limit oct4 to action on only specific cell types or stages of differentiation in embryogenesis – an important regulator function of the POU family.(35, 39)

POU transactivation often occurs with the help of co-factors. The mechanism for this action is not well understood, but POU transactivation appears to occur when the POU factor binds remotely from the target gene and is bridged by transcription factor co-activators. Adenovirus E1A expresses an oncoprotein which acts as a co-factor for oct4.(35, 37, 40)

Oct4 is expressed in pluripotent cells (germ line and stem cells) both *in vivo* and *in vitro*; down regulation of the oct4 gene expression leads to loss of the cell's totipotency and

terminal differentiation. Expression of oct 4 in the pre-implantation embryo occurs at one or two cell cycles after fertilization (8 cell stage), and transcription levels increase through the morula stage. Oct4 is expressed in the murine blastocyst and inner cell mass of the embryo. Later embryos express oct4 in the primordial germ cells, resulting in a resurgence of the gene's expression in later cell cultures. Constant levels of expression of oct4 maintain ES cells in a pluripotent state and changes in its expression drive cells to differentiate into endoderm, mesoderm, and trophoectoderm.(36) Cells in the oct4 $^{-/-}$ embryo remain totipotent, but differentiate only into trophoectoderm cells.(35, 40) Oct4 $^{-/-}$ embryos die at the time of implantation due to the lack of formation of and ICM.

C-Kit: SCF Receptor and Marker common to Hematopoietic Precursors:

Nearly all pluripotent hemangioblasts express c-Kit, (Figure 3) the receptor for SCF, which is responsible for proliferation and differentiation and of stem cells into hematopoetic cell lineages.(41, 42) The *c*-Kit proto-oncogene encodes a type III transmembrane receptor tyrosine kinase. This family includes platelet derived growth factor (PDGF), Flk1**,** Flt1**,** an the immunoglobulin superfamily.(42, 43) C-Kit contains 5 immunoglobulin-like domains (I-V) in its extracellular portion that make up the SCF binding domain and the c-Kit dimerization domain. It also has a transmembrane portion, and a split cytoplasmic kinase domain. This split of the cytoplasmic domain confers ATP-binding activation on one intracellular kinase region and phosphotransferase activity on the other region.(42, 43) Cleavage of c-Kit at the membrane-spanning region leads to the formation of soluble c-Kit, which is capable of binding SCF, and dimerizing with other c-Kit proteins. Two isoforms of

the protein are formed from alternative splicing; the functions of these two isoforms have not been determined.(42)

In vivo expression of c-Kit begins in the fetal yolk sac, liver and bone marrow, along paths of cellular migration, indicating a role in cell migration. It appears by day 8 in the yolk sac, but its expression shifts to the fetal liver by day 10. As hematopoiesis shifts from the yolk sac to the fetal liver and bone marrow, the expression of c-Kit drops off. It is also expressed by primitive T and B lymphocytes in the thymus, but its expression is lost as these cells reach terminal maturation.(41, 42) (Figure 1B)

Binding of c-Kit by SCF leads to homodimerization of the receptor and intermolecular tyrosine phosphorylation of the receptor, opening docking sites in the extracellular protein for SH2-containing signal transduction molecules.(42) The interaction of c-Kit and SCF is necessary to push HSC into the cell cycle, and sustained self-renewal of HSC is thought to be SCF dependent.(42, 44, 45) Additionally, SCF has been shown to act in synergy with IL-6, G-CSF, and IL-11 to stimulate "dormant" stem cells into blast colonies.(45)

CD45: Leukocyte Common Antigen, a Marker Common to Lymphoid Lineage

Leukocyte common antigen (LCA) or CD45 (Figure 4) is a leukocyte-specific transmembrane protein expressed on hematopoietic cells that is a member of the tyrosine phosphatase family.(46, 47) It is a 1,120 – 1,281 amino acid protein with a single transmembrane domain. CD45 is a type 1 transmembrane protein with a large extracellular glycosylated domain of 391 – 552 amino acids, and a long cytoplasmic tail (~700 amino acids) that possesses two phosphatase domains – one of which has been shown to be active.(48) The cytoplasmic region of this protein is highly conserved between species. Variable splicing of

the three exons, located in the extracellular portion of the protein, yields the formation of three isoforms, CD45RA, CD45RB, and CD45RC, and the CD45RO form, which has no extracellular domain. T-cells and B-cells have been shown to express various isoforms, with the particular isoform expressed determined by cytokine stimulation and stage of activation or differentiation of the respective cell type. (48, 49)

CD45 functions in the regulation of T-cell development, thymocyte selection and apoptosis and signal transmission from cytokine receptors and the **T-cell receptor** (TCR).(46, 48) CD45 knock-out mice have demonstrated defective thymocyte development through two pathways: inhibition of transition from double negative thymocytes to double positive cells, or failure of selection of single positive cells from double negative cells, and an increased rate of apoptosis. Additionally, these mice show decreased numbers of mature T-cells and no response to T and B cell antigen-receptor-mediated activity. (46) Interestingly, when only the intracellular domain is present, the protein's tyrosine phosphatase activity remains. (49)

Common ligands of CD45 are the mannose receptor, which functions in antigen uptake and presentation, and galectin I, a lectin that functions in apoptosis. Additionally, CD45 can bind molecules (Thy1, CD2, LFA1, the TCR complex, and CD4) on the cell's surface that is expressing CD45, resulting in cytokine expression and homodimerization.

Flk1, Flt1 & VE-cadherin: Markers of the Common Hemangioblastic Precursor and Endothelial Lineage Cells

Hemangioblastic precursors give rise to cells of the endothelioid lineage as well as lymphoid lineage. Hemangioblasts are thought to express VE-cadherin *(*Figure 5), as well as the vascular endothelial growth factor receptors 1 & 2: Flt1 and Flk1 (Figure 6). VE-cadherin and Flk1 are markers of a common precursor to endothelial and hematopoietic precursor cells;

cells that lose VE-cadherin expression, continue to express Flk1 and begin to express CD45 are considered to be committed to lymphoid lineage.(22) VE-cadherin is an endothelial-cell-specific marker that mediates homophilic adhesion at endothelial adherens junctions, and directs vasculature development in the embryo and angiogenesis in the adult. (50) Flk1 is expressed by vascular precursor cells in the lateral plate and mesoderm.(22)

It has been shown that *in vitro* ES cell differentiation proceeds through $Flk1^{+}$VE-$cadherin^{-}$ and $Flk1^{+}$VE-$cadherin^{+}$ phenotypic stages before the commitment to endothelial and hematopoietic lineages.(22) (Figure 1B) Further, it has been suggested that $Flk1^{+}$VE-$cadherin^{+}$ cells are endothelial precursors as well as an early precursor stage to definitive hematopoietic cells which are $CD45^{+}$.(22) The mesoderm is a sheet of tissue that begins as an endothelial-cadherin-expressing epithelial structure (E-$cadherin^{+}Flk1^{-}PDGFR\alpha^{-}$) and progresses to mesodermal cells expressing fetal liver kinase 1 (Flk1), becoming E-$cadherin^{-}$ $Flk1^{+}$VE-$cadherin^{-}$. Flk1 is the earliest *in vivo* marker for hemangioblastic cell lineages(24) and is up-regulated in the paraxial and lateral mesoderm to form endothelial precursors. (51, 52) $Flk1^{+}$ cells are located in the embryonic yolk sac, and have been recognized in blood islands as clusters of hematopoietic cells surrounded by endothelium. Hemangioblasts (common precursor) are $Flk1^{+}$VE-$cadherin^{+}CD45^{-}$, while the hemopoietic precursors are phenotypically $CD45^{+}$c-Kit^{+} and as differentiation and maturation progresses, become $CD45^{+}$c-Kit^{-}.(22)

VE-cadherin is a calcium dependent adhesion molecule that is constitutively expressed by endothelial cells. It is localized to the interendothelial junctions, and plays a role in vascular assembly, adhesion, morphogenesis, cell sorting and migration, and cytoskeletal organization. It is expressed as early as day 7.5 dpc in murine embryos. VE-cadherin

structure includes a cytoplasmic domain that binds β catenin, plakoglobin, and p120, intracellular proteins which anchor VE-cadherin to the actin cytoskeleton. (Figure 5) These proteins interact with intermediate filaments, and function in cell signaling. VE-cadherin has a single transmembrane region and an extracellular region containing 5 calcium binding domains of 110 amino acids each. Binding of βcatenin links VE-cadherin to actin, and participates in the Wnt signaling pathway, while binding of p120 and plakoglobin serves to recruit desmoplakin (DP) to the cell surface where it co-localizes with the intermediate filament protein vimentin and forms an adhesive complex.(53)

VE-cadherin functions in vasculogenesis and remodeling. It is expressed *in vivo* by cells in the mesoderm and the yolk sac mesenchyme, where it is restricted to the outer layers of the blood islands that give rise to endothelial cells. VE-cadherin $^{-/-}$ murine embryos die by day 9.5 dpc due to severe vascular defects. Without binding VE-cadherin, Flk1 and Flt1 cannot activate antiapoptotic cascades in the developing embryo.(54) Additionally, VE-cadherin plays a role in vascular permeability and cell migration from blood vessels.(54)

The vascular endothelial growth factor (VEGF) receptors Flk1 and Flt1 function in angiogenesis and vasculogenesis. Flk1 and Flt1 are tyrosine kinase receptors containing 7 immunoglobulin-like domains in their extracellular portion, with split tyrosine kinase domains intracellularly (Figure 6). Flt and Flk are activated when bound by one of the 5 isoforms of VEGF, but differ in ultimate function. VEGF, produced *in vivo* by the visceral endoderm of the yolk sac, as well as a multitude of other cell types in the mature animal, regulates angiogenesis and vasculogenesis through Flt1 and Flk1.

Disruption of genes that express Flt1 and Flk1 leads to abnormal development of the cardiovascular system and its cellular components. Flk1 $^{-/-}$ embryos die due to lack of

differentiation of endothelial cells and subsequent formation of the vascular system. Flt1 knockout embryos demonstrate impaired development of blood vessels. Flk1, when activated by VEGF, plays a role in migration and differentiation of endothelial cell precursors from the posterior primitive streak to the yolk sac. Flt1 promotes cell migration, and possibly plays a role in cell-cell or cell-matrix interactions responsible for embryonic cell migration to tissues where their differentiation will proceed normally.(22, 24, 55, 56)

Multiple members of the VEGF family of proteins can activate and tranduce signals through Flk1 and Flt1**,** however, only VEGF-A can actively bind and activate both Flk1 and Flt1. VEGF binding of Flk1 leads to activation of MAP kinases, endothelial cell division and migration. When bound by VEGF, both Flk1 and Flt1 induce expression of proteases which break down the basement membrane of blood vessels, one of the first steps in angiogenesis during wound healing.

VEGF is expressed during periods of hypoxia. Flt1 and Flk1 are expressed in response to elevation in VEGF, predominantly by endothelial cells, however other cell types exhibit low level expression of Flk1 and Flt1: Flt1 is expressed by trophoblasts, monocytes, and renal mesenchymal cells(55, 57) and Flk1 is expressed by hematopoietic stem cells, megakaryocytes and retinal progenitor cells.(55)

In-vitro Embryonic Stem Cell Culture Techniques: Attached –vs- Suspended

Several methods have been successfully used to culture and promote differentiation of ES cells *in vitro*. Micro-environment, cytokines, and growth factors are known to effect lineage choice and differentiation of ES cells *in-vitro*.(7, 32, 58-60) Commitment of totipotent ES cell to a cell lineage occurs at a specific period during the differentiation process,

and alterations in this commitment may result in alterations of phenotype and function of the terminally differentiated cell population.(32)

A single ES cell may give rise to one **embryoid body (EB)**, or multiple cells may adhere together to form an EB.(32) EBs are a loosely adhered conglomerate of cells with an inherent three dimensional structure that appears to be important in cell to cell interactions and control of cell division and differentiation within the EB. Studies have demonstrated that there is sufficient nutrient diffusion through the EB to address the needs of the inner cells of the mass.(32) *In-vivo* cell migration is believed to be intrinsically necessary for normal hematopoiesis.(61) The advantage of EB formation is to allow surrounding cells and the microenvironment to exert their known and unknown developmental signals on one another during crucial early stages of differentiation. The disadvantage to EB formation is the technical difficulty in handling the cultures, and observation of cells within the cell mass.(62) EB aggregation has also been shown to decrease the cell yield within a culture, so that this system is not feasible for the the generation of large numbers of cells, such as would be needed for therapeutic purposes.(32)

EBs may be cultured either attached to a tissue culture plate, adhered to a monolayer of stromal cells, or in suspension, such as occurs in a hanging drop or a methyl-cellulose medium. Co-culture techniques have been developed to address the technical difficulties presented by the hanging drop EB formation culture techniques. One such co-culture technique is the OP9 stromal cell line. The OP-9 stromal cell co-culture system selectively promotes differentiation of ES cells to lymphohematopoietic cells from embryonic stem cells (ESC).(63-68) The OP9 cell line is from newborn calavaria of (C57BL/6X C3H) F2-op/op mice that contain a mutated M-CSF gene and does not express M-CSF (inhibitory to all cell

lineages except macrophages). Additionally, the OP9 stromal cells produce SCF, which promotes differentiation of mesodermal and hematopoietic precursor cells. This co-culture system is relatively easy to use, and avoids the occasionally cumbersome formation, and maintenance, of EBs(61, 63-72)

It is evident from many studies that hanging drops and attached co-cultures provide 2 very different microenvironments that affect the resulting *in vitro* cell population. In a comparison of an adhered co-culture system to a hanging drop EB formation system, it is apparent that the two techniques give rise to vastly different cell populations. The rate of cellular proliferation appears much higher in an adhered cell culture; however, the formation of hematopoietic precursors is much reduced in this system as compared to the hanging drop technique.(32) It appears that Flk1+ cell commitment to the hematopoietic lineage occurs between days 3 and 4 in EB cultures.(22) Furthermore, attachment to the culture plate before day 4 may decrease hematopoietic precursor development. Collectively, this confirms that the three dimensional EB must possess a cell signal that affects commitment that the attached, two dimensional cultures do not express.(32) EB formation appears to give rise to more hematopoietic precursors, whereas attached co-culture techniques partially block differentiation of hematopoietic precursors. (32) Others have shown that with the addition of certain cytokines such as Notch ligand, (73) attached co-culture techniques can be used to generate a significantly greater percentage of lymphoid precursor cells.

Estrogen hormone: effects on differentiation

Estrogen, 17β estradiol, belongs to a family of steroid hormones that regulate and sustain female sexual development and reproductive function. During specific stages of development and maturity, this hormone stimulates tissue growth and cellular hypertrophy through promotion of cell proliferation, gene transcription and translation of proteins. Estrogen directly or indirectly affects all body systems, including the immune system, in all species. The effects of estrogen on cell-mediated and humoral immunity and immune cell function have been explored. Although some details of the mechanisms of these effects remain unclear, the effects are well-documented.(74-76) Estrogenic compounds may be hormonal, environmental, or synthetic in origin, and have been shown to influence development of the immune system (thymic and bone marrow development), function of T & B cells, cytokine production, and immune cell apoptosis(74-82)

There is a noted preponderance of autoimmune diseases in women: the prevalence (female : male) is 2:1 for Rheumatoid Arthritis, 9:1 for Systemic Lupus Erythematous, 9:1 for Primary Billiary Cirrhosis, 5:1 for Grave's Disease, and a 40:1 incidence in women of Hashimoto's Thyroiditis.(74) This prevalence of autoimmune dysfunction in the female is also present in mice. Specific hybrid mice which are models of autoimmune disorders similar to SLE, Lupus –like syndromes, and NOD mice all have a higher rate and faster progression of each disease in affected female mice compared to male mice.(74)

Estrogen exposure may be from several sources: endogenous hormone production, environmental sources, such as phytoestrogens in the diet (legumes, soybeans, clover, grains, fruits and vegetables containing flavones, isoflavones, and coumestan), mycotoxins (fumonisin), run-off contamination of water sources, house-hold and industrial products

(synthetic chemicals: DDT, plasticizers: bisphenol A, and industrial chemicals: polychlorinated biphenyls), and breast milk.(83-85) Estrogenic agents, regardless of whether they possess the phenolic ring structure of estrogen, bind estrogen receptors, which are present in many tissues of the body, and exert their estrogenic effects. Environmental estrogens are considered endocrine disrupting chemicals, and have been associated with developmental, reproductive and health problems in wildlife and lab-animals. Their low-level and cumulative effects on humans are yet to be determined.(85) Two estrogen receptors (ER) have been identified to date, ERα and ERβ. (Figure 7) ERs are members of the class I nuclear hormone receptor super family. They are predominantly nuclear receptors; however, a small portion of ERα has been shown to be membrane bound.(86) These receptors contain 6 functional domains. The amino-terminal A/B domain is least conserved between the two types of ER. The C-domain is a highly conserved domain containing two zinc fingers which form a helix-loop-helix motif and functions in binding the receptor to target DNA **estrogen response elements (ERE)**. The D-domain signals for nuclear localization of the ER. The E-domain is the ligand binding domain, and the F-domain is the carboxy-terminal domain that modulates activity when complexed with ligands. It is thought this later domain also functions in dimerization of the ER, as well as interacting with other co-regulatory transcription factors such as AP-1.(87, 88)

ERα is a 599 amino acid protein, while ERβ is a slightly smaller protein (485 amino acids). They share common structure of the nuclear hormone receptors, but have differences in their transactivational activities. Some anti-hormonal ligands (Tamoxifen) that block ERα actually activate ERβ.(87) Additionally, the two receptors appear to be expressed at varying levels in different tissues. ERα is expressed in ovarian thecal cells, uterus, mammary gland,

testis, pituitary, liver, kidney, heart and skeletal muscle. ERβ is located in the granulosa cells of developing follicles in the ovary, and prostate. Both receptors are present in the epididymis, thyroid, adrenals, bone and brain. There are species differences between expressions of the two receptors as well: ERβ exhibits low expression in certain tissues of mice and rats, while it is present in higher levels in humans.(88)

The inactive form of the ER is complexed with heat shock proteins and other proteins in the cell cytoplasm or nucleus.(89, 90) (Figure 8) A transformation in the receptor occurs when it binds its ligand, 17β estradiol, resulting in the formation of dimers (homodimers of ERα or heterodimers of ERα&β). These dimers associate with the cellular DNA at sequences containing a 15 bp inverted palindrome in estrogen sensitive regulatory regions of target genes, the ERE.(91) The complex can also interact with basal transcription factors, co-regulators and other transcription factors to regulate transcription of the target gene within the cell.

In addition to traditional receptor-ligand mediated pathways for mediating action, the ER has been shown to be able to activate gene transcription by ligand-independent means (increased cellular cAMP, epidermal growth factor, & dopamine), as well as by interacting with other unrelated DNA-bound transcription factors such as the AP-1 complex. (92, 93) Further studies have shown that certain factors can inhibit ER-linked gene transcription, such as Breast Cancer 1 factor (BRCA1).(94) ER occasionally acts as an anti-oncogenic agent as well. It has been shown to prevent p53 deactivation in certain cell types, which may play a role in control of the cell cycle and provide protection from neoplastic changes in certain cell types.(95) With respect to breast cancer, it has been theorized that estrogen has a mitogenic effect, and that ER activity mediates breast cancer. Recently, it was demonstrated that when

estrogen levels were high, uncontrolled cell growth did not occur. This may be due to overwhelming of the ER which inhibits estrogen from exerting its mitogenic effect at higher doses.(96)

The thymus is the main organ of T-cell development and maturation. Prothymocytes from the bone marrow enter the thymus, undergo maturation and selection or apoptosis, and are released into the periphery as functional T-cells. (Figure 1A) Stromal tissue of the thymus (epithelial cells, dendritic cells, and macrophages) plays a key role in this process. ERα & β have both been identified in thymocytes and thymic stromal tissue.(88) It is believed that estrogens exert their effects on thymic and T-cell development through binding to ERs located in the nucleus of the cells, causing gene transcription and protein expression.(81, 97)

Ligand-binding of ERs results in estrogenic effects on immune cells and effector cells of the immune system including changes in thymic size and thymocyte phenotype.(10, 81, 82, 88, 98) Studies using estrogen receptor knock-out (ERKO) mice have helped elucidate the function of ERα and the effects of estrogen on development of immune tissues. Estradiol induces thymic atrophy and cellular phenotypical alterations in mice. Chimeric mouse studies have indicated that estrogen receptors in the thymic stromal tissue are necessary for development of normal sized thymi, as opposed to ER in the hematopoietic elements of the thymus. This indicates that the final T-cell populations are perhaps affected by estrogens indirectly through estrogenic effects on supporting stromal elements.

ERα, found primarily in the thymic cortico-medullary junction and subcapsular region(81) where prothymocytes enter the thymus from the bloodstream, proliferate and differentiate respectively, provides regulatory signals for entry and maturation of early

thymocytes. Additionally, ERα activation in other stromal tissues, such as the bone marrow, may affect prothymocytes at a much earlier stage of development.

ERKO mice have smaller thymi than wild type (WT) mice with similar thymocyte phenotypes and distributions. These mice are able to respond to exogenous estradiol, but to a lesser extent than WT mice. It is possible that the small amount of thymic atrophy that occurs in response to exogenous estrogens in ERKO mice may be due to an alternative pathway, another receptor type or alternative signaling through a receptor independent pathway.(81) Indeed, an ERβ and an ERR (estrogen related receptor family) have been identified.(88)

ERKO mice also do not exhibit the phenotypic ratio changes noted in wild type mice upon treatment with estrogen. Estrogen normally affects the phenotypic switch of T cells within the thymus: CD4+CD8+ (double positive) thymocytes become selectively CD4+CD8- or CD4-CD8+ during the normal thymocyte maturation process.(74) ERKO mice fail to undergo this selection, substantiating the theory that as well as thymic atrophy, estrogens affect the phenotype of cells released from the thymus, possibly affecting immune system function through basic cell population changes.

Estrogens also affect thymic and splenic lymphocyte cytokine expression. Studies measuring IFN-γ, IL-2, and IL-4 production by lymphocytes from the thymus and spleen have shown that IFN-γ and IL-2 production is effectively up-regulated by estrogen treatment, while IL-4 production is decreased. These effects were seen in both genders of mice, but were more pronounced in intact female mice.(75, 76)

The effect of chronic exposure to estrogens on cytokine expression by T cells and therefore function of lymphocytes is yet to be determined. Increased levels of expression of IFNγ and IL-2 in stimulated thymic and splenic lymphocytes suggest that estrogens can

significantly modify both lymphoid cell function and population.(76) It has been demonstrated that estrogens increase the expression of interferon gamma (IFNγ) mRNA expression by conconavalin A stimulated splenic lymphocytes.(75) Additionally, estrogen increased expression of *CD80*, a co-stimulatory factor found on B cells and dendritic cells.(75)

Diethylstilbestrol, a synthetic estrogen with potent estrogenic activity

Diethylstilbestrol (DES) is a synthetic non-steroidal estrogenic compound that has similar estrogenic potency and action as the natural hormone 17β estradiol; it has been shown to have similar effects as estrogen on the immune system and function in animals.(75, 76) DES was developed in the 1940's and used in human medicine for estrogen replacement therapy, lactation suppression, contraception, pregnancy maintenance and prostate cancer therapy. In agriculture, DES has been used in the caponization of chickens, and as a growth promotant in feed-lot cattle. The discovery that *in-utero* exposure to DES was strongly associated with an increased incidence of vaginal clear-cell adenocarcinoma in female offspring, reproductive tract abnormalities in both male and female off-spring, an increased incidence of infertility and miscarriages in women who were exposed to DES *in-utero,* and an increased risk of prostate and testicular cancer in male offspring led to the ban of its use and an increasing concern about environmental exposure. (13, 16)

Currently, DES is used to treat estrogen-responsive prostatic tumors in humans, (99) for hormone replacement therapy during menopause, treatment of osteoporosis, and has been reported to be useful in treating benign prostatic hypertrophy in dogs.(99-101) In livestock, estrogens were used as growth promotants in the United States until 1976, when concern of meat residue and human exposure led to regulation of the hormone in food animals.

Steroid hormones function as gene transcription activation factors at all stages of development – from embryogenesis to adulthood. A critical step in cellular differentiation is the programming of differential regulation of genes; genes are turned on and off sequentially, or when the appropriate stimulation is applied to the cell at all stages of life. An example of this is the memory portion of the immune system. DNA methylation and demethylation at gene promoter sites is one mechanism for this programming.(85) DES (and estrogen) alter DNA methylation, particularly at promoter regions of several estrogen-responsive genes(85) (Bland, J, 2000) which affects mRNA and protein synthesis in the cell nucleus, and alters cytokine production. It is possible that early exposure to estrogens during embryogenesis leads to an irreversible signal that could prime the immune system for malfunction at later times during high endogenous estrogen levels, such as puberty and adulthood.(85)

DES, like estrogen, has also been shown to affect thymic development(77, 78) and alter IFNγ secretion in mice.(75, 76) Mice exposed to DES *in utero* (gd 10 – 16), harvested on gd 18, manifested thymic atrophy as decreased thymic cellularity (decreased thymic weights and cell yield per thymus). Intrathymic differentiation continued as demonstrated by normal percentages of CD4 and CD8 expressing cells. A small increase in the number of $CD4^{-}CD8^{-}$ cells was noted in this same study. (77, 78)

Prenatal mice exposed to DES *in utero* (d 14 – 17 gestation) have been shown to express normal levels of IFNγ; however, subsequent exposure to DES up to more than a year later (12 – 16 months of age) revealed a pronounced increase in the number of IFNγ secreting cells and a significantly higher IFNγ secretion in stimulated lymphocytes. This suggests that estrogens somehow alter the "cytokine programming" of lymphocytes when exposed to DES *in-utero*. (75, 76)

To date, there have been no studies characterizing the effects of DES and estrogens on the emergence of lymphoid lineage HSC in ES cell cultures. Additionally, ER expression and cellular response in ES cells has not been documented. In this study, it was hypothesized that early exposure of ES cells to DES would alter the development of the lymphoid lineage, as manifested by alterations in expression of CD45. Stem cell differentiation into HSC lineages in the face of treatment with DES were investigated utilizing an OP9 co-culture system, and an embryoid body hanging drop culture system.

Results of this study indicate the presence of estrogen receptors in differentiating ES cells as early as day three *in-vitro* (ERβ) until day 16 (ERα). DES appeared to suppress expression of mRNA for markers of common hemangioblastic and hematopoietic precursors, as well as endothelial lineage markers, but had no effect on lymphoid lineage marker mRNA expression before day 16. These effects coincided with the expression of ERα. The enduring effects of DES exposure *in-utero* may not be manifest in this ES model, or may occur at later stages of differentiation or in selected subpopulations of $CD45^{+}$ cells.

Figure 2: Structure of *oct4*, a POU transcription regulator

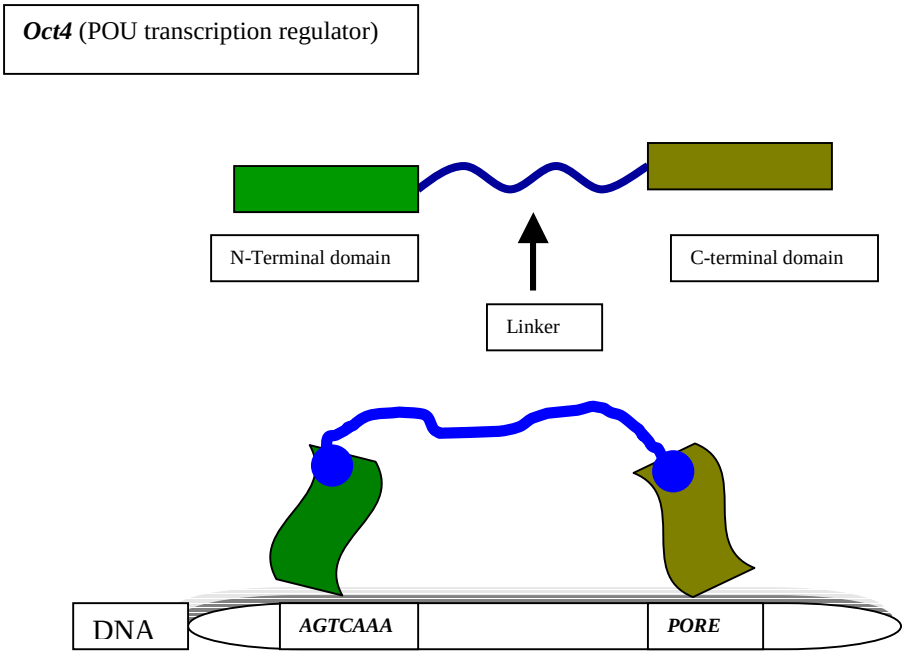


Figure 3: Structure of *c-Kit*

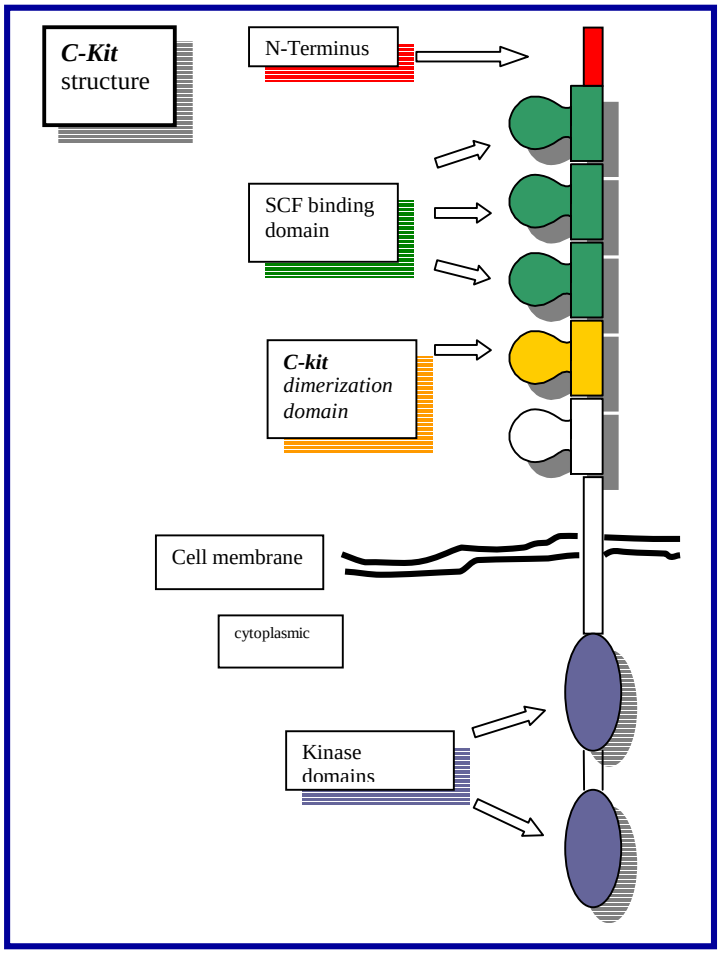


FIGURE 4: Structure of CD45, or Leukocyte Common Antigen

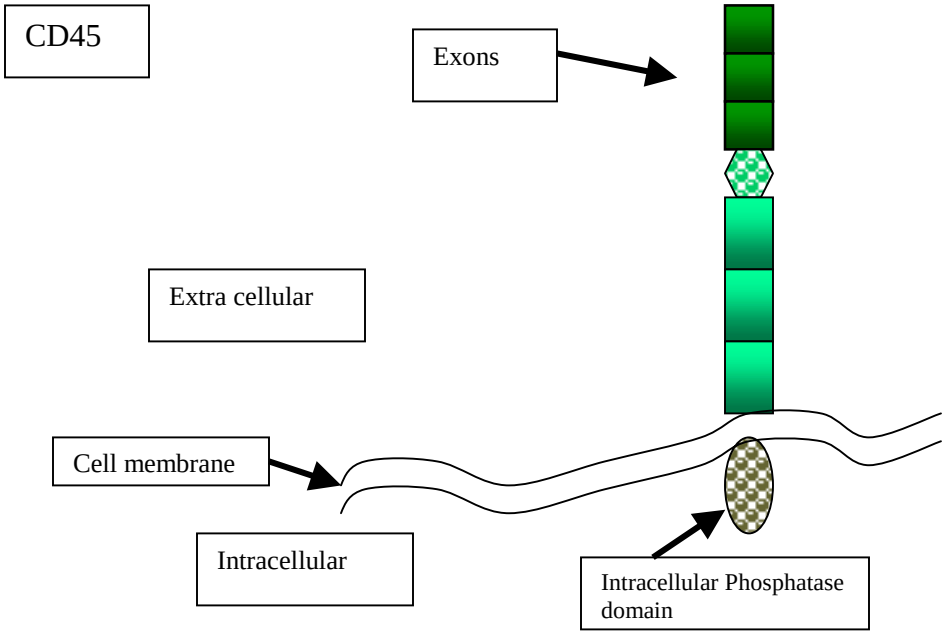


Figure 5: *VE-cadherin* structure and functional domains

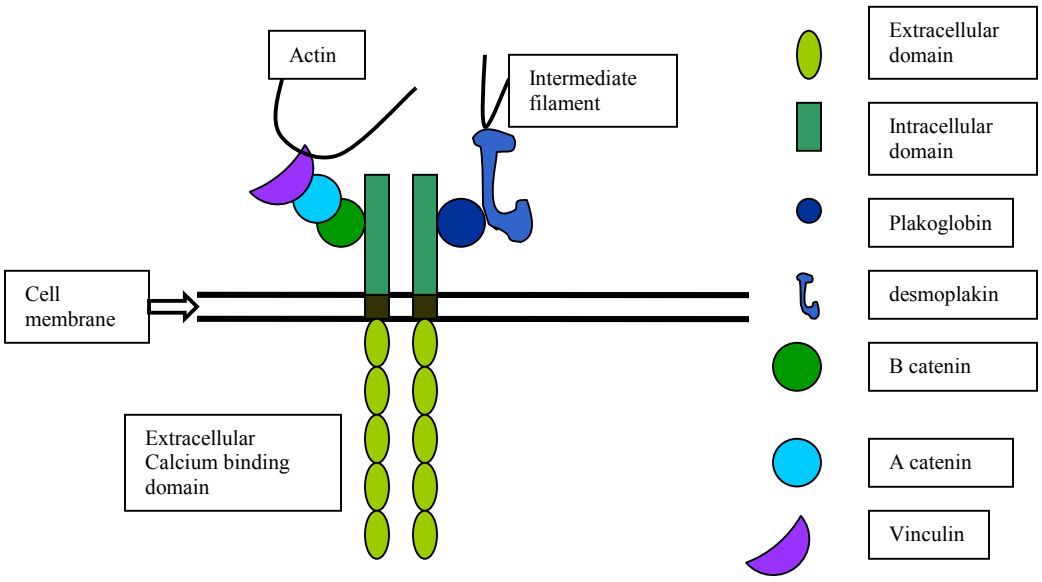


Figure 6: VEGF receptor structure and functional domains

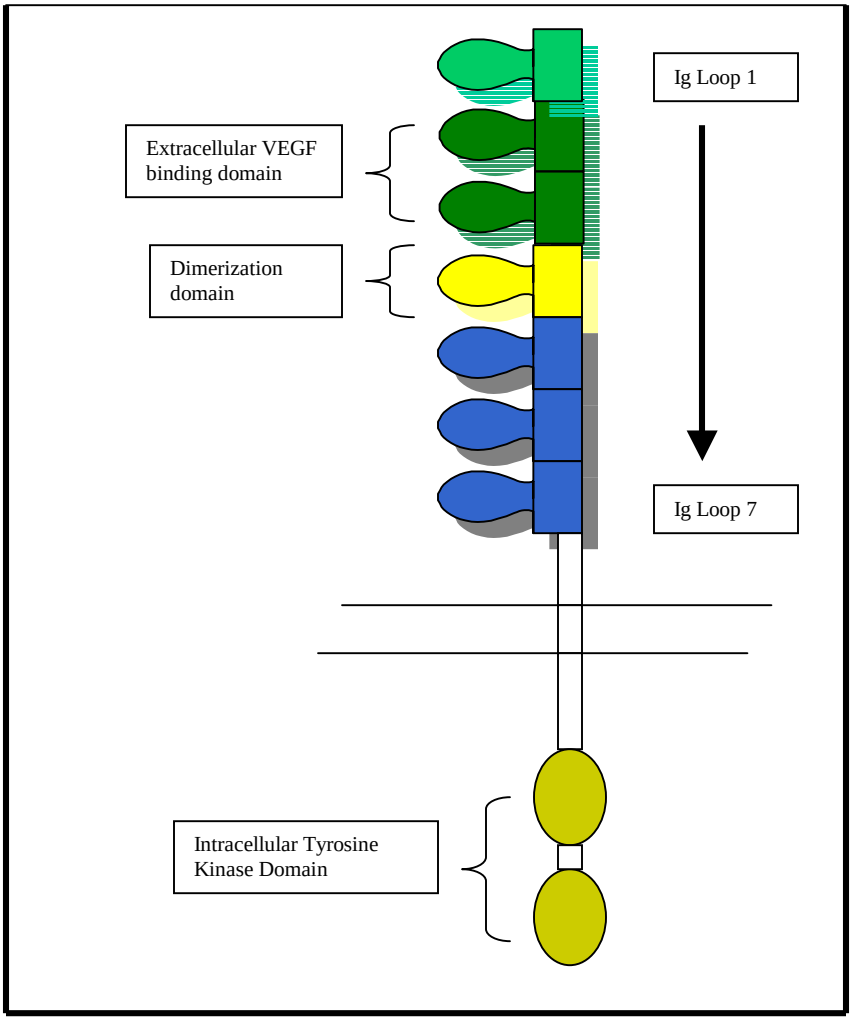


Figure 7: Estrogen Receptor Structure

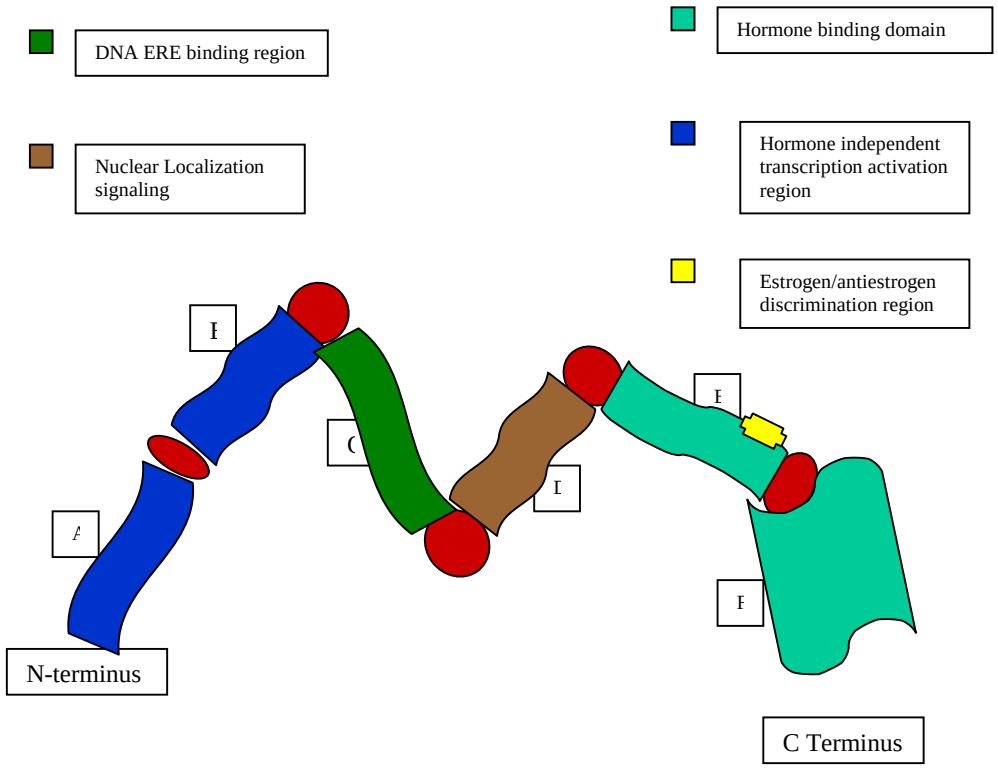
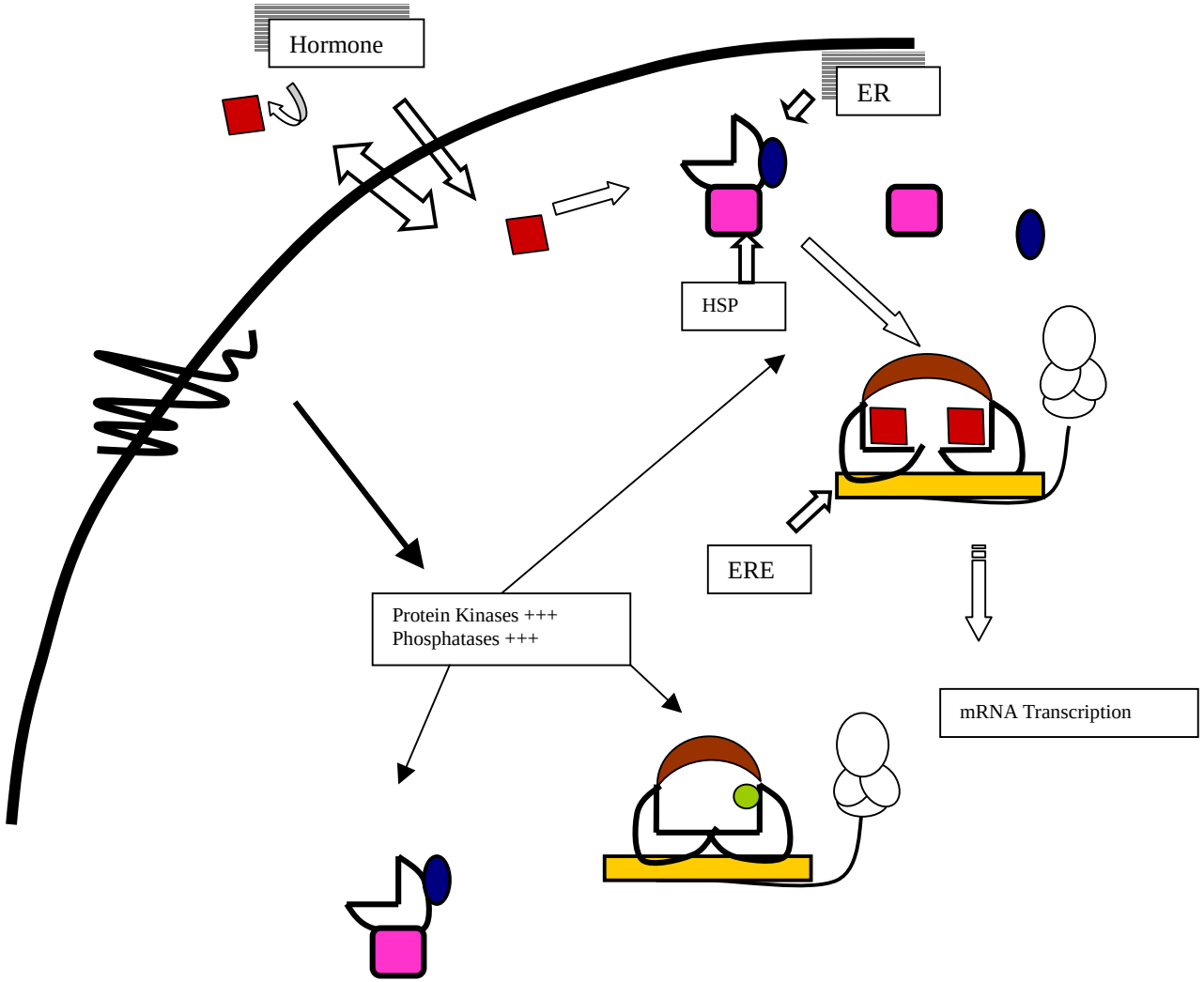


Figure 8: Estrogen Receptor Function



CHAPTER II:

MATERIALS AND METHODS

Part One: ES Culture on STO feeder layer:

Preparation of STO feeder cells & ES cells

STO feeder layers were prepared as follows. STOs (SNL2 cells, ATCC) were thawed and plated $2X10^6$ cells per 75 cm^2 flask in Dulbecco's Modification of Eagle's Medium, DMEM (Fisher) plus 10% fetal bovine serum (FBS) (Fisher), 50 µg/ml gentamicin (Invitrogen), complete medium, and incubated at 37°C, 5% CO_2. When the cells were 90% confluent, the flask was trypsinized (0.05% trypsin in 0.02% EDTA) (Fisher) and allowed to incubate for 5 minutes (37°C, 5% CO_2). The cells were transferred to two 10 cm tissue culture dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ) and into a new 75 cm^2 flask; the trypsin was quenched with STO medium. To arrest the STO feeder cells when each new plate was ~90% confluent, mitomycin C (Sigma, M4287), 10µg/ml final concentration, was added to each 10 cm plate and incubated for 2 hours (37°C). Each plate was rinsed with 10 ml PBS (Dulbecco's PBS, Ca/MG Free, Fisher), and allowed to recover overnight in STO medium before plating ES cells on the feeder layer.

Murine ES cells (ES p3, ATCC) were prepared for differentiation as follows. ES cells were thawed (2 min, 37° water bath) and centrifuged in ES medium (-LIF), 200 X G X 10 minutes, to rid the cells of freezing medium (complete medium as above plus 7.5% DMSO). Three million cells were added to each 10 cm STO feeder layer. Leukemia inhibitory factor (Chemicon ESG 1107), 1000 U/ml final concentration, was added to the plate to prevent differentiation. The cells were allowed to divide until ~60% confluent and then transferred to their respective culture conditions: OP9 co-culture or hanging drops.

Part Two: OP9 co-cultures:

Preparation of OP9 monolayers

Murine OP9 stromal cells (ATCC CRL-2749) were thawed (2 minutes, 37°C), placed in a tissue culture flask (Costar, Corning Inc., Corning, NY) containing 10 ml Alpha Modification of Eagle's Medium (AMEM) (Cellgro) with 20% fetal bovine serum (FBS) (Gibco, Invitrogen Corporation, Cat. # 16141), 2 mM L-glutamine, and gentamicin (10 mg/ml), and incubated (37°C, 5% CO_2) for three days, or until ~90% confluent. The resulting monolayer was split into three 75 cm^2 tissue culture flasks when 90% confluent. Passage was performed by trypsinization of the flasks for five minutes (0.025% trypsin and 0.05% EDTA), at 37°C, 5%CO_2. The trypsin was quenched using 10 ml OP9 AMEM medium. The cells were collected and equal volumes placed into three new recipient flasks. All flasks were incubated at the same conditions as above.

The 90% confluent flasks were once again trypsinized as above, and passage #3 OP9 cells were placed on 6 cm (3.4 X 10^4 cells/plate) and 10 cm (8.7 X 10^4 cells/plate) tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) and allowed to divide to a 90% confluent monlayer in preparation for co-culture with murine ES cells. A continuing cell population was maintained in 75 cm^2 tissue culture flasks, and passaged when ~90% confluent.

OP9 Co-culture of ES cells:

The OP9 co-culture of murine ES cells was performed following the protocol published by Cho et. al.(63) (See Figure 9) Murine ES cells were allowed to divide on STO

feeder cells as above until they reached 60% confluency. They were then plated on OP9 monolayers ($5X10^4$ ES cells per 10 cm OP9 monolayer). A stock solution of 200mM DES (Sigma-Aldrich Chemical, St. Louis, MO, 99% pure powder, Prod # D4628) in dimethylsulfoxide (DMSO) (Sigma Chemical, Prod. # D2650) was prepared. This solution was diluted 1000 fold with PBS and added to AMEM medium (final concentration $1 X 10^{-7}$ M DES in 0.1% DMSO) and applied to the appropriate plate. Treated plates received DES-containing medium, and untreated plates received 0.1% DMSO-containing medium. Every two days, the medium was changed on the plates: old medium was gently aspirated from the plate, and the medium replaced.

Trypsinization, RNA Harvest, and transfer to fresh OP9 monolayer: Day 5

After five days of differentiation, differentiating ES cells on the OP9 monolayer were trypsinized as above, and the ES cells transferred to a new OP9 monolayer. Each plate was rinsed gently with PBS. Trypsin (0.05%)/EDTA (0.02%) was added to each plate, and allowed to incubate for 5 minutes (37°C, 5%CO_2). DES treated plates were pooled on collection from trypsinization, as were the DMSO treated plates. All cells were removed from the plates by vigorous pipetting. Trypsinization was quenched with AMEM medium, each original plate rinsed, pooled samples transferred to a second plate, containing no monolayer, and allowed to incubate (30 minutes, 37°C) to allow the OP9 cells to settle and attach to the dish. The non-adherent ES cells were placed in a centrifuge tube and centrifuged (150 X G X 5 minutes). The resulting pellet of ES cells was resuspended in AMEM media, and viable cells counted. ES cells were plated ($5X10^5$ cells per 10 cm plate, +/- DES) on

fresh OP9 monolayers, and Flt3 ligand, 5 ng/ml, (PeproTech, Rocky Hill, NJ) was added to the medium of all plates to promote hematopoietic cell differentiation.

The remaining cells were harvested for RNA as follows: the cell suspension was centrifuged (150 X G X 5 minutes), resuspended gently in PBS, centrifuged again (same speed/time), and the PBS aspirated off. The resulting cell pellets were lysed with RLT lysis buffer (Quiagen Sciences, Maryland) containing 0.01% β-mercaptoethanol (βME) and the cellular contents homogenized using a QIA shredder mini spin column (Qiagen, Maryland, cat. # 79656) as per the protocol in the Qiagen RNeasy Mini Handbook, June 2001, 3rd edition. All homogenized cellular lysates were stored (-20°C) until further analysis.

Transfer to the 6 well OP9 tissue culture plates and RNA harvest: Day 8

Six OP9 monolayers were prepared by placing ~$7.2X10^4$ OP9 cells per well on a six-well tissue culture plate (Falcon 35-3046 multiwell 6 well, Becton Dickinson Labware, Franklin Lakes, NJ), and allowing them to divide to confluency (2 days). On day 8 the non-adherent population of differentiating ES cells in the plates created on day 5 was transferred to the monolayers in the 6 well plates. Each 10 cm plate was rinsed gently with the medium contained in the plate, followed by 2 mls of PBS. The medium and PBS retrieved from each plate were centrifuged (150 X G X 5 minutes), and the cell pellets resuspended in the appropriate premixed medium (AMEM +/- DES, + Flt3L, + rmIL7, 1 ng/ml, (R&D)). The loosely adhered cell contents of each 10 cm plate were applied to one well of the previously prepared six-well tissue culture plate. The remaining two wells of plates containing OP9 monolayers received only media, +/- DES (10^{-7} M, + Flt3L, 5 ng/ml, + rmIL-7, 1 ng/ml).

The remaining two 6 cm plates created on day 5, containing only OP9 monolayers were harvested for RNA by applying RLT-lysis buffer + 0.01% βME directly to the plate, and collecting the resulting suspension. The cellular contents were homogenized in a QIA shredder and stored as previously described.

The medium was again changed in the wells every two days: spent medium was collected into centrifuge tubes and centrifuged (150 X G X 5 minutes) and the resulting pellet resuspended in fresh treated or untreated medium +Flt3L (5 ng/ml) and IL7 (1 ng/ml), to promote lymphohematopoietic differentiation, and reapplied to same OP9 monolayer in the 6 well plate.

Final RNA harvest: Day 12

On day 12 all wells were harvested for RNA as follows: Each treated and untreated well was aggressively rinsed by briskly pipetting medium (2 mls) into the well to dislodge loosely adhered cells from the monolayer. The cells were collected into a centrifuge tube containing 8 mls PBS, and centrifuged (150 X G X 5 minutes). The media and PBS were aspirated from the pellet and RLT lysis buffer + 0.01% βME was added. The resulting suspension of cellular contents homogenized in a QIA shredder and stored as above. The remaining two wells containing simply OP9 cells were lysed directly in the well, shredded and stored as above.

Part Three: Hanging drops and Embryoid body formation

Hanging drop set up & EB formation: Day 0

ES cells, cultured in ES medium containing DMEM, 15% ES qualified FBS (Gibco), non-essential amino acids (1X, Fisher)), monothioglycerol (80 μg, Sigma), and gentamicin

(50 µg/ml) on STO feeder layers, were harvested with trypsin and EDTA when they reached 60% confluency, as above, and plated in hanging drops. A 10 cm plate containing ES cells and the STO feeder layer was trypsinized and gently rinsed with ES medium to preserve the ES cells in clumps. This volume was collected into a conical tube; the large clumps of ES cells were allowed to settle out, single STO and non-viable ES cells and debris in the supernatant that was subsequently discarded.

Vigorous resuspension of the settled clumps of cells with 5 ml of PBS created a single cell suspension. This suspension was plated on a fresh 10 cm plate and allowed to sit (30 minutes, 37°C, 5%CO_2) to allow remaining STO cells to attach by differential plating. The medium containing non-adherent ES cells was removed to a conical tube, and viable cells were counted. Drops of media (40µl/drop, ~500 ES cells/drop) were placed on the lid of a petri dish. The lid was quickly inverted and placed over the base, which was half filled with sterile water to maintain humidity and temperature within the plate environment. The drops were allowed to hang (37°C, 5% CO_2) undisturbed for three days. During this time period, the ES cells began differentiating and formed embryoid bodies within the drops.

Two aliquots of ES cells (5 X 10^5) were reserved for RNA harvest (day 0). These aliquots were centrifuged, the supernatant discarded, and RLT lysis buffer + 0.1%βME added to lyse the cells. The resulting cell contents were homogenized using the same procedure as described above.

24 well plate transfer and RNA Harvest: Day 3

Three days after placement in hanging drops, the EBs were transferred to 24 well plates (Falcon 351147 non-tissue culture treated multiwell plate, Becton Dickinson, Franklin

Lakes, NJ) and 2 samples of the EBs harvested for RNA. Six wells in the center of three 24 well plates were filled with ES media, in addition to two microfuge tubes. The EB from each hanging drop was aspirated into a wide-mouthed pipet tip and expelled into a well on the 24 well plate or one of the microfuge tubes. This process was performed sequentially until 15 EBs were placed in each well and each microfuge tube. This allowed enough EBs within a plate to have enough tissue (EBs) to harvest for RNA, as well as to ensure sampling of multiple types and sizes of EBs. The medium in each well was gently aspirated, and an aliquot of pre-mixed media (+ DES, 10^{-7}M, or +0.1% DMSO, vehicle) was placed into each well such that three wells were treated with DES (10^{-7}M) and three contained the vehicle only (0.1% DMSO). The remaining 18 wells on each plate were filled with 1 ml PBS to maintain humidity. The plates were allowed to incubate undisturbed (37°C, 5%CO_2), and the media changed every two days.

The medium in each microfuge tube was aspirated, leaving the EBs settled at the bottom of the tube. RLT lysis buffer + 0.1%βME was applied directly to the EBs. The resulting lysate was homogenized as above, and stored at -20°C for further analysis.

Sequential plate RNA harvests: Day 8, 12 and 16

On day 8, the first 24-well plate was harvested for RNA as follows. Spent medium was aspirated from the wells, and RLT lysis buffer + 0.1%βME applied directly to each well. The resulting lysate was removed, homogenized, and stored as above. Fresh medium was pre-mixed and applied to the remaining two plates. On day 12, the second plate was harvested for RNA as the first, and the third plate harvested on day 16. Between harvests, the

medium was changed on each well every two days by gently aspirating spent medium from the well, and replacing it with premixed, fresh medium.

Part Four: Total RNA Isolation and cDNA Synthesis:

RNA Isolation and 1st Strand cDNA Synthesis

Total RNA was isolated from the above cellular lysates using the RNeasy protocol (RNeasy Mini Handbook, June 2001, 3rd ed. Qiagen, pgs 30-35) and the RNeasy Mini Kit (250) (Qiagen, Cat # 74106, Maryland). Briefly, homogenized cellular lysates were treated with ethanol to allow selective binding of the RNA to the RNeasy silica-gel membrane present in the microfuge tubes in the kit. Contaminants were washed from the sample, leaving RNA bound to the membrane. An on-column DNase digestion was performed using RNase-free DNase set (Qiagen, cat. # 79254) to prepare the RNA samples for TaqMan RT-PCR analysis. After washing away contaminants and remaining DNase, the RNA was eluted from the column.

Spectrophotometric analysis was performed (Beckman DU 640, Beckman Coulter) to determine the concentration of RNA recovered from each sample, and first-strand cDNA synthesis was performed using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen, www.invitrogen.com, Cat. # 11904-018, Version B, pg 7). Briefly, a sample of the isolated RNA was denatured (65°C, 5 min) in the presence of nonspecific primers (random hexamers) and dNTPs, which allowed RNA in the sample to be a template for the 1st strand cDNA synthesis. The RNA was allowed to anneal (25°C, 10 minutes) with primers in place, and synthesis of cDNA catalyzed (42°C, 50 minutes) using SuperScript™ II RNase H reverse transcriptase (RT). This particular enzyme, designed to prevent degradation of mRNA during the synthesis of cDNA also has DNA polymerase activity, and is not inhibited

by ribosomal or transfer RNA, allowing it to synthesize longer cDNAs more efficiently. The reaction was terminated (70°C, 15 minutes), and the RNA removed from the new cDNA strand using RNase H digestion (37°C, 20 minutes).

Polymerase Chain Reaction

To establish the success of the previous 1st strand cDNA synthesis procedure, polymerase chain reaction (PCR) was performed using *Flt1* primers BH205 (Exon 13 ss: 5'AGAAGACTCGGGCACCTATG3') and BH206 (Exon 14, as: 5'GGCGCGGGGACACCTCTA3')(102) and a thermal cycler (Hybaid). See Table 3 for thermal cycler conditions. The amplified fragments were separated by gel electrophoresis (2% agarose gel in 1 X TBE) 100V for 25 minutes. A positive cDNA control and negative cDNA control (containing water) were also run on the gel.

Part Five: Primer/Probe design, RT-PCR & Analysis:

Primer/Probe design:

Probes and primers were designed(103) using the Primer Express software (PE Biosystems) for CD45, VE-cadherin, ERα, ERβ, and c-Kit, containing the exons noted in Table 1. Probes and primers for the targets Flt1, Flk1, and oct4 had been previously designed and tested.(102, 104) The probes contained a 5'-reporter dye (FAM, or 6-carboxyfluorescein), and a 3' quencher dye (TAMRA, or 6-carboxytetraamethylrhodamine) attached to the oligonucleotide. The proximity of the quencher on the intact probe suppressed the reporter dye fluorescence. When the probe annealed to the target sequence between the forward and reverse primers, the probe was cleaved between the reporter and quencher, and was displaced from the target, allowing fluorescence to occur. Fluorescence increased as the target was

amplified by PCR. The first appearance of fluorescence measured the starting amount of target sequence. An ABI Prism 7700 Sequence Detection System was used to record the fluorescence and generate the C_T (Applied Biosystems, Perkin-Elmer Corporation, Foster City, CA).

Amplification efficiencies were determined as described in Huckle and Roche, 2004, for each target. Briefly, triplicate 25µl reactions containing TaqMan® Universal Master Mix (PE Biosystems), 0.3 µM primers, 0.2 µM Probe, and dilutions of mouse embryo-derived cDNA created by reverse transcription with random hexamer primers (Superscript II, Life Technologies) were performed. QPCR was allowed to proceed through denaturization at 95°C for 10 minutes, followed by 40 cycles between 95°C (15s) and 60°C (1 minute) using a PE7700 Sequence Detection System. Negative controls (no template, and no reverse transcribed RNA) were included. Adequate efficiency was determined to be $\geq$ 95%. This established that the targets were detectable in mouse embryo tissue, and their amplification efficiency was comparable to the housekeeping gene for 18s rRNA, allowing normalization of the threshold counts to this value on day 0.

RT-PCR & Analysis

Real time polymerase chain reaction was performed on the OP9 samples using specifically designed primers and fluorogenic TaqMan® probes for CD45, Flt-1 ex 13/14, c-Kit and 18s r RNA (Table 1). Relative quantification of the RT-PCR results and the mRNA expression for all the markers were compared to an internal control (18s rRNA); relative changes in marker gene expression was determined using the threshold cycles and the $2^{-\Delta\Delta CT}$ method.(103) This technique describes amplification of the PCR product and expresses this as

a threshold cycle (C_T), or the cycle number at which the amplified target reaches a fixed threshold. The resulting C_T for each marker mRNA was normalized to an internal control (18s rRNA) for the amount of RNA added to the reverse transcription reactions. The value, ΔC_T, was the difference of expression of the target and the internal control. This value was calibrated to expression of a pretreatment control on day 0, obtaining the value $\Delta\Delta C_T$. The fold change in gene expression for each target was determined relative to day 0. RT-PCR was performed on the EB hanging drop samples for the following markers: oct 4, Flk-1, C-kit, CD45, estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), VE-cadherin, and Flt-1.

ANOVA (Type 3 mixed-effects analysis, SAS) was performed on the unexponentiated $\Delta\Delta C_T$ values from the real-time PCR data collected from EBs on days 8, 12, & 16 of differentiation. The data was analyzed assuming complete randomized design. The expression of mRNA for the surface markers c-Kit, oct4, Flk1, CD45, Flt1, VE-cadherin, ERα and ERβ were evaluated. The effects of DES, time, and the interaction of DES and time were analyzed. Post hoc analysis included evaluation of the effects of DES treatment on expression of each marker on day 16, when the expression of ERα was highest.

Figure 9: OP9 Co-culture system protocol

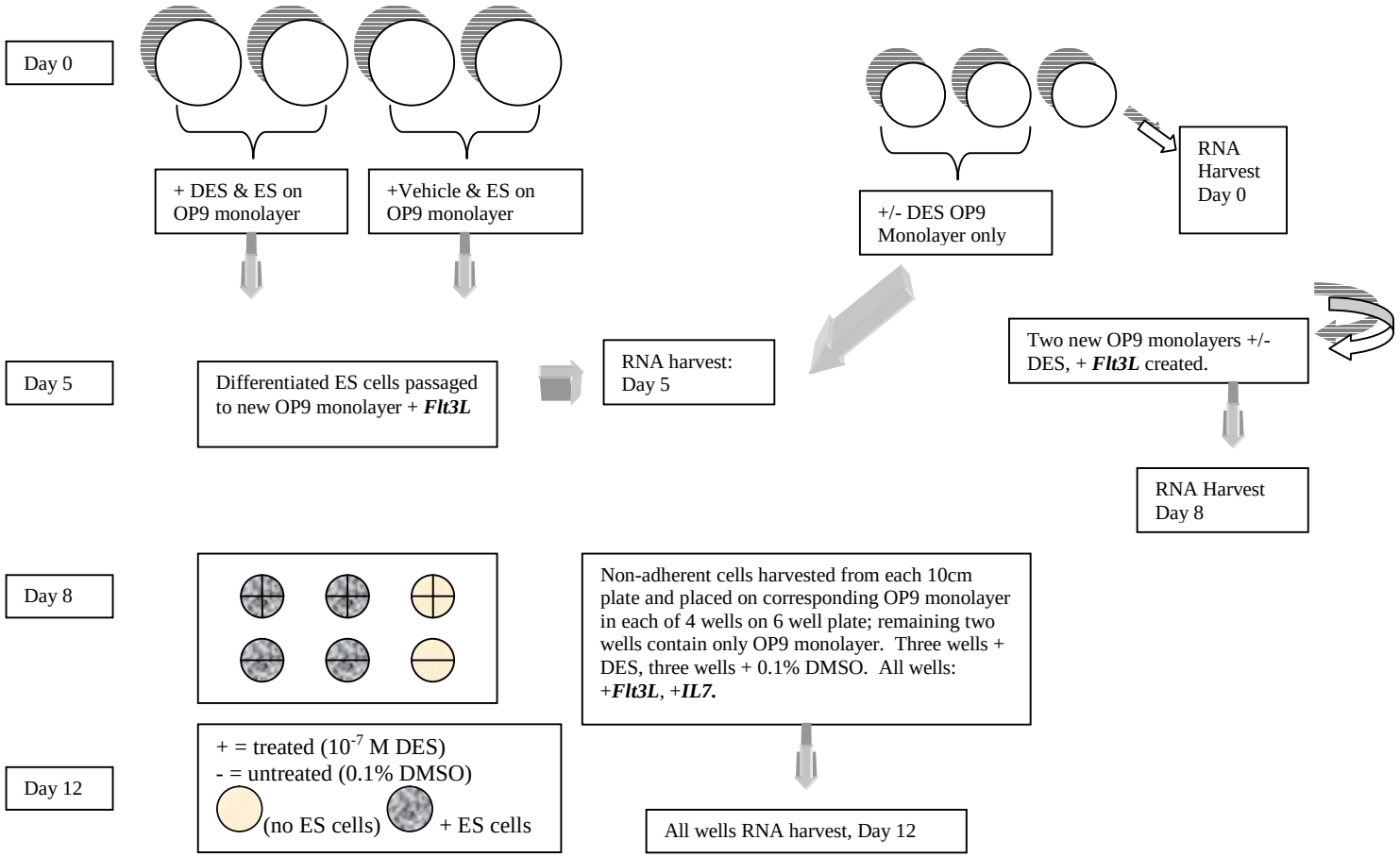


Table I: Thermal Cycler conditions: Hybaid PCR Sprint Thermal Cycler and Eppendorf Mastercycler Thermal Cycler (total time 2 hours 10 minutes).

Cycle 1	Cycle 2-29	Cycle 30	4ºC hold
94ºC X 2 min	***94ºC X 1 min***	***94ºC X 1 min***	
55ºC X 1 min	***55ºC X 1 min***	***55ºC X 2 min***	
72ºC X 2 min	***72ºC X 2 min***	***72ºC X 10 min***	

Table II: RT-PCR Primers and TaqMan® probes:

Target	Primer/Probe #	Primer Sequence: 5' → 3'	Probe sequence (5'-6FAM, 3'-TAMRA)	PCR product size (bp)
Oct4	3'BH316 5'BH317 Probe: BHTP 8	CTCACCCTGGGCGTTCTCT AGGCCTCGAAGCGACAGA	5'TGGAAAGGTGTTCAGCCAGACCACC3'	64
c-kit ex 7/8	3"BH382 5'BH383 Probe: BHTP19	GCTTCCGTGACATTCAACGTT TGCCATTTATGAGCCTGTCGTA	5'-ACGTGAACACAAAACCAGAAATCCTGACG-3'	73
Flk1	3'BH324 5'BH325 Probe: BHTP12	GGGACCTGGACTGGCTTTG CCGCATTCAGTCACCAATACC	5'- TTTCCTCAGAATCACGCTGAGCATTGG-3'	70
CD45 ex 33/34	3'BH374 5'BH375 Probe: BHTP 15	CACAAGCATGCATCCATCCT AGATTGAACAAGGCACAGAACAAC	5'-TCCACTGCAGAGATGGATCCCAGCA-3'	77
ERα ex 8/9	3'BH376 5' BH377 Probe: BHTP 16	GGCTGCGCAAGTGTTACGA TCCTCGGCGGTCTTTCC	5'-ATGCCGCCTTTCATCATGCCCA-3'	62
ERβ ex 7/8	3' BH378 5'BH379 Probe: BHTP17	GCCAACCTCCTGATGCTTCTT TCATGCTGAGCAGATGTTCCA	5'-CTCATGTCAGGCACATCAGTAACAAGGGC-3'	73
Flt1ex 13/14	3'BH228 5'BH229 Probe: BHTP 3	TTCGGAAGACAGAAGTTCTCGTT GACCTCGTAGTCACTGAGGTTTTG	5'-AGATTCGGAAGCGCCACACCTGCT-3'	
VE-cadherin ex 10/11	3'BH380 5'BH 381 Probe: BHTP 18	CCTGAAGAACGAGGACAGCAA CTGCCCATACTTGACCGTGAT	5'-AAACAACCATGACAACACCGCCAACA-3'	79

CHAPTER III: RESULTS

Primer/Probe Validation and Dilution Curves:

Dilution curves were generated for five of the targets used in this project: c-Kit, ERα, ERβ, CD45 and VE-cadherin to verify the equivalence of amplification efficiency required for the $\Delta\Delta C_T$ analysis. (Figures 10 & 11) All targets produced a linear dilution curve when the log of increasing concentrations was plotted with the resulting C_T. The efficiency calculated from the slope of each line approached 100% (Table III). Dilution curves were previously established for the markers *oct4, Flk1 & Flt1*.(102, 104) All markers were detectable by RT-PCR in undifferentitated ES cells. The ΔΔCt method of relative quantization of gene expression was used to determine relative expression of 18s rRNA and the markers of differentiation. A C_T of 40 was considered non-expression, as established by the no-template and non-reverse transcribed samples included in each RT-PCR run.

OP9 Co-culture Results

There was constant and abundant expression of mRNA for 18s rRNA, initial high expression of oct4 mRNA, and low expression of c-Kit mRNA; the later increased a small amount over the remainder of the culture time period (12 days). The expression of Flt1 mRNA increased as differentiation on the OP9 co-cultures progressed. There was minimal expression of the marker of interest, CD45, before day 12; therefore, the OP9 co-culture technique was set aside for the hanging drop culture technique, which had been previously shown to reliably produce cells expressing CD45 in untreated conditions. (Data not shown)

Hanging Drop EB Results

During the first three days in hanging drops, the ES cells began differentiating and formed EBs. Beginning on day eight of differentiation, the EBs developed rhythmic pulsations or "heartbeats", indicating continuing differentiation after transfer from hanging drops to the 24 well plates. Within the EBs, in the absence of treatment, all markers (oct4, c-Kit, Flk1, ERα, ERβ, CD45, Flt1, & VE-cadherin) were detectable. Figure 12 shows the exponentiated normalized mRNA C_T values on day 3 relative to day 0. The log scale of the Y-axis demonstrates that expression of mRNA for markers related to earlier, less differentiated cells (oct4, c-Kit, & Flk1) was decreasing by day 3 of differentiation, while mRNA expression for markers common to more differentiated lymphoid and endothelial cells (CD45, Flt1, and VE-cadherin) was increasing. Both estrogen receptor mRNAs were expressed in low but detectable amounts on day 3. As the physical number of cells increased within the EB, it is assumed there were relatively fewer numbers of undifferentiated ES cells, resulting in decreased expression of oct4.

ANOVA (Type 3 mixed-effects analysis, SAS) was performed on the unexponentiated $\Delta\Delta C_T$ values from real time PCR data collected from EBs on days 8, 12, & 16 of differentiation. The data was analyzed assuming randomized design, based on the random selection of EBs from the hanging drops followed by sequential placement into a well on the 24 well plate. Wells to be treated and untreated on each plate were organized the same way in each plate to avoid confusing which well had been treated and which had not been treated. The expression of the surface markers c-Kit, oct4, Flk1, CD45, Flt1, VE-cadherin, ERα and ERβ were evaluated. Expression of all markers changed significantly ($P<0.05$) over time with the exception of Flk1 ($P=0.7246$). Post-hoc ANOVA analysis was performed to assess the

effects of DES treatment on expression of the markers of interest on day 16. This day was chosen for post-hoc analysis because it is when the expression of ERα was the most abundant. The expression of the endothelial lineage markers c-Kit, VE-cadherin & Flt1 was significantly reduced on day 16 in DES treated EBs (Figure 16 & 17). Treatment with DES also reduced the expression of Flk1 on day 16, but this effect is not statistically significant (P=0.2121).(Figure 14)

Differentiation of the ES cells was observed within treated and untreated EBs between days 8 and 16, as demonstrated by the continued decrease in expression of oct4 mRNA compared to day 3, and an increased expression of Flt1, Flk1 and c-Kit mRNA.(Figures 13 - 16) Flk1 mRNA, the earliest marker of hemangioblasts, was detectable at all sampling points. (Figure 14) Its expression was observed to increase between days 3 & 8 then remained constant.

After an initial decrease during the first three days of differentiation in the EBs, (Figure 12) the expression of c-Kit mRNA, the marker of the common hematopoietic precursor, increased significantly between days 8 and 16 ($P<0.0001$). (Figure 15) In untreated cells, expression of c-Kit mRNA increased ~ 3.5X between day 8 and day 16, whereas its expression in treated cells increased ~1.3X. A statistically significant ($P<0.0124$) difference in expression of mRNA for this marker was noted on day 16.

The expression of Flt1 mRNA increased significantly ($P<0.0266$) between days 3 and 16. A ~2.5X increase in expression of mRNA for this marker was evident in untreated cells between day 8 and 16, while a ~1.3X increase was observed in treated cells. A statistically significant ($P<0.0254$) difference in expression between treated and untreated cells on day 16 was noted.

Expression of VE-cadherin mRNA increased significantly between days 12 and 16. (Figure 17) Expression of VE-cadherin mRNA in untreated cells increased ~5X between days 12 and 16. Its expression in treated cells increased ~2X in this same time period. There was a statistically significant ($P<0.0425$) difference in expression of mRNA for VE-cadherin between treated and untreated cells on day 16.

Expression of mRNA for the marker common to lymphoid lineage cells, CD45, also increased between days 12 -16. (Figure 18) In untreated cells there was approximately a 4X increase in mRNA expression. Treated cells exhibited ~3X increase in expression. There was, however, no statistical difference ($P=0.5505$) in expression of this marker on day 16 between untreated and treated cells.

Both estrogen receptors were detectable between days 8 and 16 of differentiation within the EBs. (Figures 19 & 20) Expression of ERα mRNA increased ~11X between days 12 and 16 in untreated cells and ~10X in treated cells. ERβ was detectable in low levels at all time points sampled. Treatment with DES was not associated with a statistically significant effect on the expression of ERα ($P=0.3778$) or ERβ ($P=0.3757$).

CHAPTER IV: DISCUSSION

OP9 co-culture discussion

The OP9 co-culture technique has been used successfully to induce differentiation of ES cells into lymphohematopoietic cells.(64-69, 71, 73, 105-107) This differentiation is dependent on binding of Notch receptors and the Notch ligand signaling pathway. The Notch signals are essential in determining the development of T and B cells.(73, 106, 108, 109) A modified line of OP9 stromal cells, the OP9-DL1 line, express Delta-like 1(DL1) ligand, which when bound to the Notch receptor, activates the Notch signaling pathway, and promotes selective differentiation of T cells.(66) Notch 1 binding directs cell fate, differentiation and maturation of thymocytes; however, the mechanism of this has not been established.(106) It stands to reason that if this protein affects later development of thymocytes, it may well serve a crucial role in early lymphoid cell commitment. The OP9 co-culture system used in this project did not express DL1 ligand, which may also have contributed to the apparent delay in the emergence of $CD45^+$ cells. Differentiation of ES cells into hemangioblastic precursors and hematopoietic precursors apparently occured in the OP9 co-culture, as demonstrated by a decreased expression of oct4 and an increased expression of Flt1 and c-Kit, as compared to their expression at day 0. (Data not shown)

Each *in-vitro* culture system used in this project had its merits; the EB culture system was chosen to evaluate ES cell differentiation because it most reliably yielded CD45 expression from the specific ES strain used in this study and time frame examined. While there was evidence of hemangioblastic, hematopoietic and endothelial differentiation within both the OP9 co-culture and EB culture systems, emergence of lymphoid precursor cell lineages only appeared to occur at a significant rate in the EB system. Allowing the ES cells

to coalesce into ball-like structures (the EBs) during the initial three days of differentiation more closely resembled *in-utero* events while allowing attachment of ES cells to the OP9 monolayer in the OP9 co-cultures more closely resembled the interaction of ES cells with the microenvironment of the bone marrow. Microenvironmental factors likely contributed to the sluggish emergence of lymphoid lineage cells within the OP9 co-culture. It has been demonstrated that attachment of ES cells to a substrate, such as a petri dish or a stromal cell co-culture monolayer before day three, alters differentiation within resulting ES colonies and inhibits the emergence of hematopoietic cells.(32) Minimal expression of CD45 within the OP9 co-cultures may have been the result of attachment of the ES cells before day three to the OP9 stromal cells, leading to suppression of differentiation of this lineage.

In this project, ES cells were allowed to differentiate on the OP9 co-cultures for 12 days. The expression of CD45 and emergence of the lymphoid lineage was barely detectable in the OP9 co-culture by day 12. If the ES cells on the OP9 co-culture system had been allowed to differentiate for a longer period of time, CD45 expression may have become more abundant.

Expression of the markers of interest on day 3 of differentiation in the EBs was consistent with expected emergence of the endothelial and lymphoid lineages. (Figures 1B and 12) The marker oct4, which is common to undifferentiated ES cells,(35-37, 39, 40, 110) Flk1, the earliest marker of the hemangioblast,(22, 24, 55, 111) or common precursor to both endothelial and hematopoietic cell lineages, and c-Kit, a marker of early hematopoietic precursors,(22) were all noted to be decreasing after three days of differentiation. (Figure 12) This would suggest that at this stage of differentiation, the earliest precursors are emerging within the EB: the common hemangioblast and very early hematopoietic precursors. The

decrease in expression of the markers of early differentiation was expected as the ES cells began to differentiate within the EB. Because oct4 is specific to germ-line cells, the emergence of terminally differentiated germ cells would result in a later small upsurge in the expression of oct4 in a developing embryo. A minor increase in expression of oct4 mRNA was noted between day 8 and 16, and could possibly have been due to emergence of germ line cells, or may simply represent a period of time when the drop in expression of mRNA for this marker plateaued.

The relative decreases in expression of markers common to the hemangioblast and hematopoietic precursor (c-Kit and Flk1) during the first three days was likely due to a dilution effect as the number of ES cells within the EB that were more mature, or other cell types whose markers were not included in this study, increased rapidly during the initial three days of differentiation. The cells within the EB are known to not differentiate at a constant rate, so this may explain apparent variations in expression of these early markers at this stage. While the evidence of differentiation at this point of the project was encouraging, there may have been functional and differentiation changes that occurred during this three-day window of time that could be influenced by treatment with DES. Waiting for three days to treat the cells with DES may have missed window of time when the cells were susceptible to the effects of DES.

Between days 3 and 8, the hemangioblastic precursor cells appeared to differentiate into hematopoietic lineages (lymphoid and myeloid) with continued differentiation of the endothelial cells as demonstrated by increasing expression of c-Kit, CD45, Flk1, Flt1, & VE-cadherin. The order of the lineage marker expression correlated with other sources.(22) The timing of emergence likely varied from other published studies due to culture conditions,

sampling times and ES strain. The expression of the markers changed rapidly between each sampling time. Whether the expression of each marker was decreasing or increasing when sampled was impossible to determine from the experiment. Another limiting factor was the measurement of mRNA, as opposed to actual protein expression. Changes in protein expression depend on the rate of translation of the protein's mRNA, protein feedback control on translation of itself, and rate of degradation or use of the protein. While the expression of mRNA suggests that the protein is being made, it doesn't indicate actual protein level or existence within the cells.

After day 8, markers common to differentiated endothelial cells (VE-cadherin, Flk1, & Flt1) began to be expressed at increased levels. Expression of VE-cadherin was significantly increased between days 12 and 16. Evidence suggests that there is a common hemangioblastic precursor to the lymphoid and endothelial lineages, which is $Flk1^{+}$VE-$cadherin^{+}$; loss of the expression of VE-cadherin indicates differentiation of hematopoietic lineage in $CD45^{+}$c-Kit^{+} cells.(22) The relatively constant expression of Flk1 between days 8 and 16 is consistent with its association with earlier, less well differentiated hemangioblasts that give rise to endothelial and lymphoid lineage cells.(22, 24, 61, 111)

While the expression of ERs is established in a wide variety of tissues within animals, and its effects on in-utero development have been investigated, (8, 10, 16, 74, 81, 82, 88, 112-114) expression of the ER within in vitro ES cultures has not been established to date. It was found in this study that estrogen receptors were expressed in the differentiating ES cells. ERβ was expressed in low, but detectable levels, throughout the sampling time. ERα was expressed between days 12 and 16 of differentiation. It is known that estrogen exerts its effects on adult cells through the ER as well as alternative signaling pathways within cells.(92,

93) The presence of ERs in early embryonic stem cells suggests that they are capable of responding to estrogens (including DES) through the ER-mediated pathways. It is impossible to rule out other affects that are mediated through alternative signaling pathways.

The expression and function of ER within certain types of cancer has been demonstrated to be affected by estrogen levels.(115-117) While the dose of DES chosen for this study was derived from other *in-vivo* studies,(75, 77) a higher or lower dose of DES may affect development of the lymphoid lineage of immune cells differently than demonstrated in this project. Additionally, repeated, or intermittent exposure to DES has significantly altered lymphocyte cytokine profiles.(75, 76) Pulsatile dosing of early ES cultures may yield much different results. Finally, it is unknown how, or if the DES is significantly metabolized or degraded within the medium. The medium in this case was replenished every two days to meet the nutrient demands of the developing EBs, but it is unknown if this interval significantly alters the exposure of the EBs to DES each time the medium was changed.

In this study, DES altered the expression on day 16 of Flt-1 and VE-cadherin as compared to vehicle-treated cells. These markers function in angiogenesis, and control the action of Flk-1. While expression of Flk1 does not appear to be directly affected by exposure to DES during the time period investigated, its function relies on both Flt-1 and VE-cadherin.(54) Alterations in the function of Flk1 and Flt1 may alter long-term development and function of not only angioblasts, but also hematopoietic precursors as well through direct signaling and indirect signaling in later stages of differentiation.

DES also appeared to alter expression on day 16 of c-Kit, a marker common to the hematopoietic lineage precursors. Exposure to DES did not affect the expression of CD45 before day 16. While this suggests that DES does not affect lymphoid lineage development

before day 16, the effects of DES on c-Kit, may indicate an earlier affect that may have long lasting, or a delayed expression in the differentiation process. Analysis of other markers common to more terminally differentiated T & B cells (CD3, CD8, CD4, or CD2) may elucidate the effect of DES exposure during earlier stages of differentiation.

Future work may include sequencing the estrogen receptor PCR products. While ERβ was expressed throughout the time period in this study, it was barely detectable. Sequencing would confirm that the PCR product is indeed consistent with known Erβ sequence. Monoclonal antibody labeling with anti-CD45 or other specific T or B cell markers and cell sorting studies would enable separation of differentiated T and B cells from the EBs at various stages, as well as confirm CD45 expression at the protein level. Labeling would allow identification of the specific cell lineages that are producing ER, as well as those lineages that are estrogen responsive. Additionally, studying specific cell populations retrieved from EBs exposed to DES would help answer the question of the effects of DES exposure on emerging cell types and relative resulting cell percentages. Finally, isolated cells could be used to repopulate an irradiated mouse, and perform in-vivo function studies of exposed precursor cells and resulting CD45+ phenotype cell populations.

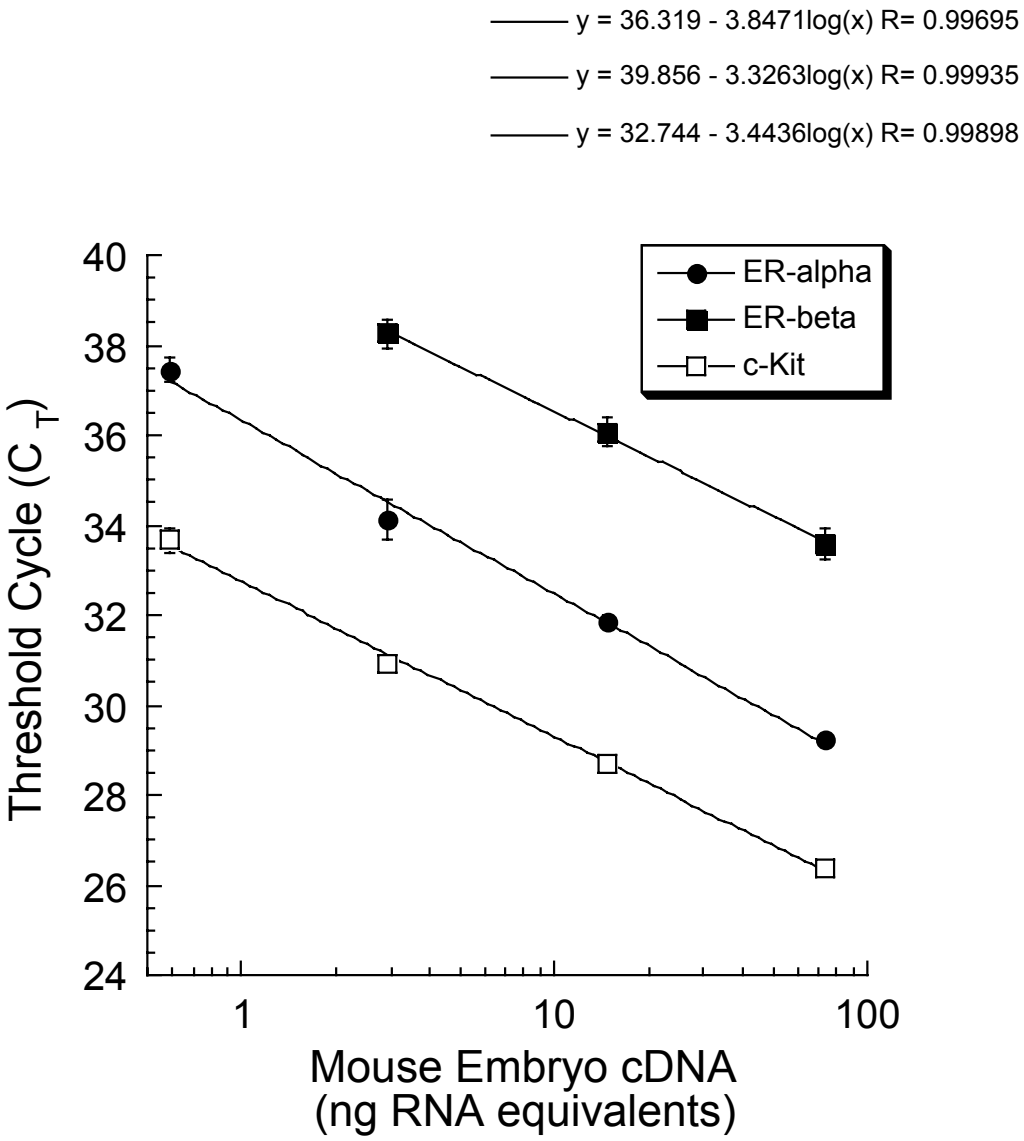


Figure 10: Dilution curves for ERα, ERβ and c-Kit. Note linear curves. Efficiencies were calculated according to the equations top right, results in Table 2.

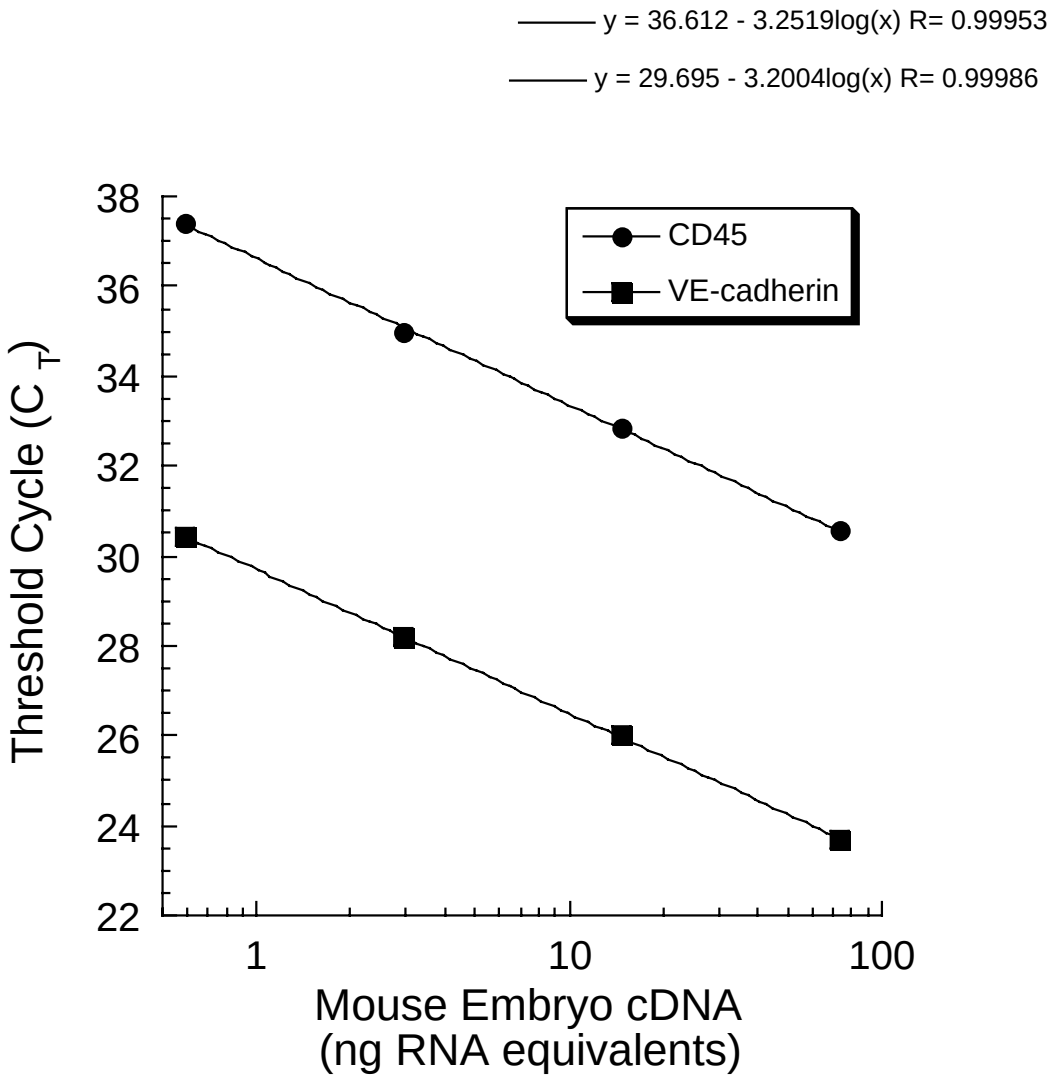


Figure 11: Dilution curves for CD45 & VE-cadherin. Note linear curves. Efficiencies calculated according to the equation in top right, results in Table 2.

Table III: Slope of line on dilution plot for each new marker; % efficiency calculated from the slope of the line.

Target	Slope	% Efficiency
ER-alpha	-3.498	96.6
ER-beta	-3.3263	99.6
c-Kit	-3.4436	97.6
CD45	-3.2519	101.5
VE-cadherin	-3.2004	102.7

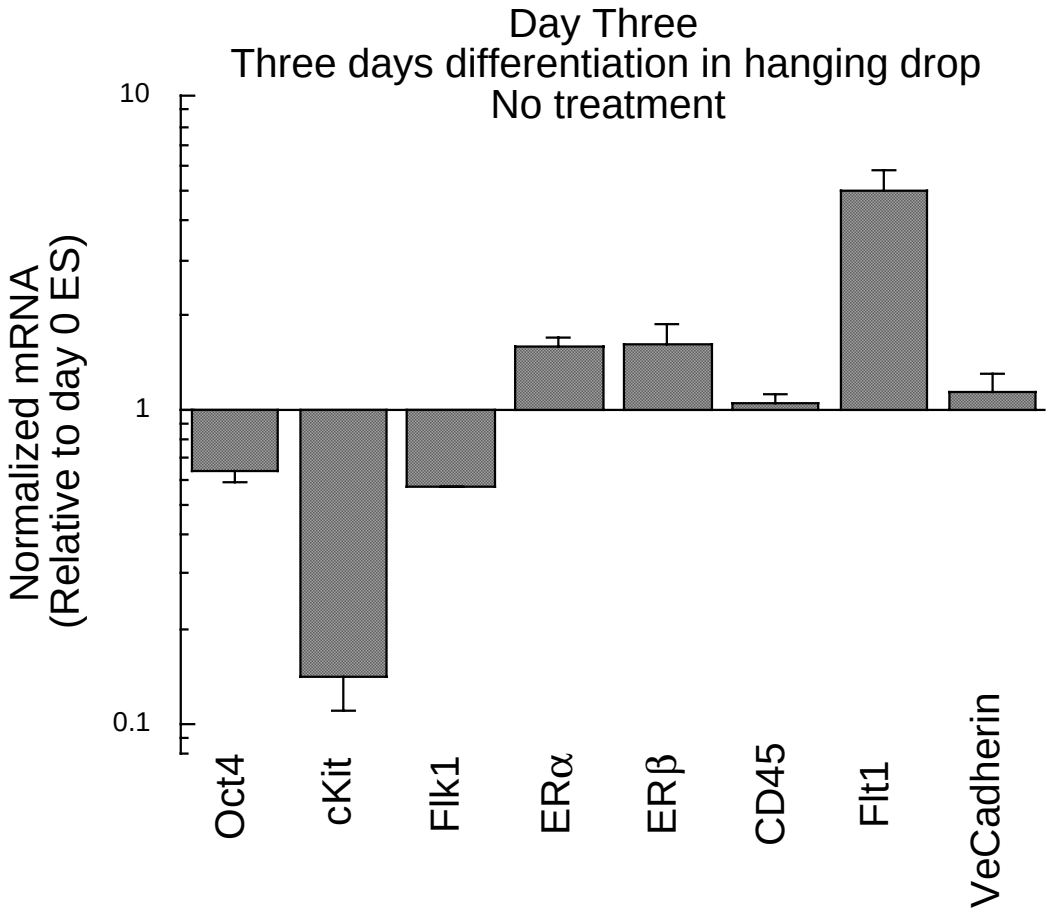


Figure 12: Normalized mRNA expression relative to day 0 for each marker after three days differentiation in hanging drops/no treatment. Values obtained by calculation of the mean of two EB samples; bars indicate range. Decreasing oct4, cKit & Flk1 compared to day 0 values.

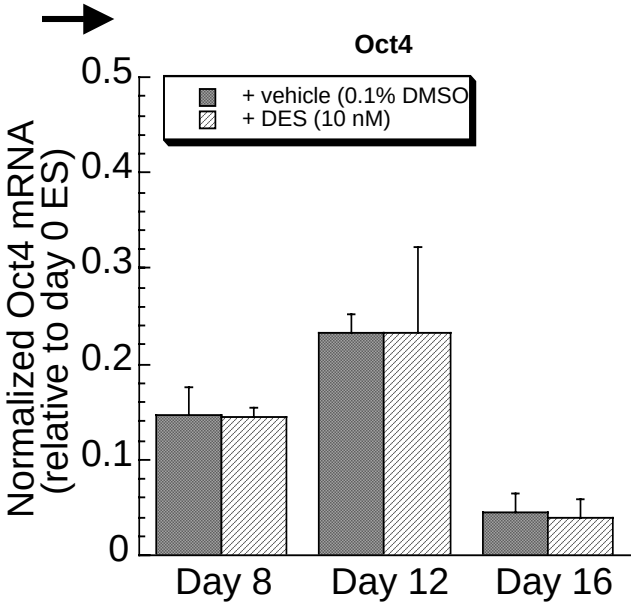


Figure 13: Normalized oct4 mRNA expression relative to day 0 for days 8, 12 & 16. Decreased expression between day 8 and 16 is indicative of differentiation and commitment of stem cells to specific lineages. Arrow indicates expression on day 3 (0.64) for reference.

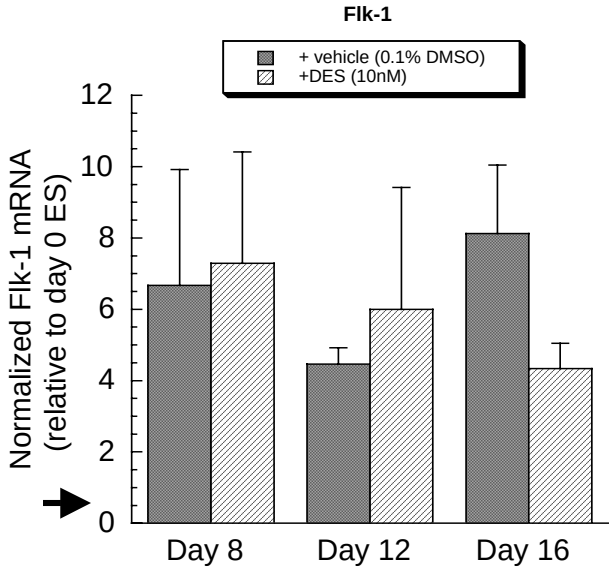


Figure 14: Normalized Flk1mRNA expression relative to day 0 for days 8, 12 & 16. No significant change in expression was noted over time, or due to treatment with DES. Arrow indicates normalized mRNA expression on day 3 (0.57).

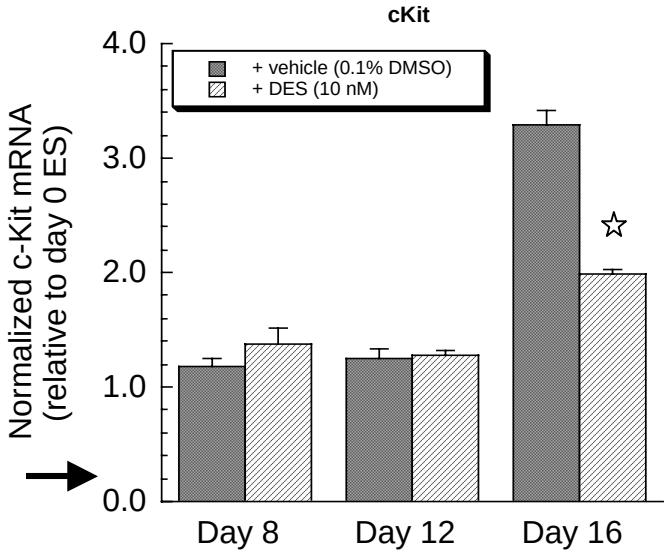


Figure 15: Normalized c-kit mRNA expression relative to day 0 for days 8, 12, & 16. ~3.5X increase in expression between d 8 – 16 in untreated cells & ~1.3X increase in treated cells. ☆=Significant effect of DES on expression & arrow indicates mRNA expression on day 3 (0.14).

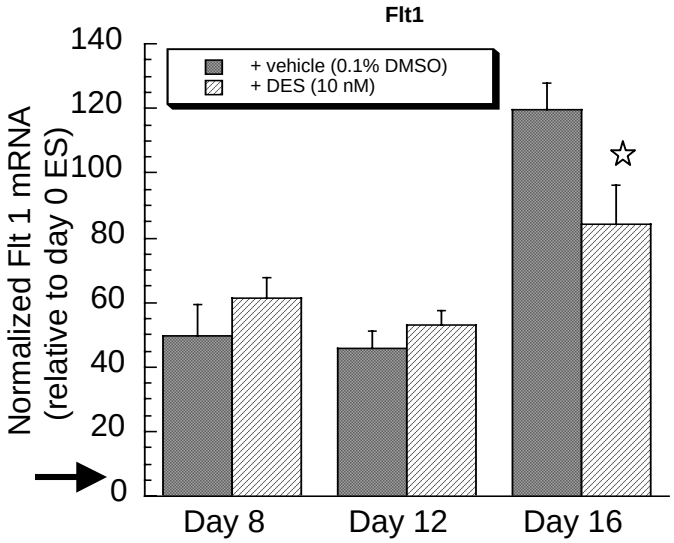


Figure16: Normalized Flt1 mRNA expression relative to day 0 for days 8, 12 and 16. Note the ~2.5X increase in expression in untreated cells and ~1.3X increase in treated cells. ☆= Significant suppression of expression by treatment with DES & arrow indicates Flt1mRNA expression on day 3 (4.98)

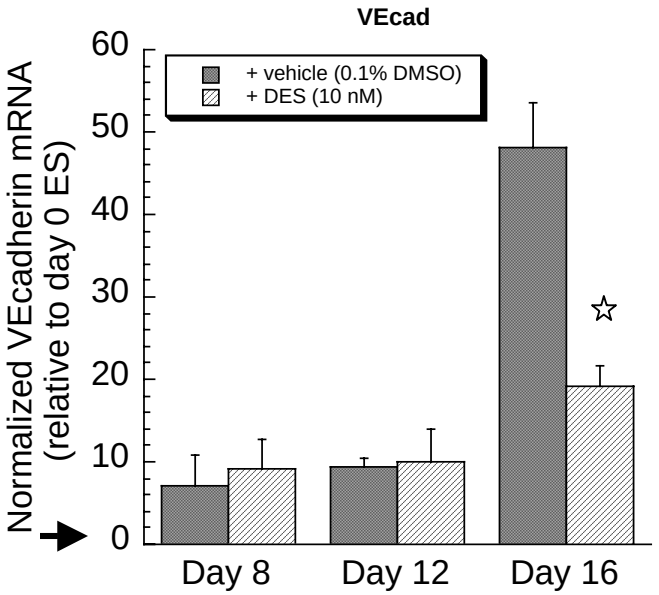


Figure 17: Normalized VE-cadherin mRNA expression relative to day 0 on days 8, 12, & 16. Note ~ 5X increase in expression between days 8 and 16 in untreated cells and ~ 2X increased in expression in treated cells. ☆=Significant effect on expression between untreated and treated cells on day 16 & arrow indicates expression on day 3 (1.16)

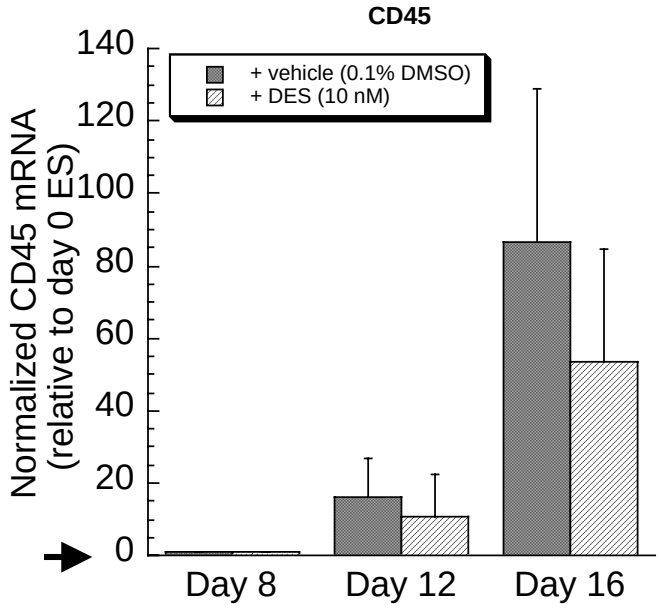


Figure 18: Normalized CD45 mRNA expression relative to day 0 for days 8, 12, &16. Note ~4X increase in expression in untreated cells compared to ~3X increase in expression in treated cells between days 12 and 16. Arrow indicates CD45 expression on day 3 (1.06)

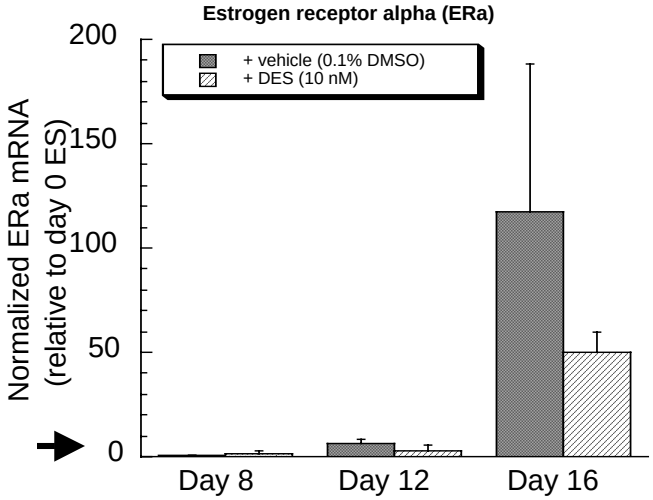


Figure19: Normalized ERα mRNA expression relative to day 0. Note ~11X change in expression and ~10X change in expression between untreated and treated cells respectively. Arrow indicates ERα expression on day 3 (1.59)

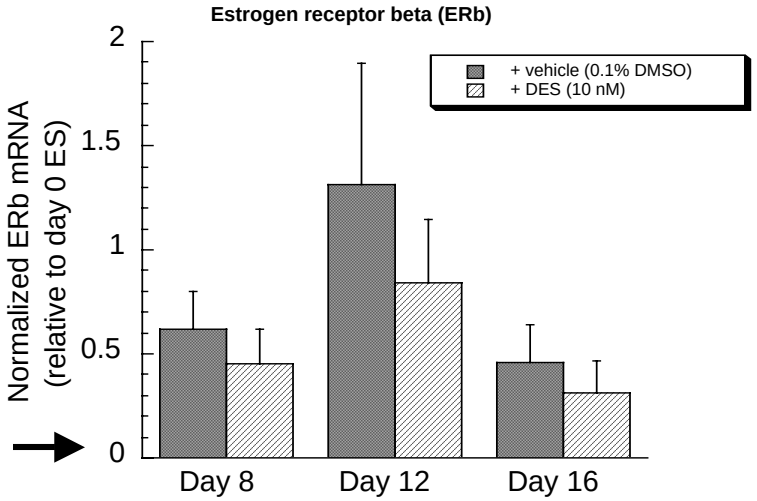


Figure 20: Normalized ERβ mRNA expression relative to day 0. Arrow indicates ERβ expression on day 3 (1.61)

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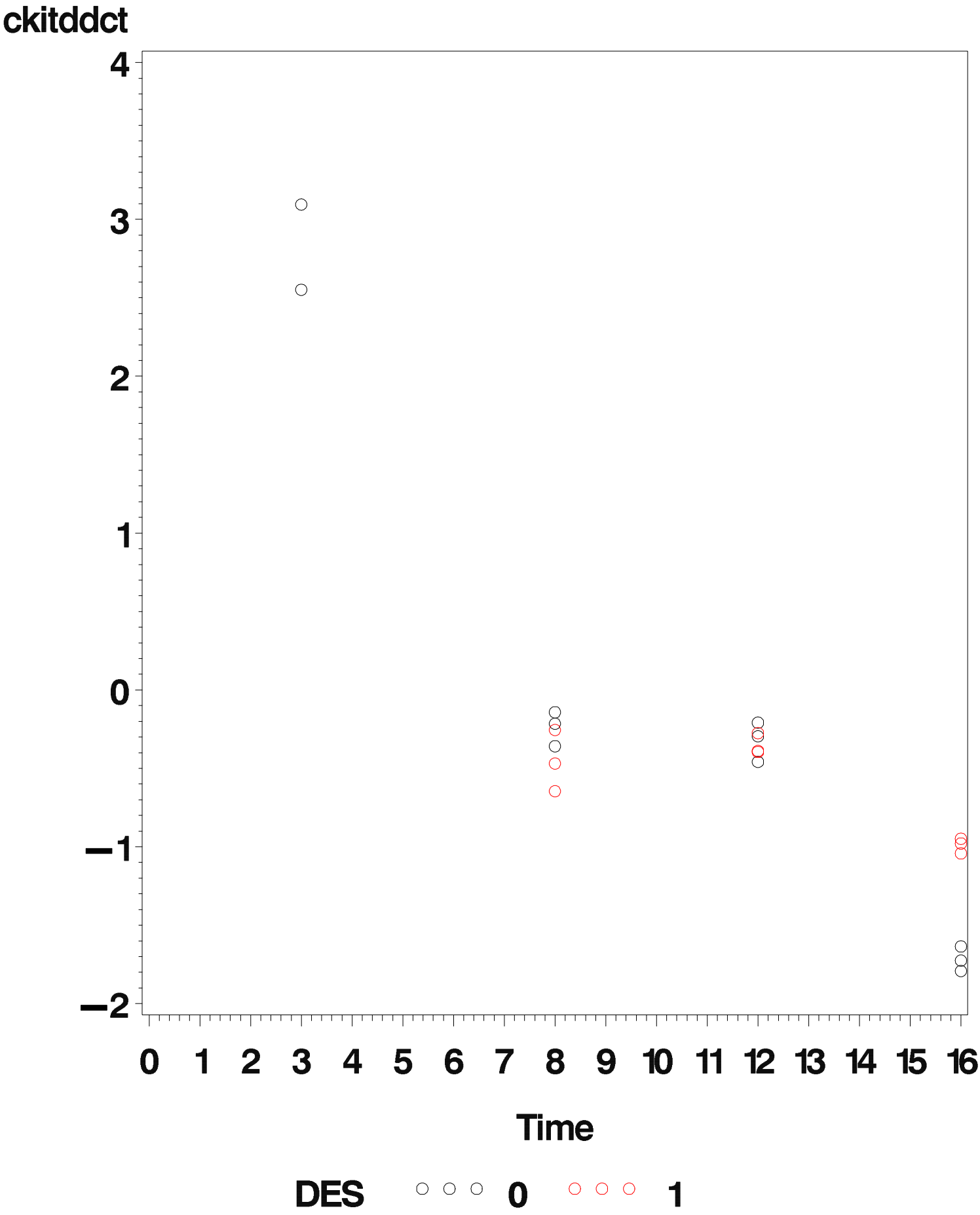
Raw data plot



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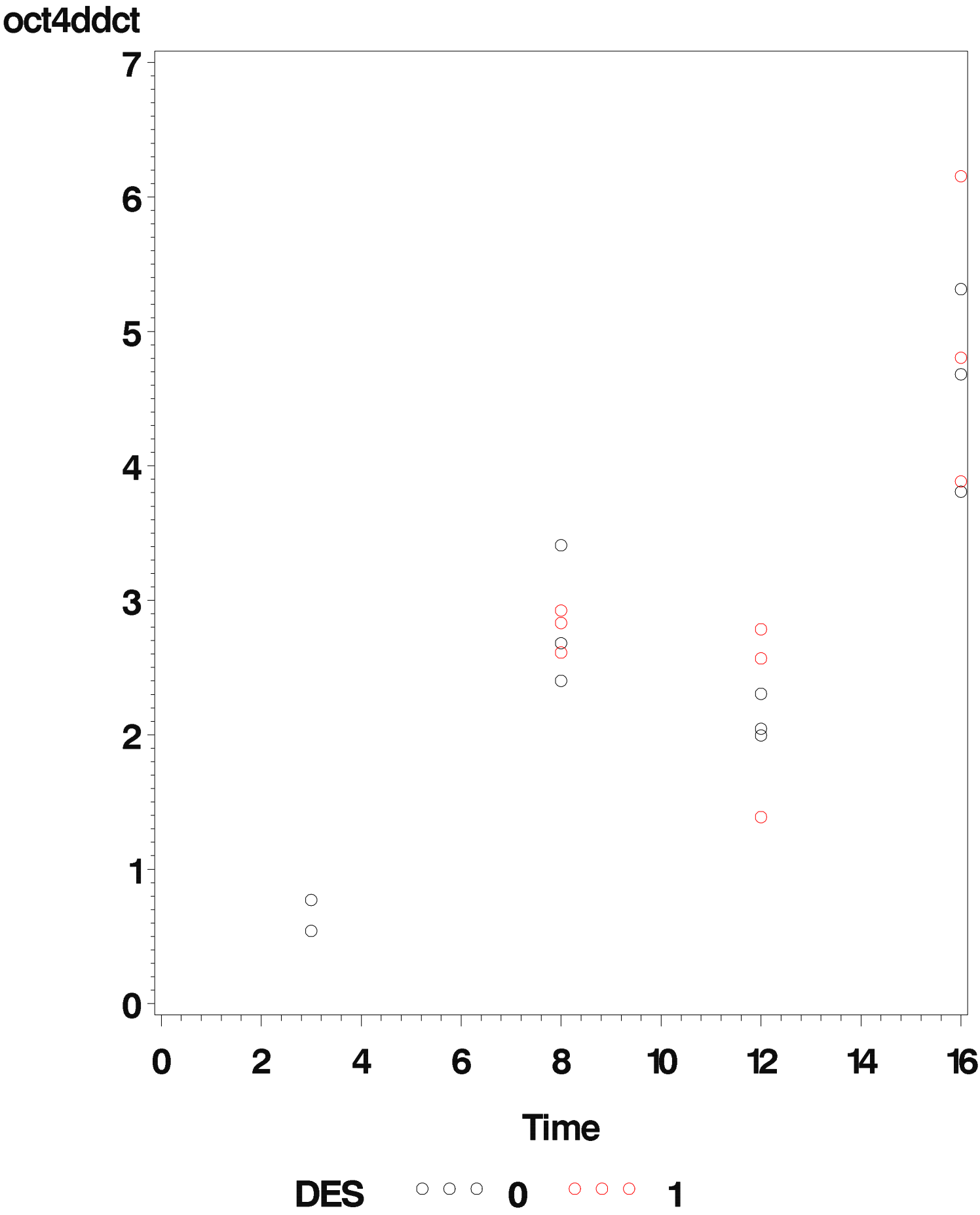

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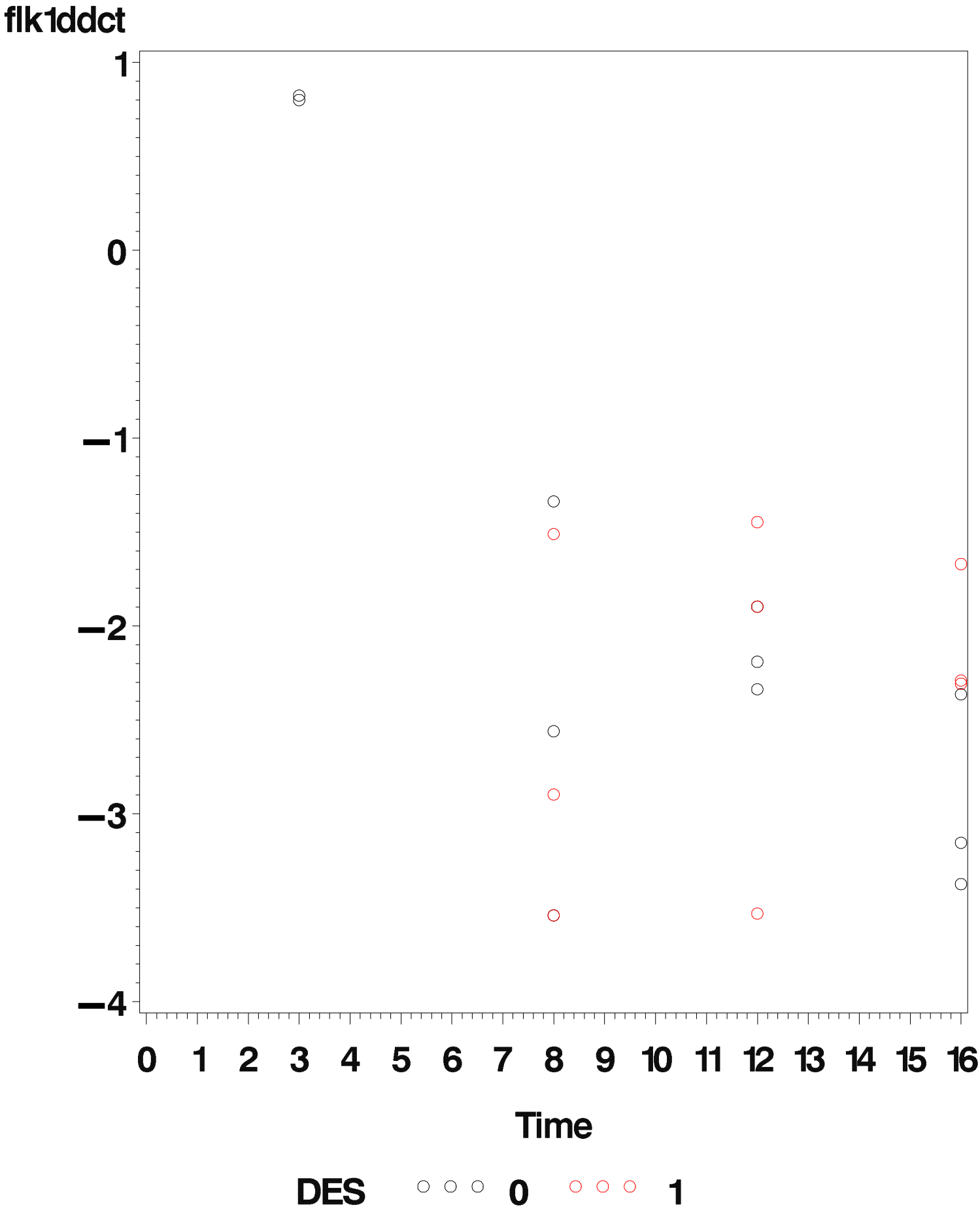

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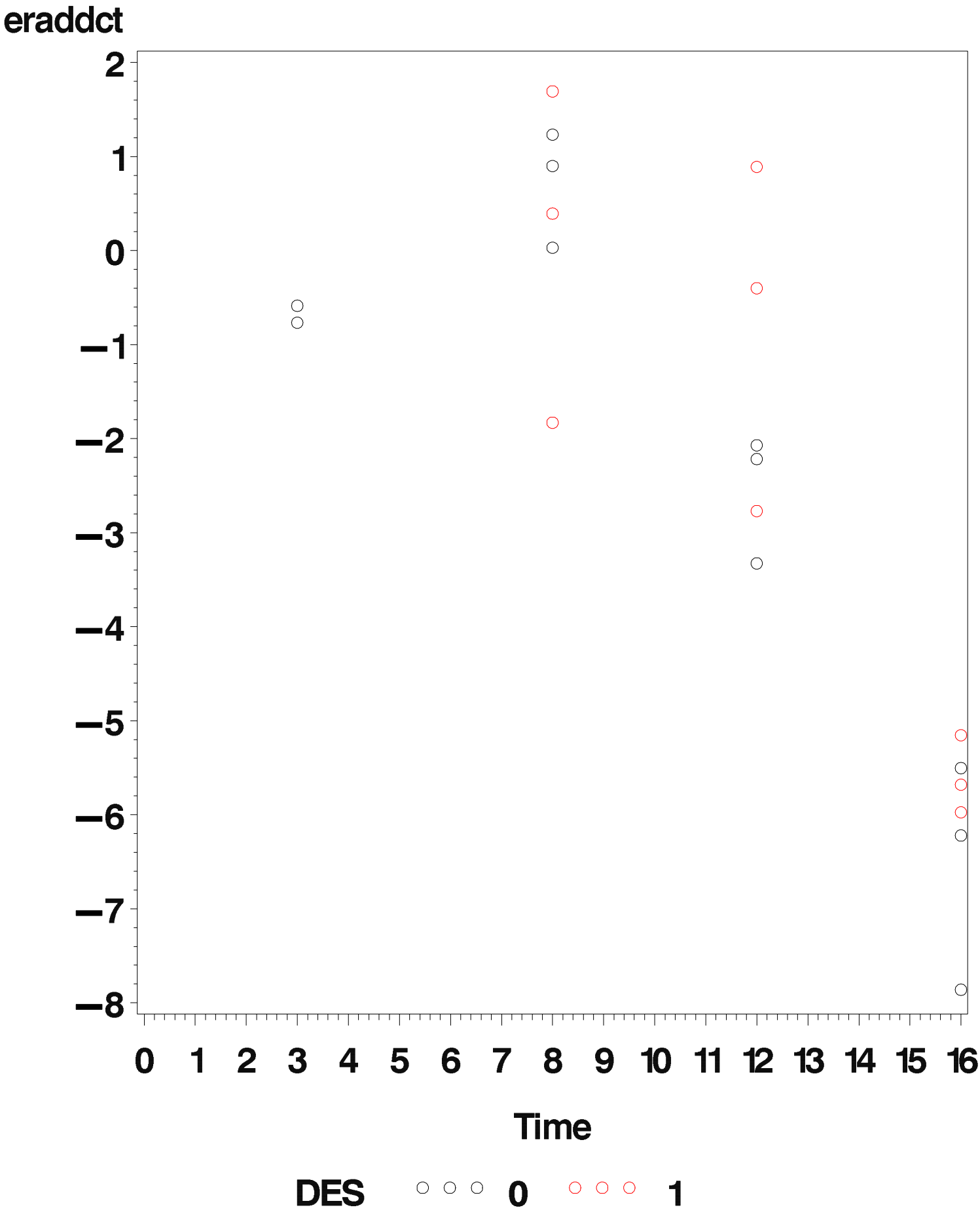

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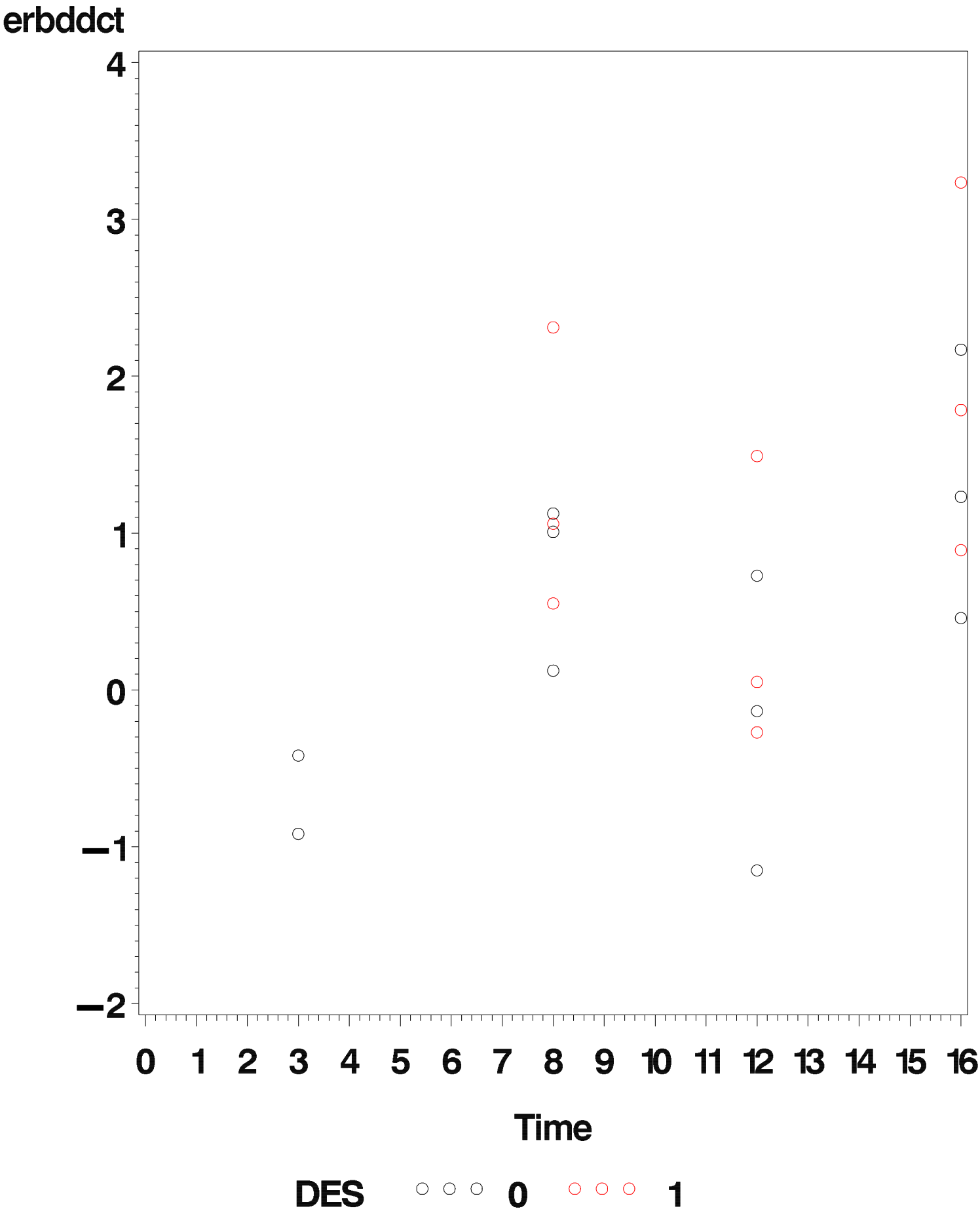

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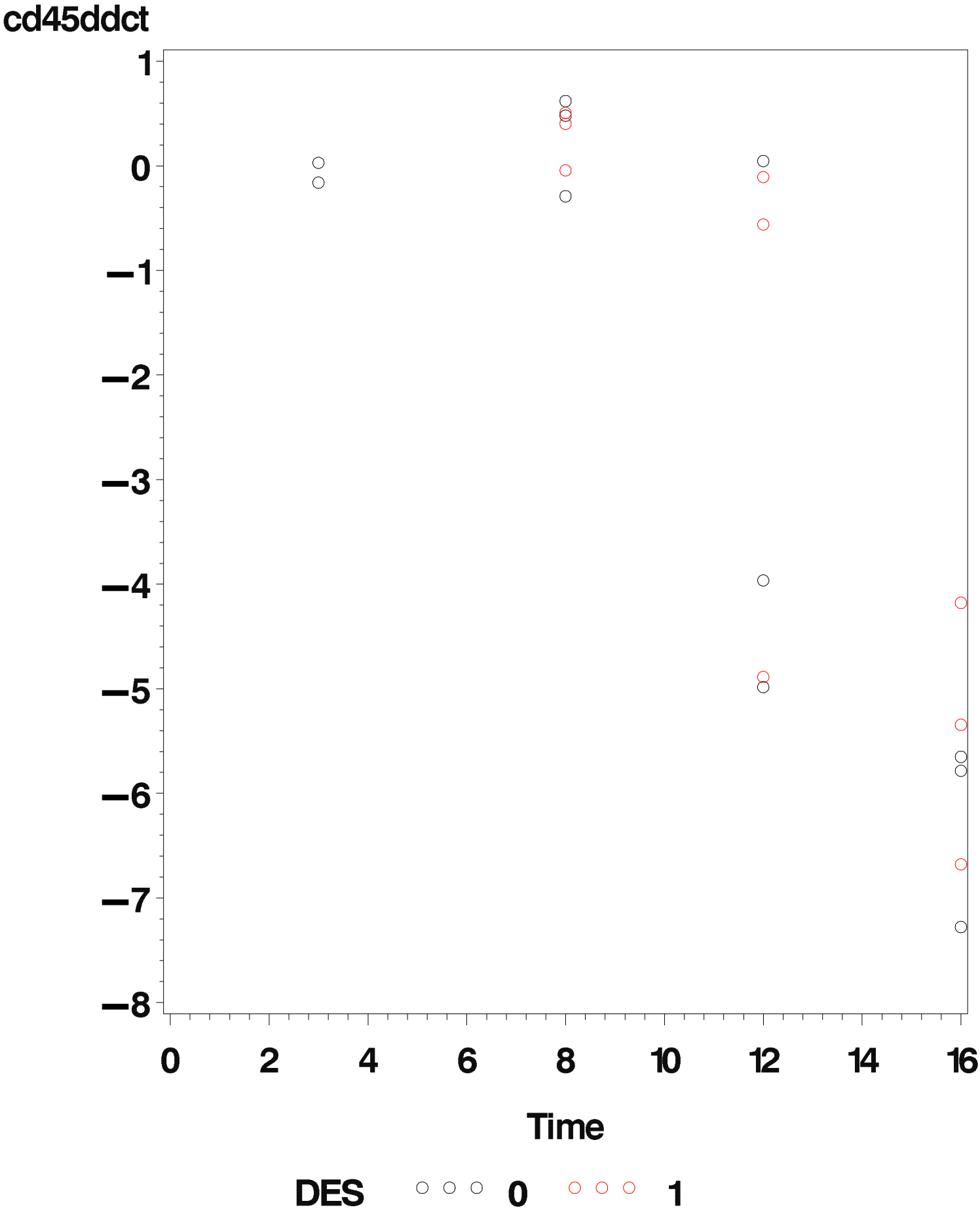

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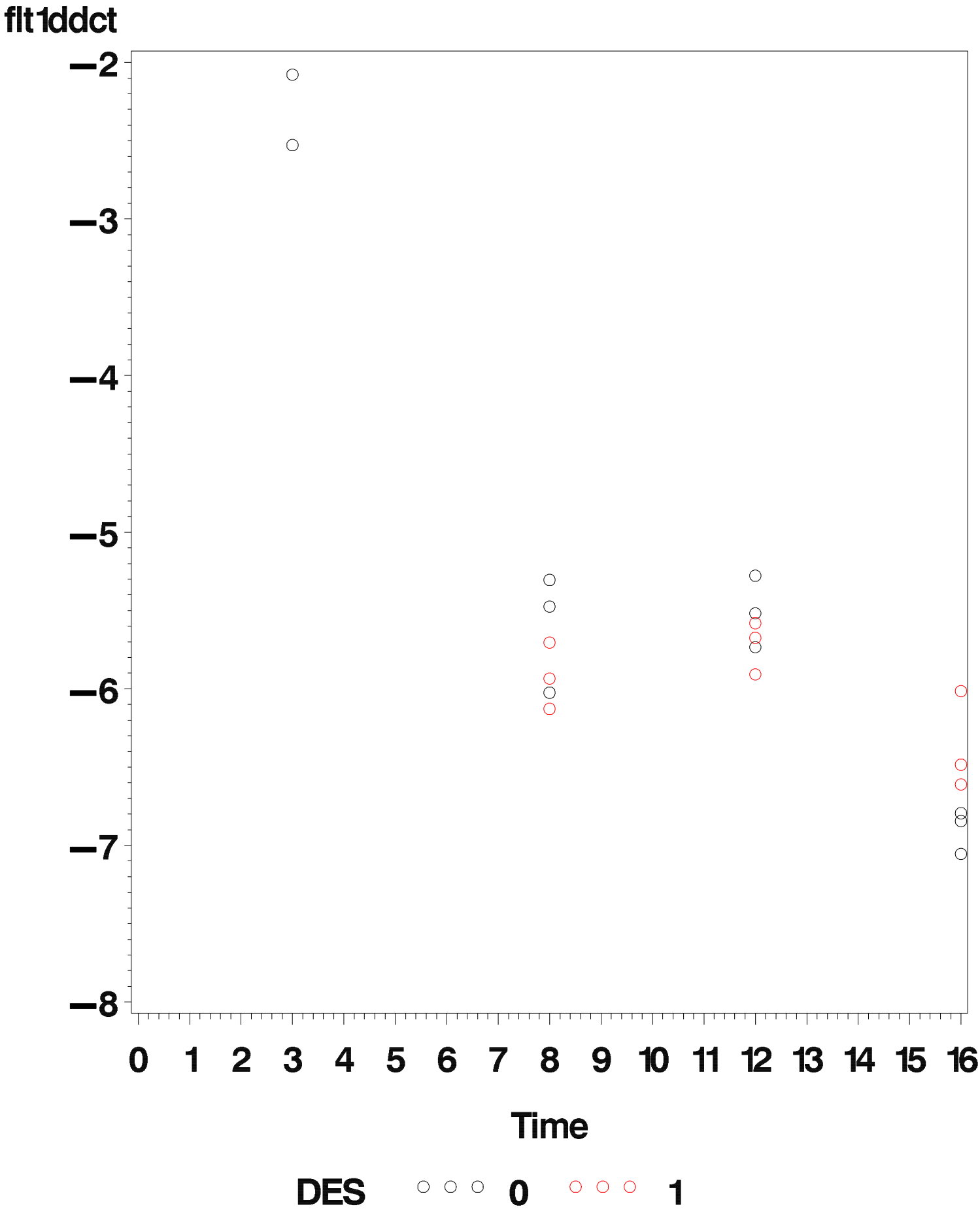

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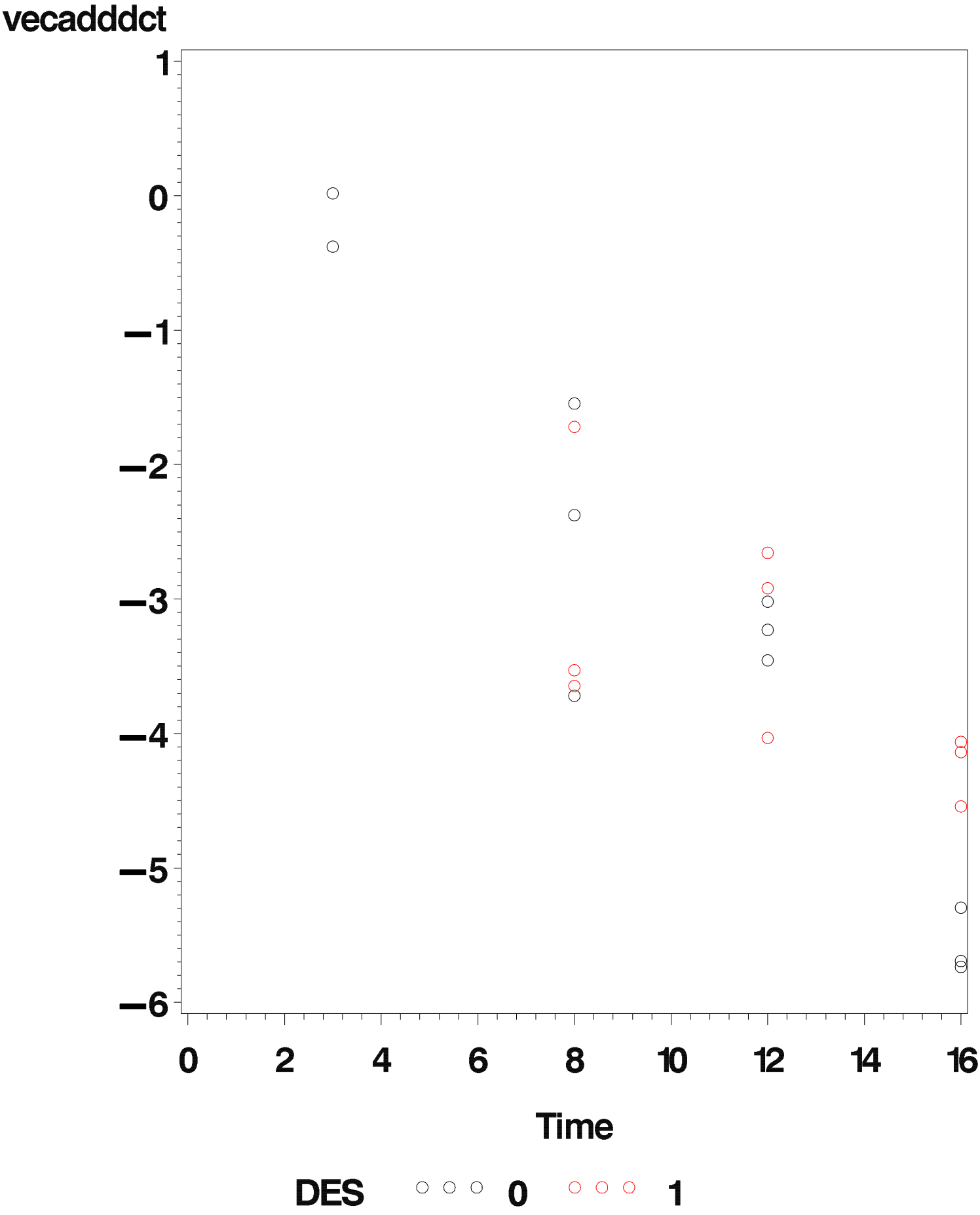

ckitddct - ANOVA for the 2 by 3

The Mixed Procedure

Model Information	
Data Set	WORK.DES
Dependent Variable	ckitddct
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information		
Class	**Levels**	**Values**
DES	2	0 1
Time	3	8 12 16

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
DES	1	12	8.63	0.0124
Time	2	12	154.62	<.0001
DES*Time	2	12	28.45	<.0001

Estimates								
Label	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES on day 16	-0.7278	0.09392	12	-7.75	<.0001	0.05	-0.9324	-0.5231
Slope from day 12 to 16	0.7600	0.1328	12	5.72	<.0001	0.05	0.4706	1.0494

Least Squares Means									
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES	0		-0.7587	0.03834	12	-19.79	<.0001	0.05	-0.8422	-0.6752
DES	1		-0.5994	0.03834	12	-15.63	<.0001	0.05	-0.6830	-0.5159
Time		8	-0.3472	0.04696	12	-7.39	<.0001	0.05	-0.4495	-0.2449
Time		12	-0.3367	0.04696	12	-7.17	<.0001	0.05	-0.4390	-0.2343
Time		16	-1.3533	0.04696	12	-28.82	<.0001	0.05	-1.4557	-1.2510
DES*Time	0	8	-0.2383	0.06641	12	-3.59	0.0037	0.05	-0.3830	-0.09363
DES*Time	0	12	-0.3206	0.06641	12	-4.83	0.0004	0.05	-0.4653	-0.1759

The Mixed Procedure

Least Squares Means										
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES*Time	0	16	-1.7172	0.06641	12	-25.86	<.0001	0.05	-1.8619	-1.5725
DES*Time	1	8	-0.4561	0.06641	12	-6.87	<.0001	0.05	-0.6008	-0.3114
DES*Time	1	12	-0.3528	0.06641	12	-5.31	0.0002	0.05	-0.4975	-0.2081
DES*Time	1	16	-0.9894	0.06641	12	-14.90	<.0001	0.05	-1.1341	-0.8447

The Mixed Procedure

Model Information	
Data Set	WORK.DES
Dependent Variable	oct4ddct
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information		
Class	**Levels**	**Values**
DES	2	0 1
Time	3	8 12 16

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
DES	1	12	0.21	0.6570
Time	2	12	23.83	<.0001
DES*Time	2	12	0.12	0.8852

Estimates								
Label	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES on day 16	-0.3467	0.5543	12	-0.63	0.5434	0.05	-1.5544	0.8611
Slope from day 12 to 16	0.2144	0.7839	12	0.27	0.7891	0.05	-1.4936	1.9225

Least Squares Means										
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES	0		3.1826	0.2263	12	14.06	<.0001	0.05	2.6895	3.6757
DES	1		3.3283	0.2263	12	14.71	<.0001	0.05	2.8353	3.8214
Time		8	2.8103	0.2772	12	10.14	<.0001	0.05	2.2064	3.4142
Time		12	2.1811	0.2772	12	7.87	<.0001	0.05	1.5772	2.7850
Time		16	4.7750	0.2772	12	17.23	<.0001	0.05	4.1711	5.3789
DES*Time	0	8	2.8311	0.3920	12	7.22	<.0001	0.05	1.9771	3.6851
DES*Time	0	12	2.1150	0.3920	12	5.40	0.0002	0.05	1.2610	2.9690

The Mixed Procedure

Least Squares Means										
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES*Time	0	16	4.6017	0.3920	12	11.74	<.0001	0.05	3.7476	5.4557
DES*Time	1	8	2.7894	0.3920	12	7.12	<.0001	0.05	1.9354	3.6435
DES*Time	1	12	2.2472	0.3920	12	5.73	<.0001	0.05	1.3932	3.1012
DES*Time	1	16	4.9483	0.3920	12	12.62	<.0001	0.05	4.0943	5.8024

The Mixed Procedure

Model Information	
Data Set	WORK.DES
Dependent Variable	flk1ddct
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information		
Class	**Levels**	**Values**
DES	2	0 1
Time	3	8 12 16

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
DES	1	12	0.23	0.6381
Time	2	12	0.33	0.7246
DES*Time	2	12	0.81	0.4674

Estimates								
Label	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES on day 16	-0.8744	0.6634	12	-1.32	0.2121	0.05	-2.3198	0.5709
Slope from day 12 to 16	1.0244	0.9382	12	1.09	0.2963	0.05	-1.0196	3.0685

Least Squares Means									
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES	0		-2.5278	0.2708	12	-9.33	<.0001	0.05	-3.1178	-1.9377
DES	1		-2.3430	0.2708	12	-8.65	<.0001	0.05	-2.9330	-1.7529
Time		8	-2.5639	0.3317	12	-7.73	<.0001	0.05	-3.2866	-1.8412
Time		12	-2.2161	0.3317	12	-6.68	<.0001	0.05	-2.9388	-1.4934
Time		16	-2.5261	0.3317	12	-7.62	<.0001	0.05	-3.2488	-1.8034
DES*Time	0	8	-2.4789	0.4691	12	-5.28	0.0002	0.05	-3.5009	-1.4569
DES*Time	0	12	-2.1411	0.4691	12	-4.56	0.0006	0.05	-3.1631	-1.1191

The Mixed Procedure

Least Squares Means										
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES*Time	0	16	-2.9633	0.4691	12	-6.32	<.0001	0.05	-3.9854	-1.9413
DES*Time	1	8	-2.6489	0.4691	12	-5.65	0.0001	0.05	-3.6709	-1.6269
DES*Time	1	12	-2.2911	0.4691	12	-4.88	0.0004	0.05	-3.3131	-1.2691
DES*Time	1	16	-2.0889	0.4691	12	-4.45	0.0008	0.05	-3.1109	-1.0669

The Mixed Procedure

Model Information	
Data Set	WORK.DES
Dependent Variable	eraddct
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information		
Class	**Levels**	**Values**
DES	2	0 1
Time	3	8 12 16

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
DES	1	12	1.41	0.2584
Time	2	12	43.17	<.0001
DES*Time	2	12	1.48	0.2664

Estimates								
Label	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES on day 16	-0.9256	1.0061	12	-0.92	0.3757	0.05	-3.1178	1.2666
Slope from day 12 to 16	-0.8522	1.4229	12	-0.60	0.5604	0.05	-3.9525	2.2480

Least Squares Means									
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES	0		-2.7815	0.4108	12	-6.77	<.0001	0.05	-3.6764	-1.8865
DES	1		-2.0922	0.4108	12	-5.09	0.0003	0.05	-2.9872	-1.1973
Time		8	0.4033	0.5031	12	0.80	0.4383	0.05	-0.6928	1.4994
Time		12	-1.6489	0.5031	12	-3.28	0.0066	0.05	-2.7450	-0.5528
Time		16	-6.0650	0.5031	12	-12.06	<.0001	0.05	-7.1611	-4.9689
DES*Time	0	8	0.7211	0.7115	12	1.01	0.3308	0.05	-0.8290	2.2712
DES*Time	0	12	-2.5378	0.7115	12	-3.57	0.0039	0.05	-4.0879	-0.9877

The Mixed Procedure

Least Squares Means										
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES*Time	0	16	-6.5278	0.7115	12	-9.18	<.0001	0.05	-8.0779	-4.9777
DES*Time	1	8	0.08556	0.7115	12	0.12	0.9063	0.05	-1.4646	1.6357
DES*Time	1	12	-0.7600	0.7115	12	-1.07	0.3064	0.05	-2.3101	0.7901
DES*Time	1	16	-5.6022	0.7115	12	-7.87	<.0001	0.05	-7.1523	-4.0521

The Mixed Procedure

Model Information	
Data Set	WORK.DES
Dependent Variable	erbddct
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information		
Class	**Levels**	**Values**
DES	2	0 1
Time	3	8 12 16

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
DES	1	12	2.04	0.1782
Time	2	12	4.14	0.0428
DES*Time	2	12	0.01	0.9926

Estimates								
Label	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES on day 16	-0.6839	0.7467	12	-0.92	0.3778	0.05	-2.3108	0.9430
Slope from day 12 to 16	0.07389	1.0560	12	0.07	0.9454	0.05	-2.2269	2.3747

Least Squares Means										
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES	0		0.6178	0.3048	12	2.03	0.0655	0.05	-0.04640	1.2820
DES	1		1.2343	0.3048	12	4.05	0.0016	0.05	0.5701	1.8984
Time		8	1.0300	0.3733	12	2.76	0.0173	0.05	0.2166	1.8434
Time		12	0.1194	0.3733	12	0.32	0.7545	0.05	-0.6940	0.9329
Time		16	1.6286	0.3733	12	4.36	0.0009	0.05	0.8152	2.4421
DES*Time	0	8	0.7522	0.5280	12	1.42	0.1797	0.05	-0.3982	1.9026
DES*Time	0	12	-0.1856	0.5280	12	-0.35	0.7314	0.05	-1.3359	0.9648

The Mixed Procedure

Least Squares Means										
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES*Time	0	16	1.2867	0.5280	12	2.44	0.0313	0.05	0.1363	2.4371
DES*Time	1	8	1.3078	0.5280	12	2.48	0.0291	0.05	0.1574	2.4582
DES*Time	1	12	0.4244	0.5280	12	0.80	0.4371	0.05	-0.7259	1.5748
DES*Time	1	16	1.9706	0.5280	12	3.73	0.0029	0.05	0.8202	3.1209

cd45ddct - ANOVA for the 2 by 3

The Mixed Procedure

Model Information	
Data Set	WORK.DES
Dependent Variable	cd45ddct
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information		
Class	**Levels**	**Values**
DES	2	0 1
Time	3	8 12 16

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
DES	1	12	0.70	0.4201
Time	2	12	20.08	0.0001
DES*Time	2	12	0.17	0.8417

Estimates								
Label	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES on day 16	-0.8378	1.3638	12	-0.61	0.5505	0.05	-3.8092	2.1336
Slope from day 12 to 16	-0.2778	1.9286	12	-0.14	0.8879	0.05	-4.4799	3.9244

Least Squares Means									
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES	0		-2.9778	0.5568	12	-5.35	0.0002	0.05	-4.1908	-1.7647
DES	1		-2.3204	0.5568	12	-4.17	0.0013	0.05	-3.5334	-1.1073
Time		8	0.2794	0.6819	12	0.41	0.6892	0.05	-1.2062	1.7651
Time		12	-2.4089	0.6819	12	-3.53	0.0041	0.05	-3.8946	-0.9232
Time		16	-5.8178	0.6819	12	-8.53	<.0001	0.05	-7.3035	-4.3321
DES*Time	0	8	0.2700	0.9643	12	0.28	0.7843	0.05	-1.8311	2.3711
DES*Time	0	12	-2.9667	0.9643	12	-3.08	0.0096	0.05	-5.0677	-0.8656

The Mixed Procedure

Least Squares Means										
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES*Time	0	16	-6.2367	0.9643	12	-6.47	<.0001	0.05	-8.3377	-4.1356
DES*Time	1	8	0.2889	0.9643	12	0.30	0.7696	0.05	-1.8122	2.3900
DES*Time	1	12	-1.8511	0.9643	12	-1.92	0.0790	0.05	-3.9522	0.2500
DES*Time	1	16	-5.3989	0.9643	12	-5.60	0.0001	0.05	-7.5000	-3.2978

The Mixed Procedure

Model Information	
Data Set	WORK.DES
Dependent Variable	flt1ddct
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information		
Class	**Levels**	**Values**
DES	2	0 1
Time	3	8 12 16

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
DES	1	12	0.00	0.9903
Time	2	12	28.36	<.0001
DES*Time	2	12	4.98	0.0266

Estimates								
Label	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES on day 16	-0.5278	0.2068	12	-2.55	0.0254	0.05	-0.9784	-0.07720
Slope from day 12 to 16	0.7389	0.2925	12	2.53	0.0266	0.05	0.1017	1.3761

Least Squares Means										
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES	0		-6.0027	0.08443	12	-71.10	<.0001	0.05	-6.1866	-5.8187
DES	1		-6.0042	0.08443	12	-71.12	<.0001	0.05	-6.1881	-5.8202
Time		8	-5.7614	0.1034	12	-55.72	<.0001	0.05	-5.9867	-5.5361
Time		12	-5.6153	0.1034	12	-54.31	<.0001	0.05	-5.8406	-5.3900
Time		16	-6.6336	0.1034	12	-64.15	<.0001	0.05	-6.8589	-6.4083
DES*Time	0	8	-5.6008	0.1462	12	-38.30	<.0001	0.05	-5.9194	-5.2822
DES*Time	0	12	-5.5097	0.1462	12	-37.68	<.0001	0.05	-5.8283	-5.1911

The Mixed Procedure

Least Squares Means										
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES*Time	0	16	-6.8975	0.1462	12	-47.17	<.0001	0.05	-7.2161	-6.5789
DES*Time	1	8	-5.9219	0.1462	12	-40.50	<.0001	0.05	-6.2406	-5.6033
DES*Time	1	12	-5.7208	0.1462	12	-39.12	<.0001	0.05	-6.0394	-5.4022
DES*Time	1	16	-6.3697	0.1462	12	-43.56	<.0001	0.05	-6.6883	-6.0511

The Mixed Procedure

Model Information	
Data Set	WORK.DES
Dependent Variable	vecadddct
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information		
Class	**Levels**	**Values**
DES	2	0 1
Time	3	8 12 16

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
DES	1	12	0.86	0.3711
Time	2	12	15.07	0.0005
DES*Time	2	12	2.40	0.1329

Estimates								
Label	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES on day 16	-1.3267	0.5847	12	-2.27	0.0425	0.05	-2.6006	-0.05274
Slope from day 12 to 16	1.2944	0.8269	12	1.57	0.1435	0.05	-0.5072	3.0961

Least Squares Means										
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES	0		-3.7855	0.2387	12	-15.86	<.0001	0.05	-4.3055	-3.2654
DES	1		-3.4718	0.2387	12	-14.54	<.0001	0.05	-3.9918	-2.9517
Time		8	-2.7558	0.2923	12	-9.43	<.0001	0.05	-3.3928	-2.1189
Time		12	-3.2186	0.2923	12	-11.01	<.0001	0.05	-3.8556	-2.5816
Time		16	-4.9114	0.2923	12	-16.80	<.0001	0.05	-5.5484	-4.2744
DES*Time	0	8	-2.5469	0.4134	12	-6.16	<.0001	0.05	-3.4477	-1.6461
DES*Time	0	12	-3.2347	0.4134	12	-7.82	<.0001	0.05	-4.1355	-2.3339

The Mixed Procedure

Least Squares Means										
Effect	**DES**	**Time**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Alpha**	**Lower**	**Upper**
DES*Time	0	16	-5.5747	0.4134	12	-13.48	<.0001	0.05	-6.4755	-4.6739
DES*Time	1	8	-2.9647	0.4134	12	-7.17	<.0001	0.05	-3.8655	-2.0639
DES*Time	1	12	-3.2025	0.4134	12	-7.75	<.0001	0.05	-4.1033	-2.3017
DES*Time	1	16	-4.2481	0.4134	12	-10.27	<.0001	0.05	-5.1489	-3.3473